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Comparative Antimicrobrial Activity of Peel and Juice Extract of Citrus Fruits Growing in Kurdistan/Iraq

Aveen Nozad Adham*

Department of Pharmacognosy, College of Pharmacy, Hawler Medical University, Erbil, Kurdisatn region, Iraq
*Corresponding author: aveennawzad@yahoo.com

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Abstract Citrus fruits is an important medicinal plant of the family Rutaceae. Qualitative and quantitative evaluation of antimicrobial activity of peel and juice ethanol 80% extract were performed by agar well diffusion method, minimum inhibitory concentration value by microtiter broth dilution method against five gram positive and two gram negative bacteria, while minimum bactericidal concentration evaluated by plate cultures. The results of antibiotic susceptibility test indicated that all isolated bacterial strains showed sensitivity to chloramphenicol, azithromysin and norfloxacin. All used citrus fruits showed antimicrobial activity either alone or in combination of peel and juice extracts against isolated bacterial strain. From isolated bacterial strain *Staphylococcus auricularis* showed highest sensitivity against *Citrus limon* juice with inhibition zone (22mm), in combination with peel (35mm) and *Citrus sinensis* in combination form showed inhibition zone (19mm). *Citrus limon* juice alone and in combination with peel extract exhibit inhibitory activity against gram positive and gram negative bacteria 100%. While *Citrus sinensis* in combination form showed activity only against gram positive bacteria 20%. On comparison of antimicrobial activity different citrus fruits the results showed that *Citrus limon* showed highest antimicrobial activity followed by *Citrus reticulata*, then *Citrus paradisi* and the lowest activity exhibited by *Citrus sinensis*.

Keywords: *citrus fruits, agar well diffusion, microtiter broth dilution, MBC*

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1. Introduction

Citrus fruits which belongs to the family Rutaceae are considered as an important constituents of human diet which contain healthy nutritional content such as vitamin C, carotinoids, limonoids, minerals, essential oil, folic acid, alkaloids, potassium, flavonoids, and dietary fibers that works wonders for the body and grown worldwide, also ranks top in world production and trade among the fruit trees [1]. The genus citrus comprises of about 140 genera and 1300 species [2], the most important include *Citrus limon*, *Citrus paradisi*, *Citrus sinensis* and *Citrus reticulata*. It is first originated from Southeast Asia then it is spread to Northeast India, Burma and China, also Iraq and Iran grows citrus fruits mainly for domestic consumption [3]. The tree is small with alternate, usually evergreen leaves, the flowers smell is strong sweet, the fruit is spherical or egg shaped. It has 8-14 juicy sections containing large, white or greenish seeds [4]. Traditionally it is used to soothe sore throats, indigestion, relieve intestinal gas and bloating, resolve phlegm and as an additive for flavoring to our foods [5,6]. Crude extracts of different part of *Citrus limon*, *Citrus paradisi*, *Citrus sinensis* and *Citrus reticulata* are mainly used to reduce high blood pressure, respiratory problems, rheumatism [7,8], anticancer, antimicrobial and antioxidant activity being associated with polyphenols such as (caffeic acid, p-coumaric acid, ferulic acid and sinapinic acid), flavonoids such as (hesperidine, narirutin, naringin, eriocitrin) [9,10], essential oil composition which includes (D-limonene, β-Myrcene, α-pinene, β-pinene, γ-terpinene, α-ter- pinolene, α-Caryophyllene, copaene, β-phellandrene) [11,12] and other constituents present in it. However, so far there is no comparative study has been reported in these varieties available in Kurdistan region for its antimicrobial properties. Hence, in the present study, we aimed to carry out a comparative investigation of its antimicrobial properties of peel, juice extract and in combination of both of them of *Citrus limon*, *Citrus paradisi*, *Citrus sinensis* and *Citrus reticulata* using agar well diffusion method and microtiter broth dilution method against gram positive and gram negative bacteria.

2. Materials and Methods

2.1. Materials

Ethanol from Scharlab S.L. (Spain), Muller Hinton agar, Nutrient agar, Nutrient broth (Merck Co. Germany), Dimethylthiazolyl diphenyl tetrazolium bromide (MTT) (Taizhou xianju pharma.co., China).

2.2. Collection and Preparation of Citrus Fruits Extract

The ripe fruits of four citrus species were collected from garden in Kurdistan region during June 2015. The plant materials were identified in department of Pharmacognosy, Collage of pharmacy, Hawler Medical University. Fruits were brought to laboratory washed with sterile distilled water. The 100 g of peel and juice of fruits were weighed then extracted successively with 200 ml 80% ethanol yielding ethanol fraction using ordinary reflex extraction. The extract was filtered using Whatman filter paper (No. 1) and concentrated in vacuum at 40°C using a rotary evaporator, then the extract kept at 4°C until used for further study.

2.3. Evaluation of Antimicrobial Activity

2.3.1. Tested Microorganism

Five gram positive *Staphylococcus aureus, Staphylococcus auricularis, Streptococcus mitis, Streptococcus salivarius, Streptococcus pneumoniae* and two gram negative *Klebseilla pneumoniae, Escherichia coli* were selected for the evaluation of antimicrobial activity and identified by using biochemical tests in the Microbiological Laboratory of the Biological department, Collage Of Education, Salahadin University..

2.3.2. Antibiotic Susceptibility Testing

The susceptibility of isolates to antimicrobial agents was examined by an agar well diffusion method using the following antibiotic concentrations: Chloramphenicol (10µg), Bacitracin (10µg), Azithromysin (10µg), Ceftriaxone (10µg), Norfloxacin (10µg).

2.3.3. Agar Well Diffusion Method

The antimicrobial activity of peel and juice 80% ethanol extracts of citrus fruits against five gram positive and two gram negative isolated pathogenic bacteria was evaluated by using agar well diffusion method [13] with slight modification. Suspensions of the isolated strains of microorganisms *Staphylococcus aureus, Staphylococcus auricularis, Streptococcus mitis, Streptococcus salivarius, Streptococcus pneumoniae, Klebseilla pneumoniae* and *Escherichia coli* were prepared using normal saline with a turbidity equivalent to that of the 0.5 ml McFarland standard, and 100µl from each suspension was then spread with sterile swabs on petridish. 6mm wells were made with sterile borer into Muller Hinton agar plates containing the bacterial inoculum. 100µl volume of the 50mg/ml peel, juice and combination of peel and juice extracts were poured into a well of inoculated plates. DMSO was used as a negative control which was introduced into a well instead of fruit extract. The plates were incubated for 18-24 hrs at 37^0C, and then examined for appearance inhibition zone. The diameter of inhibition zone (DIZ) was measured and expressed in millimeters. The mean values of the diameter of inhibition zones were calculated.

2.3.4. Microtiter Broth Dilution Method for Determination of MIC and MBC

The minimum inhibitory concentration (MIC) for ethanol 80% juice extracts were estimated using 96-flat well microtiter broth dilution method. The test was performed in sterile 96 well. Juice extracts were dissolved in 10% DMSO then the two folds serial dilution of products was added to the wells, starting from 25 mg/ml as higher active concentration. Each well included 100 µl of the growth medium, around 10 µl bacterial suspensions adjusted to 0.5 McFarland turbidity were added. Bacterial suspension were used as growth control, broth as a sterility control. The plates were covered and incubated for 24 hr at 37°C. After that 30 µl of 3- (4, 5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) at a final concentration 0.5 mg/ml freshly prepared in water was added to each well and incubated for 30 min. The change to violet colour indicated that the bacteria were biologically active. The MIC was taken to the well, where no change of colour of MTT was observed [14]. while to determine the minimum bactericidal concentration (MBC), 10µL of each of the wells without bacterial growth was placed on a sterile Muller Hinton agar plate then incubated at 37°C for 24 hours. The MBC was determined as the lowest concentration that no visible microbial growth showed.

2.4. Statistical Analysis

All procedures for antibacterial activity were repeated at least three times and the mean value were estimated using Microsoft Excel 2007.

Table 1. Multidrug Resistance Pattern of Bacteria Against Different Antibiotics

Standard antibiotics						
Bacteria	C	B	Az	Ce	N	MAR index for isolated bacteria
Bacterial strains G (+)						
Staphylococcus aureus	S	S	S	R	S	0.2
Staphylococcus auricularis	S	S	S	S	S	0
Streptococcus mitis	S	S	S	R	S	0.2
Streptococcus salivarius	S	R	S	R	S	0.4
Streptococcus pneumoniae	S	R	S	R	S	0.4
Bacterial strains G (-)						
Klebseilla pneumoniae	S	R	S	S	S	0.2
Escherichia coli	S	R	S	R	S	0.4

C:Chloramphenicol/ B:Bacitracin/ Az:Azithromysin/ Ce:Ceftriaxone/ N:Norfloxacin/MAR : Multiple antibiotic resistance.

3. Results and Discussion

With an increase in the antibiotic use by humanity increase chance of accepting resistance within a bacterial community. In the present study *Staphylococcus auricularis* was found to be sensitive to all used antibiotics, *Staphylococcus aureus*, *Streptococcus mitis* and *Klebseilla pneumoniae* were sensitive to four used antibiotics, while *Streptococcus salivarius, Streptococcus pneumoniae* and *Escherichia coli* sensitive only against three antibiotics the results showed in Table 1. These results are nearly consistent with the previous data of other researchers [15,16]. The MAR (Multiple antibiotic resistance) index is an important way to analyze the development of bacterial resistance within the environment. MAR indices were between 0.1 and 0.5 [17]. In this study observed that MAR index for *Streptococcus salivarius, Streptococcus pneumoniae* and *Escherichia coli* were 0.4in which indicated that for management of bacterial infections in human have been exposed to several antibiotics and lead to acquiring resistance to most of antibiotics.

Table 2. Antimicrobial Activity of Peel and Juice Extracts of Four Citrus Fruits

Diameter of inhibition zone (DIZ) in mm (Mean ±SD)													
Bacteria	*Citrus limon*			*Citrus paradisi*			*Citrus sinensis*			*Citrus reticulata*			DMSO
	P	J	P&J	P	J	P&J	P	J	P&J	P	J	P&J	
Bacterial strains G (+)													
Staphylococcus aureus	R	20±0.0	32±0.1	R	R	R	R	R	R	R	R	15±0.0	0
Staphylococcus auricularis	R	22±0.3	35±0.0	R	R	22±0.1	R	R	19±0.0	R	R	15±0.3	0
Streptococcus mitis	R	18±0.0	25±0.0	R	R	15±0.0	R	R	R	R	R	R	0
Streptococcus salivarius	R	17±0.2	25±0.2	R	R	R	R	R	R	R	R	20±0.1	0
Streptococcus pneumoniae	R	15±0.0	27±0.1	R	R	8±0.0	R	R	R	R	R	18±0.0	0
Bacterial strains G (-)													
Klebseilla pneumoniae	R	16±0.1	20±0.2	R	R	R	R	R	R	R	R	R	0
Escherichia coli	R	20±0.3	22±0.2	R	R	25±0.2	R	R	R	R	15±0.3	20±0.02	0

P. Peel; J. Juice; DMSO. Dimethyl sulfoxide.

Table 3. MIC of *Citrus limon* and *Citrus reticulata* Juice Extracts Against Gram Positive and Gram Negative Bacteria

Bacteria	*Citrus limon*		*Citrus reticulata*	
	MIC	MBC	MIC	MBC
Bacterial strains G (+)				
Staphylococcus aureus	3.125	6.25		
Staphylococcus auricularis	0.781257	1.56256		
Streptococcus mitis	0.781257	1.56256		
Streptococcus salivarius	3.125	6.25		
Streptococcus pneumoniae	1.56256	3.125		
Bacterial strains G (-)				
Klebseilla pneumoniae	3.125	6.25		
Escherichia coli	3.125	6.25	3.125	6.25

From four citrus fruits used in this study for evaluation of their antimicrobial activity against five gram positive and two gram negative bacteria, initially determined by agar well diffusion method followed by quantitative evaluation antimicrobial activity by microtiter broth dilution method. All of isolated bacteria showed resistance against peel extracts of four citrus fruits. Juice extracts of *Citrus limon* showed highest inhibitory effect (22 mm) against *Staphylococcus auricularis* with lowest MIC and MBC value (0.781257 and 1.56256), followed by (20 mm) against *Staphylococcus aureus* and *Escherichia coli* with MIC and MBC value (3.125and 6.25), then against *Streptococcus mitis* (18mm), *Streptococcus salivarius* (17mm), *Klebseilla pneumoniae* (16mm), *Streptococcus pneumoniae* (15mm) with MIC and MBC value ranged between (0.781257 and 6.25). Juice extracts of *Citrus reticulata* showed inhibitory effect only against *Escherichia coli* (15mm) with MIC and MBC value (3.125 and 6.25). While juice extract of *Citrus paradisi* and *Citrus sinensis* were not showed any inhibitory effect on isolated bacteria. On combination peel and juice extracts of each citrus fruits separately, *Citrus limon* and *Citrus sinensis* showed highest activity (35mm and 19mm) against *Staphylococcus auricularis* and lowest activity (20mm) against *Klebseilla pneumoniae*. *Citrus paradisi* showed highest activity (25mm) against *Escherichia coli* and lowest activity (8mm) against *Streptococcus pneumoniae.* While *Citrus reticulata* showed highest inhibitory activity (20mm) against *Escherichia coli* and *Streptococcus salivarius*, while lowest activity (15mm) against *Staphylococcus aureus* and *Staphylococcus auricularis* the results showed in Table 2 and Table 3. Our study is in agreement with the study done by Adedeji *et al*., [18] who reported on antimicrobial activity of *Citrus limon* and *Citrus paradisi* juice against gram positive and gram negative bacteria such *as Pseudomonas aeruginosa, Staphylococcus aureus, proteus, klebsiella pneumoniae, Escherichia coli* and *Streptococcuus pneumoniae.* While

in contrast to the present study Hindi and Chabuck [19] reported that Peel extract *Citrus limon* showed activity against *Klebsiella pneumoniae* and *Staphylococcus aureus* but no effect on *Streptococcuus pneumoniae* and *Escherichia coli*. According to other study done by Youseif *et al.*, *[20]* reported on the antimicrobial activity *Citrus reticulata* and *Citrus sinensis* juice against *Escherichia coli* and *Staphylococcus aureus*. Tahera *et al*., [21] reported that ethanol peel extract of *Citrus sinensis* showed inhibitory effect on *Escherichia spp, Klebsiella spp* but no effect on *Staphylococcus spp*. This antimicrobial effect of citrus fruit due to the presence of some substances with antibacterial activity including flavonoids such as hesperidin, eriocitrin, luteolin, epigenin, phenolic acid, dietary fiber, ascorbic acid, citric acid, limonene, linalool, linalyl acetate, turpinol, and cymen [22,23]. The antioxidant activities of citrus flavonoids and phenolic compounds exhibited a potent antibacterial activity which is probably due to their ability to complex with bacterial cell walls and disrupt microbial membrane [24,25]. On comparison of antimicrobial activity different citrus fruits the results showed that *Citrus limon*>*Citrus reticulata*> *Citrus paradisi*>*Citrus sinensis* the results of this study were in agreement with the study conducted by other workers on Egyptian citrus fruit species [20].

The results of Figure 1 and Figure 2 revealed that *Citrus limon* juice and combination extract showed activity against all gram positive and negative bacterial strain 100%, followed by *Citrus reticulata* combination extract effect on 80% gram positive, while juice alone and in combination with peel effect on 50% gram negative bacterial strain. Combination of peel and juice extract of *Citrus paradisi* showed effect on 60% gram positive and 50% gram negative bacterial strain. while *Citrus sinensis* only effect on 20% gram positive bacteria. The results supported by previously recorded data in which Citrus fruit have high antimicrobial activity against gram positive than gram negative bacterial strain [26].The difference in sensitivity of the Gram-positive bacteria compared to that of Gram-negative bacteria to antibiotics and antimicrobial natural products may due to differences in their cell wall composition [27].

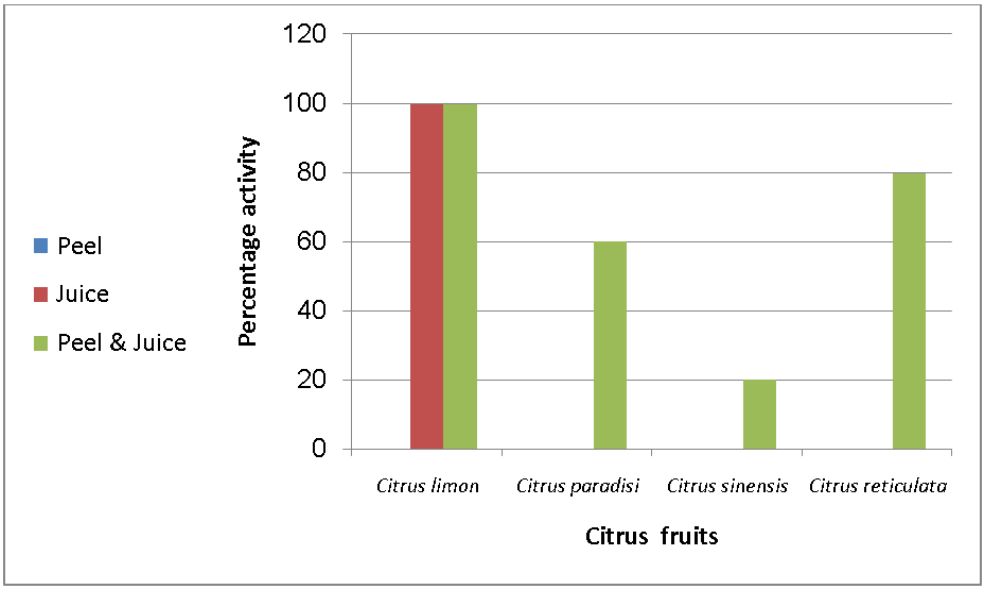

Figure 1. Percentage antimicrobial activity of citrus fruits against gram positive strains of bacteria

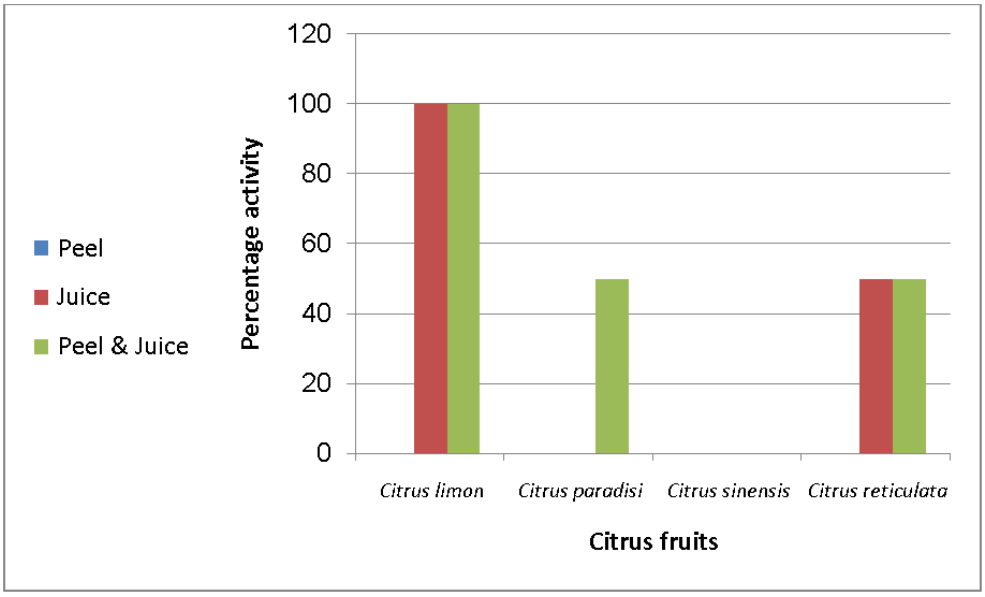

Figure 2. Percentage antimicrobial activity of citrus fruits against gram negative strains of bacteria

4. Conclusions

From the result concluded that most of isolated bacteria was highly resistance to bacitracin and ceftriaxone. As a result concluded that juice have higher antimicrobial activity than peel. On comparison of antimicrobial activity different citrus fruits the results showed that *Citrus limon* > *Citrus reticulata* > *Citrus paradisi* > *Citrus sinensis.* Citrus fruit have high antimicrobial activity against gram positive than gram negative bacterial strain.

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Conflict of Interest

Authors have declared that no competing interests exist.

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