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Phytochemical analysis and evaluation antibacterial activity of *Citrus medica* peel and juice growing in Kurdistan/Iraq

Aveen Nozad Adham

Department of Pharmacognosy, College of Pharmacy, Hawler Medical University, Erbil, Kurdisatn region, Iraq.

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ABSTRACT

Citrus medica is an important medicinal plant of the family Rutaceae Qualitative and quantitative screening were performed for evaluation bioactive constituents in *Citrus medica* fruit. The antibacterial activity of the ethyl acetate and ethanol 80% peel extracts and juice of *Citrus medica* was assessed by agar well diffusion assay, minimum inhibitory concentration (MIC) value determination by 96 well serial dilution against five gram positive and two gram negative bacteria, while minimum bactericidal concentration (MBC) evaluated by plate cultures. Qualitative investigation shows the presence of carbohydrates, flavonoids, phenols, tannins, steroids, cardioactive glycosides in peel and juice, while saponins, terpenoids, and anthraquinones absent in all fraction of *Citrus medica*. On quantitative screening peel contain highest quantity tannin as compared to juice while juice contain higher quantity phenol and *Citrus medica* fruits contain high quantity phenol than flavonoids. The juice of *Citrus medica* exhibited largest inhibition zone (12mm) at (100mg/ml) and recorded the lowest MIC and MBC value (1.5625mg/ml & 3.125mg/ml) against *Staphylococcus auricularis*. The ethyl acetate extract of peel revealed antibacterial activity against four bacterial strain while ethanol 80% only against two bacteria. Peel extracts exhibited largest inhibition zone (10 & 22mm) at (100mg/ml) and recorded the lowest MIC value (25mg/ml & 12.5mg/ml) and MBC (50 mg/ml) against *Escherichia coli*.

INTRODUCTION

Genetic ability of bacteria to acquire resistance against antibacterial drugs due to their widely use in medicine, animal and agriculture encourage the search for new antibacterial agents for the establishment alternative therapies against resistant bacteria (Abeysinghe, 2010). Generally Gram negative bacteria are more resistant than Gram positive bacteria (Parekh *et al*., 2005). Medicinal plant is the most important source for manufacturing different drugs due to their active constituents which are responsible on therapeutic activity because of their structural diversity & biological functionality constituents which is indispensable for drug discovery (Salih and Abass, 2003). Those constituents can be extracted from different part of plant like barks, leaves, fruits seeds and fruit rinds etc (Parekh and Chanda, 2007). *Citrus* is one of the most important commercial fruit crops grown in all continents of the world. Among the different species of citrus fruits the *Citrus medica* is an important medicinal plant of the family Rutaceae which is commonly known as citron or otroj is an evergreen small tree (2.4-4.5 m) high, having large fruit (20-22.5 cm. long) resembling pineapple in shape and mostly grown near the Mediterranean, Iran, Central and South America (Rafiee *et al*., 2007), India (Anonymous, 2001). *Citrus medica* traditionally used as an appetizer, carminative, refrigerant, stomachic, tonic, antispasmodic, expectorant, cardiotonic, and induration of the spleen tumors (Hartwell, 1982). *Citrus medica* peel is eaten raw with rice, also in remedy for dysentery (Fleisher, 1991; Bhuiyan *et al*., 2009). *Citrus medica* is relevant to treatment of diabetes and Alzheimer's disease (Filomena, 2007). The plant is reported to possess anthelmintic Activity (Bairagi *et al*., 2011), antioxidant activity (Al yahya *et al*., 2013), antimicrobial activity (Kabra *et al*., 2012), anti-inflammatory and pain reducing activity in rats (Sood *et al*., 2009). The fruit juice exerts antimutagenicity and anticancer effect (Entezari *et al*., 2009). There are several report on essential oil composition such as limonene, geranial, neral (Theanphong *et al*., 2008), γ-terpinene (Shiota, 2006). *Citrus medica* also known to contain coumarin compounds, p-coumaric acids, steroids, triterpenoids (Feng *et al*., 2004; Yin and Lou, 2004), scoparone, scopoletin, umbelliferone, vitamin c, citroflavonoids which is hesperidoside, naringoside and ecryodietyoside, in addition to

* *Corresponding Author*
Email: aveennawzad@yahoo.com

glucosides hesperidin and rutin (Sood *et al*., 2009; Khare, 2007). The aim of this study was to evaluate the activity of the juice and different solvent extract of *Citrus medica* peels against gram-positive and gram-negative isolated bacterial strains in vitro which some of them not previously tested, also active extracts were evaluated for determine their minimum inhibitory concentrations, minimum bactericidal concentration and analysis their bioactive constituents which are responsible for their activity.

MATERIALS AND METHODS

Collection and extraction of *Citrus medica* fruits

Fresh fruits of *Citrus medica* were collected from garden in Erbil city 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 peel of fresh fruit removed and 200 g was weighed then extracted successively with 500 ml ethyl acetate yielding ethyl acetate fraction, the residue dried and re-extracted by 80 % ethanol yielding ethanol fraction using ordinary reflex extraction. The extract was filtered using Whatman filter paper (No. 1) and then concentrated in vacuum at 40^{o} C using a rotary evaporator. While their juice obtained by hand squeezing the fruits then evaporated to dryness at 40 ^{o}C under vacuum. Peel and juice extract were kept at 4 ^{0}C until used for further study.

Phytochemical screening

Qualitative phytochemical screening

The ethyl acetate, ethanol 80% peel extracts and juice of *Citrus medica* were analysed for presence of carbohydrates, alkaloids, flavonoids, phenols, tannins, saponins, steroids, terpenoids, aminoacids, coumarin, anthraquinones, cardioactive glycosides by standard procedures (Tiwari *et al.,* 2011; Sofowra, 1993).

Quantitative phytochemical screening

Estimation of total phenolic content

The total phenolic content was determined by the spectrophotometric method (Kim *et al*., 2003) with slight modification. In brief a 1 ml of ethanol 80% peel extract and juice were mixed with 1 ml of Folin-Ciocalteu's phenol reagent. After 5 min, 10 ml of a 7% Na_2CO_3 solution was added to the mixture followed by the addition of 13 ml of distilled water and mixed thoroughly. The mixture was kept in the dark for 90 min, after which the absorbance was read at 750 nm. The total phenolic content was determined from extrapolation of calibration curve which was made by preparing gallic acid solution (20-100 µg/ GA and expressed as (µg GA/ g) of dried extract.

Estimation of total flavonoids content

The total flavonoids content of *Citrus medica* peel and juice was estimated by method described by (Zhishen *et al*., 1999) with slight modification. A volume of (1ml) of ethanol 80% peel extract and juice were mixed with 4ml of distilled water and subsequently with 0.30ml of a $NaNO_2$ solution (10 %). After 5 min, 0.30 ml $AlCl_3$ solution (10 %) was added fallowed by 2.0ml of NaOH solution (5 %) to the mixture. Immediately, the mixture was thoroughly mixed and absorbance was then determined at 510 nm versus the blank. Standard curve of quercetin was prepared (20-100 µg/ml) and the results expressed as quercetin equivalents (µg quercetin/g dried extract).

Estimation of total tannins content

The tannin content was determined using Folin-Ciocalteu assay (Tamilselvi *et al*., 2012) with slight modification. A volume of 100 µL of ethanol 80% peel extract and juice were added to 750 µL of distilled water, 500 µL Folin-Ciocateu reagent and 1000 µL of 35 % sodium carbonate (Na_2CO_3). The mixture was shaken vigorously after diluting to 10 mL of distilled water, then incubated for 30 min at room temperature and read at 725 nm using Genway 6305 UV-Vis spectrophotometer. Distilled water was used as blank. The total tannins content was calculated from the prepared standard curve with 20 - 100 µg/ GA and expressed as (µg GA/ g) dry extract.

Antibacterial activity assay

Bacterial strain and growth condition

The antibacterial activity of peel extracts and juice were assessed against seven bacteria species in which five gram positive *Staphylococcus aureus, Staphylococcus auricularis, Streptococcus mitis, Streptococcus salivarius, Streptococcus pneumoniae* and two gram negative *Klebseilla pneumoniae, Escherichia coli*. The isolated pathogenic bacteria were used in this work identified and confirmed after morphological studies and by using biochemical tests in the Microbiological Laboratory of the Biological department, Collage Of Education, Salahadin University. All bacteria were grown on Muller Hinton and blood agar at 37 ºC for 24 h, and then stored at 4 °C until used.

Agar well diffusion method

The antibacterial activity of peel (Ethyl acetate and 80% ethanol extracts) and juice of *Citrus medica* against seven isolated pathogenic bacteria was evaluated by using agar well diffusion method (Ahmad and Beg, 2001; Srinivasan *et al*., 2001) with slight modification. Petridish were inoculated with 100µl of standardized inoculum bacterium and spread with sterile swabs. 6mm wells were made with sterile borer into Muller Hinton agar plates containing the bacterial inoculum. 50µl volume of the peel extracts and juice were poured into a well of inoculated plates. DMSO and Tween 80 were used as a negative control which was introduced into a well instead of fruit extract. Commercially available standard antibiotics (Chloramphenical and bacitracin) as a positive control for comparative study. The plates were incubated for 24 hrs at 37 °C, following incubation the plates were observed. The diameter of inhibition zone (DIZ) was measured and expressed in millimeters. The mean values of the diameter of inhibition zones were calculated.

Determination of minimum inhibitory and minimum bactericidal concentration

The minimum inhibitory concentration (MIC) for the peel extracts and juice were estimated using 96- flat well microtiter broth dilution method. The test was performed in sterile 96 well. All the extracts were dissolved in 10% DMSO and 20% Tween then the two folds serial dilution of products was added to the wells, starting from 100 mg/ml as higher active concentration. 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 ^{0}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. While minimum bactericidal concentration (MBC) of extract were determined by sub-culturing the wells that showed nonturbid only. Following incubation for 24 h at 37 0C the concentration of extract that does not showing bacterial growth after sub-culturing considered as MBC (Umeh *et al*., 2005; Basri and Fan, 2005).

Statistical analysis

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

RESULTS

The phytochemical characteristics of peel extracts and juice of *Citrus medica* studied were summarized in (table 1) the results revealed presence of carbohydrates, flavonoids, phenols, tannins, steroids, cardioactive glycosides in peel extracts and juice, while saponins, terpenoids, and anthraquinones abscent in all fraction of *Citrus medica*.

Table 1: Qualitative phytochemical screening of peel and juice of *Citrus medica*.

Chemical tests	*Citrus medica* peel		*Citrus medica* Juice
	Ethyl acetate	Ethanol 80%	
Carbohydrates	+Ve	+Ve	+Ve
Alkaloids	-Ve	-Ve	+Ve
Flavonoids	+Ve	+Ve	+Ve
Phenols	+Ve	+Ve	+Ve
Tannins	+Ve	+Ve	+Ve
Saponins	-Ve	-Ve	-Ve
Steroids	+Ve	+Ve	+Ve
Terpenoids	-Ve	-Ve	-Ve
Aminoacids	-Ve	+Ve	+Ve
Coumarin	+Ve	+Ve	-Ve
Anthraquinones	-Ve	-Ve	-Ve
Cardioactive glycosides	+Ve	+Ve	+Ve

+Ve: Presence ; -Ve: Abscence

Alkaloids only present in juice, amino acids in juice and 80% ethanol fraction of peel, coumarin present in ethyl acetate and 80 % ethanol fraction of peel. The results of quantitative screening are shown in (table 2, figure 1-3) were expressed as mg/g of dry extract revealed that different amount of bioactive constituents present in peel and juice of *Citrus medica*.

Table 2: Total phenolic, flavonoid and tannin content in peel and juice of *Citrus medica*.

Phytochemicals	Peel	Juice
Total phenolic content (mg/g of dry extract)	21.18	9.38
Total flavonoid content (mg/g of dry extract)	4.59	1.44
Total tannin content (mg/g of dry extract)	32.20	8.3

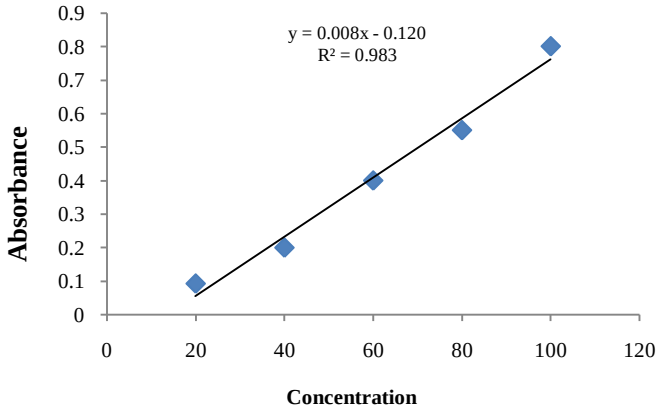

Fig. 1: Linear regression plots and correlation coefficients of total phenols contents of *Citrus medica*.

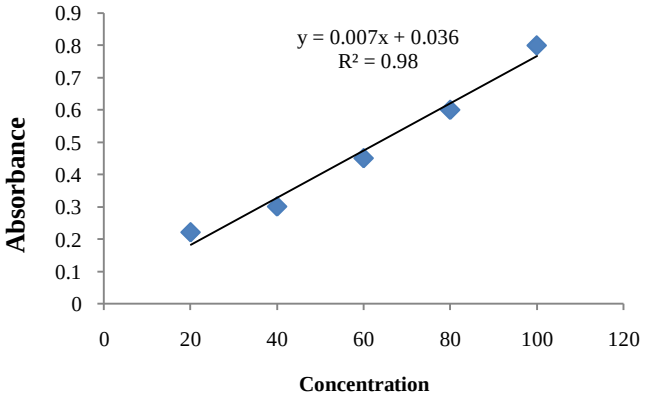

Fig. 2: Linear regression plots and correlation coefficients of total flavonoid contents of *Citrus medica*.

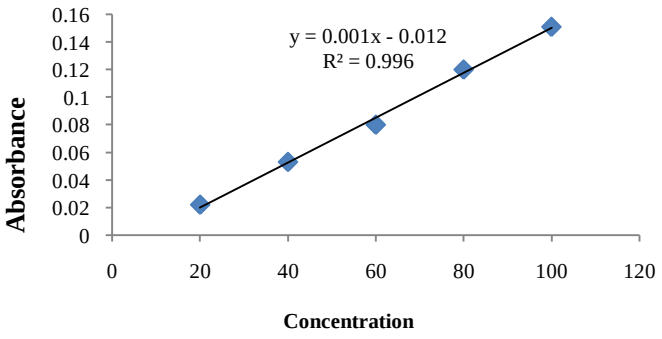

Fig. 3: Linear regression plots and correlation coefficients of total tannin contents of *Citrus medica*.

Table 3: In vitro antibacterial activity of peel extracts and juice of *Citrus medica*.

Bacteria	Diameter of inhibition zone (DIZ) in mm										
	E a (mg/ml)			E 80% (mg/ml)			J (mg/ml)			Chloram.	Bacitr.
	400	200	100	400	200	100	400	200	100	10µg/ml	10µg/ml
Bacterial strains G (+)											
Staphylococcus aureus	10	7	R	R	R	R	16	10	R	18	5
Staphylococcus auricularis	R	R	R	10	R	R	23	15	12	15	15
Streptococcus mitis	R	R	R	R	R	R	20	13	10	25	25
Streptococcus salivarius	R	R	R	R	R	R	R	R	R	10	R
Streptococcus pneumoniae	7	3	R	R	R	R	16	14	10	17	R
Bacterial strain G (-)											
Klebseilla pneumoniae	7	6	3	R	R	R	13	10	7	10	R
Escherichia coli	15	13	10	35	30	22	R	R	R	25	R

E a: Ethyl acetate extract; E 80%: Ethanol 80% extract; J: Juice; Chloram.: Chloramphenicol; Bacitr.: Bacitracin; R: Resistance.

Table 4: Determination of MIC and MBC of peel extracts and juice of *Citrus medica* .

Bacteria	MIC (mg/ml)			MBC (mg/ml)		
	E a	E 80%	J	E a	E 80%	J
Bacterial strain G (+)						
Staphylococcus auricularis	--	--	1.5625	--	--	3.125
Streptococcus mitis	--	--	6.25	--	--	12.5
Streptococcus pneumoniae	--	--	6.25	--	--	25
Bacterial strain G (-)						
Klebseilla pneumoniae	12.5	--	3.125	25	--	6.25
Escherichia coli	25	12.5	--	50	50	--

The ethanolic 80% extract of peel was highest tannin content (32.2mg/g) followed by phenolic (21.18 mg/g) and flavonoid contents (4.59mg/g), while juice contain high quantity phenol (9.38 mg/g), tannin (8.3 mg/g) then flavonoid (1.44 mg/g). The antibacterial activities of *Citrus medica* against seven isolated bacterial strains examined in the present study and their potency were qualitatively and quantitatively assessed by the presence or absence of inhibition zones and zone diameter by agar well diffusion, MIC and MBC values. The ethyl acetate, ethanol 80% peel extracts and juice showed varied antibacterial activity against the studied isolates gram positive and gram negative bacteria. As can be seen from (table 3) ethyl acetate extract exhibited highest antibacterial activity (10mm) against *Escherichia coli*, followed by (3 mm) against *Klebseilla pneumoniae* at (100mg/ml) and (7 mm& 3mm) against *Staphylococcus aureus* and *Streptococcus pneumoniae* at (200 mg/ml) while showed no activity against the other bacteria even at (400 mg/ml). The minimum inhibitory effect of ethyl acetate was recorded against *Klebseilla pneumoniae* and *Escherichia coli* (12.5 & 25 mg/ml) and minimum bactericidal concentration was (25 & 50mg/ml) respectively (table 4). The ethanol 80% extract also shown highest antibacterial activity at minimum concentration 100mg/ml against *Escherichia coli* (22mm), followed by (10mm) against *Staphylococcus auricularis* at (400mg/ml) and not exhibited activity against other bacteria. The minimum inhibitory effect of ethanol 80% was recorded against *Escherichia coli* (12.5 mg/ml) and minimum bactericidal concentration was (50mg/ml). The results of juice have shown some variation, the high antibacterial activity at (100mg/ml) was revealed against *Staphylococcus auricularis* (12mm), followed by *Streptococcus mitis*, *Streptococcus pneumoniae* (10mm) and *Klebseilla pneumoniae* (7mm), then against *Staphylococcus aur*eus (10mm) at (200mg/ml) and not showed any activity against other bacteria. The minimum inhibitory effect of juice was recorded against *Staphylococcus auricularis* (1.5625 mg/ml) with MBC (3.125mg/ml), followed by *Klebseilla pneumoniae* (3.125mg/ml) with MBC (6.25mg/ml) then *Streptococcus mitis*, *Streptococcus pneumoniae* (6.25mg/ml) with MBC (12.5& 25 mg/ml) respectively.

DISCUSSION

Plants and herbal remedies are source most important bioactive constituent should be investigated for their antibacterial activity for finding novel antibacterial drugs with less adverse effect and high ability to kill gram positive and gram negative bacteria (Nascimento *et al*., 2000). Bioactive constituent of plant are non-nutritive posses specific chemicals structure help in their disease preventive and antimicrobial activities and work with different mechanism of action (Panda and Bandyopadhyay, 2013). Results of qualitative phytochemical screening in (table 1) clearly demonstrated the presence of number important active constituents. Presence of carbohydrates, flavonoids, phenols, steroids and absence of saponins, anthraquinones, coumarin in *Citrus medica* in agreement with previously recorded data (Kabra *et al*., 2012; Chan *et al*., 2010), while presence of tannins, cardioactive glycosides in peel and juice and absence of alkaloids in peel of Iraqi species in contrast to the same study (Kabra *et al*., 2012) . The quantitative determination of plant bioactive constituents in this study the results showed in (table 2) revealed that phenolic content (21.18 & 9.38 mg/g dry extract) in *Citrus medica* was higher than flavonoid content (4.59& 1.44 mg/g dry extract) in peel and juice respectively are in accordance with those carried out in Riyadh, Saudi Arabia in which *Citrus medica* fruits showed high total phenol content (192.4 mg GA /100 g) than flavonoid values (74.1 mg quercetin /100 g) (Al-Yahya *et al*., 2013). while their levels in this study are much higher than those

measured in *Citrus medica* from Riyadh indicating that the contents can be influenced by several factors like extraction methods, harvest time, genotypic differences, geographical and climatic conditions and cultural practices (Vander *et al*., 2001). Quantitative estimation of tannin in *Citrus medica* not previously recorded. In this study the antibacterial activity of peel extracts and juice against five gram positive and two gram negative bacteria was evaluated. The diameter of inhibition zone was measured by agar well diffusion assay are presented in (table 3). The results indicated that juice showed antibacterial activity against five bacterial strain followed by ethyl acetate fraction against four bacterial strain then ethanol 80% fraction only against two bacterial strain.

The antibacterial activities of peel extracts and juice varied with the type of test organism. Peel extracts revealed stronger activity against gram negative when compared to gram positive bacteria while juice have stronger activity against gram positive than gram negative bacteria. *Escherichia coli* highly inhibited by peel extracts as largest zone of inhibitions were obtained. Gram positive bacteria *Staphylococcus auricularis*, *Streptococcus mitis, Streptococcus pneumoniae* showed complete sensitivity against juice of *Citrus medica* obvious zone of inhibition. While *Streptococcus salivarius* demonstrated the complete resistance against juice and peel extracts of *Citrus medica* among all the bacterial species examined in this study (table 3). The antibacterial activity *Citrus medica* against, *Staphylococcus auricularis, Streptococcus mitis, Streptococcus salivarius, Streptococcus pneumoniae* has not been previously described. The antibacterial activity of semi-polar solvent extract of *Citrus medica* against *Klebseilla pneumoniae, Escherichia coli* and *Staphylococcus aureus* and ethanolic extract activity against *Escherichia coli* in agreement with the study conducted by (Tumane *et al*., 2014).

Also Kabra *et al.*, 2012 reported that ethanolic extract of *Citrus medica* showed antibacterial activity when tested against *Klebseilla pneumoniae, Escherichia coli* and *Staphylococcus aureus*. These results are in accordance to the results obtained in the present study about activity against *Escherichia coli* while in contrast for *Klebseilla pneumoniae* and *Staphylococcus aureus*. Negative control using DMSO and Tween 80 used for extract preparation showed no inhibition of any bacteria, indicating that peel and juice itself and not solvent inhibited the growth of bacteria. Chloramphenicol and bacitracin (a positive control) showed variable inhibition diameters ranging from (10 -25 mm and 5-25mm) respectively. Bacitracin showed activity only against three gram positive bacteria. The MIC determined by broth micro dilution method in which gram positive bacteria most susceptible to *Citrus medica* present with lower MIC value than gram negative bacteria.

The high sensitivity of gram positive bacteria may be due to phytoconstituents present in *Citrus medica* which inhibit peptidoglycan synthesis in gram positive bacteria, or may be related to a strong permeability barrier of outer surface gram negative bacteria which due to an outer phospholipidic membrane carrying the structural lipopolysaccharide components which cannot be found in gram positive bacteria (Nostro *et al*., 2000). The lowest MIC value was for juice (1.5625 mg/ml) with MBC (3.125mg/ml) against *Staphylococcus auricularis* and the highest MIC value for ethyl acetate extract (25mg/ml) against *Escherichia coli* with MBC (50mg/ml) (table 4). Surprisingly, ethyl acetate extract have larger inhibition zones and higher MIC value against *Escherichia coli* in compared to *Klebseilla pneumoniae*; this can be probably due to the presence of compounds in this extract which are difficult to spread on the agar surface, but when they are diluted in agar present a high antibacterial activity. As a result concluded that the antibacterial activity of *Citrus medica* due to their bioactive constituent present in it because phenolic acid, tannins, flavonoid (Marjorie, 1996), alkaloids (Okwu and Okwu, 2004), steroids (Raquel, 2007) have been reported to have antibacterial properties.

CONCLUSION

The results demonstrated the presence of bioactive constituents in peel and juice of *Citrus medica* including phenols, tannins, flavonoids, cardioactive glycosides, alkaloids, steroids, and coumarins. Peel extract revealed stronger activity against gram negative while juice have stronger activity against gram positive bacteria. juice showed antibacterial activity against five bacterial strain followed by ethyl acetate fraction against four bacterial strain then ethanol 80% fraction only against two bacterial strain. The antibacterial activity of peel and juice of *Citrus medica* due to the bioactive constituents present in it.

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