Potent anti-oxidant behaviour of citrus fruit peels and their bactericidal activity against multi drug resistant organism *Pseudomonas aeruginosa*

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ABSTRACT

Fruits and vegetables are very important source in the diet because they provide several vital vitamins and minerals. In fruit processing industry, large volumes of peels were dumped and thrown as waste material. If this waste can be exploited for some beneficial purpose, it will be useful, with this idea it was thought of interest, the potent antioxidant behavior and bactericidal activity of citrus fruit peels against Multi Drug Resistant (MDR) Organism (Orange, Pineapple and Papaya) were investigated. These citrus fruit peels were sequentially extracted (as individual extract and mixed fruit peel extract) using solvents of increasing polarity such as toluene, Petroleum ether and Methanol. The antioxidant behavior was determined by DPPH radical scavenging activity assay and ferric ion reducing power assay for the individual and mixed fruit peel extract. From the results it was clear that the Mixed Methanolic Citrus Fruit Peel Extract (MMCFE) showed potential antioxidant activity to scavange DPPH radical (IC_{50}= 34.688 µg/ml), strongest reducing capacity (83.6%) and were comparable with the standard Ascorbic acid.The effective antimicrobial activity against *Pseudomonas aeruginosa*, which is MDRO, was obtained for the MMCFE having a zone of inhibition of 1.2cm (Dia). Then the phytochemical constituents of MMCFE were analysed and reports to contain Flavanoids, Terpenoids, Tannins, Anthraquinones, Proteins, and cardiac Glycosides. The TLC analysis for MMCFE revealed single compound in the extract using the solvent system Cyclohexane:Acetonitrile:Methanol (1:1:1). The functional groups in MMCFE were examined through FTIR. This study confirms the belief that the MMCFE containing potential antioxidant and antimicrobial compound can be therapeutically used as effective drug formulates.

KEY WORDS: Citrus Fruit peels, Soxhlets extraction, Antioxidant assay, Bactericidal activity and MDR

1. INTRODUCTION

The Natural Products are an important concern for human health and welfare. Natural products are economically beneficial, safe and had promising effect. The natural sources such as plants, fruits and vegetables are rich in bioactive compounds and are valuable products for pharmaceutical industry.

Fruits and Fruit juices are important dietary components which are rich in antioxidants. Synthetic antioxidants will have potential health risks. Hence an increasing attention should be paid to identify natural and possibly more economic and effective natural antioxidants. In fruit processing industry and in fruit shops the fruit peels are thrown or dumped as waste, but the real fact is that the peels are having better biological activities than other fractions (Suree, 2010). Among the fruits, Citrus fruits are considered as a valuable part of a healthy and nutritious diet and it is well established that some of the nutrients in citrus promote health and provide protection against chronic disease. The citrus production is estimated as 80 million tonnes per year, making it an important source useful to human health. The main waste of the citrus fruits after processing is the citrus peel. Hence, attention should be paid for proper extraction of potential compounds and check their suitability as therapeutics. This will increase the aggregate value of the industrial waste. As it was underlined, citrus by-products are a good source of phenolic compounds, especially the characteristic flavanone glycosides which mainly include naringin, hesperidin, narirutin, and neohesperidin (Ibrahium, 1996). Their extraction from citrus peels has attracted considerable scientific interest to use them as natural antioxidants (Karsheval, 2004) mainly in foods to prevent the rancidity and oxidation of lipids (Aleksandra, 2002). One of the main purposes of these investigations is the study of the possibility to replace synthetic additives as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), which might be liver-damaging, carcinogenic with natural low-cost antioxidants extracted from citrus by-products. The natural bioactive compounds in fruits such as carotenoids, quercetin derivatives, phenolic acids and anthocyanins were found originally in the peel and the concentration decreased towards the flesh. Recent studies also confirmed the substantially higher amount of phenolic compounds and ascorbic acids in the peel than in the pulp for most of the fruits claimed that the skin usually contain higher bioactive compounds in order to protect the inner materials from insects and microorganisms deterioration.

The aim of this study is to highlight the potent antioxidant activity of individual and mixed form of citrus fruit peels using solvents such as toluene; petroleum ether and methanol and antimicrobial activity against MultiDrug Resistent Pathogen *Pseudomonas auerginosa*. The citrus fruits selected for this study includes *Citrus sinensis* (Orange), *Ananas comosus* (Pineapple) *and Carica papaya* (Papaya).

2. MATERIALS AND METHODS

2.1. Sample Collection and Pretreatment: *Citrus sinensis* (Orange), *Ananas cosmosus* (Pineapple), *Carica papaya* (papaya) fruit peel waste were collected from fruit market, Sivakasi, Virudhunagar district, Tamilnadu state, India. Collected fruit samples were screened for dust and ravage particles, unadulterated fruit peel samples were air dried in shade at 30°C for about 1 week. Fruit peel Samples, free of moisture were grinded finely.

2.2. Sequential Extraction using Soxhlet apparatus (Mital, 2009): Pretreated fruit peel samples were taken individually and as a mixture (equal amount) was weighed (104.4g) and was packed in Soxhlet apparatus. Sequential Extraction was carried out with different solvents (toluene, petroleum ether & methanol) of increasing polarity. Extraction was continued for about 2 to 3 hrs. 50mg of collected individual extracts were weighed and dissolved in 2ml of dimethyl sulfoxide (DMSO) and it was taken for further analysis. The yield of Extracts were calculated as follows,

$$\text{Yield } (\%) = \frac{\text{Weight of Extract recovered}}{\text{Weight of Fresh Fruit peel powder}} \times 100$$

2.3. Antioxidants assays

2.3.1. DPPH free radical scavenging activity assay: The free radical scavenging activity assay was done by DPPH method (Chen et al., 2011). Fruit peel extract of 1ml was added with 1ml of DPPH (4.72mg/40ml methanol) and 1ml of methanol. The reaction mixture was left for shaking for 5min and kept in dark for 30min. After the reaction time, absorbance of the reaction mixture was measured at 517nm. 2ml of methanol with 1ml DPPH was taken as negative control. 1ml of Ascorbic acid was taken as standard for positive control. Scavenging activity was calculated from the following equation

$$\text{Scavengingactivity}(\%) = \left[\frac{\text{Abscontrol} - \text{Abssample}}{\text{Abscontrol}}\right] \times 100$$

2.3.2. Ferric Reducing Antioxidant Power (FRAP) Assay: The reducing capacity of the citrus fruit peels were determined by FRAP assay method. Fruit peel extracts of varying concentration (50, 500, 1000 µg/ml) in deionized water was mixed in 2.5ml of phosphate buffer (0.2M, pH 6.6) and 2.5ml potassium ferricyanide. The reaction mixture was incubated for 50°C for 20min. 2.5ml, 10%TCA was added to the mixture and centrifuged for 10min at 3000rpm. 2.5ml of the centrifuged clear solution was mixed with 2.5ml distilled water and 0.5ml of freshly prepared 1%$FeCl_2$. Absorbance of the final solution was measured at 700nm. Ascorbic acid was taken as standard antioxidant for positive control. %increase in reducing power was calculated as follows

$$\text{Increaseinreducingpower}(\%) = \left|\frac{(\text{Abstest} - \text{AbsBlank})}{\text{AbsBlank}}\right| \times 100$$

2.4. Phytochemical Analysis: Extract with high antioxidant potential was subjected to qualitative chemical analysis. Then the phytochemical components were determined (Goulas, 2012).

2.5. Antimicrobial activity: Extract having high antioxidant potential was checked for its antimicrobial activity against a Multi Drug Resistant Pathogen (MDRP), *Pseudomonas aeruginosa* through well diffusion method (Akhilesh, 2012). The MDR behaviour of *Pseudomonas aeruginosa* was analysed against commercial antibiotics, Gentamycin, Ampicillin, Metformin and Colistin. Different solvent extracts of mixed fruit peel and individual peel were taken in wells to evaluate its antimicrobial potential against MDRP.

2.6. TLC analysis: (Wagner and Bladt, 1996): The TLC analysis for the potent Extract was done in Glass slides (6cmx2cm) a coated with2.5mm silica gelusing different solvent system which reveals the compounds in the extract. The solvent system (individual and combinations of solvents) were chosen based on trial and error process. The gradient solvent system may also be chosen. The R_F value for the extract was determined by the following formula

$$R_F = \frac{\text{Distance moved by Solute (cm)}}{\text{Distance moved by Solvent (cm)}}$$

2.7. FTIR: FT-IR is perhaps the most powerful tool for identifying types of chemical bonds (functional groups).The IR spectra of MMCFE extracts were measured on Shimadzu FT-IR 8000 series instrument. The sample was grounded with IR grade potassium bromide (KBr) (1:10) pressed in to discs under vacuum using spectra lab pelletiser. The IR spectrum was recorded in the region 4000-400 cm^{-1} and the typical stretching frequency of the bioactive substance was recorded for further characterization analysis.

3. RESULTS AND DISCUSSION

3.1. Extraction efficiency of various solvents: Percentage yield of extracts using various solvents for fruit peels were studied by evaluating the final volume (g/ml) of extract obtained. Extraction using methanol shows a higher yield of extract on comparing with extraction by toluene and petroleum ether and it was listed in Table 1. The MMCFE shows a high yield (15.61 of % Recovery) comparing with others. The Petroleum ether extract of citrus fruit peel were neglected in further analysis due to low yield of extracts.

Table.1.Extraction efficiency of various solvents

Fruit peels	Amount of powder packed (g)	Solvents used	Amountof extract collected (g)	% yield of extracts
Orange	104.42	Toluene	1.5	1.43
		Petroleum Ether	0.02	0.019
		Methanol	11.8	11.30
Pineapple	104.42	Toluene	0.03	0.02
		Petroleum ether	0.025	0.019
		Methanol	12.43	11.90
Papaya	104.42	Toluene	1.00	0.957
		Petroleum ether	0.02	0.019
		Methanol	12.56	12.03
Mixed	104.42	Toluene	14.6	13.98
		Petroleum ether	0.02	0.019
		Methanol	16.3	15.61

3.2. Antioxidant assays: In the radical scavenging assay, when the DPPH is exposed to antioxidant compounds, the reduction capability of DPPH radicals was determined by the decrease in its absorbance at 517 nm, which is induced by antioxidants. The DPPH radical scavenging activity of toluene and methanol extract of selected fruit peels were detected and compared with Ascorbic acid. On evaluating the percentage inhibition at various concentration (50, 500, 1000 µg/ml) of toluene and methanol extract of selected fruit peel, MMCFE sample shows a high percentage of inhibition (Table 2) (i.e) about 98% equivalent to the standard antioxidant ascorbic acid (Fig.1). The percentage of inhibition was efficient for mixed methanolic citrus peel than the individual extracts and its IC_{50} is 34.688 µg/ml. (Habibur, 2003) Reducing power assay method is based on the principle that, substances which have reduction potential, react with potassium ferricyanide (Fe^{3+}) to form potassium ferrocyanide (Fe^{2+}), which then reacts with ferric chloride to form ferric ferrous complex that has an absorption maximum at 700 nm. The reductive capabilities of the Orange peel, Pineapple peel, Papaya peel and mixed extract were compared with ascorbic acid for the reduction of the Fe^{3+} to Fe^{2+}. The reducing capacity of a compound may serve as a significant indicator for its potential antioxidant activity. From the results (Table 3) it was found that the reducing power of the samples increased with the increase in concentrations (Fig.2). The Better reducing power is obtained for the MMCFE. However, this reducing power is lower than that of Ascorbic acid which was used as standard. This shows that the fruit peel extract and its constituents possess reducing power capabilities and act as a potent antioxidant.

Table.2.DPPH assay for the individual and mixed citrus fruit peel extracts

Sample/Source	**Solvents**	**% of inhibition at various concentration**		
		50 (µg/ml)	**500 (µg/ml)**	**1000 (µg/ml)**
Ascorbic acid	DMSO	**79.6**	**82.9**	**88.7**
Orange peel	Toluene	53.7	62.9	79.6
	Methanol	44.4	50.0	72.2
Pineapple peel	Toluene	46.2	61.1	75.9
	Methanol	48.14	59.26	66.62
Papaya peel	Toluene	50.03	64.8	74.07
	Methanol	48.82	66.12	72.24
Mixed fruit peel	Toluene	67.83	77.05	83.40
	Methanol	72.07	80.56	87.03

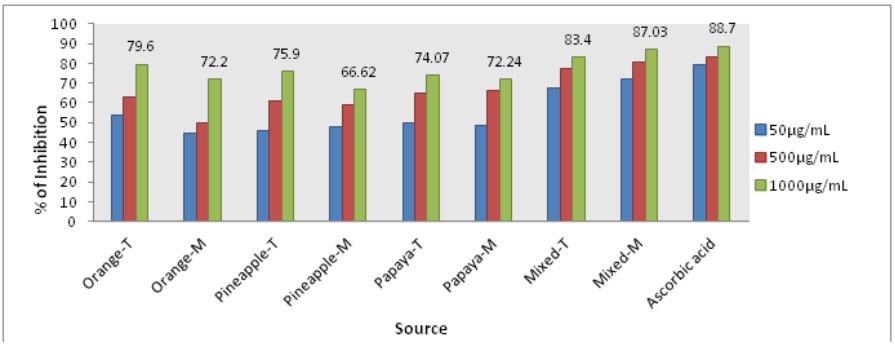

Fig 1: DPPH Radical scavenging activity of Individual and mixed citrus fruit peel extract T- Toulene extract; M-Methanol extract

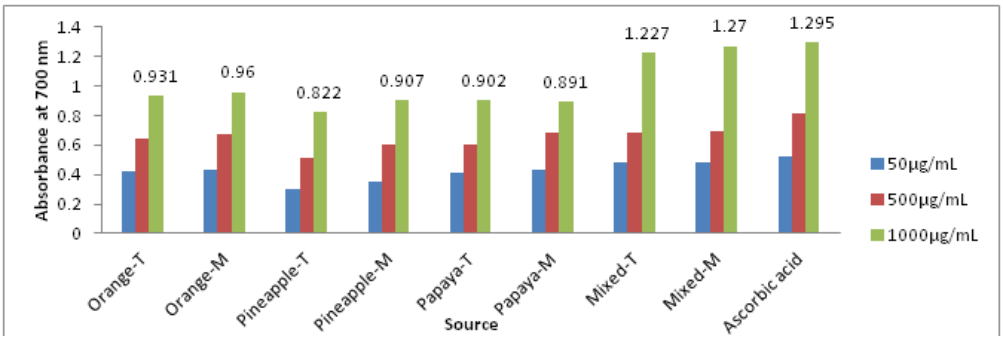

Figure.2.Reducing power activity of Individual and mixed citrus fruit peel extract

Table.3.Reducing power of individual and mixed citrus fruit peel extracts

Sample / Source	Solvents	Absorbance(700nm) at various concentration		
		50 µg/mL	500 µg/mL	1000 µg/mL
Control	-	0.015	0.015	0.015
Ascorbic acid	DMSO	0.527	0.713	1.295
Orange	Toluene	0.42	0.64	0.931
	Methanol	0.432	0.673	0.96
Pineapple	Toluene	0.302	0.516	0.822
	Methanol	0.352	0.607	0.907
Papaya	Toluene	0.412	0.603	0.902
	Methanol	0.431	0.684	0.891
Mixed	Toluene	0.480	0.685	1.227
	Methanol	0.485	0.696	1.270

3.3. Phytochemical analysis: The methanolic extract of mixed fruit peel having efficient antioxidant potential, was analysed for its phytochemical constitution such as tannin, cardiac glycosides, terpenoids, proteins etc. The phytochemical constitution of MMCFE was listed in the Table 4.

Table.4.Phytochemical analysis of MMCFE

Test performed	Result
Amino acid	+
Tannins	+
Anthraquinones	+
Terpenoids	+
Proteins	+
Cardiac glycosides	+

3.4. Antimicrobial activity: *Pseudomonas aeruginosa* is resistant to Ampicillin, Gentamycin and Colistin and thus it proves its MDR behavior (Fig.3). Antimicrobial activity of different solvent extracts of mixed fruit peel and individual fruit peels, against MDRP, *Pseudomonas aeruginosa* was evaluated by measuring the zone of inhibition formed (Table 5). Methanolic extract of mixed fruit peel extract shows a large zone of inhibition of about 1.2cm in diameter, on compared with other solvent extracts.Thus, MMCFE evidences to have potent antimicrobial activity against MDRP.

Table.5.Determination of MDRP behaviour of *pseudomonas aeruginosa* against commercial Antibiotics

Antibiotics	Zone of inhibition (cm)	Resistance/ Sensitive
Gentamycin	-	Resistant
Colistin	-	Resistant
Ampicillin	-	Resistant
Metformin	0.5	Sensitive

3.5. TLC analysis: The TLC for the MMCFE was done using the solvent systems Cyclohexane: Acetonitrile: Methanol (1:1:1) and Methanol (25%, 75% ad 100%). The direct visualization of TLC plate showed single spot in the solvent system Cyclohexane: Acetonitrile: Methanol (1:1:1) with an R_F value 0.6 which may indicate the presence of single compound in the MMCFE.

Table.6.Antimicrobial activity of individual and mixed citrus fruit peel extracts against MDRP

Extract	Solvents	Zone of inhibition In diameter (cm)
Control	-	-
Orange peel extract	Toluene	0.26
	Methanol	0.53
Pineapple peel extract	Toluene	0.07
	Methanol	0.35
Papaya peel extract	Toluene	0.39
	Methanol	0.8
Mixed fruit peel extract	Toluene	0.98
	Methanol	1.2

3.6. FTIR: In MMCFE, FTIR-Spectrum shows strong absorption peak at 3394.72 cm^{-1} which shows strong absorbency for primary and secondary amines (Table 7). The major functional groups includes alkanes, primary amines, nitro compounds, aromatics, carboxylic acids in the wave number 2926.01& 2956.58, 1629.85,1514.2, 1406.11, 923.90 respectively (Fig.3)

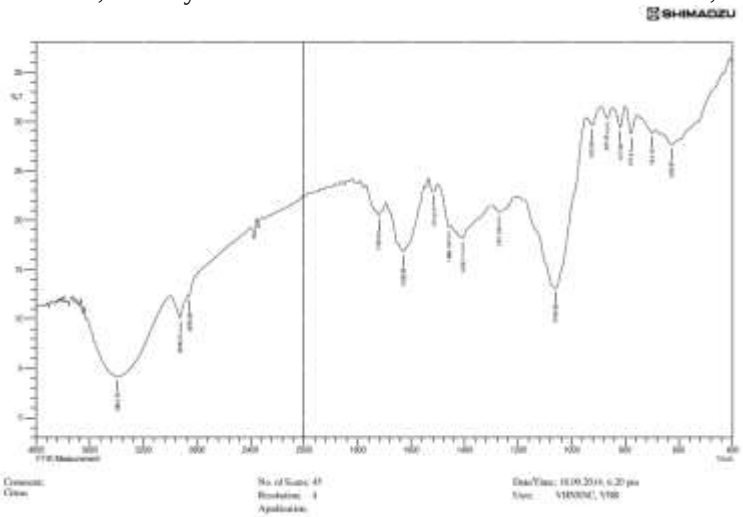

Figure.7.FTIR Analysis for MMCFE

Table.7.FTIR analysis for MMCFE

Absorption Frequency Region	Wave Number Cm^{-1}	Bond	Major Functional Group
3400-3250	3394.72	N-H stretch	Primary and secondary amines, amides
3000-2850	2926.01	C-H stretch	Alkanes
	2956.58		
1730-1715	1720.50	C=O stretch	α,β- unsaturated esters
1650-1580	1629.85	N-H bend	Primary amines
1550-1475	1514.12	N-O asymmetric stretch	Nitrocompounds
1470-1450	1458.18	C-H bend	Alkanes
1500-1400	1406.11	C-C stretch (in-ring)	Aromatics
1335-1250	1271.09	C-N stretch	Aromatic amines
1250-1020	1058.92	C-N stretch	Aliphatic amines
950-910	923.90	O-H bend	Carboxylic acids
900-675	867.97	C-H"oop"	Aromatics
	817.82		
	777.31		
	700.16		
700-610	626.87	-C=C-H: C-H bend	Alkynes

4. CONCLUSION

The study emphasis the importance of mixed citrus fruit peel waste which is also a source having potent antioxidant and antimicrobial compounds. The result of the study shows that the MMCFE exhibited efficient antioxidant and antimicrobial activity against MDR organism (*Pseudomonas aureginosa*). The TLC analysis of MMCFE revealed that it may contain one compound. The FTIRanalysis shows the major functional groups present in MMCFE.This work definitely helpful for identification and isolation of bioactive compounds which can serve as effective drug formulate for effective prevention of degenerative diseases. Further work is needed to quantify and structurally investigate the bioactive constituents present in the mixed fruit peel extract.

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