Phytochemical Study of some Flavonoids Present in the Fruit Peels of *Citrus reticulata* Grown in Iraq.

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Key words: *Citrus reticulata*, Tangeretin, Nobiletin, Hesperidin, Quercetin.

(Received : April 2012, Accepted: June 2012)

Abstract

*Citrus reticulata* (Tangerine) of the family Rutaceae is widely growing in Iraq. Literature survey reveals that there was no phytochemical study concerning *C. reticulata* fruit peels in Iraq. Flavonoids from citrus genus have been of particular interest because of their broad spectrum of biological activities, including anti-inflammatory, anticarcinogenic, and antiatherogenic properties. Therefore, the isolation and characterization of flavonoids from *C. reticulata* fruit peels will lead to new applications of the byproducts from citrus juice processes and other citrus consumption in nutraceutical and pharmaceutical products. Literature survey also reveals that *C. reticulata* fruit peels were widely used by the ancients for treatment of different kinds of diseases; therefore a research on Iraqi *C. reticulata* fruit peels will be of important value. This study is concerned with the extraction, identification, isolation and purification of some biologically important flavonoids in Iraqi *C. reticulata* fruit peels including: Tangeretin, Nobiletin, Hesperidin & Quercetin. Extraction was carried out by two methods including Soxhlet apparatus & Maceration. Two flavonoids which are Tangeretin & Nobiletin were isolated, purified & quantitatively estimated while Hesperidin & Quercetin were identified by thin layer chromatography (TLC) & this identification was further augmented by using High performance liquid chromatography (HPLC). The identification of the isolated compounds (Tangeretin & Nobiletin) was done by measuring the melting point (M.P.), TLC where different solvent systems had been used, HPLC & infrared spectroscopy (IR). The most suitable extraction & identification methods were fully described in this study. This study confirms the presence of Tangeretin, Nobiletin, Hesperidin & Quercetin in the fruit peels of *C. reticulata* grown in Iraq, these compounds have important medicinal & therapeutic values that are mentioned in this study. The percentages of Tangeretin & Nobiletin were higher in the extract obtained by soxhlet apparatus than that obtained by maceration.


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*Citrus reticulata* (Tangerine) of the family Rutaceae is widely growing in Iraq. Literature survey reveals that there was no phytochemical study concerning *C. reticulata* fruit peels in Iraq. Flavonoids from citrus genus have been of particular interest because of their broad spectrum of biological activities, including anti-inflammatory, anticarcinogenic, and antiatherogenic properties. Therefore, the isolation and characterization of flavonoids from *C. reticulata* fruit peels will lead to new applications of the byproducts from citrus juice processes and other citrus consumption in nutraceutical and pharmaceutical products. Literature survey also reveals that *C. reticulata* fruit peels were widely used by the ancients for treatment of different kinds of diseases; therefore a research on Iraqi *C. reticulata* fruit peels will be of important value. This study is concerned with the extraction, identification, isolation and purification of some biologically important flavonoids in Iraqi *C. reticulata* fruit peels including: Tangeretin, Nobiletin, Hesperidin & Quercetin. Extraction was carried out by two methods including Soxhlet apparatus & Maceration. Two flavonoids which are Tangeretin & Nobiletin were isolated, purified & quantitatively estimated while Hesperidin & Quercetin were identified by thin layer chromatography (TLC) & this identification was further augmented by using High performance liquid chromatography (HPLC). The identification of the isolated compounds (Tangeretin & Nobiletin) was done by measuring the melting point (M.P.), TLC where different solvent systems had been used, HPLC & infrared spectroscopy (IR). The most suitable extraction & identification methods were fully described in this study. This study confirms the presence of Tangeretin, Nobiletin, Hesperidin & Quercetin in the fruit peels of *C. reticulata* grown in Iraq, these compounds have important medicinal & therapeutic values that are mentioned in this study. The percentages of Tangeretin & Nobiletin were higher in the extract obtained by soxhlet apparatus than that obtained by maceration.

**Key words:** *Citrus reticulata*, Tangeretin, Nobiletin, Hesperidin, Quercetin.
Introduction:

Tangerine peel is the outermost layer of *Tangerine*, a popular fruit widely known also as a mandarin and the general botanical name for *Tangerine* is *Citrus reticulata*\(^1,2\). It’s dried and mature peel has been recorded in the Chinese Pharmacopeia as appropriate for medical use\(^3,4\). It is now known that tangerine peel contains far more flavonoids and anti-oxidants that are beneficial for good health than fruit juices do. Two major *Tangerine* peel benefits are its cholesterol lowering capabilities and more recently its use in fighting cancer. Now pharmacological research has indicated that Tangerine peel exhibits significant antimutagenic\(^5\), anti-inflammatory\(^6,7\), antioxidant\(^8,9\), antitumor\(^10,11,12,13,14,15\), antiatherosclerosis\(^16,17\), and antibacterial properties\(^18\). The disease fighting flavonoids *Tangeretin* and *Nobiletin* are found in higher concentrations in peels than in the juice we commonly drink. Other benefits of tangerine peel are a lower risk of heart disease and obesity. The flavonoids found in tangerine peels are even thought by some to effectively lower cholesterol as well as some prescription drugs\(^19,20\). *Tangeretin* and *Nobiletin* are easily absorbed into the body and metabolized. *Tangerine* peels promote weight loss, and are one of the most effective ways to fight obesity. It has also been shown to stimulate the lymph system which helps eliminate excess fluid. *C. reticulata* fruitpeel extract possess activities against human gastric cancer cell line and the inhibitory activities of tangerine peels were largely found to be higher than those of the corresponding juice parts. Peels can benefit people suffered from cardiovascular and neural diseases such as Parkinson's disease\(^21,22\).

This study confirms the presence of *Tangeretin*, *Nobiletin*, *Hesperidin* & *Quercetin* flavonoids in the fruit peels of *C. reticulata*.

*Nobiletin* seems to be a very noble phytochemical with many potential health benefits including:

**Anti-inflammatory:** *Nobiletin* acts directly as an antioxidant but also interferes with biological inflammatory processes. It inhibits the expression of genes involved in inflammation by blocking the binding of NF-kappaB with DNA.

**Anti-cancer:** *Nobiletin* acts by its antiproliferative effect without being toxic to normal cells. Favorable results have been obtained on cancer cell lines of the liver, stomach, prostate and colon\(^14\).

**Cholesterol lowering:** *Nobiletin* inhibits the formation of macrophage foam cells, macrophages loaded with lipids, which build up on artery walls.
**Acne treatment:** Nobiletin inhibits sebum production and inhibit the proliferation of sebocytes, the cells that form the sebaceous gland.(23).

![Tangeretin](image)

Tangeretin is readily absorbed in tissues and has many beneficial properties including:

**Anti-tumor:** It induces apoptosis in leukemia cells without being toxic to normal cells. Tangeretin stops the growth of cancer cell in the G1 phase.

**Neuroprotection:** Tangeretin increases the levels of dopamine and has potential neuroprotective activity. It also has Cholesterol lowering properties.

![Quercetin](image)

Quercetin has many beneficial health effects including improvement of cardiovascular health, reducing risk for cancer, protection against osteoporosis. This phytochemical has anti-inflammatory, anti-allergic and antitoxic effects. Most of these properties are linked to its strong antioxidant action but Quercetin also modulates the expression of specific enzymes. Quercetin induces apoptosis and influences protein and lipid kinase signaling pathways. Quercetin is a candidate for preventing obesity-related diseases(24).

![Hesperidin](image)

Hesperidin has antioxidant, anti-inflammatory, hypolipidemic, vasoprotective, anticarcinogenic and cholesterol lowering actions. Hesperidin can inhibit the following enzymes: phospholipase A2, lipoxygenase, HMG-CoA reductase and cyclo-oxygenase. Hesperidin improves the health of capillaries by reducing the capillary permeability. Hesperidin is used to reduce hay fever and other allergic conditions by inhibiting the release of histamine from mast cells. The possible anti-cancer activity of Hesperidin could be explained by the inhibition of polyamine synthesis(25, 26).
Materials and methods:

Plant material:
The ripe fruits of Iraqi *C. reticulata* (Tangerine) were collected in November & December (2010) from Ba‘quba in Iraq & the fruits were peeled & the peels were air dried in the shade for two weeks, then the peels were pulverized by mechanical mills & weighed. The plant (Tangerine) was identified in the department of pharmacognosy /College of pharmacy/ University of Baghdad and was authenticated by National Iraqi Herbarium, Botany Directorate at Abu-Ghraib.

Extraction method No. 1:
A 100 gm of dried powdered fruit peels of Iraqi *C. reticulata* were packed in a thimble & placed in a soxhlet extractor & a 500 ml of 80% methanol was used as a solvent & placed in a 1 liter round bottom flask fitted with a soxhlet extractor. The extraction was continued for 12 hr.s. The extract was filtered & concentrated under reduced pressure to dryness using rotary evaporator at a temperature not exceeding 40°C & the dry extract was weighed & subjected to identification & purification procedures.

Extraction method No. 2:
A 500 gm of dried powdered fruit peels of Iraqi *C. reticulata* (Tangerine) were macerated with a 1000 ml of 75% methanol in a 2000 ml flask & left for 5 days then the extract was filtered & the residual plant material was macerated again with another 1000 ml of 75% methanol in a 2000 ml flask & left for another 5 days then the extract was filtered & the methanol fractions were collected together & evaporated to dryness under reduced pressure using rotary evaporator at a temperature not exceeding 40°C, then the dry extract was weighed & subjected to identification procedures.

Identification of plant constituents by TLC:
The final products of extraction method were examined by TLC, using ready made plates of silica gel GF254 (20×20cm) of 0.25mm thickness (MERCK). Detection was done by using UV light at 254 nm & 366 nm. The following flavonoids standards were used:

- **Tangeretin** (Biopurity Phytochemicals),
- **Nobiletin** (Biopurity Phytochemicals),
- **Quercetin** (FLUKA. Austria)
- **Hesperidin** (FLUKA. Austria).

Developing solvent systems:
A 100 ml volume of solvent system was placed in a glass tank (22.5gm×22cm×7cm) and covered with a glass lid and allowed to stand for 45 minutes before use. Different solvent systems were used for the detection of flavonoids:
- S1= n-Butanol:n-Hexane (15:85).
- S2= Ethyl acetate: n- Hexane (40:60).
- S4= Toluene: Glacial acetic acid (4:1).
- S6= n-Butanol: Glacial acetic acid: Water (4:1:5)

Isolation of the active constituent:
Isolation of the active constituents (*Tangeretin* & *Nobiletin*) was done by Preparative Layer Chromatography (PLC) using ready made plates of silica gel GF254 (20×20cm) of 1mm thickness (MERCK). The final product obtained from the extraction method no. 1 was applied as a
concentrated solution in a row of spots using capillary tube. **Tangeretin & Nobiletin** standards were applied at the right side of the baseline. The application of the extract was repeated four times in each plate, one should wait after each application until all the solvent is evaporated. The mobile phase used was $S_1=n$-Butanol: $n$-Hexane (15:85). The detection was done by using UV light at a wavelength of 254 nm. The bands corresponding to the **Tangeretin & Nobiletin** standards were scrapped out and collected in a beaker and eluted with gentle heating and filtered, then the filtrate was evaporated to dryness under vacuum to give white precipitate. Recrystallization was done to get pure compounds.

**Figure (1):** Preparative layer Chromatography of **Tangeretin & Nobiletin** using $S_1 = n$-Butanol: $n$-Hexane (15:85).

**Qualitative and quantitative estimation of plant constituents by HPLC method of analysis:**

High Performance Liquid Chromatography (HPLC) method was used for qualitative and quantitative estimation of **Tangeretin & Nobiletin** and **Hesperidin & Quercetin**. HPLC analysis was carried out in the department of pharmacology, College of pharmacy, Baghdad University. Using (Knauer / Germany). Identifications were made by comparison of retention times obtained at identical chromatographic conditions of analyzed samples and authentic standards. The HPLC conditions are listed in **Table (1)**.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mobile phase</th>
<th>Column</th>
<th>Flow rate</th>
<th>Detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tangeretin</td>
<td>Acetonitrile: Water (45:55)</td>
<td>C18 150mm × 4.6mm/5um</td>
<td>1ml / min</td>
<td>UV. Detector at $\lambda$ 326 nm</td>
</tr>
<tr>
<td>Nobiletin</td>
<td>Acetonitrile: Water (45:55)</td>
<td>C18 150mm × 4.6mm/5um</td>
<td>1ml / min</td>
<td>UV. Detector at $\lambda$ 326 nm</td>
</tr>
<tr>
<td>Quercetin</td>
<td>Acetonitrile: Methanol: Glacial acetic acid (70:30:0.1)</td>
<td>C18 5mm ×150mm</td>
<td>0.5 ml / min</td>
<td>UV. Detector at $\lambda$ 306 nm</td>
</tr>
<tr>
<td>Hesperidin</td>
<td>Methanol : Water (60:40)</td>
<td>C18 5mm ×150mm</td>
<td>1.5 ml / min</td>
<td>UV. Detector at $\lambda$ 345 nm</td>
</tr>
</tbody>
</table>
Results and discussion:
Two extraction methods were tried, method no.1 was the best because the amount of the extract & the isolated compounds were higher than in method no.2 as shown in Table (2).

Table (2): Percentages of extract & isolated compounds (Tangeretin & Nobiletin) in the fruit peels of Citrus reticulata.

<table>
<thead>
<tr>
<th>Method</th>
<th>Percentage of extract</th>
<th>Percentage of Tangeretin</th>
<th>Percentage of Nobiletin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Method no.1</td>
<td>11.5</td>
<td>0.65</td>
<td>1.8</td>
</tr>
<tr>
<td>Method no.2</td>
<td>7</td>
<td>0.43</td>
<td>1.1</td>
</tr>
</tbody>
</table>

Selection of the best developing solvent systems:
The developing solvent systems S1 for (Tangeretin & Nobiletin) & S6 for (Hesperidin & Quercetin) were found to be the best & more efficient for both qualitative & quantitative analysis as shown in figures (2 & 6).

Identification of flavonoids:
TLC of the extracts confirms the presence of Tangeretin, Nobiletin, Hesperidin & Quercetin flavonoids in both extracts obtained by method no.1 (E1) & method no.2 (E2) using different solvent systems as in figures (from 2 to 8). The flavonoids appears as a single spots having the same color and Rf values as that of standards. This identification was further augmented by HPLC which also confirms the presence of these flavonoids as in figures (from 9 to 16).

Table (3): Rf values of flavonoids and their standards in different solvent systems.

<table>
<thead>
<tr>
<th>Solvent system</th>
<th>S1</th>
<th>S2</th>
<th>S3</th>
<th>S4</th>
<th>S5</th>
<th>S6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rf value of Tangeretin Standard (TS)</td>
<td>0.333</td>
<td>0.18</td>
<td>0.714</td>
<td>0.62</td>
<td>0.98</td>
<td>0.882</td>
</tr>
<tr>
<td>Rf value of Tangeretin from Extract (T)</td>
<td>0.333</td>
<td>0.18</td>
<td>0.715</td>
<td>0.62</td>
<td>0.98</td>
<td>0.882</td>
</tr>
<tr>
<td>Rf value of Nobiletin Standard (NS)</td>
<td>0.15</td>
<td>0.103</td>
<td>0.658</td>
<td>0.52</td>
<td>0.95</td>
<td>0.829</td>
</tr>
<tr>
<td>Rf value of Nobiletin from Extract (N)</td>
<td>0.15</td>
<td>0.103</td>
<td>0.658</td>
<td>0.52</td>
<td>0.95</td>
<td>0.828</td>
</tr>
<tr>
<td>Rf value of Hesperidin Standard (HS)</td>
<td>0.8</td>
<td>0.833</td>
<td>0.75</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rf value of Hesperidin from Extract (H)</td>
<td>0.8</td>
<td>0.833</td>
<td>0.75</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rf value of Quercetin Standard (QS)</td>
<td>0.108</td>
<td></td>
<td></td>
<td>0.93</td>
<td>0.606</td>
<td></td>
</tr>
<tr>
<td>Rf value of Quercetin from Extract (Q)</td>
<td>0.108</td>
<td></td>
<td></td>
<td>0.93</td>
<td>0.606</td>
<td></td>
</tr>
</tbody>
</table>
Figure (2): TLC using $S_1$ = n-Butanol: n- Hexane (15:85) as a developing solvent system, detection by UV-light at 254 nm.

Figure (3): TLC using $S_3$ = Toluene: Chloroform: Acetone (40:25:35): Acetone (40:25:35) as a developing solvent system, detection by UV-light at 366 nm.

Figure (4): TLC using $S_3$ = Toluene: Chloroform: Acetone (40:25:35): Acetone (40:25:35) as a developing solvent system, detection by UV-light at 254 nm.
**Figure (5):** TLC using S4 = Toluene

**Figure (6):** TLC using S6 = n-Butanol: Glacial acetic acid (4:1):acetic acid: Water (4:1:5) as a developing solvent system, detection by UV-light at 254 nm.

**Figure (7):** TLC using S2 = Ethyl acetate

**Figure (8):** TLC using S5 = Ethyl acetate: Formic acid: Glacial acetic acid: Water (100:11:11:27) as a developing solvent system, detection by UV-light at 366 nm.
Isolation & quantitative determination of Tangeretin & Nobiletin by preparative TLC:

It was found that Tangeretin & Nobiletin were present in both extracts obtained by method no.1 & method no.2 but in higher concentration in the extract obtained by method no.1 (E1) than that obtained by method no.2 (E2). The percentages of the isolated compounds were obtained by weighting these compounds as shown in Table (2).

Identification of the isolated compounds (Tangeretin & Nobiletin):

TLC:

Both isolated compounds appeared as a single spots having the same color & Rf values as that of reference standards in six different developing solvent systems.

Measuring melting points:

The isolated compounds were identified to be Tangeretin & Nobiletin from their sharp melting points. Since one of these compounds showed a melting point of (50~52°C) compared to Tangeretin standard melting point (50~51°C ), the other compound showed a melting point of (137~138°C) compared to Nobiletin standard melting point (137~138°C ).

HPLC:

Identification of plant materials by HPLC confirms the presence of Tangeretin, Nobiletin, Hesperidin & Quercetin flavonoids in both extracts obtained by method no.1 (E1) & method no.2 (E2) as shown in figures (from 9 to 16 ). Qualitative identifications were made by comparism of retention times obtained at identical chromatographic conditions of analyzed samples and authentic standards. The amounts of Tangeretin & Nobiletin were found to be higher in the extract obtained by method no.1 (E1) than that obtained by method no.2 (E2). The percentages of Tangeretin & Nobiletin in the fruit peels extracts of Iraqi C. reticulata (Tangerine) were summarized in Table (4).

Table (4): Percentages of Tangeretin & Nobiletin by HPLC

<table>
<thead>
<tr>
<th>Extract</th>
<th>Percentage of Tangeretin</th>
<th>Percentage of Nobiletin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extract obtained by method no.1 (E1)</td>
<td>0.65</td>
<td>1.8</td>
</tr>
<tr>
<td>Extract obtained by method no.2 (E2)</td>
<td>0.43</td>
<td>1.1</td>
</tr>
</tbody>
</table>
Figure (9): HPLC analysis of Nobiletin standard

Figure (10): HPLC analysis of Tangeretin standard

Figure (11): HPLC analysis of Nobiletin & Tangeretin sample (from E1)
Figure (12): HPLC analysis of Nobiletin& Tangeretin sample (from E2)

Figure (13): HPLC analysis of Hesperidin standard

Figure (14): HPLC analysis of Hesperidin sample (from E1)
**Figure (15):** HPLC analysis of Quercetin standard

**Figure (16):** HPLC analysis of Quercetin sample (from E1)

**IR:**

The identification of the isolated compounds (Tangeretin & Nobiletin) was further confirmed by using infrared-spectroscopy (IR). The IR spectra of the isolated compounds (Tangeretin & Nobiletin) were identical with those of the authentic standards which confirm that our isolated compounds were Tangeretin & Nobiletin as in figures (17, 18, 19 & 20).
Figure (17): IR spectrum of Nobiletin standard

Figure (18): IR spectrum of isolated Nobiletin (sample)
Figure (19): IR spectrum of Tangeretin standard

Figure (20): IR spectrum of isolated Tangeretin (sample)
Conclusion:

Soxhlet apparatus is more efficient method for the extraction of flavonoids from the fruit peels of *C. reticulata* growing in Iraq than maceration. Tangeretin, Nobiletin, Hesperidin&Quercetin were found in the fruit peels of Iraqi *C. reticulata*. Tangeretin&Nobiletin were isolated & purified, these flavonoids have important medicinal & therapeutic activities including antiinflammatory, anticarcinogenic,antioxidant and antiatherogenic properties so can be used for treatment of different kinds of diseases.

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