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Evaluation of antioxidant and antimicrobial activities *in vitro* of different citrus peels and combinations thereof

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ABSTRACT

The present study delineates antioxidant and antimicrobial activities of aqueous extracts of peels of Citrus sinensis (orange), Citrus limon (lemon) and Citrus maxima (pummelo), individually or in binary combinations, for efficient combat against detrimental principles like free radicals and microbes. The antioxidant assays performed were DPPH radical decolorization assay, ferric reducing antioxidant potential assay, hydroxyl radical scavenging assay and estimation of bioactive principles like total polyphenols and ascorbic acid. Antimicrobial activities were done against common food borne pathogens like Bacillus cereus, Escherichia coli, Staphylococcus aureus and Klebsiella pneumoniae. The major conclusions arising out from the study was that orange peel extract showed most promising activity in the experimental parameters. Orange peel, in combination with the pummelo peel, showed the most effectual activity amongst the binary combinations. The study also indicated that both hydrogen atom and electron transfer occur during the antioxidant activity of the binary combination of orange and pummelo peel extract, which delivered its' most potent activity. Although ascorbic acid one of the most important bioactives of citrus species, it did not play crucial role in the pharmacognostic activities of the extracts. On the other hand, binary combination of orange and lemon peel extract showed most promising activity against Staphylococcus aureus. A synergism was also observed in the bacteriostatic activity against all the food borne microorganisms.

Keywords: Orange, lemon, pummelo, peel, antioxidant, antimicrobial.

INTRODUCTION

Fruits and vegetables are considered to be an important part of diet in the tropical countries. Besides their delicious taste and flavor, they are known to reduce risk of several chronic and fatal diseases like cerebrovascular, cardiovascular and certain types of cancers due to the presence of a number of phytochemicals [1-3]. However, fruits and vegetables wastes and their by-products are formed in great amounts during consumption in households as well as processing in food industries [4]. Fruit by-products like peels could produce serious health problems as they turn into bad odor, dwelling to insects and germs and also could produce soil pollution [5]. It was reported that peels of orange represent between 50 to 65% of the weight which are discarded as by-product [6]. Some attempts were made to use these residues as livestock feed, although they have very low nutritional value [7]. On the other hand, extracts of peels were found to possess quite astounding radical scavenging and antimicrobial abilities [8-10]. Antimicrobial activity of the peel extracts is directly linked with the components thereof like essential oils,

terpenoids and phenolic acids and esters [11]. All these informations led to carry out extensive researches worldwide to utilize these wastes.

The genus Citrus belongs to the family Rutaceae and is native to tropical and subtropical areas in Southeast Asia. The citrus plants are grown worldwide and ranks top in world trade among the fruits trees. The citrus peels are divided into epicarp or flavedo and mesocarp or albedo. The flavedo is colored and is the outer most surface of the peel whereas the albedo is the white, soft inner layer of the peel. The citrus peels contain high quantity of phenolic compounds including several flavonoid compounds. The citrus peel extracts and essential oils are known to exhibit various biological activities such as antimicrobial and antioxidant activities [12]. The peels of *Citrus* fruits are rich sources of flavonoid glycosides, coumarins, β and γ-sitosterol, glycosides and volatile oils [13]. They also contain fibers, polyphenols and especially vitamin C, which can act as a cure in vitamin C deficiencies [14]. In general, peels contain a higher concentration of antioxidant substances than the flesh of the fruit [15]. For hundreds of years, herbalists trained in Traditional Chinese Medicine (TCM) have used orange peel to improve digestion, relieve intestinal gas and bloating, and resolve phlegm [16].

There are several *Citrus* fruits consumed as food, e.g. *Citrus limon* (lemon), *Citrus aurantium* (bitter orange), *Citrus limetta* (sweet lemon), *Citrus jambhiri* (Rough lemon) and *Citrus paradise* (grape fruit), and majority of them showed very good antioxidant activities [17]. In the Indian perspective, *Citrus sinensis* (orange) and *Citrus limon* (lemon) are two very important fruits consumed widely. A body of recent research has been focusing on the pharmacognostic evaluation of the peels on various aspects of well-being. Due to the low cost and easy availability of peels, they are now regarded as potential source for development of nutraceuticals capable of offering significant low-cost dietary supplements, although they are usually discarded as waste in the environment [18]. Critical study of recent literature indicated that antioxidant and antimicrobial studies with citrus peels are usually done after extraction with organic solvents, which sometimes might produce toxic materials [19]. Extraction with water, the universal solvent could nullify any toxic effect in the extract that could interfere with the outcome of the experiments. In light of the above discussion, the present study was undertaken to evaluate the antioxidant and antimicrobial potential of the aqueous extracts of peels of *Citrus sinensis* (orange), *Citrus limon* (lemon) and *Citrus maxima* (pummelo), individually or in binary combinations, so that an ideal product would be achieved from waste materials having all necessary ingredients needed for efficient combat against detrimental principles like free radicals and microbes. Moreover, the study was probably the first to adjudicate the efficacy of the aqueous extracts of the peels, alone or in combination, against food-borne pathogens.

MATERIAL AND METHODS

Chemicals

2,2′-Diphenyl-1-picryl hydrazyl (DPPH) were obtained from Himedia, India. Analytical grade of 2-Deoxy-D-ribose was obtained from Loba Chemie; thiobarbituric acid (TBA), ascorbic acid, gallic acid, Folin-Ciocalteau's solution, sodium hydroxide and sodium carbonate were obtained from Merck, India. Muller Hinton Agar was purchased from HiMedia. All other reagents and chemicals used were of analytical grade procured from local sources. Deionized distilled water was used in the entire study.

Preparation of samples

The samples were procured from local markets of Barasat, Kolkata. The samples were checked for dirt or any visible damages prior to the study. Such samples were discarded. 5 gms each of the samples were taken in 50 ml double distilled water, separately, for the preparation of extract. After extraction, the samples were centrifuged at 8000 rpm for 5 mins. The clear supernatants were used for *in vitro* antioxidant and antimicrobial assays. In case of the binary combinations of the peels, they were taken in 1:1 w/w ratio. The peel extracts were designated as follows – Orange – OR, Lemon – LM, Pummelo – PM, Orange + Lemon – OL, Lemon + Pummelo – LP and Pummelo + Orange – PO.

DPPH radical decolorization assay

The DPPH assay was performed using a previously described procedure [20]. 1 ml DPPH solution (0.1 mM) was mixed with 0.5 ml sample solution and the decrease in absorbance of the mixture after 20 minutes of incubation in the dark was monitored at 517 nm in a Systronics spectrophotometer (model – 2202). The concentration that causes a decrease in the absorbance of initial oxidants by 50% is defined as IC_{50} of the samples. Gallic acid was used as

positive control and comparing with its' IC_{50} and the results were expressed as Gallic acid equivalents (µM/gm fresh leaves).

Ferric reducing antioxidant power (FRAP)

Ferric reducing potentials of the samples were estimated with a previously established procedure with minor modifications [21]. Briefly, a maximum of 100 µl of extract solution or standard was mixed with 1.9 mL of FRAP reagent and incubated at 37^{o}C for 30 mins. FRAP reagent was prepared by mixing 0.1 M acetate buffer (pH 3.6), 10 mM TPTZ solution and 20 mM $FeCl_3$ solution. After the stipulated time period, absorbance was measured at 593 nm in a UV-Vis spectrophotometer (model – Systronics 2202). Gallic acid is used as standard. Results are expressed as Gallic acid equivalents (GAE).

Estimation of total phenolics content

Total phenolic compound contents were determined by the Folin-Ciocalteau method [22]. The samples (0.5 ml) were mixed with Folin-Ciocalteau reagent (5 ml, of 1:10 diluted sample with distilled water) for 5 min and aqueous sodium carbonate (4 ml, 1 M) was then added. The absorbance of the reaction mixture was then measured at 765 nm in a UV-Vis spectrophotometer (model – Systronics 2202). Gallic acid was used as standard. The results were expressed in terms of µg gallic acid equivalent/gm fresh leaves.

Estimation of ascorbic acid content

Ascorbic acid contents of the samples were estimated with a previously described procedure with minor modifications [23]. Briefly, a maximum of 100 µl sample (or standard) was mixed with 400 µl 5% metaphosphoric acid solution. Then another 500 µl of 10% metaphosphoric acid solution was added followed by 300 µl of citrate buffer (pH 4.15) and 300 µl of 2,6-DCP-IP solution. Absorbance was read at 520 nm in a UV-Vis spectrophotometer (model – Systronics 2202) after 1 min.

Hydroxyl radical scavenging assay

Hydroxyl radical scavenging potentials of the samples were estimated with a previously described procedure with minor modifications [21]. Briefly, 10 mM each of $FeSO_4.7H_2O$, EDTA, 2-deoxy-D-ribose and H_2O_2 solutions were prepared in water. 0.2 ml each of above four and 0.2 ml sample and/or standard solution was mixed in a test tube and incubated at 37^{o}C for 90 mins. H_2O_2 solution was added last. After the incubation, 1 ml of 2.8% (w/v) aqueous TCA solution and 1 ml of 1% (w/v) aqueous TBA solution were added to the reaction mixture and kept at boiling water bath for 20 mins. Development of the pink chromophore was measured at 532 nm in a UV-Vis spectrophotometer (model – Systronics 2202). Gallic acid is used as standard. Results were expressed as Gallic acid equivalents (GAE).

Antibacterial Activity Assay

The bacterial strains used in this study included *Bacillus cereus* (MTCC 1272), *Escherichia coli* (MTCC 1610), *Staphylococcus aureus* (MTCC 9542) and *Klebsiella pneumoniae* (MTCC 9544). The strains were obtained from IMTECH, Chandigarh, India and preserved at Department of Microbiology, Ramakrishna Mission Vidyamandira. The antibacterial activity was measured by agar well diffusion method. Each bacterial isolates was previously grown on sterile Muller Hinton Agar (HiMedia M173) plate at 35°C for 24 hours. Single colony of each of the isolates was grown in Muller Hinton broth (HiMedia M391) for 3 hours at 35°C. After that, each of the isolates was inoculated with 100µl of standardized inoculums of each bacterium (in triplicates) and spread with sterile cotton swabs. Wells are 6 mm sizes were made with sterile borer into agar plates containing the bacterial inoculums. Different working dilutions of extracts of orange peels, pummelo peels, lemon peels and their mixtures [(orange peel + pummel peels), (pummel peels + lemon peels), (orange peels +lemon peels)] were prepared in sterile water. For those extract, 25 mg/ml, 50mg/ml, 100mg/ml and 200mg/ml dilutions were prepared. From these different dilutions, 50µl solution was poured into the wells of the respective culture plates. The plates thus prepared were left at room temperature for ten minutes allowing the diffusion of the extract into the agar. After incubation for 24 hours at 35^{o}C, the plates were observed. If antibacterial activity was present on the plates, it was indicated by an inhibition zone surrounding the well containing the different dilutions of extract and nanoparticles. The zone of inhibition was measured and expressed in millimetres. Antibacterial activity was recorded if the zone of inhibition was greater than 6 mm. The antibacterial activity results were expressed in term of the diameter of zone of inhibition and <9mm zone was considered as inactive; 9-12mm as partially active; while13-18mm as active and >18mm as very active [24].

RESULTS AND DISCUSSION

DPPH radical decolorization assay

DPPH assay was used to determine the scavenging potential of antioxidant extracts based on their capabilities as hydrogen donor in tandem with electron transfer. The results of DPPH radical scavenging assay of citrus peels and their combinations showed that peel extract of orange was the best among the individual peel extracts, the GAE value being more than twice with respect to the other two peel extracts (Fig. 1). Among the binary combinations, PO showed the highest potential, probably due to the contribution of the orange peel bioactives.

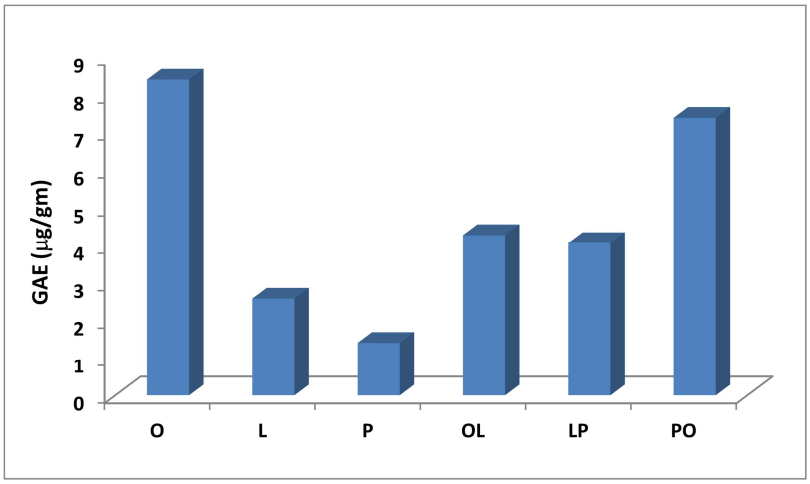

Fig. 1: DPPH radical scavenging activities of the peel extracts and their binary combinations. Results are expressed as gallic acid equivalent (GAE). All the results are mean of three values.

Ferric reducing antioxidant power (FRAP)

In this assay, the reduction power of the sample extracts were indicated by their abilities to transfer electrons towards the FRAP reagent. The result showed that orange peel extract scored well over the others regarding their reducing abilities (Fig. 2). Among the binary combinations, PO showed the highest potential, probably due to the contribution of the orange peel bioactives. It was also observed that the GAE values for the extracts were more than their DPPH scavenging values, clearly indicating that electron transfer to be the foremost phenomena for their antioxidant potentials.

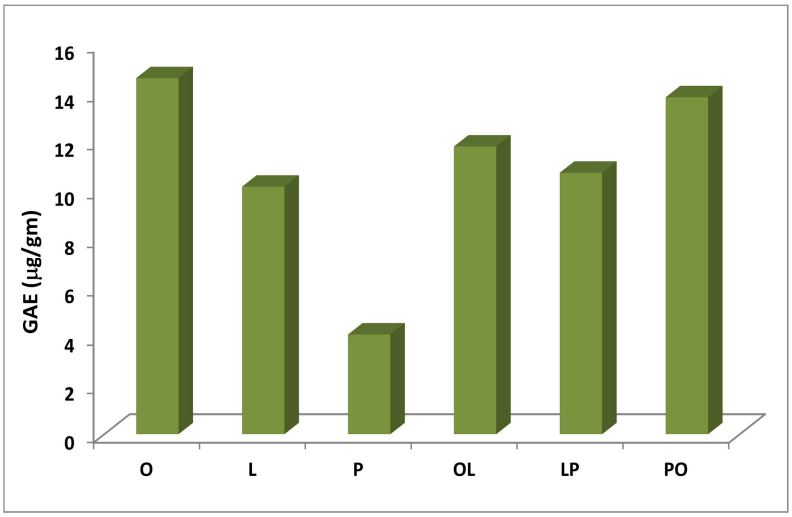

Fig. 2: Ferric reducing potential (FRAP) of the peel extracts and their binary combinations. Results are expressed as gallic acid equivalent (GAE). All the results are mean of three values.

Total phenolics content

Total phenolic contents of the three citrus peel aqueous extracts and their binary combinations commensurate with their reducing abilities as depicted in Fig. 3. The result showed that orange peel extract scored well over the others regarding their phenolic contents. Among the binary combinations, PO showed the highest potential, probably due to the contribution of the orange peel bioactives as was discussed earlier.

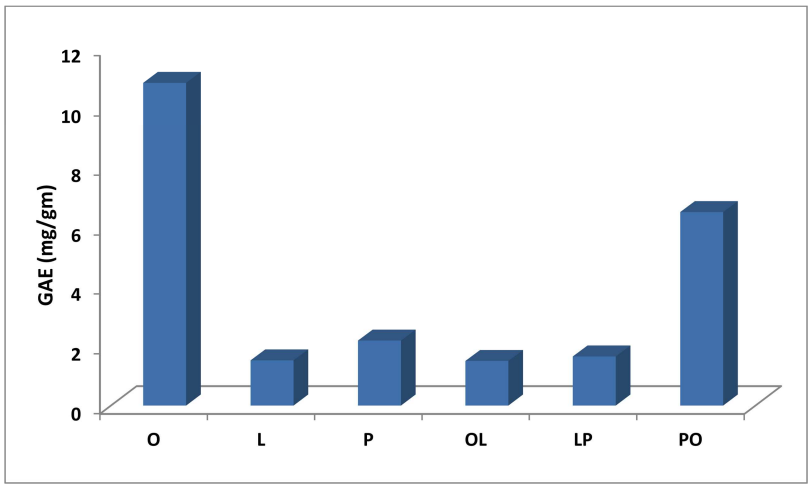

Fig. 3: Total phenolic contents of the peel extracts and their binary combinations. Results are expressed as gallic acid equivalent (GAE). All the results are mean of three values.

Estimation of ascorbic acid content

The results this showed that peel extract of orange was the best among the individual peel extracts, although the content of the Pummelo extract was very close (Fig. 4). Among the binary combinations, all of them showed similar contents. These results clearly suggest that reducing power of the extracts were not dependent upon their ascorbic acid contents solely, but also to the other polyphenolic bioactives.

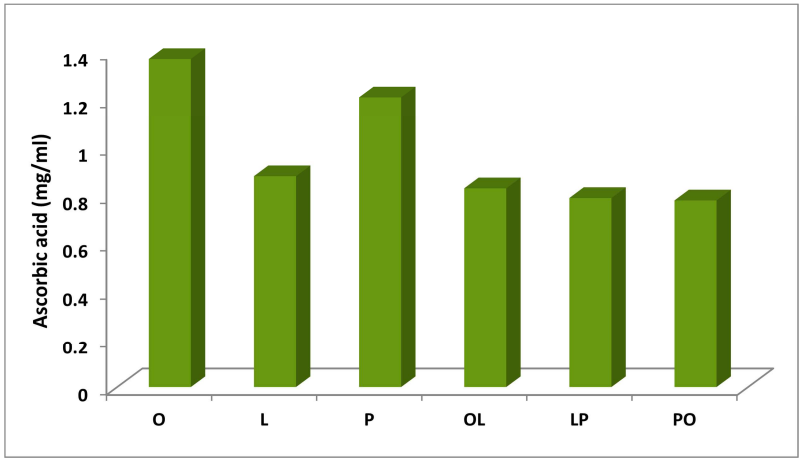

Fig. 4: Ascorbic acid contents of the peel extracts and their binary combinations. Results are expressed as mg ascorbic acid/ml aqueous extract. All the results are mean of three values.

Hydroxyl Radical Scavenging assay

The scavenging activity for the most deleterious radical was highest for pummelo peel extract (Fig. 5). Lemon peel extract showed very poor scavenging potential. However, among the binary combinations, OL showed the highest potential, although they were not better with respect to pummelo individually. This might be due to some synergism between the two peel extracts.

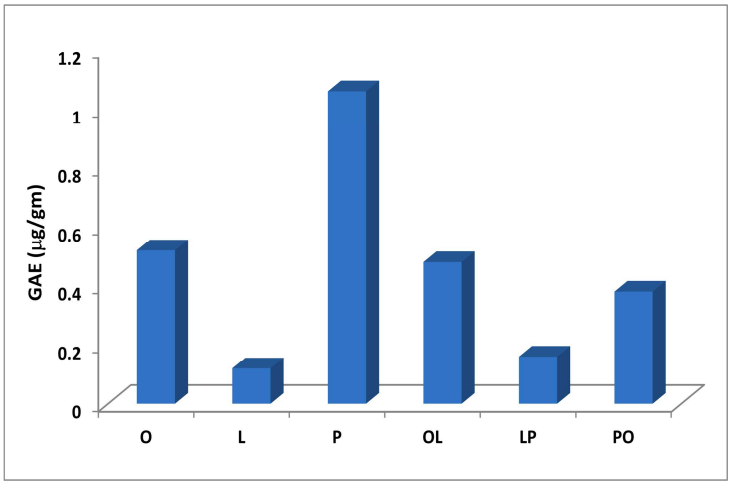

Fig. 5: Hydroxyl radical scavenging activities of the peel extracts and their binary combinations. Results are expressed as gallic acid equivalent (GAE). All the results are mean of three values.

Table 1: Bacteriostatic activities of the peel extracts and their binary combinations.

Sample	Concentration of the extracts (µg/ml)	Diameter of zone of inhibition (mm)			
		S. aureus	*E. coli*	*B. cereus*	*K. pneumoniae*
O	25	-	-	9	10
	50	11	-	12	12
	100	13	12	14	15
	200	14	14	16	16
L	25	9	-	-	-
	50	11	-	12	-
	100	14	9	14	-
	200	16	12	15	10
P	25	-	-	-	-
	50	10	-	9	-
	100	11	10	11	9
	200	13	11	14	11
OL	25	14	-	-	-
	50	16	10	13	-
	100	18	14	15	-
	200	22	16	18	14
LP	25	-	-	10	-
	50	13	-	13	-
	100	16	10	15	10
	200	18	14	16	12
PO	25	10	-	-	-
	50	14	-	12	10
	100	18	8	16	14
	200	20	12	18	16

Antibacterial Activity Assay

The antibacterial properties of extracts of peels of orange, pummelo and lemon, and their binary combinations against four common food borne bacteria were assessed quantitatively by determining the diameter of inhibition zones as shown in Table 1. All individual peels extracts and their mixtures in concentrations of 200 µg/ml showed significant inhibition towards all the selected bacteria (both gram-positive and gram-negative). Among the individual peel extracts, orange peel extract showed maximum effectiveness against all selected bacteria. Among the binary combinations, PO showed the highest potential, again probably due to the contribution of the orange peel bioactives. The study also indicated that the antioxidant principles of orange peel extracts were well active against food borne micro-biota. It can also be seen from the table that the zone of inhibition for the binary combinations were greater than the average of the individual zone of inhibitions of the components. This clearly indicated that

there might be some synergism between the two components, probably due to combination of potent bioactive principles obtained from the individual extracts.

CONCLUSION

The present study elaborated comparison of the antioxidant and antimicrobial activities of peel extracts of *Citrus sinensis* (orange), *Citrus limon* (lemon) and *Citrus maxima* (pummelo), individually or in binary combinations, to achieve an ideal extract for efficient combat against detrimental principles like free radicals and microbes. The major conclusions arising out from the study was that orange peel extract showed most promising activity in the experimental parameters. Orange peel, in combination with the pummelo peel, showed the most effectual activity amongst the binary combinations. The study also indicated that both hydrogen atom and electron transfer occur during the antioxidant activity of the binary combination of orange and pummelo peel extract, which delivered its' most potent activity. Although ascorbic acid one of the most important bioactives of citrus species, it did not play crucial role in the pharmacognostic activities of the extracts. On the other hand, binary combination of orange and lemon peel extract showed most promising activity against *Staphylococcus aureus*. Its' bacteriostatic activity against *Bacillus cereus* was identical at the highest concentration used, clearly indicating its' potential role against the food borne microorganisms. Apart from orange peel extract, their activities against *Klebsiella pneumonia* were not very prominent.

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