Phytochemical Study of the Major Flavonoid from *Punica granatum*

A Thesis Submitted in Partial Fulfillment of the Requirements for the M.Sc. Degree in Chemistry

By

Roaa Awad Abdalla Abdel Rahman

(B.Sc., Chemistry)

Supervisor: Prof. Mohamed Abdel Karim

November, 2014
Abstract

Fruit peels of punica granatum were extracted with 95% ethanol. Removal of the solvent under reduced pressure gave a crude product which was subjected to phytochemical screening.

Phytochemical screening of the alcoholic extract revealed the presence of flavonoids, steroid, glycosides and absence of alkaloids.

The ethyl acetate extract which resulted by partitioning the alcoholic with ethyl acetate gave a positive test for flavonoids. It was fractionated over silica gel plates developed by 60% acetic acid. In this way a pure flavonoid-compound A was isolated. The IR data reflected the anticipated functional groups.

UV studies indicated a benzoyl chromophore (band II of flavonoids). When investigating the isoflavone with UV shift reagents the hydroxylation pattern of the isolated component was illustrated.

The spectral studies indicated that the isolated component is a 5,4-dihydroxyisoflavone.
الخلاصة

استخلصت قشور ثمار الرومان بالإيثانول (95%) ثم بخار الإيثانول تحت ضغط منخفض لمنتج المستخلص الخام الذي أخفض لمسح فيتوكيميائي.

انتضح من المسح الفيتوكيميائي وجود الفلافونيدات، الجليكسيودات، الاستيرويدات وعدم وجود قلويات.

استخدمت كروماتوغرافيا الطبقة الرقيقة لفصل الفلافونويد الرئيسي في الهيئة النقية من مستخلص إيثيل استات باتستخدم نظام مذيب (0.6%) حمض خليك.

أوضح طيف الأشعة تحت الحمراء المجموعات الوظيفية المتوقعة لهيكل الفلافونويد. أما طيف الأشعة فوق البنفسجية فقد أوضح امتصاصاً لكروموفورد بنزويل المميز لبعض الفلافونيدات.

اتضح الهدрокسيل للفلافونويد المفصل باستخدام كواشف الإزاحة في مطيافه فوق البنفسجيه. وقد أوضحت هذه الدراسات الطيفية أن المركب المفصل هو عبارة عن: 4,5-ثنائي هيدروكسي ايزوفلافون.
1-Introduction

1.1-General Approach

Phenolics can be classified into 2 major groups:
- The flavonoids
- Non-flavonoids

The flavonoid skeleton can have a large number of substituents: hydroxyl, sugars (e.g. glucose, galactose, rhamnose). Most structures are glycosylated-methylated-prenylated (farnesylated) and cyclated. Sugars and hydroxyl groups increase the water solubility of flavonoids while methyl and isopentyl groups make flavonoids lipophilic.

Flavonoids are natural products widely distributed in plant kingdom and currently consumed in large amounts in the daily diet. These are categorised according to their molecular structures into: flavonols, flavones, flavanones, isoflavones, catechins, anthocyanidins and chalcones. Flavonoids are capable of modulating the activity of enzymes and affect the behaviour of many cell systems and exerting beneficial effects on body. This property of flavonoids has aroused considerable interest\(^1\).

Flavonoids represent a diverse group of low molecular weight natural polyphenolic compounds. The term Flavonoid has been derived from the Latin word flavus (meaning yellow). Their presence in plant is responsible for various colours and combination of colours exhibited by roots, bark, heartwood, leaves, flowers, fruits and seeds of plants. They are characterized by the flavan nucleus, consisting of two aromatic rings (A and B) interconnected by a three carbon atom heterocyclic ring C as shown below\(^2\).
In plants, flavonoids occur in different forms, such as free aglycones, glycosides, and biflavonoids. Flavonoids function throughout the plant kingdom as UV protectants, attract insects for pollination, and as antimicrobial compounds. Flavonoids and isoflavonoids may be of ecotoxicological importance because they are present in the heartwood of tree species used for wood pulp. Flavonoids are common components of human diet as they are found in foods (fruits and vegetables) and beverages (tea, and juices). Some plants contain the significant flavonoids: quercetin and rutin which reduces the risk of coronary heart disease.

The flavonoid quercetin is also known for its ability to relieve hay fever and asthma. Flavonoids can be useful in the prevention of cancer and heart diseases as they influence cell signaling pathways and gene expression. Moreover, they can induce mechanisms that can inhibit tumour invasion and kill cancer cells.
Flavonoids also have antioxidant activity. Flavonoids are becoming very popular because they have many health promoting effects. Some of the activities attributed to flavonoids include: anti-allergic, antioxidant, anti-inflammatory and anti-viral.

1.2 Optical activity of flavonoids

The flavonoids are a class of natural product that gains interest due its great variety and the number of its members. The flavonoids are often hydroxylated in positions 3, 5, 7, 3', 4', and 5' which are frequently methylated, acetylated, or sulphated\(^2\).

The actual number of flavonoids that have been found so far and for which the structure has been completely elucidated is large, but probably does not exceed 1% of the theoretical number of possible variants. This abundance of variants is further augmented by the chirality of the subunits and their connections. Since many stereoisomers do not differ significantly in their electronic or fluorescence spectra, the optical activity of the species is often a useful analytical parameter\(^3\).

1.3 Distribution of flavonoids

Flavonoids are widely distributed among the plant kingdom. Flavonoids are found in vegetables, fruits, nuts, seeds, stem, flowers, tea, wine… etc. These are an integral part of our daily diet\(^4\)\(^-\)\(^6\). The dietary intake of flavonoids is estimated to be 1-2 g/day\(^7\). The average intake of flavonols and flavones was found to be 23 mg/day, among which, flavonol quercetin contributed 16 mg/day\(^8\). The table below (Table 1.1) describes various flavonoids present in our daily dietary food source.
Table 1.1: Occurrence of flavonoids in some foods

<table>
<thead>
<tr>
<th>Flavonoid subclass</th>
<th>Food source</th>
<th>Representative flavonoids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flavonol</td>
<td>Onion, kale, broccoli, apples, cherries, berries, tea, red wine</td>
<td>Kaempherol, myricetin, quercetin, rutin</td>
</tr>
<tr>
<td>Flavones</td>
<td>Parsley, Thyme</td>
<td>Apigenin, chrysin, luteolin</td>
</tr>
<tr>
<td>Flavonones</td>
<td>Citrus</td>
<td>Hesperitin, erodictyol, naringen</td>
</tr>
<tr>
<td>Catechins</td>
<td>Apple, tea</td>
<td>Catechin, galocatechin</td>
</tr>
<tr>
<td>Anthocyanidins</td>
<td>Cherries, Grapes</td>
<td>----------------------------------------------</td>
</tr>
<tr>
<td>Isoflavones</td>
<td>Soya beans, Legumes</td>
<td>Daidzen, genistein, glyciten, formanantine</td>
</tr>
</tbody>
</table>

1.4- Structure and classification of flavonoids

The chemical structure of flavonoids is based on a \((C_6 - C_3 - C_6)\) skeleton. They differ in saturation of heteroatomic ring C, in the placement of the aromatic ring B at the positions 2,3 or 4. According to the modifications of the central C-ring, they can be divided into different structural classes including flavones, flavonols, flavan-3-ols, flavonones, isoflavones, and anthocyanins. In a few cases, the 6-membered heterocyclic ring C occurs in an isomeric open form or is replaced by a 5-membered ring as in the case of chaclones and aurones\(^9,10,11\). In plants, flavonoid aglycones (flavonoids without attached sugars) occur in a variety of structures. Most frequently encountered groups of flavonoid aglycones includes flavones, flavonols.
Flavonoid aglycones possess the chemical properties of phenolics, and thus they are slightly acidic. Those possessing a number of unsubstituted hydroxyl groups, or sugar moieties, are polar substances and soluble in polar organic solvents. The presence of sugar makes flavonoid more water soluble, while less polar aglycones like isoflavones,
flavanones, and highly methoxyalted flavones and flavonols tend to be more soluble in ether or chloroform$^{12,13}$.

Flavonoids are benzo-$\gamma$-pyrone derivatives consisting of phenolic and pyran rings and are classified according to substitutions. The polyphenolic structure allows a large number of further substitutions, including phenolic hydroxy groups, methoxy groups, O-sugars, C-sugars, and sulfates, thus producing an extremely diverse range of derivatives. Dietary flavonoids differ in the arrangements of hydroxyl, methoxy, and glycosidic side groups, and in the conjugation between the A- and B-rings. Structurally, they consist of two main groups, the 2-phenylchromans (the flavonoids, including flavanones, flavones, flavonols, flavan-3-ols, and anthocyanidins) and the 3-phenylchromans (the isoflavonoids, including isoflavones, isoflavans, and pterocarpans$^{14,15}$ (see Table 1.2)).
### Table 1.2 - Classes of flavonoids

<table>
<thead>
<tr>
<th>Class</th>
<th>Structure</th>
<th>Example (Substitution pattern)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flavonol</td>
<td><img src="image1.png" alt="Flavonol Structure" /></td>
<td>(+)-catechin (3,5,7,3',4'-OH)</td>
<td>Tea</td>
</tr>
<tr>
<td>Flavone</td>
<td><img src="image2.png" alt="Flavone Structure" /></td>
<td>chrysin (5,7-OH)</td>
<td>Fruit skins</td>
</tr>
<tr>
<td>Flavonol</td>
<td><img src="image3.png" alt="Flavonol Structure" /></td>
<td>quercetin (3,5,7,3',4'-OH)</td>
<td>Onion, Tomato</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Red wine</td>
</tr>
</tbody>
</table>
1.5-Anthocyanins

Anthocyanins (3-hydroxy-2-phenylchromenylium) are one of the most important plant pigments visible to the human eye. Anthocyanins are the largest group of colourful plant pigments and are responsible for colours ranging from red to violet and blue. These plant pigments accumulate richly in the epidermal or subepidermal cell vacuoles of flowers, fruits, vegetables and foliage. Generally, anthocyanins belong to the widespread class of polyphenolic compounds, which are collectively named flavonoids. Anthocyanins are the glycosides of flavonoids with
polyhydroxy and polymethoxy derivatives of 2-phenylbenzopyrylium or flavylium cation. Anthocyanins are conjugated aromatic systems, which are often positively charged. Anthocyanins can absorb in the visible range, hence each anthocyanin can be represented by their unique colour. Anthocyanin itself has a carbohydrate (sugar, usually glucose) esterified at the 3 position. An anthocyanidin, termed aglycone, does not have a sugar at the 3 position. Glucose (glc), galactose (gal), arabinose (arab), rhamnose (rham) and xylose (xyl) are the most common sugars that are bonded to anthocyanidins as mono-, di- or trisaccharide forms. Except for the 3-deoxyanthocyanidins, such as luteolinidin and apigeninidin in sorghum\textsuperscript{16}, aglycones are rarely found in fresh plant materials. Only six anthocyanidins: cyanidin (Cy), delphinidin (Dp), petunidin (Pt), peonidin (Pn), pelargonidin (Pg) and malvidin (Mv), are ubiquitously distributed. For example, the distributions of anthocyanidins in the edible parts of plants are: Cy (50%), Pg (12%), Pn (12%), Dlp(12%),Pt (7%) and Mv (7%). The presence of sugar in anthocyanins is important for water solubility; if their sugar (s) are hydrolyzed or lost, their solubility might decrease or be lost and the anthocyanin molecules will be destabilized and lost. The differences in function between the individual anthocyanins relate to the number of hydroxyl groups, the kinds and number of sugars attached to the molecule, the position of the attachment, and the kinds and number of aliphatic or aromatic acids attached to the sugars in the anthocyanin molecule.

The following four classes of anthocyanin glycosides (anthocyanins) are very common: 3-monosides, 3-biosides, 3,5-diglycosides and 3,7–diglycosides. 3-Glycosides occur about two and half times more frequently than 3,5-diglycosides. The most widespread anthocyanin is cyanidin-3-glucoside. Three non-methylated anthocyanidins of cyanidin (Cy), delphinidin (Dp) and pelargonidin (Pg)
are the most widespread in the plant kingdom, being present in 80% of pigmented leaves, 69% of fruits and 50% of flowers\(^\text{16}\).

1.6- The flavanols

Structures of flavanols are very similar to those of anthocyanidins: but no positive charge on the oxygen atom and no double bonds in the C ring. A common flavan is flavan-3-ol that occurs in many plants. It is found in green tea and cocoa powder. It is also a common subunit of proanthocyanidin polymers such as procyanidin. Epicatechin is another common example; it differs from catechins only in the spatial orientation of its -OH group.

![Epicatechin](image)

Green tea contains high levels of flav-3-ols such as (-) epigallocatechin gallate. Flavan-3-ols, such as epicatechin, catechin and epigallocatechin (and procyanidins their polymers) are:
- Powerful antioxidants
- Have beneficial effects on cardiac health and immunity.

1.7- Flavones

The C ring of the flavones (2-phenyl-chromen-4-one) contains a double bond between positions 2 and 3, and a keto function on position 4. Most flavones of fruits and vegetables hold a hydroxyl group on position 5 of ring A, whereas hydroxylation on other positions, most often position 7 of ring A or position 3` and 4` of ring B, can vary depending on the taxonomic classification of a particular fruit or vegetable.
The flavones are of widespread occurrence and they include apigenin, (5, 7, 4’-trihydroxyflavone) (1), luteolin (5, 7, 3’, 4’ tetrahydroxyflavone) (2) and chrysin (3). Apigenin and luteolin are the most widespread flavone aglycones. However the diverse substitution patterns make this group the largest. Glycosylation occur mostly at positions 5 and 7, and methylation and acylation usually occur on hydroxyl groups of ring B. Some flavones are polymethoxylated, such as tangeretin (4) and nobiletin (5), scutellarein (6) and sinensetin (7) and they are found almost exclusively in the peels of citrus fruits which contain a number of compounds including C-flavone glycosides in the form of isoorientin (luteolin-6-C-glucoside) (8) and orientin (luteolin-8-C-glucoside) (9). Orientin and isoorientin also occur in *Cymbopogon citrata* along with two other flavones C-glycosides, chrysoeriol-6-C-glucoside (isoscoparin) (10) and 7-O-methyl-luteolin-6-C-glucoside (swertiajaponin) (11)\(^\text{18, 19, 20}\)
1.8- The Flavonols

The flavonols are characterized by a double-bonded oxygen atom attached to position 4. They are still "-ols" because they retain the -OH group at position 3 like the flavanols. The double-bonded oxygen atom, makes them like another class of flavonoids known as "flavones". Another double bond is cited between C2 and C3 (C ring) which is involved in UV screening, due to their strong absorbance in UV-A (325-400nm) and UV-B (280-325 nm) wavelengths.

Quercetin is the most abundant flavonol in the diet and is found in hundreds of herbs and food. Onions are especially rich in quercetin which has proven its antioxidant effects. Falvonols are mostly found as O-glycosid and more than 200 different sugar conjugates of kaempferol were isolated.
1.9- Chalcones

Chalcones 1-(2-hydroxyl-phenyl)-3-phenylpropenon and dihydrochalocones (12) have a linear C₃-chain connecting the two aromatic rings. The C₃–chain of chalcones contains a double bond, whereas the C₃- chain of dihydrochalcones is saturated. Chalcones, such as butein (13), are yellow pigments in flowers. An example a dihydrochalcone is phloridzin (14), a compound found in apple leaves, and which has been reported to have anti-tumor (21) activity. Chalcones are flavonoids lacking a heterocyclic C-ring. Generally, plants do not accumulate chalcones. After its formation, narigenin chalcone is rapidly isomerized by the enzyme chalcone isomerase to form the flavanone-narigenin. The most common chalcones found in foods are phloretin and its 2-O-glucosides: chalconarigenin and arbutin.

![Chalcone structures](12, 13, 14)

1.10- Isoflavones

Isoflavones (3-phenyl-chromen-4-one) also have a diphenylpropane structure in which the B ring is located in the 3 position. Common isoflavones aglycones such as genistein (15), daidzein (16), and glycitein (17) occur, in low levels, in black beans (Phaseolus vulgaris) and green beans (Pisum sativum) together with formononetin (18) and
biochanin A (19)\textsuperscript{24}. They have structural analogies to estrogens, such as estradiol, with hydroxyl groups at the C\textsubscript{7} and C\textsubscript{4} positions\textsuperscript{(23)}

\begin{center}
\includegraphics[width=0.8\textwidth]{flavonoid_structures.png}
\end{center}

1.11-Biosynthesis of flavonoids

The biosynthesis of flavonoids is a very complex process and involves series of reactions. The basic pathways to the core flavonoid skeletons have been established both enzymatically and genetically\textsuperscript{7,8}.

The flavonoid biosynthesis, starts with general phenylpropanoid pathway. The synthesis of phenylpropanoids starts with the removal of the amino group of phenylalanine by phenyl ammonium lyase (PAL) to produce trans-cinnamic acid. The aromatic ring of trans-cinnamic acid is then hydroxylated to produce \textit{p}-coumaric acid by the enzyme cinnamate - 4-hydroxylate (C4H). Coumaric acid can then be ligated to coenzyme A by a ligase [ 4-coumaroyl-CoA ligase (4CL) ].
Coumaroyl-CoA, an intermediate formed by the phenylpropanoid pathway is a substrate for the enzyme chalcone synthase (CHS) and stilbene synthase (STS). Coumaroyl-CoA along with malonyl-CoA results in the formation of chalcones and stilbene as shown below:
Ring closure of chalcone to produce a pyran ring in naringenin occurs spontaneously. This step is catalysed by a chalcone isomerase (CHI).

Flavones and flavonols can be made from flavanone (naringenin). Flavanone 3-hydroxylase (F3H) catalyzes the hydroxylation of C-3 position of C-ring. Then the introduction of a double bond between C-2 and C-3, results in the formation of flavonol. The desaturation of C-ring is catalyzed by flavonol synthases (FLS).

Further, the reduction of keto group in the C-ring is reduced by the action of dihydroflavonol reductase (DFR), and then anthocyanidin synthase (ANS) introduces two double bonds in the C-ring forming anthocyanidin. Flavonoid 3-O-glucosyltransferase (F3GT) transfers the glucose residue from nucleotide sugar to 3-OH position forming anthocyanin.
(Naringenin) Flavanone

F3H: Flavanone 3-hydroxylase
FLS: Flavonol synthases

Dihydrokaempferol Dihydroflavonol

kaempferol Flavonol

Dihydrokaempferol Dihydroflavonol

leuco pelargonidin Leucoanthocyanidin

Pelargonidin 3-O-glucoside Anthocyanin

Pelargonidin Anthocyanidin

DFR: Dihydroflavonol reductase
ANS: Anthocyanidin synthase
F3GT: Flavonoid 3-O-glucosyltransferase
1.12-Chemical Synthesis

Baker-Venkatraman synthesis is a base-catalyzed rearrangement. The substituted o-hydroxyacetophenones on benzylation with benzoyl chloride is converted to esters. Esters undergo rearrangement in the presence of a base to the diketones, which then undergo cyclisation to flavones.\(^{25}\)

\[
\text{HO-CH(OH)CH=CH-CH(OH)OH} \xrightarrow{C_6H_5COCl} \text{HO-CH(OH)CH=CH-CH(OH)OCOC_6H_5} \xrightarrow{PhOCONa} \text{HO-CH(OH)CH=CH-CH(OH)OCOC_6H_5} \xrightarrow{H^+} \text{Flavone}
\]

In the Robinson’s synthesis flavones can be obtained from o-hydroxyacetophenone which is treated with benzoic acid anhydride and sodium benzoate and then subsequently heated.\(^{26}\)

\[
\text{HO-CH(OH)CH=CH-CH(OH)OH} + (C_6H_5CO)_{2}O \xrightarrow{C_6H_5COONa, \Delta} \text{Flavone}
\]

The Von-Konstanecki method for the synthesis of flavonols utilizes Claisen condensation between benzaldehyde and o-hydroxyacetophenone. Flavanones are obtained on acidification of condensation product, which on reaction with isoamyl nitrite and subsequent hydrolysis gives flavonols.
Biological Activity of flavonoids

Flavonoids are the largest group of naturally occurring plant polyphenolic compounds. They are known to possess diverse biological and medicinal properties. The contribution of flavonoids in warding off microbial infection and protecting plants from herbivory is well known. The biological properties of flavonoids are considered in an evaluation of the medicinal and nutritional values of these compounds. Certain plants containing flavonoids have been used for thousands of years in traditional medicine.

Increasingly, flavonoids are becoming the subject of medical research. Many drugs available today are either from plant source or modified from the plant derived compounds. Flavonoids are among the most ubiquitous polyphenolic compounds found in nature that show wide range of biological activity viz. anticancer, antiviral, antioxidant, antimalarial, anti-inflammatory, antifungal, antiallergic, antimicrobial and antiprotozoan etc.
Flavonoids and related polyphenols also protect plants from microbial invasion. They function as antifungal, antibacterial and antiviral agents. The phenolic groups in the flavonoids provide antimicrobial activity and this activity is further enhanced by additional phenolic groups. There is growing interest in their use against fungal pathogens of man. They are considered for treating human diseases and especially for controlling the HIV virus (the causative agent of AIDS). They are not only present in plants as constitutive agents but are also formed in plant tissues in response to microbial attack. The flavone baicalein is reported to be largely responsible for the plant’s antimicrobial effects.

![Baicalein](image)

Owing to the widespread ability of flavonoids to inhibit spore germination of plant pathogens, they have been proposed for use against fungal pathogens of man\(^2\)\(^8\) K. Karanjin a furano favonol obtained from the seeds of karanja tree (*Pongamia glabra* Vent.) showed promising antifungal activity. Galangin, a flavonol commonly found in propolis samples was first isolated in 1881 from the root of galangal (*Alpinia officinarum*). Galangin is a broad spectrum antimicrobial agent where it can inhibit the growth of Gram-positive bacteria, Gram-negative bacteria and a number of fungal species\(^2\)\(^8\).
Flavonoids show inhibitory activity against various viruses. Researchers are investigating inhibitory activity of some flavonoids against human immunodeficiency virus (HIV). *In vitro* studies have shown that baicalin inhibits HIV-1 infection and replication. Flavonoids can inhibit reverse transcriptases of different origin and can act as antiretroviral agents. Amentoflavone possess anti-reverse transcriptase activity\(^{30}\). Studies also revealed that common flavonols and aurones were strongly active and inhibited the tomato ringspot virus\(^{31}\). Quercetin and other flavonoids appear to interfere with an early event in the virus life cycle. Quercetin at a concentration of 5 µg ml-1 resulted in 70% inhibition of local lesion development of the virus on the test plant *Chenopodium quinoa*\(^{32}\).

Flavonoids are also known to possess antibacterial activity. The activity of the flavonoid- quercetin, has been attributed to inhibition of DNA gyrase. Also Quercetin has been reported to completely inhibit the growth of *Staphylococcus aureus*. Research groups have also isolated and identified the structure of various flavonoids possessing antibacterial
activity. Other flavonoids whose mechanisms of action have been investigated include apigenin, rutin, robinetin, myricetin and galangin. The study of these compounds may help in the development of a pharmacologically acceptable antimicrobial agent or class of agents. Antibacterial activity has been displayed by a number of flavonoids. Most of the flavonones having no sugar moiety showed antimicrobial activities whereas none of the flavonols and flavonolignans tested showed inhibitory activity on microorganisms.

Flavonoids are known to act as scavengers of various oxidizing species. Antioxidant flavonoids are naturally present in fruits, vegetables, tea and have been found in vitro to inhibit oxidation of low-density protein (LDL). The antioxidant activity of flavonoids arise from their ability to inhibit lipid peroxidation, chelate redox-active metals, and attenuate other processes involving reactive oxygen species. Multiple hydroxyl groups enhance substantial antioxidant and chelating activity. A double bond and carbonyl function in the heterocycle or polymerization of the nuclear structure increases activity by affording a more stable flavonoid radical through conjugation and electron delocalization. Antioxidant properties depends on the number of hydroxyl groups which
help in free radical scavenging. The high reactivity of hydroxyl substituents participate in the reaction given below:

\[
\text{Flavonoids-OH} + \text{R}^\cdot = \text{Flavonoids-O}^\cdot + \text{RH}
\]

Where \( R^\cdot \) is a free radical and \( O^\cdot \) is oxygen free radicals.

Certain plants species containing flavonoids have been used for thousands of years in traditional Eastern medicine. Flavonoids and their derivatives from both natural and synthetic sources have been investigated for various activities. flavonoids can inhibit carcinogenesis, they can induce mechanisms that can inhibit tumor invasion and kill cancer cells. Flavonoids also possess anti-inflammatory properties. They can be helpful in curing rheumatoid arthritis and osteoarthritis.

1.14- *Punica granatum*

**Scientific classification**

<table>
<thead>
<tr>
<th>Kingdom</th>
<th>Plantae</th>
</tr>
</thead>
<tbody>
<tr>
<td>(unranked)</td>
<td>Angiosperms</td>
</tr>
<tr>
<td>(unranked)</td>
<td>Eudicots</td>
</tr>
<tr>
<td>(unranked)</td>
<td>Rosids</td>
</tr>
<tr>
<td>Order</td>
<td>Myrtales</td>
</tr>
<tr>
<td>Family</td>
<td>Lythraceae</td>
</tr>
<tr>
<td>Genus</td>
<td>Punica</td>
</tr>
<tr>
<td>Species</td>
<td><em>P. granatum</em></td>
</tr>
</tbody>
</table>

**Binomial name**

*Punica* *granatum* L.

**Synonyms**

*Punica* *malus* Linnaeus, 1758
*Punica granatum* fruits

*Punica granatum* seeds on a plate

*Punica granatum* fruit and leaves
The pomegranate, botanical name Punica granatum, is a fruit-bearing deciduous shrub or small tree growing between 5 and 8 m (16–26 ft) tall. In the northern hemisphere, the fruit is typically in season from September to February, and in the southern hemisphere from March to May. As intact arils or juice, pomegranates are used in cooking, baking, meal garnishes and juice blends.

The pomegranate is considered to have originated in the region between the Himalayas and Egypt, and has been cultivated since ancient times in India, Persia Mesopotamia, Turkey and the Arabian Peninsula. It is mentioned in many ancient texts, notably in Babylonian texts and the Book of Exodus.

Today, it is widely cultivated throughout the Mediterranean region of southern Europe, the Middle East and Caucasus region, northern Africa and tropical Africa, the Indian subcontinent, Central Asia, and the drier parts of southeast Asia.

In the Indian subcontinent's ancient Ayurveda system of traditional medicine, the pomegranate has been used extensively as a source of traditional remedies.

The rind of the fruit and the bark of the pomegranate tree are used as a traditional remedy against diarrhea, dysentery, and intestinal parasites. The seeds and juice are considered a tonic for the heart and throat, and classified as having bitter-astringent taste plus a range of taste from sweet to sour, depending on ripeness. Thus, pomegranate is considered a healthful counterbalance to a diet high in sweet-fatty components.

Especially when sweet, pomegranate fruit is nourishing for systems and is considered a blood builder. The astringent qualities of the flower juice, rind, and tree bark are considered valuable for a variety of purposes, such as stopping nose bleeds and gum bleeds, toning skin,
(after blending with mustard oil) firming-up sagging breasts, and treating hemorrhoids. Pomegranate juice (of specific fruit strains) is also used as an eyedrop, as it is believed to slow the development of cataracts.

Pomegranate has been used as a contraceptive and abortifacient by means of consuming the seeds, or rind, as well as by using the rind as a vaginal suppository. This practice is recorded in ancient Indian literature, in medieval sources, and in modern folk medicine.

Pomegranate extracts (alkaloids) are used to treat intestinal parasite infestations in some nations38.

1.15-Aim of this study

This study was aimed to:

i) Extraction of plant phenolics

ii) Isolation of the major flavonoid from target species.

iii) Conducting UV and IR studies on the isolated component.

2-Materials and Methods

Analytical grade reagents were used. The IR spectra were run on FTIR-8400S, Infrared Spectrophotometer. The UV Spectra were recorded on a Perkin-Elmer UV-1800 Spectrophotometer.

2.1-Materials

2.1.1-Collection of plant material

Punica Granatum was collected from EI Obeid(eastern Sudan) and authenticated by the Aromatic Plants Research Institute-Khartoum.

2.2-Methods

2.2.1-Preparation of test reagents for phytochemical screening

2.2.1.1-Flavonoid test reagent

i) Aluminum chloride solution

(1g) Aluminum chloride was dissolved in (100ml) methanol

ii) Potassium hydroxide solution

(1g) Potassium hydroxide was dissolved in (100ml) water
iii) Ferric chloride solution
(0.5g) ferric chloride was dissolved in (100ml) 97% methanol.

2.2.1.2- Alkaloid test reagents
Modified Dragendeoff’s reagents
i) Stock solution (A)
(0.5g) Bismuth nitrate was dissolved in (10ml) acetic acid and 
(40ml) of water was added.
ii) Stock solution (B)
(8g) Potassium iodide was dissolved in (20ml) water.

When testing for alkaloids, (5ml) of stock solution (A) is mixed 
with (5ml) of stock solution (B), 20ml of acetic acid and (100ml) water 
were added.

2.2.2- Plant extract
Powdered air-dried fruits of Punica Granatum were macerated at 
room temperature with 95% ethanol (5 L) for 48hr.. The solvent was 
evaporated under reduced pressure and part of residue was used for the 
following tests.

2.2.3- Phytochemical screening
Punica Granatum was screened for steroids, flavonoids, alkaloids 
and glycosides.

2.2.3.1- Test for steroids

Part of the crude product was stirred with petroleum ether to 
remove most of the coloring matter. The residue was extracted with 
(20ml) chloroform and the solution was dehydrated over anhydrous 
sodium sulphate.

(5ml) Portion of the solution was mixed with (0.5ml) acetic 
anhydride, followed by two drops of concentrated sulphuric acid.
2.2.3.2- Test for alkaloids

(5ml) of 2N hydrochloric acid were added to the crude product and the solution was heated with stirring in a water bath for 10 minutes. The cooled solution was filtered. To portion (5ml) of this solution, few drops of Dragendorff's reagent were added. No precipitate was formed.

2.2.3.3- Test for flavonoids

Part of the crude product was defatted by extraction with petroleum ether. The defatted residue was dissolved in (30ml) 95% ethanol and filtered. The filtrate was used for the following tests:

i) To (3ml) of filtrate, few drops of 1% methanolic aluminium chloride were added. Formation of yellow colour indicated the presence of flavonoids.

ii. To (3ml) of filtrate, few drops of potassium hydroxide solution were added, A dark yellow colour indicated the presence of flavonoids.

iii. To (3ml) of filtrate, few drops of ferric chloride solution were added. Development of a blue colouration was taken as a positive test for flavonoids.

2.2.3.4- Test for glycosides:

Part of the powdered air-dried plant was vigorously shaken in a test tube with water. The presence of a froth that persisted for one hour indicated the existence of glycosides.

2.2.4- Isolation of flavonoids from Punica Granatum

Part of the crude product (2.2.2) was dissolved in minimum amount of ethanol and applied as narrow zone on Whatman No. 3 sheets. The sheets were irrigated with 60% acetic acid. The chromatograms were located under UV light. The major flavonoid was eluted from paper by methanol. Removal of methanol under reduced pressure gave compound I.
2.2.5-UV shift reagents

i. Sodium methoxide stock solution
   freshly cut metallic sodium (2.5g) was added cautiously in small portions to dry spectroscopic methanol (100ml). The solution was stored in a glass container with a lightly fitting plastic stopper.

ii. Aluminium chloride stock solution
   (5g) of fresh anhydrous aluminium chloride were added cautiously to spectroscopic methanol (100ml).

iii. Hydrochloric acid stock solution
   Concentrated hydrochloric acid (50ml) was mixed with water (100ml) and stored in a glass stopper bottle.

iv. Boric acid (H3BO3)
   Anhydrous powdered reagent grade H3BO3 was used.

v. Sodium acetate (NaOAC)
   Anhydrous powdered reagent grade NaOAC was used.

2.2.6- The UV spectrum of compound I in presence of UV shift reagents

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

2.2.6.2- The UV spectrum of compound I in presence of AlCl3
   Six drops of aluminum chloride were added to a solution of compound I in methanol (2ml) and the UV spectrum was recorded immediately.

2.2.6.3- The UV spectrum of compound I in presence of AlCl3/HCl
   Three drops of stock solution of hydrochloric acid were added to the solution in (2.2.6.2) and the UV spectrum was recorded immediately. The spectrum showed max (MeOH+Alcl3+HCl) 360nm

2.2.6.4- The UV spectrum of compound I in presence of sodium acetate

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

2.2.6.5- The UV spectrum of compound I in presence of boric acid /sodium acetate

Sufficient powdered anhydrous H3BO3 was added with shaken to the cuvette containing the solution in (2.2.6.4) to give a sturated solution. The UV spectrum was recorded after two minutes.
3-Results and Discussions

3.1- Extraction and purification of flavonoids

The fruits of Punica Garnatum were extracted with 90% ethanol and the crude product obtained was subjected to physiochemical screening where qualitative tests were positive for flavonoids, steroids, but negative for alkaloids.

The crude extract was then fractionated by thin paper layer chromatography where Whatman number 3 sheets were irrigated with 60% acetic acid. After the usual workup a flavonoid- compound A- was isolated.

3.2- Spectral data of compound A

The IR (KBr) spectrum of compound A (Fig 1) showed v575.04, 781.12 (C-H, bending Ar.), 10513.13-1107.06 (C-O, ether), 1600(C=O), 1730.03(α, β-unsaturated carbonyl group) and 3411.84 cm⁻¹(OH).

Compound A cannot be an anthocyanin or flavan since the IR spectrum gave a carbonyl stretching . It could be: a flavones, flavonol, chalcone, aurone, isoflavone, flavanone, dihydrochalcone or dihydroflavonol.

![Flavone](image1)

![Flavonol](image2)
Chalcone                                Aurone

Flavanone                                Isoflavone

Dihydroflavonol                          Dihydrochalcone
In their UV spectra, most flavonoids exhibit two major bands in the region 240 – 400 nm. These two peaks are commonly referred to as band I (usually 300 – 400 nm) and band II (usually 240 – 280 nm). Band I is considered to be associated with absorption due to the B-ring cinnamoyl system.
Isoflavones, flavanones and dihydroflavonols all give only band II due
to lack of conjugation between the carbonyl function and the aromatic B
ring, while flavones, flavonols, aurones and chalcones give similar UV
spectra as a result of conjugation between the A- and B- rings. Thus they
exhibit both band I and II. The UV absorption of some classes’ flavonoids
is depicted in table 3.1

<table>
<thead>
<tr>
<th>Flavonoid class</th>
<th>Band I</th>
<th>Band II</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Favone</strong></td>
<td>330-350</td>
<td>250-270</td>
</tr>
<tr>
<td><strong>Flavonol</strong></td>
<td>350-390</td>
<td>250-280</td>
</tr>
<tr>
<td><strong>Chalcone</strong></td>
<td>365-390</td>
<td>240-260</td>
</tr>
<tr>
<td><strong>Aurone</strong></td>
<td>390-430</td>
<td>240-270</td>
</tr>
</tbody>
</table>

In the UV, compound A absorbs (Fig.2) at \( \lambda_{\text{max}} \) (MeOH) 240,
300(sh)nm. Such absorption indicates loss of conjugation between the A
and the B rings which is characteristic of: isoflavones, flavanones,
dihydrochalcones and dihydroflavonols. However the shoulder which
appeared in the spectrum at 330 nm clearly indicates that this
phytochemical is an isoflavone.
Considerable structural feature has also been obtained by UV shift reagents which produce shifts in the UV absorption maxima in accordance with the location of the various hydroxyl functions in flavonoids.

Sodium methoxide is strong base and it is used for detection of free 3 – and / or 4′-hydroxy functions. It affords bathochromic shifts in presence of such groups but with decrease in intensity in case of a 3-OH function.

Fig. 2: UV spectrum of compound A

Fig. 3: Sodium methoxide spectrum of compound A
When sodium methoxide was added to a methanolic solution of compound A, a bathochromic shift (Fig 3) without decrease in intensity was observed indicating a 4′-OH function.

Sodium acetate is a weak base and as such, ionizes only the more acidic hydroxyl group’s i.e. the 7-OH function. No bathochromic shift was observed when sodium acetate was added to methanolic solution of compound A (Fig. 4).

Flavones and flavonols which contain hydroxyl groups at C-3 or C-5 from acid-stable complexes with aluminum chloride, whereas the aluminum chloride complexes with ortho-dihydroxy groups are not stable in acidic media. Such acid-stable and acid-labile complexes are shown in scheme 3. The presence of an ortho-dihydroxy groups in the B-ring of flavones and flavonols can be detected by comparison of the spectrum of the flavonoid in the presence of AlCl₃ with that obtained in AlCl₃/HCL.
Figure 5 illustrates the A[Cl] 3 spectrum of compound A where a bathochromic shift was observed indicating a 5- OH function.
On the basis of the above argument compound A is an isoflavone which is hydroxylated at the 5- and 4’- positions.
Conclusion

The fruits of *Punica granatum* were extracted with aqueous ethanol. Fractionation of the ethanolic extract by paper chromatography gave a major flavonoid- compound A. UV studies using UV shift reagents indicated that the isolated component is an isoflavone which is hydroxylated at the 5- and 4´- positions.

Recommendations

1- Other flavonoids present in fruits of *Punica granatum* may also be isolated and identified by spectral data.
2- The isolated flavonoid may be evaluated for its biological activity.
3- The structure of the isolated flavonoid may fully be elucidated via a combination of spectral techniques (IR, UV, $^1$HNMR, $^{13}$CNMR and MS).
References


