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Antibacterial activity of the glycosidic extract from *Citrus laurantifoia L.* fruits

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ABSTRACT

Glycosides of the Citrus laurantifoia L. fruits have been extracted, then number of preliminary qualitative chemical tests were carried out on the extract. It was found that the extract consist of three glycoside compounds (G_1, G_2 & G_3). Compound (G_1) has been isolated from Citrus laurantifoia L. fruits by column chromatography using n-Butanol : Acetic acid : Water (4:1:5, upper layer) as eluent. The possible antibacterial activity of glycoside compound (G_1) was assayed against the bacteria, Staphylococcus aureus, Streptococcus aureus, Pseudomonas aeruginosa and Escherichia coli using agar diffusion method. The results revealed that compound (G_1) exhibited antibacterial activity against all the bacteria tested indicating that the presence compound had broad spectrum antibiotic effect.

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Key words: *Citrus laurantifoia L.*, Glycosidic extract, Antibacterial activity.

INTRODUCTION

Medicinal plants have been of age long remedies for human diseases because of therapeutic value [1]. Although synthetic and semi synthetic antimicrobial drugs abounds in various markets today, there is need for continuous search for new ones cope with the increased evaluation of multiple antimicrobial resistant strains of organisms [2]. Contrary to the synthetic drugs, antimicrobials of plant origin are not associated with side effects and have an enormous therapeutic potential to heal many infectious diseases. For example, vincristine (an antitumor drug), digitalis (a heart regulator) and ephedrine (a bronchodilator used to decrease respiratory congestion) were all originally discovered through research on plants. The potential for developing antimicrobials from higher plants appears rewarding as it will lead to the development of a phytomedicine to act against microbes. Plant-based antimicrobials have enormous therapeutic potential as they can serve the purpose with lesser side effects that are often associated with synthetic antimicrobials [3]. The general belief that the advent of antibiotics will bring end to the occurrence of infectious diseases was cut short with the occurrence of resistance to antimicrobial drug [4]. The rate of resistance to these drugs is higher in developing countries when compared with developed countries. This may be due to the indiscriminate use of antibiotics and also self medications without prescription by physician. Furthermore, the use of antibiotics in animal feeds may induce resistance. As this resistivity increases, the need for newer and/or alternative therapy becomes very necessary [5]. This study was designed to isolate glycoside compounds from *Citrus laurantifoia L.* fruits and assess their potentials as alternative therapies in the management of infectious disease.

MATERIALS AND METHODS

Plant Material

Fruits of *Citrus laurantifoia* L. were supplied locally. The plant was botanically authenticated and voucher specimens were deposited in the Herbarium of Basrah (Iraq, Basrah, College of Science, University of Basrah). The fruits were ground by hand mill and kept polyethylene bags until time of use.

Preparation of extracts

Methanolic extract of *Citrus laurantifoia* L. fruits

Quantity (100g) of powdered plant was extracted in a Soxhelet apparatus with 80% methanol, for 24 h. The methanolic extract was filtered and evaporated to dryness under reduced pressure in a rotary evaporator to afford 7.2g of dry extract.

Glycosidic extract of *Citrus laurantifoia* L. fruits

Fifty grams of dried ground fruits were refluxed with 400mL of (2% acetic acid-water) in water bath for eight hours at 70° C**.** The residue was removed by filtration using Buchner funnel, and the filterate was extracted by (3×50 ml) n-butanol saturated with sodium chloride. Organic layer was evaporated under vacuum in a rotary evaporator to yield (1.78)g [6] .

Isolation of the components of glycosidic extract

The component G_1 (Spot with higher R_f = 0.91) of glycoside compound was separated and purified by column chromatography technique. A glass column size (3×60 cm) was plug down to the bottom with small glass wool, then packed with HCl-washed silica-gel (mesh 230-400 μm). The slurry was prepared by dissolving 125g of silica-gel in 200ml of n-Butanol **:** Acetic acid **:** Water (4**:**1**:**5, upper layer) as eluent. The solid residue was then loaded to the top of the column and fractions of 5mL were collected and monitored by TLC. Fractions with the similar R_f were collected and dried at room temperature [7].

Preliminary phytochemical analysis

The extracts obtained were subjected to Preliminary phytochemical screening, to identify the chemical constituents. The methods of analysis employed were those described by [8,9].

Tests for carbohydrates

(1) Molisch's test**:** 1ml of the extract was added to 4ml of α-naphthol and the resulting mixture shaken properly. 2ml of conc. H_2SO_4 was then poured carefully down the side of the test tube. A violet ring at the interphase indicates the presence of carbohydrate.

(2) Iodine test**:** To 3ml of the extracts was added about 1ml of iodine solution. A purple coloration at the interphase indicates the presence of carbohydrate.

Tests for glycosides

(1) Before hydrolysis**:** To 1ml of the extract was added equal volume of Benedict's reagent in test tube. Heat in boiling water bath for 5min. A formation of yellow or orange precipitate indicates the presence glycosides.
(2) After hydrolysis**:** 1ml of extract was boiled with few drops of 1% HCl on a water bath for 5min. A formation of yellow or orange precipitate indicates the presence reducing sugar. Then 1ml of the Benedict's reagent was added and heat the test tube in water bath for 5min. A formation of large amount of precipitate indicates the presence glycosides.

Test for alkaloids

2ml of extract was stirred with 2ml of 1%HCl on a steam bath. Dragendroff, Mayer's and Wagner's reagents were then added to the mixture. Turbidity of the resulting precipitate was taken as evidence for the presence of alkaloids.

Test for phenols

About 2ml of the extract was stirred with 2ml of distilled water and few drops of $FeCl_3$ solution were added. The formation of a green precipitate was an indication for the presence of phenols.

Test for flavonoids

(1) To 1ml of extract was added 1ml of 10% lead acetate solution. The formation of a yellow precipitate was taken as a positive test for flavonoids.
(2) To 1ml of extract was added 5ml of 95% ethanol. 0.5ml acetic anhydride, few drops of conc. H2SO4 and magnesium ribbon. A pink or red color indicate the presence of flavonoids.

Test for saponins

1ml of extract was shaken vigorously with 1ml of distilled water in a test tube and warmed. The formation of stable foam was taken as an indication for the presence of saponins.

Test for terpenoids
1ml of the extract was dissolved in 1ml of chloroform and evaporated to dryness. 1ml of concentrated sulphuric acid was then added and heated for about 2min. A greenish color indicates the presence of terpenoids.

Tests for steroids
A red color produced in the lower chloroform layer when 1ml of extract was dissolved in 1ml of chloroform and 1ml conc. H_2SO_4 added indicates the presence of steroids.

Thin layer chromatography (TLC)
Thin layer chromatography was carried out for glycosidic extract and the compounds (G_1) of *Citrus laurantifoia L.* fruits. 10µl of each extract was used on thin layer coated by silica gel as a stationary phase (2×10 cm) and n-Butanol **:** Acetic acid **:** Water (BAW) mixture solvent was used as mobile phase with ratio (4**:**1**:**5, upper layer) respectively. These layers were left in the solvent for 60 minutes, then they were dried by hair drier to detect on chemical families (active components) present in extracts. The spots (chemical components) were detected using long wavelength 366nm ultraviolet. The plates were also saturated with ammonium vapor. The plates were also stained with $FeCl_3$-$K_3Fe(CN)_6$ (1:1) (Bruno and Svoronos, 2003). The plates were also stained with Antimony chloride (10% in $CHCl_3$), Folin-Denis, Benedict reagent and lead acetate (basic, 25%) [10].

Determination of Antibacterial Activity
A total of four bacterial strains were selected on the basis of their clinical importance in causing diseases in humans. Two Gram positive bacteria - *Staphylococcus aureus* and *Streptococcus aureus* and Two Gram negative - *Pseudomonas aeruginosa and Escherichia coli* were chosen for evaluation of antibacterial activity. All the test microorganisms were maintained on Nutrient Agar. Various concentration (125 mg/ml, 250 mg/ml & 500 mg/ml) of the glycosidic compounds (G_1) extract of the fruits of *C. laurantifoia L.* were evaluated for antimicrobial activity by agar well diffution method [11]. All the bacterial strains were adjusted to 0.5 McFarland standard, which is visually compartable to a microbial suspension approximately 1.5×10^8 cfu/ml. 20ml of agar media was poured into each petri plate and plates were swabbed with 100µl inocula of each test bacterial strain and kept for 15 min. for adsorption. Wells of 6mm diameter were punches into seeded agar plats and loaded with a 100µl volume with different concentrations of extracts reconstituted in the dimethylsulphoxide (DMSO). All the plates were incubated at 37° C for 24 hrs. Antibacterial activity was evaluated by measuring the diameter of inhibition zone of growth minus the diameter of the disc.

Statistical analysis
The results are expressed as mean values ± SD and tested with analysis of variance followed by Student's *t*-test. P-values < 0.05, < 0.01 were considered to be statistically significant.

RESULTS AND DISCUSSION

Qualitative analysis for all extracts of *Citrus laurantifoia L.* fruits
Table (3-1) indicate the preliminary phytochemicals analysis for aqueous extract, glycosidic extract and glycosidic compounds (G_1) of *C. laurantifoia L.* leaves. The results showed the presence of alkaloids, glycosides, steroids, carbohydrates, flavonoids and saponins in the aqueous extract. Same table indicate that the glycosidic extract contains only glycosid compounds. The most phytochemical classified as secondary metabolites are produce mainly by the shoot part of the plant, often their functional in the plant is unknown but some phytochemicals are known to have structural, functional and general defense against plant pathogens [12]. Plant extract is the best source of phytochemical, which used as medical treatment but their uses as well as other alternative form of phyto-treatment [13]. The results presented in Figure(3-1) and Table(3-2) shows that the glycosidic extract of *C. laurantifoia L.* fruits contains three components. These components are relate to the flavonoid family because they give a positive test with Lead acetate (basic, 25%), Folin-Denis, Benedict reagent, $FeCl_3$-$K_3Fe(CN)_6$ (1:1) and Antimony chloride(10% in $CHCl_3$) reagents. The results revealed that the isolated glycosidic compounds (G_1) by column chromatography technique, is one compound belong to the flavonoid family.

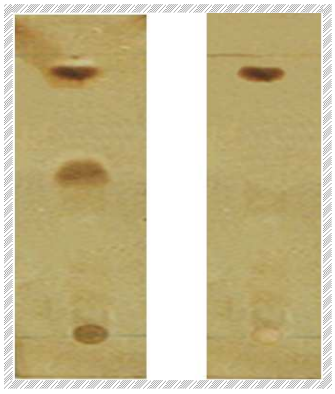

Figure (1) : Thin layer chromatography for glycosidic extract and glycosidic compounds (G_1) *C. laurantifoia L.* fruits

Table (1) : Qualitative analysis for all extracts of *C. laurantifoia L.* fruits

Test	Observation		
	Methanolic extract	Glycosidic extract	Glycosidic compound **A**
Alkaloids	+	-	-
Carbohydrates	+	+	+
Glycosides	+	+	+
Steroids	+	-	-
Flavonoids	+	-	-
Phenols	-	-	-
Saponines	+	-	-
Terpenoids	-	-	-

Table (2): Thin layer chromatography for all extracts of *C. laurantifoia L.* fruits

UV 366 nm		Lead acetate (basic, 25%)		Antimony chloride (10% in $CHCl_3$)		Folin-Denis		Benedict reagent		$FeCl_3$-$K_3Fe(CN)_6$ (1:1)	
GE	**GC A**	**GE**	**GC A**	**GE**	**GC A**	**GE**	**GC A**	**GE**	**GC A**	**GE**	**GC A**
0.91	**0.91**	**0.91** **0.32** **0.01**	**0.91**	**0.91** **0.32** **0.01**	**0.91**	**0.91** **0.32** **0.01**	**0.91**	**0.91** **0.32** **0.01**	**0.91**	**0.91** **0.32** **0.01**	**0.91**
Conjugated test		Flavonoid test		Flavonoid test		Flavonoid test		Flavonoid test		Flavonoid test	

Where, GE: Glycosidic Extract and GC A: Glycosidic compound A

Antibacterial Activity for glycosidic compounds (G_1)

The glycosidic compounds (G_1) inhibited all the tested bacterial strains with highest activity and the inhibition ranging from 1-9 mm. The results showed that the increase in concentration of extract increased the zone of inhibition against all tested bacteria. Antibiotics provide the main basis for the therapy of bacterial infections. However, the high genetic variability of bacteria enables them to rapidly evade the action of antibiotics by developing antibiotic resistance. In recent years development of multi-drug resistance in the pathogenic bacteria and parasites has created major clinical problems in the treatment of infectious diseases [3]. This and other problems such as toxicity of certain antimicrobial drugs on the host tissue triggered interest in search of new antimicrobial substances/drugs of plant origin [14]. Considering the rich diversity of plants, it is expected that screening and scientific evaluation of plant extracts for their anti-microbial activity may provide new anti-microbial substances; hence the present investigation clearly reveals the antibacterial nature of this plant and suggests that this plant could be exploited in the management of diseases caused by these bacteria in human systems. The present study is successful in identifying candidate folkloric plant with heat stable constituents, since, all the extracts were subjected to heat treatment and them subject to antibacterial activity. Constituents were also found to be bactericidal since no growth was observed even after 48 hour of incubation in zone of inhibition area. In addition, this result form a good basis for selection of the plant for further phytochemical and pharmacological investigation and suggests antibacterial properties that can be used as antimicrobial agents in new drugs for the therapy of infectious caused by pathogens. Two important pathogens viz., *E. coli* frequently associated with urinary track infection a common problem in stressed human being and office bearers who share common toilets [15] and Pseudomonas *aeruginosa* frequently associated with infant bacteria is highly susceptible to methanolic and glycosidic extract [16]. The plant is of folkloric origin and is already in use as a medicinal plant with minimum and least toxicological studies the plant could be exploited against these pathogens of highest clinical importance. Several workers have reported that many plants possess antimicrobial properties including the parts which include; flower, bark, stem, leaf, etc. It has been shown that when solvents like ethanol, petroleum ether, chloroform, isopropanol, hexane and methanol are used to extract plants, most of them are able to exhibit inhibitory effect on both gram positive and gram negative bacteria [17].

The demonstration of antimicrobial activity against both Gram-positive and Gram-negative bacteria may be indicative of the presence glycosidic compounds (G_1) had broad spectrum antibiotic effects [18].

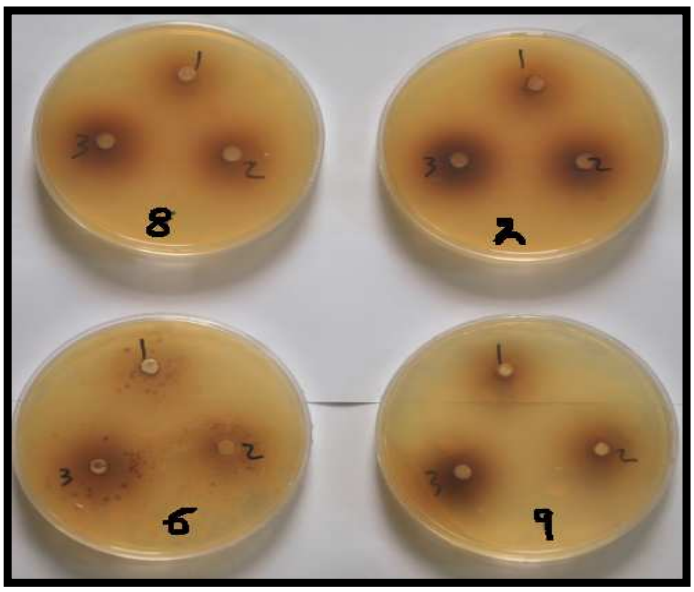

Figure (2): Effect of glycosidic compound (G_1) of *C. laurantifoia* L. fruits on tested bacteria at the concentration (125, 250 & 500 mg/mL)

Table (3): Antibacterial activity results of glycosidic compound (G_1) of *C. laurantifoia* L. fruits

Concentration (mg/ml)	Mean diameter of zone of inhibition (mm ± SD)			
	Pseudomonas aeroginosa	***Staphylococcus aurens***	***E. coli***	***Streptococcus aureus***
	S.N.=2	S.N.=6	S.N.=8	S.N.=9
125	32.4	29.8	28.2	30.1
250	36.6[a]	34.3[a]	35.2[a]	35.3[a]
500	41.7 [b]	40.5[b]	41.5[b]	41.6[b]

Values are mean inhibition zone (mm) ±S.D, S.N.= serial number, [a] P<0.05, [b] P<0.01, as compared to lower concentration (125 mg/ml)of the glycosidic compound (G_1).

CONCLUSION

This study provides the important baseline information for the use of glycoside compound (G_1) isolated from *Citrus laurantifoia L.* fruits as natural drug possesses potent antimicrobial activity towards *Staphylococcus aureus, Streptococcus aureus, Pseudomonas aeruginosa and Escherichia coli*, and can be used to treat infections caused by these pathogens after suitable clinical trials.

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