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ANTIOXIDANT AND BRINE SHRIMP LETHALITY BIOASSAY OF PECTIN FROM GRAPE FRUIT (*CITRUS PARADISI*) AND LEMON (*CITRUS LIMON*)

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ABSTRACT: Pectin is a complex polysaccharide and has been used in food and pharmaceutical industries. The current study aimed to determine antioxidant and cytotoxic activities of pectin from peels of grape fruit (GP) and lemon (LP) by ferric reducing power (FRP) assay and brine shrimp lethality bioassay respectively. In addition, antioxidant and cytotoxic activities of GP and LP were compared with food-grade pectin (FGP). GP and LP exhibited positive results of antioxidant and cytotoxic activities. The percentage FRP of GP at 10 to 100 µg/ml was found to be higher as compared to FGP while LP showed closed values to that of FGP. The observed percentage mortality of shrimps at 10 to 1000 µg/ml by LP was found to be higher as compared to percentage mortality caused by FGP. Percentage mortality of shrimps by GP and FGP showed closed values. The LC_{50} of LP was found to be lower than that of FGP but close to the LC_{50} of standard drug etoposide. The LC_{50} of GP was closed to that of FGP and higher than that of etoposide. The obtained results suggested cost-effective use of GP and LP for antioxidant and cytotoxic actions.

INTRODUCTION: The word pectin is originated from the Greek language which means curdled or congealed. Pectin is present in the primary cell wall and middle lamella of higher plants [1]. Chemically, structure of pectin belongs to heteropolysaccharide that is consisted of D-galacturonic acid, L-rhamnose, L-arabinose and D-galactose linked by 1, 4 glycosidic linkages [2]. Continuously increased production of free radicals in the body can cause mutation in DNA which can lead to production of cancer cells [3].

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The lack of antioxidants in the body also leads to other serious disorders such as CNS diseases (Alzheimer's disease, Parkinson's disease, and other neurodegenerative disorders), aging, rheumatoid arthritis, cataract; liver, lung and kidney disorders; and cardiovascular diseases [4].

Pectin is obtained from citrus peels and apple pomace on commercial scale. The pectin that serves many health beneficial roles in our body, has great economic value regarding its sources because it is also obtained from fruit waste as by-product of fruit juice industries [5]. Considering the novel structure of pectin, it has been the part of interest for the researchers to explore the antioxidant and cytotoxic activities of pectin. Previously, antioxidant activity of citrus peels pectin, and apple pomace was determined by different methods like DPPH and ABTS methods [6].

Recently, other sources of pectin are also used for evaluation of its antioxidant activity, such as hot pepper pectin exhibited antioxidant activity [7]. Pectin has also shown profound effect against cancer cells such as lung metastasis of colon cancer cell [8]. Another recent research study exhibited the anticancer potential of apple pectin against most widely spread breast cancer. In this study, for *in-vitro* study, 4T1 breast cancer cells were studied while for *in-vivo* study, animal models were used [9].

One of the previous studies showed a correlation of rhamnogalacturonan with anticancer activity of pectin [10]. Moreover, the modification in pectin structure by lowering esterification, lowering molecular weight as well as linear chain of galacturonic acids caused improved antioxidant property [11]. Pectic polysaccharide from Korean Citrus Halabong was also investigated, and the resulted data concluded that these polysaccharides had activated the macrophages and NK cells and resisted lung metastasis of colon 26-M3.1 cells [8]. The anticancer role of pectin, as well as its use as vehicle for cytotoxic drugs, were discussed in previous review article by Zhang and Zhang [10]. In this article galectin-3 role was also highlighted for anticancer potential of modified pectin. Concerning the cytotoxic activity, shrimps were selected in some research work as they behave like mammalian cells. So the lethality to shrimps can be an effective tool to evaluate the cytotoxicity of a drug [12]. Brine shrimps lethality bioassay is performed for the determination of cytotoxicity of test compound on a simple organism-brine shrimp for determination of toxicity of pesticides, heavy metals, medicines and particularly the natural plant extracts [13]. The global world, especially under developing countries, is facing many ailments among which the most common are cancer and other diseases associated with lack of antioxidants in the body such as CNS, CVD and skin diseases. Thus, the present study aimed to investigate pectin for its antioxidant and cytotoxic activities concerning its natural and cost-effective source.

MATERIALS AND METHODS: *Citrus paradisi* (CPF-04-16/19) and *Citrus limon* (CLP-04-16/19) were collected from the local market of Karachi. All the chemicals used were of analytical grade. Food grade pectin (standard pectin) was obtained from Sigma Aldrich. Weighing balance (Sartorius GmbH; type A 6801), Water bath (Thermostatic Grant Type JB2.USA), Homogenizer (Panasonic MX-JI20P. Japan), pH meter (Jenway 3510.UK), Freeze dryer (Trio, Science Co. Limited, TR-FD-BT-50 1988. Japan), FT-IR spectrophotometer (Thermo Nicolet 3s80. USA), Furnace (Gallen Kamp Scientific Tech.UK), Incubator (BINDER GmbH. Germany) and UV/Vis Spectrophotometer (Beckman Coluter, DU730, Life Science) were utilized in this study.

Extraction of Pectin: Pectin was extracted fromlemon and grape fruit peels with a little modification in the method followed by Siddiqui *et al*. [14] The fruits were washed with distilled water and then air-dried. Fruits were peeled off and cut into small pieces of about 1 cm with the help of knife. 200 g of each citrus fruit peels were weighed and dipped in 1000 ml of industrial methylated spirit (IMS) in beaker. Then these peels were boiled in water bath for 5 min. After that the solvent was decanted carefully. The boiled peels were then blended with 600 ml of distilled water for 30 sec. The loose slurry was obtained which was then boiled for 10 min on Bunsen burner. The mixture was left at room temperature to cool down. The pH was adjusted with the help of 0.1N hydrochloric acid solution. The mixture was then filtered with muslin cloth. Ethanol (95%) was added to the filtrate at the ratio of 1:3 to precipitate pectin. The precipitated pectin was filtered with the help of Buchner funnel. The beaker was weighed and then the pectin was transferred into it. The beaker was reweighed. The wet pectin was freeze-dried by tray dryer and the beaker was weighed again and yield was calculated for wet and dried pectin by the following formulae respectively.

$$\% \text{ Yield} = \text{Weight of wet pectin} / \text{ weight of peels} \times 100$$

$$\%\text{Yield} = \text{Weight of dried pectin} / \text{ weight of peels} \times 100$$

The dried pectin from both citrus sources was sieved with mesh number 60 and then desiccated.

FTIR Spectral Analysis: The extracted citrus pectin (GP and LP) and the standard pectin samples were subjected for FTIR spectroscopic analysis. 1 g of each sample was ground and then through Smart Acr attachment, the samples were analyzed and scanned within the range of 4000-400 cm^{-1} [15].

Antioxidant Activity: The reducing power assay of extracted pectin (LP and GP) as well as of standard pectin was performed with reference of the method followed by Xu, Tai, Wei, Yuan, Gao [7]. 1ml of different concentrations (10 to 100 µg/ml) of each sample solution was taken in test tubes. 5ml of phosphate buffer (2 M, pH 6.6) was added in all test tubes including blank. 2.5 ml of 1% solution of potassium ferricyanide was added in all test tubes. The solutions were then incubated at 50 °C for 20 min. 5 ml of 10% solution of trichloroacetic acid was added in all test tubes. 5 mL of each solution was taken from all test tubes into other respective test tubes and 5 ml distilled water was added in these test tubes. Then 1 ml of 1% of ferric chloride solution was added in the test tubes and left for 10 min.

The whole experiment was conducted in three replicates. Finally, the treated solutions were analyzed at 700 nm and percentage reducing power was calculated for extracted and food grade pectin with reference of ascorbic acid as standard by the following formula.

$$\text{Percentage reducing power} = [1 - (1 - At / As)]$$

At = Absorbance of the test sample
As = Absorbance of standard (ascorbic acid) at maximum concentration

Brine Shrimp Lethality Bioassay: The brine shrimp lethality bioassay was performed according to Rahman, Choudhary and Thomsen [16]. 20 mg of standard and extracted pectin (LP and GP) was dissolved in 2 ml of distilled water to make solution of a concentration of 10 mg/ml. From each solution 5, 50 and 500 µl were transferred to different vials to make the concentrations of 10, 100, 1000 µg/ml respectively, and total of 12 vials was obtained. After 2 days when hatching of shrimps was taken place, 10 larvae were transferred to each vial with the help of Pasteur pipette in three replicates. The volume was made up to 5 ml with seawater. Then the solutions were incubated at 25-27 ºC for 24 h under illumination. The negative control was prepared by solvent only. After 24 h, number of survivors in each vial was counted by using magnifying glass. At 95% confidence of interval, LC_{50} was determined by Probit analysis. Etoposide (LC_{50} 7.6425 µg/ml) was considered a positive control.

Statistical Analysis: The mean ± standard error mean of three replicates was determined by SPSS, version 22 and LC_{50} was determined at 95% of confidence of interval by Probit analysis using Minitab [17].

RESULTS AND DISCUSSION:

Yield of Extracted Citrus Pectin: The current study was designed to extract pectin from lemon and grapefruit peels by hot water extraction method using IMS for bleaching purposes. Previously, pectin was extracted at different pH from 1 to 7 [14]. In the current study pH was adjusted using 0.1N solution of HCl. **Table 1** shows the yield of pectin on wet and dry form. The yield of LP and GP on wet basis was 11.57% and 17.28% respectively; whereas on dry basis, the pectin yield was found 1.48% and 1.94%, respectively. The result showed highest percentage yield in case of GP both on wet and dry basis. In the previous study, percentage yield of LP was recorded as 16.71% and 2.76% whereas the percentage yield of GP was observed as 15.70% and 1.68% on wet and dry basis respectively [17]. The variation in percentage yield among previous and current study may be due to different factors such as temperature, pH and duration of extraction [18, 19]. Method of extraction and type of acid used also influence the yield of pectin from its source [20].

TABLE 1: PERCENTAGE YIELD OF PECTIN FROM LEMON AND GRAPE FRUIT PEELS

Source	Percentage Yield of pectin on a wet basis	Percentage yield of pectin on a dry basis
Lemon (*Citrus limon*)	11.57	1.48
Grape fruit (*Citrus paradisi*)	17.28	1.94

FTIR Spectral Analysis: The FTIR spectra of LP **Fig. 1**, GP **Fig. 2** as well as of standard pectin **Fig. 3** were comparatively studied and their important identified functional groups were observed.

The pectin structure may be changed during extraction, storage, and other processes, so exact structure determination is quite difficult [21]. The spectra of the standard pectin as well as of tested extracted pectin showed peaks in the range of 3344.7 cm^{-1} to 3274.3 cm^{-1} representing sharp and strong stretching of O-H group indicating presence of hydrogen bond and carboxylic acid.

The medium sharp stretching peaks of tested extracted pectin at 3626.4 cm^{-1} and standard pectin at 3627.0 cm^{-1} indicated the presence of free O-H groups that may be due to the presence of carboxylic acid and ester. Identification of O-H group by the presence of strong and sharp peak at 3595.31 cm^{-1} was also observed in the previous study [22]. Carbohydrate ring is also represented by the presence of medium stretching of C-H group in the range of 2934.2 to 2925.7 cm^{-1}.

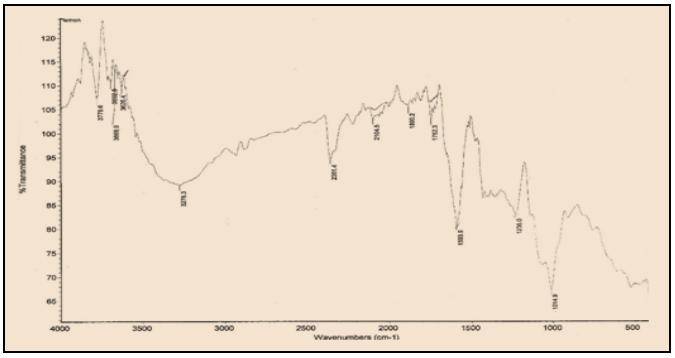

FIG. 1: FTIR SPECTRUM OF PECTIN FROM PEELS OF LEMON (LP)

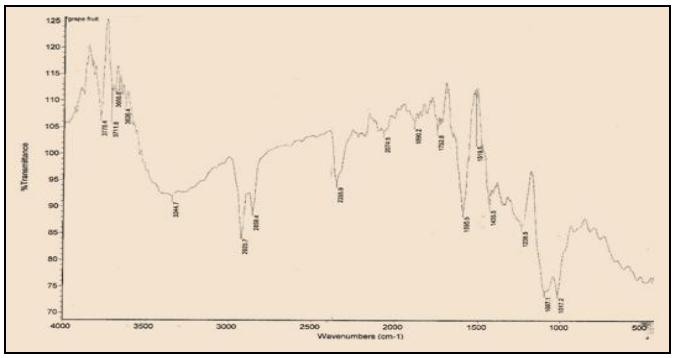

FIG. 2: FTIR SPECTRUM OF PECTIN FROM PEELS OF GRAPE FRUIT (GP)

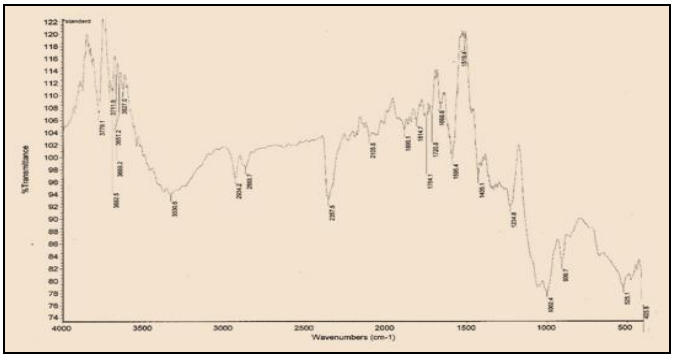

FIG. 3: FTIR SPECTRUM OF PECTIN FROM PEELS OF STANDARD PECTIN

The weak stretching of C≡C from 2105.8 to 2104.5 cm^{-1} showed alkyne group [22]. All the spectra of extracted citrus pectin showed C=O group due to strong stretching from 1752.8 to 1752.3 cm^{-1} and the same type of peak is also represented by the spectrum of standard pectin at 1754.1 cm^{-1} which indicated the presence of carboxylic acid and esters.

The medium stretching for the C=C group from 1595.5 to 1593.9 cm^{-1} showed conjugated alkene group. The medium stretching CH_3 group was present at 1435.5 cm^{-1} in GP, while this medium stretching of same group in standard pectin was found at wavelength 1435.1 cm^{-1} showed the existence of aromatic methyl group.

The strong stretching of C-O appeared from 1238.9 to 1014.9 cm^{-1} showed the presence of carboxylic acid and ester group and also confirmed the pyrenoid ring structures in the tested extracted and standard pectin as observed in the previous study Xu, Tai, Wei, Yuan, Gao [7].

Antioxidant Activity: Fig. 4 represents the antioxidant activity of LP, GP and standard pectin in comparison of ascorbic acid (in terms of absorbance at 700 nm). It was observed that ascorbic acid showed highest absorbance compared to extracted pectin as well as from standard pectin which was found to be from 0.043 ± 0.002 to 0.158 ± 0.05. All the extracted pectin showed absorbance in increasing order with increase in concentrations from 10 to 100 µg/ml.

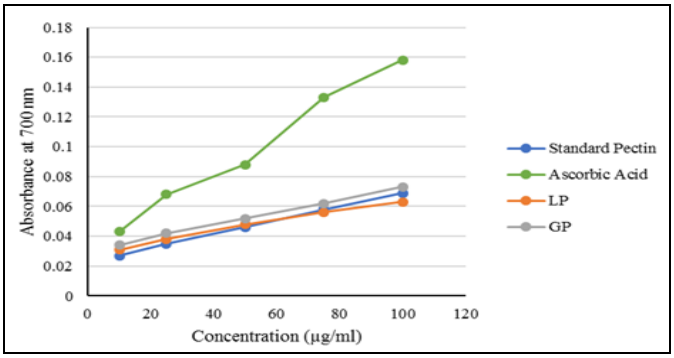

FIG. 4: ANTIOXIDANT ACTIVITY OF PECTIN EXTRACTED FROM LEMON AND GRAPE FRUIT

The percentage increase in reducing power was calculated for all the extracted and food grade pectin by taking absorbance of ascorbic acid at maximum concentration.

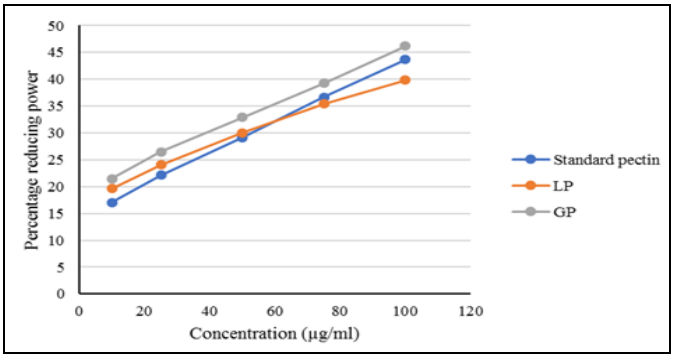

FIG. 5: FERRIC REDUCING POWER OF PECTIN EXTRACTED FROM LEMON AND GRAPE FRUIT

GP exhibited comparatively good antioxidant activity than that of standard pectin. GP exhibited absorbance in the range of 0.034 ± 0.002 to 0.073 ± 0.002 and percentage reduction power in between 21.50 to 46.20 which was higher than absorbance (0.027 ± 0.004 to 0.069 ± 0.004) **Fig. 4** and percentage reducing power (17.08 to 43.67%) **Fig. 5** of standard pectin.

The comparative results of LP with standard pectin showed higher absorbance and percentage reduction at all concentrations with exception of two concentrations (75 µg/ml and 100 µg/ml) where LP showed slightly lower absorbance and percentage reduction than that of standard pectin **Fig. 4** and **5**. The absorbance of LP at 75 and 100 µg/ml was found to be 0.056 ± 0.008 and 0.063 ± 0.002 respectively whereas the standard pectin showed 0.058 and 0.069 respectively which was slightly higher than that of LP. Similarly, the percentage reducing power of LP at 75 and 100 µg/ml was 35.44 and 39.87% respectively and standard pectin exhibited 36.70% and 43.67% respectively at same concentrations. GP also exhibited higher percentage reducing power (21.50 to 46.20%) than that of LP (19.62 to 39.87%) **Fig. 5**.

The positive results of the antioxidant activity of pectin by reducing power assay also supported through another study [7]. The overall mechanism behind the reducing power assay is the donation of electron from the antioxidant which causes reduction of Fe^{+3}/ferricyanide complex to ferrous form which causing Perl Prussian blue formation at 700 nm. The amount of Fe^{+2} formation can be monitored by this Perl Prussian blue formation [23]. This complex formation was quantified in case of extracted as well as of standard pectin. All the solutions showed green color with Prussian blue shade. The favorable structure of pectin towards reducing power is due to the presence of abundant of -OH and -COOH groups [7].

Brine Shrimp Lethality Bioassay: Researchers have been working to further explore the toxicity of pectin against the tumor cells. Pectin has already shown toxicity against colon and breast cancer cells [8, 9].

TABLE 2: BRINE SHRIMP LETHALITY BIOASSAY OF EXTRACTED AND STANDARD PECTIN

Sample	Conc. (µg/ml)	No. of survivors			Total no. of survivors	SEM	% Mortality	LC_{50} (µg/ml)	Lower limit	Upper limit
		T1	T2	T3						
LP	10	9	3	2	14	±2.1	53.33	8.1965	2.3991	14.5353
	100	0	0	0	0	±0.00	100			
	1000	0	0	0	0	±0.00	100			
GP	10	8	10	9	27	±0.5	10	218.608	117.28	448.1354
	100	9	6	7	22	±0.88	26.66			
	1000	1	1	3	5	±0.6	83.33			
Standard pectin (FGP)	10	9	8	8	25	±0.5	16.66	209.5	105.55	485.94
	100	9	5	10	24	±2.6	20			
	1000	3	2	0	5	±1.5	83.33			

LC_{50} of Etoposide (standard drug) = 7.4625µg/ml [24]

Table 2 represents the cytotoxicity of extracted pectin and standard pectin by brine shrimp lethality bioassay. The cytotoxic results of LP showed 53.33% mortality of shrimps at 10 µg/mL but 100% mortality at 100 and 1000 µg/mL which was higher than the percentage mortality of shrimps by standard pectin (16.66 to 83.33%). The LC_{50} of LP is 8.19 µg/mL which was closed to the LC_{50} value of etoposide (7.46 µg/mL) and but lower than that of LC_{50} of standard pectin (209.5 µg/ml).

The GP behaved slightly similar to standard pectin regarding percentage mortality and LC_{50}. The percentage mortality of shrimps by GP at 10 and 100 µg/ml was 10 and 26.66% respectively whereas the standard pectin showed close values 16.66 and 20% mortality at the same concentrations at 100 µg/ml both GP and standard pectin showed 83.33% mortality. The LC_{50} of GP was found to be 218.6 µg/ml which was near to the LC_{50} of standard pectin (209.5 µg/ml). According to percentage mortality and LC_{50}, GP and standard pectin were closely related to each other. While comparing the LC_{50} of GP with that of etoposide (7.4625 ug/ml), GP was found to be less toxic than etoposide. Moreover, LC_{50}<1000 µg/ml is considered significantly active and LC_{50}>1000 µg/ml is considered as inactive compounds [25]. While observing the LC_{50} of LP and GP and standard pectin in current study, all the pectin behaved as cytotoxic.

Different researchers have proved the cytotoxic effect of pectin by different mechanisms. Pectin inhibits galectin-3 mediated actions to decrease cancer cells progression [26].

The previous study also exhibited inhibition of galectin-3 response and cancer cell cycle arrest after treating with citrus and apple pectin [27]. RG-1 part of pectin plays an important role in suppressing the galectin-3 dependent responses because of having short side chains of galectin [28]. Another research data also confirmed the correlation of antiproliferative action of pectin with its RG-1 fragment. Whereas HG component of pectin and neutral sugars side chains also participate in the inhibition of cell proliferation. The immunostimulatory mechanisms of pectin play positive role against cancer cells due to enhancement of proliferation of lymphosides, implifying macrophage activity and increasing natural killer (NK) cells [29]. All the extracted pectin (LP and GP) showed positive results concerning antioxidant activity and brine shrimp lethality bioassay.

CONCLUSION: Cancer and diseases causing due to lack of antioxidants in the body are widely spread all over the world. Thus, focusing on cost-effective and natural sources, the study was designed to investigate antioxidant and cytotoxic activities of pectin. Among lots of natural compounds, pectin is also proved for its anticancer and antioxidant activities. In current work, LP and GP exhibited antioxidant and cytotoxic activity by ferric reducing power assay and brine shrimp lethality bioassay, respectively. The results also showed that antioxidant activity of GP was higher than that of FGP and LP exhibited closed values to that of FGP. However, LP exhibited higher cytotoxicity than FGP and GP showed closed values to that of FGP. Further investigation is required for the confirmation of the observed results.

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CONFLICTS OF INTEREST: All the authors have no conflict of interest in this study.

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