Phytochemical Analysis and Inhibitory Effect of *Citrus aurantium* L. (Bitter Orange) Leaves on some Bacterial Isolates *in vitro*

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Abstract

This study has been done in order to investigate the phytochemicals constitution and determine *in vitro* antimicrobial activity of aqueous and methanolic *Citrus aurantium* L. leaves extract against bacteria. The organic solvent extraction done by used Soxhlet apparatus, solvents used were methanol (80%), while aqueous extract was prepared by using magnetic stirrer. The phytochemical analysis of plant extract was performed using precipitation and coloration methods. Antibacterial activity of methanolic and aqueous of *Citrus aurantium* L. leaves extract determined by disc diffusion and agar well diffusion method. The different concentrations of aqueous and methanolic *Citrus aurantium* L. leaves extract exhibited potent antibacterial activity against selected bacteria. The phytochemical screening of aqueous and methanolic *Citrus aurantium* L. leaves extract revealed the presence of steroids, flavonoids, saponins, alkaloids, phenols and tannins, while the glycosides detectable in the methanolic extract only, and the terpenoids not detectable. Both aqueous and methanolic *Citrus aurantium* L. leaves extract at concentrations (5% and 10%) showed considerable antibacterial activity against (*Staphylococcus epidermidis* and *Staphylococcus aureus, E. coli, Pseudomonas aerugenosa* and *Proteus mirabilis*). To conclude**,** the antimicrobial activity of the aqueous and methanolic extracts of *Citrus aurantium* L. leaves was efficiency against some bacteria strains.

Keywords: - *Citrus aurantium* L., Phytochemical, Antibacterial, Phenols, Alkaloids.

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تحليل مركبات الايض الثانوية والتأثير التثبيطي لأوراق نبات النارنج على بعض انواع البكتيريا خارج الجسم الحي

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الخلاصة

أجريت هذه الدراسة لغرض الكشف عن وجود مركبات الايض الثانوية وتقييم الفعالية ضد البكتريا في المختبر لكل من المستخلص المائي والكحولي لاوراق النارنج. استخدم جهاز السوكسليت لأستخلاص بالمذيبات العضوية فقد أستخدم الميثانول بنسبة (80%). بينما حضر المستخلص المائي بوساطة استخدام جهاز الرجاج المغناطيسي. تم الكشف عن مركبات الايض الثانوية باستخدام الطرائق اللونية وطريقة الترسيب . اما الفعالية المضادة للبكتريا لكل من المستخلص المائي والكحولي لاوراق النارنج فقد قيمت بطريقة الأنتشار فِي الأكار بواسطة الحفر وطريقة الاقراص المشربة. حيث أظهرت التراكيز المختلفة لكل من المستخلص المائي والكحولي لاوراق النارنج فعالية ضد البكتريا. التحليل النوعي للمكونات الايض الثانوية لكل من المستخلصين المائي والكحولي لأوراق النارنج أظهرت وجود كل من الستيرويدات والفلافونيدات والصابونينات والقلويدات والفينولات والعفصيات بينما الكلايكوسيدات حددت في المستخلص الكحولي فقط و التيربينويدات لم تحدد في المستخلص المائي والكحولي . كلا المستخلصان المائي والكحولي بتركيز (5% ، 10%) أظهرت فعالية كبيرة ضد (*Staphylococcus epidermidis* and *Staphylococcus aureus, E. coli,* *Pseudomonas aerugenosa* and *Proteus mirabilis*). الاستنتاج ، الفعالية المضادة للبكتريا لكل من المستخلص المائي والكحولي لاوراق النارنج كانت كفوءة ضد بعض العتر البكترية.

الكلمات المفتاحية:- النارنج , مركبات الايض الثانوية , مضاد للبكتريا , الفينولات , القلويدات.

Introduction

Citrus aurantium L., previously known as *Fructus aurantii*, is the botanical name of a plant known as green orange, bitter orange or sour orange.[1,2] The extract of the immature fruit or peel of *C. aurantium* L. has been widely used in weight loss dietary supplements and in sports performance products[3]. In addition, *Citrus aurantium* L. is among the species that have been used for medical purposes on account of the numerous bioactive compounds that it contains

such as phenolic, essential oils, vitamins and flavonoids. *Citrus aurantium* L. used for medicinal purposes such as the leaves, peel and flowers of the plant.[4] Many active phytoconstituents such as flavonoids have a large spectrum of biological activity including antibacterial, antiviral, anticancer, antifungal and antidiabetic activities. [5, 6] The phytochemicals are also known as secondary metabolites that are derived from primary metabolites and are used as drugs.[7] The phytochemicals also contribute to the flavor, colour and other characteristics of plant parts.[8] Phytochemical analysis is benefit in determination of some active biological constituents of some vegetables and medicinal plants.[9] Phytochemicals are not essentially required for the sustenance of life but confer extra health benefits against bacteria.[10] In the last three decades, a numerous of antibiotics and other synthetic drugs were produced in the world with an aim of eradicating the microorganisms which were responsible for many diseases.[11] Drugs or antibiotics induced mutations in the genetic composition of these microorganisms rendering them resistant to several drugs or antibiotics.[12] Moreover, the side effects associated with the long-term use of the synthetic drugs may cause serious damages to many of human organs. Therefore, to overcome this limitation of synthetic drugs, researchers have shifted their focus towards medicinal plants which are recognized as rich sources of antibacterial agents and are widely used by different countries for medicinal purposes. Traditionally used medicinal plants are known to produce a variety of compounds with therapeutic properties, such as antidiabetic, antioxidant, antibacterial, anti-inflammatory, antipyretic, gastroprotective effects, etc.[13] The aim of this study was to investigate the phytochemicals constitution and determine *in vitro* antimicrobial activity of *Citrus aurantium* L. leaves extract against *Staphylococcus aureus, Staphylococcus epidermidis, E.coli, Pseudomonas aerugenosa* and *Proteus mirabilis.*

Materials and Methods

Collection of Plant

Fresh leaves of *Citrus aurantium* L. plant were collected from Baqubah, Diyala Province, Iraq. Which was identified, authenticated by Assist. Prof. (dr.) Khazal Dhbea Wadi,

College of Sciences, Diyala University. The leaves were cleaned, washed then dried in room temperature at shade. The leaves were then crushed by using mechanical grinding machine.

Preparation of Extracts

Aqueous Extract: Thirty grams of leaves powder was suspended in 300 ml of distilled water. Aqueous extraction was done at 70˚C for 30 minutes, after that filtering of the extracts by using Whatman filter paper No.1. Then evaporated the extracts at 45˚C to form a paste and after that transferred into sterile bottles and refrigerated 4˚C until use.

Methanolic Extract: Organic solvent extraction of the *Citrus aurantium* L. leaves powder was carried out by using methanol (80%). This was done by using Soxhlet apparatus. The extraction was carried out for 24 hours by heating temperature that kept the solvent at 50-60˚C until a colorless and clear solvent appeared in the extracting chamber. After that, the extract was dried by electric oven at temperature 40-45˚C until dry extract was obtained. The final extract was kept frozen at (–18˚C) until use.

Phytochemical Analysis (Qualitative Analysis):

Phytochemical analysis was carried out to evaluate the qualitative of chemical composition of crude extracts to identify the secondary metabolites groups such as glycosides, phenolic, compounds alkaloids, tannins, saponins, terpenoids, steroids and flavonoids by using coloration and precipitation methods. This analysis revealed the presence or absence of the phytochemicals constitution in the aqueous and methanolic extract.[14-18]

Microbial Isolates

Different five clinical microbial isolates (Gram positive and Gram negative) were collected and identified by using conventional biochemical tests [19] and cultivated in pure culture, at Microbiological Laboratory/ College of Veterinary Medicine / Diyala University. These include *(Staphylococcus epidermidis, Staphylococcus aureus, E. coli, Pseudomonas aerugenosa and Proteus mirabilis).*

Antibacterial Activities

Agar Well Diffusion Method

Determination of extract activities by spreading of 0.1 ml of bacterial suspension prepared according [20] which contain $1.5*10^8$ cell/ml over the surface of Muller – Hinton agar plate to obtain uniform growth, left the plate to dry for five minutes. Then the wells were prepared by using Cork borer five mm diameter. These wells filled by 50 microliter concentrated extract of either methanolic or aqueous extract according to concentration used 2.5%, 5% and 10%, the medium was left for 1 hour in laboratory condition, then incubated for 24 h at 37 ˚C and the inhibition zone of antibacterial activity appeared around the well were measured the diameter by centimeter scale in mm (millimeter), each treatment consists of 4 repeat. [21-23]

Disc Diffusion Method

Antibacterial activity of aqueous and methanolic extracts were determined by disc diffusion method on Muller –Hinton agar by (Steel and Torrie).[24] Sterile Whatman filter disc (5 mm diameter) were made, these disc impregnated in the 50 microliter of aqueous or methanolic extract. Placed in Petri dishes according to concentrations (2.5%, 5% and 10%) for 24 hours. Inoculums containing $1.5*10^8$ CFU / ml of bacteria were spread, with sterile swab moistened with the bacterial suspension. The disc also impregnated in 50 μL of solvent distilled water served as a standard control.[23] Standard antibiotic disc; Tetracycline (T); Streptomycin (S); Penicillin (P); Amoxicillin (AUG) for antibacterial activity tests were carried out against bacterial strains.

Statistical Analysis

The values are expressed as the mean ± the standard error of the mean (M±SEM). The values were analyzed by using one way analysis of variance ANOVA, and then the test of the least significant difference LSD applied to find the significant differences between the means of inhibitory zones .[25] The significant level of test was $P< 0.05$.

Results and Discussion

Phytochemical Analysis

The results of the preliminary phytochemical screening of aqueous and (80%) methanolic *Citrus aurantium* L. leaves extract revealed the presence of a wide range of phytochemicals constitution including steroids, tannins, alkaloids, flavonoids, saponins, phenol while the glycosides are detected only in the *Citrus aurantium* L. methanolic extract but the terpenoid don't detectable in the aqueous and methanolic extract. Table (1).

Table 1: Phytochemical screening of aqueous and (80%) methanolic *Citrus aurantium* L. leaves extract

No.	Test	*Citrus aurantium* L. Aqueous Extract	*Citrus aurantium* L. 80% methanolic Extract
1	**Alkaloid**	+	+
2	**Flavonoid**	+	+
3	**Steroid**	+	+
4	**Terpenoid**	–	–
5	**Phenol**	+	+
6	**Saponins**	+	+
7	**Tannins**	+	+
8	**Glycosides**	–	+

[+ = Present, — = Absent]

In the present study, we evaluated the phytochemical compound of aqueous and methanolic extract of *Citrus aurantium* L. leaves. The analysis of phytochemicals constitution conducted on the plant extracts revealed the presence of phytochemicals constitution which are known to exhibit medicinal as well as physiological activities.[26] The citrus peels are rich in nutrients and contain many phytochemicals with strong potential for use in drug production or as food supplements.[27,28] Our results are in agreement with these assertions as a range of phytochemicals; alkaloids, tannins, flavonoids, saponins, cardiac glycosides, steroids were detected in the orange peels and seeds extracts. (Swapnil Mehta *et al*., 2011)[29], has reported that phytochemical analysis of *Citrus maxima* showed the presence of important classes of

phytoconstituents like alkaloids, saponins and carbohydrates, this is similar to the results obtained in the present study.

Antibacterial Activity

The present study shown that the inhibitory zones were more at (10%) in comparison with (2.5%, 5%) of aqueous and methanolic *Citrus aurantium* L. leaves extract in each disc diffusion and well diffusion method against all bacteria species. On the other hand, the present study show significant difference at $P<0.05$ between susceptibility of bacteria species to 5% and 10% concentration of methanolic extract of *Citrus aurantium* L. leaves extract in well diffusion as in (Table 2). The methanolic extract of *Citrus aurantium* L. leaves extract in well diffusion method shown inhibition zone 5% and 10% concentration more than in disc method.

Table (2): Comparison the effect of methanolic extract of *Citrus aurantium* L. leaves extract in Well diffusion and disc diffusion methods against some bacteria species.

Bacteria species	Methanolic extract of *Citrus aurantium* L. Well diffusion Concentration of extract			disc diffusion Concentration of extract		
	2.5% M±SE (mm)	5% M±SE (mm)	10% M±SE (mm)	2.5% M±SE (mm)	5% M±SE (mm)	10% M±SE (mm)
Staphylococcus aureus	0.0±0.0	8 ±0.0 -b-	12.6±0.35 -c-	0±0.0	7±0.0 -b-	10.3±0.35 -c-
Staphylococcus epidermidis	7±0.0 -a-	10.6±0.35 -b-	12.3±0.35 -c-	0±0.0	0±0.0 -b-	10±0.33 -c-
E. coli	0±0.0	9±0.0 -b-	11±0.0 -c-	0±0.0	7±0.0 -b-	10±0.33 -c-
Pseudomonas aeruginosa	0±0.0	7±0.0 -b-	9±0.0 -c-	0±0.0	0±0.0 -b-	7±0.33 -c-
Proteus mirabilis	0±0.0	7.6±0.33 -b-	9.6±0.33 -c-	0±0.0	0±0.0 -b-	8±0.0 -c-

Values: Mean ± Standard error of the mean (M± S.E.M). a, b, c; significantly different level of P < 0.05
-a- mean the significance was between concentration 2.5% and 5%.
-b- mean the significance was between concentration 5% and 10%.
-c- mean the significance was between concentration 2.5% and 10%.

The aqueous extract of *Citrus aurantium* L. leaves in agar well diffusion method exhibit a significantly higher zones of inhibitions against gram positive bacteria (*Staphylococcus epidermidis* and *Staphylococcus aureus*) and gram negative bacteria (*E. coli, Pseudomonas aerugenosa* and *Proteus mirabilis*) in used at 10%. Comparison with 2.5%, 5% and 10% concentration (Table 3). On the other hand the aqueous extract of *Citrus aurantium* L. in well diffusion method shown inhibition zone 10% and 5% concentration more than in disc method.

Table (3): Comparison the effect of aqueous extract of bitter orange in Well diffusion and disc diffusion methods against some bacteria species.

Bacteria species	Aqueous extract of *Citrus aurantium* L. Well diffusion Concentration of extract			disc diffusion Concentration of extract		
	2.5% M±SE (mm)	5% M±SE (mm)	10% M±SE (mm)	2.5% M±SE (mm)	5% M±SE (mm)	10% M±SE (mm)
Staphylococcus aureus	0.0±0.0	7±0.0 -b-	11.6±0.35 -c-	0.0±0.0	0±0.0 -b-	10±0.33 -c-
Staphylococcus epidermidis	0.0±0.0 -a-	8.6±0.35 -b-	13.6±035 -c-	0.0±0.0 -a-	9.6±0.33 -b-	12.3±0.35 -c-
E. coli	0.0±0.0	8.6±0.35 -b-	12.6±0.5 -c-	0.0±0.0	0±0.0	11±0.0
Pseudomonas aeruginosa	0.0±0.0	7.6±0.5 -b-	10±0.33 -c-	0.0±0.0	0±0.0	7±0.0
Proteus mirabilis	0.0±0.0	7±0.0 -b-	9±0.0 -c-	0.0±0.0	0±0.0	8±0.0

Values: Mean ± Standard error of the mean (M± S.E.M). a, b, c ; significantly different level of P < 0.05
-a- mean the significance was between concentration 2.5% and 5%.
-b- mean the significance was between concentration 5% and 10%.
-c- mean the significance was between concentration 2.5% and 10%.

Antibacterial efficacy of aqueous and methanolic extract of *Citrus aurantium L.* (Bitter Orange) leaves against five bacteria by agar well diffusion and disc diffusion method showed excellent inhibitory action against all tested bacteria at concentration (5%, 10%), but

don't shown inhibition at concentration (2.5%)[Table 1,2]. Citrus fruit products such as *Citrus aurantium L.* act as potent antimicrobial agents against micro-organism (bacteria and fungus) because citrus fruits are rich source of flavonones and many polymethoxylated flavones, in other plants this phytoconstituents are very rare.[30]

In the study, Kirbaşlar *et al.* [31] evaluated antibacterial activity of peel oil from Citrus fruits. It was observed that peel oil of *C. sinensis* and *C. aurantium* exhibited more or less similar inhibition of Gram-positive and Gram-negative bacteria.

Bouabdelli *et al.* [32] showed that *S. aureus, Proteus mirabilis* and *E. coli* were sensitive to aqueous extract of *C. aurantium* L. leaves while *P. aeruginosa* was resistant to it. In addition, Gopal illustrated that extracts of *C. aurantium* L. leaves were effective against both Gram-negative and Gram-positive organisms with inhibition zone (12-14 mm). Preliminary phytochemical analysis of the *C. aurantium* L. leaf extracts showed the presence of tannins, terpenoids, sterols, alkaloids, flavonoids and carbohydrates.[33] Also Kabra, *et al.*, [34] reported that ethanolic extract of *Citrus medica* showed antibacterial activity when tested against *Klebseilla pneumoniae, Escherichia coli and Staphylococcus aureus.* Cano, *et al*., [35] reported the higher antimicrobial activity of *Citrus paradise* due to the presence of essential oils, vitamin C and flavonoids. Dhanavade, *et al*., [36] reported the presence of alkaloids, lactons, polyacetylene, essential oils, corydaline, pseudohypericin, protopine, hypericin and acyclic sesquiterpenes compounds from lemon peel, this compounds are effective against many strains of bacteria.

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