SHORT COMMUNICATION

In vitro assessment of antioxidant, antibacterial and phytochemical analysis of peel of *Citrus sinensis*

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Abstract: Antibacterial effect of *Citrus sinensis* peel extracts was evaluated against several pathogenic bacteria associated with human and fish infections *viz*., *Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumonia, Staphylococcus aureus, Streptococcus pyogenes, Staphylococcus epidermidis*, *Serratia marcesnces, Shigella flexneri, Enterobacter amnigenus, Salmonella* Typhimurium and *Serratia odorifera*. Methanol, ethanol, chloroform and diethyl ether solvents were used for extraction. *In vitro* antibacterial activity was analyzed by agar well and agar disc diffusion methods. It was found that ethanol extract showed highly significant inhibition of *E. coli* and *K. pneumonia* (12.6±0.94 mm and 11.6±1.2 mm) whereas methanol extract of *C. sinensis* also showed high zone of inhibition of *S. odorifera* (10.0±2.16 mm). The potential activity of active extracts was assessed and also compared with standard antibiotics through activity index formulation. The order of antioxidant activity through ABTS·+ and DPPH free radical scavenging activity was ethanol>methanol>chloroform>diethyl ether. Phytochemical screening of all solvents had determined the presence of terpenoids, alkaloids, steroids, glycosides and flavonoids. It was also found that Chloroform/Methanol (5:5) and Butanol/Ethanol/Water (4:1:2.2) solvent systems showed significant separation of active phytochemical constituents. These findings reveal the potential use of *C. sinensis* peel to treat infectious diseases, which are being caused by microorganisms.

Keywords: Antibacterial, Antioxidant, Agar disc diffusion method, Bacterial pathogens, *Citrus sinensis*

INTRODUCTION

Infectious diseases are serious health problem worldwide. The use of commercial anti-microbial drugs subjectively in the treatment of many infectious diseases is being led to multiple drug resistance phenomenons in human pathogenic microorganisms (Das *et al*., 2010; Sokmen *et al*., 2004). Because this problem is prevailing at very high magnitude there is an unmet need to find and develop alternative eco-friendly methods to treat bacterial or other infectious pathogens without or with minimal toxicity. In the last few years, researchers had conducted studies on the use of medicinal plants as anti-microbial agents having bioactive compounds (Thenmozhi and Rajeshwari, 2010; Duraipandiyan *et al*., 2006).

Citrus is one of the most essential commercial fruit crops which are grown in all continents of the world (Tao *et al*., 2008). In Pakistan it is cultivated on an area of approx.197, 000 ha with annual production of approx. 1816, 000 tones (Anonymous, 2004). Pakistan is one of the largest citrus producing countries of the world. Citrus peel is used as fodder at fisheries, raw material for traditional paper, activated carbon, cosmetic products and bio-ethanol production (Kim *et al*., 2008; Kim *et al*., 2007; Sharma *et al*., 2007). Likewise, essential oil of citrus peel has been identified to exhibit antibacterial activity (Upadhyay *et al*., 2010; Palakawong *et al*., 2010; Ayoola *et al*., 2008). Due to high anti-microbial effect it is abundantly used in pharmaceutical products (Njoroge *et al*., 2005). It has also been used as an anti-diabetic, larvicidal, anti-microbial, anti-fungal, hypotensive agent, antioxidant, antiviral, insect repellent, antibacterial, anti-mutagenic and anti-yeast agent (Ghasemi *et al*., 2009; Kanaze *et al.,* 2008; Hamendra and Anand 2007; Proteggente *et al.,* 2003; Caccioni *et al.,* 1998; Han, 1998; Stange *et al.* 1993; Kumamoto *et al.,* 1986).

Bacterial food poisoning is the most common problem (Soković *et al*., 2007). Free radicals formed in human metabolism by two ways: 1) naturally released by human body to deactivate the viral and bacterial presence, or 2) due to environmental factors like pollution, smokes, and others. According to Mantena *et al*. (2008) radical chain reactions with DNA, proteins and cell membrane cause harmful effects to human body. On the other hand, antioxidants, enzymes and vitamins are naturally available anti-free radical defense system that is used to

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prevent oxidative damage and also to protect the body from harmful pathogens (Nabavi *et al*., 2013). Hence, the current investigation was aimed to determine the antibacterial and antioxidant activities of various extracts of *Citrus sinensis* against pathogenic bacterial strains.

MATERIALS AND METHODS

Plant material

Citrus sinensis were purchased from the supermarket of Muzaffarabad and brought to Microbial Biotechnology lab, Department of Zoology, University of Azad Jammu and Kashmir (UAJ & K), Muzaffarabad, Pakistan. The fruits were rinsed with running tap water and dried with tissue papers.

Preparation of powder

The peel of ripened fruit was removed with sterilized knife and placed on white papers under shade for drying for 2 weeks. Dried peel was crushed to make fine powder. The powder was stored at room or at normal refrigerator temperature before analysis.

Preparation of extracts

Powder (20g) was homogenized with various solvents *viz.,* ethanol, methanol, chloroform, and diethyl ether according to high polarity for two weeks and used as 100% concentrated extract against tested pathogens. The homogenized mixtures in different solvents were collected, filtered and evaluated for antibacterial and antioxidant activities, and phytochemical screening.

Isolation and culturing of bacterial pathogens

Bacterial pathogens such as *Shigella flexneri, Serratia odorifera, Salmonella* Typhimurium and *Enterobacter amnigenus* (isolated from spoiled fish), *Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumonia, Staphylococcus aureus, Streptococcus pyogenes, Staphylococcus epidermidis* and *Serratia marcesnces* (P236) were isolated from human infected samples like blood, urine and pus at Microbial Biotechnology lab, Department of Zoology, UAJ&K, and their identification was carried out at Combined Military Hospital (CMH), Muzaffarabad, Pakistan.

Antibacterial activity of extracts

Agar disc and agar well diffusion methods were used to determine the antibacterial activity of various extracts of *C. sinensis* peel as described by (Kumar *et al*., 2011; Nwanebu *et al*., 2011). Nutrient agar and Nutrient Broth Media (NAM; Oxide CMOO3 and NBM; CM1) were used for bacterial culture. The overnight culture was mixed with freshly prepared nutrient agar medium (NAM) at 45°C and was poured into the sterilized Petri dishes. All Petri dishes were kept at room temperature in laminar flow for solidification. The 5 mm discs were soaked with various prepared peel extracts and placed on agar surface in case of disc diffusion whereas wells were formed in well diffusion method. The Petri dishes were incubated at 37°C for 48 h. Discs, soaked with chloroform, ethanol, methanol, and diethyl ether were used as control. Microbial growth inhibition was assessed by calculated the zone of inhibited diameter (mm) after 48h of incubation. Before each experiment, the optimal density (OD) of bacterial growth 10^7 colony forming units (cfu)/ml was measured through spectrophotometer at the wavelength 600 nm (Seeley *et al.,* 2001). Each experiment was performed in triplicate. The results of sensitivity tests were denoted as (0) for no sensitivity, (1-3) for low sensitivity, (4-8) for moderate sensitivity and (9 or bigger than 9) for high sensitivity. The antibacterial activity of extracts with standard antibiotics was also assessed by (AI) activity index (Shekhawat and Vijayvergia, 2010).

Sensitivity test of antibiotics

Sensitivity of antibiotics including amoxicillin (10 µg), penicillin G (10µg), ampicillin (10µg) and trimethobrim (10µg) against all tested bacterial strains was assessed by agar disc diffusion method (Baker and Pallister, 1998; Perez *et al*., 1990).

Preliminary Phytochemical screening

The phytochemical analyses of four extracts were carried out according to the methods described by Harborne, 1993; Sofowora, 1986; Trease and Evans, 1983.

Total Phenolic content

Phenolic contents (mg/100ml of extracts) were determined using the Folin-Ciocalteu reagent method described by Zhou and Yu with slight modifications (Zhou and Yu, 2006). The reaction mixture was made with extract (100µl), Folin-Ciocalteu reagent (100µl) and 20% sodium carbonate (3 ml). Reaction mixture was incubated at room temperature for 1h and the absorbance of deep blue complex was measured at 765 nm. Gallic acid was used as a standard with varied concentration from 200ppm to 1000ppm. The total phenolic content was expressed as mg gallic acid equivalents per gram extract weight (mg/100gm).

Total flavonoid content

Estimation of total flavonoid contents of extracts was quantified by the method illustrated by Zou *et al*., (2004). The reaction mixture cocktail containing extracts (500 µl), distilled water (2ml), and 5% $NaNO_2$ (0.15ml) was incubated at room temperature for 6 min. After incubation, 10% $AlCl_3$ (0.15ml) solution was added, the kept for 6 min at room temperature, followed by the addition of 2ml of 4% NaOH solution. After the addition of water to the sample to make the final volume to 10 ml, the mixture was mixed immediately and kept for 15 min further. The absorbance of the reaction mixture was measured at 510 nm. Rutin was used as standard for the calibration curve. Total flavonoid content of extracts and fractions was expressed as mg rutin equivalents (RE) per gram of sample (mg/g).

Table 1: Antibacterial activity of extracts of *Citrus sinensis* peel

Pathogens	Mean±Standard deviation of extracts			
	Diethyl ether (mm)	Chloroform (mm)	Ethanol (mm)	Methanol (mm)
Pseudomonas aeruginosa	3.6±0.46*	4±0.81**	4.33±1.24**	5.33±2.05**
Klebsiella pneumoniae	6.3±0.47**	4.6±0.94**	11.6±1.25***	9.3±1.24***
Serratia marcesnces	3.6±0.47*	4±0.81**	4.6±1.24**	4.6±1.7**
Staphylococcus epidermidis	2.6±0.94*	4.6±0.47**	6±2.14**	5.6±0.94**
Streptococcus Pyogenes	4.6±1.7**	4.6±0.47**	5.8±0.47**	4.3±0.47**
Escherichia coli	4±0.81**	4±1.84**	12.6±0.94***	9±0.81***
Staphylococcus aureus	5.6±0.47**	4.6±0.47**	7.6±0.47**	6±0.81**
Salmonella Typhimurium	3.3±0.47*	5.66±1.24**	4.33±0.94**	6.0±0.81**
Shigella flexneri	2.6±0.94*	1.6±0.47*	7±2.16**	6.3±2.49**
Enterobacter amnigenus	5.3±0.47**	1.3±0.47*	4.3±0.47**	4.3±1.24**
Serratia odorifera	2.6±0.47*	3.0±0.81*	3.3±0.47*	10.0±2.16***

The results of the sensitivity tests were expressed as (0) for no sensitivity, (below 4; *) for low sensitivity, (4-8; **) for moderate sensitivity and (9-14; ***) for high sensitivity.

Thin layer chromatography

The determination of major phytochemicals was further confirmed by thin layer chromatography (TLC) using pre-coated Silica gel 60F264 plates (Wagner and Bladt, 2004). In order to get better resolution of components different screening systems were used (Table 4). The developed plates were observed under UV light (254-336 nm). Retention factor (R_f) value of each spot was calculated as R_f = distance travelled by the solute/ distance travelled by the solvent.

ABTS$^{\cdot+}$ free radical scavenging activity

ABTS$^{\cdot+}$ or 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) free radical scavenging activity was carried out to evaluate the antioxidant potential of *C. sinensis* extracts according to the method described by Re *et al*. (1999). The ABTS stock solution was prepared by reacting potassium persulphate (2.5mM) and ABTS (7mM), then the mixture was kept for at least 16 h to generate $ABTS^+$ free radicals and their absorbance were recorded at 734 nm ($Ao_{Control}$). For tests, 1ml of ABTS running solution was mixed with 20-200 µg/ml of different compounds. The absorbance of test samples (Ai_{Sample}) was also observed at 734 nm. The percentage radical scavenging activity (% RSC) was calculated using the formula: $\%RSC=[(Ao_{Control}-Ai_{Sample})/ Ao_{Control}]\times100\%$.

DPPH Free Radical Scavenging Activity

DPPH [(di(phenyl)-(2,4,6-trinitrophenyl)iminoazanium] free radical scavenging activity of *C. sinensis* extracts was determined with slight modifications (You *et al*., 2006). Half ml of MeOH (0.1mM) solution of DPPH was mixed with 20-150µl of extracts, mixed with vortex vigorously and left for 30 min at 37°C in an incubator The volume was made up to 2ml by adding methanol. Methanol used as a baseline control. The absorbance was recorded at 517nm. Water (20-150 µl) was used as a control and the percentage scavenging activity was calculated by formula: %=[(Ao-Ai)/Ao]*100; where Ao is the absorbance of control and Ai is the absorbance with extracts.

STATISTICAL ANALYSIS

Each experiment was repeated in triplicates and mean ± standard deviation (M±SD) from absolute data were measured through on line Standard deviation calculator http://easycalculation.com/statistics/standard-deviation. php. The percentage of bacterial growth was analyzed statistically with One-way analysis of variance (ANOVA) through http://www.danielsoper.com/statcalc3/calc.aspx?id=43 to distinguish the treatments means (Gomez and Gomez, 2007).

RESULTS

Since decades, herbs, trees and shrubs have used/applied by humans in many ways such as drugs, foods and flavors. Our research is therefore aimed for the verification of the antibacterial activity of *C. sinensis* peel extracts against some food borne organisms such as *E. coli, P. aeruginosa, K. pneumonia, S. aureus, S. Pyogenes, S. epidermidis*, *S. marcesnces (*P236), *S.* Typhimurium (KL2*), S. flexneri* (KL2), *E. amnigenus* (F1), and *S. odorifera* (F). This *in vitro* study was done to provide a direction for the awareness of *C. sinensis* extracts to the population who utilize them to treat various diseases caused by these bacterial pathogens. These pathogens were isolated and identified by the researchers of Microbial Biotechnology Laboratory, Department of Zoology, Muzaffarabad, Pakistan (Ume-Kalsoom *et al*., 2013; Andleeb *et al*., 2013; Rafique *et al*., 2012). The results of the sensitivity tests were expressed as (0) for no sensitivity, (below 4*) for low sensitivity, (4-8**) for moderate sensitivity and (9-14***) for high sensitivity.

Table 2: Activity index analysis against human pathogenic bacteria

Effect of *Citrus sinensis* on human pathogens through activity index					
Pathogens	Solvents				Antibiotics
	Diethyl ether	Chloroform	Ethanol	Methanol	
Pseudomonas aeruginosa	0.6	0.66	0.72	O.88	Amoxillin (6)
	0.6	0.66	0.72	0.88	Penicillin G (6)
	0.17	0.19	0.20	0.25	Trimethobrim (21)
	E>A	E>A	E>A	E>A	Ampicillin (0)
Klebsiella pneumonia	1.05	0.76	1.9	1.55	Amoxillin (6)
	E>A	E>A	E>A	E>A	Penicillin G (0)
	0.9	0.65	1.65	1.32	Trimethobrim (7)
	1.05	0.76	1.93	1.55	Ampicillin (6)
Serratia marcescens	0.6	0.66	0.76	0.76	Amoxillin (6)
	0.45	0.5	0.57	0.57	Penicillin G (8)
	0.45	0.5	0.57	0.57	Trimethobrim (8)
	0.45	0.5	0.57	0.57	Ampicillin (8)
Staphylococcus epidermidis	E>A	E>A	E>A	E>A	Amoxillin (2)
	E>A	E>A	E>A	E>A	Penicillin G (0)
	0.07	0.13	0.18	0.16	Trimethobrim (33)
	E>A	E>A	E>A	E>A	Ampicillin (0)
Streptococcus Pyogenes	E>A	E>A	E>A	E>A	Amoxillin (0)
	E>A	E>A	E>A	E>A	Penicillin G (0)
	E>A	E>A	E>A	E>A	Trimethobrim (0)
	E>A	E>A	E>A	E>A	Ampicillin (0)
Escherichia coli	E>A	E>A	E>A	E>A	Amoxillin(0)
	E>A	E>A	E>A	E>A	Penicillin G (0)
	2.0	2.0	6.3	4.5	Trimethobrim (2)
	E>A	E>A	E>A	E>A	Ampicillin (0)
Staphylococcus aureus	E>A	E>A	E>A	E>A	Amoxillin (0)
	E>A	E>A	E>A	E>A	Penicillin G (0)
	0.25	0.20	0.34	0.27	Trimethobrim (22)
	0.8	0.65	1.08	0.85	Ampicillin (7)
Salmonella Typhimurium	E>A	E>A	E>A	E>A	Amoxillin (0)
	0.36	0.62	0.48	0.66	Penicillin G (9)
	0.47	0.80	0.61	O.85	Trimethobrim (7)
	0.47	0.80	0.61	0.85	Ampicillin (7)
Shigella flexneri	E>A	E>A	E>A	E>A	Amoxillin (0)
	E>A	E>A	E>A	E>A	Penicillin G (0)
	E>A	E>A	E>A	E>A	Trimethobrim (0)
	E>A	E>A	E>A	E>A	Ampicillin (0)
Enterobacter amnigenus	E>A	E>A	E>A	E>A	Amoxillin (0)
	E>A	E>A	E>A	E>A	Penicillin G (0)
	0.24	0.12	0.39	0.24	Trimethobrim (11)
	E>A	E>A	E>A	E>A	Ampicillin (0)
Serratia odorifera	E>A	E>A	E>A	E>A	Amoxillin (0)
	0.65	0.75	0.82	2.5	Penicillin G (4)
	0.32	0.37	0.41	1.25	Trimethobrim (8)
	E>A	E>A	E>A	E>A	Ampicillin (0)

E > A and > 1 values indicate extracts have higher effect against bacterial pathogens compared to antibiotics; less than 1 values indicate antibiotics have higher effect against bacterial pathogens compared to extracts.

Antibacterial activity of extracts

The conventional solvent extraction of the citrus peel using different solvents had yielded different results in each of the experiment conducted in this study. Hence solvents of different polarity were employed in research work as polar: ethanol and methanol; and non-polar: chloroform and diethyl ether. The antibacterial activity of a *C. sinensis* extracts was assayed *in vitro* by agar disc

Table 3: Phytochemical screening of extracts of *Citrus sinensis* peel

Phytochemicals	Solvents			
	Ethanol	Methanol	Diethyl ether	Chloroform
Saponins	+	+	-	-
Tannins	+	+	-	-
Terpenoids	+	+	+	+
Alkaloids	+	+	+	+
Steroids	+	+	+	+
Cardiac glycoisdes	+	+	+	+
Flavonoids	+	+	+	+

+ indicates presence, - indicates absence

Table 4: Thin Layer Chromatography of *C. sinensis* peel extracts

Extracts used	Used Solvent systems							
	CM (5:5)		BAW (4:1:5)		BEW (4:1:2. 2)		BAEW (9:6:5:1)	
	Rf value	Spot color	Rf value	Spot color	Rf value	Spot color	Rf value	Spot color
Diethyl Ether	S1= 0.3 S2=0.7	Yellow Purple	S1= 0.4 S2= 0.7	Yellow Purple	S= 0.8	Yellow	No result	
Chloroform	S1= 0.2 S2= 0.5	Blue Yellow	S= 0.3	Blue	S1= 0.7 S2= 0.8	Purple Blue	No result	
Ethanol	S= 0.3	Yellow	S= 0.3	Pink	S1= 0.2 S2= 0.4	Yellow Purple	No result	
Methanol	S1= 0.5 S2= 0.5 S3= 0.9	Yellow Purple Purple	S= 0.4	Pink	S1= 0.7 S2= 0.4	Yellow Pink	0.6	Purple

Solvents: CM= Chloroform/ Methanol (5:5), BAW = Butanol / Acetic acid / Water (4:1: 5),
BEW = Butanol /Ethanol / Water (4:1:2. 2), BAEW = Butanol / Acetic acid / Ether / Water (9:6:5:1).

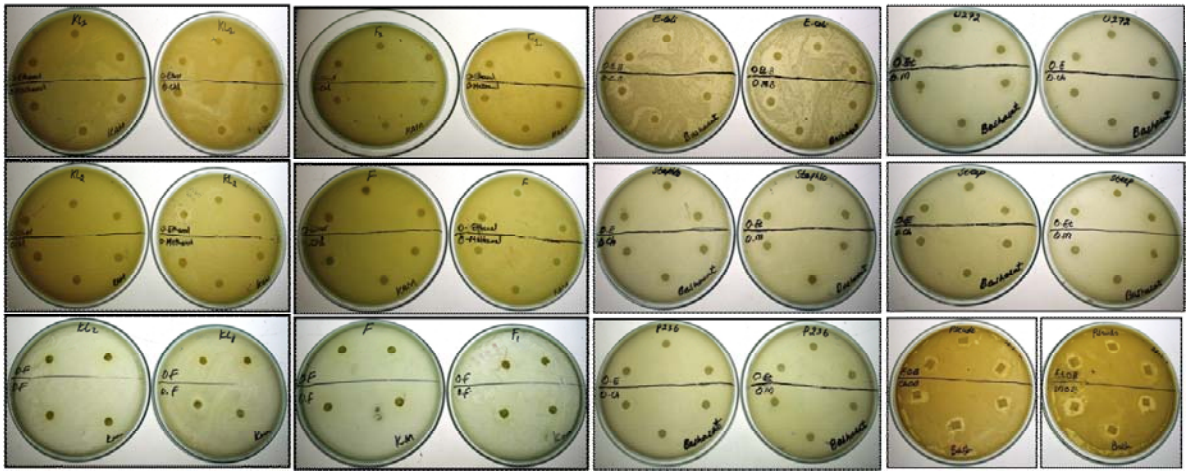

Fig. 1: Anti-bacterial activity of extracts of *C. sinensis* peel against food borne pathogens through agar disc diffusion method. *Salmonella typhimurium* (KL1), *Shigella flexneri* (KL2), *Enterobacter amnigenus* (F1), *Serratia odorifera* (F), *Pseudomonas aeruginosa* (Psuedo), *Staphylococcus aureus* (U272), *Streptococcus Pyogenes* (Strep), *Escherichia coli, Staphylococcus epidermidis* (Staphlo) and *Seratia marcesnces* (P236)

and well diffusion against food-borne bacterial pathogens (fig. 1). Table 1 summarizes the microbial growth inhibition of all extracts of citrus peel. There was no inhibition zone formed in control *viz*., ethanol, methanol, chloroform, and diethyl ether. Among the solvent extracts, ethanol and methanol extracts exhibited the highest antibacterial activity. These extracts were effective against *K. pneumoniae* (11.6±1.25 mm, 9.3±1.24 mm) and *E. coli* (12.6±0.94 mm, 9±0.81 mm; table 1). Methanolic extract of *C. sinensis* also showed significant activity against *S. odorifera* (10.0±2.16 mm) whereas ethanol extract had low effect (3.3±0.47 mm) (table 1). Chloroform extracts exhibited reasonable bioactivity between 4-8 mm against all tested bacterial pathogens except *S. flexneri*, *E. amnigenus* and *S. odorifera* (table 1).

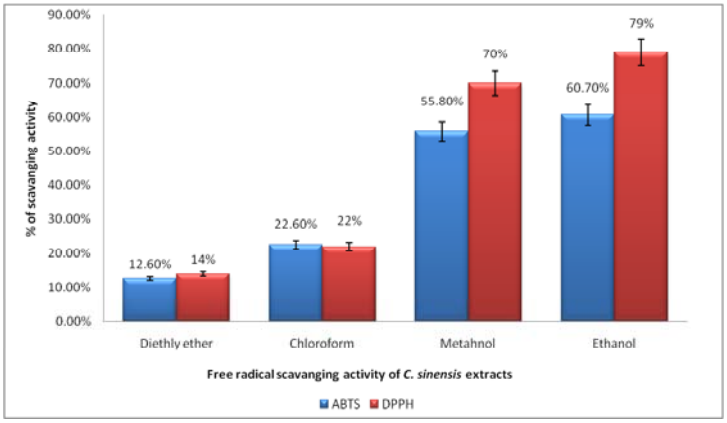

Fig. 2: Scavenging activities of peel extracts of *Citrus sinensis* towards $ABTS^{-+}$ and DPPH free radicals.

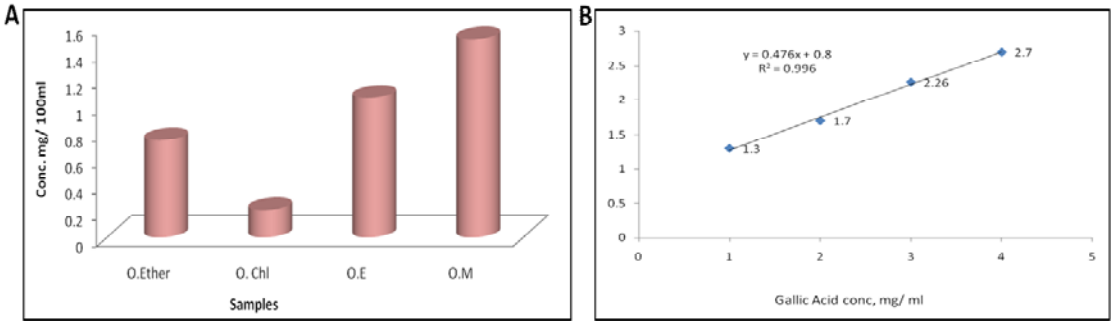

Fig. 3: Gallic acid equivalent phenolic contents in mg/100 ml of *Citrus sinensis* extracts absorbance recorded at 765 nm. A) Gallic acid equivalent phenolic contents of *C. sinensis* extracts. B) Calibration Curve using gallic acid as a standard. O. Ether (extract of *C. sinensis* in diethyl ether); O. Chl. (extract of *C. sinensis* in chloroform); O.E. (extract of *C. sinensis* in ethanol); O.M. (extract of *C. sinensis* in methanol).

Activity index (AI) of extracts

Four antibiotics were used against all tested bacterial pathogens. The maximum inhibition was recorded by trimethobrim against *P. aeruginosa, S. epididermis, S. aureus, and E. amnigenus.* On the other hand, ampicillin, amoxillin, and penicillin G showed both moderate and low effects against all tested pathogens as mentioned in table 2. The sensitivity of antibiotics was compared with different *C. sinensis* extracts through activity index (table 2). Results confirmed the potential use of all extracts as compared to antibiotics except trimethobrim.

Phytochemical screening of extracts

Phytochemical screening indicated the presence of terpenoids, alkaloids, steroids, glycosides and flavonoids in all solvents (table 3). On the other hand, saponins and tannins were present in polar solvents as compared to non-polar. The results of thin layer chromatography of various *C. sinensis* peel extracts showed a common occurrence of different phytochemicals when separated using four solvent systems such as Chloroform/ Methanol (CM: 5:5), Butanol / Acetic acid / Water (4:1: 5), Butanol /Ethanol / Water (BEW: 4:1:2.2), and Butanol / Acetic acid / Ether / Water (BAEW: 9:6:5:1). CM and BEW systems showed significantly high separation followed by BAW system which revealed moderate separation. BAEW system was not favorable for this separation as only methanolic extract of orange had showed separation in this system (table 4).

Antioxidant activity of extracts

The antioxidant activity of methanol and ethanol extracts of *C. sinensis* peel showed a significant free radical scavenging activity generated by ABTS (55.8% and 60.7%) as compared to other solvent extracts i.e chloroform and diethyl ether (fig. 2). Similar results were also found by DPPH assay. The methanolic and ethanolic extracts of *C. sinensis* peel showed 70 to 80% DPPH scavenging activity based on its capability as hydrogen donator (fig. 2).

Total phenolic contents

Phenolic antioxidants are included in the category of free radical terminators. Natural antioxidants are primarily plant phenolics and polyphenolic compounds that may present in different parts of plants. Current research determined the concentration of phenolic contents in *C. sinesis* extracts as these contents are higher in methanol extracts, followed by ethanol extracts, moderate in ether extracts and very low in chloroform extracts (fig. 3A). Calibration Curve using gallic acid as a standard is also shown in fig. 3B.

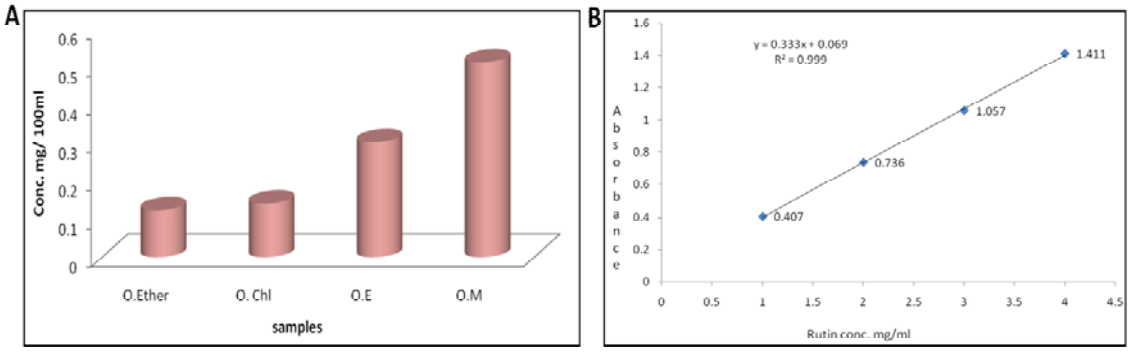

Fig. 4: Rutin equivalent flavonoid contents in mg/100 ml of *Citrus sinensis* extracts absorbance recorded at 510 nm. **A)** Rutin equivalent flavonoid contents in mg/100 ml of *C. sinensis* extracts. **B)** Calibration Curve using rutin as a standard. O. Ether (extract of *C. sinensis* in diethyl ether); O. Chl. (extract of *C. sinensis* in chloroform); O.E. (extract of *C. sinensis* in ethanol); O.M. (extract of *C. sinensis* in methanol)

Total flavonoid content

A reasonable quantity of flavonoids was found in all tested *C. sinensis* extracts (fig. 4A). The presence of flavonoids was gradually increased as moving towards more polar solvent extracts as compared to non-polar. Methanolic extracts expressed higher flavonoid contents than rest of all tested extracts (fig. 4A). The calibration Curve using rutin as a standard was also shown in fig. 4B.

DISCUSSION

Antibacterial activity

In current research the extracts of *C. sinensis* were prepared through conventional solvent extraction method using both polar: ethanol and methanol; and non-polar: chloroform and diethyl ether solvents which revealed the significant results. These extracts showed considerable antibacterial activity against tested pathogens. Similar results were obtained by Ekwenye and Edeha, (2010) against *K. pneumoniae, E. coli, and S. aureus*. On the other hand, moderate bacterial growth inhibition was recorded with diethyl ether extract against *K. pneumoniae*, *S. Pyogenes, S. aureus, E. coli* and *E. amnigenus* (table 1). It was found that diethyl ether extract showed low sensitivity of *S. epidermidis, S. marcesnces*, and *P. aeruginosa*. Kabra *et al*. (2012) also found that the ethanolic extract of citrus peel was effective against *S. aureus, K. pneumonia*, *B. subtilis*, *P. vulgaris, E.* coli and *P. aeruginosa.* Our results are not agreed with other studies such as non-polar solvents did not show any significant effect against bacterial pathogens except *E. coli.* This may indicate that these solvents have the capacity of extracting the antibacterial agent, which may be extremely toxic to *K. pneumoniae*, *S. Pyogenes, S. aureus*, *E. coli*, and *E. amnigenus*. Thus, it points out that diverse extracts may have different antibacterial agent, which may have different modes of action. *C. sinensis* peel oil showed inhibition of *E. coli and B. subtilis* (Amandeep *et al.,* 2009).

Alterable antibacterial activity of different extracts of *Citrus* was in concurrence with previous studies (Palakawong *et al*., 2010; Pandey *et al*., 2010; Nannapanenl *et al*., 2008; Soković *et al*., 2007). The difference in anti-bacterial activity could be due to variation in chemical compositions and their penetration through the cell wall and cell membrane of bacterial pathogens (Palakawong *et al*., 2010; Soković *et al*., 2007). In previous literatures, it has been demonstrated that citrus plants have been used as an antidiabetic (Hamendra and Anand (2007), antioxidant (Kanaze *et al.,* 2008; Proteggente *et al.,* 2003), insect repellent, antibacterial, antiviral, antiyeast, and anti-mutagenic agent (Han, 1998).

Phytochemical screening

The most important bioactive compounds of plants are alkaloids, flavonoids, tannins and phenolic compounds (Duraipandiyan *et al*., 2006; Edeoga *et al*., 2005). Our results were consistent with Ahmad *et al*. (2006) that the peel of *Citrus* fruit was a rich source of phytochemicals, which were very rare in other plants and were active against various pathogens. The difference in the antibacterial activity with the same source when extracted with different solvent proves that not all phytochemicals that are responsible for antibacterial activity are soluble in a single solvent.

Antioxidant activity

Similar results were obtained by both $ABTS^{\cdot+}$ and DPPH free radical scavenging methods. Our results were consistent with the findings of Kanaze *et al.* (2008). Foods rich in antioxidant phytochemicals were important for the prevention of diseases related to oxidant stress i.e. heart and cancer (Nabavi *et al*., 2013; Seerama *et al*., 2005).

CONCLUSIONS AND FUTURE WORK

In conclusions, *C. sinensis* peel extracts have a wide range of antibacterial and antioxidant activity. These

extracts provide the possibilities to discover new effective antibacterial agents. Further studies are required to purify and characterize the bioactive compounds of both polar and non-polar extracts of the *C. sinensis* peel.

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