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Qualitative and Quantitative Investigation of Iraqi Grapefruit (*Citrus padisi*) Flavonoids From Peel and Seeds and Comparing Their Aqueous Extracts for Antimicrobial Activity

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Abstract

Iraqi grapefruit (*Citrus paradisi*, family Rutaceae) flavonoids were investigated qualitatively and quantitatively. The total isolated flavonoids from seeds and peel were 3.6 mg and 12.53 mg respectively in each gram of powder. The antimicrobial activity of aqueous extracts and total isolated flavonoids from seeds and peel were assessed against strains of Gram positive bacteria(S*taphylococcus aureus*, *Staphylococcus epidermidis*), Gram negative bacteria(*Escherichia coli*, *Pseudomonas aeruginosa*) and yeast(*Candida albicans*). The aqueous extracts lacked antimicrobial activity against all bacteria and yeast, while the total flavonoids showed a moderate inhibitory effect against test bacteria and yeast. This difference in inhibitory activity between aqueous extract and total flavonoids may be due to structurally-related aspects.

Key words: *Citrus paradisi*, Flavonoids, Gram positive bacteria, Gram negative bacteria, *Candida paradisi*.

التحري نوعياً وكمياً عن فلافينويدات الكريب فروت العراقي المستخلصة من القشور والبذور ومقارنة مستخلصهم المائي نسبة إلى النشاط المضاد للميكروبات

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الخلاصة

تم التحري نوعياً وكمياً عن الفلافينويدات في الكريب فروت العراقي. الكمية الكلية للفلافينويدات المعزولة من البذور والقشور كانت 3,6 ملغ و12,53 ملغ على التوالي لكل غرام واحد من المسحوق. الفعالية المضادة للميكروبات للمستخلص المائي والفلافينويدات المعزولة كلياً من البذور والقشور قد تم إختبارها على بكتريا إيجابية الغرام (المكورات العنقودية الذهبية, المكورات العنقودية البشروية), وعلى بكتريا سلبية الغرام (الإشريكية القولونية, الزائفة الزنجارية) وعلى الخمائر (المبيضات البيض) . يفتقد المستخلص المائي الفعالية المضادة للميكروبات ضد جميع البكتريا والخمائر , بينما الفلافينويدات الكلية أظهرت تأثير تثبيطي متوسط ضد بكتريا الاختبار والخمائر. هذا الاختلاف في التأثير التثبيطي بين المستخلص المائي والفلافينويدات الكلية قد يرجع إلى جوانب متعلقة بالتركيب الجزيئي.

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Introduction

Medicinal plants have been used as traditional treatments for numerous human diseases for thousands of years and in many parts of the world [1]. Citrus fruits are a group of plants of great medicinal importance [2]. The therapeutic efficacy of citrus fruits such as grapefruit (*Citrus paradisi*) is a member of Rutaceae family contain different classes of polyphenolic flavonoids that have been shown to exert antibacterial, antifungal and antioxidant activities [3-4]. The aim of the study is the qualitative and quantitative phytochemical investigation of the Iraqi grapefruit flavonoids from peel and seeds and compare their aqueous extracts for determination of their antimicrobial activity.

Materials and Methods

Preparation of *Citrus paradisi* seed and peel aqueous extracts:

The grapefruits were purchased from a farm in Diyala Provence, Iraq. The grapefruit seeds and peel were air dried for three weeks. Then the dried material were grinded using electrical grinder.

About four grams of each seed and peel powder were placed separately in 25 milliliters of distilled water and digested for 30 minutes. The solution was cooled and filtered by filter paper (Whatmans No.1)then by 0.22µm Millipore filter to produce a final concentration of 160 mg/ ml aqueous extract[5].

Phytochemical screening of *Citrus paradisi* seed and peel aqueous extracts:

Phytochemical investigation were carried out for the aqueous extracts [5-7]

1- Detection of alkaloids: Dragendroff"s test
2- Detection of glycosides: Benidect's test
3- Detection of flavonoids: Alkaline potassium hydroxide test
4- Detection of polyphenols and tannins: Ferric chloride test
5- Detection of saponins: Foam test

Determination of Total Flavonoids:

A. Quantitative Determination

A quantity of ten grams from both powdered seed and peel of grapefruit were reflected in 10% hydrochloric acid for 8 hours. The solution was cooled and filtered. The filtrate was transferred to a separatory funnel and the aglycon moiety was extracted with ethyl acetate three times. The collected lower organic layers were washed from the excess acid then evaporated to dryness to be re-dissolved in 50% ethanol and stored in cold place till further investigations. The stock solution final content was 10 mg/ ml.

Rutin standard stock solution was prepared at concentration of 10mg/ml in 50% ethanol, then serial dilutions were made to obtain different rutin standard solutions at concentrations represented by (0.2,0.5,1, 2.5 and 5) mg/ml in 50% ethanol. Aliquot of 1ml from each concentrations of standard solution and from the re-dissolved extracted residue(total flavonoid) were transferred into a glass tubes, then 0.75 ml of 5% sodium nitrite solution was added and mixed well and left to stand at room temperature for 5 minutes. To all tubes 1.5 ml of 10% $AlCl_3$ in 50% ethanol was added, shaken well and left to stand at room temperature for another 5 minutes. Finally 5ml of 1N NaOH solution was added to all tubes. The absorbance was read by spectrophotometer at 510 nm, and a standard curve was plotted between each concentration and the absorbance, then the amount of total flavonoid was calculated as rutin from the equation of straight line that obtained from the plotted curve[8].

B. Qualitative Determination of total flavonoid:

The qualitative determination of total flavonoids of *Citrus paradisi* seeds and peel achieved by using thin layer chromatography(TLC) in solvent systems composed of *n*-Hexane: Ethyl acetate: Glacial acetic acid (30:20:1.5).Thin layer chromatography was performed on silica gel Gf254 aluminum sheets by applying one spot of each standard solution for rutin, quercetin and luteolin prepared at concentration 0.1mg/ml and from the extracted total flavonoids, in a mobile phase that is used for developing the sample until reaching about one centimeter beneath the upper chromatogram margin[9].

Antimicrobial Activity of *Citrus paradisi* seed, peel and total flavonoids

Eighteen to 24 hours single colonies on agar plates were used to prepare the bacterial suspension with turbidity of 0.05 McFarland(equal to 1.5×10^8) cells/ml. Aqueous extracts were evaluated using well diffusion method on Muller- Hinton agar[10]. The reference bacteria and fungus used for antimicrobial assay of aqueous extracts were *Staphylococcus aureus*(ATCC:6538P), *Staphylococcus epidermidis*, *Escherichia coli*(ATCC:10536), *Pseudomonas aeruginosa*(ATCC:15442), *Candida albicans*(ATCC:10231) supplied by Research and Production for Drugs and Medical Supplies, Baghdad, Iraq. Positive controls were Clindamycin(2µg) for Gram positive bacteria, Gentamicin(10µg) for Gram negative bacteria, Clotrimazole(5mg/ml) and Nystatin(5000IU/ml) for fungus.

The Muller- Hinton agar plates were inoculated with bacterial stains and dextrose agar plates were swabbed with fungal culture under aseptic condition.. A 6 millimeter diameter wells were made using a sterile cork borer and 50µl of each extract(250mg/ml for seed and peel extracts and 10mg/ml for total isolated flavonoids of seed and peel extracts) was introduced into the well. The agar plates of bacterial and fungal strains were incubated at 37˚C for 24 hours and 28˚C for 48 hours respectively. After the incubation period, a caliper was used to measure the diameters of inhibition zones [11]. The experiment was repeated twice and the average values were recorded.

Results

Phytochemical Investigation:

Phytochemical screening test of grapefruit seeds and peel aqueous extracts revealed the following results as shown in Table -1.

Table 1- Phytochemical screening tests of grapefruit seed and peel aqueous extracts

Phytochemicals	**Seeds**	**Peel**
Alkaloids	Negative	Negative
Glycosides	Negative	Positive
Flavonoids	Trace amount	Positive
Polyphenols & Tannins	Negative	Positive
Saponins	Trace amount	Positive

Determination of Total Flavonoids Content:

The dried powdered *Citrus paradisi* seeds and peel (10g) yield a quantity of (1.5mg/g for seed extract and 1.4 mg/g for peel extract) residue which involved the aglycone part of the total flavonoid. The residue was dissolved in 30 ml of 50%v/v ethanol for following the investigations.

A. Quantitative Determination:

The spectrophotometric analysis of dried powdered *Citrus paradisi* seeds and peel total flavonoids with different standard concentrations and the plotted curve shown in Figure- 1 and Table -2 .

Table 2- Spectrophotometer analysis for standard rutin solutions and the extracted total flavonoids from peel and seeds of *Citrus paradisi*.

Rutin concentration (mg\ml)	Absorption (510nm)
0.2	0.12
0.5	0.478
1	0.65
2.5	1.645
5	2.16
The extracted *Citrus paradisi* peel total flavonoids(double diluted)	1.0147
The extracted *Citrus paradisi* seed total flavonoids(double diluted)	0.577

From the straight line equation the total flavonoids quantity was calculated. Each gram of dried powdered *Citrus paradisi* peel contains 12.53 mg of total flavonoids calculated as rutin and each gram of powdered *Citrus paradisi* seed contains 3.6 mg of total flavonoids calculated as rutin.

B. Qualitative Determination:

Chromatographic analysis for flavonoids was done by TLC on silica Gf254 plate. Efficient separation of the components of the extracts appeared on the chromatogram due to the use of efficient mobile phase, as shown in Figure- 2. Table- 3 showed the relative fraction values (R_f) for different flavonoids standard and the extracted flavonoids from *Citrus paradisi* in different solvent systems

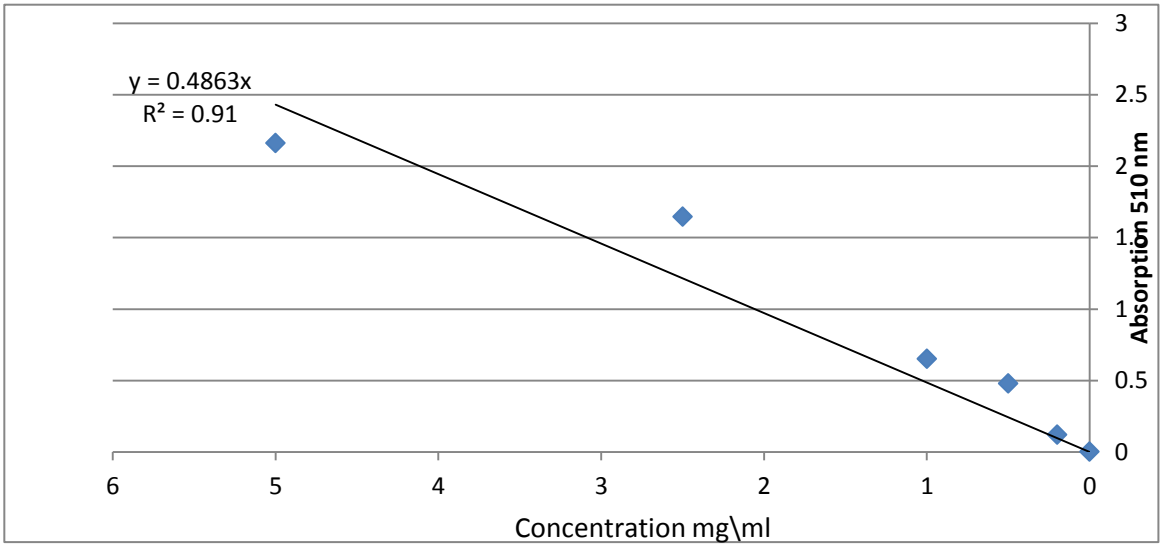

Figure 1-Standard curve for Rutin as determined by spectrophotometer at 510 nm. R2=0.9

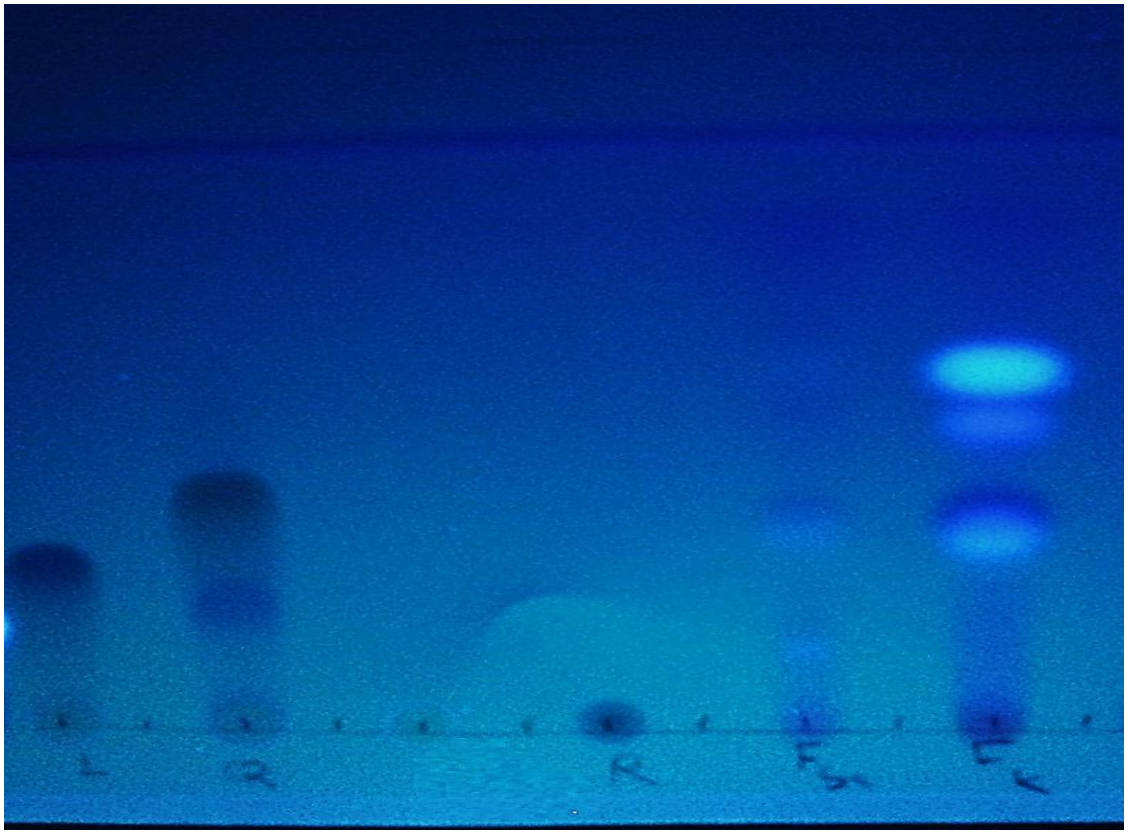

Figure 2- TLC Chromatogram on Slica gel Gf $_{254}$ sheet. Standard solutions including :Rutin(R), Quercetin(Q with two spots), Luteolin (L) and *Citrus paradisi* total flavonoids extracts of seed and peel (FS and FK).

Table 3- R_f Values for different flavonoid standards and the extracted flavonoid

Flavnoid Standard&Test	R_f value for Different Mobile Phase	R_f value
R	Rutin	Baseline
Q	Quarecetin	0.26&0.44
K	Luteolin	0.32
S_F and S_K	*Citrus paradisi* flavonoid extract(Total)	All spots are present &others

Antimicrobial assay of aqueous extracts and total isolated flavonoids from seeds and peel of *Citrus paradisi*:

The results revealed that aqueous extracts of seeds and peel of *Citrus paradisi* did not show any antimicrobial activity, while the total isolated flavonoids of seeds and peel showed a moderate antimicrobial activity compared to positive controls. The results of the antimicrobial assay are summarized in Table- 4.

Table 4- Antimicrobial assay of *Citrus paradisi* seed and peel aqueous extracts and their total flavonoid content.

Sample	***S.aureus*** (mm)	***S.epidermidis*** (mm)	***E.coli*** (mm)	***P.auerginosa*** (mm)	***C. albicans*** (mm)
Peel aq. extract	0	0	0	0	0
Seed aq. extract	0	0	0	0	0
Total flavonoids (peel)	12	12.5	12.5	14.5	0
Total flavonoids(seed)	11	11.5	11	12.5	13
Positive Controls					
Clindamycin(2µg)	22	24	0	0	0
Gentamicin(10µg)	0	0	26	34	0
Clotrimazole(5mg/ml)	0	0	0	0	17
Nystatin(5000IU/ml)	0	0	0	0	26

The total flavonoids of peel extract showed a greater degree of inhibition than the total flavonoids isolated from seed extract for bacterial strains. The total flavonoids isolated from the seed extract showed a high degree of inhibition of Candida albicans while the total flavonoids of peel extract did not show any inhibition to the same fungus. Both seeds and peel aqueous extracts of grapefruit failed to inhibit any microbial growth. The inhibition zones of seed and peel extracts and the isolated total flavonoids are shown in Figure- 3.

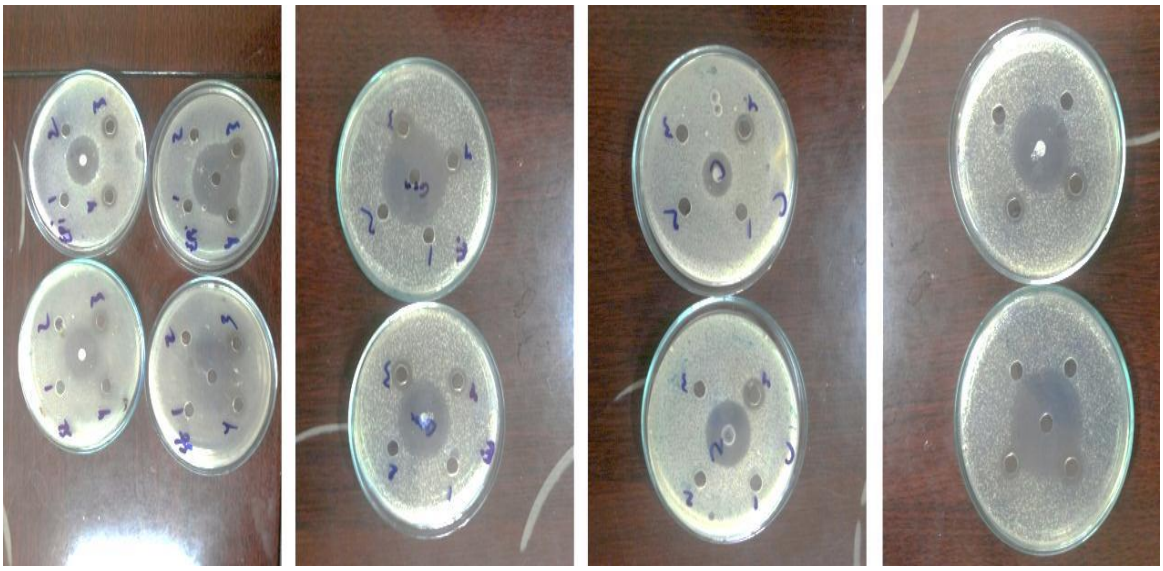

Figure 3- Antimicrobial assay using well diffusion method

Discussion:

Numerous studies have been conducted with extracts of various plants, screening antimicrobial activity as well as for the discovery of new antimicrobial compounds[12-13]. Phytochemicals derived from plant products serve as a prototype to develop less toxic and more effective medicines in controlling the growth of microorganisms[14-15]. In the current study, the aqueous extracts of grapefruit seed and peel lacked antimicrobial activity, while the total isolated flavonoids showed antimicrobial activity. This difference in antimicrobial activity between the aqueous extracts and total flavonoids may be related to the chemical structure. In aqueous extracts the flavonoids existed as glycosides in which a sugar moiety is conjugated at the carbon 3 of the C ring and/or at carbon 7 of the A ring of the basic structure of flavonoids. This may contribute to lack of antimicrobial activity. The total flavonoids isolated as an aglycon the carbon 3 and/or the carbon 7 were substituted by a hydroxyl group that may increase the antimicrobial activity. A study conducted by Osawa *et al.* [16]. assessed the activity of a number of structurally different flavonoids including flavones, flavanones, isoflavones and isoflavanones based on agar diffusion assay. It was shown that 5- hydroxyflavonones and 5- hydroxyisoflavonones with one, two or three additional hydroxyl groups at the 7,2' and 4' positions inhibited the growth of *Streptococcus mutans* and *Streptococcus sobrinus*. Osawa also showed that the lack of activity may be due to the poor diffusion of flavones and isoflavon (compared to flavanones and isoflavanones) through the medium [16].The total flavonoids of grapefruit peel failed to inhibit yeast growth, while total flavonoids of seeds showed antifungal activity and this may be due to lack of structural features of flavonoids that are necessary to target different components and functions of yeast cells[17-19].

It is generally accepted that phytochemicals are less potent anti-infective than agents of microbial origin i.e. antibiotics [20]. Flavonoids may be used to develop new classes of antimicrobial drugs through structural alterations to produce potent chemotherapeutics and combat the multi-drug resistance developed by microorganisms.

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