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

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

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Abstract

Citrus reticulata (**Tangerine**) of the family***Rutaceae***is widely growing in Iraq, Literature survey reveal that there was no phytochemical study concerning ***C.reticulata***fruitpeelsin 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 reveal that ***C.reticulata*** fruitpeels were widely used by the ancients for treatment of different kinds of diseases; therefore a research on Iraqi ***C.reticulata***fruitpeels will be of important value. This study is concerned with the extraction, identification, isolation& purification of some biologically important flavonoids in Iraqi ***C.reticulata***fruitpeels 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 **o**f 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** inthe fruit peels of ***C.reticulata*** grownin Iraq, these compounds have important medicinal & therapeutic values that are mentioned in this study. The percentages of **Tangeretin &Nobiletin** werehigher in the extract obtained by soxhlet apparatus than that obtained by maceration.

دراسة كيميائية لبعض الفلافينويداتالموجودة في قشور ثمار اليوسفي الذي ينمو في العراق.

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مفتاح البحث: نبات اليوسفي، التانجرتين، النوبلتين، الكورستين، الهسبريدين

الخلاصة

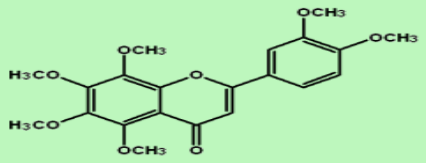
ينمو نبات اليوسفي (*Citrus reticulata*) في العراق ونظرا لعدم وجود دراسات حول قشورثمارااليوسفي في العراق ولاحتواء هذه القشور على العديد من المركبات ذات التاثيرات الطبية والعلاجية المهمة ومنها التاثيرات المضادة للالتهاب والمضادة للسرطان ولهذا فان من المهم دراسة هذه القشور وكشف وفصل مكوناتها الفعالة وما يترتب على ذلك من تطبيقات جديدة لقشور ثمارااليوسفي الناتجة من صناعة العصائر والصناعات الغذائية الاخرى. بينت الدراسات ان قشور ثمارااليوسفي قد استخدمت في الماضي لعلاج بعض الامراض وهذايزيد من اهمية هذه الدراسة. تتضمن هذه الدراسة استخلاص وكشف وفصل وتنقيةبعض الفلافينويدات ذات التاثيرات الطبية والعلاجية المهمة ومنها مادة التانجرتين (Tangeretin) والنوبلتين (Nobiletin)والكورستين (Quercetin) والهسبريدين (Hesperidin). تمت عملية الاستخلاص بطريقتين وهما عملية التنقيع (maceration) باستخدام مذيب الميثانول (75% methanol) والاستخلاص بجهاز السوكسليت (soxhlet apparatus) ومذيب الميثانول (80% methanol).تم فصل وتنقية مادتي التانجرتين والنوبلتين من قشورثمارااليوسفي العراقي واثبتت هذه الدراسة وجود مادتي الكورستين والهسبريدين في قشورثمارااليوسفي العراقي.تم الكشف عن هذه المركبات باستخدام تقنية

كروماتوغرافيا الطبقة الرقيقة وكذلك باستخدام تقنية كروماتوغرافيا الاداء العالي السائلة. تم تحديد الطرقالافضل للاستخلاص والكشف في هذه الدراسة كما استخدمت مجموعة تقنيات للتحقق من نوعية المركبات المفصولة ودرجة نقاوتها والتي شملت: درجة الانصهار للمركب المفصول , مطياف الاشعة تحت الحمراء , كروماتوغرافيا الطبقة الرقيقة وكروماتوغرافيا الاداء العالي السائلة. وتم التحقق من صحة و نقاوة المركبات المفصولة(التانجرتين والنوبلتين) باستخدام التقنيات المذكورة اعلاه والتي تطابقت نتائجها مع نتائج المركبات القياسية. النتائج التي تم الحصول عليها باستخدام تقنية كروماتوغرافيا الاداء العالي السائلة كانت متطابقة مع النتائج التي تم الحصول عليها باستخدام تقنية كروماتوغرافيا الطبقة الرقيقة. اثبتت هذه الدراسة ان كمية المواد الفعالة كانت اكثر في المستخلص بطريقة السوكسليت منها بطريقة التنقيع.

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 andmature peel has been recorded in the Chinese Pharmacopoeia 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]andantibacterialproperties [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** flavonoidsinthe fruit peels ofIraqi ***C.reticulata*** .



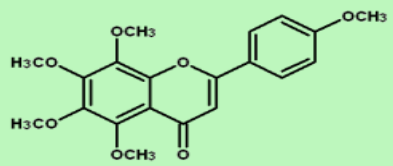
Nobiletin

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].

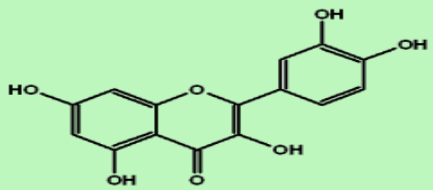
Cholesterol lowering: Nobiletin inhibits the formation of macrophage foam cells, macrophages loaded with lipids, which build up on artery walls.

Acne treatment: Nobiletin inhibits sebum production and inhibit the proliferation of sebocytes, the cells that form the sebaceous gland [23].



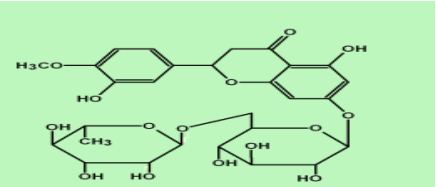
Tangeretin

Tangeretinis 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 neuroprotectiveactivity. It also has Cholesterol lowering properties.



Quercetin

Quercetin has many beneficial health effectsincluding 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

Hesperidin has antioxidant, anti-inflammatory, hypolipidemic, vasoprotective, anticarcinogenic and cholesterol lowering actions. Hesperedin can inhibit the following enzymes: phospholipase A2, lipoxygenase, HMG-CoA reductase and cyclo-oxygenase. Hesperedin improves the health of capillaries by reducing the capillary permeability.Hesperedin 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 Hesperedin could be explainedby 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 andwasauthenticated 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 [(27)].

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 another1000 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 methodswere 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[(28,29)]:

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 thedetection of flavonoids:

S1= n-Butanol:n-Hexane (15:85).**S2**=Ethyl acetate: n- Hexane (40:60).
S3=Toluene: Chloroform: Acetone (40:25:35).**S4**= Toluene: Glacial acetic acid (4:1).**S5**=Ethyl acetate: Formic acid: Glacial acetic acid: Water (100:11:11:27).**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**)usingready 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** standardswere 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 **S1**=n-Butanol: n-Hexane (15:85). The detection was done by using UV light at a wave length of 254 nm. The bands corresponding to the **Tangeretin** &**Nobiletin** standardswere 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.

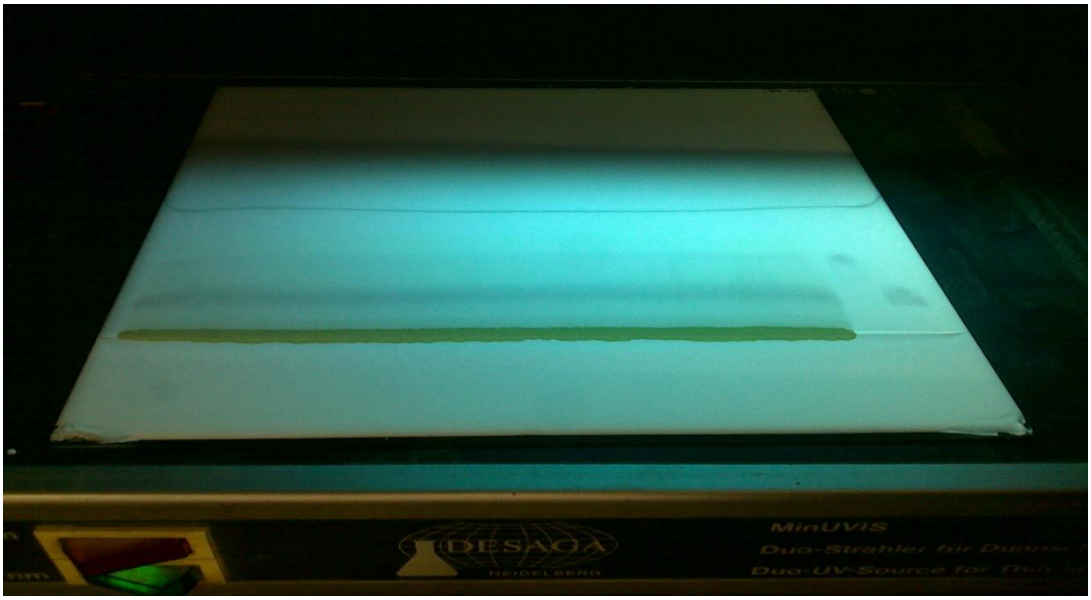


Figure (1): Preparative layer Chromatography of **Tangeretin** &**Nobiletin**using **S1** =n-Butanol: n-Hexane (15:85).

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

Table (1): HPLC conditions[29, 30]

Sample	Mobile phase	Column	Flow rate	Detection
Tangeretin	Acetonitrile: Water (45:55)	C18 150mm × 4.6mm/5um	1ml / min	UV. Detector at ƛ 326 nm
Nobiletin	Acetonitrile: Water (45:55)	C18 150mm × 4.6mm/5um	1ml / min	UV. Detector at ƛ 326 nm
Quercetin	Acetonitrile: Methanol: Glacial acetic acid(70:30:0.1)	C18 5mm ×150mm	0.5 ml / min	UV. Detector at ƛ 306 nm
Hesperidin	Methanol : Water (60:40)	C18 5mm ×150mm	1.5 ml/ min	UV. Detector at ƛ 345 nm

Results and discussion:

Two extraction methods were tried, method no.1was the best because the amount of the extract & the isolated compounds were higher than in method no.2as shown in**Table(2).**

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

Method	Percentage of extract	Percentage of **Tangeretin**	Percentage of **Nobiletin**
Method no.1	11.5	0.65	1.8
Method no. 2	7	0.43	1.1

Selection of the best developing solvent systems:

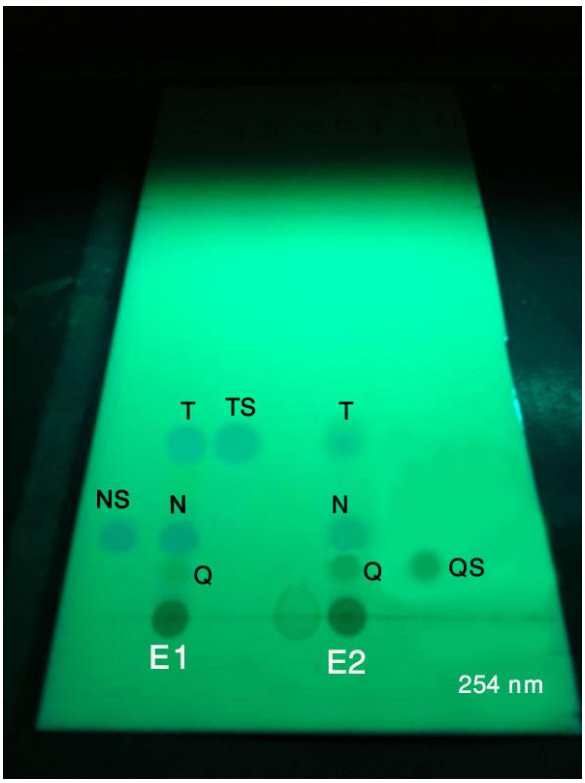
The developing solvent systems **S1**for (**Tangeretin** &**Nobiletin)**&**S6**for (**Hesperidin**&**Quercetin)** were found to be the best & more efficient for both qualitative & quantitative analysis as shown in figures (2&6).

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 appearas 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.

Solvent system	**S1**	**S2**	**S3**	**S4**	**S5**	**S6**
Rf valueof**Tangeretin** Standard (**TS**)	0.333	0.18	0.714	0.62	0.98	0.882
Rf valueof**Tangeretin**from Extract(**T**)	0.333	0.18	0.715	0.62	0.98	0.882
Rf valueof**Nobiletin** Standard (**NS**)	0.15	0.103	0.658	0.52	0.95	0.829
Rf valueof**Nobiletin**from Extract (**N**)	0.15	0.103	0.658	0.52	0.95	0.828
Rf valueof**Hesperidin** Standard (**HS**)				0.8	0.833	0.75
Rf valueof**Hesperidin**from Extract (**H**)				0.8	0.833	0.75
Rf valueof**Quercetin** Standard (**QS**)	0.108				0.93	0.606
Rf valueof**Quercetin**from Extract (**Q**)	0.108				0.93	0.606



TS =Tangeretin Standard

T = Tangeretin from Extract

NS = Nobiletin Standard

N = Nobiletin from Extract

HS = Hesperidin Standard

H = Hesperidin from Extract

QS = Quercetin Standard

Q = Quercetin from Extract

E1 = Extract obtained by soxhlet apparatus (method no.1)

E2 = Extract obtained by maceration (method no.2)

Figure(2): TLC using**S1**=n-Butanol: n- Hexane (15:85) as a developing solvent system, detection by UV-light at **254** nm.

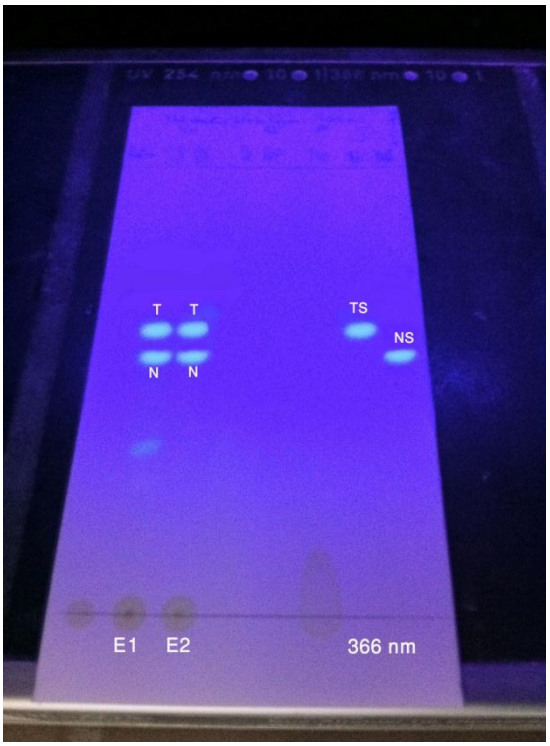


Figure (3):TLC using **S3**=Toluene: Chloroform : Acetone (40:25:35): Acetone (40:25:35) as a developing solvent system, detection by UV-light at **366** nm.

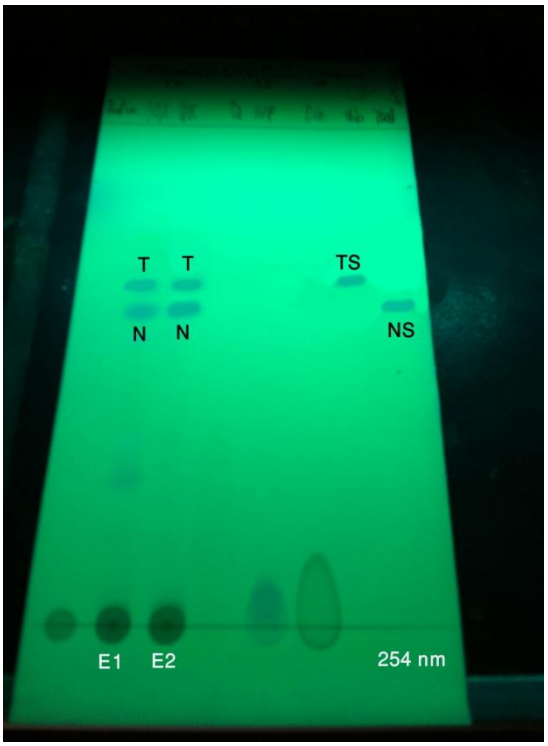


Figure (4):TLC using **S3**=Toluene: Chloroform as a developing solvent system, detection by UV-light at **254** nm.

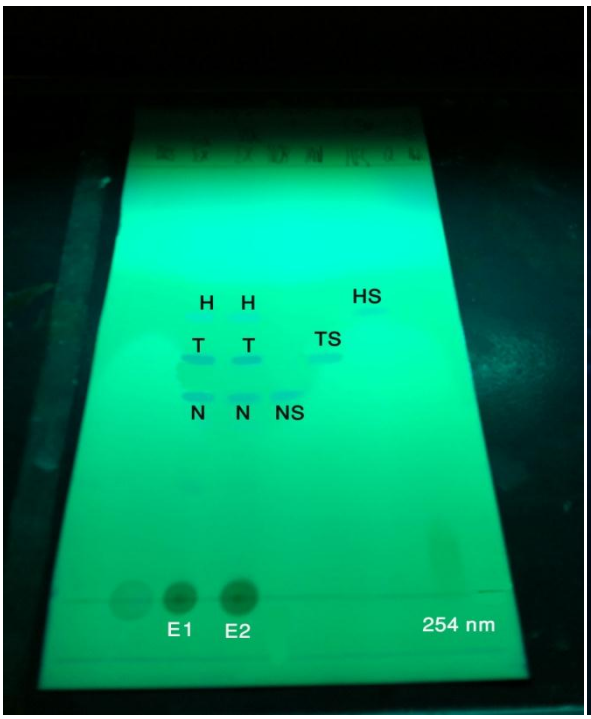
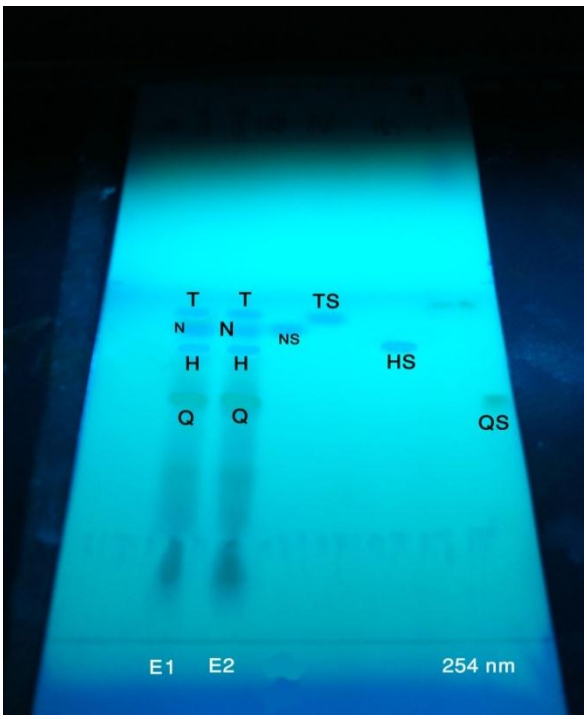


Figure (5):TLC using **S4**= Toluene: Glacial acetic acid (4:1)acetic acid: Water (4:1:5) as a developing solvent system,as a developing solvent system, detection by UV-light at **254** nm.



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

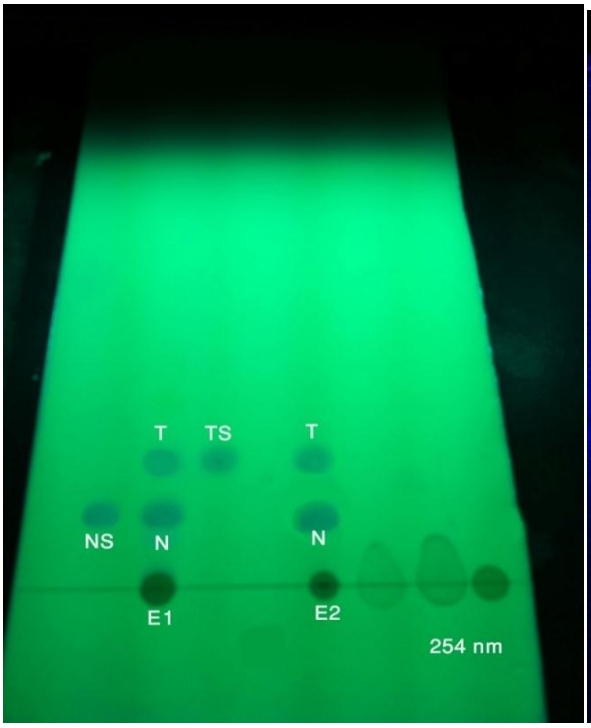


Figure (7): TLC using **S2**=Ethyl acetate: n- Hexane (40:60)as a developing solvent system, detection by UV-light at **254** nm.

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 extractobtainedby 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**fromtheir 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 inthe extractobtainedby method no.1 (**E1**) than that obtained by method no.2 (**E2**). The percentages of **Tangeretin** &**Nobiletin**in thefruit peels extracts of Iraqi ***C. reticulata***(**Tangerine**) were summarized in **Table(4)**.

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

Extract	Percentage of **Tangeretin**	Percentage of **Nobiletin**
Extractobtainedby method no.1(**E1**)	0.65	1.8
Extractobtainedby method no.2 (**E2**)	0.43	1.1

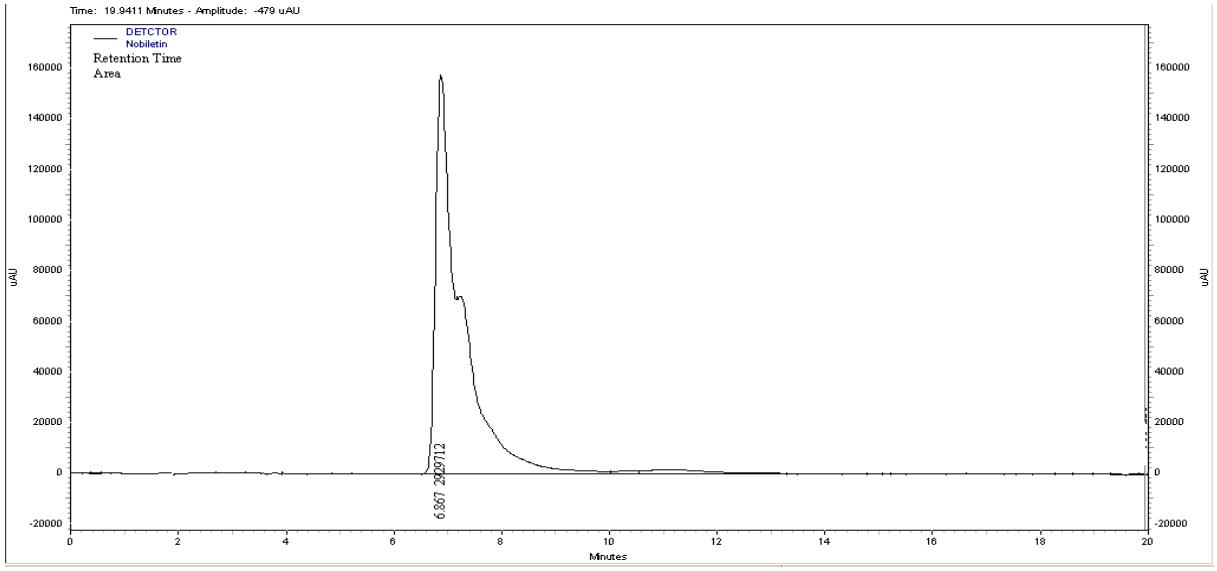


Figure (9): HPLC analysis of **Nobiletin** standard

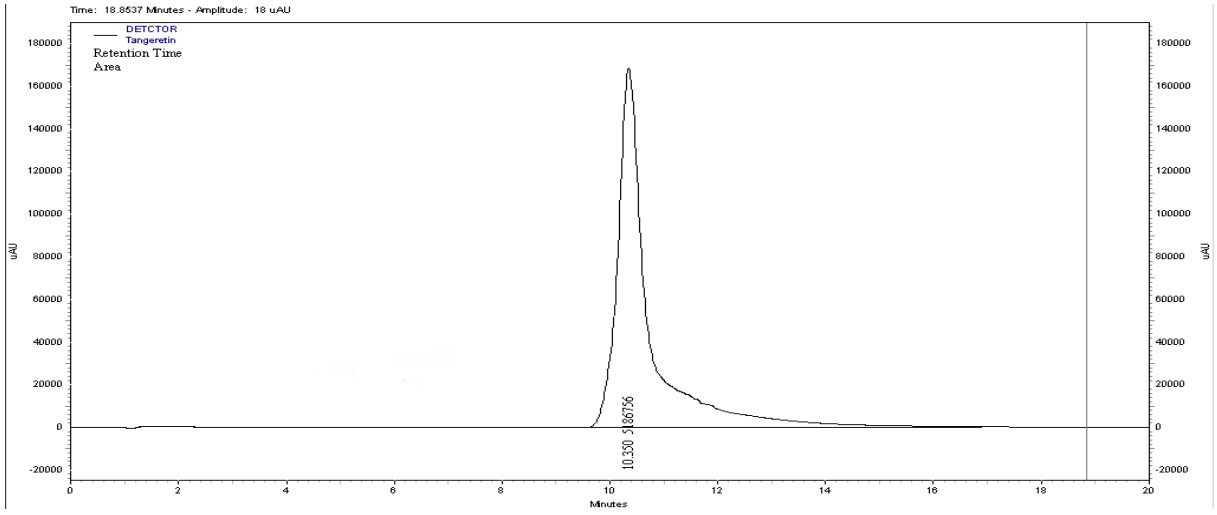


Figure (**10**): HPLC analysis of **Tangeretin** standard

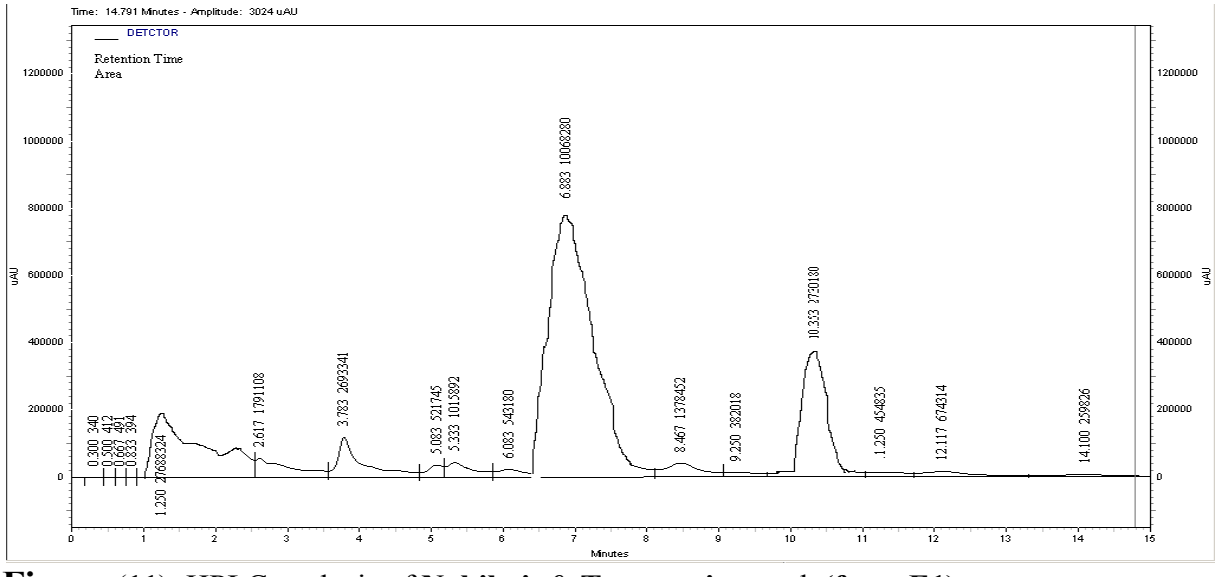


Figure (**11**): HPLC analysis of **Nobiletin**& **Tangeretin**sample**(from E1)**

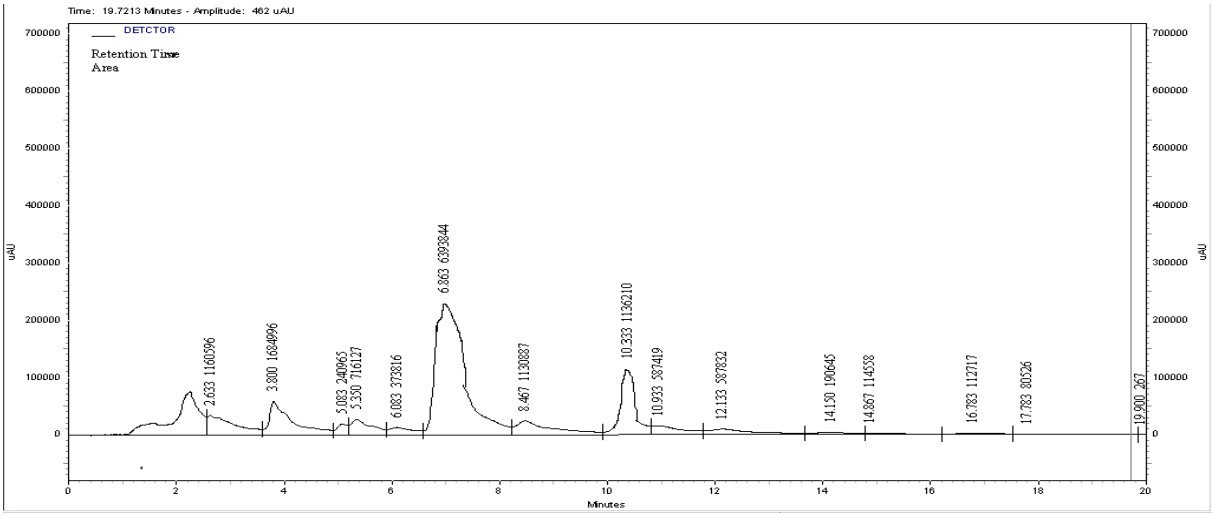


Figure (**12**): HPLC analysis of **Nobiletin**& **Tangeretin**sample**(**from **E2)**

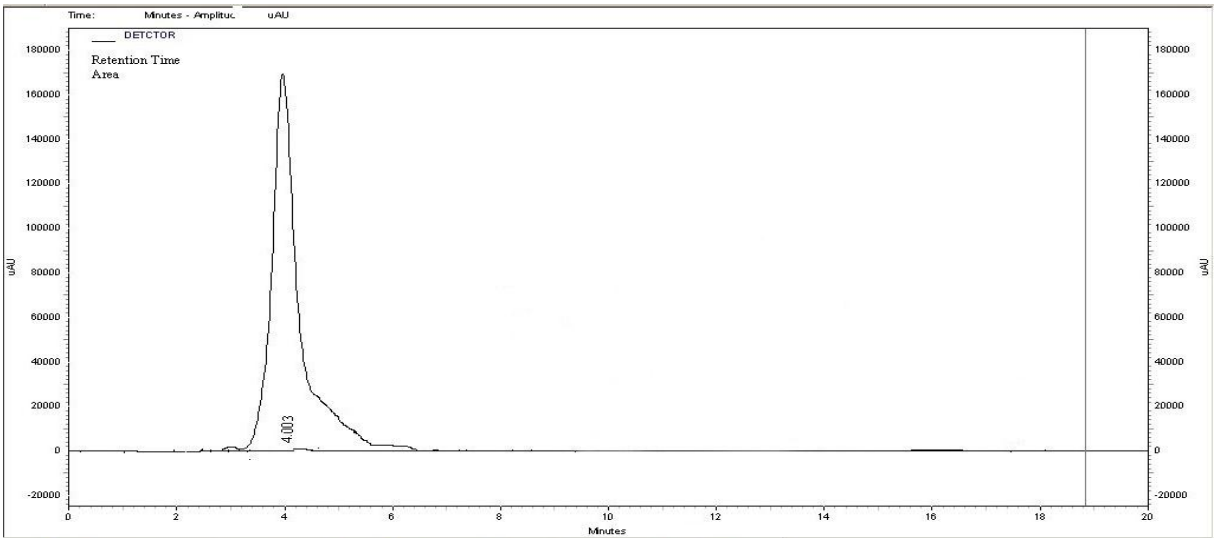


Figure (**13**): HPLC analysis of **Hesperidin** standard

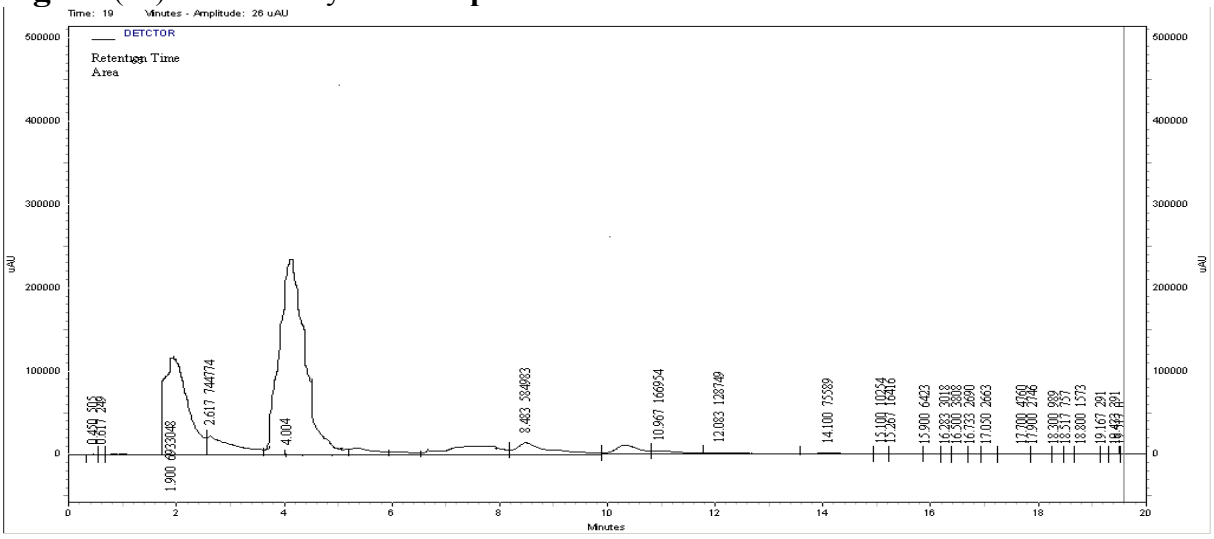


Figure (**14**): HPLC analysis of **Hesperidin**sample (from**E1)**

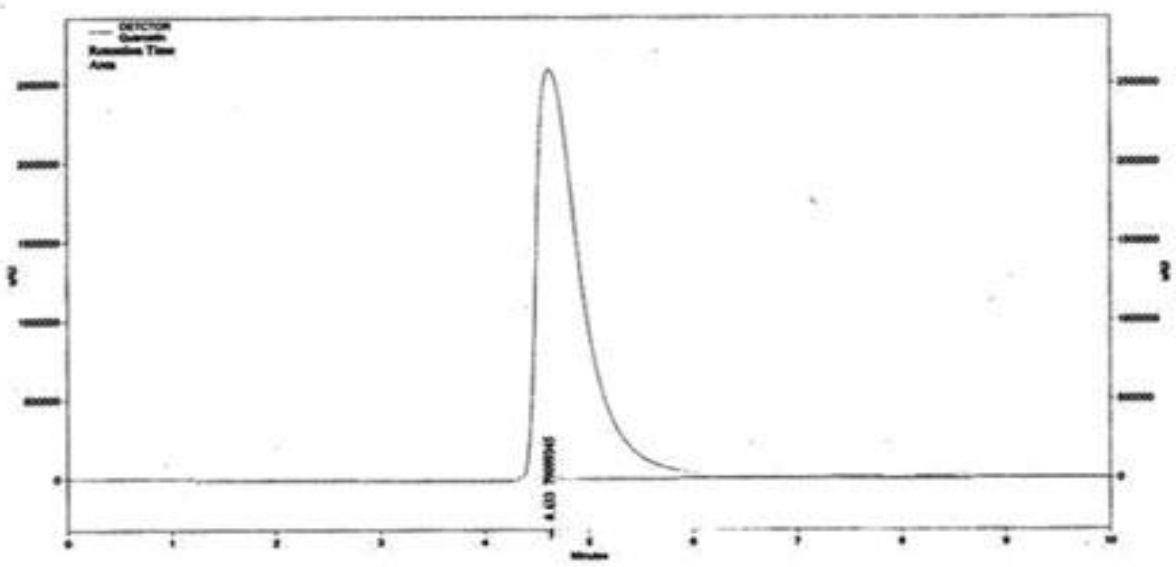


Figure (**15**): HPLC analysis of **Quercetin** standard

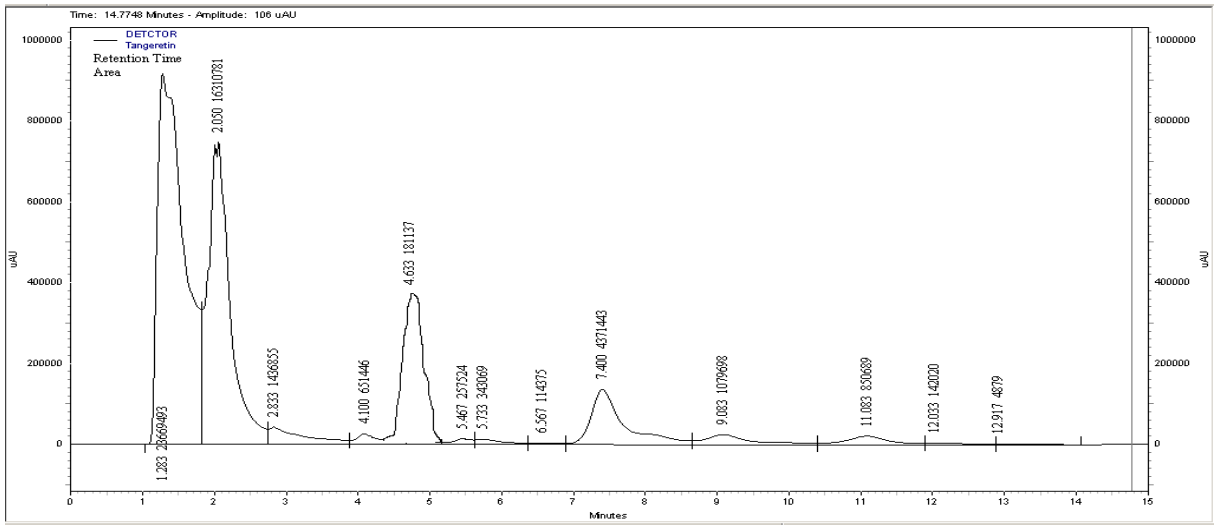


Figure (**16**): HPLC analysis of **Quercetin**sample**(**from **E1)**

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).

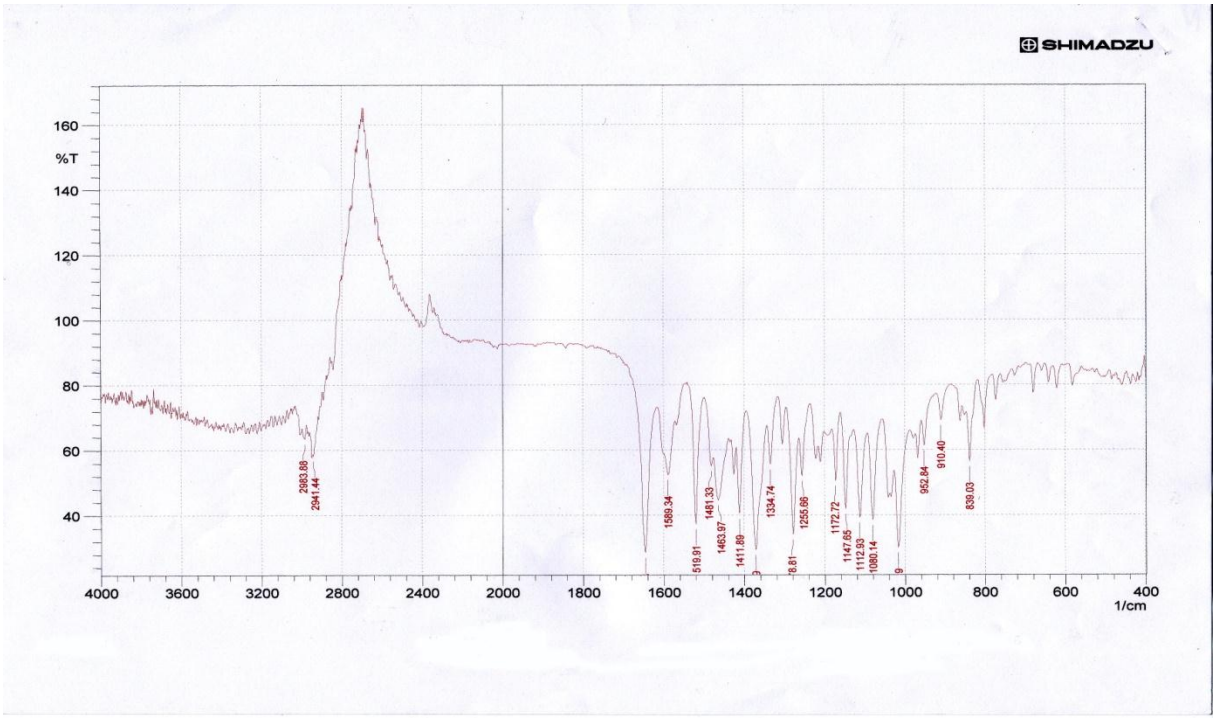


Figure (17): IR spectrum of Nobiletin standard

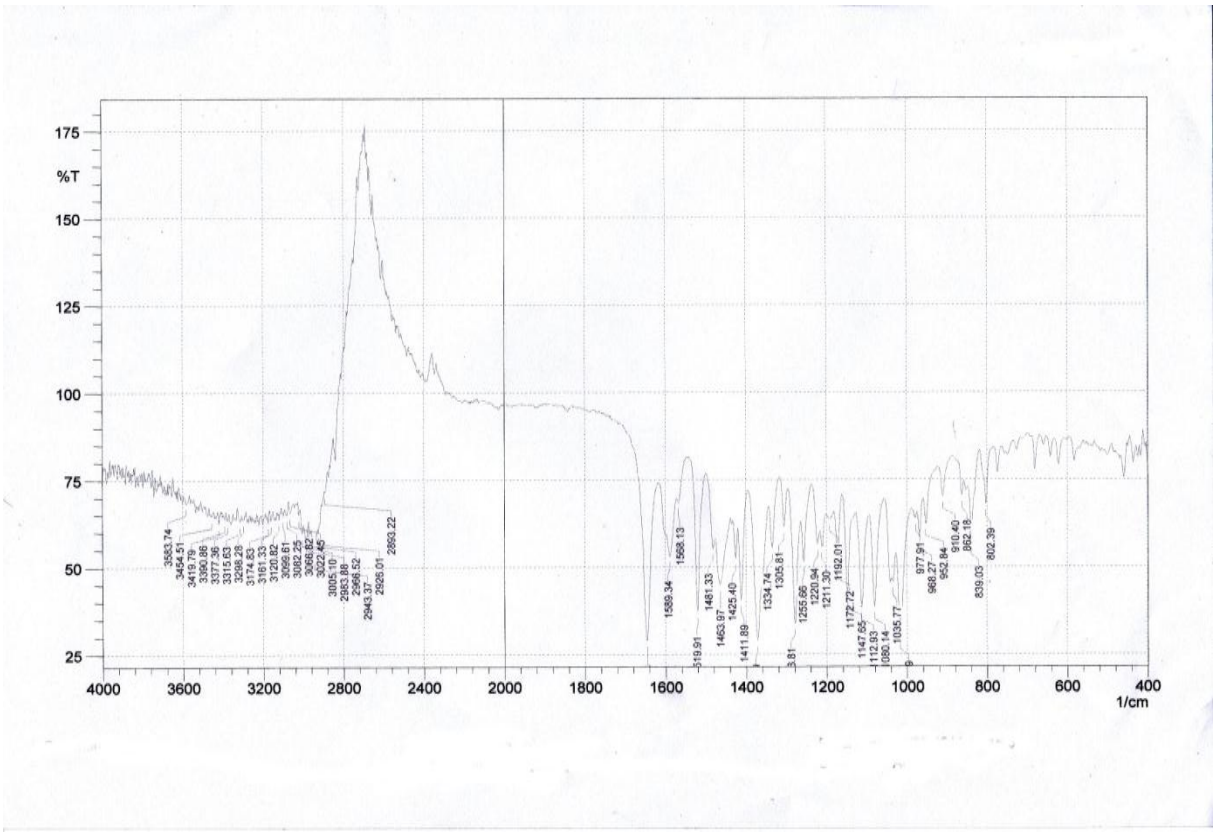


Figure (18): IR spectrum of isolated Nobiletin (sample)

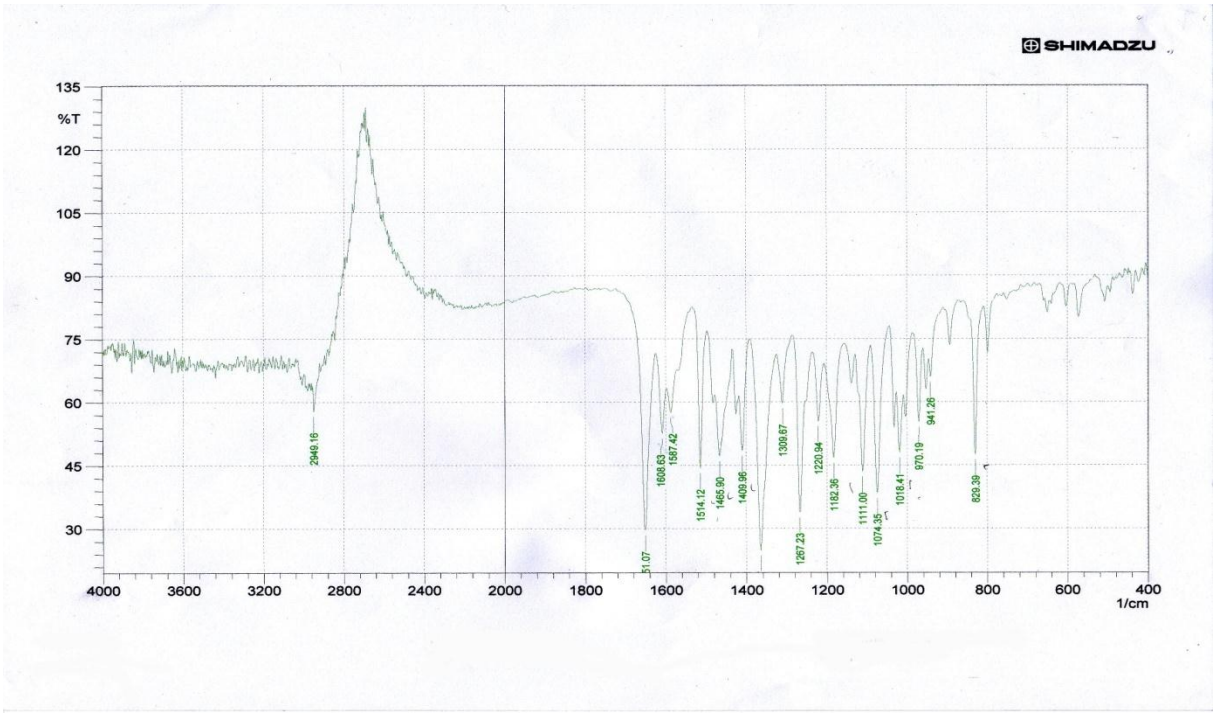


Figure (19): IR spectrum of Tangeretin standard

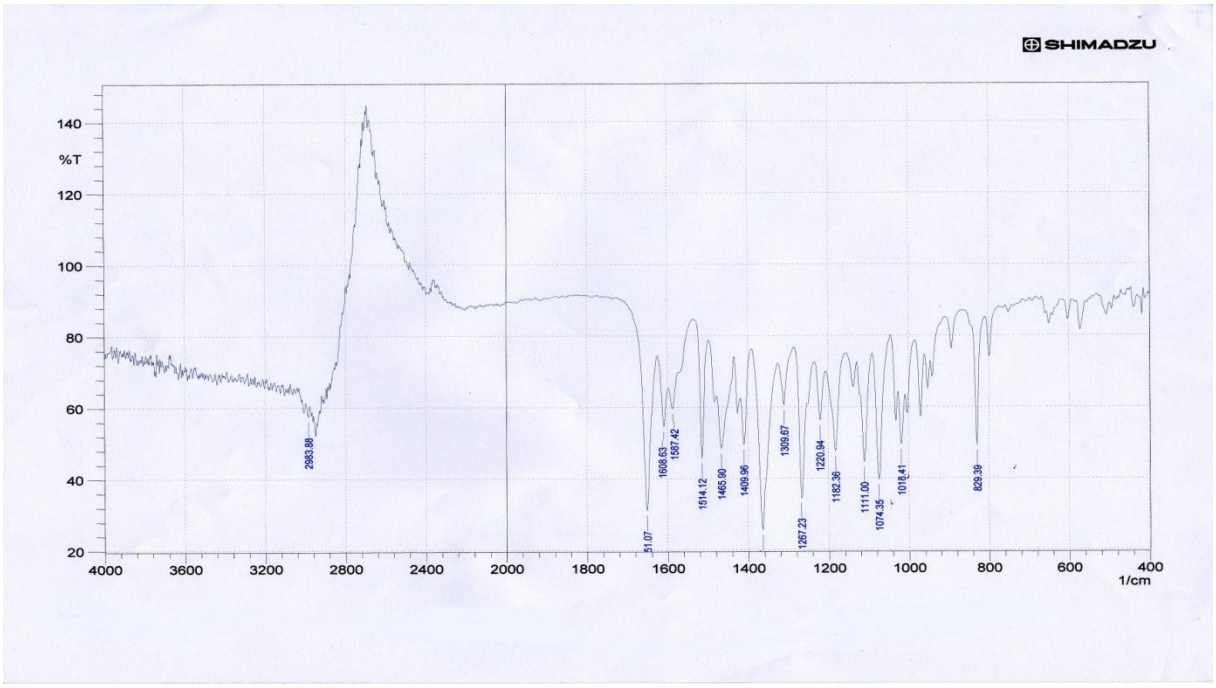


Figure (20): IR spectrum of isolated Tangeretin (sample)

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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