CONTRIBUTION TO THE STUDY OF POLYPHENOLIC COMPOUNDS OF FOUR BIOLOGICALLY ACTIVE PLANT SPECIES: *RUMEX CHALEPENSIS, R. NEPALENSIS (POLYGONACEAE), INDIGOFERA HEBEPETALA* AND *I. CASSIOIDES (LEGUMINOSAE)*

A DISSERTATION SUBMITTED
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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF **DOCTOR OF PHILOSOPHY** IN

ORGANIC CHEMISTRY

BY

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DEDICATED

TO

MY PARENTS, SISTERS, BROTHER, WIFE,
MY LOVING SON ASIM AND
LITTLE MARIA

DECLARATION

This is to certify that this dissertation submitted by IFTIKHAR AHMAD is accepted in its present form by the Department of Chemistry, Quaid-i-Azam University, Islamabad, Pakistan, as satisfying the dissertation partial requirement for the degree of **DOCTOR OF PHILOSOPHY** in organic chemistry.

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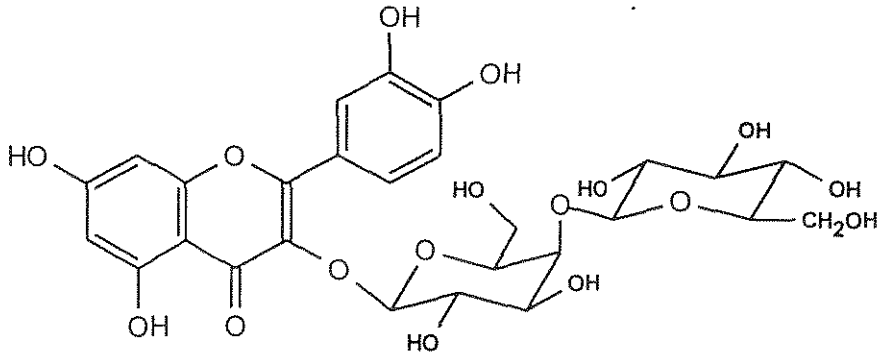
SUMMARY

Qualitative, quantitative and anti-bacterial activity analysis of flavonoids of 20 plant species of Islamabad and its surroundings were carried out. On the basis of results obtained after these analysis the plant species namely *Rumex chalepensis* and *Indigofera hebepetala* were selected for detailed study of their flavonoid contents. In addition, to the two plant species cited above, another two species belonging to the same genera i,e. *Rumex nepalensis* and *Indigofera cassioides* were also selected for similar studies.

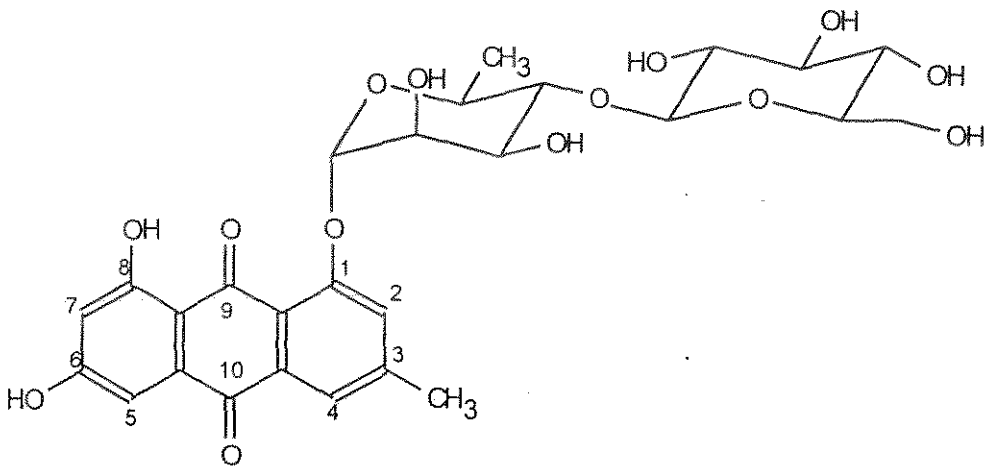
Apart from the chapter on the methodology used during the course of this study the present dissertation is further divited into three chapters. The first is devoted to the isolation and structural elucidation of flavonoids and related compounds from the leaves of *R. chalepensis* and *R. nepalensis*, whereas the second chapter deals with the similar work done on *I. hebepetala* (flowers and leaves) and leaves of *I. cassioides*. In the course of these studies a total of 6 new and 13 known/rare compounds were isolated and identified from the following four plant species under investigation:

Rumex chalepensis

a. New compounds



Quercetin 3-O-β-D-glucopyranosyl(1→4)-β-D-galactopyranoside (RC-1)



6,8-dihydroxy-3-methyl-anthraquinone-1-O-ß-D-glucosyl(1→4)-α-L-rhamnoside (RC-5)

b. Known rare compounds

Kaempferol 3-O-α-L-rhamnopyranosyl(1→6)-β-D-galactopyranoside (RC-2)

c. Known common compounds

Quercetin 3-O-α-L-rhamnopyranosyl(1→6)-β-D-glucoyranoside (RC-3)

Quercetin 3-O-α-L-rhamnopyranoside (RC-4)

3, 5, 7, 4'-tetrahydroxy-3', 5'-dimethoxy anthocyanidin (Malvidin) (RC-1A)

Rumex nepalensis

a. Known rare compounds

Quercetin 3-O-β-D-glucopyranosyl(1→4)-β-D-galactopyranoside (RN-1)

Kaempferol 3-O-α-L-rhamnopyranosyl(1→6)-β-D-galactopyranoside (RN-2)

b. Known common compounds

Quercetin 3-O-α-L-rhamnopyranosyl(1→6)-β-D-glucoyranoside (RN-3)

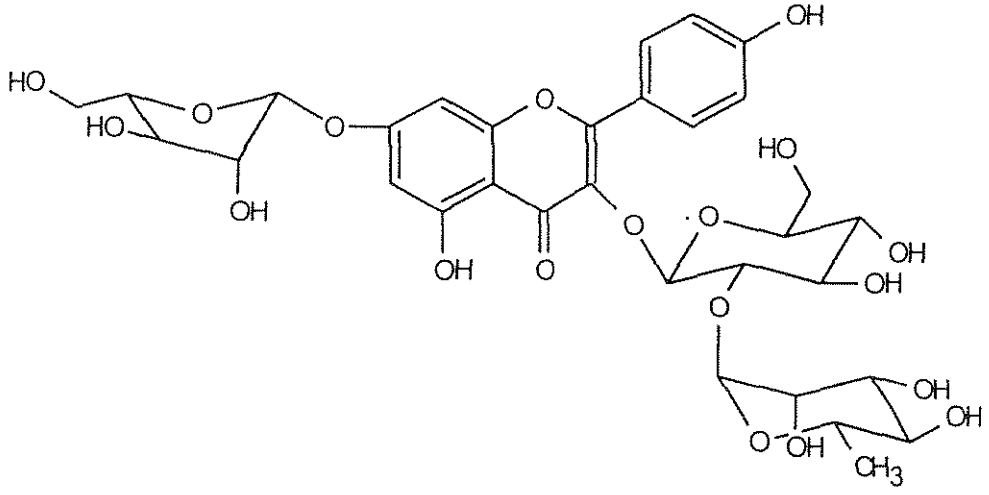
Quercetin 3-O-α-L-rhamnopyranoside (RN-4)

1, 6, 8-trihydroxy-3-methyl anthraquinone (RN-5)

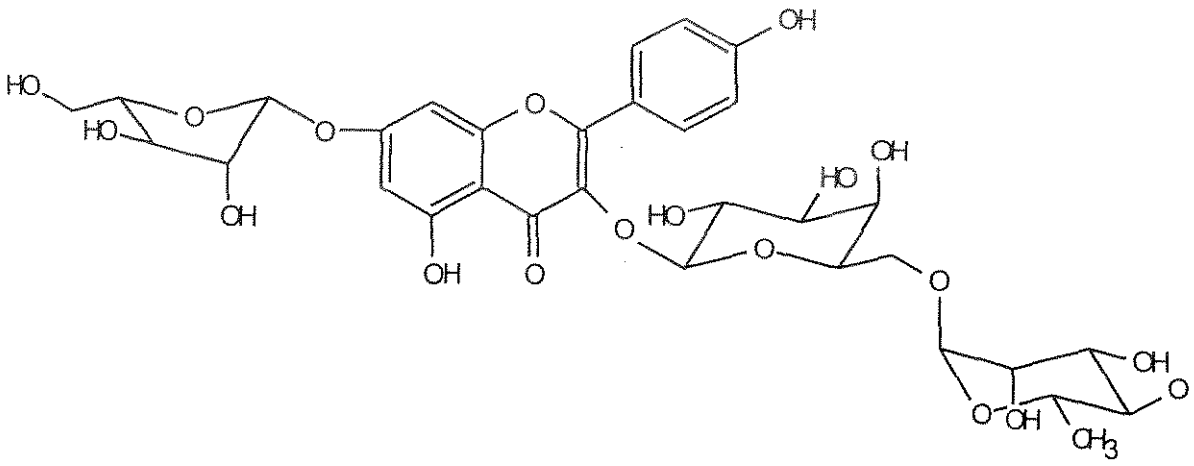
3, 5, 7, 4'-tetrahydroxy-3', 5'-dimethoxy anthocyanidin (Malvidin) (RN-1A)

***Indigofera hebepetala* (Flowers)**

a. New compounds



Kaempferol 3-O-α-L-rhamnopyranosyl(1→2)-β-D-galactopyranoside-7-O-α-L-arabinofuranoside (IHF-1)



Kaempferol 3-O-α-L-rhamnopyranosyl(1→6)-β-D-galactopyranoside-7-O-α-L-arabinofuranoside (IHF-2)

b. Known rare compounds

Kaempferol 3-O-α-L-arabinopyranoside-7-O-α-L-rhamnopyranoside (IHF-3)

Kaempferol 3-O-α-L-rhamnopyranoside-7-O-α-L-arabinopyranoside (IHF-4)

c. Known common compounds

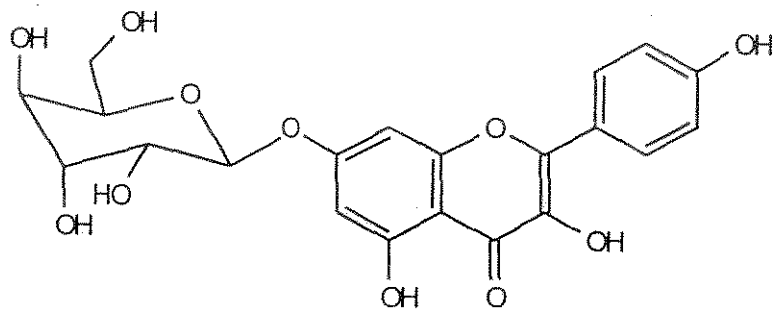
Kaempferol 7-O-α-L-rhamnopyranoside (IHF-5)

3, 5, 7, 3', 4'-pentahydroxy anthocyanidin (Cyanidin) (IHF-1A)

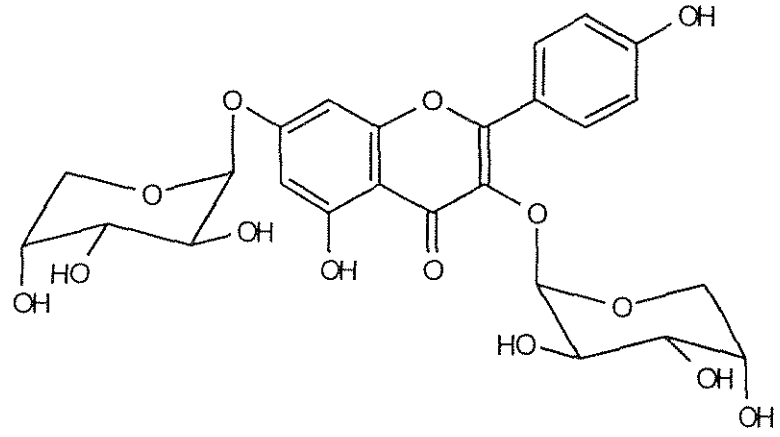
3, 5, 7, 3', 4', 5'-hexahydroxy anthocyanidin (Delphinidin) (IHF-2A)

Indigofera hebepetala **(Leaves)**

a. New compounds



Kaempferol 7-O-β-D-allopyranoside (IHL-1)



Kaempferol 3, 7-O-α-L-diarabinopyranoside (IHL-2)

b. Known rare compounds

Kaempferol 3-O-α-L-arabinopyranoside-7-O-α-L-rhamnopyranoside (IHL-3)

Kaempferol 3-O-α-L-rhamnopyranoside-7-O-α-L-arabinopyranoside (IHL-4)

c. Known common compound

3, 5, 7, 3', 4'-pentahydroxy anthocyanidin (Cyanidin) (IHL-1A)

Indigofera cassioides

a. Known common/rare compounds

Kaempferol 7-O-α-L-rhamnopyranoside (IC-2)

Kaempferol 3-O-(Galactose + Rhamnose) (IC-3)

Quercetin 3-O-β-D-galactopyranoside (IC-4)

Quercetin 3-O-(Glucose + Arabinose) (IC-5)

Structural determinations were mainly based on NMR [^{1}H NMR, ^{13}C (B.B and DEPT) NMR] spectral assignments which were confirmed by homo and hetero COSY experiments and by homonuclear NOE difference experiments. The conclusions drawn from NMR spectral analysis were further confirmed by the mass spectra (EIMS and FABMS), UV analysis in methanol before and after the addition of shift reagents and co-chromatography.

Amount of flavonoids in mg/gm as well as the number of flavonoids oresent in each plant specie are tabulated in the final portion of the dissertation. Antibacterial activity of the global flavonoid extracts as well as of the isolated purified flavonoids is also listed in the same section..The antibacterial activity analysis exhibited significant antibacterial activity for the global flavonoid extracts of *Rumex chalepensis* and *Indigofera hebepetala*. However this activity was enhanced when the purified flavonoids

of *Rumex chalepensis* and *Indigofera hebepetala* were tested against different bacterial strains (*Staphylococcus aureus*, *Sarcina lutea*, *Bacillus subtilis*, *Escherchia coli and Psudomonas aueroginosa*).

INTRODUCTION

GENERAL INTRODUCTION

Science is based on hypothesis, facts, ideas, imaginations, observations and careful experimentation. By significant observations science opens a world of thought, vision and opinion leading to the discovery of new facts and drawing of novel conclusions. Since disease and death have always coexisted with life from ancient time. The primitive man must have used therapeutic agents and remedial sources for the treatment of diseases. Man as a savage would have learned from experience how to relieve his sufferings by the use of herbs growing around him. Records of ancient civilizations show that a considerable number of drugs used as modern medicine were already in use in the form of plant materials in ancient times. For instance , Dioscorides, a Greek physician described about 600 plants in his book "*De Meterea Medica*" that were known to have medicinal properties (Tyler *et al*, 1988). Galen (131-200 A.D.) a Greek pharmacist physician described the method of preparing formulas containing plant and animal drugs (Tyler *et al*, 1988).

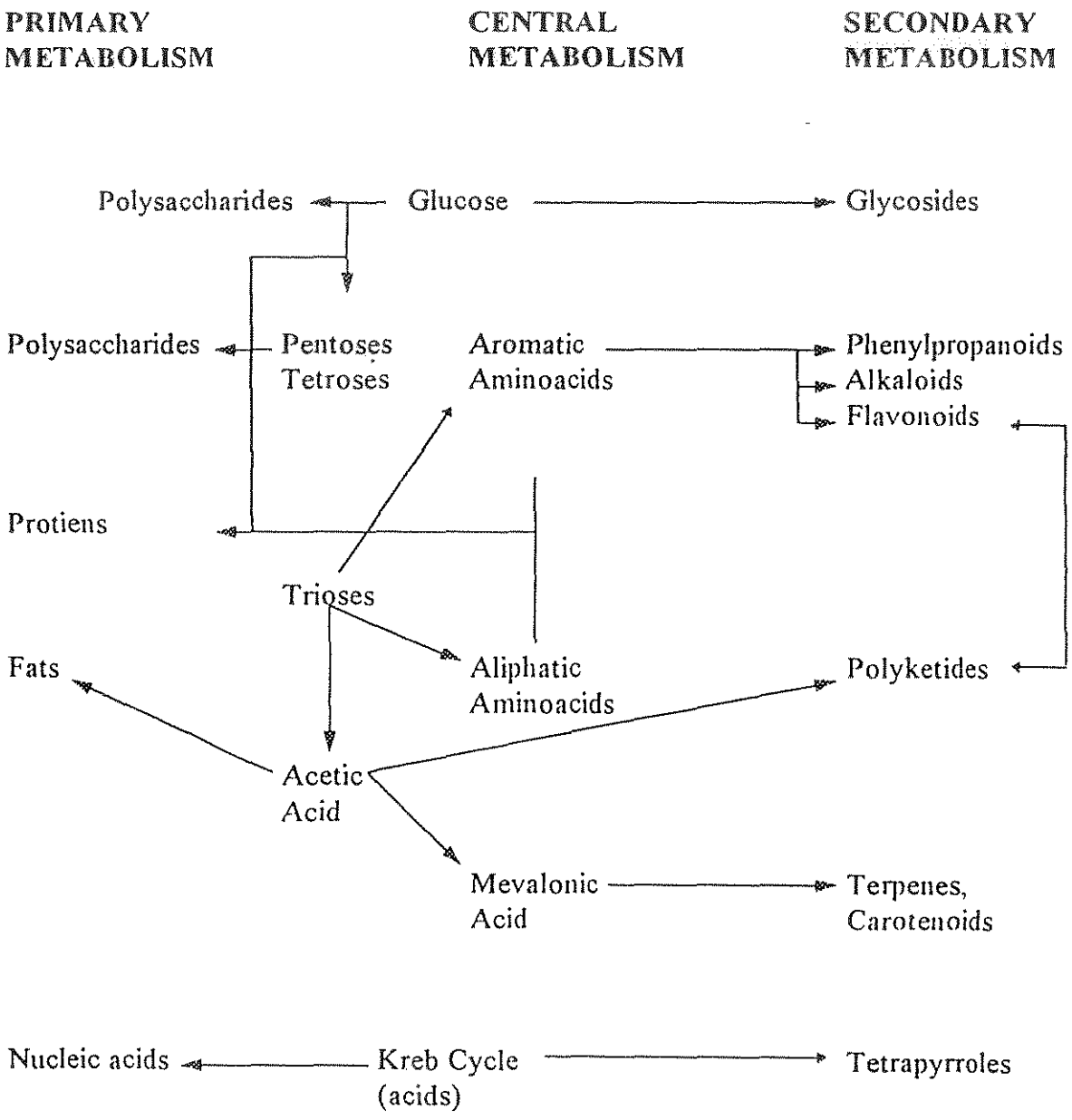
A significant contribution was made by Arab physicians in the uses of medicinal plants. Rhazes (Abu Bakar Mohammad bin Zakaria) is credited with having written nearly 250 works in his book entitled "*History of Medicine*". Avicenna (Sheikh Bu Ali Sina, 980-1037 A.D.) was the world famous author of the "*Al Qanoon Fil Tibb*" (The laws in medicine). He introduced the process of gilding and silvering of pills. Ibn al-Baitar (1197-1214 A.D.) wrote a massive work, "*Jame-ul-Mufradat*" in which he assembelled the remarks of the Greek physician, as well as of Galen, Rhazes, Avicenna and others on drugs. It deals with 2000 drugs of which 1700 are herbs.

The progress became more rapid in the nineteenth century when a large number of natural products were discovered in pure state. These natural products were mostly organic

compounds that had physiological and pharmaceutical properties and some of which are still in use as valuable drugs. The most significant pharmacological applications of these natural products are their use as anti-inflammatory agents (Pegel *et al*, 1990), anti-tumor agents (Rogers *et al*, 1979), immunostimulents, drugs affecting the cardiovascular and central nervous system (Mahato *et al*, 1982), analgesics (Cardenas *et al*, 1993) etc.

Until the middle of 20th century the main emphasis in the field of natural products chemistry remained on isolation and structure determination of a wide variety of compounds. By this time, the attention of natural product chemists was turning to the elucidation of the actual biosynthetic pathways found in the plants. The study of biochemical pathways has assumed a very important role in recent years. Plants synthesize primary metabolites from simple substances such as water, CO_2, N_2, and a number of inorganic salts in small amounts. These primary metabolites are transformed to secondary metabolites which are used as drugs. The transformation of primary to secondary from central metabolites is out lined in scheme 1.

Polysaccharides, proteins, fats and nucleic acids are the fundamental building blocks of living matter and are thus considered to be primary metabolites. The whole range of process by which organisms synthesize and demolish these substances, in order to survive, constitute the primary metabolic processes. Other chemical processes take place only in certain species or give different products according to the type of species. Such reactions do not appear to be essential for the existence of the organism and hence are called secondary metabolic processes. Major secondary metabolites include alkaloids, steroids, flavonoids and terpenoids, which often play a key role in the survival of species over others. Among these different types of secondary metabolites, synthesized by the plants, FLAVONOIDS is

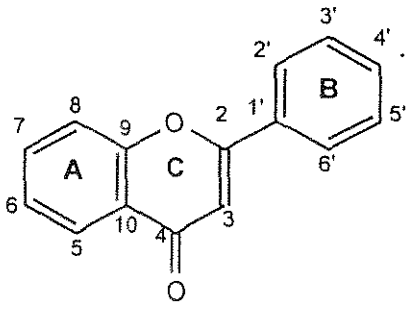


Scheme.1: Interrelationship of biosynthetic pathways leading to secondary constituents in plants.

one of the biggest class of natural products and has shown to have immense pharmaceutical, economic and agricultural importance.

Nature as it surrounds us is predominantly green. It is not surprising therefor that plants and parts of plants which are in strong contrast to this over whelming greenness have always attracted man and other animals. Almost all the brilliant colours such as yellow, red, blue, violet, even white and brown or black which are found in flowers and fruits are due to the presence of one or another type of flavonoids.

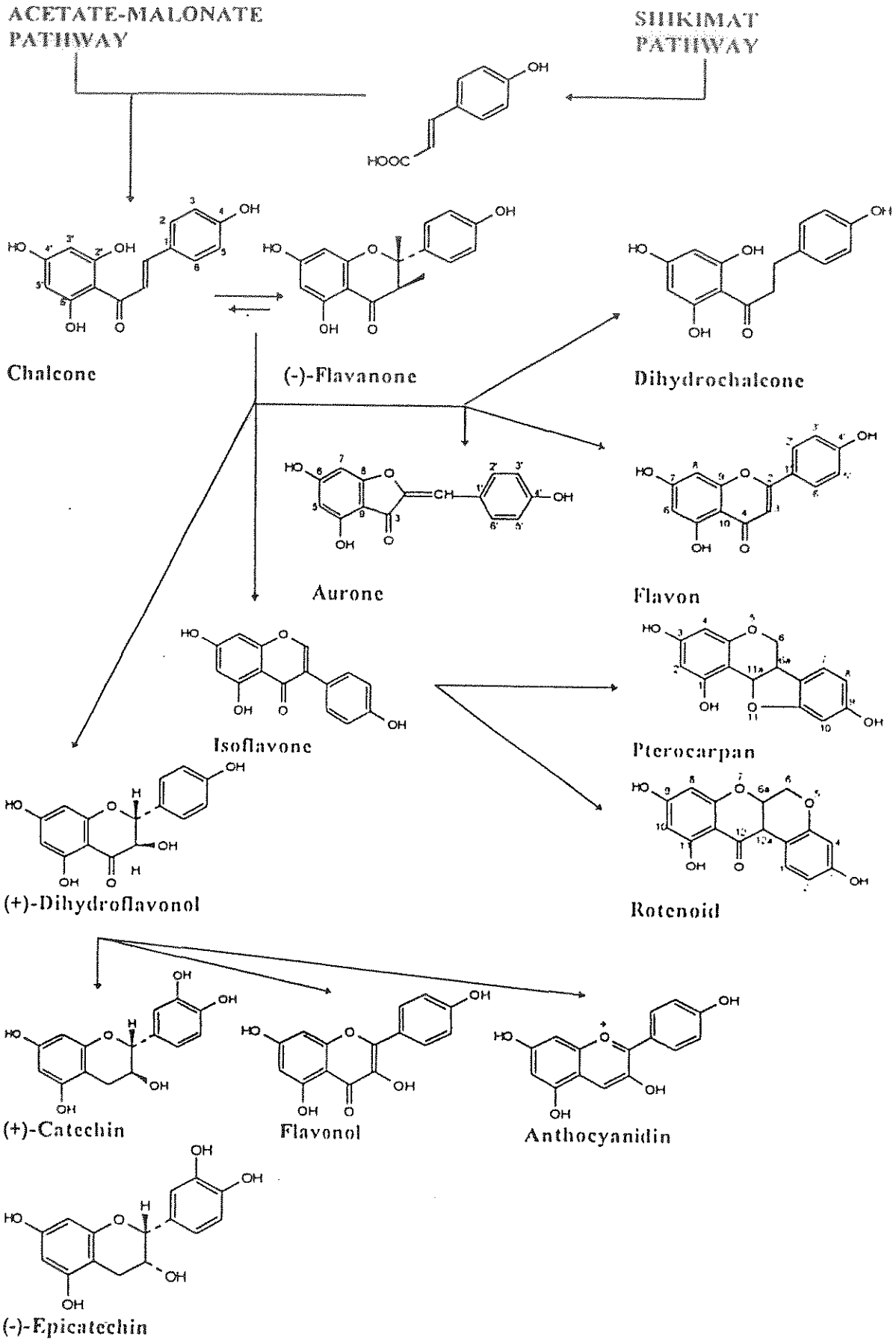
The name "FLAVONOID" has its origin from the Greek word "FLAVUS" meaning yellow. The name was formally applied to yellow pigments but to day, with a number of known structures the term flavonoid is used to embrace all those plant pigments having their structure based upon a fifteen carbon atoms skeleton arranged in C_6-C_3-C_6 configuration (Geissman, 1955). For convenience, it is customary to label the rings as A, B and C. The individual carbon atoms are referred to by a numbering system which utilizes ordinary numerals for the A- and C-ring and "primed" numerals for the B-ring as shown below.



The range of structural variation found in the known compounds of the flavonoid type is associated primarily with variation in the oxidation level of C_3 linkage of the molecule. The range of oxidation level extends from highly reduced catechin type to highly oxidized flavonols. The flavonoid variants are all related by a common biosynthetic pathway that incorporates precursors from both the "Shikimate" and "Acetate Malonate" pathways (Wong, 1976), the first flavonoid being formed immediately following the confluence of the two pathways is the chalcone. All other forms are derived from this by a variety of routs as shown in Scheme 2.

Further modification of the flavonoid may occur at various stages resulting in additional hydroxylation; methylation of hydroxyl groups or of the flavonoid nucleus; dimrization; bisulphate formation; and most important by glycosylation of hydroxyl groups or of the flavonoid nucleus. Acylated glycosides have one or more of their sugar(s) hydroxyls derivatized with an organic acid to form ester linkage. And indeed this accounts for the further diversification of flavonoids.

The analysis of flavonoids started in the early sixties when paper chromatography (PC) was first applied for the separation of flavonoids (Bate-Smith, 1949), but with the advent of modern analytical techniques of separation and identification, flavonoid analysis gained more momentum during the past two decades. Chromatography has played a vital role in the separation and purification of flavonoids. Although advanced chromatographic techniques have helped a lot to resolve complex flavonoid mixtures, conventional chromatographic techniques are still in use and are often used in conjunction with the advanced chromatographic techniques.



Scheme-2: Biosynthesis of flavonoids.

Paper chromatography, first applied for the separation of phenolics in the middle of the present century, is still used for the separation of flavonoids both in mono and bi-dimensional modes. Averett and Raven (1983) have isolated three flavonol glycosides, kaempferol-3-O-α-L-rhamnoside, quercetin 3-O-α-L-rhamnoside by two dimensional paper chromatography (2DPC) in tertiary-butanol (*t*-BuOH)-Acetic acid (HOAc)-Water (H_2O) (3:1:1) and 15% HOAc. Luteolin 6,8-di-C-rhamnoside (Imperato, 1992), apigenin 7-O-rhamnoside-4'-O-glucorhamnoside (Malan, 1993), Kaempferol 7-alloside and kaempferol 3,7-diarabinoside (Hasan *et al*, 1994) have been isolated by 2DPC using normal-butanol (*n*-BuOH)-Acetic acid (HOAc)-Water (H_2O) (4:1:5) BAW and 15% HOAc as the solvent system. Although other chromatographic techniques have been introduced , PC still remains one of the important method for preliminary separation of phenolic compounds.

In addition to PC, thin layer chromatography (TLC) also has a significant role in the separation of flavonoids. As compared to PC, TLC is more rapid , versatile and exhibit a high degree of resolution. According to Barberan *et al* (1985) 6-hydroxy flavones and 8-hydroxy flavones can easily be distinguished by difference in their chromatographic behavior on cellulose coated plates with 30% HOAc, the former have lower R_f values than the latter compounds. Becker *et al* (1986) has reported a new system for the separation of biflavonoids, employing polyamide as the adsorbent and Ethyl acetate(EtOAc)-Methyl ethyl ketone (MeCOEt)-Formic acid (HCO_2H)-Water (H_2O) (5:3:1:1) as the eluent. This system yields compact spots and reasonable R_f values for more highly hydroxylated biflavonoids. Iinuma *et al* (1993), Tahara *et al* (1993) and Tanee *et al* (1994) have reported the isolation of novel isoflavones and known phenolic compounds from *Sophora fraseri*, root bark of *Piscidia erythrina* and bark of *Erthrina sengalensis* with the help of preprative thin layer chromatography (PTLC) using $CHCl_3$-MeOH (10:1), hexane-Me_2CO-EtOH and CH_2Cl_2-MeOH (19:1) as the solvent systems. Separation by TLC has been accelerated

further by making use of its modified version called high pefermance thin layer chromatography (HPTLC). Recent research has shown that HPTLC can provide much batter separation than TLC of complex flavonoid mixtures. Heimler (1986) has reported the use of HPTLC for the separation of selected flavonoid aglycones on ready-for-use layers of silanized silica gel. Bilia *et al* (1993) isolated two flavone and three flavanones from roots of *Pyracantha coccinea* by means of RP-8 HPTLC using MeOH-H_2O (3:1) as the solvent system.

Column chromatography (CC), is yet another conventional chromatographic method with the help of which isolation of flavonoids can be scaled up almost to industrial scale. Polyclar AT column has been used for the isolation of some less polar and non-polar flavonoids from the leaves of *Perityl vaseyi* (Bohm *et al*, 1986) and cabbage (Nielsen *et al*, 1993). Markham *et al* (1990) has reported isolation of a new flavonoid, kaempferol-3-sphorosid-7-α-L-arabinofuranoside by cellulose column. The use of Sephadex LH 20 as adsorbent now widely used for the final purification of flavonoids, has been reported for the successful separation of complex flavonoids (Sekine *et al*, 1991, Allais *et al*, 1991, Dange *et al*, 1992, and Mizuno *et al*, 1992). Liu *et al* (1993) and Okamura *et al* (1993) achieved the successful separation of flavonoid aglycones and novel flavonoid glycosides from the aerial parts of *Baccharis thesioides* and *Eucalyptus rostrata* on Sephadex LH 20 column using MeOH as the eluent. Boza *et al* (1993) isolated two flavonoids, dihydroquercetin-3-O-ß-D-xyloside and caryatin 7-O-ß-D-glucoside from twigs of *Eucryphia glutinosa* by CC over silca gel. Isolation of two new flavonoids, 5, 8-dihydroxy-4'methoxy-6, 7-methyllenedioxy isoflavone and carthamidin 7-O-α-L-rhamnopyranoside from *Sartium junceum* was achieved by CC on silica gel (Bilia *et al*, 1993).

In order to reduce the separation time, a modified form of column chromatography called flash chromatography (FC) has been introduced. By making use of FC Peteraka and Feuwick (1988) and Dominguez *et al* (1983) isolated glucosinol from *Mustard* and Mixtecacin from the root of *Tephorsia woodi* respectively. Isolation of a novel bridged isoflavan (ferrugin) from the bark of *Aglaia ferruginaea* has also been reported by FC on silica gel (Merck 9385) using 10% EtOAc-90% $MeCl_2$ as solvent system (Dean et al, 1993).

Although CC remains a very useful technique for priliminary purification of flavonoids from crude plant extracts. Rcent reports have shown that High Performance Liquid Chromatography (HPLC) is one of the most useful technique available to day for separating complex mixture of flavonoid compounds. The range of flavonoid compounds that have been successfully separated by HPLC continue to expand at very rapid rate. With in the last decade HPLC has been applied to highly complex separations in the field of natural product chemistry. Kingston (1979) and Van Sumere *et al*, (1979) have published very useful reviews about the applications of HPLC to the separation of plant phenolics. Useful informations on the theory and relationship of theory to practice in HPLC has been published by Snyder (1974) and Engelhardt (1979). The proper choice of a column plays a significant role in the flavonoid separation. Silica gel columns are often used for non-polar flavonoid (Piretti and Doghieri, 1990) while chemically bonded octadecyl silane (ODS) columns exhibit excellent result for the separation of polar compounds (Zeng *et al*, 1990, Gao *et al*, 1990, Kaouadji , 1990, Slacanin *et al*, 1991, Pietta *et al*, 1991, Lamaison *et al*, 1991, Finger *et al*, 1991, Tomas-Lorente *et al*, 1992 and D'Agestino *et al*, 1992). Acetonitrile-water or Methanol-Water containing small amount of acetic acid are commonly used solvents. A novel chalcone glycoside, okanin-4-methoxy-4'-O-(6"-O-acetylglucopyranoside) (Redl *et al*, 1993) was isolated by HPLC on Hamilton PRP-1 (305 x

7.0, 7μm) column with MeCN-H_2O gradient system (15-40% MeCN in 40 min., flow rate 2.0 ml/min.). An excellent separation of dihydroflavonol glycosides, isolated from *Erica cinerea*, was achieved by HPLC on μ-Bondapack C-18 column with 40% aq. MeOH. Triglycosides of kaempferol and quercetin have been isolated efficiently by HPLC from tea with HOAc-MeCN-MeOH used in gradient mode (Bennini *et al*, 1993). Isolation of seventeen flavonoid glycosides from *Equisetum arvense* by HPLC using Supersphere RP 18(e) column ; and solvent system with solution profile: A= 0.3% H_3PO_4 in water; B= MeOH has been reported by Veit *et al* (1990). Krause *et al* (1991) have stereospecifically analyzed enantiomeric flavanones by gradient elution on a column of triacetate sported on silica gel diol. Optical resolution of six monosubstituted and one diglycoside flavanone has been made possible by HPLC on Chiracel OD, Chiralpal OP(+), Chia Sphere, Cyclobond and acetalated cyclobond I column (Krause *et al*, 1990).

Despite the advance of refine and sophisticated equipment, real progress in HPLC has suffered from the lack of a universal UV detector that allows the analyst to optimize response, detection limits and resolution. To over come this difficulty HPLC has been coupled with a new sophisticated detection system called Photo Diode Array (PDA). There has been one of the most important advances in HPLC detector during the last few years. Using this method the sample is scanned every few millisecond, generating the absorbance maximum. In addition the purity of each peak can be examined providing different spectra for the possible impurity and the pure component. HPLC-PDA detection has recently been successfully used in analysis of five anthocyanins from *vitis unifera* using acetonitrile, water and formic acid as solvent system in the gradient mode, while detection was performed simultaneously at 525, 330, 310 and 280 nm. (Hebrero *et al*, 1988). Similarly three isoflavones, genistein, formononetin and biochanin A, were identified from *Ononis spinosa* L. (Pietta and Ceva, 1990) with the help of HPLC-PDA on a C-8 Aquapore RP

300 column using 2-propanol-tetrahydrofuran-water (28:2:70) as the solvent system in isocratic mode. Revilla *et al* (1991) has reported the isolation and identification of catechins and proanthocyanidins from grape seeds by HPLC- PDA. The isolation was carried out on C-18 SEP-PAK column while using 10% acetic acid (solvent A) and water (solvent B) at a flow rate of 0.8 ml/min. Isolation and identification of eight flavonol glycosides from *Althaea officinales* (Dzido *et al*, 1991) has also been reported with the help of HPLC-PDA on Li Chrosorb RP-18 (250 x 4.6 mm I.D) column using H_2O-MeOH or acetonitrile as solvent system. Using the same technique Pietta *et al* (1991) analyzed naringenin-4'-glucoside, kaempferol-3-glucoside and 4, 2', 4', 6'-tetrahydroxy chalcone-2'-glucoside from *Helichrysum italicum*. Sabatier *et al* (1992) reported the isolation and identification of chrysin, quercetin, tectochrysin and kaempferol in honey by HPLC-PDA technique using orthophosphoric acid (solvent A) and acetonitrile (solvent B) in linear gradient elution. Rigaud *et al* (1993) successfully applied HPLC-PDA technique to the analysis of procyanidin extract from Cacao beans and grape seeds using normal phase silica column and a gradient elution of CH_2Cl_2-MeOH-HCO_2H-H_2O. Guedon and Pasquier (1994) isolated 7-O-rutinside, hesperidin and rosmarinic acid from *Piperita folium* by employing similar HPLC-PDA conditions as applied by Rigaud *et al* (1993).

Capillary Electrophoresis (CE) is a new tool in the separation science which provides high separation efficiency. This technique was first described by Hjerten in 1967 but the initial demonstration of the power of capillary zone electrophoresis was done by Jorgenson (1981) starting around 1979. Since the separation takes place in an aqueous medium, the use of CE has been focussed on the separation of proteins, amino acids and polyphenols etc. Terabe *et al* (1984) developed the technique of micellar electrokinetic capillary chromatography (MECC) which has further widened the applications of CE to include separation of natural substances. Delgado *et al* (1994) has reported the isolation and

identification of fourteen flavonoids from honey with the help of CE by using 200 mM boric acid/50 M SDS pH 8.5 as electrophoretic solution and 20.9 KV as average voltage.

Isolation and purification of natural products is generally followed by their structural analysis. In addition to the wide range of separation techniques cited above, a number of identification techniques are available to the phytochemists which mainly include spectroscopic methods.

Ultraviolet spectrophotometry (UV) is one and probably the most useful technique for flavonoid identification because of two reasons. Firstly only a very small amount of pure flavonoid compound is needed. Secondly sufficient amount of structural informations are gained from a UV spectrum which are further enhanced by the use of specific shift reagents which react with the functional groups on the flavonoid nucleus. Addition of each of these shift reagents to an alcoholic solution of flavonoid induces significant shift in UV spectrum and help to a great deal in determining the flavonoid class as well as the nature and position of most of the substituents on the flavonoid nucleus. A number of reviews on UV-Visible spectrophotometry have appeared in the past e,g Jurd (1962) Mabry *et al* (1970), Markham (1982) and Voirin (1983). The most comprehensive being those of Jurd (1962) and Mabry *et al* (1970). The article by Jurd gives detailed references of the spectroscopic work on flavonoids up to 1960. The book of Mabry *et al* (1970) provides a detailed catalogue of the UV spectra of 175 flavonoids. UV-Visible spectrophotometry still remains the prime and inevitable tool of flavonoid identification. This is evident by the number of reports appearing from all parts of the globe e.g. Rao *et al* (1993) has reported the use of this technique for identification of two new flavanones, spinoflavanones A & B isolated from *Tephrosia spinosa*. UV spectrophotometry has also been used in establishing the structure of rare dihydroquercetin 3-O-ß-D-xyloside and caryatin 7-O-ß-D-glucoside isolated from

Eucryphia glutinosa (Boza *et al*, 1993). Liu *et al* (1993) have used UV to establish the structure of a new flavone triglycoside, isolated from *Baccharis thesioides*. Structures of two flavonols, quercetin 3-O-α-L-arabinofuranoside and quercetin 3-O-α-L-arabiopyranoside have been established by UV spectrophotometric analysis (Pistelli *et al*, 1993).

Infrared (IR) spectrophotometry of flavonoids is not as advanced as other spectroscopic techniques. This method is however valueable for identification of flavonoids which are complex because each flavonoid has its own characteristic spectrum. Nia *et al* (1992) have reported the absorption bands of two new flavonoids at 3425 (chelated hydroxyl), 1630 (conjugated carboyl), 1610 (C=C) and 1360 cm^{-1} (gemdimethyl). An other phenolic compound isolated from *Epimedium acuminatum* showed a chelate carbonyl absorption at 1640 cm^{-1}, while aromatic nature of the compound was established due to the occurance of absorption bands at 1620 and 1510 cm^{-1} (Liang *et al*, 1993).

Mass Spectrometry (MS) has been successfully employed for the structural determination of flavonoids. The main advantage of MS over most other spectroscopic techniques is that only very small quantities (less than 1.0 mg) of the flavonoid compound is required for analysis. However the main disadvantage of this technique is the requirement of sufficiently volatile flavonoid compounds at the prob temperature. In such cases mostly polar flavonoids have to be derivatized to render them sufficiently volatile for MS analysis.

Electron Impact Mass Spectrometry (EIMS) has been applied for the structural determination of both flavonoid aglycones and glycosides. Structures of four new chalcones, isolated from *Angelica keiskei* were established by using EIMS (Baba *et al*, 1990). After permethylation Budzianowski and Pakulski (1991) determined the structre of

two C and O-glycosylflavones by EIMS which were isolated from *Srellaria media.* EIMS. Four new flavonid glycosides were isolated from *Arnica. montana* and their molecular structure was determined by means of EIMS after perdeuteromethylation (Merfort and Wendisch, 1992).

Field Desorption Mass Spectrometry (FDMS) has also been successfully applied to a number of studies on phenoilc methyl ether derivatives. Structure of quercetin 3-glucoside-2"-gallate, a new flavonoid isolated from *Polygonum nodosum*, was determined by FDMS (Isobe *at al*, 1979). Yoshida *et al* (1987) were able to established the structure of an acylated flavonol glucoside, isolated from *Allium tuberosum*, by means of FDMS.

Fast Atomic Bombardment Mass Spectrometry (FABMS) is currently the method of choice for mass determination of the flavonoids. The molecular weights and sugar sequences of four flavonol glucosides, isolated from *Moghania faginea*, were determined with the help of FABMS (Soicke *et al*, 1990). The FABMS technique was efficiently used to determine the structure of a new isoflavone glycoside (auriculatin 4'-O-glucoside) isolated from the stem bark of *Erythrina eriotricha* (Nkengfack *et al*, 1991). Redl *et al* (1993) isolated two chalcone glycosides from *Bidens campylotheca* and determined their molecular weight as well as sugar linkage with the help of FABMS. Structure of two novel flavonoids, obtained from rhizomes of *Selligguea feei*, was established by using FABMS technique (Baek *et al*, 1994)

Thermo Spray Mass Spectrometry (TSPMS) ia a soft ionization technique that has recently been used for the structure determination of flavonoid glycosides. TSPMS mainly forms adduct ions such as $[M+H]^+$ with molecules. These adduct molecular ions allow a rapid determination of the molecular mass of a component directly after its elution from LC

column. The use of TSPMS has already provided on line molecular mass information in the analysis of polyphenols and polyphenol glycosides (Schroeder and Mefort, 1991). Wolfender and Hostettmann (1993) have employed TSPMS technique for the structure determination of flavonoids, isolated from different *Chironia* species.

Nuclear Magnetic Resonance Spectrometry (NMR) is another useful identification technique and in the word of Mabry *et al* (1964) the structure of flavonoids can be assigned almost solely on the basis of their NMR spectrum. Proton magnetic resonance (^{1}HNMR) spectroscopy has been widely employed for flavonoid structure analysis. Until 1964, ^{1}H NMR studies were limited to relatively non-polar flavonoids such as aceylated or methylated flavonoids which are soluble in deuterochloroform or carbontetra chloride (Massicot *et al*, 1962 and Schwarz, 1964). Batterham *et al* (1964) introduced hexadeuterodimethylsulfoxide (DMSO-d_6) as the solvent for more polar flavonoids. The ^{1}H NMR spectrum appears predominantly in the range of 0-10 ppm down field than the reference signal of trimethylsilane (TMS). Since only protons produce signals in this range so the type of protons present in the flavonoid compound can thus be determined. It also helps to define oxygenation pattern (in all three rings), the determination of the number and position of methoxy groups and distinguish clearly isoflavones, flavanones, dihydroflavanol, flavones and flavonols from each other. Further more it aids in the determination of the number of sugars present, their linkage (α or ß) and the detection of side chain such as C-linked CH_3. Application and significance of ^{1}H NMR in solving the problems of structural elucidation of flavonoids, has been reported by a number of workers (Imperato, 1992, Malan, 1993, Tahara *et al*, 1993, Iinuma *et al*, 1993 and Tanee *et al*, 1994).

^{13}C NMR spectroscopy has emerged as one of the most powerful and indispensable technique for the study of molecular structure and dynamics in chemistry (Bloch and Burcell, 1946). The first report dealing with ^{13}C NMR study of flavonoid compounds appeared in 1974 (Buddurus and Bauer, 1987). Since then, large number of publications reporting ^{13}C NMR data on flavonoids have been published. Subsequently ^{13}C NMR spectroscopy of flavonoids has been the subject of several reviews (Joseph-Nathan *et al*, 1974, Chari *et al*, 1977, Sim, 1978, Chri *et al*, 1979, Agrawal and Rastogi, 1981, Markham and Chari, 1982). Several NMR techniques are known for establishing the number of directly bonded hydrogen atoms to individual carbon. Among these, the classical technique is single frequency off-resonance decoupling (SFORD), and several new techniques such as spin-echo fourier transform (SEFT), attached proton test (APT), selective population transfer (SPT), insensitive nuclei enhancement by polarization transfer (INEPT) and distortionless nuclei enhancement by polarization transfer (DEPT) have been employed in which signal phases and signal intensities depend on multiplicity.

Along with the 1D NMR techniques, 2D NMR techniques such as homonuclear proton COSY (^{1}H, ^{1}H-COSY) (Shirataki *et al*, 1990), heteronuclear carbon COSY (^{1}H, ^{13}C-COSY) (Markham *et al*, 1992), heteronuclear multiple quantum coherence (HMBC) (Martin and Crouch, 1991), heteronuclear multiple-bond connectivity (HMQC) (Martin and Crouch, 1991) and homonuclear Hartmann-Hann (HOHAHA) (Kondo *et al*, 1991) spectroscopy are also being used to solve the structural elucidation problems of complex flavonoid compounds.

In the constant efforts to improve the effeciency of Western medicine, researchers are increasingly turning their attention to folk medicine for new drugs. The medical practices of non-Western culture has contributed a lot to establish a scientific basis

for the success of these remedies. The development in this respect is being made beginning with an anthropological approach in order to appreciate how and why a particular medical practice became established and then to explore the chemical and biological reasons for the success of this practice. In broad sense botanical survey has become a predominant factor which led to the search for the active ingredients in a specific medicinal plant. The greater emphasis in this respect is being given to the isolation and identification of the chemical constituents of the plants.

Herbal medicine is now practiced worldwide and has been recognized by World Health Organization (WHO) as an essential building block for primary health care (Onayade *et al*, 1990, Mossa *et al*, 1991, Blesken, 1992). Consequently there are numerous herbal health store, even in Europe, where a wide range of mainly herbal medicines from various parts of the world are sold over the country.

South East Asia is rich in medicinal plants, several species of them are found in Pakistan which are the sources of active principles. In the Indo-pak subcontinent, records of the indigenous system of medicine known as Ayur-Veda, go back to 700 B. C. Some of the major classes of medicinal significance contain FLAVONOIDS which are responsible for their therapeutic properties. The following paragraphs elaborate thie biological, economical and industrial importance of these natural products.

Sigmodin (I), a permethylated flavanone and Sigmodin (II) isolated from *Erythria sigmodea* bark were assyed against *Staphylococcus aureus*. Sigmosdin (I) exhibited antibacterial activity with minimum inhibition concentration (MIC) 20 μg/ml as compared to 0.06 μg/ml for ampicillin (Fomum *et al*, 1986). Mori *et al* (1987) and Nishino *et al* (1987) observed the effect of a number of flavonoid aglycones against different bacterial

strains as test organisms. Five flavonols (kaempferol, quercetin, myricetin, morin and galangin) possesed antibacterial activity. Isorhamnitin 3-rutinoside, hispidulin and 5, 7, 4'-trihydroxy 6, 3'-dimethoxyflavone, isolated from *Artimisia sublessingiana*, were tested in vitro against gram negative and gram positive bacteria, dermatophytic fungi and other microorganisms. 5, 7, 4'-trihydroxy 6, 3'-dimethoxyflavone suppressed growth of *Trichophyton gypseum* at MIC 6.1 ug/ml (Manadilova *et al*, 1987). Ivancheva *et al* (1992) studied the effect of flavonoid extracts, obtained from *Geranium macrorrhizum* and *G. sanguineum*, for in vitro inhibition of the growth of some gram-negative bacteria (*Klebsiella pneumoniae*, *Escherichia coli*, *Pseudomonas aeruginosa*) , Gram-positive bacterium (*Staphylococcus aureus*), and fungus (*Candida alhicans*). Some *geranium* extracts caused a strong increase of the survival rate in an infection with *K. pneumoniae* in mice. 5, 6, 7-trisubstituted flavones and isorhamnitin 3-O-ß-D-robonoside, isolated from *Gomphrena martiana* and *G. boliviana* were tested against 20 microorganisms, including gram-positive bacteria, an acid-fast bacterium, a fungus and two yeasts, which showed high activity against gram-positive and gram-negative bacteria at MIC 75 and 50 µg/ml respectively (Pomilio *et al*, 1992).

4'-hydroxy 3-methoxyflavones are natural compounds with known antiviral activity against picorna virusses, such as poliomyelitis and rhino virusses. The analogs of 4'-hydroxy 3-methoxyflavone were prepared and was observed that substitution at position 5 and polysubstituted A-ring appeared to be essential for high activity. The most interesting compound was 4', 7-dihydroxy 3-methoxy 5, 6-dimethoxyflavone possessing in vitro TI_{99} value (therapeutic index) of 1000 and 200 against poliovirus type I and rhino virus type 15 (De Meyer *et al*, 1991). Ono *et al* (1991) studied the effect of isoflavones on human immunodeficiency virus and human T. cell leukimia. Luteolin at 10 µg/ml inhibited 88.7% reverse transcriptase of murine leukemia virus. The lyophilized infusion from flowers of

Verbascum thapsiforme (FVI) showed antiviral activity in vitro studies against Flow plague virus, several influenza A strains, influenza B strains as well as Herpes simplex virus. FVI has shown virucidal activity on H. simplex virus at 300 μg/ml. Phytochemical investigations of FVI have shown the presence of flavonoids (Zgorniak *et al*, 1991). Isosuctellarein (5, 7, 8, 4'-tetrahydroxyflavone) from the leaves of *Scutellaria baicalensis* inhibited the replication of influenza virus A/WSN/33 in Madin Darby bovine kidney cells with 50% virus inhibitory dose at 16 nmol/well and influenza virus A/PR/34 in the allantoic sac of embryonated egg with little toxic effects. Similarly isoscutellarein-8-methyl ether (F 36) prevented proliferation of mouse adapted influenza virus A/PR/8/34 in mouse lung by the intranasal (0.5 mg/kg) and intraperitoneal (4 mg/kg) administration, and it was more potent than the known anti-influenza virus substances, amantadine. Intranasal administration of F 36 (0.5 mg/kg) also protected mice against a lethal influenza virus A/PR/8/34 infection (Nagai *et al*, 1992).

A series of isoflavanes , isoflavonones, pterocarpans, isoflavones and coumestan, obtained from five *Phaslus* species, were tested for their fungitoxicity against *Aspergillus niger* and *Caladosporium cacuerium*. A high level of lipophilicity and presence of ≥1 phenolic function appear important to isoflavonoid fungitoxicity (Stoessel, 1985). Murai and Tsushita (1987) studied the effect of methylated catechol on *Pyricularia aryzae* and *Cochlibolus miyabeanus*. A highly fungicidal effect was observed at 100 ppm and 500 ppm concentration respectively. Apigenin 7-O-glucoside, echinacin I & II were isolated from *Echinops echinatus*. The two derivatives, apigenin 5-4'-dimethyl ether and echinacin permethyl ether were assayed against germination of conidia of *Alternaria tenvissima* , which is responsible for leaf blight disease in pigeon pea. These compounds showed antifungal effect at the concentration of 25-150 μg/ml (Singh *et al*, 1988). Four flavones, three flavanones and catechin were tested for fungicidal activity in malt extract both against

5 storage isolates of *Aspergillus*. Unsubstituted flavones and flavoanones were highly active while highly hydroxylated flavonoids possesed only weak activity (Weidenboerner *et al*, 1990).

Liu *et al* (1985) isolated jaceosidin and hispidulin from *Saussurea involucrata* and tested them as antitumor agents against mouse S180 and ascites hepatoma cell culture. The inhibition was higher in ascites hepatoma than in S180 cells. The 50% inhibitory concentration of jaceosidin and hispidulin on DNA synthesis in ascites hepatoms cell was 70.8 and 116 μg/ml. A novel plant flavonoid, prenylated apigenin glycoside, was shown to have cytotoxic effects on six cancer cell lines. IC_{50} on the cell tested ranged from 2.8 to 7.5 g/ml (Li *et al*, 1990). Seven flavans, three flavones and two biflavans, isolated from *Muntingia calabura* roots showed a considerable cytotoxicity against various murine and human solid tumors in vitro and in vivo (Kaneda *et al*, 1991). Twelve flavonoids, isolated from *Eriodictyon californicum*, inhibited metabolism of the carcinogen benzo[a]pyrene by hamster embryo cells in tissue culture. Four active flavones, cirsimaritin, chrysoeriol, hispidulin and chrysin showed remarkable inhibition of benzo[a]pyrene metabolism at a concentration of only 10 μg/ml (Liu *et al*, 1992).

Quercetin, myricetin and luteolin, were tested for their anti-ulcer activity in immobilized mice. All of these showed a significant effect against ulcer. Luteolin prolonged the relaxant effect of adernalin in the isolated rabbit intestine . Free OH-groups at 3' and 4' positions, as well as nonsubstituted OH-group at 3-position, appeared to be associated with antiulcer and adernalin-enhancing activity (Barnaulov *et al*, 1985). The similar effect of flavonoids have been reported by Martin *et al* (1988). Flavonoids such as luteolin-7-glycoside and genistine isolated from *Genista rumelica* were tested in rats with gastric ulcer induced by pyloric ligature, reserpine, phenylbutazone and serotonin. The mixture of the

above flavonoids + linseed gel + antacid exhibited a good antiulcer effect and low toxicity (Rainova *et al*, 1988). The flavonoid extract of *Bidens campylotheac*, consist of okanin-4-methoxy-4'-O-(6"-O-acetyl-glucoside), okanin-4-methoxy-4'-O-glucoside and okanin-4-methoxy-4'-O-primveroside, has been used to cure sore throat, asthma and stomach disorder (Redl *et al*, 1993).

Middleton *et al* (1987) studied fifteen selected citrus flavonoids presenting several different classes for the effect on experimentally induced histamine release by human basophils and chemoattractant peptide induced ß-glucuronidase human neutrophils. A number of flavonoids proved to be active inhibitors of basophills histamine release and neutrophil ß-glucuronidase. Five polymethoxy flavones, isolated from orange peel showed marked inhibitory activities in passive cutaneous anaphlexis reaction test (Chen *et al* , 1986).

Khalid *et al* (1986) studied 21 flavonoids, isolated from 9 medicinal plants used in traditional medicine in Sudan and other African countries, in vitro for their antimalarial activity against *plasmodium falciparum*. Quercetin, obtained from *Diosma pilosa* was found to have same activity as the commercially available antimalarial drugs. Similarly a flavonoid extract of *Uvaria dependens* showed significant antimalarial activity (Nkunya *et al*, 1993).

Intra venus (i.v.) injection of the total flavonoids of *Rosa dayvrica* fruit decreased blood pressure and brain vascular resistance in mice, rats and rabbits. In isolated guinea pig and rabbit hearts, the flavonoids increased coronary flow and decreased heart rate and contraction. The i. v. LD_{50} of flavonoids in mice was 956 mg/kg. (Han and Han, 1987). The acute hemodynamic effect of two flavonoids with different electrophysiological

characteristics, rhoifolin and vitexin, were studied in anesthetized dogs. Rohoifolin and vitexin decreased the mean aortic pressure, arterial and pulmonary capillary pressure and heart rate (Occhiuto *et al*, 1990).

Chrysin, morin and rutin were examined for their analgesic activity by the acetic acid writhing test in mice. All of these flavonoids showed analgesic effect (Sambantham *et al*, 1985). Flavones and flavones glucosides derivatives were tested for their analgesic activity in mice employing acetic acid-induced writhing and tail immersion methods. All the tested compounds exhibited significant analgesic activity with varying potencies (Thiugnanasambantham *et al*, 1990). Flowers of *Carthamus tinctorius* containing, 6-hydroxy kaempferol, kaempferol 3-glucoside, quercimertin and kaempferol 3-rutinoside are used in the treatment of utrine congestion, cardiovascular diseases and as analgesic (Kim *et al*, 1992). Cardenas *et al* (1993) isolated kaempferol 3-O-α-L-rhamnosyl (1-6)-ß-D-glucoside and kaempferol 3-O-ß-D-glucoside from the leaves of *Hedyosmum bonplandianum* which exhibited analgesic activity in mice.

Skakum *et al* (1985) studied tetracycline (0.5 g/kg/day orally for five days) which inhibited hepatic bile secretion, increased hepatic lipid peroxidation and alternation in blood enzyme level which were all indicative of hepatotoxic action. The three biflavonoid preparations, flamin, flacumin and transflon exhibited protective effect against tetracycline hepatotoxicity. The flavonoid drugs, Legalon, Silibor, Flamin and Flacumin, have also been reported much potent against tetracycline toxicity (Skakum, *et al*, 1988). Ethanolic extract of *Cochlospermum tinctorium*, containing flavonoids, showed significant hepatoprotective activity (Diallo, *et al*, 1992).

The antioxidant and radical scavenging activities of a large number of polyphenolic compounds isolated from medicinal palnts were studied in various experimental systems: stabilization of ascorbic acid, inhibition of lipid peroxidation in rat liver mitochondria and in ocular lens, effects on arachidonic acid metabolism and inhibition of cytotoxicity in primary cultured hepatocytes. (-)-Epigallocatechin gallate and pentagalloylglucose showed a strong inhibitory effect against tumor which may be related to their radical scavenging activity.

The flavonoids, isolated from *Thymus vulgaris* were tested for their antioxidant properties. The antioxidant activity of flavones were greater than α-tocopherol (Miura and Nakatani, 1989). Five flavonoid preparations were tested for antioxidant activity by measuring their ability to scavenge O_2 produced in a model system and to inhibit lipid peroxidation by rat liver microsome in vitro. For the O_2 scavenging system, the decreasing order of potency of the preparation was (a) *Vitis vinifera* procyanidol oligomers, (b) *Cupressus sempervirens* procyanidol, (c) *Vaccinium myrtillus* anthocyanins, (d) *Ribes nigrum* anthocyanins, and (e) *Vitis vinifera* anthocyanins. For inhibition of lipid peroxydation, the order of decreasing potency was a, b, c, e, d (Meunier *et al*, 1989). The effectiveness of α-tocopherol, two flavonoids (quercetin and rutinic acid) and a cinnamic acid (chlorogenic acid) as inhibitors of peroxidase were evaluated. Four concentrations (125, 250, 500, and 1000 mg of each compound) were used. Crude extracts of tomayoes, carrots and egg plant were the sources of enzyme activity. Peroxidase activity of carrots and egg plant was inhibited more than of tomato by all antioxidants. Chiorogenic acid was most effective, followed by quercetin and rutinic acid (Hemeda and Klein, 1990).

The significance of flavonoids as food preservative and to improve the quality of food is well known. Turbidity of fruit juice is prevented by mixing with H_2O-soluble

flavonoids. Sucrose 126, citric acid 1.5, grape juice 100 parts were mixed with 1.0% aqueous solution containing 10% flavonoids I, mixed with H_2O to 1000 parts, sterilized at 85 ^{o}C, and left at room temperature for ten days showed no turbidity, where as there was sever turbidity without I (Nishimura *et al*, 1990). Anthraquinone type dyes in food are stabilized by the presence of flavonoids. The stability of cochineal red dye in a beverage was demonstrated by adding rutin. Eighteen other flavonoids are claimed as stabilizers (Nishimura *et al*, 1990). Coffee beverages with better and long lasting flavour contain H_2O-soluble flavonol glycosides. Ground roasted coffee bean (10kg) were extracted with H_2O containing 0.1% H_2O soluble flavonol glycosides at 100 C^0 to make 30 kg coffee beverage, which was mixed with 30 gm H_2O-soluble flavonol glycosides and preserved in a refrigerator for 1 week. The coffee had a better flavor before and after preservation than did a control (Soejima *et al*, 1990).

Dima (1952) studied the effect of quercetin on germination of seeds. It stimulated the germination by acting as auxin synergisis. Different flavonoids have shown their plant growth regulatory effect by retarding destruction of chlorophyll and proteins in bean leaves (Karanov and Kimenov, 1970, Naef, 1971).

Biological nitrogen fixation and its great importance in soil fertility is well known. In the process microorganisms either free living or in symbiosis with plant and found mainly in their root nodules, reduce nitrogen to ammonia. Flavonoid extracts of alfalfa variety *Hairy peruvian* (HP 32) seedling roots contain 77% higher concentration of luteolin than the roots of other species (HP). Luteolin is responsible for inducing nitrogen fixation in a bacterium *Rhizobium meliloti* (Van Nostrand, 1976).

Solar ultraviolet radiation has been known to cause the disease of Jonathan Spot in apples. Dean (1963) observed that flavones protect plants from harmful UV rays. Many plants combat attack by worms and insects by producing flavonoids which kill the adults or larvae (Rajaratnam and Hock, 1975).

The flavonoid compounds are useful genetic markers in the investigation of interspecific hybridization with in the genus. The best documented research on the leaf phenolics to investigate hybridization in *Beptisia* is by Alston and Turner (1963b) where chemical compounds are inherited additively in hybrids. Of the 125 flavonoids used by Alston and Turner, many are species-specific markers which enable them to find out a hybrid origin of plants which were not exactly intermediate between their parents, but were the result of introgression. Alston *et al* (1965) found a new quercetin glycoside in hybrids between the white flowered *Beptisia leucantha* and the yellow flowered *B. sphaerocarpa*. Hull and Nicholl (1982) pointed out hybridization between *Rumex aquaticus* and *R. obtusifolius* in Britian. Evidence of introgressive hybridization in R. acetocella from the 4 x into 6 x ploidy level has been found in Czech Socialist Republic and a part of lower Austria especially in the region of the well known migration rout through the Moravian Gate (Den-Nijs *et al*, 1982).

In view of the immense importance of the flavonoids as pharmacological and agricultural agents as well as chemotexonomic markers, the present work was undertaken to explore the flavonoid contents of some medicinal plants of Pakistan. For this purpose 20 plant species were collected and subjected to qualitative and quantitative analysis of their flavonoid compounds. At the same time global flavonoid extracts were tested for their anti-bacterial properties. Results revealed that amongst the 20 plant species under investigation *Rumex chalepensis* (Polygonaceae) and *Indigofera hebepetala* (Leguminosae) had the

larger number of flavonoids and significant % yields. Further more both these species showed pronounced antibacterial activities.

It has been shown that member of the family Polygonaceae are quite rich in flavonoids and related natural products. Yoshitama *et al* (1987) carried out the analysis of 9 Polygonum species and reported a number of anthocyanin glycosides. The presence of C-glycosyl flavonoids in *Muehlenbeckia platyclada* and *Polygonum amplexicaule* was reported by Kawasaki et al (1986) and Pathak and Manral (1987). Investigation of Rumex acetosa exhibited the presence of acylated C-glycosyl flavonoids (Kato and Morita, 1990). Some rare flavonoids such as methylenedioxyflavones and flavonols were found in *Polygonum minus* (Urones *et al*, 1990). Diversity of flavonoid compounds can be observed by the presence of flavonols in *Polygonum flaccidum* (Ahmad *et al*, 1991), flavonoid glycosides in *Fagopyrum cymosum* (Saxena and Samaiya, 1987), prenylated flavonoid glycosides in *Polygonum tinctorium* (Kohda *et al*, 1990), dihydrokaempferol glycosides in *Fagopyrum esculentum* (Samaiya and Saxena, 1989) and dihydroquercetin glycosides in *Polygonum orientale* (Zhang *et al*, 1990).

It is well known that the family Leguminosae is one of the most important family of angiosperm that contains flavonoids in almost each of its members even at the level of sub specie and varieties. Moreover presence of number of each class of flavonoids weather simple or complex have been reported from plants species belonging to the family Leguminosae (Jay *et al*, 1978a, 1978b, 1980). Presence of anthocyanins glycosides has been reported by Ishikura *et al* (1978) and Singh *et al* (1979). Markham *et al* (1989) and Katanaka *et al* (1989) have reported the presence of C-glycosyl flavonoids in *Abrus pecatorius* and *Cassia torosa* respectively. Some species of this family such as *Trigonella foenumgreacum*, *Crotalaria retusa* and *Lespedza davurica* have shown the presence of C and O-flavonoid glycosides (Adamska and Lutomski, 1971, Srinivasan and Subramanian

and Matsuzaki *et al*, 1990). The phytochemical analysis of *Puerasia tuberosa* and *Sarothamnos scoparius* resulted in the presence of acylated C-glycosyl flavonoids (Bhutani *et al*, 1969 and Prum-Bousquet *et al*, 1977). Flavone and flavonol glycosides are found in a number of leguminous plant species such as *Sesbanis rostrata* (Messens *et al*, 1989), *Thermopsis alternifolia* (Yuldashev *et al*, 1989), *Cassia alata* (Gupta and Singh, 1991), *Moghania faginea* (Soicke *et al*, 1990) and *Cassia biflora* (Ahmed *et al*, 1991).

It seemed evident that a detailed study of the two selected plant species i,e *Rumex chalepensis* and *Indigofera hebepetala*, belonging respectively to Polygonaceae and Leguminosae families may lead to the isolation and characterization of new and/ or rare flavonoid compounds probably having potential biological applications. In order to have a broader prospective two additional species of the same genera namely: *Rumex nepalensis* and *Indigofera cassiodes* were also chosen for the determination of their flavonoid contents.

The present dissertation consists of four chapters. In the first chapter the techniques of analysis used in this work are presented. The following two capters i, e chapter 2 and chapter 3 present and discuss respectively results obtained after the complete phytochemical analysis of Rumex (*R. chalepensis* and *R. nepalensis*) and Indigofera (*I. hebepetala* and *I. cassioides*) species. The last chapter is devoted to the qualitative and quantitative analysis of the global flavonoid extracts of 20 selected plant species as well as the anti-bacterial assays of the same flavonoid extracts. Results obtained after the anti-bacterial assays of the isolated and purified flavonoid compounds of *R. chalepensis* and *I. hebepetala* are also included in this chapter.

CHAPTER I

METHODS AND MATERIALS

CHAPTER 1

METHODS AND MATERIALS

CONTENTS:

Electron impact mass spectrometry (EIMS)

Fast atomic bombardment mass spectrometry (FABMS)

1.3.3.5 Nuclear magnetic resonance spectroscopy (NMR)

Proton resonance spectroscopy (^{1}H NMR)

C-13 resonance spectroscopy (^{13}C NMR)

Nuclear overhauser effect (NOE)

^{1}H, ^{1}H-COSY (Homonuclear) NMR

^{1}H, ^{13}C-COSY (Heteronuclear) NMR

1.3.3.6 Identification of sugars

1.1 PLANT MATERIAL

1.1.1 GENUS *RUMEX*

The genus *Rumex* is one of the genera belonging to the tribe *Rumiceae* of the *Polygonaceae* family. Polygonaceae is divided into thirty genera, comprising of about 800 species which are widely distributed in the world (Hooker, 1885). In Pakistan this family is represented by 12 genera and about 100 species (Bhopal and Chaudhri, 1977).

The genus Rumex consists of annual or perennial erect herbs with a woody suffrutescent base, or subshrubs, often stout. Ochreae +-tubular. Leaves alternate. Flowers polygamous, arranged in glomerules or fascicles. Perianth segments 6, in 2 whirls, the outer ones remaining small, the inner becoming enlarged and hardened in fruit. Stamens 6. Styles 3; stigmas penicillate. Nut triquetrous, included in the persistent perianth.

RUMEX CHALEPENSIS (Mill.)

It is an erect, perennial herb, ca. 26-85 cm high; internode 2-8 cm long, striate. Ochreae 0.7-1.5 cm long. Lower leaves petioled , lamina 5-20(25.5) x 2.5-6(-10) cm, ovate-oblong, obtuse, cordate at base; upper leaves similar through smaller, with turncate base. Glomerules 0.7-2 cm in diam, remotelysituated, lower ones leafy. Flowers 1.5-3 mm long; bract 1-2.5 mm long; pedicel 3-6 mm long, swollen just below the perianth, jointed. Outer perianth segments 1-1.5 mm long, oblong, inner 2-3 mm long, ovate. Stamens 6. Ovary trigonous; styles 3; stigma penicillate. Fruiting valves 4-6 x 2.5-5 mm, broadly cordate, + - acute, margin unequally 4-10 dentate, reticulately nerved. Nut ca. 3 mm long, triquetrous, brown. The flowering season of the plant is from March to June and it is found at an altitude of 300-4000 meter. In Pakistan, the plant is distributed in Punjab, N. W. F. P. , Gilgit agency and Azad Kashmir. For present investigations leaves of R. chalepensis were collected from Islamabad in April 1993. The leaves were dried under shade and a voucher

specimen (No. 66845) was preserved in the herbarium of Department of Biology, Quaid-i-Azam University, Islamabad.

RUMEX NEPALENSIS (Sprengel)

It is an erect, glabrous, perennial herb, ca. 30-70 cm high; internodes 2.5-9 cm long. Ochreae 1.5-3 cm long. Leaves petioled; lamina 5-17.5 x 2.5-11 cm, ovateo-blong, pbtuse, base cordate, margin sinuate. Glomerules 1.3-3 cm in diam; lower remote and leafy, upper contiguous and leafless. Flowers perianth segments ca. 1.5 mm long, linear-oblong, inner 2-2.5 mm long, ovate-oblong. Stamens 6. Ovary trigonous; styles 3; stigmas penicillate. Fruiting valves 3.5-5 x 2-3 mm, ovate-cordate, 12-20-deeply dentate, teeth with prominently hooked tips or apices. It is found at an altitude of 600-3500 meter and its flowering season is from May to August. In Pakistan it is distributed in Punjab, N.W.F.P., Gilgit Agency and Azad Kashmir. It was collected from Murree hills on 3rd June 1991. Leaves were dried under shade and a specimen (No. 148) was deposited in the herbarium of Department of Biology, Quaid-i-Azam University Islamabad.

1.1.2 GENUS *INDIGOFERA*

Genus *Indigofera* belongs to the sub family papilionatae of the family *Leguminosae* and comprises more than 300 species. It is widely distributed in tropics and subtropics of both new and old world, with center of distribution in tropical Africa. In Pakistan, this genus is represented by 24 species (Ali, 1977). The genus consists of shrubs and herbs, generally pubescent. Leaves pinnate or imparipinnate or palmately trifoliate or unifoliate or simple. Leaflets entire, stipule present. Calyx lobes or teeth are subequal or lower most longer. The flower's vexillum consists of sessile clamed. Wings are slightly cohering with the keel. Stamens are 9+1, diadelphous, anther uniform, terminating in a short, sharp and flexible point. Ovary have numerous or 1-2 ovules, style glabrous, stigma capitate. The fruit is linear or globose, terrete, 3-4 seeded or compressed and septate between the seeds. The seeds are globose to cylindrical, truncate compressed or quadrate.

INDIGOFERS HEBEPETALA (Benth. ex Baker)

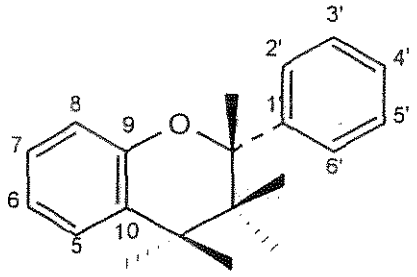
It is deciduous shrub and the branches are separsely pubescent when young. The leaves are about 10-25 cm long. the leaflets are 5-11, 2.5-10 cmlong. The inflorescence is about 10-25 cm long raceme. The calyx is about 2.5 mm long with pubescent short teeth. Vexillum is about 10-12 mm. The fruit is about 2.5-5.5 cm long, straight and glabrous. The flowering season of the plant is from May to September. In pakistan, the plant is distributed in Punjab, N.W.F.P. and Kashmir. For present investigation leaves of I. hebepetala were collected from Murree hills (Kashmir Point) on 30th September 1991. The leaves were dried under shade and a voucher specimen (No. 63662) was preserved in the herbarium of Department of Biology, Quaid-i-Azam University, Islamabad.

INDIGOFERA CASSIOIDES (Rottl. ex DC)

It is erect shrub, 10-15 cm tall, young branches pubescent. Leaf imparipinnately compound, c. 7-15 cm long; leaflets 11-2.4 cm long, c. 7-15 mm on both sides; stipules c. 2-4 mm long, deciduous. Inflorescencea 5-17 cm long raceme, covered with sterile scales at the base. Bract c. 2-10 mm long. Pedicel c. 1-2 mm long. Calyx cup c. 2-3 mm long, teeth subequal, c. 1-2 mm long. Corolla bright pink, fading to violet. Vexillum c. 1.2-1.8 cm long. Fruit 2.4-4.3 cm long, c. 4 mm broad, straight, glabrous, dehiscent, 8-12-seeded. The flowering season of the plant is from March to May. In pakistan, the plant is distributed in Punjab. For present investigation leaves of *I. cassioides* were collected from Pir sohawa (near Islamabad) on 12th August 1993. The leaves were dried under shade and a voucher specimen (No.160) was preserved in the herbarium of Department of Biology, Quaid-i-Azam University, Islamabad.

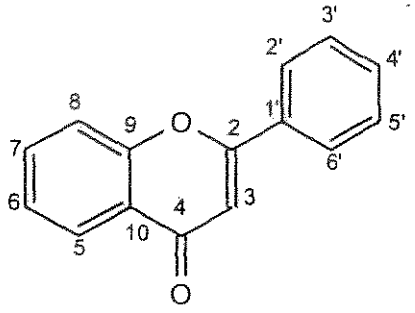
1.2 STRUCTURES OF MOST COMMON FLAVONOIDS

FLAVANS



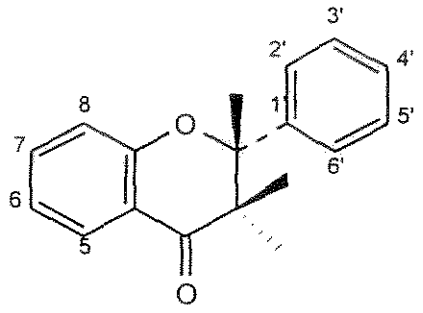
1.	Cassiaflavan	7, 4'-dihydroxy flavan
2.	Apigeniflavan	5, 7, 4'-trihydroxy flavan
3.	Luteoliflavan	5, 7, 3', 4'-tetrahydroxy flavan
4.	Tricetiflavan	5, 7, 3', 4', 5'-pentahydroxy flavan
5.	Distenin	3, 5, 7-trihydroxy flavan
6.	Afzelechin	3, 5, 7, 4;-tetrahydroxy flavan
7.	Catechin	3, 5, 7, 3', 4'-pentahydroxy flavan
8.	Gallocatechin	3, 5, 7, 3', 4', 5'-hexahydroxy flavan
9.	Fisetinidol	3, 7, 3', 4'-tetrahydroxy flavan
10.	Robinetinidol	3, 7, 3', 4',5'-pentahydroxy flavan

FLAVONES



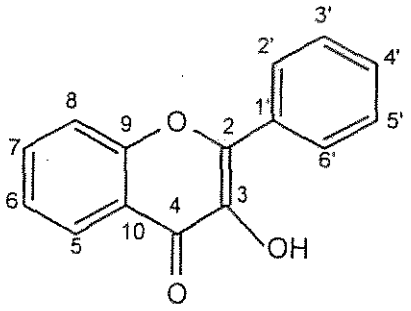
1.	Chrysin	5, 7-dihydroxy flavone
2.	Baicalein	5, 6, 7-trihydroxy flavone
3.	Apigenin	5, 7, 4'-trihydroxy flavone
4.	Acacetin	5, 7-dihydroxy 4'-methoxy flavone
5.	Scutellarien	5, 6, 7, 4'-tetrahydroxy flavone
6.	Hispidulin	5, 7, 4'-trihydroxy 6-methoxy flavone
7.	Luteolin	5, 7, 3', 4'-tetrahydroxy flavone
8.	Chrysoeriol	5, 7, 4'-trihydroxy 3'-methoxy flavone
9.	Diosmetin	5, 7, 3-trihydroxy 4'-methoxy flavone
10.	Tricetin	5, 7, 3', 4', 5'-pentahydroxy flavone

FLAVANONES OR DIHYDROFLAVONES



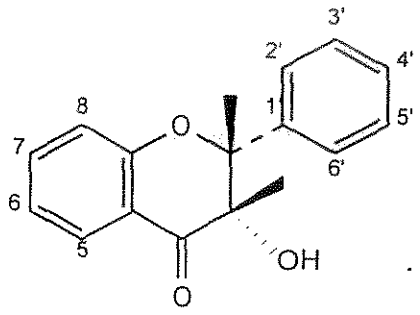
1.	Pinocembrin	5, 7-dihydroxy flavanone
2.	Pinostrobene	5-hydroxy-7-methoxy flavanone
3.	Alpinotin	7-hydrocxy-5-methoxy flavanone
4.	Liquiritigenin	7, 4'-dihydroxy flavanone
5.	Butin	7, 3', 4'-trihydroxy flavanone
6.	Plathymenin	6, 7, 3', 4'-tetrahydroxy flavanone
7.	Isokenin	7, 8, 3', 4'-tetrahydroxy flavanone
8.	Eriodictyol	5, 7, 3', 4'-tetrahydroxy flavanone
9.	Hesperetin	5, 7, 3'-trihydroxy-4'-methoxy flavanone
10.	Naringenin	5, 7, 4'-trihydroxy flavanone

FLAVONOL



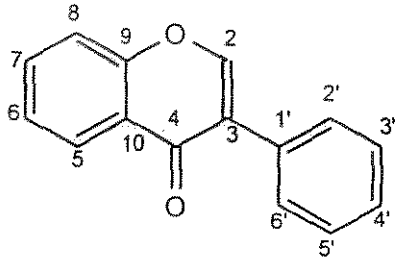
1.	Galangin	5, 7-dihydroxy flavonol
2.	Fisetin	7, 3', 4'-trihydroxy flavonol
3.	Kaempferol	5, 7, 4'-trihydroxy flavonol
4.	Kaempferide	5, 7-dihydroxy-4'-methoxy flavonol
5.	Robinetin	7, 3', 4', 5'-tetrahydroxy flavonol
6.	Herbacetin	5, 7, 8, 4'-tetrahydroxy flavonol
7.	Quercetin	5, 7, 3', 4'-tetrahydroxy flavonol
8.	Rhamnetin	5, 3', 4'-trihydroxy, 7-methoxy flavonol
9.	Isorhamnetin	7, 5, 4'-trihydroxy-3'-methoxy flavonol
10.	Muricetin	5, 7, 3', 4', 5'-pentahydroxy flavonol

FLAVANONOLS OR DIHYDROFLAVONOLS



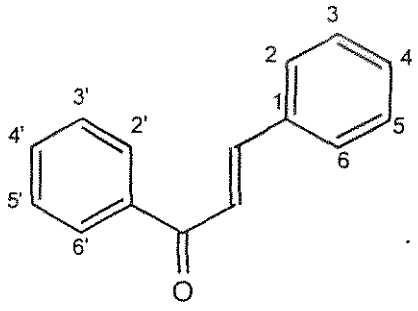
1.		7-hydroxy flavanonol
2.	Pinobanksin	5, 7-dihydroxy flavanonol
3.	Alpinone	5, 6-dihydroxy 7-methoxy flavanonol
4.	Garbenzol	7, 4'-dihydroxy flavanonol
5.	Fustin	7, 3', 4'-trihydroxy flavanonol
6.	Sepinol	7, 3', 5'-trihydroxy 4'-methoxy flavanonol
7.	Taxifolin	5, 7, 3', 4'-tetrahydroxy flavnonol
8.	Aromadendron	3, 5, 7, 4'-tetrahydroxy flavanonol
9.	Dihydrobinitin	7, 3', 4', 5'-tetrahydroxy flavnonol
10.	Dihydromyricetin	5, 7, 3', 4', 5'-pentahydroxy flavnonol

ISOFLAVONES



1.	Daidzein	7, 4'-dihydroxy isoflavone
2.	Formononetin	7-hydroxy-4'-methoxy isoflavone
3.		7, 4'-dimethoxy isoflavone
4.	Cabreuvin	7, 3', 4'-trimethoxy isoflavone
5.	Baptigenin	5, 7, 3', 4', 5'-pentahydroxy isoflavone
6.	Reutusine	7, 8-dihydroxy-4'-methoxy isoflavone
7.	Genistein	5, 7, 4'-trihydroxy isoflavone
8.	Biochanin-A	5, 7-dihydroxy-4'-methoxy isoflavone
9.	Prunetin	5, 4'-dihydroxy-7-methoxy isoflavone
10.	Orobol	5, 7, 3', 4'-tetrahydroxy soflavone

CHALCONE



1 2', 4'-Dihydroxy chalcone

2. 2', 4'-dihydroxy 5'-methoxy chalcone

3. 2', 6'-dihydroxy 4'-methoxy chalcone

4. 2', 4'-dihydroxy 6'-methoxy chalcone

5. 2', 4', 6'-trihydroxy chalcone

6. 2', 3', 4', 5', 6'-pentahydroxy chalcone

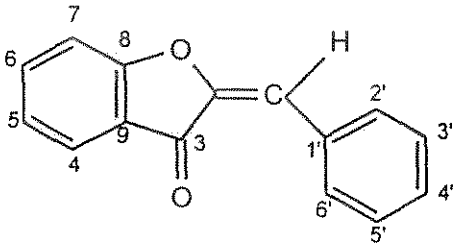
7. 2', 5'-dihydroxy 3', 4', 6'-trimethoxy chalcone

8. 4, 2', 4'-trihydroxy chalcone

9. 3, 4, 2', 4'-tetrahydroxy chalcone

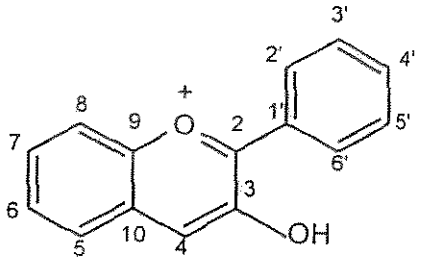
10. a, 3, 4, 2', 4'-pentahydroxy chalcone

AURONES



1.	Hispidol	6, 4'-dihydroxy aurone
2.	Sulfuretin	6, 3', 4'-trihydroxy aurone
3.	Carpusin	2, 6, 4'-trihydroxy 4-methoxy aurone
4.	Aureusidin	4, 6, 3', 4'-tetrahydroxy aurone
5.	Cernuoside	6, 3', 4'-trihydroxy-4-methoxy aurone
6.	Maritimetin	6, 7, 3', 4'-tetrahydroxy aurone
7.	Leptosidin	6, 3', 4'-trihydroxy-7-methoxy aurone
8.	Bracteatin	4, 6, 3', 4', 5'-petahydroxy aurone

ANTHOCYANIDINS



1.	Apigenidin	5, 7, 4'-trihydroxy anthocyanidin
2.	Luteolinidin	5, 7, 3', 4'-tetrahydroxy anthocyanidin
3.	Tricetinidin	5, 7, 3', 4', 5'-pentahydroxy anthocyanidin
4.	Pelargonidin	3, 5, 7, 4'-tetrahydroxy anthcyanidin
5.	Aurantinidin	3, 5, 6, 7, 4'-pentahydroxy anthocyanidin
6.	Cyanidin	3, 5, 7, 3', 4'-pentahydroxy anthocyanidin
7.	Peonidin	3, 5, 7, 4'-tetrahydroxy-3'-methoxy anthocyanidin
8.	Delphinidin	3, 5, 7, 3', 4', 5'-hexahydroxy anthocyanidin
9.	Petunidin	3, 5, 7, 4', 5'-pentahydroxy-3'-methoxy anthocyanidin
10.	Malvidin	3, 5, 7, 4'-tetrahydroxy-3', 5'-dimethoxy anthocyanidin

1.3 CHEMICAL ANALYSIS

1.3.1 EXTRACTION

Since the flavonoids occur virtually in all parts of a plant (roots, bark, leaf, fruit, flowers, sapwood, heart wood etc), the method of isolation therefore depends to some extent both on the source, material and the type of flavonoids being isolated. In cases where flavonoids occur in the surface oils or waxes they may be obtained simply by washing the surface with an appropriate solvent.

Generally extraction is a process in which the plant material is ground up or macerated prior to the extraction. The possible enzymic action during this early period of isolation that would lead to the hydrolysis of glycosides can be checked by plunging the plant material into boiling solvent or by rapid drying before extraction. Pre-drying of plant material generally appears to increase the yield of extraction. Solvents employed for extraction are selected according to the polarity of the flavonoids under study. The less polar solvents are particularly useful for the extraction of flavonoid aglycones while the more polar solvents are used if flavonoid glycosides are sought. The less polar aglycones such as isoflavones, flavanones and dihydroflavonols or flavones and flavonols which are highly methylated, are usually extracted with solvents such as benzene, chloroform and ether.

A pre-extraction with light petroleum or chloroform is frequently carried out to rid the plant material of sterols, carotenoids, chlorophyll etc. Flavonoid glycosides and more polar aglycones such as hydroxylated flavones, flavonols, biflavonoids, aurones and chalcones are generally isolated from the plant material by extraction with acetone, alcohol, water or a combination of these. Perhaps the most useful solvent for the extraction of these type of compounds is a 1:1 mixture of methanol and water. Boiling water has been found suitable for the extraction of polyglycosides. In recent years the use of fresh starting material has greatly been recommended as it is believed that fresh vegetation provides the

ideal plant material for flavonoid analysis. With fresh plant material it is recommended that after a selective sample has been set aside as a voucher specimen, the remainder be dried rapidly in an oven at 100 °C. The dried plant material can then either be stored in sealed bags for further use or ground to a fine powder for solvent extraction. It is often a wise precaution to extract a sample of undried plant material for a latter chromatographic check that the drying process has not altered the flavonoid composition.

Flavonoids can be separately isolated as glycosides and aglycones. Separate methods are employed for the extraction of each of these forms of flavonoids. The former are extracted directly from plant the material whereas the latter can be isolated after hydrolysing the plant tissues.

1.3.1.1 EXTRACTION OF FLAVONOID GLYCOSIDES

Pre-weighed, dried plant material is subjected to extraction in four steps;

1.	EtOH	(100 %)
2.	EtOH : H_2O	(90:10)
3.	EtOH : H_2O	(50:50)
4.	EtOH : H_2O	(50:50) + heating

At each step sufficient solvent was added to make a liquid slurry and the mixture was stirred overnight. After filtration all the extracts were combined and evaporated until all of the EtOH had been removed. The resultant aqueous extract was then cleared off low polarity contamination such as fats, terpenes, chlorophyll, xanthophyll etc. by extracting with chloroform in a separating funnel. The aqueous layers containing the bulk of flavonoid was extracted successively by ethyl acetate (EtOAc). Occasionally another extraction by *n*-butanol is also carried out.

The above procedure is suitable for the extraction of most of flavonoid glycosides but not for anthocyanins. For anthocyanins, fresh leaves or petals are not dried but crushed with MeOH containing 1% hydrochloric acid (HCl). Extraction is almost immediate as evidenced by the colour in solution. Chromatographic and spectroscopic analysis of the extract is carried out as soon as possible in order to minimize the risk of decomposition or glycosidic hydrolysis.

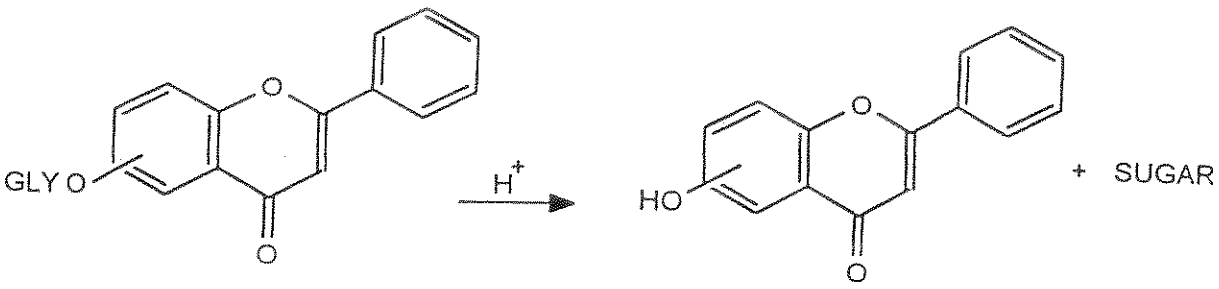
1.3.1.2 EXTRACTION OF FLAVONOID AGLYCONES

ACID HYDROLYSIS

For the extraction of flavonoid aglycones a small amount of dried plant material (in portion of 3.0 gm) was treated with 2N HCl and heated in a water bath at 100 °C for one hour. By this treatment normally glycosides are converted to aglycones, anthocyanins to anthocyanidins where as the C-glycosides remain unaffected (scheme.3). After cooling, the aqueous phase was extracted with diethyl ether (Et_2O) for the removal of the flavonoid aglycones. A second series of extraction by n-butanol quantitatively removed the anthocyanidins.

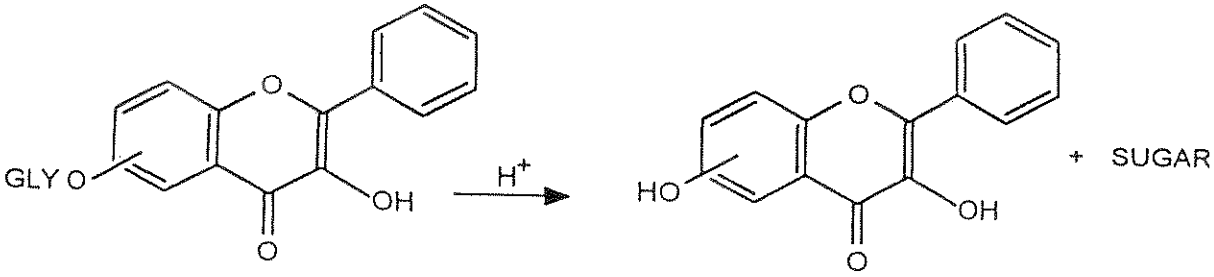
After the completion of acid hydrolysis it is possible to estimate quantitatively the total amount of flavonoids and anthocyanidins present in the plant tissue. The method adopted for quantative estimation was that described by Bate-Smith (1949) and afterward modified by Lebreton (1967). The Et_2O extract so obtained was allowed to evaporat to dryness at room temperature. The dry residue was taken up in a known volume (10 ml) of MeOH and was diluted. Its optical density (O.D) was measured between 350-450 nm by a UV spectrophotometer. Amount in mg/gm of flavonoid aglycone per gram of plant material was calculated by the following equation.

$$\text{Amount of flavonoid aglycone mg/gm} = 1.3 \times 10^{-2} \times \text{O.D. Vxd/W}$$



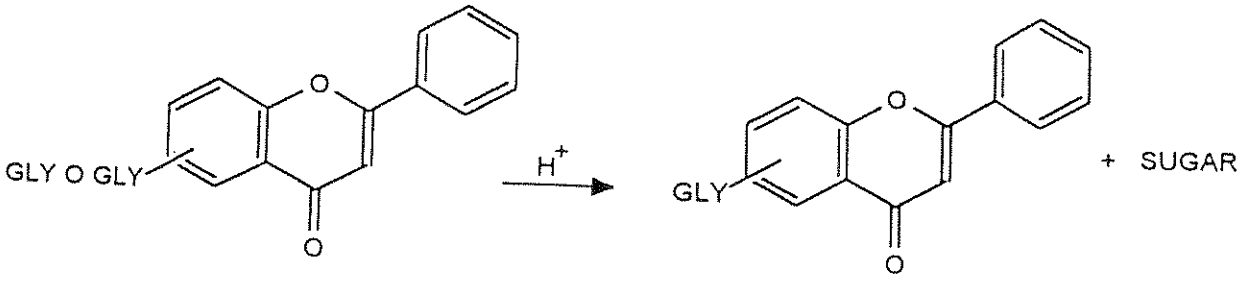
Flavone-O-glycoside

Flavone



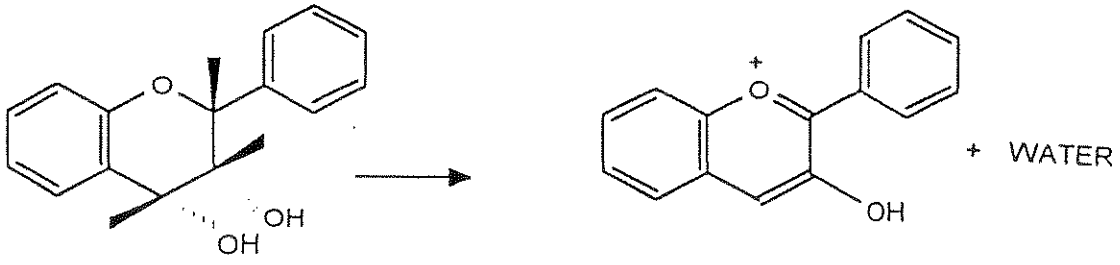
Flavonol-O-glycoside

Flavonol



Flavone-C-glycoside

Flavone-C-glycoside



Leucoanthocyanidin

Anthocyanidin

Scheme-3: Acid hydrolysis of flavonoids.

where

V = Original volume of the extract.

d = Dilution

W = Weight of plant hydrolysed

For the quantitative analysis of anthocyanidins, the n-BuOH extract was adjusted to a known volume by repeated washing with 2N HCl. The n-BuOH extract was appropriately diluted and its optocal density was measured between 500-600 nm by a UV spectrophotometer. Amount in mg/gm of anthocyanidins in the extract can be calculated by the following equation.

$$\text{Amount of anthocyanidins mg/gm} = 5.2 \times 10^{-2}(\text{O.D}) \times \text{Vxd/W}\ .$$

Acid hydrolysis was also used to determine the nature of the aglycone and the sugar(s) of our purified flavonoid glycosides.

Alkaline and enzymatic hydrolysis are two other methods to determine the nature of the aglycone and the sugar(s) of pure flavonoid glycosides.

ALKALINE HYDROLYSIS

Alkaline hydrolysis is rarely used with flavonoid glycosides. However it does offer a method for selectively removing a sugar (or sugars) from the 7-(or 4') hydroxyl group in the presence of one attached to the 3-OH. Care must be taken to exclude air from the reaction mixture as many flavonoid oxidized in the presence of alkali and oxygen.

To determine the presence of organic acid linkage to a sugar (acyl linkage) or flavonoid nucleus, purified flavonoid glycosides were subjected to alkaline hydrolysis. For this purpose ca 5 mg of the glycoside was dissolved in 10 ml of 0.5% KOH and heated for half an hour on a steam bath in N_2 atmosphere. The solution was then neutralized with 2M

HCl and chromatographed on paper in 15% AcOH. The developed chromatograms were revealed under UV and UV/NH_3 for the detection of organic acids.

ENZYMIC HYDROLYSIS

Enzymic hydrolysis is a useful method for establishing the nature and type of the sugar linkage (i.e. α or ß) attached to the flavonoid aglycone. Some of the commonly available enzymes employed for enzymic hydrolysis of various glycosides included:

1.	ß-Glucosidase :	hydrolyses ß-D-glucosides and xylosides.
2.	ß-Galactosidase:	hydrolyses ß-D-galactosides.
3.	ß-Glucuronidase:	hydrolyses ß-D-glucuronides.
4.	Anthocyanase:	hydrolyses Anthocyanidin glycosides.
5.	Pectinase:	hydrolyses ß-D-glucosides and α-L-rhamnoside.
6.	Rhamnodiastase:	removes many oligosaccharides intact from glycosides.
7.	Naringinase:	hydrolyses naringenin 7-O-neohesperidoside to naringenin.
8.	Takadiastase:	hydrolyses α-L-rhamnoside.

For enzymic hydrolysis a small amount of the purified flavonoid glycoside (0.5 mg) was taken in 50 ml round bottom flask and dissolved in distilled water (0.5 ml, pH 4-5). Solution was kept at 18-37 °C for a period of 60 min. to 48 hours (depending upon the nature of the glycoside). After the incubation, water was evaporated to dryness under reduced pressure and the residue was again dissolved in distilled water and transfered to a 50 ml separating funnel. The aglycone was extracted by EtOAc while the sugar remained in the aqueous phase.

Acylated sugars and C-glycosides are resistant to enzymic hydrolysis. It is often recommended that enzymic hydrolysis should be carried out in acidic buffers of about pH 5, distilled water is generally satisfactory with flavonoid glycosides and has the advantage that it does not complicate the work-up.

1.3.2 SEPARATION AND PURIFICATION TECHNIQUES

The separation and purification of plant constituents is normally carried out using one or another or a combination of chromatographic techniques such as paper chromatography (PC), thin layer chromatography (TLC) and column chromatography (CC). With in the last decade, numerous new chromatographic techniques have become available to the flavonoid chemists. They not only reduce the separation time, but simplify the isolation of previously unknown constituents from crude plant extracts. High performance liquid chromatography (HPLC), vacuum liquid chromatography (VLC) and high performance liquid chromatography coupled with photo diode array (HPLC-PDA) are the major innovations. However there is no single method of separation capable of solving all isolation problems. Classical techniques such as PC, TLC and CC are still very useful.

The constant need in natural product chemistry to separate both large and small quantities of mixtures efficiently, rapidly and inexpensively is unfortunately seldom satisfied by the use of one ideal chromatographic technique. The long way from a crude extract to a pure constituent often requires several separation steps involving a selection of techniques. In the following some of the most common chromatographic techniques employed for flavonoid separation and purification are described.

1.3.2.1 CONVENTIONAL CHROMATOGRAPHIC TECHNIQUES

PAPER CHROMATOGRAPHY (PC)

Preliminary analysis of a plant extract for the presence of flavonoids is conveniently performed by this technique which consists of applying an alcoholic solution of the plant extract to a chromatographic paper (Whatman No.1). The separation of flavonoid is commonly carried out by one or two dimenssional PC in the descending mode. In the case of two dimensional paper chromatography (2DPC) the global flavonoid extract was applied evenly to the paper at one of its corners in the form of a 1.0 cm diameter circle.

The chromatogram thus prepared was developed in an all glass chromatography tank, by suspending the paper in from a tray, containing the developing solvent. For preliminary assessment of the flavonoid glycosides extract, the PC was first run in an alcoholic solvent in the larger dimension. After development the chromatogram was removed from the chromatocab and air dried in a fume cupboard. The chromatogram was then run in the other dimension in an aqueous solvent (15% AcOH) for which a separate chromatocab was used.

Most flavonoids are not visible on the paper chromatograms the main exception being anthocyanins (red), chalcones, aurones and 6-hydroxy flavonols (yellow). For this reason the chromatograms were viewed under UV light 366 nm for spot detection. Once all visible spots were pencilled out consideration was given to the chemical nature of each spot. The typical flavone and flavonol glycosides spot appeared dark purple in the UV that turned yellow when exposed to NH_3 vapours. A number of other colour combination are also possible together with possible flavonoid structure relationship (Table-1). Invisible spots can also be detected by spraying the completed chromatogram with different reagents. Some of the most commonly used spray reagents are mentioned below:

i. **$AlCl_3$:** 5% $AlCl_3$ solution in MeOH when sprayed on a PC reveals all the 5-hydroxyflavonoids as fluorescent yellow sports when viewed under UV.

ii. **Diazotized Sulphanilic Acid:** The paper chromatogram is sprayed with a solution of diazotized sulphanllic acid and then by 20% Na_2CO_3 solution. Most compounds with free phenolic hydroxyls appear as yellow orange or red spots.

iii. **Naturstoffreagent A:** Application of 1% MeOH solution of diphenylboric acid - ethanolamine complex to the P.C reveals (after drying) all 3', 4'-dihydroxyflavones and flavonols as orange spots and 4'-hydroxy equivalents appear yellow green.

iv. **Venillin-HCl:** 5% vanillin in ethanol is mixed with conc. HCl in the ratio 4:1

Table.1 INTERPRETATION OF SPOT COLOUR IN TERM OF FLAVONOID STRUCTURE

Spot colour in UV light		Possible flavonoid type
UV light without NH_3	UV light with NH_3	
Dark	Yellow Yellow green or green	a. Commonly 5-OH flavones or flavonols (3-O-substituted with 4′-OH) b. Occasionally 5-OH flavanones and 4′-OH chalcones with no Bring-OH.
	Little or no colour change	a. Commonly flavones or 3-O-substituted flavonols with 5-OH but lacking a free 4′-OH b. Some 6- or 8-OH flavones and 3-O-substituted flavonols with 5-OH c. Isoflavones, dihydroflavonols, biflavonoids and some flavanones with 5-OH d. Chalcones with 2′- or 4-OH.
	Light blue Red or orange	Some 5-OH flavanones Chalcones with a free 2- and/ or 4-OH.
Fluorescent light blue	Fluorescent yellow green blue green	a. Flavones and flavanones lacking a free 5-OH, e.g. 5-O-glycosides b. Flavonols lacking a free 5-OH but with the 3-OH substituted.
Invisible	Florescent light-blue	Isoflavones lacking a free 5-OH
Dull and Yellow, or Orange fluorescence	Little or no colour change	Flavonols with a free 3-OH and with or without a free 5-OH (sometimes Yellow originating from the dihydroflavonol)
Fluorescent yellow	Orange or red	Aurones with a fre 4′-OH and some 2- or 4-OH chalcones
Yellow-green blue-green	Little or no colour change	a. Aurones lacking a free 4′-OH and flavanones lacking a free 5-OH b. Flavonols with a free 3-OH and with ot without a free 5-OH
Dull orange red or mauve	Blue	Anthocyanidin 3-glycosides
Cerise pink or fluore-scent yellow	Blue	Most Anthocyanidin 3, 5-diglycosides

just prior to use. Red spots are produced by catechin and proanthecyanidins immediately on spraying and warming where as flavanones and dihydroflavonols appear more slowly.

An other valuable source of information is the position of the spot on the paper chromatogram. A guide as to where a particular flavonoid type be expected to appear using the standard chromatographic conditions is given in figure.1.

In order to isolate the individual flavonoids for further study the 2DPC was loaded to its maximum capacity. By running a large number of them each flavonoid could be separated quantitatively. Identical spots from each paper chromatogram was penciled out, cut into small pieces and eluted by MeOH. After several hours of contact, the liquid was filtered and evaporated to dryness. Further purification was achieved by PC employing other solvent systems or by other chromatographic techniques.

In case of one dimentional paper chromatography (1DPC) instead of applying the extract as a circular spot at the origin, the extract was streaked right across the paper to produce a 0.5-1.0 cm wide band. By this method separation of flavonoids can be increased 10-15 times. This technique was used for the separation of flavonoid aglycones where as 2DPC is preferred for the separation of somewhat complex mixtures of flavonoid glycosides.

COLUMN CHROMATOGRAPHY (CC)

By means of column chromatography the separation of flavonoids can be increased to industrial level. CC consists of depositing a flavonoid mixture in solution to a column of powdered adsorbent and subsequently eluting the individual flavonoid with appropriate solvents. The column is simply a glass tube fitted with a tap at one end , with dimensions such as that the diameter to length ratio is in the range of 1:10 to 1:30. Before packing the column it was thoroughly washed with water, acetone and finally with methanol. The neck

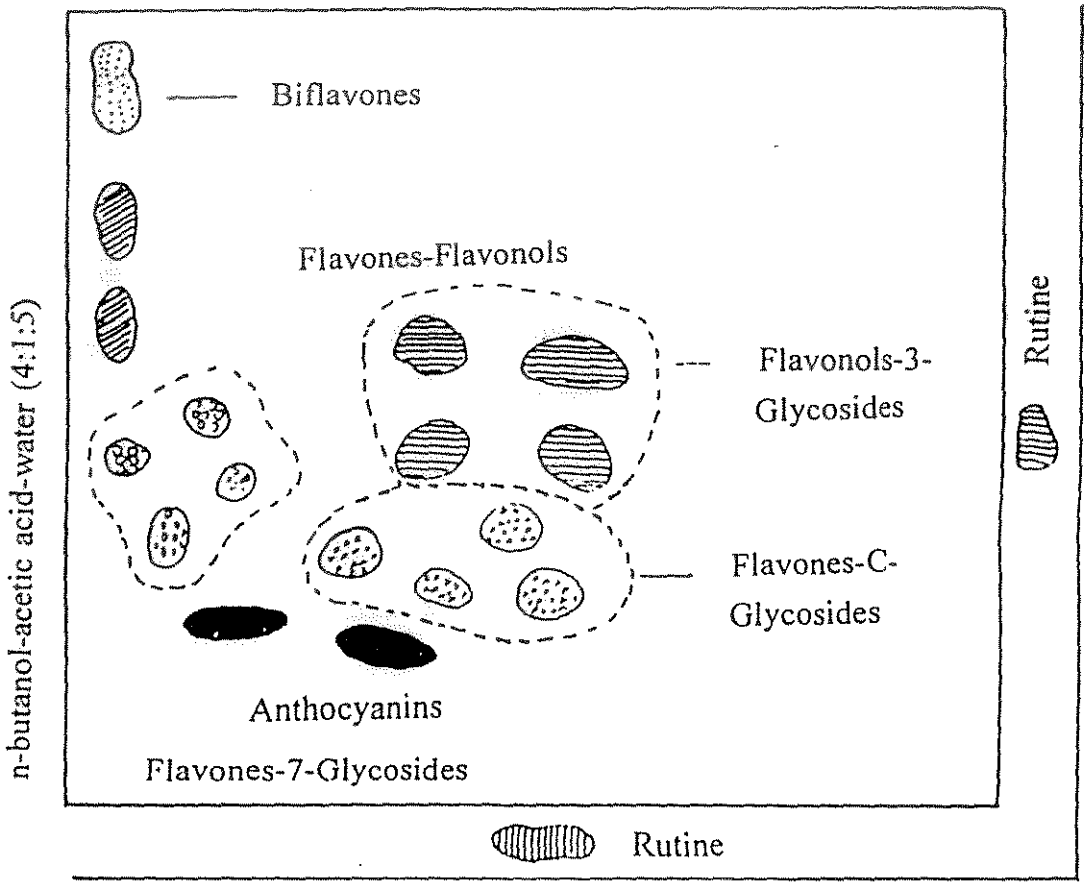


Figure-1: Two dimensional paper chromatogram of flavonoids.

of the column was pluged with glass wool and was covered with the eluting solvent to a height of 10.0 cm.

Packing of the column was carried out with great care so as to prepare a homogeneously packed column. The column packing was then slurried in a beaker with the same solvent and poured carefully into the column all in one continuous process to avoid layering. The packing was then allowed to settle for which the column's glass tube was occasionally taped with a rubber tubing. Finally the column was thoroughly washed when packed.

To commence the process of separation by column chromatography, the flavonoid extract was deposited on to the top of the packed column with the help of a pippette. The sample concentrate was then allowed to seep slowly into the column packing by opening slightly the column tap. Once the top of the column was exposed again, the tap was closed and some of the eluting solvent was carefully introduced by the pipette. This too was allowed to seep into the column before the bulk of the eluting solvent was added and the chromatographic process started.

For the priliminary separation of flavonoid glycosides a column of polyamide (MN SC6, grain < 0.07 mm, Macherey Nagel, Germany. 60 x 3.0 cm) was prepared. For this purpose the adsorbant was presocked in MeOH , stirred overnight and allowed to stand for about an hour before decanting the upper layer. This process was repeated two to three times to eliminate the finer particals. The adsorbant was finally stirred in H_2O before packing the column. The column was eluted in the gradient mode from H_2O to MeOH. In cetain cases polyamide was replaced by silica gel (70-230 mesh, 0.200 mm, Merck, Germany) where as the eluting solvent was a mixture of CH_3Cl:MeOH:H_2O (12:4:1). For final purification of flavonoid glycosides Sephadex HL-20 was used and the column was run in pure MeOH.

All the column fractions were evaporated to dryness under reduced pressure in a rotary evaporator. Each dried fraction was taken up with small amount of distilled MeOH. The composition of each fraction was checked under UV light 366 nm either on precoated or self prepared polyamide TLC plates using the reported solvent system as used for column elution.

THIN LAYER CHROMATOGRAPHY (TLC)

TLC is a technique which has developed rapidly to a great extent and it has some what replaced PC in analytical and small scale separation of flavonoids. However it is complementary to PC 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 reagents. The main value of TLC in flavonoid work is as a rapid analytical method which requires very small amount of the material.

TLC is particularly useful in flavonoid analysis for the investigation of solvents for possible use in CC, identification of flavonoids by co-chromatography and small scale isolation of pure flavonoids. The adsorbent and solvents used are generally similar to those discussed for PC and CC. The adsorbent of choice for the separation of flavonoids are silica and polyamide.

The technique of TLC consists of applying the flavonoid sample either on commercially available pre-coated or self made glass plates coated with an adsorbent. For analytical work pre-coated aluminum or plastic backed TLC plated are especially recommended as they are transparent to UV light and can easily be cut to any size. Such plates are coated with silica gel, polyamide or cellulose. For preprative as well as analytical work TLC plates can also be easily prepared in the laboratory by spreading with the help of a spreader, a slurry of any of the adsorbent in an adequate solvent or mixture of solvents, on 20 x 20 cm glass plates. In each case chromatography can be carried out by placing these plates in a covered glass tank.

Preprative TLC is carried out for the separation of milligram quantities of material. However it can be upgraded to handle upto a gram when layers of 1-5 mm thick are used in conjunction with plates of upto 20 x 100 cm size. Separated constituents are recovered after the development of a chromatogram, by scraping off the adsorbent at the appropriate places on the developed plate, eluting the powder with a solvent and finally centrifuging to remove the adsorbent.

For analytical work, pre soacked polyamide F 11_{254} (Merck) plates were used where as for the preparative separation of flavonoids, glass plates were coated with polyamide DC-6 (Polyamide 6F, Riedel-de-hae'n). 10 gm of the adsorbent was slurried in 100 ml of ethanol for preparation of five 20 x 20 cm glass plates. Thickness of the coating varried between 0.25 to 0.50 mm depending on the amount of flavonoid mixture.

1.3.2.2 ADVANCED CHRMATOGRAPHIC TECHNIQUES

VACUUM LIQUID CHROMATOGRAPHY (VLC)

VLC is an innovated version of conventional column chromatography where as a vacuum is created at the exit of the column by employing a suction pump. The creation of vacuum accelerates the flow rate hence reduces the elution time. For the separation of flavonoid glycosides by VLC the column was packed by silica gel (70-230 mesh, 0.200 mm, Merck). The flavonoid extract was deposited on top of the VLC column after mixing it with small amount of the adsorbant and evaporating the solvent. Elution was carried out by Et_2O which was enriched with EtOAc and finally by MeOH as indicated in the following chart.

Fr. No.	Ether %	EtOAc %	MeOH %	Fr. No.	Ether %	EtOAc %	MeOH %
1	100	0	0	16	25	75	0
2	95	5	0	17	20	80	0
3	90	10	0	18	15	85	0
4	85	15	0	19	10	90	0
5	80	20	0	20	5	95	0
6	75	25	0	21	0	100	0
7	70	30	0	22	0	99	1
8	65	35	0	23	0	98	2
9	60	40	0	24	0	97	3
10	55	45	0	25	0	96	4
11	50	50	0	26	0	95	5
12	45	55	0	27	0	94	6
13	40	60	0	28	0	93	7
14	35	65	0	29	0	92	8
15	30	70	0	30	0	91	9

The fractions thus collected were treated similar to those collected from the conventional column.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

HPLC has proved to be one of the most useful technique available for the separation of complex mixture of flavonoid glycosides. In this technique mixture of flavonoid glycoside is first dissolved in a liquid solvent and then forced to flow through a chromatographic column under high pressure. In this column, the mixture is resolved into its individual components. The resolution of the components depends upon the extant of interaction between the solute components and the stationary phase. The stationary phase is the immobile packing material in the column. The moving part of the system is the mobile phase, which is a liquid. The interaction of the solute with mobile phase and stationary phase can be manipulated through different choices of solvent and columns.

The basic equipment consists of a column, injector, solvent pumps, detector and data acquisition system. The column is an open tubular structure usually made of either stainless steel or glass. Length of the column vary between 20-50 cm while the inner diameter varies from 5 to 20 mm. The column is packed with the stationary phase, having uniform particle size which may vary from 4 to 10 μm. The uniformity of the particle size is a particular feature upon which the resolution power of the system depends. Smaller particle size is important because it improve the column efficiency. Larger particle size, upto 10 microns, are also widely used, but with an increase in the particle size efficiency of the column decreases. The material most commonly used as stationary phase are simple and bonded silica gel, porous glass and polystyrene divinylbenzene. In order to protect the main HPLC column a guard column is normally fixed between the injection system and the analytical column. The function of the guard column is to remove the impurities which would otherwise be strongly retained on the packing material of the main column and ultimately decrease its life time and efficiency.

All separations depend on getting the solute onto the column. This requires an injector, which is located between the column and the high pressure pump. The injection system is often the loop type. Loop injection requires a multiple port, rotary switching valve. The solution mixture is first loaded into a fixed volume loop. Using the switching valve, this loop is switched into the path of the following mobile phase, empltying the loop contents onto the column.

Fluid flow, essential for HPLC separation, is accomplished with a high pressure pump. The high pressure is needed to overcome the resistance to fluid flow arising from the microporous stationary phase packed in a narrow bore column. Because fluid flow should remain constant, independent of the back pressure, pumps must be regorously designed. The pressure of the solvent is mintained by the pumping system upto a value of 6000 p.s.i. while the elution of the solvent may vary from 1.0 to 10.0 μl/ml.

The composition of the mobile phase during a run is important for controlling the separation and retention time. A HPLC system is termed ISOCHRATIC whenever the solvent does not change composition during a given run. If it is desired to change the mobile phase composition during a run, a GRADIENT SYSTEM is used. In gradient system, two precision pumps operate in conjunction with a gradient controller. This gradient controller, regulates the percent flow of each pump. The controller offers a variety of percent options, including rate of gradient change, total flow rate, starting and final conditions and choice of gradient profile.

After injection the solution in the loop is released in the main solvent flow and passes through the column where after separation the compounds pass into the detector. The most commonly used detection system is a UV-VIS detector. It operates by measuring the absorption of either visible or ultra-violet light through the column effluent. The typical transmittance wave length of HPLC UV detector is 254 nm. Additional wave lengths of 214, 229, 280, 308, 360, 410 and 440 nm are commonly available for the specific applications. Since the detector signal is electronic, use of modern data acquisition technique can aid in the signal analysis. In addition system can store data in a retrievable form for highly sophisticated computer analysis at a later time. HPLC offers the researchers a method of qualitatively and quantitatively analyzing the flavonoid compounds of a mixture at high level of resolution sesitivity (<50 ng) for checking the purity of flavonoids and for chemotexanomic comparison.

Being the most sensitive technique HPLC was used to monitor the purity of each isolated flavonoid compound. For this purpose a SHIMADZU HPLC, SCL-6A equiped with a UV-Visible detector DPD-6A, high pressure pump LC-6A, auto injector SIL-6A and C-R3A data processor were used. Purified flavonoid glycosides dissolved in MeOH were injected on to a ODS Zorbax HPLC column (25 cm x 4.6 mm i.d.), eluted with H_2O:MeOH:AcOH (80:16:04) at a flow rate of 1.7 ml/min. and detected at a fixed wave length of 280 nm. In some cases HPLC/PDA and preprative HPLC (PHPLC) were also

used for the priliminary detection of the flavonoid glycosides. HPLC conditions were similar as mentioned above.

1.3.3 IDENTIFICATION TECHNIQUES

1.3.3.1 R_f VALUES

The mobility of any one compound in a particular solvent is referred to as its R_f value in that solvent. A comparison of the R_f values of an unknown flavonoid with authentic markers, provide preliminary information about the structure of the unknown flavonoid.

The flavonoids isolated in the pure state from the global extract of each sample were subjected to a control of R_f values by chromatography on Whatman No.1 by solvent (15% AcOH, 60% AcOH, BAW) and by TLC on polyamide $11F_{254}$, Merck, solvent 4:3:3 in the presence of standered markers. The results obtained were also compared with the data reported in the literature (Harborne, 1959, Jay et al, 1975).

Although R_f values in different solvents provide preliminary informations about the structure, spectroscopic methods provide concrete informations about the complete structural analysis of all types of flavonoids. Spectroscopic methods generally used for structural elucidation of flavonoids include ultraviolet-visible spectrophotometry (UV), Infrared spectrophotometry (IR), Mass spectroscopy (MS) and Nuclear magnetic resonance spectroscopy (NMR) etc.

1.3.3.2 ULTRAVIOLET-VISIBLE SPECTROPHOTOMETRY

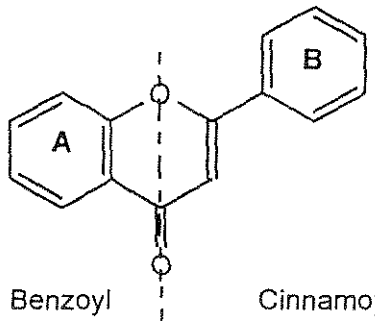
Ultraviolet-visible absorption spectrophotometry is perhaps the single technique available for flavonoid analysis. This technique is employed to aid, both, identification of the flavonoid type and definition of oxygenation pattern. In addition, the citing on the

flavonoid nucleus of the unsubstituted phenolic hydroxyl groups may be established by adding shift reagents to the sample solution and observing the resultant shifts in the absorption peaks. Thus, indirectly, the technique may be useful in determining the location of a sugar or methyl group attached to the phenolic hydroxyls.

A major advantage of this technique is the very small amount of flavonoid required for complete analysis. Normally a stock solution of 0.1 mg/ml in MeOH is prepared and then further diluted until the absorbance of the major peak is about 0.6 (O.D). This stock solution is than used for all subsequent measurements.

UV SPECTRUM OF FLAVONES AND FLAVONOLS IN MeOH:

UV spectra in MeOH of flavones and flavonols exhibit two major absorption peak in the region between 240 - 400 nm. These two peaks are commonly referred to as band-I (between 300 - 380 nm) and band-II (between 240 - 280 nm). Band-I is considered to be associated with absorptions due to the B-ring cinnamoyl system and band-II with absorptions involving the A-ring benzoyl system.



The MeOH spectra, particularly the position of band-I, provides information about the type of flavonoid as well as its oxygenation pattern. On increasing oxygenation of B-ring in flavonoids, bathochromic shift in band-I occurs with each additional oxygen

function. Increasing hydroxylation of A-ring in flavones and flavonols produces a notable bathochromic shift in band-II. Typical spectra of the major flavonoid types with equivalent (5, 7, 4') oxygenation patterns are given in Fig.2.

The presence or absence of hydrogen bonded 5-OH group has a marked effect both on band-I and band-II in the UV spectra of flavonoids. If 3, 5, or 4'-OH groups on the flavonoid nucleus are methylated or glycosylated hypsochromic shift, especially, in band-I is observed. the shift associated with the substitution of 3-OH group is usually of the order of 12 - 17 nm but reaches 22 - 25 nm in flavonols which do not contain a free 5-OH group. Methylation of the 5-OH group results in a 9-15 nm hypsochromic shift in both band-I and band-II, and methylation or glycosylation of the 4'-OH group produces a 3-10 nm hypsochromic shift in band-I. A guide to the principal maxima for each flavonoid type is given in Table-2.

Table.2 ULTRAVIOLET-VISIBLE ABSORPTION RANGES FOR FLAVONOIDS

Band II (nm)	Band I (nm)	Flavonoid type
250-280	310-350	Flavone ·
250-280	330-360	Flavonol (3-OH substituted)
250-280	350-385	Flavonols (3-OH free)
245-275	310-330 shoulder	Isoflavones
245-275	320 peak	Isoflavones (5-deoxy-6,7-dioxygenated)
275-295	300-330 shoulder	Flavanones and dihydroflavonols
230-270 (low intensity)	340-390	Chalcones
230-270 (low intensity)	380-430	Aurones
270-280	465-560	Anthocyanidins & Athocyanins

Interpretation of the changes induced in the MeOH spectrum of flavones and flavonols by various shift reagents is as follow:

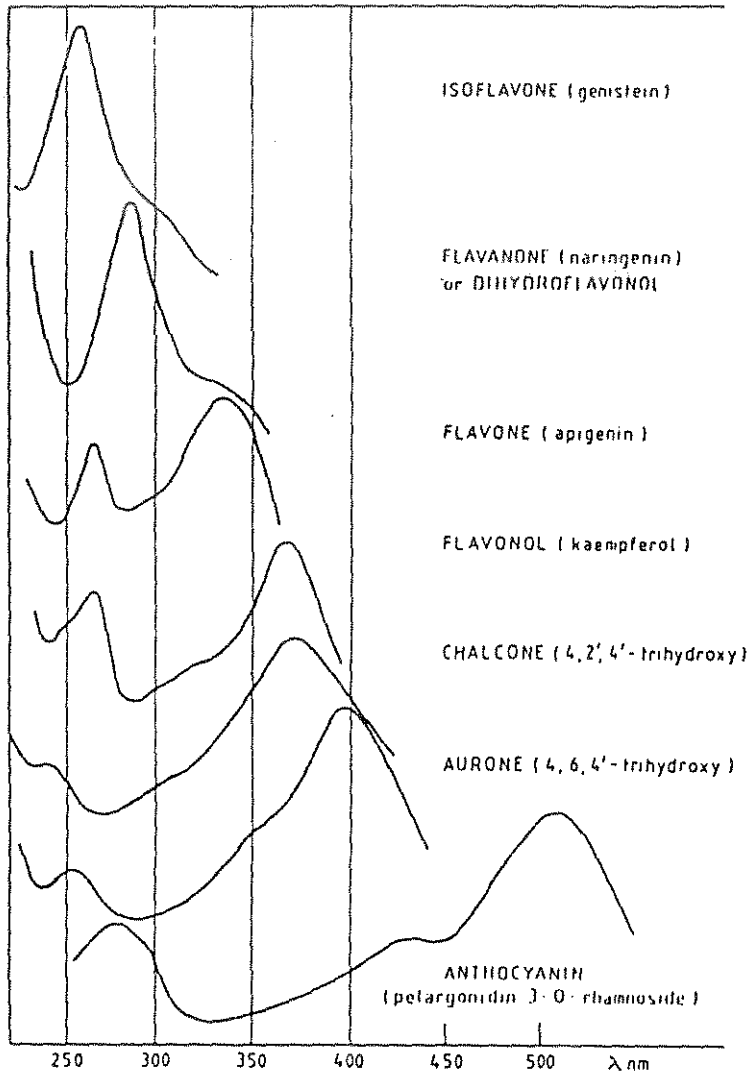


Figure-2. Ultraviolet-visible absorption spectra of different flavonoid types with equivalent hydroxylation patterns.

UV SPECTRUM OF FLAVONES AND FLAVONOLS IN THE PRESENCE OF NaOMe:

Sodium methoxide (NaOMe) is a strong base and ionizes to some extant all hydroxyl groups on the flavonoid nucleus. For this reason, it is difficult to correlate the spectral shifts observed on the addition of NaOMe with the flavonoid hydroxylation pattern. However, use has been made of the effect of NaOMe on the UV spectra of flavonoids for the detection of free 3- and 4'-OH groups.

The addition of NaOMe to flavonoids in MeOH usually produce bathochromic shifts in all absorption bands. However, a large bathochromic shift of 40-60 nm without decrease in intensity, is diagnostic for the presence of 4'-OH group. Although flavonols lacking free 4'-OH group also give a 50-60 nm bathochromic shift in band-I, there is usually a decrease in intensity of the peak. In these compounds bathochromic shift results due to the presence of a free 3-OH group.

Flavonols which have free -OH groups at both 3 and 4'-positions are unstable in NaOMe and the absorption peaks in the NaOMe spectrum degenerate in a few minutes. Flavonols which contain a 3, 3', 4'-trihydroxyl system decompose even faster than those having the 3, 4'-dihydroxyl system. Although alkali instability is generally associated with flavonols having the 3, 4'-dihydroxyl grouping. Other hydroxylation patterns in flavones, notably 5, 6, 7; 5, 7, 8 and 3', 4', 5' and also cause alkali sensitivity.

UV SPECTRA OF FLAVONES AND FLAVONOLS IN THE PRESENCE OF NaOAc:

Sodium acetate (NaOAc) is a weaker base than MeONa and as such ionizes only the more acidic hydroxyl groups in flavones and flavonols i,e the 3, 7 and 4'-OH groups . Because ionization of the 7-OH group mainly affects band-II where as ionization of 3 and /

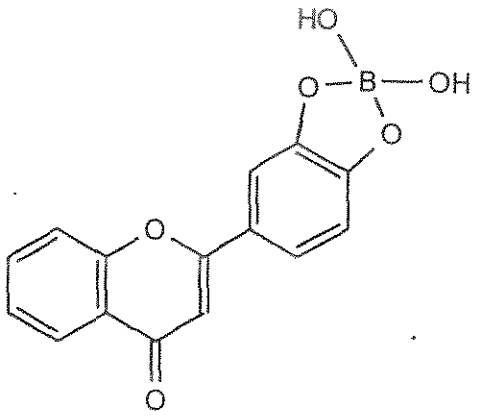
or 4'-OH groups mainly affect band-I. NaOAc is particularly useful diagnostic reagent for the specific detection of 7-OH group.

The UV spectra of flavones and flavonols containing free 7-OH groups exhibit a diagnostic 5-20 nm bathochromic shift of band-II in the presence of NaOAc. However, when 6 and 8 oxygen substituents are present in flavones the bathochromic shift with NaOAc is often small or imperceptible presumably because of the reduced acidity of 7-OH group.

Flavones and flavonols which possess a 4'-OH group and no free 3- or 7-OH groups usually show a pronounced shoulder on the long wave length side of band-I in the presence of NaOAc. If the NaOAc spectrum of a flavone or flavonol changes after several minutes then the flavonoid has decomposed due to the presence of an alkali-sensitive grouping.

UV SPECTRUM OF FLAVONES AND FLAVONOLS IN THE PRESENCE OF $NaOAc/H_3BO_3$:

In the presence of NaOAc, boric acid (H_3BO_3) chelates with orthodihydroxyl groups at all locations on the flavonoid nucleus. Flavones and flavonols containing a B-ring ortho-dihydroxyl group show a consistent 12-30 nm bathochromic shift of band-I in the presence of $NaOAc/H_3BO_3$. A-ring ortho-dihydroxyl groups at C-6, 7 or C-7, 8 in flavonoids are also detectable by the affect of $NaOAc/H_3BO_3$ on the UV spectra. It appears that a bathochromic shift of about 5-10 nm in band-II is diagnostic for the flavones and flavonols containing either 6, 7 or 7, 8 dihydroxyl groups.

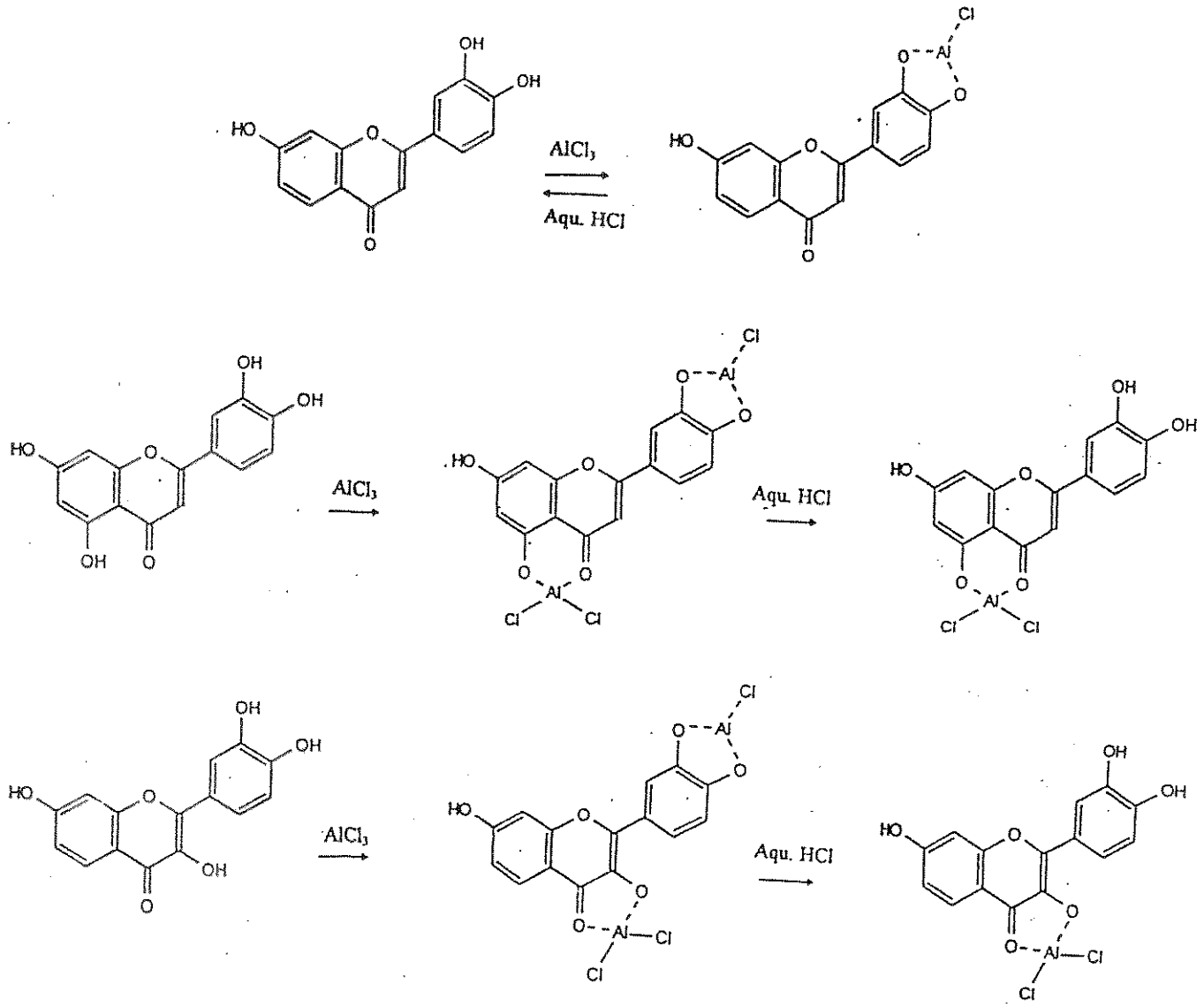


UV SPECTRA OF FLAVONES AND FLAVONOLS IN THE PRESENCE OF $AlCl_3$ AND $AlCl_3$/HCl:

With $AlCl_3$ flavones and flavonols which contain hydroxyl groups at C-3 or C-5 form acid stable complexes, in addition, $AlCl_3$ form acid labile complexes with flavonoids which contain orthodihydroxylation. The complexes formed between $AlCl_3$ and the A- and B-ring ortho-dihydroxyl groups, decompose in the presence of acid. In contrast, the $AlCl_3$ complex between the C-4 keto function and either the 3- or 5-OH group is stable in the presence of acid (scheme-4).

The presence of an orthodihydroxylation in the B-ring of flavonoids can be detected by a comparison of the spectrum of the flavonoid in the presence of $AlCl_3$ with that obtained in $AlCl_3$/HCl.A hypsochromic shift of 30-40 nm indicates the presence of an orthodihydroxylation grouping.

The addition of acid to a methanolic solution of flavone or flavonol which already contains $AlCl_3$ decomposes complex between $AlCl_3$ and ortho-dihydroxy groups. Therefore any shift still remaining in band-I or band-II relative to the methanolic spectrum will be due to the presence of 3- and or 5-OH groups in the flavonoid. The $AlCl_3$/HCl spectrum of a 5-OH flavone typically consists of four major absorption peaks, band-Ia, Ib, IIa and IIb, which are all shifted bathochromically relative to their bands of origin in the methanolic spectrum.



Scheme-4: Complexation of $AlCl_3$ with flavones and flavonols in the presence and absence of acid.

There is a clear distinction between the magnitude of the $AlCl_3$/HCl bathochromic shifts associated with 5-OH flavones and those observed for 3-OH flavones. The bathochromic shift of band-I in MeOH to band-Ia in the spectra of 5-hydroxyflavones and 3-substituted flavonols are in the range of 35-55 nm. In contrast, the shift is consistently around 60 nm for 3-hydroxyflavones. The bathochromic shift of band-I to Ia in 3, 5-dihydroxyl flavone range from 50-60 nm and is usually intermediate between those observed for their 3-OH and 5-OH equivalents.

PREPARATION OF STOCK SOLUTION AND SHIFT REAGENTS:

In order to measure the UV spectra of isolated and purified flavonoids, the stock solution of flavonoids and the standered shift reagents were prepared according to the following procedure.

1. Stock Solution: A stock solution of the flavonoid was prepared by dissolving a small amount of the compound (about 1.0 mg) in about 10 ml of spectral grade MeOH. The concentration was then adjusted so that the optical density of the major absorption peaks between 250 and 400 nm was in the region 0.6-0.8.

2. Sodium Acetate (NaOAc): Powdered, anhydrous, A. R grade NaOAc was used after its fusion to remove the residual HOAc (Mabry *et* at, 1970).

3. Boric Acid (H_3BO_3) Powdered anhydrous A. R grade H_3BO_3 was used.

4. Aluminium Chloride ($AlCl_3$): About 5 gm of fresh, dry $AlCl_3$, which should fizz when added to H_2O, was added cautiously to 100 ml of freshly distilled MeOH and stored in plastic stopped bottle prior to use.

5. Hydrochloric Acid (HCl): Concentrated reagent grade HCl (50 ml) was added to 100 ml of distilled H_2O.

6. Sodium Methoxide (NaOMe): Freshly cut metallic sodium (2.5 g) was added cautiously in small portions to 100 ml of dry spectroscopic grade MeOH.

STEPWISE PROCEDURE FOR THE MEASUREMENT OF UV SPECTRA IN THE PRESENCE OF SHIFT REAGENTS:

1. All the spectra were recorded on BECKMAN DU-7 UV-Spectrophotometer from a spectral grade MeOH solution of flavonoids.

2. After measurement of the spectrum of the flavonoid in MeOH, small amount (ca 1.0 mg) of powdered NaOAc was then added in the UV cuvette. The mixture was thoroughly shaken before the NaOAc spectrum was recorded. Recording of the spectrum was repeated after a lapse of 5.0 minutes to monitor any sample decomposition due to the presence of alkali sensitive groupings. The "NaOAc/H_3BO_3" spectrum was subsiquently measured after the addition of a small amount of H_3BO_3.

3. A second series of UV measurements was made by adding 6 drops of $AlCl_3$ reagent to a fresh methanolic solution of flavonoid. To the sample UV cuvette 3 drops of Hcl were added. After mixing, the"$AlCl_3$/HCl" spectrum was measured.

4. 3 drops of NaOMe solution were added to the methanolic solution of flavonoid and after mixing the "NaOMe" spectrum was recorded. To check for sample decomposition the "NaOMe" spectrum was rerun after 5 minutes.

1.3.3.3 INFRARD (IR) SPECTOPHOTOMETRY

IR spectrophtometry is not as useful as UV-spectrophotometry. However, it helps in the identification of flavonoid compounds. The IR spectra of all the flavonoids and isoflavonoids show absorption bands in the region of 1500-1600 cm^{-1} due to aromatic rings, along with a carbonyl band at 1620-1670 cm^{-1}. The carbonyl absorption does not appear in

flavanoids, isoflavanoids, pterocapanoids and chalcanoids. The presense of hydroxyl groups in hydroxyflavonoids is evidenced by absorption in the region 3300-3450 cm^{-1}. An absorption at 925 cm^{-1} in indicative of a methylenedioxy group and the presence of gem-dimethyl group is indicated by the appearance of a band at 1400 cm^{-1}. The glycosidic nature of a flavonoid is exhibited by broad bands at 3250 and 1060 cm^{-1}.

Since all the purified flavonoid glycosides were obtained as crystalline solids, 1.0 mg of each was mixed in KBr and IR spectra were measured on PERKIN ELMER 1710 Infrared Forier Transform Spectrophotometer.

1.3.3.4 MASS SPECTROSCOPY (MS)

ELECTRON IMPACT MASS SPECTROMETRY:

Electron Impact Mass Spectroscopy (EI-MS) of both flavonoid aglycones and glycosides serves as a valuable aid in determining their structure especially when only very small quantity (i.e; less than 1.0 mg) of a compound is available. A mass spectrum comprises of a series of signals each of which represents a charged fragment of the parent flavonoid, produced by the electron impact with in the spectrometer. The signals are displayed as a series of lines on a chart paper or in numerical from and are arranged according to mol. wt. per unit charge (m/z) of the fragments they represent. In the spectrum signal intensities are proportional to the level of individual charged species produced by electron impact. The signal of greater intensity is referred to as the base peak and is assigned an arbitrary intensity of 100%. All other signals are expressed as a percentage of the base peak.

A prerequisition for successful MS is that the flavonoid should be sufficiently volatile in the high vacuum within the mass spectrometer. Most aglycones are sufficiently volatile at prob temperature of 100-300°C. However, the polyhydroxy-flavones and flavonol glycosides, anthocyanidins and biflavonoids requires higher temperature, hence

they, must be derivatized to have improved volatility. The standard method is that of per-methylation, per-deuteromethylation or trimethylsilylation.

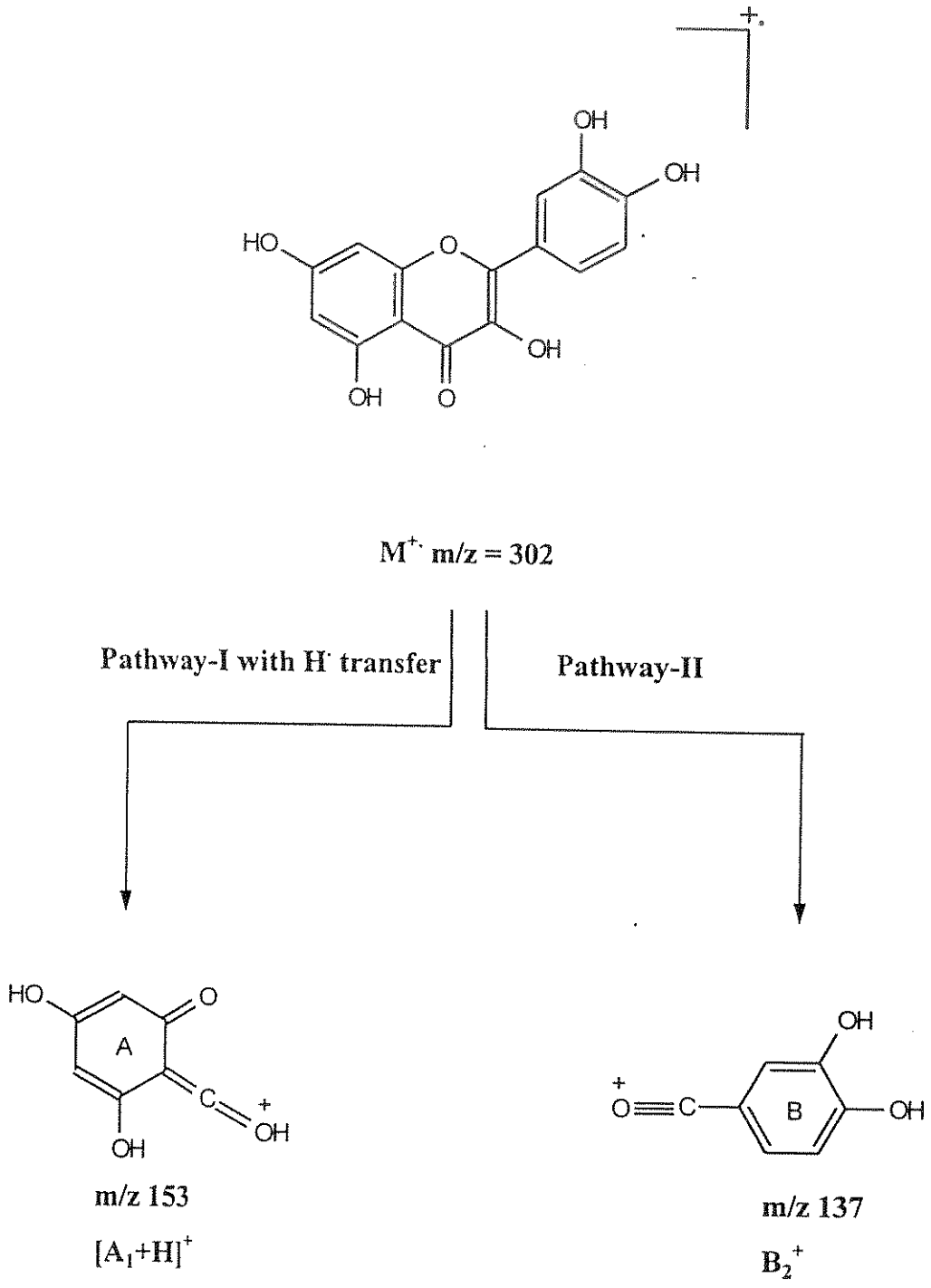
$R\text{-}(OH)_x \longrightarrow R\text{-}(OCH_3)_x$ per-methylation

$R\text{-}(OH)_x \longrightarrow R\text{-}(OCD_3)_x$ perdeutromethylation

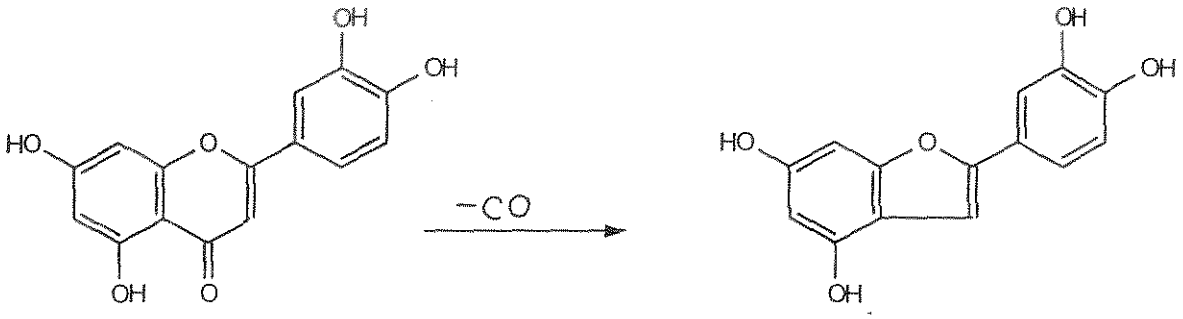
$R\text{-}(OH)_x \longrightarrow R\text{-}[OSi(CH_3)_3]_x$ trimethylilyiation

With underivatized flavonoid glycosides, however, the molecular ion is rarely observed, and even permethylated or perdeuteromethylated derivatives give a peak of only low intensity. Recognition of M^+ is aided by the fact that it must be an even number. Furthermore, it must represent a reasonable molecular weight of basic flavonoid nucleus i.e. flavones, isoflavones and aurones, 222; flavonones and chalcones 224; flavonols, 238; and dihydroflavonols, 240; and 18 mass unit (m.u) must be added for each -OH, the 30 m.u. for each $-OCH_3$ and 33 m.u. for each OCD_3.

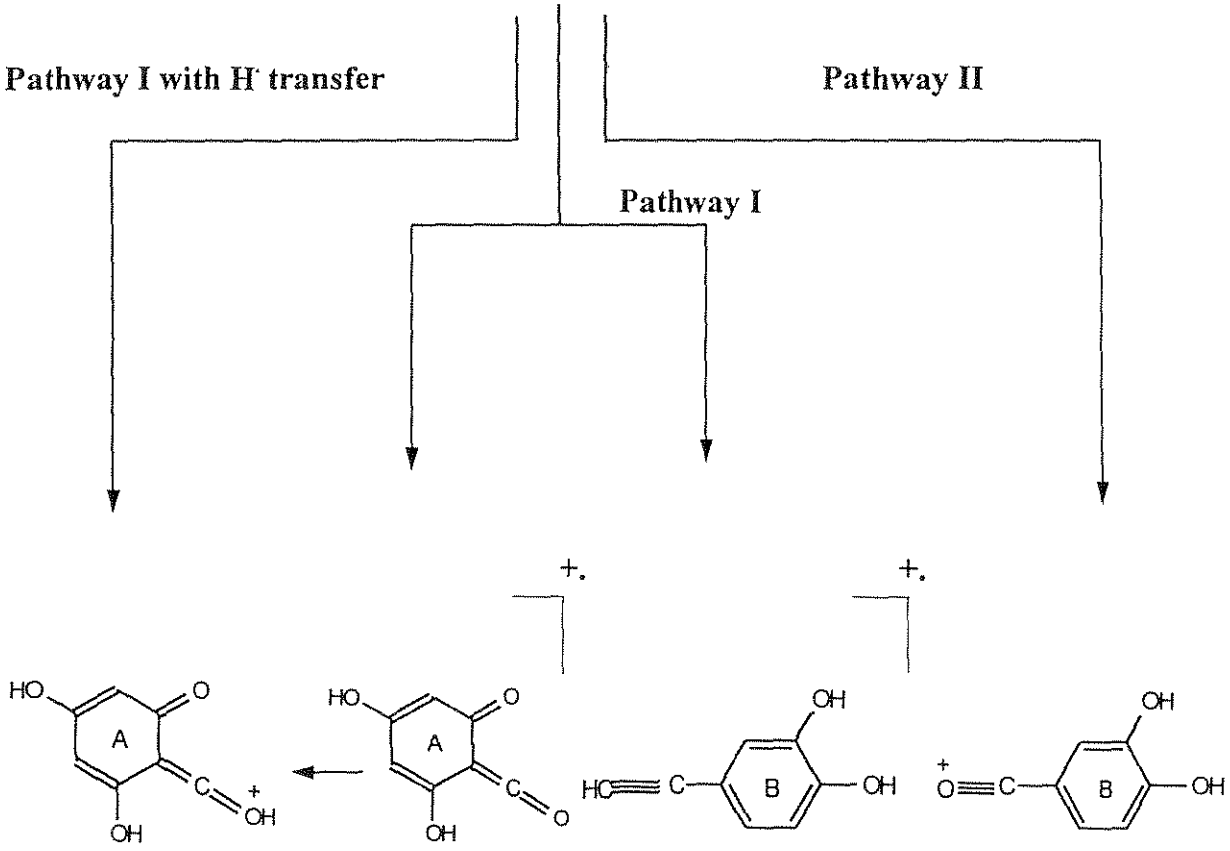
In addition to the molecular ion, flavonoid aglycones usually afford major peaks for (M^+-H) and, when methylated (M^+-CH_3). If the flavonoid contains a methoxy at C-6 or C-8, the loss of CH_3 radical produces an intense ion (M^+-15) which may be more intense than M^+. 6- and 8-methoxy flavonoids can be clearly distinguished from one another by the relative intensities of these and other ions. Lower intensity M^+-15 ions can originate from flavonoids O-methylated at other sites. Loss of $OH^·$ is indicated by the presence of M^+-17 ion. This usually involves internal ring formation and is commonly associated with 2'-hydroxylation in flavones, flavonols, isoflavones etc. A loss of 31 m.u i.e; (M-31) may indicate loss of OCH_3 from a 2'-methxoylated flavonoid by the same mechanism. M^+-18 represents the loss of H_2O which is common in flavonols, flavan 3,4-diols and C-glycosides. M^+-28(29) indicate loss of CO(CHO) from the 4-keto function to from a 5-membered ring (especially with 3-hydroxyflavones and dihydroxyflavonols).



Scheme-5: Mass fragmentation pattern of flavonols



$M^{+\cdot}$ m/z 286 m/z 258



m/z 153 m/z 152 m/z 134 m/z 137

$[A_1+H]^+$ $A_1^{+\cdot}$ $B_1^{+\cdot}$ B_2^+

Scheme-6: Mass fragmentation pattern of flavones.

The most useful fragmentations in term of flavonoid identification are those which involve cleavage of intact A- and B-ring fragments. These fragmentations usually involve one of the two competing pathways as shown in scheme-5 and 6. The dominant pathway is determined by the aglycone type, although on occasions neither pathway produces detectable fragments. Flavonols produce $(A^1+H)^+$ and B_2^+ fragment ions while flavones and isoflavones tend to produce A_1^+, $(A_1+H)^+$ plus B_1^+ fragments; flavanones, A_1^+, $(A_1+2H)^+$ plus $(B_1+H_2O)^+$ fragments and dihydroflavonla A_1^+ plus $(B_1+H_2O)^+$ fragments.

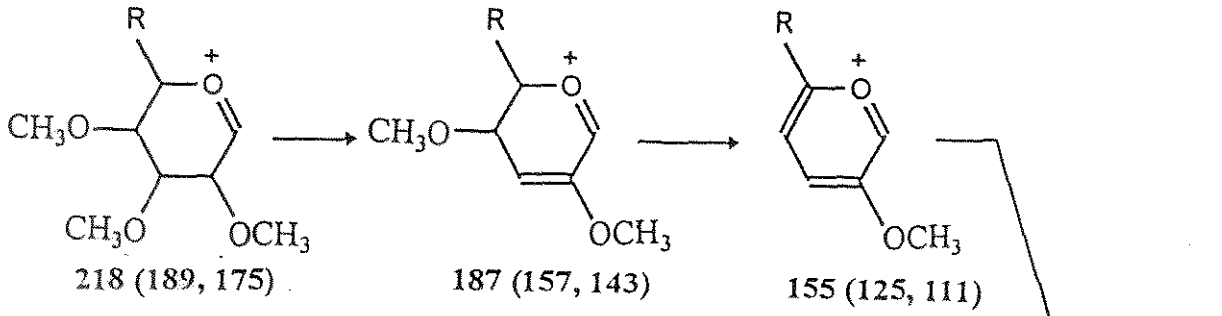
MS of Flavonoid Glycosides

a) Flavonoid C-glycosides: Mass spectroscopy of underivatized flavonoid C-glycosides rarely produces an observable molecular ion. The base peak is usually the aglycone fragment containing a $-CH_2$-remnant of each original C-linked sugar. This ion subsequently fragments by the same process observed for other flavonoid aglycones (Pathway I, Pathway II).

The permethylated -mono- and di-C-glycosides of flavonoids give well defined mass spectra including a sizable M^+ ion (15-100%). If the aglycone moiety is known, the molecular wight of the M^+ ion defines the type(s) of sugar(s) linked to the flavonoid.

b) Flavonoid O-glycosides: Most permethylated and perdeuteromethylated ethers of 3,5 and 4'-O-glycosides give low intensity molecular ion (0.1-2%) due to rapid loss of sugar, the equivalent 7-O-glycosides normally give molecular ions of high intensity (10-90%). The mass spectra are characterized by the presence of the aglycone ion in which the sugar moiety is replaced by a hydrogen. The sugar fragments from permethylated mono-O-glycoside are produced mainly by sequential loss of methanol and reveal the type of sugar involved (scheme.7). Thus two types of sugars i.e. hexoses and pentoses can be differentiated by mass spectra.

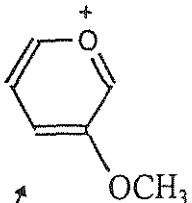
a: Pentosides (R = H) and hexosides (R = CH_3 or CH_2OCH_3)



218 (189, 175)

187 (157, 143)

155 (125, 111)



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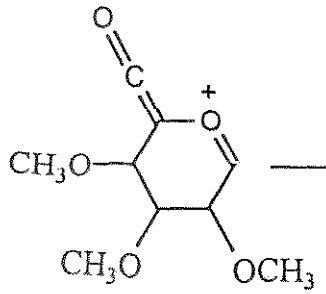
b: Glucuronides



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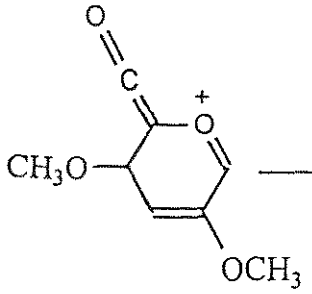

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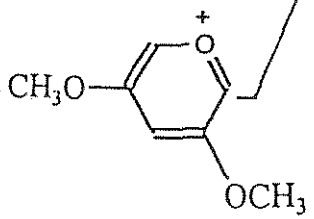

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Scheme.7: Mass fragmentation pattern of permethylated sugars.

FAST ATOM BOMBARDMENT MASS SPECTROMETRY (FABMS):

Fast atom bombardment (FAB) is a soft ionization technique. It is obtained from an ion gun, by allowing the accelerated ion beam to enter a collision chamber and exchange energy with neutral atoms.

$$Xe^{+}\ (fast) + Xe \longrightarrow Xe^{+} + Xe\ (fast)$$

Argon is often used, but the grater mass of xenon means that the fast atom beam has higher energy and results in the production of more ions from the sample. The resultant fast atoms are directed on to the sample, held in a liquid matrix on a metal target. The liquid matrix is most commonly glycerol or lactic acid. The metal target is held at a potential difference with respect to the source slit, so that ions formed, at or just above the surface of the sample on impact of the fast atoms, are expelled from the source. By reversing the nature of the potential, either positively or negatively charged ions may be examined. The method is extremely simple and gives rise to spectra at near room temperature.

Generally, abundant quasi-molecular ions [$(M+1)^{+}$ or $(M-1)^{-}$] plus a few diagnostic fragment ions result. The main advantage of this technique is that the molecules such as peptides and flavonoid glycosides don,t need prior derivatization. FABMS shows the sequential loss of sugars in case of flavonoid di- or tri-glycosides which is very helpful in determining the structure of the molecule.

1.3.3.5 NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY (NMR)

The application of NMR to the structure analysis of flavonoids is now well established. It is one of the prime important non-destructive analytical technique which is being used efficiently to solve the structural elucedation problems in the field of natural products as well as synthetic chemistry. One dimensional NMR (^{1}H NMR, ^{13}C (B.B & DEPT) NMR, NOE) and two dimensional NMR (^{1}H, ^{1}H-COSY, ^{1}H, ^{13}C-COSY, ^{13}C, ^{13}C-

COSY) are the most commonly available techniques to the chemists. For structure determination of flavonoid glycosides. One dimensional NMR (^{1}H NMR, ^{13}(B.B & DEPT) NMR, NOE measurements) and two dimentsional NMR (^{1}H, ^{1}H-COSY, ^{1}H, ^{13}C-COSY) techniques were employed on AM 300 and 400 (BRUKER) NMR machines.

^{1}H NMR

Proton megnatic resonance (^{1}H NMR) spectroscopy has been widely used as a method for the structural analysis of flavonoids. The ^{1}H NMR spectrum appears predominently in the range of 0-10 ppm downfield from the reference signal of tetramethylsilane (TMS). The integeral of the signal is proportional to the number of protons it represents and the chemically identical protons are represented by the same signal. The chemical shift establishes the nature of the hydrogen, and coupling constants determine the presence of the ortho, meta and vicinal protons. The ortho and meta couplings have ranges of 6.5-9.0 Hz and 1.5-2.5 Hz respectively. ^{1}H chemical shifts of some flavonoids as well as sugars are presented in Table-3.

^{13}C NMR

^{13}C NMR spectra are recorded in the proton-noise (broad band) decoupled mode in order to avoid the extensive dignal overlap which can occur due to the large one bond carbon hydrogen coupling constants (120-250 Hz). The multiplets are thus reduced to single lines and it is possible to determine the number of carbons in an unknown natural product by direct observation of the ^{13}C spectral peaks. Usually, the carbon resonances representing the A- and C-ring are not superimposed and give rise to independent signals. However, the six B-ring carbons can give rise to fewer than six signals when the B-ring is unsubstituted or possesses symmetrical substitution. In proton decoupled spectra, all carbon 13 resonances appear as single lines. These, in most flavonoids, occur in the

Table.3 CHEMICAL SHIFT OF ^{1}H NMR OF FLAVONOIDS

A-RING PROTONS			
Flavone, Flavonol & Isoflavone	(C-5) δ ppm	(C-6) δ ppm	(C-8) ppm
5, 7-di-OH	-	6.0 - 6.2 (d)	6.3 - 6.5 (d)
7-O-glycoside 5-OH	-	6.2 - 6.4 (d)	6.5 - 6.0 (d)
7-OH	-	6.7 - 7.1 (q)	6.7 - 7.0 (d)

B-RING PROTONS			
Compounds	C-2', C-6' δ ppm	C-3', C-5' δ ppm	C-5' δ ppm
C-4' -OH	7.1 - 8.0	6.65 - 7.1	-
C-3', C-4' di-OH	7.2 - 7.9	-	6.7 - 7.1 (d)
C-3', C-4'C-5'	6.5 - 7.5 (s)	-	-
Flavananones (C-4'-OH)	7.1 - 7.3	6.65 - 7.1	-
Dihydroflavonols (C-4'-OH)	7.2 - 7.4	6.65 - 7.1	-
Isoflavones (C-4'-OH)	7.2 - 7.5	6.66 - 7.1	-
Chalcones(C-4'-OH)	7.4 - 7.6	6.65 - 7.1	-
Aurones (C-4'-OH)	7.6 - 7.8	6.65 - 7.1	-
Flavones (C-4'-OH)	7.7 - 7.0	6.65 - 7.1	-
Flavonols (C-4'-OH)	7.9 - 8.1	6.65 - 7.1	-

C-RING PROTONS		
Compound	C-2 δ ppm	C-3 δ ppm
Flavone	-	6.3
Isoflavone	7.7 - 7.8	-

SUGAR PROTONS		
Compound	C-1" δ ppm	Other protons δ ppm
4', 5, 7-O-glycosides	5.0	3.5 - 4.3
3-O-glycosides	5.8	3.5 - 4.3
7-O-glycosides	4.8 - 5.2	3.5 - 4.3
3-O-glucosides	5.8 - 6.0	3.5 - 4.3
Rhamnosyl- Me	0.8 - 1.2	3.5 - 4.3

SUBSTITUTED PROTONS	δ ppm
MeO	3.5 - 4.1
CH_3CO	2.25- 2.5
TMS	0.1 - 0.5
C-6-Me	4.04 - 2.27
C-8-Me	2.14 - 2.45

chemical shift range of 0-220 ppm downfield from TMS. The chemical shift directly reflects the electron density at the particular carbon nucleus and is, therefore, a sensitive probe for the structural characterization of the molecule. The carbon resonances at lowest field are generally those of carbonyl and oxygenated aromatic carbons.

The decoupled ^{13}C NMR spectrum of an unknown flavonoid permits ready differentiation between 4-keto flavonoids, flavonoids lacking a keto group, 2,3-unsaturated flavonoids and 2,3-saturated flavonoids. The 4-keto flavonoids possess a carbonyl carbon resonance in the range 170-205 ppm which is not present in flavonoids lacking the 4-keto function. All fifteen signals due to the flavonoid nucleus resonate in a region 90-200 ppm in the case of 2,3-unsaturated flavonoids and isoflavonoids whereas 2,3-saturated flavonoids and isoflavonoids possess only thirteen signals in this region, the two additional signals resonating at higher field. The flavonoids, petrocarpanoid and chalcanes possess three aliphatic resonances and twelve aromatic resonances in above mentioned chemical shift range. The presence of many signals in the 60-80 ppm region is generally indicative of glycosidic carbons. Approximate chemical shift ranges for each carbon encountered in flavonoids are outlined in table No.4.

NUCLEAR OVERHAUSER EFFECT (NOE)

NOE measurement have gained much importance for solvong the structural elucidation problems, especially in natural products chemistry. In this technique a nuclei, close to another nuclei in space, is subjected to strong decoupling which stimulate an alternative relexation path for the other nucleus resulting in an increase in the population of the lower state of that nucleus. The increase in population is accompained by a corresponding increase in signal intensity.

The NOE can be more carefully assessed with difference spectra, in which a spectrum is first recorded without double irridiation, and then with double irridiation of proton, or group of protons. Substraction of the former from the latter leaves residue of

Table.4 CHEMICAL SHIFT OF ^{13}C NMR OF SOME FLAVONOIDS

C	Kaempferol DMSO-d_6	Quercetin DMSO-d_6	K-3-O-Glu DMSO-d_6	Q-3-O-Glu DMSO-d_6	K-3-O-Rh-(1→6)-Gal DMSO-d_6
C-2	146.8	148.3	156.3	156.5	156.5
C-3	135.6	136.7	133.0	133.7	133.3
C-4	175.9	176.7	177.5	177.6	177.5
C-5	160.7	157.5	161.1	161.3	161.2
C-6	98.2	99.6	98.7	98.8	98.8
C-7	163.9	164.5	164.1	164.2	164.0
C-8	93.5	95.0	93.6	93.8	93.6
C-9	156.2	161.4	156.3	156.5	156.5
C-10	101.3	104.4	104.1	104.2	103.8
C-1'	121.7	123.7	121.0	121.4	120.9
C-2'	129.5	116.5	130.7	115.2	131.0
C-3'	115.4	145.7	115.0	144.8	115.1
C-4'	159.2	148.0	159.8	148.5	160.0
C-5'	115.4	116.9	115.0	116.5	115.1
C-6'	129.5	122.0	130.7	121.6	131.0
C-1"	-	-	100.7	101.4	102.1
C-2"	-	-	74.2	74.3	71.1
C-3"	-	-	77.3	76.8	73.0
C-4"	-	-	69.8	70.3	68.1
C-5"	-	-	76.4	77.5	73.6
C-6"	-	-	60.8	61.3	68.7
C-1"'	-	-	-	-	100.1
C-2"'	-	-	-	-	70.7
C-3"'	-	-	-	-	70.4
C-4"'	-	-	-	-	72.0
C-5"'	-	-	-	-	68.3
C-6"'	-	-	-	-	17.9

K-3-O-Glu = Kaempferol 3-O-glucoside.

Q-3-O-Glu = Quercetin 3-O-glucoside.

K-3-O-Rh-(1→6)-Gal = Kaempferol 3-O-rhamnosyl (1→6)-galactoside.

signals on where that signal from a nearby proton has been NOE enhanced, since all other signals will cancel out. The method has been used to establish the steriochemistry and interglycosidic linkage of flavonoid glycosides.

^{1}H, ^{1}H-COSY (Homonuclear) NMR

Proton-proton correlation spectroscopy is one of the most important technique which sets out the proton NMR spectrum of an organic molecule along the X-axis, and repeats it along the Y-axis, with the signals repeated yet in the countours of the diagonal peaks. ^{1}H, ^{1}H-COSY spectra are symmetrical with respect to the diagonal, since both frequency domains contain the same ^{1}H-chemical shift informations. Symmetrization of the 2D data matrix facilitates interpretation of the spectra and, in addition, leads to an improvement in the signal/noise ratio by a factor of $\sqrt{2}$. Usually two different types of signals are observed in ^{1}H, ^{1}H-COSY spectra. Those at diagonal (diagonal peaks) present in the orignal spectrum, as obtained in 1D experiment, and the off-diagonal signals (cross peaks) which prove the existance of scalar (through bond) couplings.

An ^{1}H, ^{1}H-COSY measurement can be considered as equivalent to a series of selective decouplings during which all chemically different protons are decoupled consecutively. Selective decoupling experiments are laborious and, because of signal overlap, often inconclusive, so the two dimensional technique is clearly superior. ^{1}H, ^{1}H-COSY spectra of samples upto a substance 0.3 to 0.5 mM or more can be obtained in relatively shorter time. The minimal substance requirement depends on a number of additional factors such as ^{1}H-relexation time and the sensitivity of the spectrometer.

^{1}H, ^{13}C-COSY (Heteronuclear) NMR

Like ^{1}H, ^{1}H-COSY, ^{1}H, ^{13}C-COSY is also a very important technique which show the ^{13}C-NMR spectrum along one axis and the ^{1}H-NMR spectrum along the other. H-C shift correlations are expressed by "Spots" in 2D display. ^{1}H, ^{13}C-COSY identifies protons and

carbons that are coupled. It is also of particular use in assighning proton resonances where the carbon resonances of the same sites are known, and vice virsa. Thus ^{1}H, ^{1}H-COSY and ^{1}H, ^{13}C-COSY techniques are often compementary to one another.

1.3.3.6 IDENTIFICATION OF SUGARS

All of the commonly encountered sugars resulting from the hydrolysis of the glycosides can readily be analyzed by PC (Markham, 1991). For this purpose a standard solutions of sugars such as glucose, galactose, rharmnose, arabinose etc. were made (100mg sugar in 20 ml water containing 10% 2-propanol as preservative) for reference purposes. Aliquots (4ul) of these reference solutions were then applied at points along the base line of a sheet of Whatman No.1 chromatographic paper (57 cm length), and between these points the unknown sugars were spotted with a variety of different loadings (e.g; 1,3 and 5 applications per spot). Several chromatograms were prepared to enable development in a range of different solvent systems such as:

1. BAW n-BuOH:AcOH:H_2O (4:1:5,top)
2. BEW n-BuOH:EtOH:H_2O (4:1:2.2)
3. BTPW n-BuOH:Tol,:Pyr.:H_2O (5:1:3:3)
4. EPAW EtOAc:Pyr.:AcOH:H_2O (36:36:7:21)
5. PhOH Phenol saturated with water.

The paper chromatograms, after being developed in descending mode, were dried in the fume cupboard and then sprayed with the following reagent.

Aniline hydrogen phthalate: Dissolve 0.92 ml aniline and 1.6 g phthalic acid in 49 ml of each of n-BuOH and ether and 2 ml of water.

Other spray reagents which can also be used for sugar detection on PC are cited below:

p-Anisidine hydrochloride: Dissolve p-anisidine-HCl (1.0 g) in MeOH (10 ml) add n-BuOH (100 ml) and sodium dithionite (0.1 g).

Diphenylamine/aniline/H_3PO_4: To 1 part of 85% orthophosphoric acid, 10 parts of a solution of, 1g each of diphenylamine and aniline in 100 ml of acetone, are added just prior to spraying.

To render sugars spots visible the paper chromatogram was then heated in an oven at 100-120°C for about 3 minutes, until brownish spots appeared. Most of the separations achieved on paper can also be obtained by TLC on microcrystalline cellulose using the same solvent systems and spray reagents. Silica gel, a popular TLC adsorbent, is often modified by pretreatment with a suitable buffer (phosphate or borate) before it is used for sugar separations. An effective sugar separation can be achieved by TLC on silica gel using n-BuOH:AcOH:Ether:H_2O ((:6:3:1) as solvent system. R_f (x100) values for the most common sugars are as under

Sucrose	09	Fructose	27
Ribose	47	Glucose	22
Xylose	40	Rhamnose	55

Gas-Liquid Chromatography (GLC) is a more sensitive and quantitative method of sugar analysis. For GLC analysis the sugars, obtained after the acid hydrolysis of flavonoid glycosides, were converted to their TMS derivatives by the standared procedure (Markham, 1982). 2 µl of TMS derivative of each sugar was injected on to the top of the column (Mecherey Nagel SE-54, 25 m, I.D = 0.25 mm; column temperature 208°C). Nitrogen was used as a carrier gas at a flow rate of 3-4ml/min. and detection was made by flame ionization detector. Under these conditions, retention times measured for some of the standared sugars are shown in fig.3. While the structure of the common sugars are presented in fig.4.

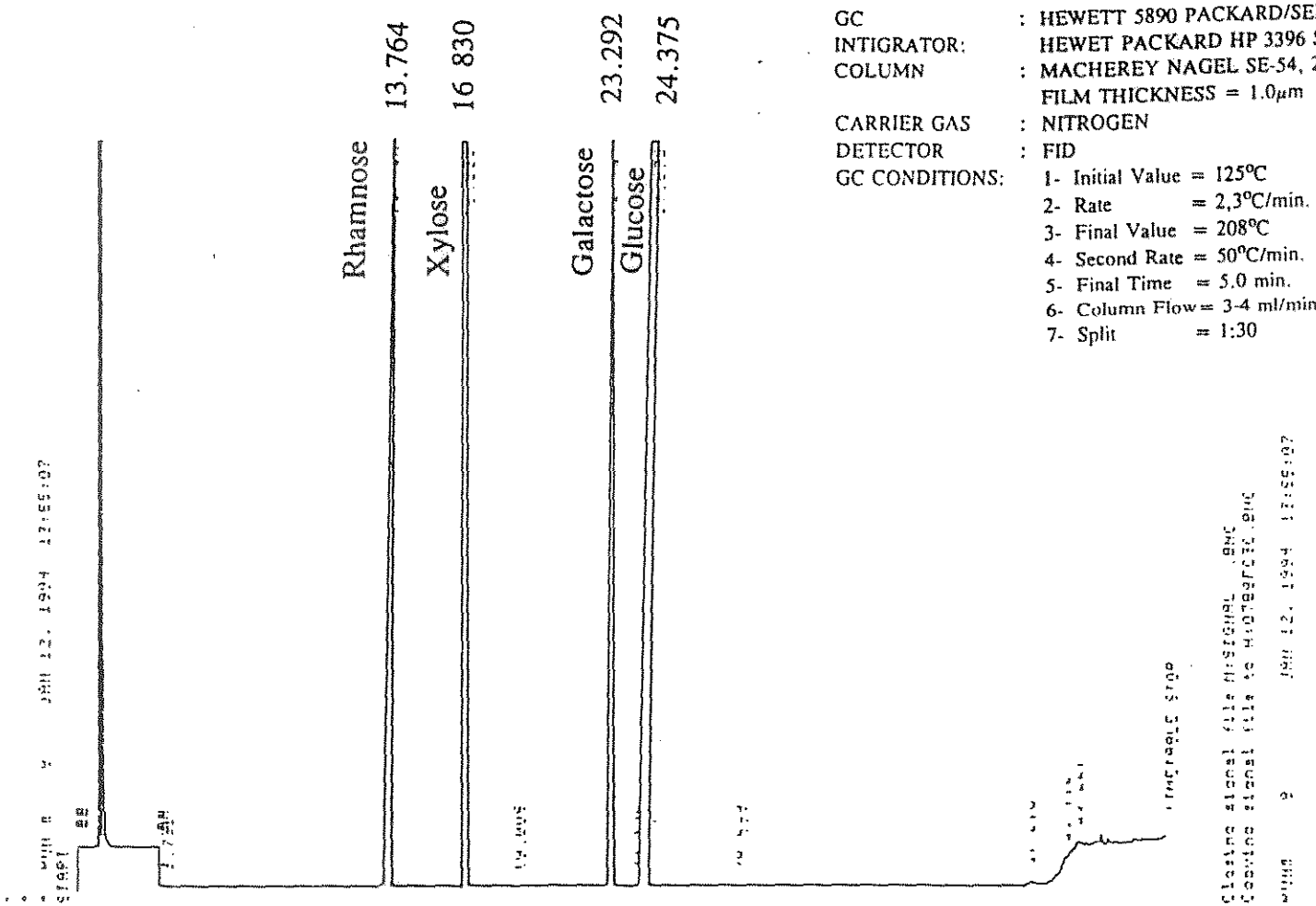
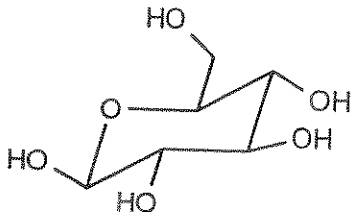
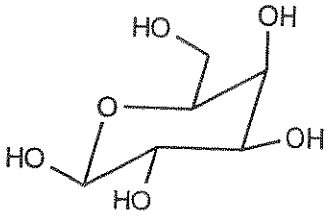


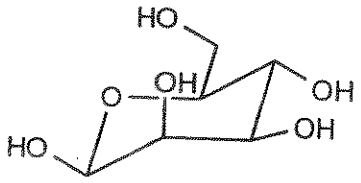
Figure-3. Gas chromatogram of standared sugars.



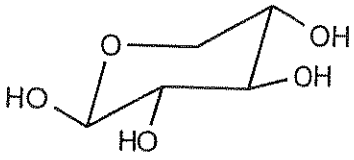
Glucose



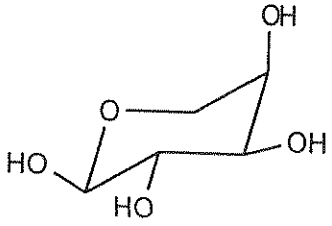
Galactose



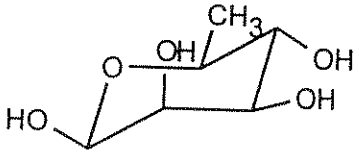
Mannose



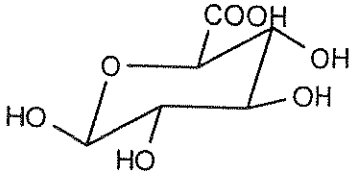
Xylose



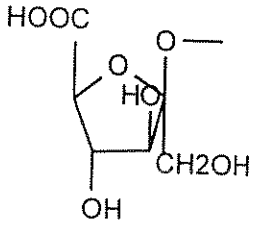
Arabinose



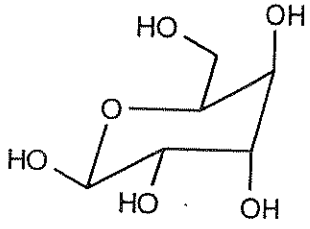
Rhamnose



Glucuronic acid



Fructose



Allose

Figure-4: Structure of common sugars.

CHAPTER 2

PHYTOCHEMICAL ANALYSIS OF RUMEX

CHAPTER 2

PHYTOCHEMICAL ANALYSIS OF *RUMEX*

CONTENTS

2.1 LITERATURE SURVEY OF THE GENUS *RUMEX*

A detailed literature survey was carried out to investigate the presence of different polyphenolic compounds in the genus *Rumex*. The survey revealed that all the plant species belonging to the genus *Rumex* are rich in flavonoids as well as in anthraquinones.

The C-flavonoids: vitexin, isovitexin, orientin and isoorientin have been isolated from *Rumex acetosa, R. cyprius* and *R. chalepensis* (Aritomi et al, 1965, Zhapu and Jiazhu, 1982, Masaru et al, 1986 and El-Fattah, 1989). The presence of flavonols such as quercetin, isoquercetin and their glycosides have been reported in *R. confertus*, *R. acetosa*, *R. dentatus*, *R. obtusifolius*, *R. Crispus*, *R. conglomeratus*, *R. japonicus*, *R. longifolius*, *R. hastatus* and *R. acetosella* (Horhammer and Volz, 1955, Bagrii and Mrivenchuk, 1964, Volkhonskaya *et al*, 1964, Aritomi *et al*, 1965, Bargman, 1972, Mukhamed and Chumbalov, 1979, Tiwari and Sinha, 1980, Tamano and Koketsu, 1982, Masaru *et al*, 1986 and El-Fattah, 1989). Alyukina *et al* (1969) and Tamano and Kokestu (1982) isolated kaempferol and their 3-O-substituted glycosides from *R. acetosa* and *R. rechingerianus*. Masaru *et al* (1986) reported the presence of a flavone 7-O-substituted leuteolin in *R. acetosella*. Bilbao and Rodriguez (1978) isolated naringenin a flavonone from *R. congomeratus*. Along with flavonid aglycones and flavonoid glycosides different types of anthocyanidins have also been reported by different authors. e,g. Hansel and Horhammer (1954) isolated pelargonidol from *R. trianschanicus*. The presence of cyanidol and delphinidol has also been reported in *R. trianschanicum* (Chumbalov and Mukhamed, 1962). A large number of anthraquinones such as emodin dianthrone, hyperin, emodin, emodin glycosides, emodin glycoside sulphates, chrysophanein, chrysophanol and aloe emodin have been isolated from *R. acetosa*, *R. acetosella*, *R. conglomeratus*, *R. confertus*, *R. nepalensis*, *R. pulcher*, *R. hastatus*, *R. chalepensis* and *R. dentatus* (Bagrii et al, 1964, Suri *et al*, 1976, Bhadoria and Gupta, 1977, Harborn and Mokhtari, 1977, Bilbao and Rodriguez, 1978, Paul *et al*, 1978, Mukhamed and Chumbalov, 1979, Tiwari and sinha, 1980 and Zhapu and Jiazhu, 1982).

2.2 PHYTOCHEMICAL ANALYSIS OF *RUMEX CHALEPENSIS*

2.2.1 NATURAL GLYCOSIDES

2.2.1.1 EXTRACTION

Flavonoid glycosides from leaves of *R. chalepensis* were obtained by extracting successively 2.5 kg of the air dried plant material, by ethanol and ethanol/water (as shown below) by stirring at room temperature, for 24 hours in each solvent.

1- Ethanol
2- Ethanol : Water (80:20)
3- Ethanol : Water (50:50)
4- Ethanol : Water (50:50) + heating

The combined hydroalcoholic extracts were evaporated between 30-35 °C under reduced pressure to remove the ethanol. To remove fats, terpenes, carotenoids and chloropyhll, the remaining aqueous phase was treated with chloroform (3x150 ml). The defatted aqueous layer was then repeatedly extracted with ethyl acetate (3x200 ml). The ethyl acetate extract was evaporated to dryness and the dried extract was found to be 10.12 gm.

2.2.1.2 SEPARATION AND PURIFICATION

The dried ethyl acetate extract was dissolved in about 10 ml of a water/methanol (95:5) mixture. It was loaded on to the top of a polyamide column (MN-polyamide SC 6, grain size <0.07 mm, Macherey Nagel, Germany). The column was run in the gradient mode from H_2O to MeOH according to the following chart.

POLYAMIDE COLUMN AND ELUTION CHART

Fr. No.	H_2O %	MeOH %	Fr. No.	H_2O %	MeOH %
	100	0	21	40	60
2	95	5	22	38	62
3	90	10	23	36	64
4	85	15	24	34	66
5	80	20	25	32	68
6	75	25	26	30	70
7	70	30	27	28	72
8	68	32	28	26	74
9	66	34	29	24	76
10	64	36	30	22	78
11	62	38	31	20	80
12	60	40	32	18	82
13	56	44	33	16	84
14	54	46	34	14	86
15	52	48	35	12	88
16	50	50	36	10	90
17	48	52	37	9	91
18	46	54	38	8	92
19	44	56	39	6	94
20	42	58	40	4	96

Composition of each fraction (after completely drying and then dissolving in small amount of MeOH) was monitored on silica gel precoated plates (Merck) which were developed in $CHCl_3$:MeOH:H_2O (13:3:2) solvent system.

Table.5 SPECTRAL AND CHROMATOGRAPHIC DATA OF GLYCOSIDES ISOLATED FROM *RUMEX CHALEPENSIS* LEAVES

UV λmax	RC- 1			RC-2				RC-3			RC-4				RC-5			
MeOH	257	327	355	265		315	351	257	299	362	245	265	301	351	211	275	274	377
NaOAc	274	327	382	274		318	384	274	325	374	271		322	377	211	257	268	388
$NaOAc/H_3BO_3$	260	297	377	268		320	354	262	298	377	260		300	372	214	257	277	377
$AlCl_3$	274	304	431	274	301	354	400	274	303	440	274	304	333	434	208	377	417	468
$AlCl_3$/HCl	268	303	402	275	299	348	400	268	300	400	271	303	352	394	208	377	417	467
NaOMe	273	327	408	274	301	339	397	271	327	410	268		326	391	211	257	268	391
FAB^+MS	m/z			m/z				m/z			m/z				m/z			
$[M+H]^+$	627			595				611			449				579			
$[M+H\text{-pentose}]^+$	-			449				465			303				-			
$[M+H\text{-hexose}]^+$	465			-				-			-				417			
$[M+H\text{-pentose-hexose}]^+$	303			287				303			-				-			
$[M+H\text{-hexose-pentose}]^+$	-			-				-			-				271			
AGLYCONE	Quercetin			Kaempferol				Quercetin			Quercetin				Emodin			
SUGARS	Galactose+Glucose			Galactose+Rhamnose				Glucose+Rhamnose			Rhamnose				Rhamnose+Glucose			
R_f x 100																		
BAW	51			77				35			76				87			
15% HOAc	29			30				57			52				68			
60% HOAc	68			76				72			74				82			
$CHCl_3$-MeOH-H_2O(12/4/1)	54			68				2			60				72			
C_6H_6-MEK-MeOH(25/10/11)	05			08				06			14				24			

As a result similar fractions were combined as follows for the further purification.

A =	Fraction No. 01 - 05	..... Four spots
B =	Fraction No. 0 5 -15	..... Two spots
C =	Fraction No. 16 - 20	..... Three spots
D =	Fraction No. 21 - 28	..... Two spots
E =	Fraction No. 29 - 33	..... Three spots
F =	Fraction No. 34 - 40	..... Three spots

Flavonoid glycosides present in each fraction were purified by a combination of PTLC on silica gel and CC on Sephadex LH-20. The four flavonoid glycosides were finally obtained in pure form. Purity of each isolated flavonoid glycoside was checked by HPLC using a C18 ODS (Zorbex) column in H_2O:MeOH:HOAc (80:16:4) as the solvent system in the isocratic mode.

1.	RC-1	=	16.0 mg
2.	RC-2	=	9.0 mg
3.	RC-3	=	35.0 mg
4.	RC-4	=	87.0 mg

2.2.1.3 IDENTIFICATION:

Identification of the four purified flavonoid glycosides was carried out by recording their R_f values in 3 solvent systems, acid hydrolysis to aglycones and sugars and spectroscopic techniques (UV, IR, EIMS and FAB^+MS). Results obtained are grouped in Table-5. where as data obtained by NMR spectroscopy [^1H NMR, ^{13}C (DEPT & B.B) NMR, ^1H, ^1H-COSY, ^1H, ^{13}C-COSY and NOE measurments] are presented in Table-6 to 19. Sugar identification was carried out by paper chromatography and Gas Chromatography.

COMPOUND RC-1

RC-1 flavonoid glycoside from *Rumex chalepensis* was obtained as a yellow crystalline solid. It appeared purple under UV (366 nm) light and turned yellow when exposed to NH_3 vapours which indicated it to be a 3-O-substituted flavonol (Markham, 1982). R_f values recorded after co-chromatography of RC-1 with authentic marker Rutin (Quercetin 3-O-α-L-rhamnopyranosyl(1→6)-ß-D-glucopyranoside) in solvent system H_2O:EtOH:MEK:Acetyl Acetone (13:3:3:1) suggested this compound to be a 3-O-substituted flavonol diglycoside (Hasan, 1976). In order to identify the aglycone and the sugar residue, compound RC-1 was subjected to acid hydrolysis.

Acid Hydrolysis:

3.0 mg of RC-1 was dissolved in 5 ml of MeOH:2N HCl (1:1) and refluxed on a water bath at 100 °C for one hour. After hydrolysis the aglycone was extracted by EtOAc while the sugar present in the aqueous extract was neutralized by Na_2CO_3.

Alkaline Hydrolysis:

On alkaline hydrolysis with 0.5% KOH, the compound RC-1 remained unchanged and no organic acid was detected, showing that it was not acylated.

Identification of Aglycone:

After complete evaporation of the ethyl acetate extract the aglycone was obtained as a yellow solid. It appeared yellow under UV (366 nm) light and remained yellow when exposed to NH_3 vapours, indicating it to be a flavonol. UV spectrum recorded in MeOH gave two absorption maxima at 370 nm (Band-I) and 259 nm (Band-II), diagnostic bands of a flavonol aglycone (Markham, 1982). Addition of NaOAc produced a bathochromic shift of 15 nm in band-II with respect to MeOH spectrum, showing the presence of a free

hydroxyl at postion 7 of ring A. A bathochromic shift of 15 nm was observed in band-I after the addition of H_3BO_3 in the methanolic solution of aglycone containing NaOAc. This bathochromic shift is typical for flavonols having a 3', 4'-orthodihydroxylation. A strong bathochromic shift of about 80 nm was observed in band-I when 5% alcholic solution of $AlCl_3$ was added to the methanolic solution of aglycone, indicating the formation of acid stable complex between 5 and or/3 OH of the keto group at 4-position, as well as acid labile complex with orthodihydroxy groups. A hypsochromic shift of 23 nm in band-I in the presence of $AlCl_3$/HCl as compared to $AlCl_3$ spectrum cofirmed 3', 4'-orthodihydroxylation in ring B. Addition of NaOMe caused a rapid decomposition of band-I, which is diagnostic for the presence of 3, 3', 4'-trihydroxy alkali sensitive system on the flavone nucleus.

EIMS of aglycone exhibited a molecular ion peak at m/z 302 (100%) along with other diagnostic fragment ions such as m/z 153$(A_1+H)^+$, 137$(B_2)^+$ and 109 (B_2^+-28). Mass fragmentation pattern of aglycone supported the penta hydroxy flavone with dihydroxylation on ring A and orthodihydroxylation at ring B (Harborn *et al*, 1975).

^{1}H NMR (CD_3OD, 300 MHz) recorded a doublet at δ 6.15 (1H, *d*, $J_{6,8}$= 2.0 Hz) corresponding to H-6 of ring A, showing a meta coupling with H-8 which resonated at δ 6.34 (1H, *d*, $J_{8,6}$= 2.0 Hz). A sharp doublet appeared at δ 7.35 (1H, *d*, $J_{2',6'}$=2.0 Hz) was assigned to H-2' of ring B, meta coupled with H-6'. A doublet with relatively large coupling constant observed at δ 6.90 (1H, *d*, $J_{5',6'}$=8.0 Hz) was due to H-5' of ring B having an ortho-coupling with H-6' which appeared as a doublet of a doublet at δ 7.29 (1H, *dd*, $J_{6',2',5'}$=2.0, 8.0 Hz) because of ortho-coupling with H-5' and a meta coupling with H-2' respectively. On the basis of co-chromatography, UV, EIMS and ^{1}H NMR data the structure of aglycone was identified as 5, 7, 3, 3', 4'-pentahydroxy flavone or Quercetin.

Identification of sugars:

The neutralized sugar extract was concentrated under reduced pressure on rotary evaporator and subjected to PC on Whatman No.1 along with standard sugars in five solvent systems: BAW, BEW, BTPW, EPAW and PhOH. The R_f values revealed the presence of glucose and galactose in RC-1. Silyl derivative of the sugars obtained from acid hydrolysis of RC-1 were prepared along with those of standared sugars for GC analysis. Retention times of the unknown sugars when compared to that of the standared sugars confirmed the results obtained by PC and also confirmed the presence of glucose and galactose in RC-1.

Identification of glycoside:

In order to determine the position of attachment of the sugars to the aglycone UV spectrum of flavonoid glycoside RC-1 was recorded in MeOH which showed two absorption bands at 359 nm and 257 nm. This range of absorption is typical for 3-O-substituted flavonol. Presence of a free hydroxyl group at position 7 of ring A was indicated by the addition of NaOAc which exhibited a 17 nm bathochromic shift in band II with respect to the methanol spectra. A bathochromic shift of 12 nm in the presence of $NaOAc/H_3BO_3$ showed the presence of 3', 4'-orthodihydroxylation. A bathochromic shift of 66 nm with 5% methanolic solution of $AlCl_3$ and a decrease of 29 nm after the addition of HCl indicated the presence of free hydroxyl group at position 5 of ring A and 3', 4'-orthodihydroxylation at ring B. UV spectra recorded in MeOH and on addition of diagnostic shift reagents suggested copmound RC-1 to be a 3-O-substituted flavonol with both the sugars (glucose and galactose) attached at position 3 of the aglycone.

The IR spectrum in KBr revealed intense absorption bands at 3392 cm^{-1} (free OH), 1652 cm^{-1} (conjugated carbonyl group), 1604-1447 cm^{-1} (aromatic rings) and 1073 cm^{-1} (C-O-C).

EIMS of RC-1 displayed a molecular ion peak for aglycone M^+ at m/z 302 (100%) corresponding to the aglycone part. Other diagnostic fragments were same as mentioned in the case of the aglycone. To determine the sugar sequence FABMS of RC-1 was recorded in positive mode in lactic acid, which showed a molecular ion peak at m/z 627 $(M+H)^+$. Other diagnostic fragments ions appeared at m/z 465 $(M+H-162)^+$ and m/z $(M+H-324)^+$ indicating successive loss of two hexoses.

^1H NMR (CD_3OD,300 MHz) of RC-1 showed the chemical shifts of two meta coupled protons of ring A i,e. H-6 and H-8 at δ 6.15 (1H, *d*, $J_{6,8}$=2.0 Hz) and δ 6.31 (1H, *d*, $J_{8,6}$=2.0 Hz) respectively. H-2', H-5' and H-6' of ring B resonated at δ 7.60 (1H, *d*, $J_{2',6'}$=2.0 Hz), δ 6.90 (1H, *d*, $J_{5',6'}$=8.0 Hz), δ 7.32 (1H, *dd*, $J_{6',2',5'}$=2.0, 9.0 Hz) respectively. A doublet appeared at δ 5.12 (1H, *d*, $J_{1'',2''}$=6.0 Hz) was assigned to the anomeric proton (C-1") of galactose, showing a ß-configuration, on the basis of ^1H, ^1H-COSY and ^1H, ^{13}C-COSY experiments. H-2" and H-3" resonated as double doublet at δ 3.56 (1H, *dd*, $J_{2'',1''}$6.0 , $J_{2'',3''}$8.0 Hz) and δ 3.36 (1H, *dd*, $J_{3'',2''}$=8.0, $J_{3'',4''}$=3.3 Hz) respectively. Chemical shift of H-4" was observed as broad doublet at δ 3.65 (1H, *br.d*, J3.2 Hz). H-5" and H-6"$_a$ appeared as multiplet at δ 3.33 (2H, m) while H-6"$_b$ resonated as double doublet at δ 3.46 (1H, *dd*, $J_{6''b,6''a}$ =9.5, $J_{6b'',5''}$=5.0 Hz). The signal observed at δ 5.20 (1H, *d*, $J_{1''',2'''}$=6.0 Hz) was attributed to anomeric proton of glucose having a β configuration. H-2"' resonated as double doublet at δ 3.55 (1H, *dd*, $J_{2''',1'''}$ =6.0, $J_{2''',3'''}$ =9.0 Hz). A triplet was observed for H-3"' and H-4"' at δ 3.50 (1H, *t*, J=9.0 Hz) and δ 3.40 (1H, *t*, J=9.4 Hz) respectively.H-5"' appeared as multiplet at δ 3.73 (1H, *m*). The H-6"'$_a$ resonated as double doublet at δ 3.85 (1H, *dd*, $J_{6'''a,6'''b}$=11.8, $J_{6'''a,5'''}$ =5.2 Hz) while H-6"'$_b$ appeared at δ 3.65 (1H, *dd*, $J_{6'''b,6'''a}$=11.8, $J_{6'''b,5'''}$=2.2 Hz).

In order to confirm the coupling interactions between different protons COSY-45^0 experiments were carried out . A strong cross peak was observed between H-6 (δ 6.15) and H-8 (δ 6.31) of ring A. While connectivities between H-2' (δ 7.60), H-5' (δ 6.90) and H-6' (δ 7.32) were established by this homonuclear technique. The coupling interactions between

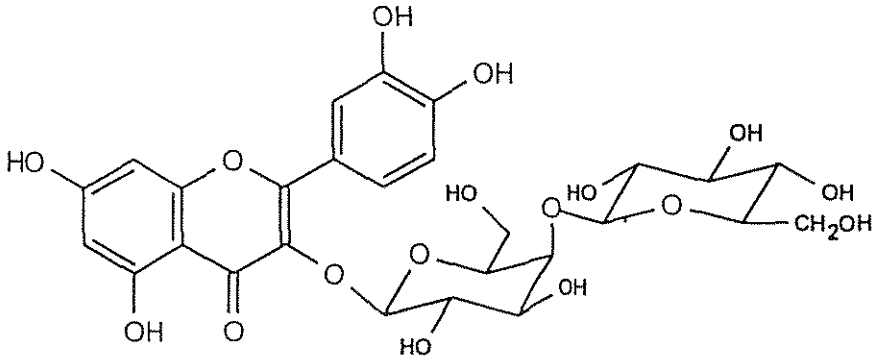
sugar protons were confirmed by measuring magnified ^{1}H, ^{1}H-COSY spectra of sugar part, which were in accord with those of glucose and galactose.

The ^{13}C NMR spectrum (CD_3OD, 75.5 MHz) of RC-1 showed 27 carbon resonances, presented in Table-8. The multiplicities were determined by (DEPT) experiments with the polarization angle $\theta = 45^{\circ}$, 90° and 135°. The assignments revealed the presence of 10 quaternary, 15 methine and 2 methylene carbons. C-2, C-3 and C-4 of ring C appeared at δ 158.2, 135.8 and 178.9 respectively, while the chemical shifts observed at δ 162.7, 101.3, 166.3 and 95.7 were attributed to C-5, C-6, C-7 and C-8 of ring A. The quaternary carbons C-9 and C-10 showed their signals at δ 158.9 and δ 104.5. The chemical shifts observed at δ 123.1, 116.6, 145.8, 150.0, 116.7 and δ 122.9 were assigned to C-1', C-2', C-3', C-4', C-5' and C-6 of ring B. A downfield shift of 9.9 ppm in C-2 and 2.2 ppm in C-4 while an upfield shift of 0.99 ppm in C-3 as compared to ^{13}C chemical shifts of quercetin (Teral and Markham, 1976) indicated the site of glycosylation at C-3 of ring-C. Two anomeric carbons belonging to galactose and glucose, exhibited their chemical shifts at δ 101.3 and δ 104.8, indicated their C-O-linkage to the aglycone and among themselves. The signals observed at δ 75.2, 77.1, 70.0, 78.1 and δ 62.5 were assigned to C-2''', C-3''', C-4''', C-5''' and C-6''' of glucose. While . C-2", C-3", C-4", C-5" and C-6" of galactose resonated at δ 71.2, 73.2, 78.3, 75.1 and δ 61.9. A downfield shift of 8.3 ppm in C-4" of galactose proved glucose to be the terminal sugar and its site of linkage to be C-4".

In order to established one-bond proton-carbon connectivities, hetero-COSY experiments were carried out . The hetero-COSY spectrum showed the carbon at δ 101.3 (C-6) was coupled with proton at δ 6.15 (C-6H). Similarly, the carbon resonated at δ 95.7 (C-8) showed cross peak with proton at δ 6.31 (C-8H), while the signals at δ 116.6 (C-2'), 116.7 (C-5') and 122.9 (C-6') were coupled with the protons at δ 7.60 (C-2'H), 6.90 (C-5'H) and 7.32 (C-6'H) respectively. The anomeric carbon of galactose (C-1") resonated at δ 101.3 showed a cross peak with the proton at δ 5.12 (C-1"-H) The signals at δ 71.2 (C-2"), 73.2 (C-3"), 78.3 (C-4"), 75.1 (C-5"), and δ 61.9 (C-6"), due to galactose carbons, were found to be coupled with their respective protons at δ 3.56 (C-2"H), 3.36 (C-3"H), 3.65 (C-

4"H), 3.33 (C-5"H, 6"$_a$H) and 3.46 (C-6"$_b$H). A cross peak for the anomeric carbon C-1"' of glucose, resonated at δ 104.8, was observed with the proton at δ 5.20 (C-1"'H). Other carbons of glucose resonated at δ 75.2 (C-2"'), 77.1 (C-3"'), 70.0 (C-4"'), 78.1 (C-5"'), and δ 62.5 (C-6"') showed cross peaks with the protons at δ 3.55 (C-2"' H), 3.50 (C-3"' H), 3.40 (C-4"' H), 3.73 (C-5"' H), 3.65 (C-6"'$_a$ H), and δ 3.85 (C-6"'$_b$ H). On the basis of chromatographic data, acid hydrolysis, UV, IR, Mass, ^{1}H NMR, ^{13}C (B.B & DEPT) NMR, ^{1}H, ^{1}H-COSY and ^{1}H, ^{13}C-COSY experiments the structure of RC-1 was identified as quercetin 3-O-β-D-glucopyranosyl(1→4)-β-D-galactopyranoside.

The structure of RC-1 was further confirmed by partial hydrolysis (Markham, 1982) which afforded quercetin 3-O-β-D-galactopyranoside and glucose. Structure of quercetin 3-O-β-D-galactopyranoside was confirmed by co-chromatography and UV spectral analysis.



Quercetin 3-O-β-D-glucopyranosyl(1→4)-β-D-galactopyranoside

Table.6 ^{1}H NMR SPECTRAL DATEA OF COMPOUND RC-1 (CD_3OD, 300 MHz)

H	(δ)	Multiplicity	J (Hz)
H-6	6.15	*d*	2.0
H-8	6.31	*d*	2.0
H-2'	7.60	*d*	2.0
H-5'	6.90	*d*	8.0
H-6'	7.32	*dd*	2.0/8.0
H-1"	5.12	*d*	6.0
H-2"	3.56	*dd*	8.0/6.0
H-3"	3.36	*dd*	8.0/3.0
H-4"	3.65	*br.d*	3.2
H-5"	3.33	*m*	-
H-6"$_a$	3.33	*m*	-
H-6"$_b$	3.46	*dd*	9.5/5.0
H-1"'	5.20	*d*	6.0
H-2"'	3.55	*dd*	9.0/6.0
H-3"'	3.50	*t*	9.0
H-4"'	3.40	*t*	9.4
H-5"'	3.73	*m*	-
H-6"'$_a$	3.65	*dd*	11.8/2.2
H-6"'$_b$	3.85	*dd*	11.8/5.2

Table.7 ^{1}H NMR AND COSY 45° SPECTRAL DATA OF COMPOUND RC-1 (CD_3OD, 300 MHz)

H	(δ)	Multiplicity	Coupled to H (δ)
H-6	6.15	*d*	H-8 (6.31)
H-8	6.31	*d*	H-6 (6.15)
H-2'	7.60	*d*	H-6'(7.32)
H-5'	6.90	*d*	H-6'(7.32)
H-6'	7.32	*dd*	H-2'(7.60), H-5'(6.90)
H-1"	5.12	*d*	H-2"(3.56)
H-2"	3.56	*dd*	H-1"(5.12), H-3"(3.36)
H-3"	3.36	*dd*	H-1"(5.12), H-2"(3.56), H-4" (3.65)
H-4"	3.65	*br.d*	H-3"(3.36), H-5"(3.33)
H-5"	3.33	*m*	H-4"(3.65), H-6"$_a$(3.33),H-6"$_b$(3.46)
H-6"$_a$	3.33	*m*	H-5"(3.33), H-6"$_b$(3.46)
H-6"$_b$	3.46	*dd*	H-5"(3.33), H-6"$_a$(3.33)
H-1"'	5.20	*d*	H-2"'(3.55)
H-2"'	3.55	*dd*	H-1"'(5.20), H-3"'(3.50)
H-3"'	3.50	*t*	H-1"'(5.20), H-2"'(3.55), H-4"'(3.40)
H-4"'	3.40	*t*	H-3"(3.50), H-5"'(3.73)
H-5"'	3.73	*m*	H-4"'(3.40),H-6"'$_a$(3.65)H-6"'$_b$(3.85)
H-6"'$_a$	3.65	*dd*	H-5"'(3.73), H-6"'$_b$(3.85)
H-6"'$_b$	3.85	*dd*	H-5"'(3.73), H-6"'$_a$(3.65)

Table.8 DEPT MULTIPLICITIES, ^{13}C NMR (CD_3OD, 75.5 MHz), ONE BOND ^{1}H, ^{13}C-NMR CONNECTIVITIES AND J_{HH}(Hz) OF COMPOUND RC-1

C	DEPT Multiplicity	^{13}C NMR (δ)	^{1}H NMR (δ)	J_{HH}(Hz)
C-2	-C-	158.2	-	-
C-3	-C-	135.8	-	-
C-4	-C-	175.9	-	-
C-5	-C-	162.7	-	-
C-6	CH	101.3	6.15	*d* (2.0)
C-7	-C-	166.0	-	-
C-8	CH	95.7	6.31	*d* (2.0)
C-9	-C-	158.9	-	-
C-10	-C-	104.5	-	-
C-1'	-C-	123.1	-	-
C-2'	CH	116.0	7.66	*d* (2.0)
C-3'	-C-	145.8	-	-
C-4'	-C-	150.0	-	-
C-5'	CH	116.7	6.90	*d* (8.0)
C-6'	CH	122.9	7.32	*dd* (2.0/8.0)
C-1"	CH	101.3	5.12	*d* (6.0)
C-2"	CH	71.2	3.56	*dd* (8.0/6.0)
C-3"	CH	73.2	3.36	*dd* (8.0/3.0)
C-4"	CH	78.3	3.65	*br.d* (3.2)
C-5"	CH	75.1	3.33	*m*
C-6"$_a$	CH_2	61.9	3.33	*m*
C-6"$_b$	CH_2	61.9	3.46	*dd* (9.5/5.0)
C-1'"	CH	104.8	5.20	*d* (6.0)
C-2'"	CH	75.2	3.55	*dd* (9.0/6.0)
C-3'"	CH	77.1	3.50	*t* (9.0)
C-4'"	CH	70.0	3.40	*t* (9.4)
C-5'"	CH	78.1	3.73	*m*
C-6'"$_a$	CH_2	62.5	3.65	*dd* (11.8/2.2)
C-6'"$_b$	CH_2	62.5	3.85	*dd* (11.8/5.2)

COMPOUND RC-2

Flavonoid glycoside RC-2 was obtained as a yellow crystalline solid. It appeared purple under UV (366 nm) light and turned yellow in NH_3 vapours, which suggested it to be a 3-O-substituted flavonol. Mobilitiy of RC-2, along with standared compounds in solvent system H_2O:EtOH:MEK:Acetyl acetone (13:3:3:1) indicated it to be a 3-O-substituted flavonoid diglycoside.

Acid hydrolysis:

For the identification of the aglycone part and the sugar moieties, 3.0 mg of RC-2 were subjected to acid hydrolysis following the same procedure as described for compound RC-1.

Alkaline hydrolysis:

On alkaline hydrolysis RC-2 remained unchanged and no organic acid was detected, showing that it was not an acylated flavonoid glycoside.

Identification of aglycone:

After acid hydrolysis, the aglycone was obtained as yellow solid. It appeared dull yellow under UV (366 nm) light and remained unchanged in NH_3 vapours, suggested it to ba a flavonol. Its UV spectrum in MeOH exhibited two absorption bands at 367 nm (band-I) and 266 nm (band-II), indicating it to be a flavonol aglycone. A bathochromic shift of 8.0 nm was obsrved in band-II by the addition of NaOAc with respect to MeOH spectrum, indicating the presence of a free hydroxyl group at position 7 of ring A. Addition of H_3BO_3 in the methanolic solution of the aglycone, containing NaOAc, produced a non-significant bathochromic shift (3.0 nm) in band-I indicating the absence of orthodihydroxylation in ring B. A prominent bathochromic shift of 57 nm was observed in band-I when 5%

alcoholic solution of $AlCl_3$ was added to the methanolic solution of the aglycone, indicating the formation of complex between 5 and/or 3-hydroxyl and the keto group at 4-position. No hypsochromic shift was observed in band-I on the addition of HCl in the methanolic solution of the aglycone containing $AlCl_3$, which confirmed the presence of free hydroxyl groups at position 5 and 3 and the absence of orthodihydroxylation at ring B (Markham, 1982). A bathochromic shift of 49 nm in band-I with an increase in intensity with respect to the methanolic spectrum of the aglycone was observed after the addition of NaOMe. Slow degeneration of the spectrum after 5 minutes suggested the presence of 3, 4' hydroxyl grouping in the aglycone nucleus.

In EIMS a molecular ion peak for the aglycone was observed at m/z 286. The other important fragment ions appeared at m/z 153 $(A_1+H)^+$, 121 $(B_2)^+$ and 93 (B_2^+-28). This typical fragmentation pattern of the aglycone suggested it to be a 3, 5, 7, 4′-tetrahydroxy flavone.

^{1}H NMR (CD_3OD, 300 MHz) spectrum of the aglycone exhibited two doublets at δ 6.05 (1H, *d*, $J_{6,8}$=2.0 Hz) and δ 6.20 (1H, *d*, $J_{8,6}$=2.0 Hz) representing absorption due to two meta coupled protons i,e. H-6 and H-8 of ring A. Chemical shift for H-3' and H-5' was observed at δ 6.89 (2H, *d*, $J_{2',3'}$, $J_{5',6'}$= 8.0 Hz) showing an ortho coupling with H-2' and H-6' which resonated at δ 8.09 (2H, *d*, $J_{3',2'}$, $J_{6',5'}$= 8.0 Hz) respectively. On the basis of co-chronatography, UV, EIMS and ^{1}H NMR data the aglycone part of the compound RC-2 was identified as 3, 5, 7, 4'-terahydroxy flavone or kaempferol.

Identification of sugars:

The nuteralized aqueous sugar extract , obtained after the acid hydrolysis of RC-2, was subjected to PC on Whatman paper No.1 along with standared sugars. The chromatogram was developed in five solvent system: BAW, BTPW, EPAW and PhOH. Rhamnose and galactose were identified after making a comaprison of R_f values of the RC-

2 sugars with those of standared sugars. GC analysis of the nutralized aqueous sugars extract was performed which also confirmed the results of PC.

Identification of glycoside:

In order to investigate the position of the attachment of the sugars to the aglycone UV spectrum of flavonoid glycoside RC-2 was measured in MeOH which displayed two absorption maxima at 351 nm (band-I) and 265 nm (band-II). The range of absorption bands suggested it to be a 3-O-substitiuted flavonol. Addition of NaOAc to the methanolic solution of RC-2 caused a bathochromic shift of 9.0 nm in band-II as compared to MeOH spectrum, indicated the presence of a free 7-hydroxyl group at position 7 of ring A. A non-significant bathochromic shift of 2.0 nm in band-I showed the absence of dihydroxylation in ring B. On addition of 5% methanolic solution of $AlCl_3$ a pronounced bathochromic shift of 49.0 nm was observed in band-I, which indicated the presence of free hydroxyl group at position 5 of ring A. Addition of HCl to the methanolic solution of RC-2,containing $AlCl_3$, did not show any hypsochromic shift in band-I as compared to $AlCl_3$ spectrum, which confirmed the absence of dihydroxylation at ring B. A bathochromic shift of 46 nm, in the presence of NaOMe, suggested the presence of free 4'-OH at ring B. UV spectra of RC-2 recorded in MeOH and on addition of diagnostic shift reagents suggested compound RC-2 to ba a 3-O-substituted flavonol with both the sugars (rhamnose and galactose) attached at position 3 of the aglycone.

IR spectrum recorded in KBr showed important absorption bands at 3432 cm^{-1} (free OH), 1652 cm^{-1} (Conjugated carbonyl), 1574-1447 cm^{-1} (aromatic ring) and 1080 cm^{-1} (C-O-C).

EIMS of RC-2 exhibited a molecular ion peak for aglycone at m/z 286 (100%) which could be attributed to the aglycone.The other important fragments were identical to those mentioned in the case of aglycone. The sugar sequence was deduced by FABMS recorded in positive mode in lactic acid. A molecular ion $(M+H)^+$ was observed at m/z 595.

The other important fragment ions appeared at m/z 449 $(M+H-146)^+$ and m/z 287 $(M+H-308)^+$, showing the sequential loss of a pentose (rhamnose) and hexose (galactose) respectively. The mass fragmentation pattern obtained after FAB^+MS confirmed the terminal position of rhamnose.

^1H NMR (CD_3OD, 300 MHz) of RC-2 showed two set of doublets at δ 6.10 (1H, *d*, $J_{6,8}$=2.0 Hz) and 6.25 (1H, *d*, $J_{6,8}$=2.0 Hz) corresponding to H-6 and H-8 of ring A. The small value of coupling constant indicated that they were at meta position with each other. H-2', H-6' and H-3', H-5' resonated at δ 8.09 (2H, *d*, $J_{2',3'}$, $J_{6',5'}$=9.0 Hz) and 6.90 (2H, *d*, $J_{3',2'}$, $J_{5',6'}$=9.0 Hz) respectively. Chemical shift of two anomeric protons, belonging to galactose and rhamnose, was observed at δ 5.03 (1H, *d*, $J_{1'',2''}$=6.0 Hz) and 5.38 (1H, *d*, $J_{1''',2'''}$=2.0 Hz) showing a β and α configuration. H-2'' and H-3'' of galactose appeared at δ 3.65 (1H, *dd*, $J_{2'',1''}$=6.0, $J_{2'',3''}$=8.0 Hz) and 3.42 (1H, *dd*, $J_{3'',2''}$=8.0, $J_{3'',4''}$=3.4 Hz) respectively. Chemical shifts of H-4'' and H-5'' were observed at δ 3.80 (1H, *br.d*, J=3.20 Hz) and 3.33 (1H, *m*) while H-6''a appered at δ 3.33 (1H, m). H-$6''_b$ resonated as double doublet at δ 3.46 (1H, *dd*, $J_{6''b,6''a}$=9.5, $J_{6''b,''5}$=5.0 Hz). Protons belonging to rhamnose i,e. H-2''' and H-3''' showed their chemical shifts as double doublets at δ 3.90 (1H, *dd*, $J_{2''',1'''}$=2.0, $J_{2''',3'''}$ 6.0 Hz) and 3.65 (1H, *dd*, $J_{3''',2'''}$ =2.0, $J_{3''',4'''}$ =10.0 Hz). A triplet at δ 3.43 (1H, *t*, $J_{4''',3''',5'''}$ =10.0 Hz) and a multiplet at δ 3.0-3.7 (1H, *m*) was observed for H-4''' and H-5''' respectively. Three protons of CH_3 appeared as doublet at δ 0.95 (3H, *d*, $J_{6''',5'''}$=6.0 Hz).

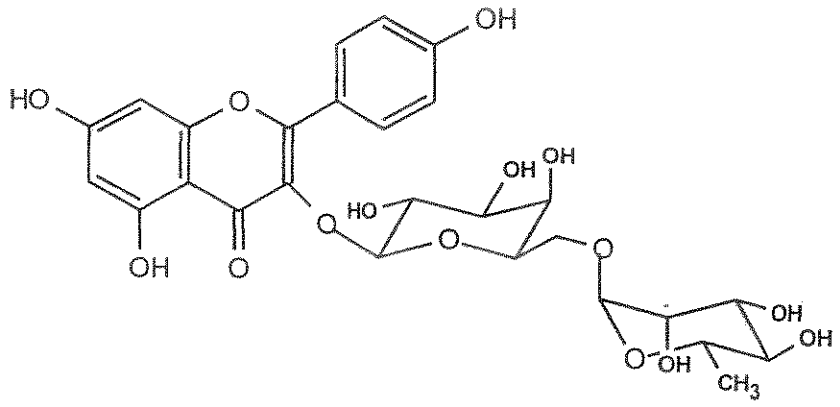
COSY 45° experiments were carried out to confirm the coupling interactions between different protons present in RC-2. A strong cross peak was observed between H-6 (δ 6.10) and H-8 (δ 6.25) of ring A. While off diagonal peaks appeared at δ 6.90 and 8.09 confirmed the ortho-couplings between H-3' and H-5' and H-2' , H-6' respectively. Proton-proton decoupling and ^1H, ^1H-COSY experiments led to the assignments of all proton resonances for the two sugars, which were in accord with those of galactose and rhamnose (Table-10).

^{13}C (DEPT) NMR (CD_3OD), 75.5 MHz) showed 27 signals, representing 9 quaternary, 6 methine, one methylene and one methyl carbon atom. Three quaternary

carbons i,e. C-2, C-3 and C-4 belonging to C-ring of the aglycone, appeared at δ 157.7, 135.2 and 178.5 respectively. The shielding for the C-3 (δ 135.2) and deshielding for both C-4 (δ 178.5) and the C-2 (δ 157.7) as compared to the ^{13}C chemical shift of kaempferol (Agrawal, 1981) indicated glycosylation at the C-3 position. C-5, C-6, C-7 and C-8 of ring A resonated at δ 162.1, 102.5, 166.0 and δ 96.0 respectively. Signals appeared at δ 122.6 and δ 162.0 were assigned to two quaternary carbons i,e. C-1' and C-4' of ring B. C-2' and C-6' resonated at δ 132.0 because of the same chemical environment. Similarly C-3' and C-5' exhibited their resonances at δ 116.4. The anomeric carbon belonging to galactose and rhamnose showed their resonences at δ 102.5 and 101.7 indicating their C-O-linkage to the aglycone part and with each other. The chemical shifts observed at δ 72.1, 71.9, 72.3 and 69.0 were sssigned to C-2"', C-3"', C-4"' and C-5"' of rhamnose. CH_3 of rhamnose resonated at δ 17.7. ^{13}C-chemical shift for galactose carbons i,e. C-2", C-3", C-4", C-5" and C-6" were observed at δ 71.9, 73.2, 69.0, 66.9. A down field shift of 5.0 ppm in C-6" of galactose proved rhamnose to be the terminal sugar and its site of attachment to be at C-6".

On the basis of chromatographic data, acid hydrolysis, UV, IR, Mass, ^{1}H NMR, ^{13}C (B.B & DEPT) NMR, and ^{1}H, ^{1}H-COSY experiments the structure of RC-2 was identified as kaempferol 3-O-α-L-rhamnopyranosyl(1→6)-β-D-galactopyranoside.

The structure of RC-2 was further confirmed by partial hydrolysis which afforded kaempferol 3-O-β-D-galactopyranoside and rhamnose. Structure of kaempferol 3-O-β-D-galactopyranoside was confirmed by co-chromatography and UV spectral analysis.



Kaempferol 3-O-α-L-rhamnopyranosyl(1→6)-β-D-galactopyranoside

Table.9 ^{1}H NMR SPECTRAL DATA OF COMPOUND RC-2 (CD_3OD, 300 MHz)

H	(δ)	Multiplicity	J (Hz)
H-6	6.10	*d*	2.0
H-8	6.25	*d*	2.0
H-2'	8.09	*dd*	9.0
H-3'	6.90	*dd*	9.0
H-5'	6.90	*dd*	9.0
H-6'	8.09	*dd*	9.0
H-1"	5.03	*d*	6.0
H-2"	3.65	*dd*	6.0, 8.0
H-3"	3.42	*dd*	8.0, 3.4
H-4"	3.80	*br.d*	3.20
H-5"	3.33	*m*	-
H-6"$_a$	3.33	*m*	-
H-6"$_b$	3.46	*dd*	9.5, 5.0
H-1"'	5.38	*d*	2.0
H-2"'	3.90	*dd*	2.0, 6.0
H-3"'	3.65	*dd*	2.0, 10.0
H-4"'	3.43	*t*	10.0
H-5"'	3-3.7	*m*	-
CH_3	0.90	*d*	6.0

Table.10 ^{1}H NMR AND COSY 45° SPECTRAL DATA OF COMPOUND RC-2 (CD_3OD, 300 MHz)

H	(δ)	Multiplicity	Coupled to H (δ) COSY 45°
H-6	6.10	*d*	H-8 (6.25)
H-8	6.25	*d*	H-6 (6.10)
H-2'	8.09	*dd*	H-3' (6.90), H-6' (8.09)
H-3'	6.90	*dd*	H-2' (8.09), H-5' (6.90)
H-5'	6.90	*dd*	H-6' (8.09), H-3' (6.90)
H-6'	8.09	*dd*	H-5' (6.90), H-2' (8.10)
H-1"	5.03	*d*	H-2" (3.65)
H-2"	3.65	*dd*	H-1" (5.03), H-3" (3.42)
H-3"	3.42	*dd*	H-1" (5.03). H-2" (3.65), H-4" (3.80)
H-4"	3.80	*br.d*	H-3" (3.42), H-5" (3.33)
H-5"	3.33	*m*	H-4" (3.80), H-6" (3.33), H-6"$_a$ (3.46)
H-6"$_a$	3.33	*m*	H-5" (3.33), H-6"$_b$ (3.46)
H-6"$_b$	3.46	*dd*	H-5" (3.33), H-6"$_a$ (3.33)
H-1"'	5.38	*d*	H-2"' (3.90)
H-2"'	3.90	*dd*	H-1"' (5.38), H-3"' (3.65)
H-3"'	3.65	*dd*	H-1"' (5.38), H-2"' (3.90), H-4"' (3.43)
H-4"'	3.43	*t*	H-3"' (3.65), H-5"' (3.49)
H-5"'	3-3.7	*m*	H-4" (3.43), H-6"' (0.95)
H-6"'	0.95	*d*	H-5"' (3-3.7)

Table.11 ^{13}C (DEPT) NMR SPECTRAL DATA OF COMPOUND RC-2 (CD_3OD, 75.5 Hz)

C	Multiplicity	(δ)
C-2	-C-	157.7
C-3	-C-	135.2
C-4	-C-	178.5
C-5	-C-	162.1
C-6	CH	102.5
C-7	-C-	166.0
C-8	CH	96.0
C-9	-C-	159.1
C-10	-C-	105.1
C-1'	-C-	122.6
C-2'	CH	132.0
C-3'	CH	116.4
C-4'	-C-	162.0
C-5'	CH	116.4
C-6'	CH	132.0
C-1"	CH	102.5
C-2"	CH	71.9
C-3"	CH	73.2
C-4"	CH	69.0
C-5"	CH	75.7
C-6"	CH	66.9
C-1"'	CH	101.7
C-2"'	CH	72.1
C-3"'	CH	71.9
C-4"'	CH	72.3
C-5"'	CH	69.0
C-6"'	CH_3	17.7

COMPOUND RC-3

Compound RC-3 was obtained as a pale yellow crystalline solid. Under UV (366 nm) it appeared violet and turned yellow in NH_3 vapours, indicating it to be a 3-O-substituted flavonol. Mobility of RC-3 along with standared flavonoid glycoside in solvent system H_2O:EtOH:MEK:Acetyl acetone (13:3:3:1) suggested it to be a 3-O-substituted diglycoside.

Acid hydrolysis:

In order to find out the aglycone part and the sugar moities, RC-3 was subjected to acid hydrolysis following the same procedure as described under the compound RC-1.

Alkaline hydrolysis:

The same method was adopted for alkaline hydrolysis as described for compound RC-1. The compound RC-3 remained unchanged and no organic acid was detected, indicating it to be a non acylated flavonoid glycoside.

Identification of aglycone:

The aglycone was identified as quercetin with the help of UV, ^{1}H NMR and EIMS, as describerd for compound RC-1.

Identification of sugars:

Glucose and rhamnose were identified as sugar moities by making use of PC and GC, as described for compound RC-1.

Identification of glycoside:

UV spectrum of RC-3 was recorded in spectral grade MeOH which exhibited two absorption maxima at 362 nm and 257 nm indicating it to be a 3-O-substituted flavonol. Addition of NaOAC showed a bathochromic shift of 17 nm in band-II as compared to methanolic spectrum indicating a free -OH group at position 7 of ring A. 12 nm bathochromic shift of band-I in the presence of $NaOAc/H_3BO_3$ indicated the presence of 3', 4'-orthodihydroxylation. A bathochromic shift of 78 nm was observed in band I with 5% methanolic solution of $AlCl_3$ which decreased by 40 nm after addition of HCl, giving a clear indication of orthodihydroxylation on ring B. Addition of NaOMe revealed a bathochromic shift of 38 nm in band-I with an increased intensity showing the presence of free hydroxyl group at 4' position of ring B. UV spectra of RC-3 recorded in MeOH and on addition of diagnostic shift reagents suggested it to be a 3-O-substituted flavonol with both the sugars i.e. glucose and rhamnose, attached at position 3 of the aglycone.

IR spectrum in KBr displayed absorption bands at 3401 cm^{-1} (free OH), 1656 cm^{-1} (conjugated carbonyl group). Other bands were observed at 1606-1455 cm^{-1} (aromatic ring) and 1067 (C-O-C) cm^{-1}.

EIMS exhibited a molecular ion peak for the aglycone at m/z 302 (100%). Other diagnostic fragments appeared at m/z 153 $(A_1+H)^+$, 137 $(B_2)^+$ and 109 (B_2-28) indicating a pentahydroxy flavone with dihydroxylation at ring A and orthodihydroxylation at ring B. FAB^+MS showed a molecular ion peak at m/z 611 $(M+H)^+$. The fragment ion at m/z 465 $(M+H-146)^+$ amd 303 $(M+H-308)^+$ indicated successive loss of rhamnosyl and glucosyl moiety. FABMS confirmed rhamnose to be the terminal sugar.

^1H NMR in CD_3OD (300 MHz) displayed two anomeric protons at δ 5.17 (1H, *d*, $J_{1'',2''}$= 7.0 Hz) and 4.53 (1H, *d*, $J_{1''',2'''}$= 2.0 Hz) indicating ß and α configuration of two sugars. Other sugar protons appeared in the range of 3.20 to 4.30 ppm. A signal at δ 1.12 (3H, *d*, $J_{6''',5'''}$= 7.0 Hz) was observed for rhamnosyl CH_3 protons. H-6 of ring A appeared at

δ 6.18 (1H, *d*, $J_{6,8}$= 2.0 Hz) showing meta coupling with H-8 which resonated at δ 6.35 (1H, *d*, $J_{8,6}$= 2.0 Hz). H-5' of ring B resonated at δ 6.68 (1H, d, $J_{5',6'}$= 8.0 Hz) showing an ortho coupling with H-6' which appeared at δ 7.62 (1H, *dd*, $J_{6',2'}$= 2.0, $J_{6',5'}$= 8.0 Hz). A signal for H-2' was observed at δ 7.65 (1H, *d*, $J_{2',6'}$= 2.0 Hz).

^{1}H, ^{1}H-COSY spectra showed the connectivities between different protons present in RC-3. A cross peak was observed between H-6 and H-8 of ring A which confirmed the presence of meta coupling between them. Similarly cross peaks were observed at 6.68, 7.62 and 7.65 showing connectivities between H-5'; H-6' and H-2' respectively. ^{1}H, ^{1}H-COSY experiment also revealed the connectivities of sugar protons (Table-13).

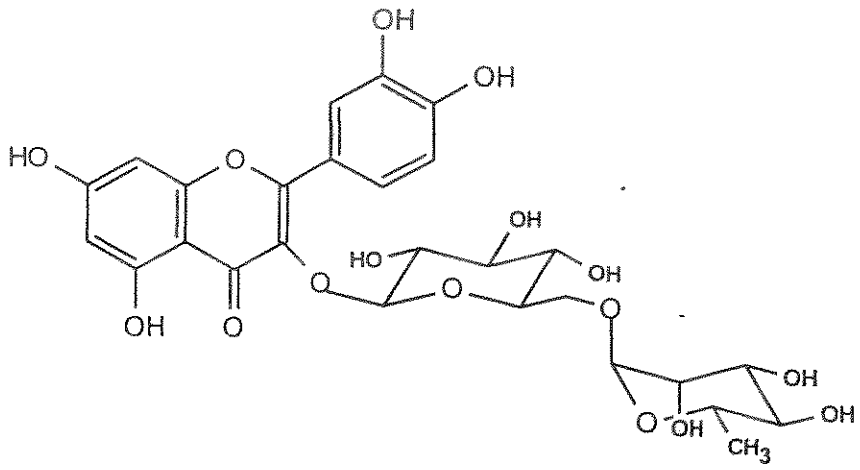
^{13}C NMR (DEPT) recorded total 27 signals. 10 of these belonged to quaternary carbons, 15 to methine, one to methylene and one to methyl carbon atom. Chemical shifts of C-2, C-3 and C-4 of aglycone appeared at δ 157.6, 135.6 and 178.5. C-5, C-6, C-7 and C-8 of ring A resonated at δ 162.2, 99.6, 165.6 and 94.5 respectively. The carbon atoms belonging to the ring B of the aglycone exhibited their resonances at δ 122.7, 117.8, 146.5, 150.4, 116.2 and 122.3. ^{13}C NMR shifts of the aglycone corresponded well to the chemical shifts of quercetin, the only significant difference being a downfield shift of 9.30 ppm for C-2, a downfield shift of 2.30 ppm for C-4 and an upfield shift of 1.10 ppm for C-3 with respect to the chemical shift of quercetin (Teral and Markham, 1976) showing the only site of glycosylation at C-3 position.

The two anomeric carbons of glucose and rhamnose appeared at δ 104.8 and 102.5 showing an O-linkage between sugar moiety and aglycone. C-2", C-3", C-4", C-5" and C-6" of the glucose resonated at δ 75.6, 78.5, 71.1, 77.4 and 68.4 while carbon atoms of rhamnose appeared at δ 72.3, 71.9, 73.8, 69.5 and 18.4 respectively. C-6" of glucose appeared about 7.4 ppm downfield indicating the attachment of terminal sugar i,e rhamnose at C-6" position. So ^{13}C NMR established a 1→6 linkage between rhamnose and glucose. Further confirmation of sugar linkage was established by NOE experiments. Each sugar proton was irriadiated one by one and its effect on other sugar protons was recorded.

Irridiation of H-6" exhibited a marked effect on H-1'" and vice virsa which is the clear indication of 1→6 linkage between rhamnose and glucose.

^{1}H, ^{13}C-COSY experiment confirmed the connectivities between protons and respected carbons of the compound RC-3. The hetero-COSY spectrum showed the carbon at δ 99.6 (C-6) was coupled at δ 6.18 (C-6H). The carbon resonated at δ 94.5 (C-8) showed cross peak with proton at δ 6.35 (C-8H) while the signals at δ 117.8 (C-2'), 116.2 (C-5') and 122.3 (C-6') were found to becoupled with the protons at δ 7.65 (C-2'H), 6.68 (C-5'H) and 7.62 (C-6'H) respectively. The anomeric carbon of glucose (C-1") resonated at δ 104.8, showed a cross peak with the proton at δ 5.17 (C-1"H). The signals at δ 75.6 (C-2"), 78.5 (C-3"), 71.1 (C-4"), 77.4 (C-5") and 68.4 (C-6") due to glucose carbons, coupled with their respected protons at δ 3.54 (C-2"H), 3.55 (C-3"H), 3.40 (C-4"H), 3.30 (C-5"H), 3.22 (C-$6"_{a}$H) and 3.60 (C-$6"_{b}$H). The connectivity of the anomeric carbon of rhamnose, resonated at δ 102.5, with the anomeric proton at δ 4.53 (C-1'") was established on the basis of a cross peak between these two nuclei. The signals at δ 72.3 (C-2'"), 71.9 (C-3'"), 73.8 (C-4'"), 69.5 (C-5'") and 18.4 (C-6'"), due to rhamnose carbons, were found to be coupled with their corresponded protons at δ 3.69 (C-2'"H), 3.65 (C-3'"H), 3.43 (C-4'"H), 3.49 (C-5'"H) and 1.12 (C-6'"H).

On the basis of acid hydrolysis, UV, IR, Mass, ^{1}H NMR, ^{13}C (B.B & DEPT) NMR, ^{1}H, ^{1}H-COSY and ^{1}H, ^{13}C-COSY experiments the structure of RC-3 was identified as quercetin 3-O-α-L-rhamnopyranosyl(1→6)-ß-D-glucopyranoside.



Quercetin 3-O-α-L-rhamnopyranosyl(1→6)-ß-D-glucopyranoside.

Table.12 ^{1}H NMR SPECTRAL DATA OF COMPOUND RC-3

(CD_3OD, 300 MHz)

H	(δ)	Multiplicity	J (Hz)
H-6	6.16	*d*	2.0
H-8	6.35	*d*	2.0
H-2'	7.65	*d*	2.0
H-5'	6.68	*d*	8.0
H-6'	7.62	*dd*	8.0/2.0
H-1"	5.17	*d*	7.0
H-2"	3.54	*dd*	9.3/7.0
H-3"	3.55	*t*	9.0
H-4"	3.40	*t*	9.4
H-5"	3.30	*m*	-
H-6"$_a$	3.22	*dd*	12.5/2.2
H-6"$_b$	3.60	*dd*	12.5/5.2
H-1"'	4.53	*d*	2.0
H-2"'	3.69	*dd*	5.0/2.0
H-3"'	3.65	*dd*	10.0/2.0
H-4"'	3.43	*t*	10
H-5"'	3.49	*m*	-
CH_3	1.12	*d*	7.0

Table.13 ^{1}H NMR AND COSY 45° SPECTRAL DATA OF COMPOUND RC-3 (CD_3OD, 300 MHz)

H	(δ)	Multiplicity	Coupled to H (δ)
H-6	6.18	*d*	H-8 (6.35)
H-8	6.35	*d*	H-6 (6.18)
H-2'	7.65	*d*	H-6'(7.62)
H-5'	6.68	*d*	H-6'(7.62)
H-6'	7.62	*dd*	H-2' (7.65), H-5'(6.68)
H-1"	5.17	*d*	H-2" (3.54)
H-2"	3.54	*dd*	H-1"(5.17), H-3"(3.55)
H-3"	3.55	*dd*	H-1"(5.17), H-2"(3.54), H-4"(3.40)
H-4"	3.40	*dd*	H-3"(3.55), H-5"(3.30)
H-5"	3.30	*m*	H-4"(3.40), H-6"$_a$(3.22),H-6"$_b$(3.60)
H-6"$_a$	3.22	*dd*	H-5"(3.30), H-6"$_b$(3.60)
H-6"$_b$	3.60	*dd*	H-5"(3.30), H-6"$_a$(3.22)
H-1"'	4.30	*d*	H-2"'(3.69)
H-2"'	3.69	*dd*	H-1"'(4.30), H-3"'(3.65)
H-3"'	3.65	*dd*	H-1"'(4.30), H2"'(3.69), H-4"'(3.43)
H-4"'	3.43	*t*	H-3"'(3.65), H-5"'(3.49)
H-5"'	3.49	*m*	H-4"(3.43), H-6"'(1.12)
H-6"'	1.12	*d*	H-5"(3.49)

Table.14 DEPT MULTIPLICITIES, ^{13}C NMR (CD_3OD, 75.5 MHz). ONE BOND ^{1}H-^{13}C NMR CONNECTIVITIES AND J_{HH}(Hz) OF COMPOUND RC-3.

C	DEPT Multiplicity	^{13}C NMR (δ)	^{1}H NMR (δ)	J_{HH}(Hz)
C-2	-C-	157.6	-	-
C-3	-C-	135.6	-	-
C-4	-C-	178.5	-	-
C-5	-C-	162.2	-	-
C-6	CH	99.6	6.18	*d* (2.0)
C-7	-C-	165.7	-	-
C-8	CH	94.5	6.35	*d* (2.0)
C-9	-C-	158.1	-	-
C-10	-C-	105.1	-	-
C-1'	-C-	122.7	-	-
C-2'	CH	117.8	7.65	*d* (2.0)
C-3'	-C-	146.5	-	-
C-4'	-C-	150.4	-	-
C-5'	CH	116.2	6.68	*d* (8.0)
C-6'	CH	122.3	7.62	*dd* (2.0/8.0)
C-1"	CH	104.8	5.17	*d* (7.0)
C-2"	CH	75.6	3.54	*dd* (9.3/7.0)
C-3"	CH	78.5	3.55	*t* (9.0)
C-4"	CH	71.1	3.40	*t* (9.4)
C-5"	CH	77.4	3.30	*m*
C-6"a	CH	68.4	3.22	*dd* (12.50/2.2)
C-6"b	CH	68.4	3.60	*dd* (12.5/5.2)
C-1"'	CH	102.5	4.53	*d* (2.0)
C-2"'	CH	72.3	3.69	*dd* (2.0/5.0)
C-3"'	CH	71.9	3.65	*dd* (2.0/10.0)
C-4"'	CH	73.8	3.43	*t* (10.0)
C-5"'	CH	69.5	3.49	*m*
C-6"'	CH_3	18.4	1.12	*d* (7.0)

COMPOUND RC-4

Compound RC-4 was obtained as yellow crystalline solid which appeared violet under UV (366 nm) light and turned to yellow in NH_3 vapours, indicating it to be a 3-O-substituted flavonol. Mobility of RC-4 with standared compound in solvent system H_2O:EtOH:MEK:Acetyl acetone (13:3:3:1) suggested it to be a 3-O-substituted mono glycoside. In order to find out the aglycone and the sugar part, acid hydrolysis of the compound RC-4 was carried out.

Acid hydrolysis:

Acid hydrolysis of the RC-4 was carried out by the procedure as described under the compound RC-1.

Alkaline hydrolysis:

The same procedure was adopted for alkaline hydrolysis as described for compound RC-1. The compound RC-4 remained unchanged and no organic acid was detected, indicating it to be a non acylated flavonoid glycoside.

Identification of aglycone:

The aglycone part was identified as quercetin with the help of UV, ^{1}H NMR and EIMS, as describerd for compound RC-1.

Identification of sugars:

Rhamnose were identified as the only sugar by making use of PC and GC, as described for the compound RC-1.

Identification of glycoside:

UV spectrum of RC-4 recorded in MeOH exhibited two prominent absorption bands at 351 nm (band-I) and 265 nm (band-II) indicating it to be a 3-O-substituted flavonol. A bathochromic shift of 6.0 nm was observed by the addition of NaOAc which showed the presence of a free -OH at position 7 of ring A. Addition of few drops of 5% methanolic $AlCl_3$ solution produced a bathochromic shift of 83 nm in band I which was decreased by 40 nm after the addition of HCl with respect to $AlCl_3$ spectrum, which proved the presence of 5-OH at ring A and orthodihydroxylation at ring B. UV specta recorded in MeOH and after addition of diagnostic shift reagents suggested compound RC-4 to be a 3-O-substituted flavonol.

IR spectrum recorded in KBr displayed absorption bands at 3431 cm^{-1} (fre OH), 1656 cm^{-1} (conjugated carbonyl group), 1609-1447 cm^{-1} (aromatic ring) and 1088 cm^{-1} (C-O-C).

EIMS of the compound gave a molecular ion peak $M^{+\cdot}$ for its aglycone part at m/z 302. Other Retro-Diels Alder fragments appeared at m/z 153 $(A_1+H)^+$, 121 $(B_2)^+$ and 93 (B_2-28). FAB^+MS exhibited a molecular ion peak $(M+H)^+$ at m/z 449. An other fragment appeared at m/z 303 $(M+H-146)^+$ showing the loss of one pentose.

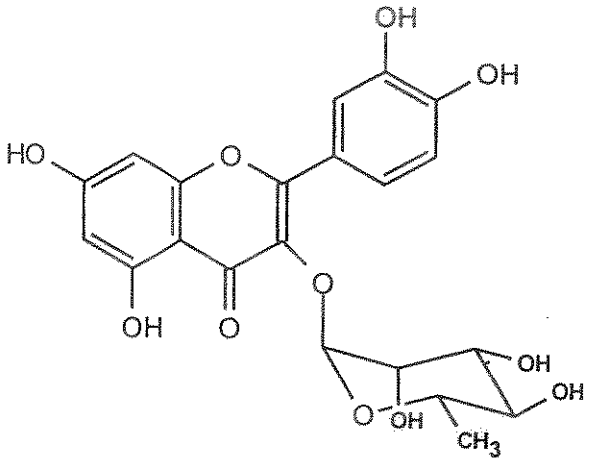
^1H MMR showed a doublet at δ 6.12 (1H, *d*, $J_{6,8}$= 2.0 Hz) corresponding to H-6 of ring A indicating meta coupling with H-8 which resonated at δ 6.31 (1H, *d*, $J_{8.6}$= 2.0 Hz). H-2' of ring B resonated at δ 7.32 (1H, *d*, $J_{2',6'}$= 2.0 Hz) showing meta coupling with H-6'. A signal for H-5' was observed at δ 6.90 (1H, *d*, $J_{5',6}$= 8.0 Hz) indicating ortho coupling with H-6' which appeared at δ 7.30 (1H, *dd*, $J_{6',5'}$= 8.0 $J_{6',2'}$= 2.0 Hz). Anomeric proton of rhamnose exhibited its chemical shift at δ 5.32 (1H, *d*, $J_{1'',2''}$= 1.9 Hz) indicating its α-configuration. The other sugar protons i,e H-2", H-3", H-4" and H-5" showed their resonances at δ 4.22 (1H, *dd*, $J_{2'',1''}$= 2.0 $J_{2'',3''}$= 5.0 Hz), 3,75 (1H, *dd*, $J_{3'',4''}$=2.0, $J_{3'',4''}$= 8.0

Hz), 3.35 (1H, *t*, $J_{4'',3'',5''}$= 8.0 Hz) and 3.40 (1H, *m*). Protons belonging to CH_3 of rhmnosyl moiety resonated at 0.93 (3H, *d*, $J_{6'',5''}$= 7.0 Hz).

^1H, ^1H-COSY experiment gave a view of coupling interactions between different protons present in the molecule. A cross peak was observed between H-6 (δ 6.12) and H-8 (δ 6.31). Interactions between H-2', H-5' and H-6' were confirmed through strong cross peak observed in ^1H, ^1H-COSY spectrum. Coupling interaction between sugar protons were studied with the help of ^1H, ^1H-COSY experiment and are given in Table-16.

^{13}C NMR (DEPT) helped to move insite of the structure. Total 21 signals were observed in which 10 belonged to quarternary carbons, 10 to methine carbons and one to methyl carbon atom. Quaternary carbons of ring C 1,e C-2, C-3 and C-4 showed their resonances at δ 158.6, 136.0 and 179.4 respectively. Signals for C-5, C-6, C-7 and C-8 of ring A appeared at δ 163.0, 100.2, 167.2 and 95.6. Two quaternary carbons C-9 and C-10 exhibited their chemical shift at δ 159.0 anf 105.5. Chemical shifts observed at δ 122.9, 116.9, 146.4, 149.8, 116.3 and 122.8 could assign to the C-1', C-2', C-3', C-4', C-5' and C-6' of ring B. Anomeric carbon of rhamnose appeared at δ 103.4 while other sugar carbons resonated at δ 71.9, 72.0, 73.3, 71.9 corresponding to C-2", C-3", C-4" and C-5'. Signal for methyl carbons of rhamnose was observed at δ 17.81. Connectivities of protons to their respective carbons were studied by ^1H, ^{13}C-COSY experiment which are given in Table-17.

On the basis of chromatographic data, acid hydrolysis, UV, IR, Mass, ^1H NMR, ^{13}C (B.B & DEPT) NMR, ^1H, ^1H-COSY and ^1H, ^{13}C-COSY experiments the structure of RC-4 was identified as quercetin 3-O-α-L-rhamnopyranoside.



Quercetin 3-O-α-L-rhamnopyranoside

Table.15 ^{1}H NMR SPECTRAL DATA OF COMPOUND RC-4 (CD_3OD, 300 MHz)

H	(δ)	Multiplicity	J (Hz)
H-6	6.12	*d*	2.0
H-8	6.31	*d*	2.0
H-2'	7.32	*d*	2.0
H-5'	6.90	*d*	8.0
H-6'	7.30	*dd*	8.0/2.0
H-1"	5.32	*d*	1.9
H-2"	4.22	*dd*	2.0/5.0
H-3"	3.75	*dd*	2.0/8.0
H-4"	3.35	*t*	8.0
H-5"	3.40	*m*	-
CH_3	0.93	*d*	7.0

Table.16 ^{1}H NMR AND COSY -45° SPECTRAL DATA OF COMPOUND RC-4 (CD_3OD, 300 MHz)

H	(δ)	Multiplicity	Coupled to H (δ)
H-6	6.12	*d*	H-8 (6.310
H-8	6.31	*d*	H-6 (6.12)
H-2'	7.32	*d*	H-6'(7.30)
H-5'	6.90	*d*	H-6'(7.30)
H-6'	7.30	*dd*	H-2'(7.32), H-5'(6.90)
H-1"	5.32	*d*	H-2"(4.22)
H-2"	4.22	*dd*	H-1"(5.32), H-3"(3.75)
H-3"	3.75	*dd*	H-2"(4.22), H-4"(3.35)
H-4"	3.35	*t*	H-3"(3.75), H-5"(3.40)
H-5"	3.40	*m*	H-4"(3.35), H-6"(0.90)
CH_3	0.93	*d*	H-5"(3.40)

Table.17 DEPT MULTIPLICITIES, ^{13}C NMR (CD_3OD, 75.5), ONE BOND ^{1}H, ^{13}C-COSY CONNECTIVITIES AND J_{HH} (Hz) OF COMPOUND RC-4.

C	DEPT Multiplicity	^{13}C NMR (δ)	^{1}H NMR (δ)	J_{HH} (Hz)
C-2	-C-	158.6	-	-
C-3	-C-	136.0	-	-
C-4	-C-	179.4	-	-
C-5	-C-	163.0	-	-
C-6	CH	100.2	6.12	*d* (2.0)
C-7	-C-	167.2	-	-
C-8	CH	95.6	6.31	*d* (2.0)
C-9	-C-	159.0	-	-
C-10	-C-	105.5	-	-
C-1'	-C-	122.9	-	-
C-2'	CH	116.9	7.32	*d* (2.0)
C-3'	-C-	146.4	-	-
C-4'	-C-	149.8	-	-
C-5'	CH	116.3	6.90	*d* (8.0)
C-6'	CH	122.8	7.30	*dd* (8.0/2.0)
C-1"	CH	103.4	5.32	*d* (1.9)
C-2"	CH	71.9	4.22	*dd* (2.0/5.0)
C-3"	CH	72.0	3.75	*dd* (2.0/8.0)
C-4"	CH	73.3	3.35	*t* (8.0)
C-5"	CH	71.9	3.40	*m*
C-6"	CH	17.8	0.93	*d* (7.0)

2.2.2 ANTHRAQUINONE GLYCOSIDE

2.2.2.1 EXTRACTION

Extraction of anthraquinone glycoside was done by the same procedure as described for the flavonoid glycosides.

2.2.2.2 SEPARATION AND PURIFICATION

Separation of anthraquinone glycoside was achieved by using the same chromatographic techniques (Polyamide column, Sephadex LH-20 column, PTLC, TLC and PC) as used for the separation and purification of flavonoid glycosides.

2.2.2.3 IDENTIFICATION

Structure elucidation of isolated anthraquinone glycoside (RC-5) was carried out with the help of R_f values, acid hydrolysis, UV and IR spectrophotometry. Different types of NMR techniques such as ^{1}H NMR (200, 300 MHz), ^{1}H, ^{1}H-COSY, and ^{13}C (DEPT & B.B) NMR (75.5 MHz) were employed. For mass spectrometry EIMS and FAB$^+$MS techniques were used. Sugar identification was carried out by TLC, PC and GC.

COMPOUND RC-5

A yellow orange coloured compound (RC-5) was obtained along with the separation of flavonoid glycosides. It appeared flourescent yellow under UV (366 nm) light and turned red under NH_3 vapours which suggested it to be an anthraquinone glycoside (Thomson, 1971). To identify the aglycone part and the sugar residue, compound RC-5 was subjected to acid hydrolysis.

Acid hydrolysis:

5.0 mg of RC-5 was hydrolysed according to the same procedure described for compound RC-1. After hydrolysis the aglycone was extracted by EtOAc while the aqueous extract, containg the sugars, was neutralized by Na_2CO_3.

Alkaline hydrolysis:

Alkaline hydrolysis of the compound RC-5 was carried out by the same method as described for compound RC-1. The compound RC-5 remained unchanged, indicating it to be a non acylated anthraquinone glycoside.

Identification of aglycone:

Complete evaporation of ethyl acetate extract under vacuum resulted in an orange crystalline solid. It appeared orange under UV (366 nm) light and turned red in NH_3 vapours, indicating it to be an anthraquinone. R_f values recorded in BAW and BEW were very close to the R_f values of 1, 6, 8-trihydroxy-3-methyl anthraquinone (Harborn, 1977). UV spectrum recorded in MeOH gave four absorption bands at 435, 284, 265, and 253 nm, typical of an anthraquinone (Thomson, 1971). An absorption band at 435 nm appeared due to weak benzenoid absorption while the intese benzenoid absorption was observed at 253 nm.

EIMS of the aglycone showed a molecular ion peak at m/z 270 (100%). Other important fragments were observed at m/z = 255 (M^+-CH_3), 242 (M^+-CO), 241 (M^+-CHO) and 213 (M^+-CHO-CO). The mass fragmentation pattern was similar to that reported for 1, 6, 8-trihydroxy-3-methyl anthraquinone (Khetwal, 1987).

^{1}H NMR (Acetone-d_6, 300 Mhz) recorded a doublet at δ 6.44 (1H, *d*, $J_{2,4}$= 2.0 Hz) corresponding to H-2, showing a meta coupling with H-4 which resonated at δ 7.15 (1H,*d*, $J_{4,2}$= 2.0 Hz). A sharp doublet appeared at δ 7.50 (1H, *d*, $J_{5,7}$= 2.0 Hz) which was due to H-5, meta coupled with H-7 which resonated at δ 7.05 (1H, *d*, $J_{7,5}$= 2.0 Hz). Chemical shift of CH_3 was observed in the form of a singlet at δ 2.42 (3H, *s*). On the basis of R_f values, UV, EIMS and ^{1}H NMR data the structure of the aglycone was indicated as 1, 6, 8-trihydroxy-3-methyl anthraquinone.

Identification of sugars:

For sugar analysis the same procedure was adopted as described for compoun RC-1. On the basis of PC and GC, rhmnose and glucose were identified as the sugar moities.

Identification of glycoside:

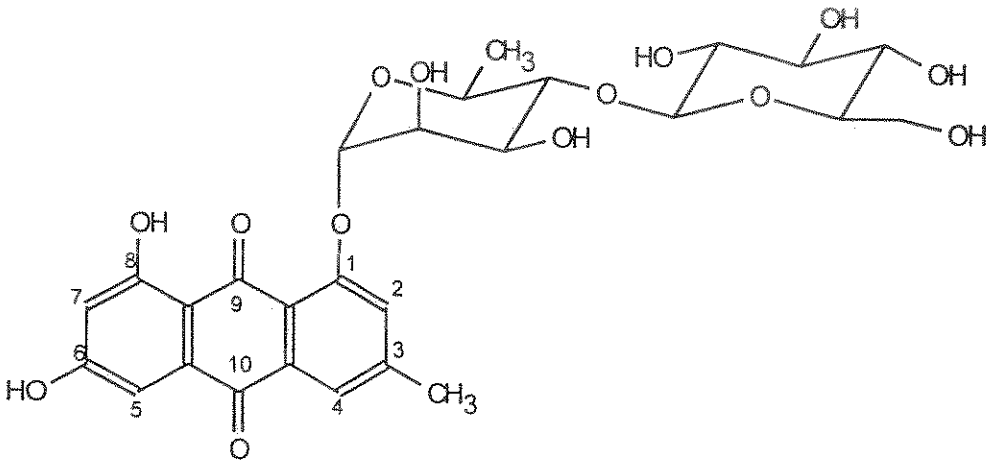
UV spectra recorded in MeOH showed absorption bands at 211, 257, 274 and 377 nm. The UV spectrum recorded after the addition of 5% methanolic solution of $AlCl_3$ and $AlCl_3$/HCl exhibited the same pattern of bands at 208, 377, 417 and 469(sh) indicating the absence of orthodihydroxylation in the molecule.

IR ν_{max} cm^{-1} recorded in KBr exhibited absorption bands at 3418, 1665, 1631, 1607, 1557, 1539, 1505, 1471, 1374, 1282, 1184, 1091, 720 and 618, indicating the presence of free OH and conjugated carbonyl group.

EIMS displayed a molecular ion peak for the aglycone at m/z = 270 (M^+). Other important fragments were observed at m/z = 255 (M^+-CH_3), 242 (M^+-CO), 241 (M^+-CHO) and 213 (M^+-CHO-CO). The sugar sequence was deduced by FABMS recorded in positive mode in lactic acid. A molecular ion peak was observed at m/z = 579 $(M+H)^+$. The fragment ion 417 $(M+H-162)^+$ showed sequential loss of hexose thus indicating rhamnose as the inner and glucose as the terminal sugar.

^{1}H NMR (CD_3OD, 300 MHz) recorded two doublets at δ 6.0 (1H, *d*, $J_{2,4}$=2.0 Hz) and δ 7.15 (1H, *d*, $J_{4,2}$=2.0 Hz) corresponding to H-2 and H-4 protons of ring A, meta coupled to each other. H-5 of ring C resonated at δ 7.23 (1H, *d*, $J_{5,7}$=2.0 Hz) showing a meta coupling with H-7 which resonated at δ 6.70 (1H, *d*, $J_{7,5}$=2.0 Hz). Coupling interaction of the above protons were also revealed by ^{1}H, ^{1}H COSY spectra. The chemical shift of CH_3, present in ring A was observed at δ 2.42 (3H, *s*). The anomeric protons appeared at δ 4.62 (1H, *d*, J=2.0 Hz) and δ 5.30 (1H, *d*, J=7.5 Hz) which showed α and ß configurations of rhamnose and glucose respectively. All other protons of rhamnose and glucose resonated in the range of 3.0 - 4.2 ppm. CH_3 of rhamnose appeared at δ 0.91 (3H, *d*, $J_{6,5}$=7.0 Hz). To find out the position of attachment of the inner sugar to the aglycone, NOE experiments were performed. Irridiation of H-1' showed a NOE effect on H-2 of aglycone while the same effect was observed when H-2 of aglycone was irridiated. This NOE interaction between H-1' and H-2 (being close to each other in space due to the free rotation of the sugar) indicated that rhamnose is attached to at position 1 of emodin. Irridiation of H-7, H-6 and H-4 did not show any NOE effect on the anomeric proton of rhamnose. In ^{13}C (DEPT) NMR two anomeric carbons belonging to rhamnose and glucose appeared at δ 102.5 and δ 103.0 respectively indicating C-O linkage. The other carbons of the sugars resonated in their normal range (Table-19) except C-4' of rhamnose which appeared 8.0 ppm further downfield than its normal chemical shift, indicating the site of attachment of the terminal glucose.

On the basis of chromatographic data, acid hydrolysis and spectroscopic analysis the structure of compound RC-5 was established as 6,8-dihydroxy-3-methyl-anthraquinone-1-O-ß-D-glucopyranosyl(1→4)-α-L-rhamnopyranoside.



6,8-dihydroxy-3-methyl-anthraquinone-1-O-ß-D-glucosyl(1→4)-α-L-rhamnoside

Table.18 ^{1}H NMR SPECTRAL DATA OF COMPOUND RC-5

(CD_3OD, 200 MHz)

H	(δ)	Multiplicity	(J Hz)
H-2	6.0	*d*	2.0
H-4	7.15	*d*	2.0
H-5	7.23	*d*	2.0
H-7	6.70	*d*	2.0
CH_3	2.42	*s*	-
H-1'	4.62	*d*	2.0
H-2'-H-5'	3.0-4.2	*m*	-
CH_3	0.91	*d*	7.0
H-1"	5.3	*d*	7.5
H-2"-H-6"	3.0-4.2	*m*	-

Table.19 ^{13}C (DEPT) NMR SPECTRAL DATA OF COMPOUND RC-5 (CD_3OD, 75.5 MHz)

C	Multiplicity	(δ)
C-1	-C-	162.4
C-2	CH	120.0
C-3	-C-	148.3
C-4	CH	124.2
C-5	CH	108.6
C-6	-C-	166.6
C-7	CH	108.1
C-8	-C-	164.5
C-9	-C-	189.7
C-10	-C-	180.0
C-9a	-C-	117.5
C-4a	-C-	135.1
C-10a	-C-	134.0
C-8a	-C-	113.7
Me	CH_3	22.2
C-1'	CH	102.5
C-2'	CH	71.7
C-3'	CH	72.3
C-4'	CH	81.5
C-5'	CH	69.7
C-6'	CH_3	18.0
C-1"	CH	103.0
C-2"	CH	74.3
C-3"	CH	76.4
C-4"	CH	71.0
C-5"	CH	76.8
C-6"	CH_2	63.0

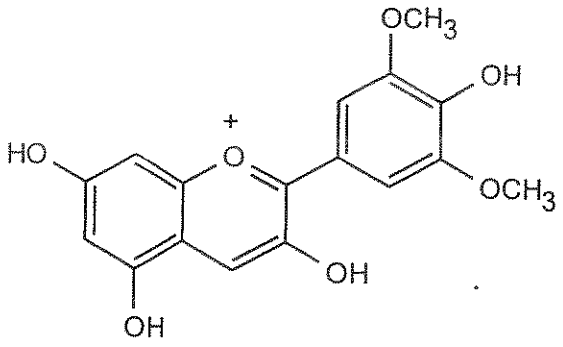
2.2.3 ANTHOCYANIDINS

2.2.3.1 EXTRACTION

3.0 grams of ground air dried leaves of *R. chalepensis* were hydrolyzed with 200 ml of 2N HCl in a conical flask. After 20 minutes of contact, the contents of the flask were heated on a water bath for 60 minutes. The heating was accompained by blowing in of the air at regular time intervals. The flask was then cooled under tap and the contents were transfered to a separating funnel. The hydrolytic solution was then successively exhausted with 3x50 ml of diethyl ether (flavonoid aglycones) and then by 3x25 ml of *n*-butanol (anthocyanidin fraction). The n-butanol extract was concentrated to a known volume by repeated washing with 2N HCl.

2.2.3.2 SEPARATION, PURIFICATION AND IDENTIFICATION OF ANTHOCYANIDIN

The concentrated *n*-BuOH extract , obtained after the hydrolysis of 3.0 gm of air dried plant material, was subjected to purification by descending preprative chromatography on Whatman No.1 chromatographic paper employing BAW as the developing solvent. Only one band was observed on the paper chromatogram. 5 similar paper chromatograms were developed and the band of anthcyanidin was cut into small pieces and eluted in MeOH : 1% HCl (99:1). After several hours of contact, the eluent was filtered and concentrated at low temperature under reduced pressure. Purity of the compound was checked in three solvent systems. A single spot was observed in each case. R_f values were calculated and compared with reference substances. UV spectra of the compound was recorded in MeOH and then in 5% $AlCl_3$. Chromatographic and UV/Visible spectral data are presented in Table-20. On the basis of R_f values, UV-Visible spectral data the compound was identified as 3, 3', 5, 7-tetrahydroxy 3', 5'-dimethoxy anthocyanidin (Malvidin).



Malvidin

Table.20 UV SPECTRAL/QUALITATIVE ANALYSIS OF ANTHOCYANIDINS

Compound	R_f (x100)			Colour under UV	Colour under day light	λ_{max} MeOH			λ_{max} MeOH/$AlCl_3$		
RC-1A	58	26	56	Mauve	Purple	541	359	272	540	360	272
Delphinidin	32	13	42	Mauve	Purple	546			+		
Petunidin	46	20	52	Mauve	Purple	543			+		
Cyanidin	49	22	68	Pink	Magenta	535			+		
Peonidin	63	30	71	Pink	Magenta	532			-		
Pelargonodin	68	33	80	Red	Purple	520			-		
Malvidin	60	27	58	Mauve	Purple	542			-		

Solvent system 1 = FORESTAL ($HOAc$-H_2O-HCl)
Solvent system 2 = HCO_2H-HCl-H_2O (5:2:3)
Solvent system 3 = BAW [*n*-BuOH-$HOAc$-H_2O (4:1:5)]

2.3 PHYTOCHEMICAL ANALYSIS OF *RUMEX NEPALENSIS*

2.3.1 NATURAL GLYCOSIDES

2.3.1.1 EXTRACTION

To obtain flavonoid glycosides, 3.0 Kg of air dried leaves of *R. nepalensis* were repeatedly extracted by ethanol and ethanol/water mixture. Extraction of flavonoid glycosides was achieved by the same procedure as described for *R. chalepensis*. The dried ethyl acetate extract was found to be 6.50 gram.

2.3.1.2 SEPARATION AND PURIFICATION

Separation of flavonoid glycosides was carried out by a polyamide column. The column was run in the gradient mode from H_2O to MeOH according to the same elution scheme as mentioned for *R. chalepensis*.

Composition of each fraction was monitored on silica gel precoated plates (Merck) which were developed in $CHCl_3$:MeOH:H_2O (13:3:2) solvent system. As a result similar fractions were combined as follows:

A =	Fraction No. 01 - 08	..... Five spots
B =	Fraction No. 0 9 -13	..... Three spots
C =	Fraction No. 14 - 18	..... Two spots
D =	Fraction No. 19 - 29	..... Two spots
E =	Fraction No. 30 - 35	..... Three spots
F =	Fraction No. 36 - 40	..... Three spots

Purification of flavonoid glycosides, present in each fraction was achieved by a combination of PTLC on silica gel and CC on Sephasex LH-20. Four flavonoid glycosides namelt RN-1 (12.0 mg), RN-2 (7.0 mg), RN-3 (28.0 mg) and RN-4 (42.0 mg) were obtained in pure form. Purity of each flavonoid glycoside was checked by HPLC using a C_{18} ODS (Zorbex) column in H_2O:MeOH:HOAc (80:16:4) as the solvent system.

2.3.1.3 IDENTIFICATION

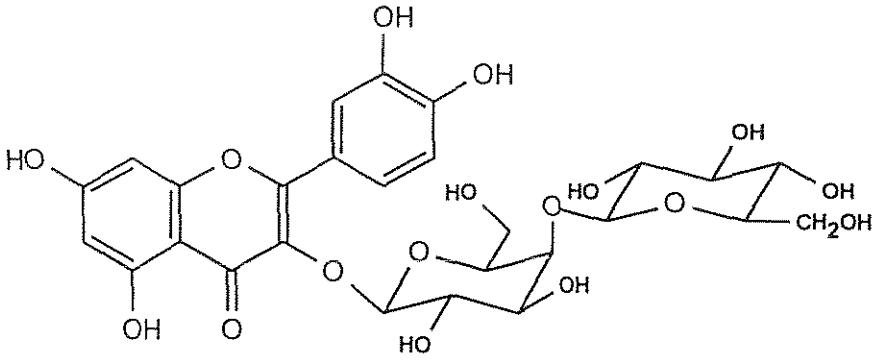
Identification of the four flavonoid glycosides was carried out by recording their R_f values on pre-coated TLC glass plates (20 x 20) of silica gel, cellulose and polyamide [Solvent A: $CHCl_3$-MeOH-H_2O (12:4:1) for silica gel; Solvent B: 15% HOAc; Solvent C: 60% HOAc; Solvent D: *n*-BuOH-HOAc-H2O (4:1:5); Solvent E: EtOAc-Pyridin-HOAc-H_2O (36:36:7:21); Solvent F: *n*-BuOH-C6H6-Pyridin-H2O (5:1:3:3) for cellulose; Solvent. G: H2O-Methanol-*n*-BuOH-AcOH (75:15:10:2) for polyamide], acid hydrolysis to aglycone and sugars and spectroscopic techniques (UV, IR, EIMS, FAB^+MS, ^1H NMR. ^{13}C (B.B & DEPT) NMR, ^1H, ^1H-COSY, ^{13}C, ^1H-COSY and NOE measurements).

Interpretation of the results obtained after the chromatographic and spectral analysis revealed that the flavonoid glycosides (RN-1 to RN-4) isolated from *R. nepalensis* leaves were identical to those isolated from *R. chalepensis*. Four flavonoid glycosides isolated from the leaves of *R. nepalensis* established the following chromatographic and spectral data:

COMPOUND RN-1

UV: Violet, R_f, Sol. A: 0.54; Sol. B: 0.29; Sol. C: 0.68; Sol. D: 0.51; Sol. G: 0.05. UV λ_{max}(nm): Methanol: 257, 327(sh), 355; +$AlCl_3$: 274, 304(sh), 431; +HCl: 268, 303,(sh), 402; +CH_3COONa: 274, 327, 408. IR $\nu_{max}(cm^{-1})$: 3400, 1652, 1604, 1494, 1445, 1358, 1301, 1200, 1166, 1083, 1055. EIMS: 302(100%), 301(26.0%), 285(3.5%), 247(1.9%), 153(15.5%), 152(3.9%), 137(19.7%), 124(3.4%), 109(11.9%). FAB^+MS: 627$[M+H]^+$,

465[M+H-162]$^+$, 303[M+H-324]$^+$. ^{1}H NMR(CD_3OD): 6.20 (1H, *d*, J=2.0 Hz)H-6, 6.35 (1H, *d*, J=2.0 Hz)H-8, 7.63 (1H, *d*, J= 9.0 Hz)H-2', 6.95 (1H, *d*, J=9.0 Hz)H-5', 7.30 (1H, *dd*, J=2.0, 9.0 Hz)H-6', 5.10 (1H, *d*, J=6.0 Hz)H-1", 5.22 (1H, *d*, J=6.0 Hz)H-1"', 3.2-4.30 (10H, m)H-2"-H-6"'. ^{13}C NMR(CD_3OD): 158.1 (C-2), 135.5 (C-3), 178.4 (C-4), 162.5 (C-5), 101.2 (C-6), 166.7 (C-7), 95.9 (C-8), 158.7 (C-9), 104.3 (C-10), 123.3 (C-1'), 166.8 (C-2'), 145.9 (C-3'), 150.0 (C-4'), 116.5 (C-5'), 122.9 (C-6'), 101.5 (C-1"), 71.3 (C-2"), 73.2 (C-3"), 78.0 (C-4"), 75.2 (C-5"), 61.8 (C-6"), 104.5 (C-1"'), 75.2 (C-2"'), 77.0 (C-3"'), 70.2 (C-4"'), 78.5 (C-5"'), 62.2 (C-6"').

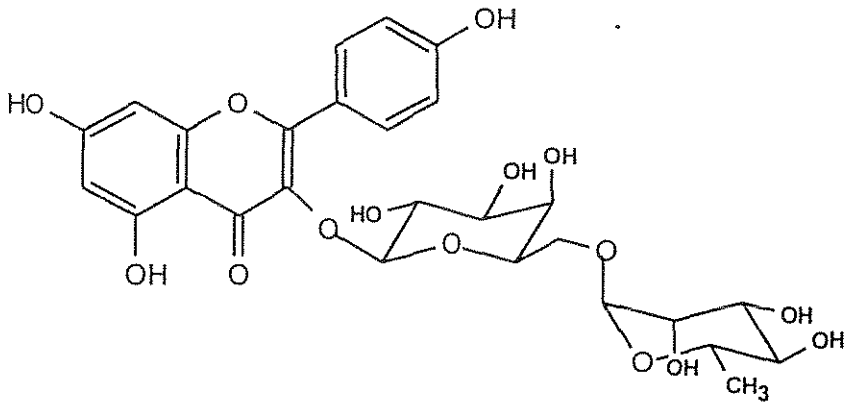


Quercetin 3-O-β-D-glucopyranosyl(1→4)-β-D-galactopyranoside

COMPOUND RN-2

UV: Violet, R_f, Sol. A: 0.68; Sol. B: 0.30; Sol. C: 0.76; Sol. D: 0.77; Sol. G: 0.14. UV λ_{max}(nm): Merthanol: 265, 315,(sh), 351; +$AlCl_3$: 274, 301, 354, 400; +HCl: 275, 299, 248, 400; +CH_3COONa: 274, 318,(sh), 384,; +H_3BO_3: 268, 319(sh), 354; +NaOH: 274, 301(sh), 397. IR ν_{max}(cm^{-1}): 3432, 1652, 1495, 1447, 1364, 1299, 1180, 1080. EIMS: 286(100%), 285(33%), 258(12.6%), 152(13.3%), 153(8.7%), 121(30.0%), 93(4.6%). FAB$^+$MS: 595[M+H]$^+$, 449[M+H-146]$^+$, 287[M+H-308]$^+$. ^{1}H NMR (CD_3OD, 300 MHz): 6.15 (1H, *d*, J=2.0 Hz)H-6, 6.30 (1H, *d*, J=2.0 Jz)H-8, 8.09 (1H, *d*, H=9.0 Hz)H-2', 6.95

(1H, *d*, J=9.0Hz)H-3', 6.95 (1H, *d*, J=9.0 Hz)H-5', 8.09 (1H, *d*, J=9.0 Hz)H-6', 5.12 (1H, *d*, J=6.0 Hz)H-1", 5.40 (1H, *d*, J=2.0 Hz)H-1"', 3.2-4.35 (10H, m)H-2"-H-6"', 0.95 (3H, *d*, H-6.0 Hz) CH_3 of rhmnose. ^{13}C NMR (CD_3OD): 157.9 (C-2), 135.5 (C-3), 178.2 (C-4), 162.3 (C-5), 101.2 (C-6), 166.0 (C-7), 96.4 (C-8), 159.0 (C-9), 104.1 (C-10), 122.3 (C-1'), 132.0 (C-2'), 116.2 (C-3'), 162.1 (C-4'), 116.2 (C-5'), 132.0 (C-6'), 101.9 (C-1"), 71.9 (C-2"), 73.3 (C-3"), 69.5 (C-4"), 75.5 (C-5"), 66.5 (C-6"), 100.7 (C-1"'), 72.1(C-2"'), 71.8 (C-3"'), 72.5 (C-4"'), 69.0 (C-5"'), 18.2 (C-6"').

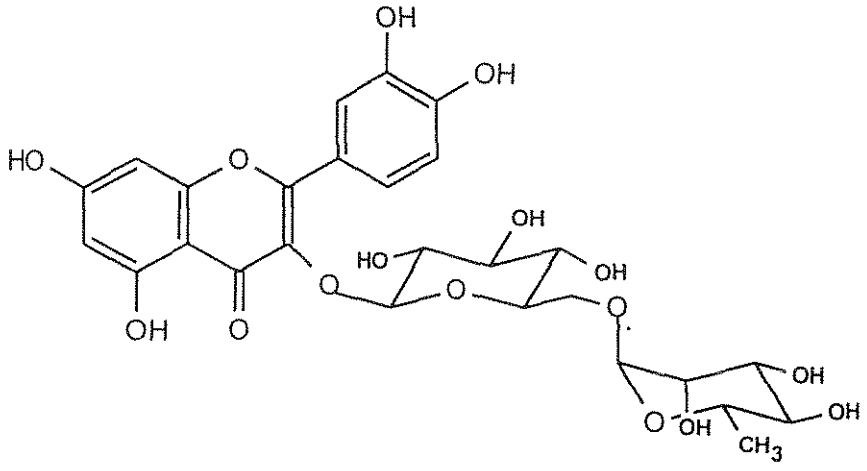


Kaempferol 3-O-α-L-rhamnopyranosyl(1→6)-β-D-galactopyranoside

COMPOUND RN-3

UV: Violet, R_f, Sol. A: 0.42; Sol. B: 0.57; Sol. C: 0.72; Sol. D: 0.35; Sol. G: 0.06. UV λ_{max}(nm): Methanol: 257, 266, 299, 359; +$AlCl_3$: 274, 305(sh), 440; +HCl: 268, 300, 400; +CH_3COONa: 274, 325, 391; +H_3BO_3: 262, 298, 377; +NaOH: 271, 327, 410. IR $\nu_{max}(cm^{-1})$: 3431, 1656, 1609, 1505, 1447, 1360, 1305, 1201, 1167, 1088, 1058. EIMS: 302(100%), 301(26.4%), 285(4.9%), 247(9.9%), 153(14.8%), 152(1.9%), 137(17.5%), 124(2.9%), 109(9.9%). FAB^+MS: 611$[M+H]^+$, 465$[M+H-146]^+$, 303$[M+H-308]^+$. ^1H NMR (CD_3OD): 6.20 (1H, *d*, J=2.0 Hz)H-6, 6.38 (1H, *d*, J=2.0 Hz)H-8, 7.67 (1H, *d*, J=2.0 Hz)H-2', 6.69 (1H, *d*, J=8.0 Hz)H-5', 7.60 (1H, *dd*, 2.0, 8.0Hz)H-6', 5.20 (1H, *d*,

J=7.0 Hz)H-1", 4.59 (1H, d, J=2.0 Hz)H-1"', 3.31-4.32 (10H, m)H-2"-H-5"', 0.90 (3H, *d*, J=6.0 Hz)CH_3 of rhamnose. ^{13}C NMR (CD_3OD): 157.2 (C-2), 135.4 (C-3), 175.6 (C-4), 162.1 (C-5), 99.6 (C-6), 165.3 (C-7), 94.9 (C-8), 158.1 (C-9), 105.0 (C-10), 122.9 (C-1'), 117.5 (C-2'), 146.9 (C-3'), 150.1 (C-4'), 116.5 (C-5'), 122.3 (C-6'), 103.8 (C-1") 75.5 (C-2"), 78.3 (C-3"), 71.3 (C-4"), 77.5 (C-5"), 68.6 (C-6"), 101.5 (C-1"'), 72.4 (C-2"'), 71.8 (C-3"'), 73.7 (C-4"'), 69.5 (C-5"'), 18.0 (C-6"').

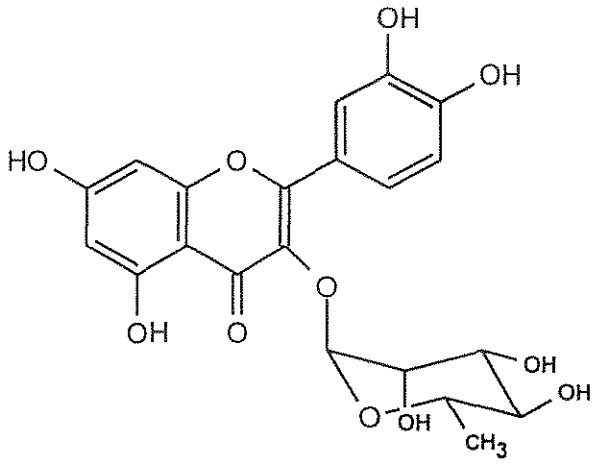


Quercetin 3-O-α-L-rhamnopyranosyl(1→6)-ß-D-glucopyranoside

COMPOUND RN-4

UV: Violet, R_f, Sol. A: 0.76; Sol. B: 0.52; Sol. C: 0.74; Sol. D: 0.60; Sol. G: 0.11. UV λ_{max}(nm): Methanol: 254, 265(sh), 301(sh), 351; +$AlCl_3$: 274, 304(sh), 333, 434; +HCl: 271, 303(sh), 353, 394; +CH3COONa: 271, 322(sh), 377; +H_3BO_3: 260, 300(sh), 372; + NaOH: 268, 326, 391. IR ν_{max}(cm^{-1}): 3431, 1656, 1505, 1447, 1360, 1201, 1168, 1088. EIMS: 302(100%), 301(26.8%), 285(2.5%), 247(1.8%), 153(13.5%), 152(2.9%), 137(18.4%), 124(2.8%), 109(11.3%). FAB^+MS(in lactic acid): 449$[M+H]^+$, 303[M+H-146]$^+$. ^1H NMR (CD_3OD, 300 MHz): 6.16 (1H, *d*, J=2.0 Hz)H-6, 6.29 (1H, *d*, J=2.0 Hz)H-8, 7.34 (1H, *d*, J=2.0 Hz)H-2', 6.92 (1H, *d*, J=8.0 Hz)H-5', 7.33 (1H, *dd*, J=2.0, 8.0

Hz)H-6', 5.33 (1H, *d*, J=2.0 Hz)H-1", 3.35-4.22 (4H, m)H-2"-H-5", 0.95 (3H, *d*, J=6.0 Hz) CH_3 of rhamnose. ^{13}C NMR (CD_3OD, 75.5 MHz): 158.3 (C-2), 136.0 (C-3), 179.2 (C-4), 163.1 (5-5), 99.9 (C-6), 166.5 (C-7), 95.3 (C-8), 159.1 (C-9), 105.0 (C-10), 122.9 (C-1'), 116.8 (C-2'), 146.8 (C-3'), 149.9 (C-4'), 116.7 (C-5'), 122.8 (C-6'), 102.5 (C-1"), 71.7 (C-2"), 72.1 (C-3"), 73.2 (C-4"), 71.9 (C-5"), 17.9 (C-6").



Quercetin 3-O-α-L-rhamnopyranoside

2.3.2 ANTHRAQUINONE

Phytochemical analysis of *R. nepalensis* gave an anthraquinone instead of an anthraquinone glycoside.

2.3.2.1 EXTRACTION

Extraction of the anthraquinone was carried out by the same procedure as described for flavonoid glycosides.

2.3.2.2 SEPARATION AND PURIFICATION

The anthraquinone was separated and purified by polyamide and Sephadex LH-20 column and preparative TLC, on self made polyamide plates.

2.3.2.3 IDENTIFICATION

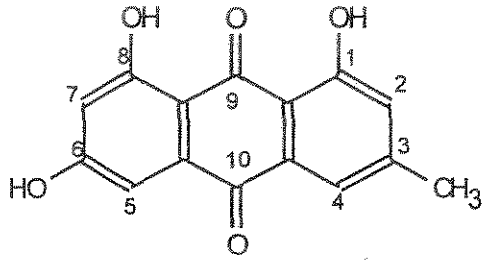
Structure of the anthraquinone (RN-5) was determined with the help of R_f values, UV, IR, EIMS and ^1H NMR techniques.

COMPOUND RN-5

Anthraquinone (RN-5) was obtained as an orange crystalline solid. It appeared orange under UV (366 nm) light on polyamide TLC plate and turned red in NH_3 vapours, indicating it to be an anthraquinone (Thomson, 1971). R_f values recorded in BAW and BEW were very close to the R_f values of 1, 6, 8-trihydroxy-3-methyl anthraquinone (Harborn, 1977). UV spectrum recorded in MeOH gave four absorption bands at 435 nm, 284 nm, 265 nm and 253 nm, typical for an anthraquinone (Suri, 1976).

R_f values. UV, EIMS and ^1H NMR of RN-5 was identical to the chromatographic and spectral data of the aglycone (1, 6, 8-trihydroxy-3-methyl anthraquinone) obtained after the acid hydrolysis of compound RC-5. On the basis of chromatographic and spectroscopic data (outlined below) RN-5 was identified as 1, 6, 8-trihydroxy-3-methyl anthraquinone (Emodin)

UV: Orange, R_f, Sol. A: 0.89; Sol. B: 0.08; Sol. C: 0.63; Sol. D: 0.71; Sol. E: 0.89. UV λ_{max}(nm): Methanol: 253, 265, 284, 435; +$AlCl_3$: 261, 305, 380, 510; +HCl: 261, 290, 360, 504; +CH_3COONa: 253, 265, 310, 475. EIMS: 270(100%), 255(3.19%), 242(14.97%), 214(9.66%), 213(12.89%), 185(7.48%). ^1H NMR (Acetone-d_6): 6.44 (1H, *d*, J=2.0 Hz)H-2, 7.15 (1H, *d*, J=2.0 Hz)H-4, 7.50 (1H, *d*, J=2.0 Hz)H-5, 7.05 (1H, *d*, J=2.0 Hz)H-7, 2.42(3H, *s*).



1, 6, 8-trihydroxy-3-methyl anthraquinone (Emodin)

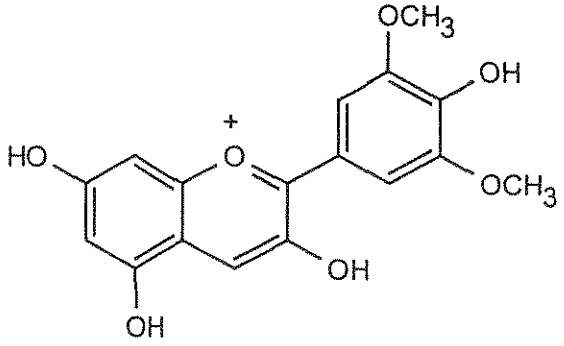
2.3.3 ANTHOCYANIDIN

2.3.3.1 EXTRACTION

Extraction of anthocyanidin from the leaves of *Rumex nepalensis* was done by the same procedure as described for *Rumex chalepensis.*

3.3.3.2 SEPARATION, PURIFICATION AND IDENTIFICATION

Separation, purification and identification of an anthocyanidin was carried out by the same chromatographic (PPC) and spectroscopic (UV-Vis.) methods. On the basis of Rf values in three solvent system and UV spectral analysis (Table-20) the compound IHL-1A was identified as Malvidin.



Malvidin

CHAPTER 3

PHYTOCHEMICAL ANALYSIS OF INDIGOFERA

CHAPTER 3

PHYTOCHEMICAL ANALYSIS OF *INDIGOFERA*

CONTENTS

3.4.2 Anthocyanidin

3.4.2.1 Extraction

3.4.2.2 Separation, purification and identification

3.1 LITERATURE SURVEY OF GENUS INDIGOFERA

A detailed literature survey revealed that genus indigofera is quite rich in a variety of organic acids and flavonoids. Phytochemical investigation of *I. heterantha* has led to the isolation and characterization of flavonoid aglycones (Anila *et al*, 1982). Rao and Hanumaiah (1984) isolated a new flavonol glycoside, Kaempferol 3, 5-O-β-D-digalactoside from the leaves of *I. hirsuta*. The isolation and characterization of certain coumarins and flavonoids has been reported from the leaves of *I. tinctoria* (Dahuraev *et al*, 1986). Kamal and Mangla (1987) have reported the presence of a number of rotenoids in *I. tinctoria*. The polyphenolic constituents of *I. mysorensis* leaves were investigated by Adinaryana ans Sarada (1987). They have isolated and charaterized procatechuric acid, *p*-hydroxy benzoic acid, apigenin 7, 4'-O-β-D-diglucoside, apigenin 7-O-rhamnoglucoside and kaempferol 3-O-neohesperiodoside. Hasan *et al* (1989) have rported the presence of a number of phenolic acids in *I. heterantha*. Phytochemical analysis of *I. arrecta* has revealed the presence of flavonoid glycosides and some phenolic acids (Dadson and Opoku-Boahen, 1992). This survey shows the presence of a vast variety of polyphenols in the genus Indigofera.

3.2 PHYTOCHEMICAL ANALYSIS OF *INDIGOFERA HEBEPETALA* FLOWERS

3.2.1 NATURAL GLYCOSIDES

3.2.1.1 EXTRACTION

Flavonoid glycisides from flowers of *Indigofera hebepetala* were obtained by extracting successively 150 gm of the air dried petals with ethanol and ethanol/water mixtures, as shown below by stirring for 24 hours at room temperature.

1- Ethanol

2- Ethanol : Water (80:20)

3- Ethanol : Water (50:50)

4- Ethanol : Water (50:50) + heating

The combined hydroalcoholic extracts were evaporated to remove alcohol, between 30-35 ^{0}C under reduced pressure. To get rid of the fats, terpenes, carotenoids and chlorophyll, the resulting aqueous phase was treated with chloroform (3x150 ml). The defatted aqueous layer was then repeatedly extracted with *n*-BuOH (3x200 ml). The n-BuOH extract was evaporated to dryness and the dried extract was found to be 15.0 gm.

3.2.1.2 SEPARATION AND PURIFICATION

The crude n-BuOH extract was adsorbed on silica (Merck) and subjected to VLC on a silica gel column. The column was run in the gradient mode from Et_2O which was progressively enriched with EtOAc and MeOH as shown in the following chart

COMPOSITION OF THE VLC ELUTING SOLVENTS

Fr. No.	Et_2O %	EtOAc%	MeOH %	Fr. No.	Et_2O%	EtOAc %	MeOH %
1	100	0	0	31	10	90	0
2	100	0	0	32	10	90	0
3	100	0	0	33	10	90	0
4	95	5	0	34	5	95	0
5	90	10	0	35	5	95	0
6	85	15	0	36	5	95	0
7	75	25	0	37	0	100	0
8	75	25	0	38	0	100	0
9	70	30	0	39	0	99	1
10	65	35	0	40	0	99	1
11	60	40	0	41	0	98	2
12	60	40	0	42	0	98	2
13	60	40	0	43	0	97	3
14	55	45	0	44	0	97	3
15	50	50	0	45	0	96	4
16	50	50	0	46	0	96	4
17	50	50	0	47	0	95	5
18	45	55	0	48	0	95	5
19	40	60	0	49	0	94	6
20	40	60	0	50	0	94	6
21	40	60	0	51	0	93	7
22	35	65	0	52	0	93	7
23	30	70	0	53	0	92	8
24	30	70	0	54	0	92	8
25	30	70	0	55	0	91	9
26	25	75	0	56	0	91	9
27	20	80	0	57	0	90	10
28	20	80	0	58	0	90	10
29	20	80	0	59	0	89	11
30	15	85	0	60	0	0	100

Table.21 SPECTRAL AND CHROMATOGRAPHIC DATA OF FLAVONOID GLYCOSIDES ISOLATED FROM *INDIGOFERA HEBEPETALA* FLOWERS

UV λ_{max}	IHF-3				IHF-2				IHF-3				IHF-4				IHF-5			
MeOH	263			347	262			346	264			343	263			345	263			366
NaOAc	264			391	263			394	264			381	264			395	263	333		417
$NaOAc/H_3BO_3$	267			350	266			349	264			347	265			348	263	323		368
$AlCl_3$	270	299	349	396	272	299	348	396	272	299	346	394	272	299	350	396	265	354		425
$AlCl_3/HCl$	271	300	345	394	273	298	343	393	271	300	340	393	271	298	346	395	266	352		423
NaOMe	268			384	268			385	264			379	266			386	264	335		418
FAB^+MS	m/z				m/z				m/z				m/z				m/z			
$[M+H]^+$	727				727				-				-				-			
$[M+H-2xpentose]^+$	449				449				-				-				-			
$[M+H-hexose]^+$	287				287				-				-				-			
AGLYCONE	Kaempferol				Kaempferol				Kaempferol				Kaempferol				Kaempferol			
SUGARS	Galactose+Rhamnose + Arabinose				Galactose+Rhamnose+ Arabinose				Rhamnose+Arabinose				Rhamnose+Arabinose				Rhamnose			
R_f x 100																				
BAW	63				53				78				79				87			
15% HOAc	74				73				66				63				15			
60% HOAc	78				77				76				76				58			

Composition of each fraction (after completely drying and then dissolving in small amount of MeOH) was monitored on silica gel precoated plates which were developed in $CHCl_3$:MeOH:H_2O (13:3:2) solvent system. As a result similar fractions were combined as follows:

A =	Fraction No. 01 - 08	..... Five spots
B =	Fraction No. 0 9 -16	..... Four spots
C =	Fraction No. 17 - 24	..... Three spots
D =	Fraction No. 25 - 35	..... Two spots
E =	Fraction No. 36 - 41	..... Two spots
F =	Fraction No. 42 - 48	..... Three spots
G =	Fraction No. 49 - 60	..... Three spots

Flavonoid glycosides present in each fraction were purified by a combination of PTLC on silica gel and CC on polyamide and Sephadex LH-20. The following five flavonoid glycosides obtained in pur foem. Purity of each flavonoid glycoside was checked by HPLC using a C18 ODS (Zorbex) column in H_2O:MeOH:HOAc (80:16:4) as the solvent system in the ischratic mode.

1.	IHF-1	=	34.6 mg
2.	IHF-2	=	30.5 mg
3.	IHF-3	=	88.9 mg
4.	IHF-4	=	9.0 mg
5.	IHF-5	=	28.5 mg

2.2.1.3 IDENTIFICATION

Identification of the five purified flavonoid glycosides was carried out by co-chromatography in 3 solvent systems, acid hydrolysis to aglycones and sugars and

spectroscopic techniques (UV, IR, EIMS and FAB$^+$MS). Results obtained are grouped in Table-21. where as data obtained by NMR spectroscopy [^{1}H NMR, ^{13}C (DEPT & B.B) NMR, ^{1}H, ^{1}H-COSY, and NOE difference measurements] are presented in Table-22 to 28. Identification of the sugars was carried out by paper chromatography and GC.

COMPOUND IHF-1

Compound IHF-1 was obtained as a dark yellow crystalline solid. It appeared violet under UV (366 nm) light and turned brownish yellow in NH_3 vapours, indicating it to be a 3-O-substituted flavonol. Chromatographic data comparative to authentic flavonoid glycosides suggested this compound to be a 3-O-substituted triglycoside (Hasan, 1976). For the identification of aglycone and the sugars, acid hydrolysis was carried out by 2N HCl.

Acid Hydrolysis:

3.0 mg of IHF-1 was dissolved in 5.0 ml of MeOH:2NHCl (1:1) and refluxed on a water bath at 100 °C for 60 minutes. After hydrolysis the aglycone was extracted by EtOAc while the sugars present in the aqueous extract were neutralized by Na_2CO_3.

Identification of Aglycone:

Complete evaporation of the EtOAc extract afforded a yellow solid. It appeared yellow under UV (366 nm) light and remained unchanged in NH_3 vapours, indicating it to be a flavonol. UV spectrum in MeOH exhibited two absorption maxima at 371 nm (band-I) and 259 nm (band-II), typical for flavonol aglycones (Markham, 1982). On addition of NaOAc to the methanolic solution of the aglycone, a bathochromic shift of 12 nm in banf-II was observed with respect to MeOH spectrum, indicating the presence of a free hydroxyl group at position 7 of ring A. Addition of H_3BO_3 to the methanolic solution of the aglycone, containing NaOAc, produced an insignificant bathochromic shift of 4.0 nm in band-I indicating the absence of orthodihydroxylation in ring B. A significant bathochromic

shift of 55 nm was observed in band-I by the addition of 5% alcoholic solution of $AlCl_3$. Addition of HCl in the methanolic solution of the aglycone, containing $AlCl_3$, did not exhibit a hypsochromic shift in band-I, which indicated the presence of a free hydroxyl group at position 5 and 3 and the absence of orthodihydroxylation in ring B. A bathochromic shift of 50 nm in band-I was observed after the addition of NaOMe with respect to MeOH spectrum of the aglycone indicating the presence of a free 4'-OH. Slow decomposition of the spectrum after 5 minutes indicated the presence of a 3, 4' hydroxylation pattern.

In EIMS a molecular ion peak for the aglycone was observed at m/z 286. The other important fragment ions appeared at m/z 153 $(A_1+H)^+$, 121 $(B_2)^+$ and 93 (B_2^+-28). This typical fragmentation pattern of the aglycone suggested it to be a 3, 5, 7, 4'-tetrahydroxy flavone.

^1H NMR (CD_3OD, 300 MHz) spectrum of the aglycone exhibited two doublets at δ 6.12 (1H, *d*, $J_{6,8}$=2.0 Hz) and δ 6.24 (1H, *d*, $J_{8,6}$=2.0 Hz) representing absorption due to two meta coupled protons i,e. H-6 and H-8 of ring A. Chemical shifts for H-3' and H-5' was observed at δ 6.91 (2H, *d*, $J_{2',3'}$, $J_{5',6'}$= 8.0 Hz) showing an ortho coupling with H-2' and H-6' which resonated at δ 8.04 (2H, *d*, $J_{3',2'}$, $J_{6',5'}$= 8.0 Hz).

On the basis of co-chromatography, UV, EIMS and ^1H NMR data the aglycone of the compound IHF-2 was identified as 3, 5, 7, 4'-terahydroxy flavone or kaempferol.

Identification of sugars:

The neutralized aqueous sugar extract , obtained after acid hydrolysis of IHF-1, was subjected to PC on Whatman paper No.1 along with standard sugars. The chromatogram was developed in five solvent system: BAW, BTPW, EPAW and PhOH. Galactose, rhamnose and arabinose were identified which had the same R_f values in all solvent systems as standared corresponding sugars. Trimethyl silyl derivatives of the sugars were subjected

to GC analysis. The same three sugars were detected by this tecnique also and confirmed the results obtained by PC.

Identification of glycoside:

UV spectrum of IHF-1 exhibited absorption bands at 263 and 347 nm, typical of 3-O-substituted flavonols. Addition of NaOAc to the methanolic solution did not produced any bathochromic shift in band-II with respect to the methanolic spectrum of IHF-1, indicating a 7-O-substituted flavonol. A pronounced shoulder and non-significant bathochromic shift in band-I in the presence of NaOAc and NaOAc-H_3BO_3, advocated the presence of a free hydroxyl group at 4'-position of ring B. On addition of 5% alcoholic solution of $AlCl_3$, a bathochromic shift of 49 nm was observed in band-I, indicating the presence of a free hydroxyl group at position 5 of ring A. Addition of HCl to the methanolic solution of IHF-1, containing $AlCl_3$, showed a non-significant hypsochromic shift of 3.0 nm which confirmed the absence of orthodihydroxylation in ring B. Spectrum recorded in the presence of NaOMe exhibited a bathochromic shift of 38 nm in band-I with respect to methanolic spectrum and further confirmed the presence of a free 4'-OH at ring B. UV spectra recorded in MeOH and on addition of classical shift reagents suggested compound IHF-1 to be a 3, 7-O-disubstituted flavonol.

IR spectrum recorded in KBr displayed intense absorption bands at 3415 cm^{-1} (free OH), 1656 cm^{-1} (conjugated carbonyl), 1570-1446 cm^{-1} (aromatic ring) and 1065 cm^{-1} (C-O-C).

EIMS of IHF-1 showed a molecular ion peak at m/z 286 (100%) which could be assigned to the aglycone. The other diagnostic fragments were same as mentioned in the case of aglycone. FABMS recorded in positive mode in lactic acid displayed molecular ion peak at m/z 727 $[M+H]^+$. The fragments from the subsequent losses of a pentose and hexose (m/z 449 $[M-132-146+1]^+$), one hexose (m/z 287 $[M-132-146-162+1]^+$) were observed, indicating the attachment of either two interlinked hexoses at position C-3 and a

pentose at C-7 or one hexose interlinked to a pentose at position C-3 and a hexose at position C-7 of the aglycone or vise virsa.

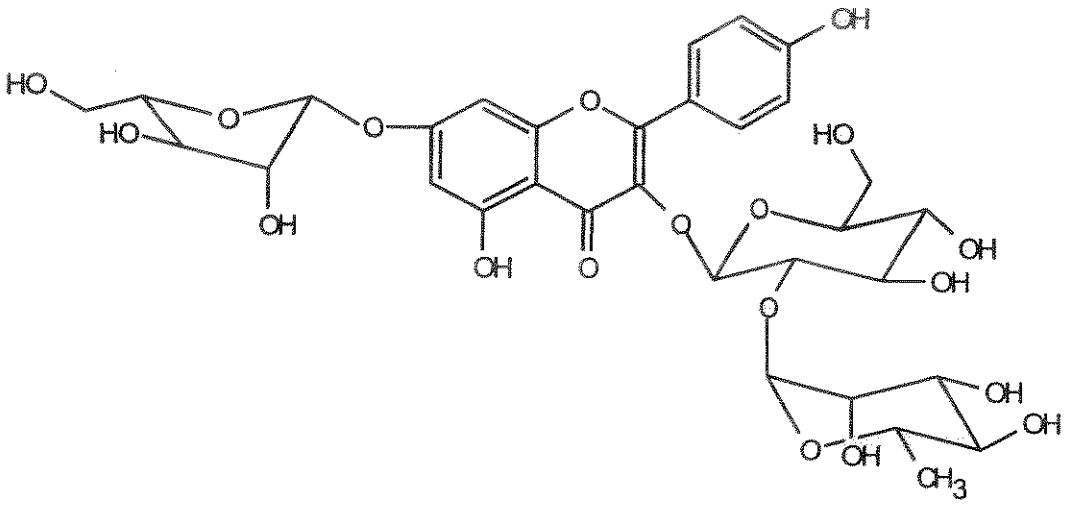
^{1}H NMR (CD_3OD, 300 MHz) of IHF-1 displayed two doublets at δ 6.45 (1H, d, $J_{6,8}$=2.2 Hz) and 6.75 (1H, d, $J_{8,6}$=2.2 Hz) due to H-6 and H-8 of ring A, showing meta coup;ing with each other. Signal for H-2', H-6' and H-3', H-5' were observed at δ 8.01 (2H, d, $J_{2',3'}$=9.1, $J_{6',5'}$=9.1 Hz) and 6.90 (2H, d, $J_{3',2'}$=9.1, $J_{5',6'}$=9.1 Hz) respectively. Three one proton signals at δ 5.20 (1H, d, $J_{1'',2''}$=5.5 Hz), 5.70 (1H, d, $J_{1''',2'''}$=1.2 Hz) and 5.59 (1H, d, $J_{1'''',2''''}$=1.0 Hz), showing β and α configurations, were assigned to the anomeric protons of galactose, rhamnose and arabinose respectively. Rest of the sugar protons resonated in the range of 3.33-4.50 ppm. CH_3 of rhamnose appeared at δ 1.60 (1H, d, J=6.0 Hz). To confirm the attachment of the sugars at C-7 and C-3 positions, NOE measurements were carried out. Irradiation of H-8 showed a NOE effect on anomeric proton of arabinose, indicating its attachment at C-7. Similarly a marked NOE effect was observed on anomeric proton of galactose by the irradiation of H-2' and H-6' of ring B, showing its attachment at C-3 of the aglycone. In order to establish the interglycosidic linkage between rhamnose and either sugar, anomeric proton of rhamnose was irrupted. A strong NOE effect connecting anomeric proton of rhamnose to H-2 of galactose was clearly detected which supported 1→2 type of linkage (Homans, 1990).

^{13}C NMR spectrum (CD_3OD, 75.5 Mhz) of compound IHF-1 showed 32 carbon resonances, presenting in Table-23. The multiplicities were determined by DEPT experiments. The assignments revealed the presence of 9 quaternary, 20 methine, 2 methylene and one methyl carbon. C-2, C-3 and C-4 of ring C resonated at δ 158.2, 135.4 and 178.5 respectively, while signals appeared at δ 163.6, 99.9, 163.4 and 95.5 were assigned to C-5, C-6, C-7 and C-8 of ring A. Chemical shifts of C-9 and C-8 were observed at δ 158.2 and 104.5. The chemical shift observed at δ 121.7, 132.3, 117.2, 160.0, 117.2 and 132.2 were attributed to C-1', C-2', C-3', C-4', C-5' and C-6' of ring B respectively. Two anomeric carbons, belonging to galactose and rhamnose appeared at δ 99.5 and 100.6 respectively, indicating their C-O-linkage to the aglycone and among themselves. The

signals observed at δ 78.1, 73.7, 71.2, 75.2 and 61.9 were assigned to C-2", C-3", C-4", C-5" and C-6" of galactose. C-2", C-3", C-4", C-5" and C-6" of rhamnose exhibited their chemical shifts at δ 71.8, 72.2, 73.7, 68.0 and 18.1. A downfield shift of 6.8 ppm in C-2 of galactose confirmed 1→2 linkage between rhamnose and galactose. ^{13}C NMR signals additional to those of kaempferol 3-O-rhmnogalactoside, were observed at δ 109.9, 82.4, 78.2, 86.2 and 63.1 equate clearly with arabinifuranoside rather than arabinopyranoside (Markham and Chari, 1982, Agrawal *et al*, 1985 and Yasukawa *et at*, 1990). The conclusions drawn from $FAB^{+}MS$, ^{1}H NMR, NOE difference measurements and ^{13}C (B.B and DEPT) NMR were also confirmed by ^{1}H, ^{1}H-COSY experiments.

On the basis of chromatographic data, acid hydrolysis, UV, IR, Mass, ^{1}H NMR, ^{13}C (B.B & DEPT) NMR, NOE measurements and ^{1}H, ^{1}H-COSY experiments the structure of IHF-1 was determined as kaempferol 3-O-α-L-rhamnopyranosyl(1→2)-β-D-galactopyranoside-7-O-α-L-arabinofuranoside.

The structure of IHF-1 was further confirmed by alkaline hydrolysis with NaOH which converted it to a product with a free 7-hydroxyl group and arabinose. Structure of kaempferol 3-O-α-L-rhamnopyranosyl(1→2)-β-D-galactopyranoside was confirmed by UV and ^{13}C NMR. The ^{13}C NMR spectra of kaempferol 3-O-α-L-rhamnopyranosyl(1→2)-β-D-galactopyranoside was similar to the reported data (Markham and Chari, 1982).



Kaempferol 3-O-α-L-rhamnopyranosyl (1→2)-β-D-galactopy-ranoside-7-O-α-L-arabinofuranoside

Table.22 ^{1}H NMR SPECTRAL DATA OF COMPOUND IHF-1

(CD_3OD, 300 MHz)

H	(δ)	Multiplicity	J (Hz)
H-6	6.45	*d*	2.2
H-8	6.75	*d*	2.2
H-2'	8.01	*d*	9.1
H-3'	6.90	*d*	9.1
H-5'	6.90	*d*	9.1
H-6'	8.01	*d*	9.1
H-1"	5.25	*d*	5.5
H-1"'	5.56	*d*	1.5
H-1""	5.55	*br.s*	1.0
CH_3	1.25	*d*	6.0

Table.23 ^{13}C (DEPT) NMR SPECTRAL DATA OF COMPOUND IHF-1

(CD_3OD, 75.5 Hz)

C	Aglycone DEPT Multiplcity	(δ)	C	Sugars DEPT Multiplicity	(δ)
C-2	-C-	158.2	C-1"	-CH-	99.8
C-3	-C-	135.4	C-2"	-CH-	78.1
C-4	-C-	178.5	C-3"	-CH-	73.7
C-5	-C-	163.6	C-4"	-CH-	71.2
C-6	-CH-	99.9	C-5"	-CH-	75.2
C-7	-C-	163.6	C-6"	$-CH_2-$	61.9
C-8	-CH-	95.5	C-1'''	-CH-	100.6
C-9	-C-	158.2	C-2'''	-CH-	71.8
C-10	-C-	104.5	C-3'''	-CH-	72.2
C-1'	-C-	121.7	C-4'''	-CH-	73.7
C-2'	-CH-	132.3	C-5'''	-CH-	68.0
C-3'	-CH-	117.2	C-6'''	$-CH_3-$	18.1
C-4'	-C-	160.0	C-1''''	-CH-	109.9
C-5'	-CH-	117.2	C-2''''	-CH-	82.4
C-6'	-CH-	132.3	C-3''''	-CH-	78.2
			C-4''''	-CH-	86.2
			C-5''''	$-CH_2-$	63.1

COMPOUND IHF-2

Compound IHF-2 was also obtained as a dark yellow crystalline solid, which appeared violet under UV (366 nm) light and turned yellow when exposed to NH_3 vapours. Chromatographic behaviour of IHF-2 was identical to that of IHF-1, indicating it to be a flavonol triglycoside. In order to identify its aglycone and sugar parts it was subjected to acid hydrolysis.

Acid Hydrolysis:

5.0 mg of IHF-2 were hydrolyzed by 2 NHCl according to the standard procedure (Markham, 1982).

Identification of aglycone:

Kaempferol was identified as the aglycone part by means of co-TLC, UV, EIMS and ^{1}H NMR.

Identification of sugars:

Galactose, rhamnose and arabinose were identified as sugar moieties by using PC and GC techniques as described for compound IHF-1.

Identification of glycoside:

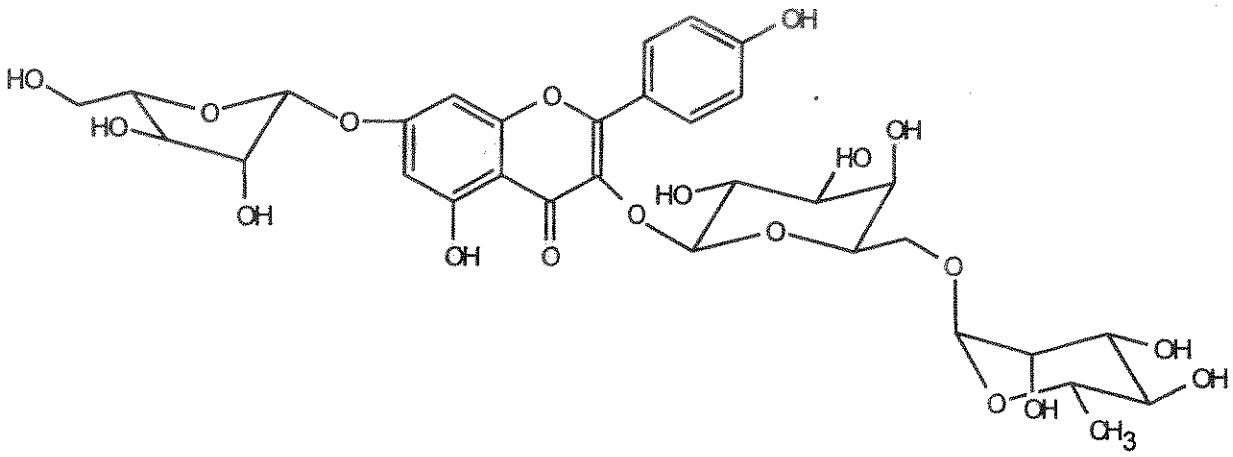
UV spectrum recorded in MeOH and in the presence of diagnostic shift reagents, EIMS and FAB$^+$MS spectral data were quite similar to that of IHF-1, indicating IHF-2 isomeric to IHF-1. NOE difference measurements confirmed the attachment of arabinose at C-7 and glactose at C-3 of the aglycone. Irradiation of the anomeric proton of rhamnose

exhibited a marked NOE effect on H-6 of galactose, indicating a 1→6 linkage between rhamnose and galactose.

^{13}C (B.B & DEPT) NMR (CD_3OD, 75.5 MHz) showed the identical resonances as reported for the kaempferol (Nielsen *et al*, 1993). Anomeric carbons of galactose and rhamnose appeared at δ 99.9 and 100.7 respectively. Chemical shifts observed at δ 71.3, 74.3, 69.1, 77.4 and 65.6 were assigned to C-2", C-3", c-4", C-5" and C-6" of galactose. C-2"', C-3"', C-4"', C-5"' and C-6"' of rhamnose resonated at δ 71.8, 72.1, 73.6, 69.4 and 18.0 respectively. A further 4.6 ppm downfield shift of C-6" of galactose confirmed the 1→6 linkage between the rhamnose and galactose. ^{13}C NMR signals appeared at δ 108.1, 80.5, 78.2, 87.5 and 62.5 were attributed to the sugar, arabinofuranoside, attached at C-7 position of the aglycone.

Mild hydrolysis with NaOH converted IHF-2 to a product with a free 7-hydroxyl group (UV, ^{13}C NMR data) and arabinose, The ^{13}C NMR spectra of hydrolyzed product was indistinguishable from that reported for kaempferol 3-O-α-L-rhamnopyranosyl(1→6)-β-D-galactopyranoside (Markham and Chari, 1982). Arabinose was identified by co-TLC and GC.

On the basis of chromatographic data, acid hydrolysis, UV, IR, Mass, ^1H NMR, ^{13}C (B.B & DEPT) NMR, NOE measurements and ^1H, ^1H-COSY experiments the structure of IHF-2 was established as kaempferol 3-O-α-L-rhamnopyranosyl(1→6)-β-D-galactopyranoside-7-O-α-L-arabinofuranoside.



Kaempferol 3-O-α-L-rhamnopyranosyl(1→6)-β-D-galactopy-ranoside-7-O-α-L-arabinofuranoside

Table.24 ^{1}H NMR SPECTRAL DATA OF COMPOUND IHF-2

(CD_3OD, 300 MHz)

H	(δ)	Multiplicity	J (Hz)
H-6	6.44	*d*	2.2
H-8	6.75	*d*	2.2
H-2'	7.95	*d*	9.1
H-3'	6.90	*d*	9.1
H-5'	6.90	*d*	9.1
H-6'	7.95	*d*	9.1
H-1"	5.20	*d*	5.5
H-1"'	5.70	*d*	1.5
H-1""	5.59	*br.s*	1.0
CH_3	1.20	*d*	6.5

Table.25 ^{13}C (DEPT) NMR SPECTRAL DATA OF COMPOUND IHF-2 (CD_3OD, 75.5 Hz)

C	Aglycone DEPT Multiplcity	(δ)	C	Sugars DEPT Multiplicity	(δ)
C-2	-C-	158.2	C-1"	-CH-	99.9
C-3	-C-	135.1	C-2"	-CH-	71.3
C-4	-C-	180.0	C-3"	-CH-	74.3
C-5	-C-	162.6	C-4"	-CH-	69.1
C-6	-CH-	95.4	C-5"	-CH-	77.4
C-7	-C-	163.6	C-6"	$-CH_2-$	65.6
C-8	-CH-	95.4	C-1"'	-CH-	100.7
C-9	-C-	158.2	C-2"'	-CH-	71.8
C-10	-C-	103.2	C-3"'	-CH-	72.1
C-1'	-C-	122.0	C-4"'	-CH-	73.6
C-2'	-CH-	132.1	C-5"'	-CH-	69.4
C-3'	-CH-	116.9	C-6"'	$-CH_3-$	18.0
C-4'	-C-	159.8	C-1""	-CH-	108.1
C-5'	-CH-	116.9	C-2""	-CH-	80.5
C-6'	-CH-	132.1	C-3""	-CH-	78.2
			C-4""	-CH-	87.5
			C-5""	$-CH_2-$	62.5

COMPOUND IHF-3

Flavonoid glycoside (IHF-3) was obtained as light yellow crystalline solid. It appeared violet under UV (366 mn) light and turned yellow in NH_3 vapours, indicating it to be a 3-O-substituted flavonol. Chromatographic profile with respect to the standard flavonol glycosides suggested it to be a flavonol diglycoside. In order to identify the aglycone and the sugars, compound IHF-3 as subjected to acid hydrolysis.

Acid Hydrolysis:

Acid hydrolysis of IHF-3 was carried out similarly as described for compound IHF-1.

Alkaline hydrolysis:

On alkaline hydrolysis IHF-3 remained unchanged and no organic acid was detected, showing that it was not an acylated flavonoid glycoside.

Identification of aglycone:

Kaempferol was identified as the aglycone part by means of co-TLC, UV, EIMS and ^{1}H NMR.

Identification of sugars:

Rhamnose and arabinose were identified as the sugars of IHF-3 by using PC and GC techniques.

Identification of glycoside:

UV spectrum recorded in MeOH showed absorption maxima at 264 nm (band-II) and 343 nm (band-I), which are characteristic absorption limits for 3-O-substituted flavonols. On addition of NaOAc, no bathochromic shift was observed in band-II as compared to the methanolic spectrum, which indicated a substitution at position 7. No significant effect was observed in the presence of NaOAc-H_3BO_3 in band-I showing the absence of orthodihydroxylation in the molecule. On addition of 5% alcoholic solution of $AlCl_3$, a bathochromic shift of 51 nm was observed in band-I, indicating the presence of a free 5-OH at ring A. Addition of HCl to the methanolic solution of IHF-3, containing $AlCl_3$, exhibited a hypsochromic shift of 9.0 nm which confirmed the absence of orthodihydroxylation in ring B. A bathochromic shift of 37 nm with an increase of intensity of band-I was produced by NaOMe with respect to the methanolic spectrum, indicating the presence of a free 4'-OH. UV spectra recorded in MeOH and on addition of diagnostic shift reagents indicated IHF-3 to be a 3, 7-O-disustituted flavonol.

IR spectrum (KBr) exhibited important absorption bands at 3400 cm^{-1} (free OH), 1657 cm^{-1} (conjugated carbonyl), 1546-1449 cm^{-1} (aromatic ring) and 1069 cm^{-1} (C-O-C).

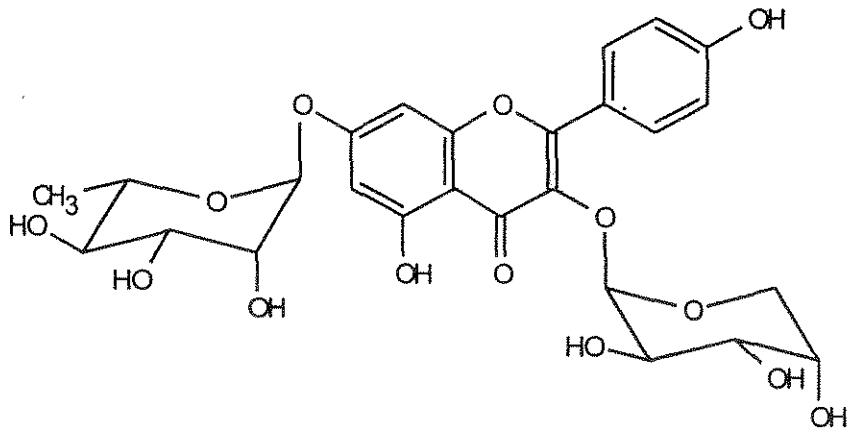
In EIMS the molecular ion peak for the aglycone was observed at m/z 286 (100%). The other characteristic fragments were similar as described for the kaempferol.

^{1}H NMR (CD_3OD, 300 MHz) displayed two signals at δ 6.41 (1H, *d*, $J_{6,8}$=2.0 Hz) and 6.67 (1H, *d*, $J_{8,6}$=2.0 Hz) corresponding to H-6 and H-8 of ring A, present at meta position with each other. Two set of doublets observed at δ 6.90 (2H, *d*, $J_{2',6'}$=9.0 Hz) and 7.78 (2H, *d*, $J_{3',5'}$=9.0 Hz) were assigned to 2',6' and 3',5' protons of ring B respectively. Two anomeric protons, belonging to rhamnose and arabinose, appeared at δ 5.54 (1H, *d*, $J_{1'',2''}$=1.5 Hz) and 5.38 (1H, *d*, $J_{1'',2''}$=1.8 Hz) respectively, indicating their α-configurations. H-2", H-3", H-4" of arabinose resonated at δ 4.04 (1H, *dd*, J=5.7, 1.8 Hz), 3.31 (1H, *dd*,

J=5.7, 2.0 Hz) and 3.38 (1H, *m*) respectively. Signals for H-5"$_a$ and H-5"$_b$ were observed at δ 3.35 (1H, *dd*, J=11.3, 5.0 Hz) and 3.30 (1H, *dd*, J=11.3, 2.0 Hz). The chemicalshifts observed at δ 4.22 (1H, *dd*, J=2.0, 5.0 Hz), 3.85 (1H, *dd*, J=2.0, 10.0 Hz), 3.48 (1H, *t*, J=10.0 Hz) and 3.63 (1H, *m*) were assigned to H-2"', H-3"', H-4"' and H-5"' of rhamnose. CH_3 of rhamnose appeared at δ 0.93 (3H, *d*, J=7.5 Hz).

In order to confirm the position of attachment of rhamnose and arabinose, IHF-3 was subjected to partial acid hydrolysis (Markham, 1982). The hydrolyzed product had violet colour under UV and subsequent UV analysis revealed the cleavage of sugar from position 7 (bathochromic shift in band-II) of NaOAc spectrum). Rhamnose was identified by means of PC and GC. After partial acid hydrolysis the position of rhamnose was confirmed at C-7 and arabinose at C-3 of the aglycone.

On the basis of R_f values, acid hydrolysis, UV, IR, EIMS and ^{1} NMR spectral data, the structure of IHF-3 was identified as kaempferol 3-O-α-L-arabinopyranoside-7-O-α-L-rhamnopyranoside.



Kaempferol 3-O-α-L-arabinopyranoside-7-O-α-L-rhamnopyranoside.

Table.26 ^{1}H NMR SPECTRAL DATA OF COMPOUND IHF-3 (CD_3OD, 300 MHz)

H	(δ)	Multiplicity	J (Hz)
H-6	6.41	*d*	2.0
H-8	6.67	*d*	2.0
H-2'	6.90	*d*	9.0
H-3'	7.78	*d*	9.0
H-5'	7.78	*d*	9.0
H-6'	6.90	*d*	9.0
H-1"	5.38	*d*	1.8
H-2"	4.04	*dd*	5.7/1.8
H-3"	3.31	*dd*	5.7/2.0
H-4"	3.38	*m*	-
H-5"$_a$	3.35	*dd*	11.3/5.0
H-5"$_b$	3.30	*dd*	11.3/2.0
H-1"'	5.54	*d*	1.5
H-2"'	4.22	*dd*	2.0/5.0
H-3"'	3.85	*dd*	2.0/10.0
H-4"'	3.48	*t*	10.0
H-5"'	3.63	*m*	-
CH_3	0.93	*d*	7.5

COMPOUND IHF-4

Compound IHF-4 was obtained as yellow crystalline solid. It appeared violet under UV (366 nm) light and turned yellow when exposed to NH_3 vapours, showing it to be a 3-O-substituted flavonol. Its chromatographic behaviour in three solvent systems (BAW, 15% HOAc, 60% HOAc) was similar to the flavonol glycoside IHF-3. The nature of aglycone and the sugar was determined after its acid hydrolysis.

Acid Hydrolysis:

Acid hydrolysis of IHF-4 was carried out according to the standard procedure.

Alkaline hydrolysis:

Alkaline hydrolysis was carried out according to the standared procedure. No organic acid was detected, showing that it was not an acylated flavonoid glycoside.

Identification of aglycone:

Kaempferol was identified as the aglycone by co-TLC, UV, EIMS and ^1H NMR techniques..

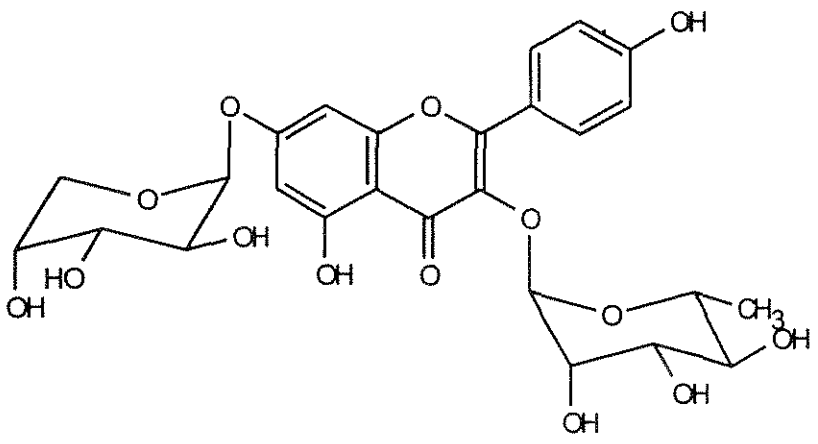
Identification of sugars:

Rhamnose and arabinose were identified as the sugar moieties by means of PC and GC.

Identification of glycoside:

UV, IR, EIMS and ^{1}H NMR spectra of IHF-4 were quite similar to those of compound IHF-3, indicating them to be the isomeric with each other. Partial acid hydrolysis of IHF-4 resulted in a product, kaempferol 3-O-rhamnoside with free 7-OH and arabinose. Kaempferol 3-O-rhamnoside was identified by UV, EIMS and ^{1}H NMR while arabinose was identified by means of PC and GC. Thus partial acid hydrolysis established the site of glycosylation of rhamnose at C-3 and that of arabinose at C-7 of the kaempferol.

On the basis of chrmatographic data, UV, IR, EIMS and ^{1}H NMR the structure of IHF-4 was identified as kaempferol 3-O-α-L-rhamnopyranoside-7-O-α-L-arabinopyranoside.



Kaempferol 3-O-α-L-rhamnopyranoside-7-O-α-L-arabinopyranoside.

Table.27 ^{1}H NMR SPECTRAL DATA OF COMPOUND IHF-4 (CD_3OD, 300 MHz)

H	(δ)	Multiplicity	J (Hz)
H-6	6.45	*d*	2.0
H-8	6.74	*d*	2.0
H-2'	6.87	*d*	9.0
H-3'	7.95	*d*	9.0
H-5'	7.95	*d*	9.0
H-6'	6.87	*d*	9.0
H-1"	5.57	*d*	1.5
H-2"	4.02	*dd*	2.0/5.0
H-3"	3.83	*dd*	2.0/10.0
H-4"	3.56	*t*	10.0
H-5"	3.65	*m*	-
CH_3	1.25	*d*	7.5
H-1"'	5.50	*br.s*	1.o
H-2"'	4.32	*dd*	5.7/1.5
H-3"'	3.35	*dd*	5.7/2.0
H-4"'	3.40	*m*	-
H-5"'$_a$	3.37	*dd*	11.2/5.0
H-5"'$_b$	3.32	*dd*	11.2/5.0

COMPOUND IHF-5

Flavonoid glycoside (IHF-5) was obtained as yellow crystalline solid. It appeared yellow under UV (366 nm) light and remained yellow on exposure to NH_3 vapours, indicating it to be a 7-O-substituted flavonol. R_f values recorded along with the standard flavonol glycosides in solvent system H_2O:EtOH:MEK:Acetyl acetone (13:3:3:1) suggested this compound to be a 7-O-substituted monoglycoside (Hasan, 1976). In order to identify the aglycone and the sugar residue, compound IHF-5 was subjected to acid hydrolysis.

Acid Hydrolysis:

Acid hydrolysis of IHF-5 was carried out by the same procedure as described for the compound IHF-4.

Alkaline hydrolysis:

On alkaline hydrolysis IHF-4 remained unchanged and no organic acid was detected, showing that it was not an acylated flavonoid glycoside.

Identification of aglycone:

Kaempferol was identified as the aglycone by means of co-TLC, UV, EIMS and ^1H NMR.

Identification of sugar:

Rhamnose was identified as the only sugar in IHF-5 by using PC and GC techniques.

Identification of glycoside:

In order to find out the position of attachment of the sugar to the aglycone, UV spectrum of IHF-5 was recorded in MeOH which showed two absorption bands at 365 nm (band-I) and 263 nm (band-II), a typical absorption range for a flavonol. Absence of a bathochromic shift in band-II, on addition of NaOAc to the methanolic solution of IHF-5, indicated a substitution at C-7 of ring A. On addition of H_3BO_3 no hypsochromic shift was observed in band-I as compared to the spectrum recorded in the presence of NaOAc, which indicated the absence of an orthodihydroxylation at ring B. A bathochromic shift of 59 nm was recorded by the addition of 5% alcoholic solution of $AlCl_3$, which confirmed the presence of a free hydroxyl groups at position 5 and 3. Addition of HCl to the methanolic solution of IHF-5, countering $AlCl_3$, did not show a hypsochromic shift in band-I as compared to $AlCl_3$ spectrum, which indicated the absence of orthodihydroxylation in ring B. Addition of NaOMe caused a bathochromic shift of 53 nm in band-I showing the presence of a free 4'-OH at ring B. UV spectra recorded in MeOH and on addition of shift reagents suggested compound IHF-5 to be a 7-O-substituted flavonol, with the sugar (rhamnose) attached at position 7 of the aglycone.

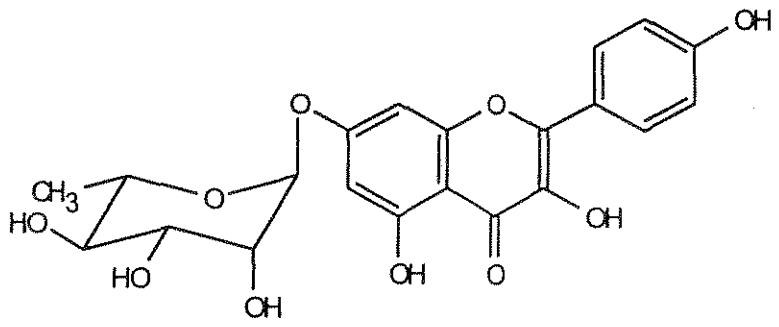
IR spectrum recorded in KBr exhibited intense absorption bands at 3412 cm^{-1} (free OH), 1654 cm^{-1} (conjugated carbonyl), 1560-1451 cm^{-1} (aromatic ring) and 1065 cm^{-1} (C-O-C).

EIMS of IHF-5 displayed a molecular ion peak at m/z 286 (100%). The other characteristic fragments were similar as described for the kaempferol.

^{1}H NMR (CD_3OD, 300 MHz) of IHF-5 exhibited two signals at δ 6.42 (1H, *d*, $J_{6,8}$=2.0 Hz) and 6.73 (1H, *d*, $J_{8,6}$=2.0 Hz) due to meta coupled H-6 and H-8 of ring A. The chemical shifts corresponding to H-2', H-6' and H-3', H-5' were observed at δ 8.10 (2H, *d*,

$J_{2',3'}$=9.0 Hz) and 6.89 (2H, *d*, $J_{5',6'}$=9.0 Hz) respectively. A signal for anomeric proton of rhamnose appeared at δ 5.55 (1H, *d*, $J_{1'',2''}$=1.5 Hz). NMR signals for H-2" and H-3" were observed at δ 4.01 (1H, *dd*, $J_{1'',2'',3''}$=2.0, 5.0 Hz) and 3.82 (1H, *dd*, $J_{3'',2'',4''}$=2.0, 10.0 Hz) while H-4" and H-5" resonated at δ 3.47 (1H, *t*, $J_{4'',3'',5''}$=10.0 Hz) and 3.5-3.66 (1H, *m*). CH_3 of rhamnose appeared at δ 1.25 (1H, *d*, $J_{6'',5''}$=7.0 Hz).

On the basis of chromatic data, acid hydrolysis, UV, IR, EIMS and ^{1}H NMR spectral data, structure of IHF-5 was identified as kaempferol 7-O-α-L-rhamnopyranoside.



Kaempferol 7-O-α-L-rhamnopyranoside

Table.28 ^{1}H NMR SPECTRAL DATA OF COMPOUND IHF-5 (CD_3OD, 300 MHz)

H	(δ)	Multiplicity	J (Hz)
H-6	6.42	*d*	2.0
H-8	6.73	*d*	2.0
H-2'	8.10	*d*	9.0
H-3'	6.89	*d*	9.0
H-5'	6.89	*d*	9.0
H-6'	8.10	*d*	9.0
H-1"	5.55	*d*	1.5
H-2"	4.01	*dd*	2.0/5.0
H-3"	3.82	*dd*	2.0/10.0
H-4"	3.47	*t*	10.0
H-5"	3.5-3.6	*m*	-
CH_3	1.25	*d*	7.0

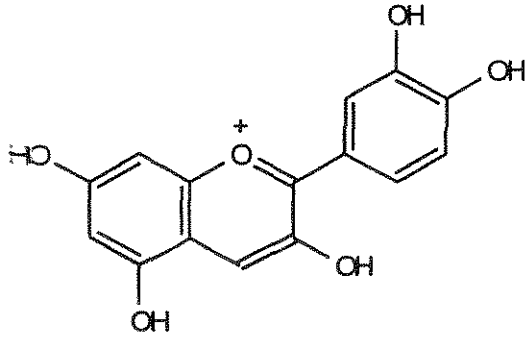
3.2.2 ANTHOCYANIDINS

3.2.2.1 EXTRACTION

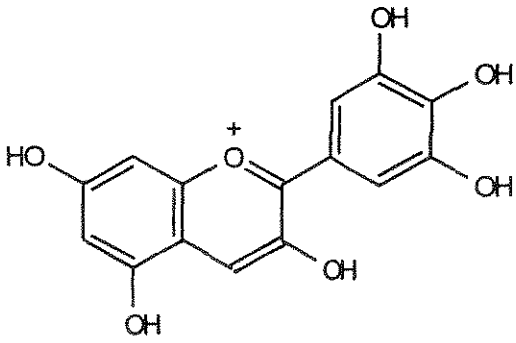
Extraction of anthocyanidin was carried out by the same procedure as described under *Rumex chalepensis*.

3.2.2.2 SEPARATION, PURIFICATION AND IDENTIFICATION

The separation and purification of anthocyanidins was carried out by means of preparative PC on Whatman No.1. The concentrated n-BuOH extract, containing anthocyanidins, was streaked at one end of the chromatography paper and the paper was developed in a descending mode employing BAW as the solvent system. The developed chromatograms revealed two bands. The separated strips of two bands were further cut in to small pieces and eluted with MeOH-1% Hcl (99:1). After 24 hours contact, the eluent was filtered and concentrated on a rotary evaporator at low temperature. The purity of these two fractions was checked in three solvent systems. A single spot was detected in each case. R_f values in three solvent systems and UV spectra of these two fractions, recorded first in MeOH and then in 5% $AlCl_3$, were compared with the standared substances (Table-29). On the basis of R_f values and UV-visible spectral data the compound IHF-1A and IHF-2A were identified as Cyanidin and Delphinidin.



Cyanidin



Delphinidin

Table.29 UV SPECTRAL/QUALITATIVE ANALYSIS OF ANTHOCYANIDINS

Compound	R_f (x100)			Colour under UV	Colour under day light	λ_{max} MeOH			MeOH/$AlCl_3$		
IHF-1A	48	21	66	Pink	Magenta	537	-	364	556	-	422
IHF-2A	33	14	41	Mauve	Purple	546	-	361	580	-	359
Delphinidin	32	13	42	Mauve	Purple	546			+		
Petunidin	46	20	52	Mauve	Purple	543			+		
Cyanidin	49	22	68	Pink	Magenta	535			+		
Peonidin	63	30	71	Pink	Magenta	532			-		
Pelargonodin	68	33	80	Red	Purple	520			-		
Malvidin	60	27	58	Mauve	Purple	542			-		

Solvent system 1 = FORESTAL ($HOAc$-H_2O-HCl)
Solvent system 2 = HCO_2H-HCl-H_2O (5:2:3)
Solvent system 3 = BAW [*n*-BuOH-$HOAc$-H_2O (4:1:5)]

3.3 PHYTOCHEMICAL ANALYSIS OF *INDIGOFERA HEBEPETALA* LEAVES

3.3.1 NATURAL GLYCOSIDES

3.3.1.1 EXTRACTION

Extraction of flavonoid glycosides from *Indigofera hebepetala* leaves (1.0 Kg) was carried out by the same procedure as described for the *Indigofera hebepetala* flowers. Extraction resulted in a dry EtOAc extract of 12.1 g.

3.3.1.2 SEPARATION AND PURIFICATION

The methanolic solution of EtOAc was dissolved in MeOH and was applied as a single spot near one corner of Whatman No.1 chromatography paper. The chromatogram was suspended in an all-glass PC tank containing BAW as the developing solvent. After complete development (18 hrs), the chromatogram was removed from the tank and dried in a fume cupboard. The folded portion of the dried chromatogram was trimmed off and the chromatogram refolded for descending chromatography in the second dimension. 15% HOAc was used as the second developing solvent. In this way a total of 100 chromatograms were developed, dried and viewed under UV (366 nm) light. Four prominent spots, one dull yellow and three violet, were penciled out. The identical spots from each paper were then cut out, combined and socked in distilled MeOH. After 24 hours of contact with occasional shaking, the MeOH extract was filtered. This extraction procedure was repeated thrice and the combined extract of each spot was filtered and then evaporated to dryness.

Final purification of the above flavonoids was carried out by 2DTLC (polyamide 6F, Riedel-de-Hae'n) using C_6H_6-MeCOEt-MeOH (4:3:3) and 15% HOAc as the solvent system. The final purification resulted in the following four flavonol glycosides

Table.30 SPECTRAL AND CHROMATOGRAPHIC DATA OF FLAVONOID GLYCOSIDES ISOLATED FROM *INDIGOFERA HEBEPETALA* LEAVES

UV λ_{max}	IHL-1				IHL-2				IHL-3				IHL-4			
MeOH	264		323	365	244	266	315	347	243	266	316	345	246	266	316	345
NaOAc	265		325	375	266	307		365	265	303		370	265	303		370
NaOAc/H_3BO_3	261		323	367	266	300		349	266	304		345	266	304		345
$AlCl_3$	275	306	350	397	275	308	352	400	276	309	352	397	276	309	352	397
$AlCl_3$/HCk	276	305	348	397	274	304	348	399	275	307	350	397	275	307	350	397
NaOMe	245	267	335	429	246	274	301	387	243	274	302	382	243	274	302	382
EIMS	m/z 286 (100%)				m/z 286 (100%)				m/z 286 (100%)				m/z 286 (100%)			
AGLYCONE	Kaempferol				Kaempferol				Kaempferol				Kaempferol			
SUGARS	Allose				Arabinose				Rhamnose+Arabinose				Arabinose+Rhamnose			
R_f x 100 (TLC on Polyamide 6F)																
C_6H_6-MeCOEt-MeOH (4:3:3)	59				61				89				85			
H_2O-MeOH-*n*-BuOH-HOAc (75:15:10:2)	13				80				71				76			
H_2O-EtOH-MeCOEt-Acet.Me_2CO (13:3:3:1)	21				66				56				65			

1- IHL-1 = 5.0 mg

2- IHL-2 = 6.0 mg

3- IHL-3 = 8.0 mg

4- IHL-4 = 7.0 mg

3.3.1.3 Identification:

The four purified flavonoid glycosides were identified by means of R_f values, acid hydrolysis and UV spectral data.

COMPOUND IHL-1

Compound IHL-1, a pale yellow solid, appeared fluorescent yellow under UV (366 nm) light and remained unchanged in NH_3 vapours. Position of spot on 2DPC suggested it to be a 7-O-monoglycoside. The mobility of IHL-1 in the solvent system H_2O-EtOH-MeCOEt-Acetyl acetone (13:3:3:1) as compared to the authentic markers also indicated it to be a flavonol monoglycoside. Identification of the aglycone and the sugar was achieved by acid hydrolysis.

Acid Hydrolysis:

Acid hydrolysis of IHL-1 was carried out according to the standard procedure.

Alkaline hydrolysis:

On alkaline hydrolysis IHL-1 remained unchanged and no organic acid was detected, showing that it was not an acylated flavonoid glycoside.

Identification of aglycone:

Kaempferl was identified as the aglycone by means of co-TLC and UV analysis.

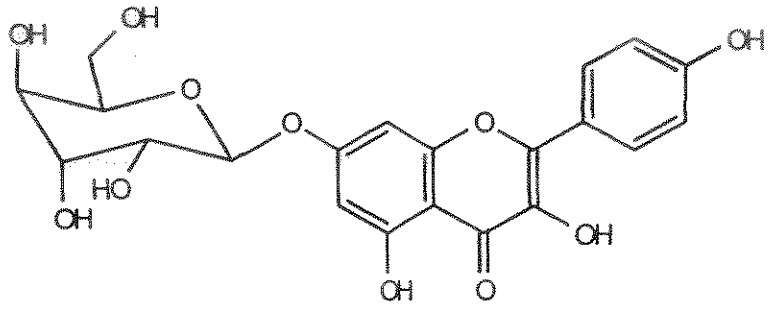
Identification of sugar:

Allose was identified as the sugar moiety by recording its R_f values in five solvent systems along with standard sugar samples (Harborne, 1984).

Identification of glycoside:

In order to find out the site of glycosylation, UV spectra of IHL-1 were recorded in MeOH and in the presence of diagnostic shift reagents. UV spectrum in MeOH showed two absorption bands at 365 nm (band-I) and 264 nm (band-II), indicating it to be a flavonol monoglycoside. UV spectra recorded after the addition of shift reagents indicated the presence of three hydroxyls at 4' (64 nm bathochromic shift of band-I with NaOMe, increased intensity), 3 and 5 (59 nm bathochromic shift with $AlCl_3$). No hypsochromic shift in $AlCl_3$/HCl and no bathochromic shift of band-I and II with NaOAc/H_3BO_3 revealed the absence of an orthodihydroxylation in ring A or B. Presence of sugar at position 7 was inferred by the absence of a bathochromic shift in band-II with NaOAc.

On the basis of R_f values, acid hydrolysis and UV spectral analysis the structure of IHL-1 was established as kaempferol 7-O-β-D-allopyranoside.



Kaempferol 7-O-β-D-allopyranoside

COMPOUND IHL-2

IHL-2 was obtained as pale yellow crystalline solid. It appeared violet under UV (366 nm) light and turned yellow in NH_3 vapours, indicating it to be a 3-O-substituted flavonol. R_f values recorded after co-chromatography with authentic markers in solvent system H_2O-EtOH-MEK-Acetyl acetone (13:3:3:1) suggested this compound to be a 3-O-substituted flavonol diglycoside. In order to identify the aglycone and the sugar residue, compound IHL-2 was subjected to acid hydrolysis.

Acid Hydrolysis:

Acid hydrolysis was performed according to the standard procedure by 2N HCl.

Alkaline hydrolysis:

On alkaline hydrolysis IHL-2 remained unchanged and no organic acid was detected, showing that it was not an acylated flavonoid glycoside.

Identification of aglycone:

Kaempferol was identified as the aglycone with the help of co-TLC and UV analysis.

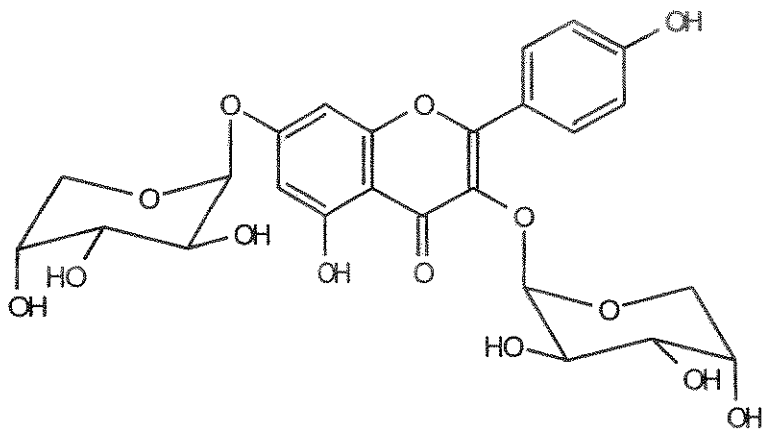
Identification of sugars:

Arabinose was identified as the only sugar present in IHL-2 bymeans of PC.

Identification of glycoside:

The position of attachment of the sugars became clear after recording the UV spectra of IHL-2 in MeOH and in the presence of shift reagents. Spectrum recorded in MeOH exhibited two absorption maxima at 347 nm (band-I) and 266 nm (band-II), indicating it to be a 3-O-substituted flavonol. On addition of NaOAc no bathochromic shift was observed which indicated a substitution at position 7 of ring A. Presence of free 4'-OH (40 nm bathochromic shift of band-I with NaOMe, with increased intensity) and 5-OH (53 nm bathochromic shift with $AlCl_3$) was detected by the addition of diagnostic shift reagents. Addition of NaOAc-H_3BO_3 did not show any bathochromic shift in band-I, indicating the absence of orthodihydroxylation in ring A or B. The typical absorption range in MeOH and the absence of bathochromic shift in band-II after the addition of NaOAc confirmed the site of attachment of two arabinose at C-3 and C-7 of the aglycone.

On the basis of R_f values, acid hydrolysis and UV spectral analysis the structure of IHL-2 was established as kaempferol 3, 7-O-α-L-diarabinopyranoside.



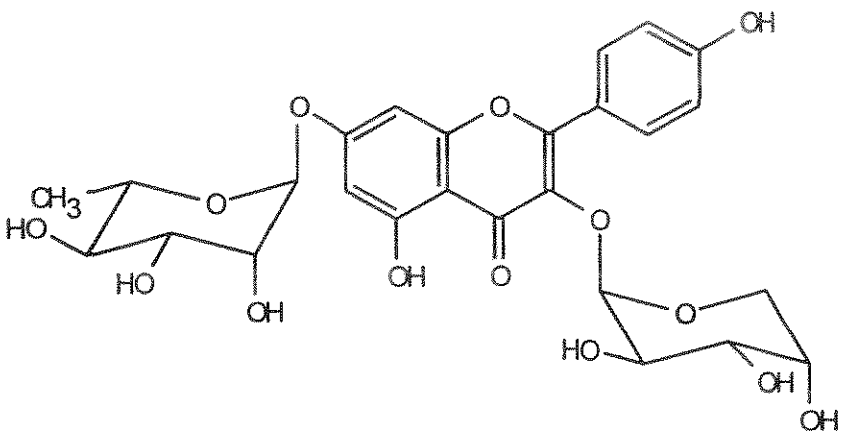
Kaempferol 3, 7-O-α-L-diarabinopyranoside

COMPOUND IHL-3, IHL-4

Flavonoid diglycosides (IHL-3 and IHL-4) were similar to the compounds IHF-3 and IHF-4. Their structures were determined by recording their R_f values in three solvent systems (BAW, 15% HOAc and 60% HOAc), acid hydrolysis and UV spectral analysis. Chromatographic and UV spectral data of compound IHL-3 and IHL-4 is presented below:

COMPOUND IHL-3

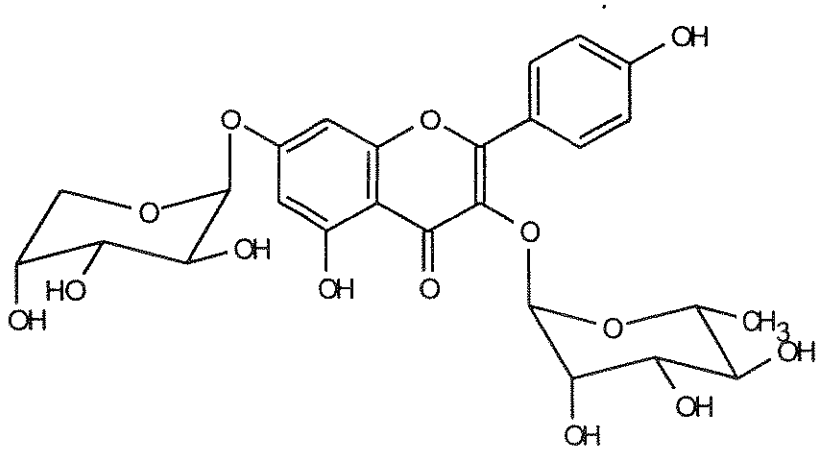
UV: violet, R_f, BAW: 0.78; 15% HOAc: 0.66; 60% HOAc: 0.77. UV λ_{max}(nm): MeOH: 265, 345; + $AlCl_3$: 272, 299, 350, 396; + HCl: 271, 298, 346, 395; + CH_3COONa: 265, 395; + H_3BO_3: 265, 348; + NaOMe: 266, 387.



Kaempferol 3-O-α-L-arabinopyranoside-7-O-α-L-rhamnopyranoside.

COMPOUND IHL-4

UV: violet, R_f, BAW: 0.79; 15% HOAc: 0.63; 60% HOAc: 0.77. UV λ_{max}(nm): MeOH: 264, 343; + $AlCl_3$: 272, 299, 346, 394; + HCl: 271, 300, 340, 393; + CH_3COONa: 264, 381; + H_3BO_3: 265, 348; + NaOMe: 264, 379.



kaempferol 3-O-α-L-rhamnopyranoside-7-O-α-L-arabinopyranoside.

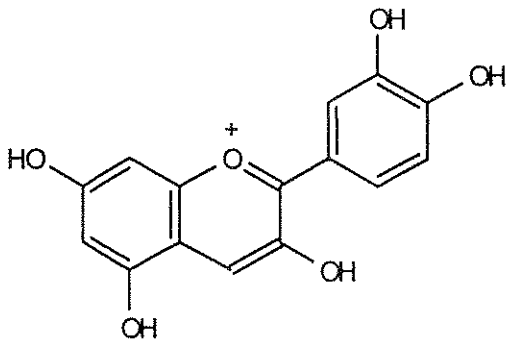
3.3.2 ANTHOCYANIDIN

3.3.2.1 EXTRACTION

Extraction of anthocyanidin from the leaves of *Indigofera hebepetala* was done by the same procedure as described for *Rumex chalepensis.*

3.3.2.2 SEPARATION, PURIFICATION AND IDENTIFICATION

Separation, purification and identification of the anthocyanidin was carried out by the same chromatographic (PC) and spectroscopic (UV-Vis.) methods. On the basis of R_f values in three solvent system and UV spectral analysis (Table-29) the compound IHL-1A was identified as Cyanidin.



Cyanidin

3.4 PYTOCHEMICAL ANALYSIS OF *INDIGOFERA CASSIOIDES* FLOWERS

3.4.1 NATURAL GLYCOSIDES

3.4.1.1 EXTRACTION

500 grams air dried leaves of *I. cassioides* were succesively extracted by ethannol and ethanol/water mixture. The extraction of flavonoid glycosides was carried out by the same procedure as described for *Rumex chealepensis*. The dried ethyl acetate extract was found to be 4.0 gm.

3.4.1.2 SEPARATION AND PURIFICATION

The EtOAc residue, dissolved in MeOH, was applied as spot near one corner of Whatman No.1 chromatography paper. The chromatogram was suspended in an all-glass PC tank containing BAW as the developing solvent. After complete development (18 hrs), the chromatogram was removed from the tank and dried in a fume cupboard. The folded portion of the dried chromatogram was trimmed off and chromatogram refolded for descending chromatography in the second dimension. 15% HOAc was used as the developing solvent. In this way a total of 100 chromatograms were developed, dried and viewed under UV (366 nm) light. Four prominent spots of flavonoid glycosides, one dull yellow and three violet, were penciled out. The equivalent spots from each paper were then cut out, combined and socked in distilled MeOH. After 24 hours of contact with occasional shaking, the MeOH was filtered. This extraction procedure was repeated thrice and the combined extract of each spot was filtered and then evaporated to dryness.

In order to determine the purity of the isolated flavonoid glycosides, each spot extract was checked by HPLC using the followoing conditions.

Column	= Nucleosil C-18 250 x 4.6 mm
Flow rate	= 1.0 ml/min.
Detection	= 340 nm
Solvent A	= H_2O-HOAc (100:2)
Solvent B	= Acetonitril-H_2O-HOAc (80:20:2)

Gradient Programme:

Time	A. Concentration	B. Concentration
0	95	5
5	95	5
45	70	30
60	55	45
75	40	60
89	95	5
90	End	

Under these conditions the pattern of HPLC chromatograms were as follows:

SPOT 1	Peak 1a	Rt = 47.45 ..... A
	Peak 1b	Rt = 65.53 ..... B
SPOT 2	Peak 2a	Rt = 45.87 C
	Peak 2b	Rt = 47.19 ..... A
SPOT 3	Peak 3a	Rt = 43.30 ..... D
SPOT 4	Peak 4a	Rt = 52.48 ..... E
	Peak 4b	Rt = 57.97 ..... F
	Peak 4c	Rt = 60.79 ..... G
	Peak 4d	Rt = 67.70 ..... H

Photodiode array (PDA) spectrum of each peak was recorded (Fig.5-8) and compared with PDA spectrum of the known flavonoid glycosides [Hasan unpublished data]. It was found that the peaks of each spot correspond to the following flavonoid glycoside types.

Peak 1a ..... Quercetin 3-O-monoglycoside

Peak 1b ..... Kaempferol 7-O-monoglycoside

Peak 2a ..... Kaempferol 3-O-diglycoside

Peak 2b ..... Quercetin 3-O-monoglycoside

Peak 3a ..... Quercetin 3-O-monoglycoside

Peak 4a ..... Quercetin 3-O-diglycoside

Peak 4b ..... Kaempferol 3-O-diglycoside

Peak 4c ..... Quercetin 3-O-monoglycoside

Peak 4d ..... Quercetin 3-O-monoglycoside

As a result of the photo diode array analysis it was determined that the following 8 types of flavonoid glycosides were present in the leaves of *Indigofera cassioides*.

A =	IC-1	E =	IC-5
B =	IC-2	F =	IC-6
C =	IC-3	G =	IC-7
D =	IC-4	H =	IC-8

Compounds from B to E were the major while A, F, G and H were the minor flavonoid glycosides. Since the flavonoid glycosides appeared as a single spot on paper chromatograms, preprative high performance liquid chromatography (PHPLC) was used for their ultimate purification the above mentioned separation conditions were used. PHPLC

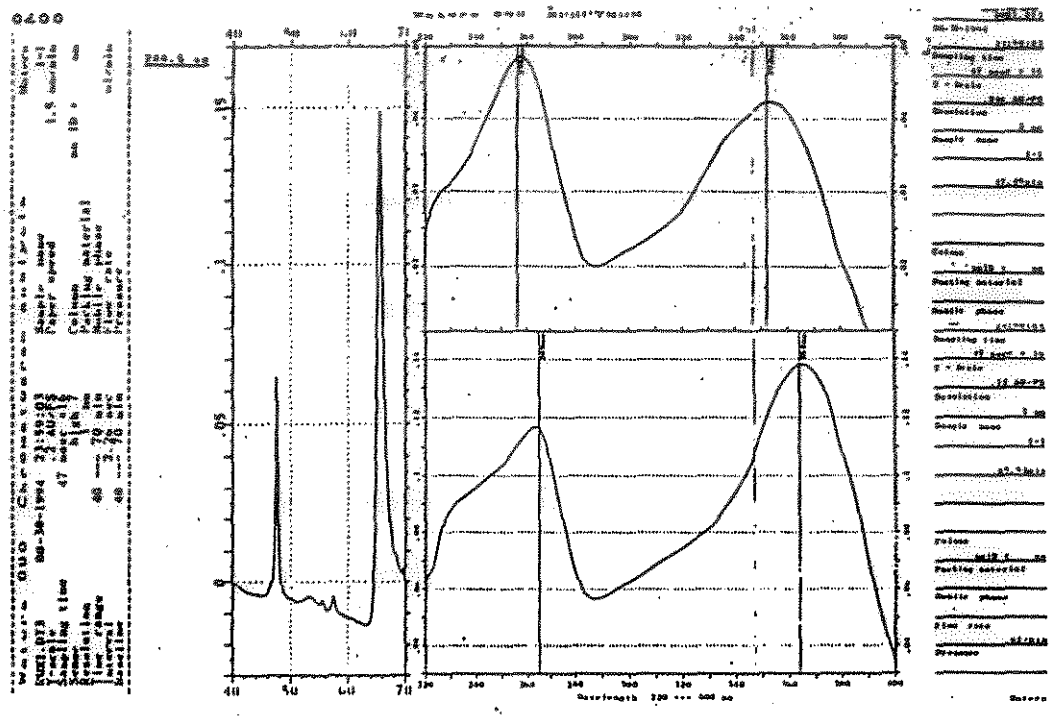


Figure-5: HPLC/PDA OF SPOT 1

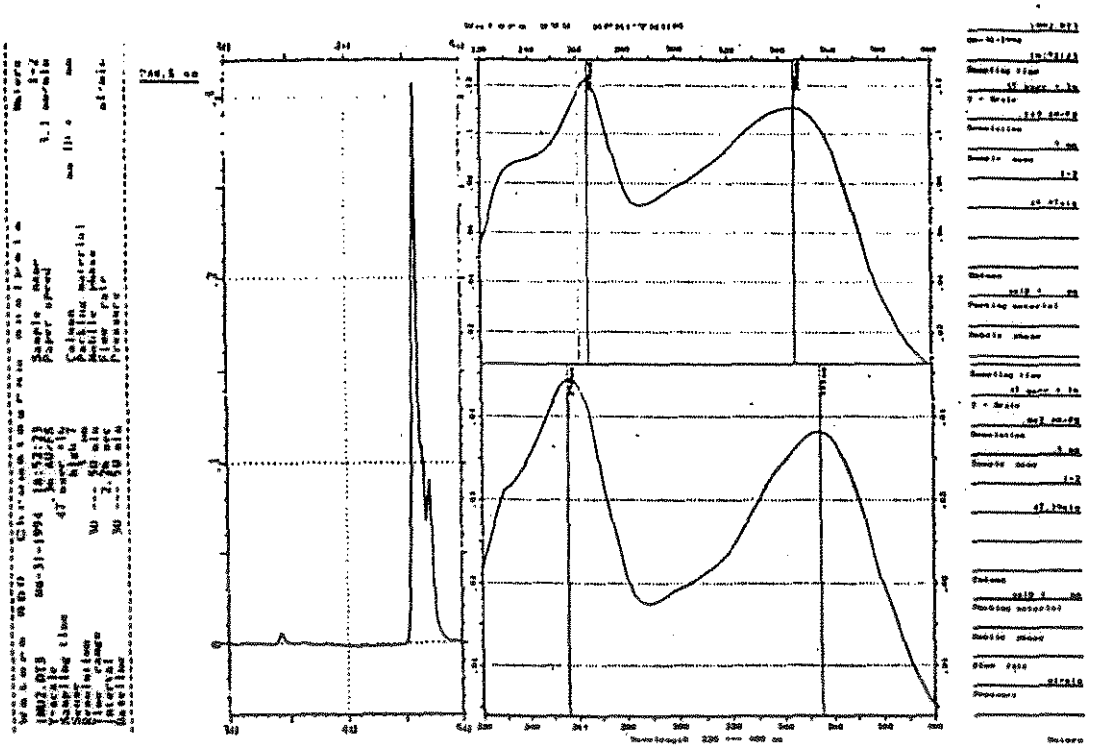


Figure-6: HPLC/PDA OF SPOT 2

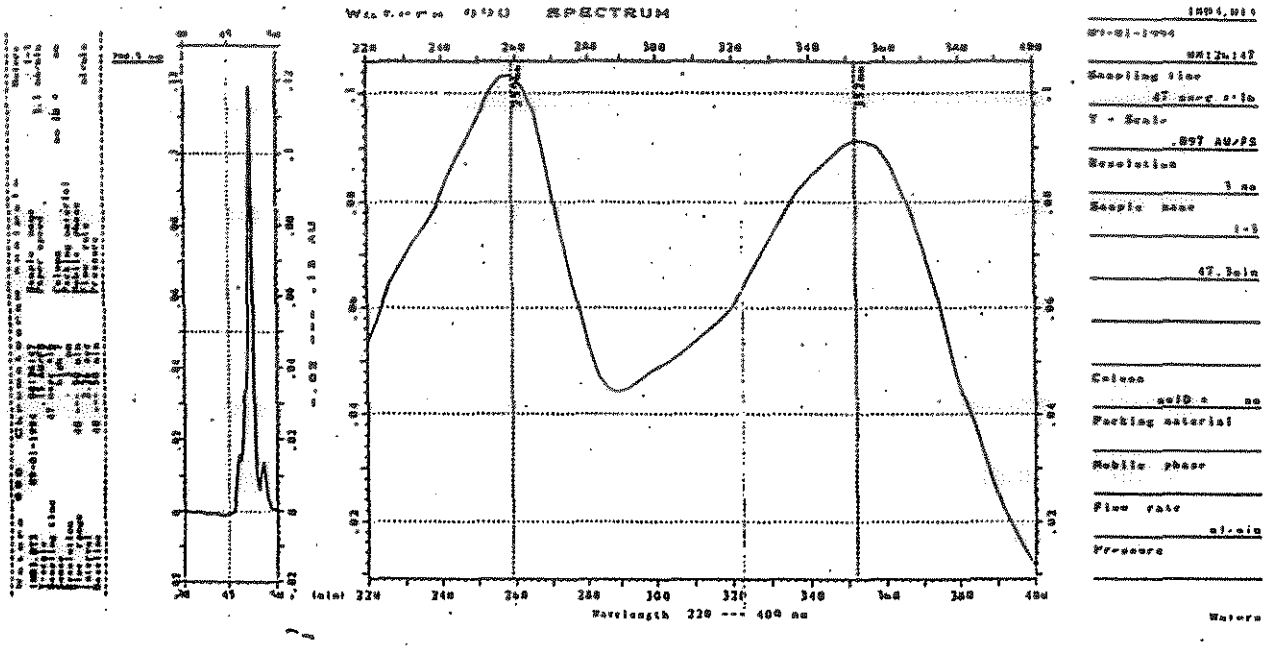


Figure-7: HPLC/PDA OF SPOT 3

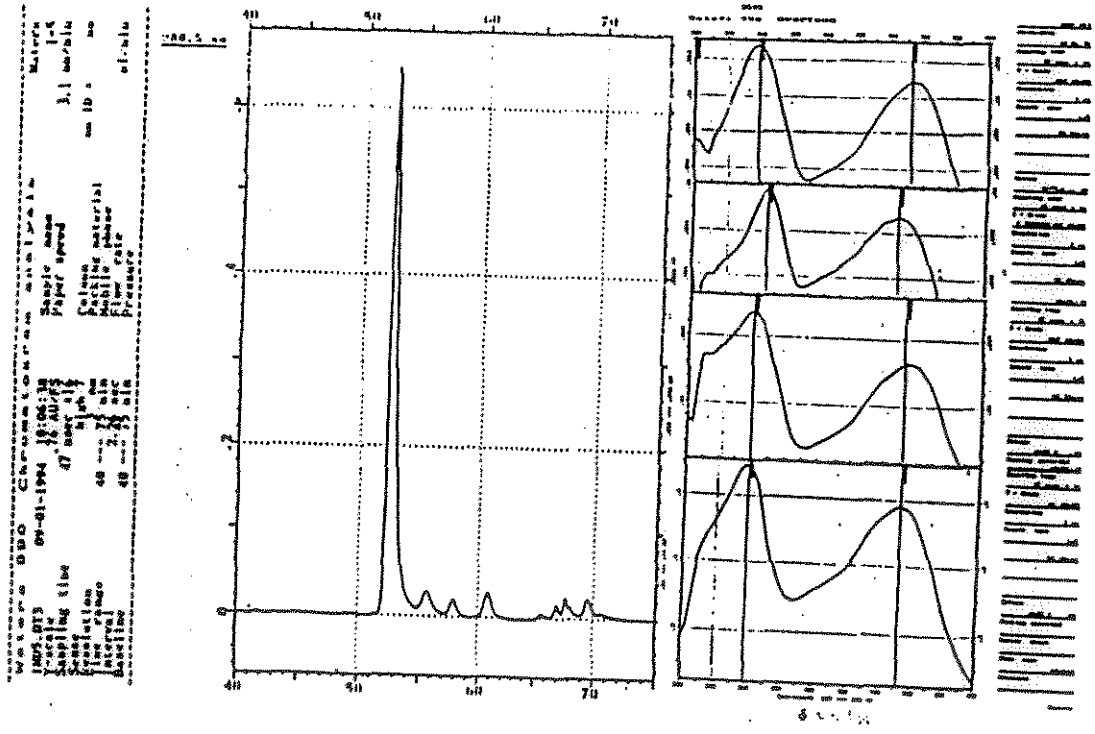


Figure-8: HPLC/PDA OF SPOT 4

Table.31 SPECTRAL AND CHROMATOGRAPHIC DATA OF FLAVONOID GLYCOSIDES ISOLATED FROM *INDIGOFERA CASSIOIDES* LEAVES

UV λ_{max}	IC-1			IC-2				IC-3				IC-4			
MeOH	261		365	262			347	262			362	262			358
NaOAc	263	330	412	267			376	267		320	402	267		321	391
$NaOAc/H_3BO_3$	264	321	370	262			350	263		299	377	263		304	379
$AlCl_3$	266	353	421	265	304	355	412	273	300	330	432	272		310	431
$AlCl_3$/HCk	267	352	419	265	300	353	410	267	300	362	399	266	300	362	399
NaOMe	264	333	416	271		328	394	270		326	412	271		324	414
EIMS	m/z 286 (100%)			m/z 286 (100%)				m/z 302 (100%)				m/z 302 (100%)			
AGLYCONE	Kaempferol			Kaempferol				Quercetin				Quercetin			
SUGARS	Rhamnose			Galactose+ Rhamnose				Galactose				Glucose+ Arabinose			
R_f x 100															
BAW	86			78				52				44			
15% HOAc	17			35				45				60			
60% HOAc	59			76				68				76			

separation resulted in the obtention of the following four major highly purified flavonoid glycosides.

1. IC-2 = 4.0 mg
2. IC-3 = 3.5 mg
3. IC-4 = 2.0 mg
4. IC-5 = 4.5 mg

The remaining four minor flavonoid glycosides could not be obtained in sufficient quantities necessary for their complete structural determination.

3.4.1.3 IDENTIFICATION

Identification of the four flavonoid glycosides was carried out by co-chromatography, in 3 solvent systems, acid hydrolysis to aglycones and sugars and spectroscopic techniques (UV, EIMS and ^{1}H NMR). Results obtained are grouped in Table-31 where as ^{1}H NMR spectral data is presented from Table-32-33.

COMPOUND IC-2

Flavonoid glycoside (IC-2) was obtained as yellow crystalline solid. It revealed yellow under UV (366 nm) light and remained yellow on an exposure to NH_3, indicating it to be a 7-O-substituted flavonol. R_f values recorded along with the standard flavonol glycosides in solvent system H_2O:EtOH:MEK:Acetyl acetone (13:3:3:1) suggested this compound to be a 7-O-substituted monoglycoside. In order to identify the aglycone and the sugar residue, compound IC-2 was subjected to acid hydrolysis.

Acid Hydrolysis:

Acid hydrolysis of IHF-5 was carried out by 2N HCl according to the satndared procedure.

Identification of aglycone:

Kaempferol was identified as the aglycone part by means of co-TLC, UV and EIMS.

Identification of sugar:

Rhamnose was identified as the only sugar in IC-2 by using PC and GC techniques.

Identification of glycoside:

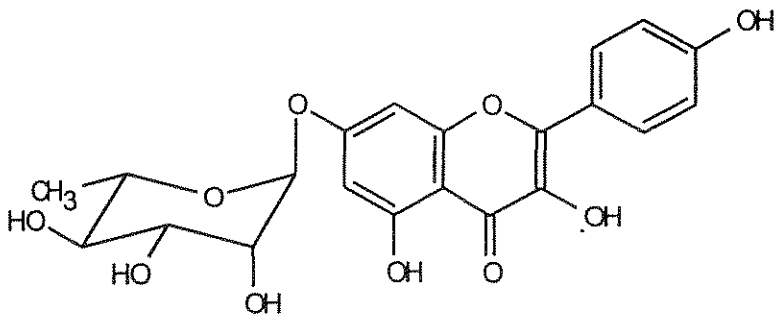
In order to find out the position of attachment of the sugar to the aglycone, UV spectrum of IC-2 was recorded in MeOH which showed two absorption bands at 365 nm (band-I) and 261 nm (band-II), a typical absorption range for a flavonol. Absence of a bathochromic shift in band-II, on addition of NaOAc to the methanolic solution of IC-2, indicated a substitution at C-7 of ring A. On addition of H_3BO_3 a significant hypsochromic shift was observed in band-I as compared to the spectrum recorded in the presence of NaOAc, which indicated the absence of an orthodihydroxylation at ring B. A bathochromic shift of 56 nm was recorded by the addition of 5% alcoholic solution of $AlCl_3$, which indicated the presence of a free hydroxyl group at position 5 of ring-A. Addition of HCl to the methanolic solution of IC-2, countering $AlCl_3$, did not show a hypsochromic shift in band-I as compared to $AlCl_3$ spectrum, which indicated the absence of orthodihydroxylation at ring B. Addition of NaOMe caused a bathochromic shift of 51 nm in band-I showing the presence of a free 4'-OH at ring B. UV spectra recorded in MeOH

and on addition of shift reagents suggested compound IC-2 to be a 7-O-substituted flavonol, with the sugar (rhamnose) attached at position 7 of the aglycone.

In EIMS a molecular ion peak for the aglycone was observed at m/z 286. The other important fragment ions appeared at m/z 153 $(A_1+H)^+$, 121 $(B_2)^+$ and 93 (B_2^+-28).

^{1}H NMR (CD_3OD, 300 MHz) exhibited two signals at δ 6.35 (1H, *d*, $J_{6,8}$=2.0 Hz) and 6.69 (1H, *d*, $J_{8,6}$=2.0 Hz) due to meta coupled H-6 and H-8 of ring A. The chemical shifts corresponding to H-2', H-6' and H-3', H-5' were observed at δ 8.0 (2H, *d*, $J_{2',3'}$=9.0 Hz) and 6.86 (2H, *d*, $J_{5',6'}$=9.0 Hz) respectively. A signal for anomeric proton of rhamnose appeared at δ 5.51 (1H, *d*, $J_{1'',2''}$=1.5 Hz). NMR signals for H-2" and H-3" were observed at δ 4.01 (1H, *dd*, $J_{1'',2'',3''}$=2.0, 5.0 Hz) and 3.82 (1H, *dd*, $J_{3'',2'',4''}$=2.0, 10.0 Hz) while H-4" and H-5" resonated at δ 3.47 (1H, *t*, $J_{4'',3'',5''}$=10.0 Hz) and 3.5-3.66 (1H, *m*). CH_3 of rhamnose appeared at δ 0.95 (1H, *d*, $J_{6'',5''}$=7.0 Hz).

On the basis of chromatic data, acid hydrolysis, UV, IR, EIMS and ^{1}H NMR spectral data, structure of IC-2 was identified as kaempferol 7-O-α-L-rhamnopyranoside.



Kaempferol 7-O-α-L-rhamnopyranoside

Table.32 ^{1}H NMR SPECTRAL DATA OF COMPOUND IC-2

(CD_3OD, 300 MHz)

H	(δ)	Multiplicity	J (Hz)
H-6	6.35	*d*	2.0
H-8	6.69	*d*	2.0
H-2'	8.00	*d*	9.0
H-3'	6.86	*d*	9.0
H-5'	6.86	*d*	9.0
H-6'	8.00	*d*	9.0
H-1"	5.51	*d*	1.5
H-2"	4.01	*dd*	2.0/5.0
H-3"	3.82	*dd*	2.0/10.0
H-4"	3.47	*t*	10.0
H-5"	3.5-3.6	*m*	-
CH_3	1.25	*d*	7.0

COMPOUND 1C-3

Flavonoid glycoside IC-3 was obtained as yellow crystalline solid. It appeared purple under UV (366 nm) light and turned yellow in NH_3 vapours, which suggested it to be a 3-O-substituted flavonol. Mobilitiy of IC-3, along with standared compounds in solvetn system H_2O:EtOH:MEK:Acetyl acetone (13:3:3:1) indicated it a 3-O-substituted flavonoid diglycoside.

Acid hydrolysis:

To find out the aglycone part and the sugar moieties, 1.0 mg of IC-3 was subjected to acid hydrolysis according to the standared procedure.

Identification of aglycone:

Kaempferol was identified as the aglycone part by means of co-TLC, UV and EIMS.

Identification of sugars:

Galactose and rhamnose were identified as the sugar moities by PC and GC.

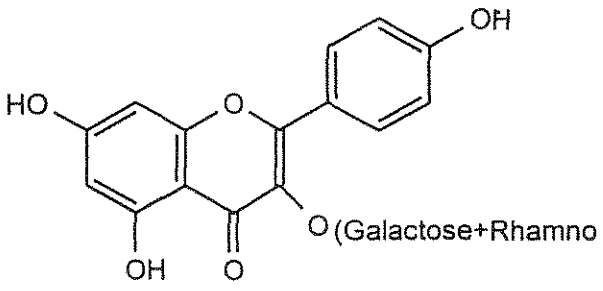
Identification of glycoside:

In order to investigate the position of the attachment of the sugars to the aglycone UV spectrum of flavonoid glycoside RC-2 was measured in MeOH which displayed two absorption maxima at 347 nm (band-I) and 262 nm (band-II). The range of absorption bands suggested it to be a 3-O-substitiuted flavonol. Addition of NaOAc to the methanolic solution of IC-3 caused a bathochromic shift of 5.0 nm in band-II as compared to MeOH spectrum, indicating the presence of a free 7-hydroxyl group at position 7 of ring A. On

addition of H_3BO_3 a non-significant bathochromic shift of 2.0 nm, with respect to methanolic spectrum, in band-I was observed which indicated the absence of dihydroxylation in ring B. On addition of 5% methanolic solution of $AlCl_3$ a pronounced bathochromic shift of 55.0 nm was observed in band-I, which indicated the presence of free hydroxyl group at position 5 of ring A. Addition of HCl to the methanolic solution of IC-3, containing $AlCl_3$, did not show any hypsochromic shift in band-I as compared to $AlCl_3$ spectrum, which confirmed the absence of dihydroxylation at ring B. A bathochromic shift of 47 nm, in the presence of NaOMe, suggested the presence of free 4'-OH at ring B. UV spectra of IC-3 recorded in MeOH and on addition of diagnostic shift reagents suggested compound IC-3 to ba a 3-O-substituted flavonol with both the sugars (galactose and rhamnose) attached at position 3 of the aglycone.

EIMS of IC-3 exhibited a molecular ion peak for aglycone at m/z 286 (100%) which could be attributed to the aglycone.The other important fragments were same as mentione in the case compound IC-2.

Due to small amout of the compound NMR techniques could not be used which obstracted to find out the sugar sequence. On the basis of above data and HPLC/PDA results, the structure of the compound could be as follow.



Kaempferol 3-O-(Galactose + Rhamnose)

COMPOUND IC-4

Compound IC-4 was obtained as yellow crystalline solid which revealed violet under UV (366 nm) light and changed to yellow in NH_3 vapours, indicating it to be a 3-O-substituted flavonol. Mobility of IC-4 with standared compound in solvent system H_2O:EtOH:MEK:Acetyl acetone (13:3:3:1) suggested it to be a 3-O-substituted mono glycoside. In order to find out the aglycone and the sugar part, acid hydrolysis of the compound IC-4 was carried out.

Acid hydrolysis:

Acid hydrolysis of the IC-4 was carried out by the standared procedure.

Identification of aglycone:

The aglycone part was identified as quercetin with the help of co-TLC, and EIMS.

Identification of sugars:

Rhamnose were identified as the only sugar by making use of PC and GC.

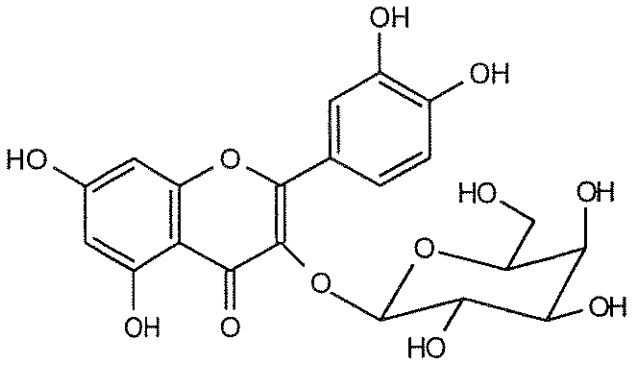
Identification of glycoside:

UV spectrum of IC-4 recorded in MeOH exhibited two prominent absorption bands at 362 nm (band-I) and 262 nm (band-II) indicating to be a 3-O-substituted flavonol. A bathochromic shift of 5.0 nm was observed by the addition of NaOAc which confirmed the presence of free -OH at position 7 of ring A. 5% methanolic $AlCl_3$ solution produced a bathochromic shift of 70 nm in band I which was decreased by 33 nm after the addition of HCl with respect to $AlCl_3$ spectrum, which proved the presence of 5-OH at ring A and

orthodihydroxylation at ring B. UV specta recorded in MeOH and after addition of diagnostic shift reagents suggested compound IC-4 to be a 3-O-substituted flavonol.

EIMS of the compound gave a molecular ion peak $M^{+\cdot}$ for its aglycone part at m/z 302. Other Retro-Diels Alder fragments appeared at m/z 153 $(A_1+H)^+$, 121 $(B_2)^+$ and 93 (B_2-28).

On the basis of chromatographic data (PC and HPLC/PDA), acid hydrolysis, UV, and EIMS structure of IC-4 was identified as quercetin 3-O-β-galactopyranoside.



Quercetin 3-O-β-galactopyranoside

COMPOUND IC-5

Flavonoid glycoside IC-5 was obtained as yellow crystalline solid. It appeared purple under UV (366 nm) light and turned yellow in NH_3 vapours, which suggested it to be a 3-O-substituted flavonol. Mobilitiy of IC-5, along with standared compounds in solvetn system H_2O:EtOH:MEK:Acetyl acetone (13:3:3:1) indicated it a 3-O-substituted flavonoid diglycoside.

Acid hydrolysis:

To find out the aglycone part and the sugar moieties, the compound IC-5 was subjected to acid hydrolysis following the standared procedure.

Identification of aglycone:

Quercetin was identified as the aglycone by means of co-TLC, UV and EIMS.

Identification of sugars:

Glucose and arabinose were identified as the sugar moities by PC and GC.

Identification of glycoside:

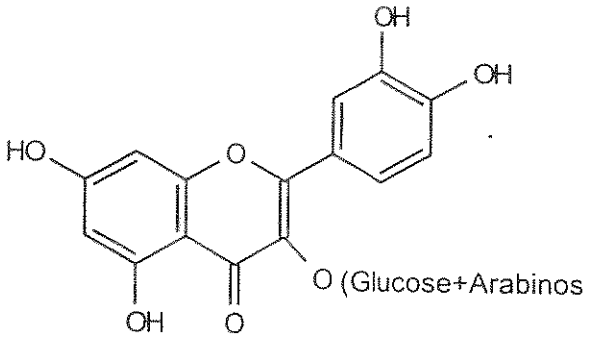
UV spectrum of IC-5 was recorded in MeOH which exhibited two absorption maxima at 360 nm (band-I) and 262 nm (band-II), indicating it to be a 3-O-substituted flavonol. Addition of NaOAc showed a bathochromic shift of 5.0 nm in band-II as compared to the methanolic spectrum indicating a free OH at position 7 of ring A. 19.0 nm bathochromic shift of band-I in the presence of $NaOAc/H_3BO_3$ indicated the presence of 3',

4'-orthodihydroxylation. A bathochromic shift of 71 nm was observed in band-I with 5% methanolic solution of $AlCl_3$ which decreased by 32 nm after the addition of HCl, giving a clear indication of an orthodihydroxylation on ring B. Addition of NaOMe revealed a bathochromic shift of 54 nm in band-I with an increased intensity showing the presence of free hydroxyl group at 4'-position of ring B. UV spectra of IC-5 recorded in MeOH and on addition of diagnostic shift reagents suggested it to be a 3-O-substituted flavonol with both the sugars (glucose and arabinose) attached at position 3 of the aglycone, which confirmed the results obtained by HPLC/PDA analysis.

EIMS exhibited a molecular ion peak for the aglycone at m/z 302 (100%). Other diagnostic fragments appeared at m/z 153 $(A_1+H)^+$, 137 $(B_2)^+$ and 109 (B_2-28) indicating a pentahydroxy flavone with dihydroxylation at ring A and orthodihydroxylation at ring B.

^{1}H NMR (CD_3OD,300 MHz) of IC-5 showed the chemical shift of two meta coupled protons of ring A i,e. H-6 and H-8 at δ 6.02 (1H, *d*, $J_{6,8}$=2.0 Hz) and δ 6.19 (1H, *d*, $J_{8,6}$=2.0 Hz) respectively. H-2', H-5' and H-6' of ring B resonated at δ 7.67 (1H, *d*, $J_{2',6'}$=2.0 Hz), δ 6.65 (1H, *d*, $J_{5',6'}$=8.0 Hz), δ 7.46 (1H, *dd*, $J_{6',2',5'}$=2.0, 9.0 Hz) respectively. The NMR signals appeared at δ 5.21 (1H, *d*, $J_{1'',2''}$=5.5 Hz) and δ 5.25 (1H, *d*, $J_{1''',2'''}$=2.5 Hz) were assigned to the anomeric protons of glucose and arabinose, showing a ß and α configuration respectively. Rest of the sugar protons appeared in the range of 3.1 to 4.2 ppm. ^{1}H NMR further confirmed the results of HPLC/PDA and UV, but due to the smaller amout of the compound sequence of the sugars could not be determined.

On the basis of above data and HPLC/PDA results, the structure of the compound could be as follow.



Quercetin-3-O-(Glucose + Arabinose)

Table.33 ^{1}H NMR SPECTRAL DATEA OF COMPOUND IC-5

(CD_3OD, 300 MHz)

H	(δ)	Multiplicity	J (Hz)
H-6	6.02	*d*	2.0
H-8	6.19	*d*	2.0
H-2'	7.67	*d*	2.0
H-5'	6.65	*d*	8.0
H-6'	7.46	*dd*	2.0/8.0
H-1(Glc)	5.21	*d*	5.5
H-1(Arb)	5.25	*d*	2.5

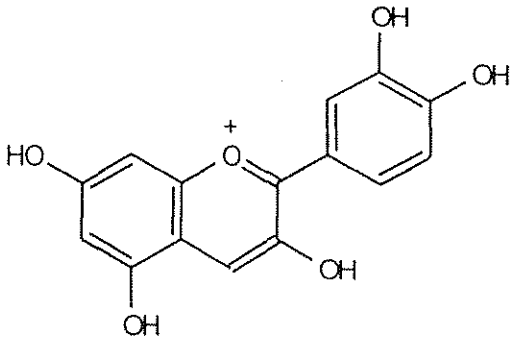
3.4.2 ANTHOCYANIDIN

3.4.2.1 EXTRACTION

Extraction of anthocyanidin from the leaves of *Indigofera cassioides* was done by the same procedure as described for *Rumex chalepensis*.

3.4.2.2 SEPARATION, PURIFICATION AND IDENTIFICATION

Separation, purification and identification of an anthocyanidin was carried out by the same chromatographic (PPC) and spectroscopic (UV-Vis.) methods. On the basis of Rf values in three solvent system and UV spectral analysis (Table-29) the compound IC-1A was identified as Cyanidin.



Cyanidin

CHAPTER 4

QUALITATIVE, QUANTITATIVE AND ANTIBACTERIAL ANALYSIS OF FLAVONOIDS OF 20 PLANT SPECIES OF ISLAMABAD AND ITS SURROUNDINGS

CHAPTER 4

QUALITATIVE, QUANTITATIVE AND ANTI-BACTERIAL ACTIVITY ANALYSIS OF FLAVONOIDS OF 20 PLANT SPECIES OF ISLAMABAD AND ITS SURROUNDINGS

CONTENTS:

4.1 INTRODUCTION:

In view of the immense pharmacological and chemotexonomic importance of flavonoids and their wide occurance in plant kingdom, 20 plant species of Islamabad and its surroundings were collected (Table-34). Qualitative, quantitative and anti-bacterial analysis of flavonoids of these plant species were carried out and the results obtained are presented in the following section.

4.2 METHOD AND MATERIAL

4.2.1 PLANT MATERIAL

Small amount of each plant specie was collected to render 20-50 gm dry weight of the plant material. After depositing a voucher specimen (Table-34) of each plant at the Herbarium, Department of Biology, Quaid-i-Azam University, Islamabad, all plants were air-dried under shade. Air-dried plants were then ground and stored in labelled air-tight glass jars. Literature survey of each plant specie was carried out and the reports of presence or absence of flavonoids from these plant species are presented in Table-35.

4.2.2 QUANTITATIVE ANALYSIS

Flavonoids in nature occur as glycosides and on acid hydrolysis liberate sugar free aglycones. The sugar free aglycones are quantitatively analyzed by the method of chelation with $AlCl_3$ (Markham, 1982). For this purpose 3 grams of air-dried plant material was hydrolysed for one hour on a hot boiling water bath, with 2N HCl. After cooling the hydrolyzed mixture was extracted with diethyl ether. Ether extract was then allowed to evaporate freely at room temperature. The dry residue from each extract composed mainly of flavonoid aglycones, was dissolved in 10 ml of spectral grade MeOH. After chelation with $AlCl_3$, optical density of this solution was measured between 240 - 440 nm on a

Table.34 PLANT SPECIES COLLECTED FROM ISLAMABAD AND ITS SURROUNDINGS

S.No.	Name of Plant	Family	Area of collection	Voucher specimen No.
1.	Asperprugo procumbens	Boraginaceae	Kalarkahar	00006
2.	Astragalus subumbllatus	Leguminoseae	Islamabad	00210
3.	Caeusrina glauca	Casurinaceae	Islamabad	00671
4.	Fumaria indica	Fumariaceae	Islamabad	00476
5.	Indigofera hebepetala	Leguminoseae	Murree	63662
6.	Linum corymbolosum	Linaceae	Islamabad	00004
7.	Malcolmia africana	Crucifereae	Islamabad	00146
8.	Mallotus philipensis	Euphorbiaceae	Islamabad	00052
9.	Mirabilis jalapa	Nictaginaceae	Mansehra	00072
10.	Nasturtium officinale	Cruciferae	Islamabad	00514
11.	Oxalis pes-caprae	Oxalidaceae	Kalarkahar	00551
12.	Plantago lanceolata	Plantaginaceae	Kalarkahar	00007
13.	Rubus ulmifolius	Rosaceae	Mansehra	00068
14.	Rumex chalepensis	Polygonaceae	Islamabad	66845
15.	Saussurea heteomalla	Compositiae	Islamabad	00470
16.	Silybum marianum	Compositiae	Islamabad	00367
17.	Taraxacum officinale	Compositiae	Islamabad	00009
18.	Tecomella undulata	Bignoniaceae	Nizampur	00421
19.	Trichodesma indica	Boraginaceae	Islamabad	00028
20.	Vitex negundo	Verbenaceae	Mansehra	00073

Table.35 REPORTED FLAVONOIDS IN TWENTY PLANT SPECIES

S.No.	Plant Species	Flavonoids reported	refrences
1.	Asperprugo procumbens	No report	
2.	Astragalus subumbllatus	No report	
3.	Caeusrina glauca	Cyanidin, Quercetin Kaempferol, Myricetin & Ellagic acid	Salah Nabiel *et al* (1979)
4.	Indigofera hebepetala	Flavonoid aglycones	Hasan *et al* (1993)
5.	Fumaria indica	No report	
6.	Linum corymbolosum	No report	
7.	Malcolmia africana	No report	
8.	Mallotus philipensis	Prenylated flavonoids, Chalcones and flavanones	Malina *et al* (1967)
9.	Mirabilis jalapa	No report	
10.	Nasturtium officinale	No report	
11.	Oxalis pes-caprae	No report	
12.	Plantago lanceolata	No report	
13.	Rubus ulmifolius	No report	
14.	Rumex chalepensis	Anthraquinones	Fang *et al* (1982)
15.	Saussurea heteomalla	No report	
16.	Silybum marianum	No report	
17.	Taraxacum officinale	No report	
18.	Tecomella undulata	No report	
19.	Trichodesma indica	No report	
20.	Vitex negundo	Anthocyanins, C-glycosides, Flavonol glycosides and flavone glycosides.	Prema *et al* (1978) Subramanian *et al* (1979) Misra *et al* (1980) Achari *et al* (1984) Firdous *et al* (1984) Bhargara (1984)

Shimadzu 260 Ultraviolet Spectrophotometer and the amount of flavonoids in mg/gm was calculated by the following formula:

Amount of flavonoid mg/gm = 1.3×10^{-2} (O.D) V x d / W

where	O. D	=	Optical Density
	V	=	Volume
	d	=	Dilution
	w	=	Plant weight

Amount of flavonoids present in each plant specie is listed in Table-36.

4.2.3 QUALITATIVE ANALYSIS

4.2.3.1 QUALITATIVE ANALYSIS OF FLAVONOID AGLYCONES

Flavonoid aglycone extracts of each plant species were concentrated to dryness and redissolved in small amount of MeOH. A concentrated spot of each extract along with three standard markers, were deposited on a 20 x 20 x 0.25 cm precoated polyamide $11F_{254}$ plates. The plate was developed in the solvent system C_6H_6 : MEK : MeOH (4:3:3). After development, the plate was viewed under 366 nm UV light. The flavonoid pattern of each extract was noted and is shown in Table-37

4.2.3.2 QUALITATIVE ANALYSIS OF FLAVONOID GLYCOSIDES

Ethyl acetate extract of each plant specie was concentrated to dryness and redissolved in MeOH. A concentrated spot of each extract was deposited on a corner of Whatman No.1 chromatographic paper. The papers were first developed in the solvent

system BAW and then with 15% HOAc in the second dimenssion. Developed chromatograms were viewed under 366 nm UV light. The pattern of flavonoid glycosides,

Table.36 QUANTITATIVE ANALYSIS OF FLAVONOID AGLYCONES

Plant No	V	d	W	λ max	O.D	Amount mg/gm
1.	10 ml	100	3 gm	361	1.290	5.59
2.	10 ml	100	3 gm	359	1.041	4.51
3.	10 ml	100	3 gm	380	1.184	8.70
4.	10 ml	100	3 gm	360	1.637	7.09
5.	10 ml	100	3 gm	427	0.723	3.13
6.	10 ml	100	3 gm	371	1.158	5.02
7.	10 ml	100	3 gm	361	1.068	4.63
8.	10 ml	100	3 gm	394	1.198	5.19
9.	10 ml	100	3 gm	422	0.262	1.14
10.	10 ml	100	3 gm	354	0.173	0.75
11.	10 ml	100	3 gm	359	0.909	3.94
12.	10 ml	100	3 gm	360	1.592	6.89
13.	10 ml	100	3 gm	404	1.870	8.10
14.	10 ml	100	3 gm	368	1.590	6.89
15.	10 ml	100	3 gm	462	0.045	0.20
16.	10 ml	100	3 gm	455	0.086	0.37
17.	10 ml	100	3 gm	421	0.245	1.10
18.	10 ml	100	3 gm	432	1.021	4.42
19.	10 ml	100	3 gm	362	1.476	6.39
20.	10 ml	100	3 gm	366	1.379	5.97

Table.37 QUALITATIVE ANALYSIS OF FLAVONOID AGLYCONES

Plant No.	Rf value/Colour under UV light					
	1	2	3	4	5	6
1	0.218 (B)	0.358 (Y)	0.558 (B)			
2	0.189 (B)	0.351 (Y)	0.645 (B)	0.735 (Y)	0.919 (V)	
3	0.094 (B)	0.186 (V)	0.281 (Y)	0.352 (Y)	0.412 (B)	0.483 (Y)
4	0.636 (B)	0.735 (Y)	0.773 (Y)	0.883 (B)		
5	0.195 (B)	0.219 (B)	0.350 (Y)	0.482 (Y)	0.559 (B)	
6	0.094 (B)	0.544 (B)	0.744 (Y)			
7	0.094 (B)	0.215 (B)	0.350 (Y)	0.700 (B)	0.938 (V)	
8	0.098 (Y)	0.247 (B)	0.409 (V)	0.579 (B)	0.927 (B)	
9	0.651 (V)	0.779 (V)	0.872 (Y)			
10	0.286 (Y)	0.411 (B)	0.483 (Y)			
11	0.352 (Y)	0.483 (Y)	0.576 (B)	0.909 (V)		
12	0.194 (B)	0.624 (B)	0.933 (V)			
13	0.273 (Y)	0.303 (B)	0.352 (Y)	0.503 (B)		
14	0.094 (Y)	0.259 (V)	0.350 (Y)	0.484 (Y)	0.571 (B)	
15	0.352 (Y)	0.568 (B)				
16	0.481 (Y)	0.500 (B)	0.600 (V)	0.659 (Y)		
17	0.274 (B)	0.483 (Y)	0.659 (Y)			
18	0.297 (Y)	0.394 (Y)	0.497 (B)	0.583 (Y)	0.703 (V)	
19	0.218 (B)	0.835 (B)	0.882 (Y)	0.941 (V)		
20	0.529 (B)	0.782 (V)	0.872 (Y)	0.923 (Y)		
Quercetin	0.352 (Y)					
Kaempferol	0.483 (Y)					
Apigenin	0.607 (V)					

B = Blue Y = Yellow V = Violet

Table.38 QUALITATIVE ANALYSIS OF FLAVONOID GLYCOSIDES

Plant No.	Number of spots							
	1	2	3	4	5	6	7	8
1	++ (Y)	++(P)	+ (V)	++ (P)				
2	+++ (V)	++ (B)	++ (B)	++ (B)	++ (B)	++ (YG)	+++(V)	
3	++ (V)	+ (V)	++ (B)	+ (Y)				
4	+++ (Y)	++ (B)	++ (Br)	++ (Br)	++ (V)			
5	++++(V)	+++ (V)	+++ (YG)	+++ (B)	+++ (V)	++ (V)	+++ (Y)	+ (B)
6	++++ (B)	+++ (V)	++ (V)	++ (V)	+ (V)			
7	+++ (B)	+ (Y)	+++ (B)	+++ (V)	++ (B)	+ (V)		
8	++ (Br)	+ (Br)	+ (Br)					
9	+++ (G)	+++ (B)	++ (G)	++ (Y)	+++ (G)			
10	+ (Y)	++ (GY)	+++ (Y)	++ (Y)				
11	++ (GY)	++++ (B)	+++ (B)	++ (B)	++ (GY)	+ (G)		
12	++ (V)	++ (B)	+++ (B)	++ (Y)	+ (Y)			
13	+++ (Y)	+++ (Y)	++++ (V)	+++ (Y)	+++ (V)	++++ (B)		
14	++++ (Y)	++ (Br)	+++ (V)	++++ (R)	+++ (V)	++ (V)	++++ (R)	++++ (B)
15	+++ (Y)	+++ (V)	++++ (B)	+++ (B)	++ (Y)	++ (Y)	++ (B)	
16	+++ (Y)	++ (Y)	+++ (BY)	+++ (GY)	+++ (BY)	+++ (Y)	++ (V)	
17	+++ (V)	++ (V)	++ (Br)	+ (Br)	+++ (B)	++ (B)		
18	+ (Br)	++ (P)	+++ (Br)	+++ (V)	++ (V)	+ (Y)		
19	+++ (V)	++ (GB)	++ (V)	+++ (V)	++++ (B)	++ (B)	+ (V)	
20	++ (B)	+ (YB)	+++ (Y)	++++ (B)	++ (B)	++ (Y)	+++ (V)	

B = Blue Br = Brown GY = Greenish yellow P = Pink

present in each extrat, i.e. colour and concentration of each spot was noted and is presented in Table-38.

4.2.4 ANTIBACTERIAL ACTIVITY ANALYSIS

4.2.4.1 ANTIBACTERIAL ACTIVITY ANALYSIS OF GLOBAL FLAVONOID AGLYCONES

Disc diffusion method (Barry *et al*, 1976) was used for this analysis. For this purpose, nutrient agar was prepared and poured in petri dishes. Inorder to check the sterility these petri dishes were kept in the incubator at 37 ^{o}C for 24 hours.. These petri dishes were then streaked with bacterial strains (*Staphylococcus aureus, Sarcina lutea, Escherichia coli* and *Pseudomonas aueroginosa*). Sterilized discs, containing 50, 500 and 1000 µg of aglycone extracts were placed on the agar along with disc of standard drug (Tetracyclin). Petri dishes were incubated at 37 ^{o}C for 24 hours and zone of inhibition were measured . The results noted are grouped in Table-39.

4.2.4.2 ANTIBACTERIAL ACTIVITY ANALYSIS OF GLOBAL FLAVONOID GLYCOSIDES

Flavonoid glycosides extracts of twenty plant species were tested against the above mentioned bacterial strains in the concentration of 50, 500 and 1000 µg/disc. The results obtained are shown in Table-40.

4.2.4.3 ANTIBACTERIAL ACTIVITY ANALYSIS OF PURIFIED FLAVONOID GLYCOSIDES

Antibacterial activity of the purified flavonoid glycosides isolated from *Rumex chalepensis* and *Indigofera hebepetala* was carried out by following the same procedure as described for the global extracts. The concentration of flavonoid glycosides varied between

Table.39 ANTIBACTERIAL ACTIVITY OF GLOBAL FLAVONOID AGLYCONE EXTRACTS

Plant No	*Steph aureus*			*Sarcina lutea*			*Escherchia coli*			*Psudomonas aueruginosa*		
	50 µg	500 µg	1000 µg	50 µg	500 µg	1000 µg	50 µg	500 µg	1000 µg	50 µg	500 µg	1000 µg
1	-	-	-	-	-	8	-	-	-	-	-	6
2	-	-	-	-	-	-	-	-	-	9	-	11
3	-	-	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-	-	7
5	-	-	11	-	6	12	-	-	8	-	-	10
6	-	-	-	-	-	-	-	-	-	-	-	-
7	-	-	-	-	-	-	-	-	-	-	-	-
8	-	-	10	-	8	11	-	-	-	-	-	6
9	-	-	-	-	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-	-	-	-	-
11	-	-	-	-	-	8	-	-	6	-	-	-
12	-	-	-	-	-	-	-	-	-	-	-	-
13	-	-	8	-	-	9	-	-	-	-	-	11
14	-	-	-	-	9	12	-	-	11	-	7	10
15	-	-	-	-	-	-	-	-	-	-	-	-
16	-	-	-	-	-	-	-	-	-	-	-	-
17	-	-	-	-	-	-	-	-	-	-	-	-
18	-	-	9	-	-	-	-	-	10	-	-	-
19	-	-	-	-	-	7	-	-	-	-	-	8
20	-	-	-	-	-	-	-	-	8	-	-	-

Zone of inhibition is indicated in mm.

Tetracyclin (10 µg/disc) = 18 mm (*S. aureus*), 20 mm (*S. lutea*), 18 mm (*E. coli*), 15 mm (*P. aeroginosa*)

Table.40 ANTIBACTERIAL ACTIVITY OF GLOBAL FLAVONOID GLYCOSIDES EXTRACTS

Plant No	*Steph aureus*			*Sarcina lutea*			*Escherchia coli*			*Psudomonas aueruginosa*		
	50 µg	500 µg	1000 µg	50 µg	500 µg	1000 µg	50 µg	500 µg	1000 µg	50 µg	500 µg	1000 µg
1	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-	-	-
5	-	-	8	-	9	11	-	-	-	-	-	10
6	-	-	-	-	-	-	-	-	-	-	-	-
7	-	-	-	-	-	-	-	-	-	-	-	-
8	-	-	10	-	-	7	-	-	-	-	-	8
9	-	-	-	-	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-	-	-	-	-
11	-	-	-	-	-	-	-	-	9	-	-	11
12	-	-	-	-	-	-	-	-	-	-	-	-
13	-	8	11	-	-	7	-	-	-	-	-	10
14	-	9	12	-	8	11	-	10	13	-	-	8
15	-	-	-	-	-	-	-	-	-	-	-	-
16	-	-	-	-	-	-	-	-	-	-	-	-
17	-	-	-	-	-	-	-	-	-	-	-	-
18	-	-	8	-	-	-	-	-	9	-	-	-
19	-	-	-	-	-	7	-	-	-	-	-	10
20	-	-	-	-	-	9	-	-	7	-	-	11

Zone of inhibition is indicated in mm.

Tetracyclin (10 µg/disc) = 18 mm (*S. aureus*), 20 mm *(S. lutea*), 18 mm (*E. coli*), 15 mm (*P. aeroginosa*)

100µg to 300 µg/ disc. Zone of inhibition of each purified flavonoid glycoside of *R. chalepensis* and *I. hebepetala* are presented in Table-41 and 42 respectively.

4.3 RESULTS AND DISCUSSION

R_f values and colour of spots under UV light of flavonoid aglycones of the 20 plant species along with three authentic markers (supplied by Sigma Chemicals, USA) are listed in Table-36. Interpretation of these results suggest that Apigenin (4', 5, 7-trihydroxy flavone) is present only in plant 17, kaempferol (3, 4', 5, 7-tetrahydroxy flavone) in 1, 3, 4, 5, 9, 10, 17 and 18. Whereas quercetin (3, 3', 4', 5, 7-pentahydroxy flavone) is present in 2, 7, 8, 11, 13, 15, 19 and 20.

The colour of spots in UV light also give some indication about the class of flavonoids. Spots yellow in colour are characteristic of flavonol aglycones whereas those violet in colour, represent either 3 methoxy flavonol or simple flavones. Spots blue in colour are due to the presence of phenolic acids.

Global aglycone extracts of plant No. 1, 2, 4, 5, 8, 11, 13, 14, 18, 19 and 20 showed antibacterial activity against *S. aureus, S. lutea, E. coli and P. aueroginosa*. The antibacterial activity was found to be significant between 500 µg to 1000 µg in plant No. 5, 8, 13 and 14 as compared to the standared antibiotic Tetracyclin. Global flavonoid glycoside extracts of plant No. 5, 8, 11, 13, 14, 18, 19 and 20 exhibited antibacterial activity against the above mentiones test organisms and were also found quite significant as compared to Tetracyclin. However the extent of activity was found to be pronounced between 500 µg to 1000 µg in plant No. 5 (*I. hebepetala*) and plant No. 14 (*R. chalepensis*).

Purified flavonoid glycoside RC-1 showed significant activity against *S. aureus*, *S. lutea* and *E. coli* at a concentration of 300 µg while RC-2 was only active against *E. coli* at the same concentration. RC-3 exhibited significant activity against *S. aureus* and weak activity against *S. lutea* at a concentration of 300µg. Flavonoid glycoside RC-4 showed

Table.41 ANTIBACTERIAL ACTIVITY OF PURIFIED FLAVONOID GLYCOSIDES FROM *RUMEX CHALEPENSIS* LEAVES

Compound	*Staphylococcus* aureus	*Sarcina lutea*	*Bacillus subtilis*	*Escherchia coli*	*Psudomonas aueruginosa*
RC1-1	12	8	-	8	-
RC1-2	-	-	-	-	-
RC1-3	-	-	-	-	-
RC2-1	-	-		8	-
RC2-2	-	-	-	-	-
RC2-3	-	-	-	-	-
RC3-1	11	6	-	-	-
RC3-2	-	-	-	-	-
RC3-3	-	-	-	-	-
RC4-1	10	-	10	12	-
RC4-2	6	-	7	10	-
RC4-3	-	-	-	-	-
RC5-1	12	-	-	-	6
RC5-2	8	-	-	-	-
RC5-3	-	-	-	-	-
TR	18	20	12	18	14
GT	20	21	14	18	9

The values indicate zone of inhibition in mm.

RC1-1 - RC5-1 = 300 µg/disc TR = Tetracycline (10 µg/disc)

RC1-2 - RC5-2 = 200 µg/disc GT = Gentamicin (10 µg/disc)

RC1-3 - RC5-3 = 100 µg/disc - = No inhibition

Table.42 ANTIBACTERIAL ACTIVITY OF PURIFIED FLAVONOID GLYCOSIDES FROM *INDIGOFERA HEBEPETALA* FLOWERS

Compound	*Staphylococus* aureus	*Sarcina lutea*	*Bacillus subtilis*	*Escherchia coli*	*Psudomonas aueruginosa*
IHF1-1	-	-	-	7	-
IHF1-2	-	-	-	-	-
IHF1-3	-	-	-	-	-
IHF2-1	-	-		9	-
IHF2-2	-	-	-	-	-
IHF2-3	-	-	-	-	-
IHF3-1	-	-	9	8	-
IHF3-2	-	-	-	-	-
IHF3-3	-	-	-	-	-
IHF4-1	-	-	7	-	-
IHF4-2	-	-	-	-	-
IHF4-3	-	-	-	-	-
IHF5-1	-	-	12	-	-
IHF5-2	-	-	8	-	-
IHF5-3	-	-	-	-	-
TR	18	20	12	18	14
GT	20	21	14	18	9

The values indicate zone of inhibition in mm.

IHF1-1 - IHF5-1 = 300 µg/disc TR = Tetracycline (10 µg/disc)

IHF1-2 - IHF5-2 = 200 µg/disc GT = Gentamicin (10 µg/disc)

IHF1-3 - IHF5-3 = 100 µg/disc - = No inhibition

pronounced activity against *S. aureus*, *B. subtilis* and *E. coli* at a concentration of 300 and 200μg. RC-5 was only active against *S. aureus* and *P. auerοginosa* at a concentration of 300 and 200 μg.

Flavonoid glycosides IHF-1 and IHF-2 were found to be active only against *E. coli* at a concentration of 300 μg while IHF-3 exhibited an inhibitory effect against *B. subtilis* and *E. coli* at 300 μg. IHF-4 showed a slight activity against *B. subtilis* at 300 μg. IHF-5 exhibited a marked effect at 300 μg and slight activity at 200 μg against *B. subtilis*.

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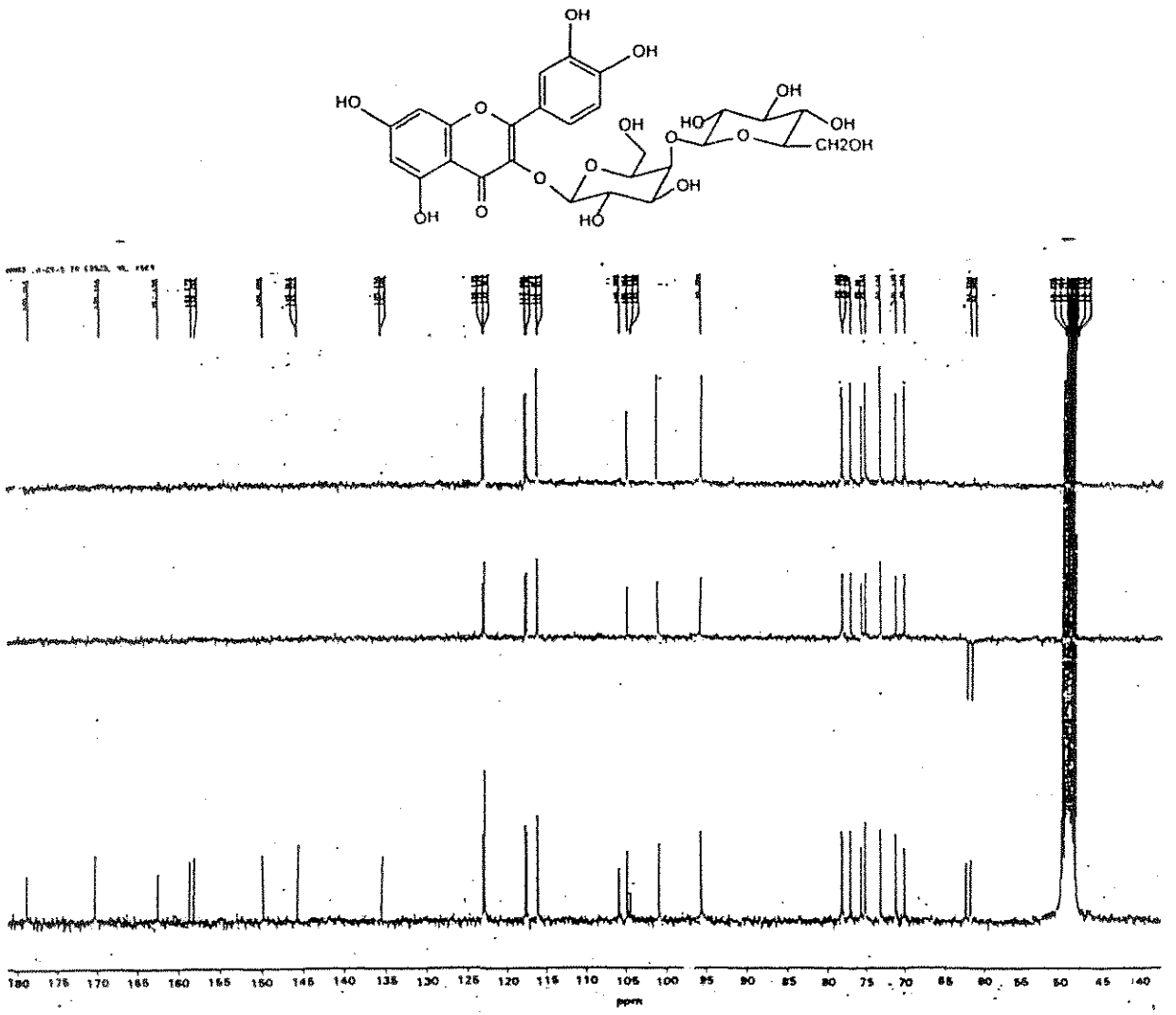
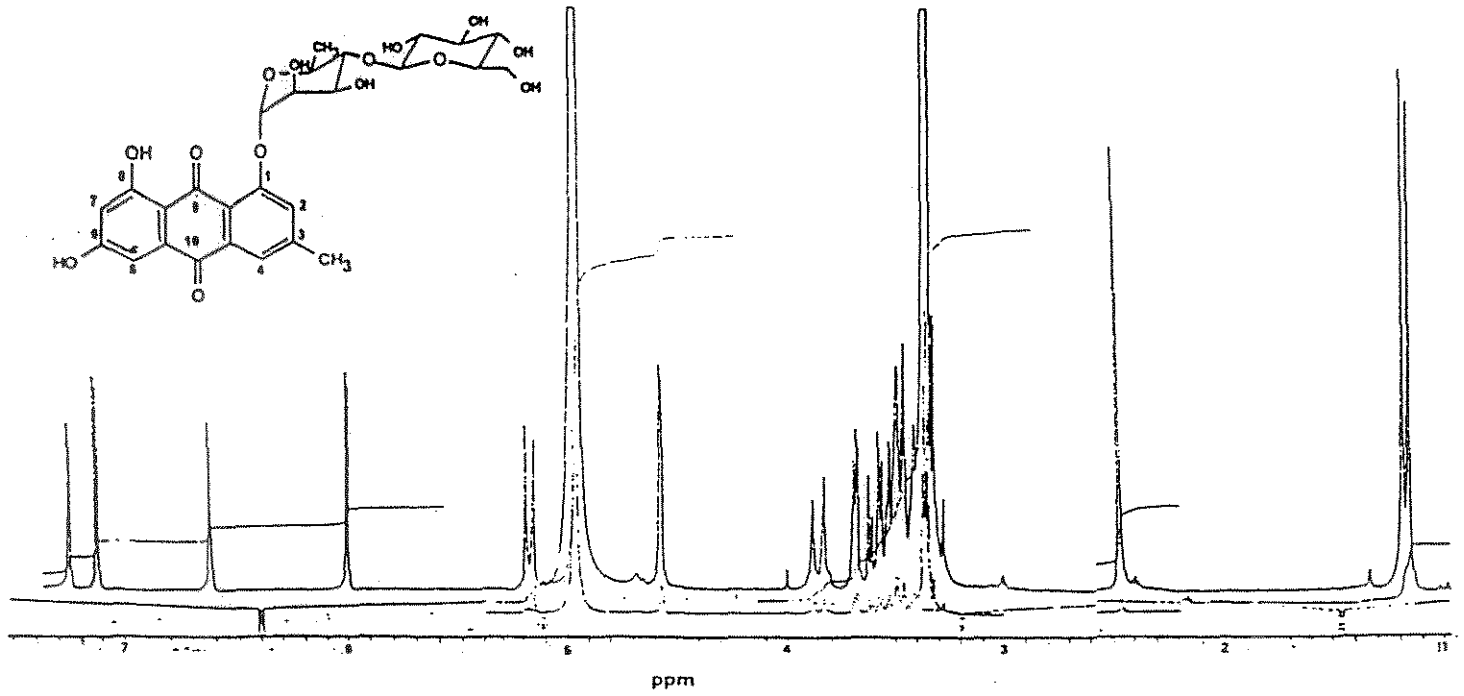
APPENDIX

^1H NMR (CD_3OD, 300MHZ) OF RC-1

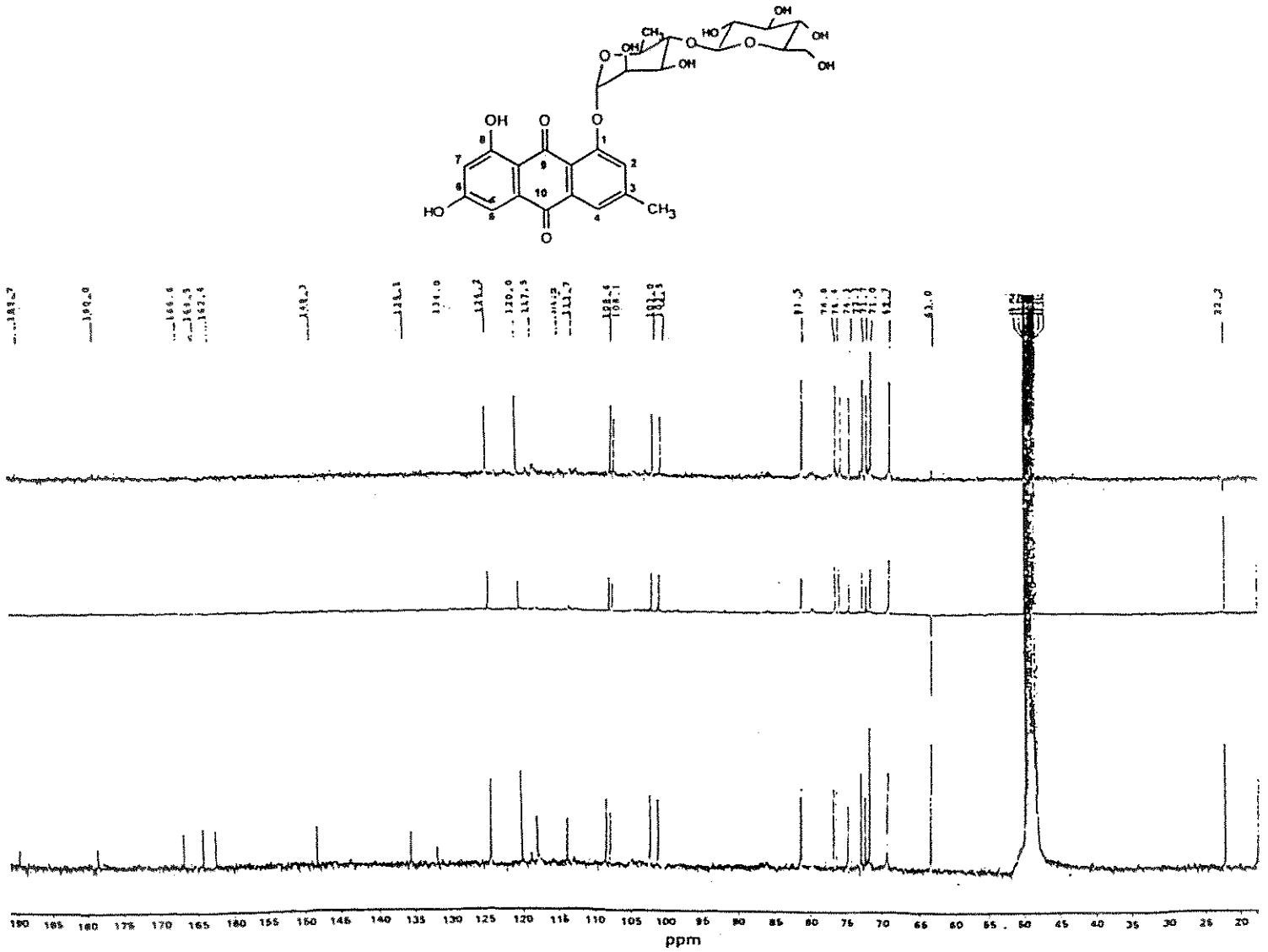


^{13}C NMR [(DEPT),CD_3OD, 75.5MHZ] OF RC-1

^{13}C NMR [(B.B),CD_3OD, 75.5MHZ] OF RC-1



^{1}H NMR (CD_3OD, 300MHZ) OF RC-5



^{13}C NMR [(DEPT),CD_3OD, 75.5MHZ] OF RC-5

^{1}H NMR (CD_3OD, 300MHZ) OF IHF-1

^{13}C NMR CD$_3$OD, 75.5MHz, OF THE I

^{1}H NMR (CD_3OD, 300MHZ) OF IHF-2

^{13}C NMR CD_3OD, 75.5MHZ) OF IHF-2