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Characterization and Modification of Pectin Extracted from Orange Peel

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GRAPHICAL ABSTRACT

In the present study pectin was extracted from orange peel and its purification, modification and characterization was done. Pectin was extracted with an acid i.e. EDTA (ethylene diaminetetracetic acid) by using microwave method at 460W, for 15 mins. and pH (2-3). Purification of pectin was done by using partial oxidation with iodine solution and modification of pectin was done by using base hydrolysis. Yield of extracted pectin, purified pectin and modified pectin were 34.02, 38.88, 83.33% respectively. Pectin was identified with the help of stiff gel test, test with iodine solution. Extracted pectin contains many phytochemicals which were identified chemically. The characterization of pectin was done through TLC data and FTIR analysis. The FTIR spectra of extracted pectin, purified pectin, modified pectin samples were determined using a computerized FTIR spectrometer in the range of 44000-400 cm-1 by KBr pellet technique. Three outstanding features of infrared spectra of of pectin band were at 1600- 1680 cm-1(for C=O), 3700 cm-1 (for -O-H) and 1000 cm-1 above (for –C-O-R).

Keywords: Citrus fruits, Pectin, Peel, Polygalcturonic acid, Methoxylated hetropolysaccharide.

INTRODUCTION

Pectin is a naturally occurring biopolymer which has many applications in the pharmaceutical and biotechnology industry. It is a complex mixture of polysaccharides (hetero polysaccharides) found in the primary cell walls of terrestrial plants and the highest concentration of pectin is found in the middle lamella of cell wall. It is used as thickening agent in many food and beverages industries. Sweet oranges are citrus fruits which consist of two parts namely the peels and pulp. These two parts easily separated from each other with the pulp serving the edible parts of the fruit while the peels as a good source of pectin. Pectin is purified carbohydrate product obtained from the inner portion of the peels of citrus fruits. It consists chiefly of partially methoxylatedpolygalcturonic acid. Pectin is capable of forming gels with sugar and acid under suitable conditions. It is formed almost universally in plant cell of all species suitable for using the production of sugar jellies and industrial production of apple pomace, citrus peels and sugar beat chips. Although pectin occurs commonly in most of the plant tissues as a cementing substance in the middle lamella and as a thickening on the cell wall, the no. of sources that may be used for the commercial manufacture of pectin very limited. Citrus pectin are light cream or light tan in color; apple pectin are often darker. The pectin is separated as a stringy gelatinous mass, which is pressed and washed to remove the mother liquor, dried and ground. Pectin is an essentially linear polysaccharide. Like most other plant polysaccharides, it is both polydisperse and polmolecular and its composition varies with the sources and the conditions applied during isolation. In any sample of pectin parameters such as the molecular weight or the content of particular subunit will differ from molecule to the molecule. The structure of pectin is very difficult to determine because pectin can change during isolation from plants, storage and processing of plant material. Pectin is a solid powder, off white in color; pectin is soluble in pure water. Pectin is practically odorless. It is mucilaginous. Pectin is stable at ordinary conditions, becomes unstable in excess heat. Pectin is soluble in pure water, partially soluble in cold water. Pectin is combustible at higher temperature, because of this it must be kept away from heat and any source of ignition. Dry powder pectin, when added to water, has a tendency to hydrate, very rapidly to forminggel [1-6].

Pectin contain three major forms: homogalacturonan (HG), rhamnogalacturonan I (RGI), and rhamnogalacturonan II (RGII). The major component of all the forms of pectin is a linear chain of D-galacturonic acid units, in which varying proportions of the acid groups are present as methyl esters [2].

The presence of non-sugar substituents, methyl and acetyl groups, is a very important feature of pectin polymers, affecting their functionalities. Pectin producers traditionally divide pectin polymers into highand low-methoxylpectins (HM and LM pectins). The boundary level between the two types is 50%, meaning that if more than 50% of a pectins carboxyl groups are methylated, it is termed HM pectin, and if this proportion is below 50%, it is LM pectin.

MATERIALS AND METHODS

Materials

Orange peel, Acid (HCl / EDTA / HNO_3), 95% Ethanol.

Sample preparation

Orange fruits were purchased from local market, Kasana Greater Noida (U.P.). The Orange peel were split/cut into four parts and the peel removed (a soft white substance inside the skin of citrus fruits), then the peels were further cut into smaller pieces for easy drying and washed with large quantity of water to remove the Glycosides the bitter taste of the peels and then weighed with a digital weighing balance and air dried.

Method

The 50 g dried peels were separately transferred into a Round bottom flask (500 mL) containing 250 mL of water 1.5 mL hydrochloric acid was added to give a acidic pH of 2. Orange peel was then boiled or reflux for 15 min at 460 W or 90°C. Thereafter, the peels were removed from the extracts by filtering through a filter paper. The cake was washed with 150 mL boiled water and the combined filter allowed to cool to 25°C to minimize heat degradation of the pectin. The extracted pectin was precipitated by adding 200 mL 95% ethanol to 100 mL of the extracted pectin with thorough stirring with the help of magnetic stirrer, left for 30-60 min to allow the pectin float on the surface. The gelatinous pectin flocculants was then skimmed off. The extracted pectin was purified by washing in 200 mL ethanol and then pressed on a nylon cloth to remove the residual HCl and universal salt. The resulting pectin was weighed and shredded into small pieces and was air dried. Finally, the dried pectin was further reduced into smaller pieces using a pestle and mortar and weighed using a digital weighing balance [2].

Figure 1: Extracted pectin

Purification of pectin

Norman and Norris [1930] had observed that pectin is very stable to oxidation with bromine in presence of calcium carbonate. Block and Cowgill [1932] had already used a method of oxidation to remove gums etc. from antineuritic vitamin extracts.Iodine and bromine are usually employed as oxidising agents in alkaline solution. The presence of any alkali is evidently ruled out in this case on account of the extreme susceptibility of the ester groups in pectin to alkalis. However, it was found that oxidation of the impurities readily took place in a neutral or faintly acid medium without any appreciable effect on the pectin. Dilute solution of hydrogen peroxide was also tried without success [6]

Method

Purification by partial oxidation with iodine solution

Pectin solution, about 0 5 %, was prepared by dissolving the pectin in boiling water. To 50 mL.of the cooled pectin solution about 3 mL of N/10 iodine solution were added. The oxidation was allowed to proceed for varying periods and at different temperatures. The excess of iodine was then removed by shaking two or three times with ether until the aqueous layer was colorless. The excess could also be removed by warming in vacuo at 40°C, or by bubbling air through the solution at the same temperature. Pectin was then precipitated by 95 % alcohol and after two hours it was filtered, finally passed through various grades of alcohol and dried in the oven at 50 for 20 h.

Figure 2: Purified pectin

Modification of pectin

Plant pectin are highly branched complex molecules and, therefore, their solubility is low. This fact creates a large obstacle in regards to the application of pectin in the medicinal field. Scientists have developed Modified Citrus Pectin (MCP), which increases the ability of pectin to biologically interact with biomolecules such as proteins and lipids. Baldwin and Shah (1993) and Pienta*et al*. (1995) have prepared modified citrus pectin by treating the pectin molecules with different pH levels and temperatures. First, citrus pectin is briefly solubilized as a 1.5% solution in distilled water and its pH is increased to 10.0 with NaOH (3N) for 1 h at 50-60°C. The solution is then cooled to room temperature, while its pH was adjusted to 3.0 with 3 N HCl and stored overnight. Samples are precipitated the next day with 95% ethanol and incubated at 20°C for 2 h. Lastly, the samples are filtered, washed with acetone and dried on Whatman filters (Figure 13). Generally, MCP has a molecular weight of 15400 g/mol and is a mostly linear homogalacturonan with a 3.8% esterification and about 10% rhamnogalacturonan MCP can be used for experimental purposes or given to patients. MCP is more easily absorbed by the digestive tract than normal citrus pectin and is then absorbed

into the bloodstream. MCP is available as a powder or a capsule. The dose suggested by manufacturers for the powder is five grams (nearly a fifth of an ounce) mixed with water or juice and taken three times a day with meals (Nicholas, 2009). As suggested by American Cancer Society (http://www.cancer.org/) the suggested dose for capsules (pecta-Sol) is 800 mg three times a day with meals [3].

Figure 3: Modified pectin

Identification test of pectin

Stiff gel test: 1 g of pectin was heated with 9 mL of water on a water bath till a solution is formed, on cooling stiff gel formed was taken as positive sample [7].

Test with 95% Ethanol: On adding an equal volume of ethanol (95%) to 1% w/v solution of pectin sample, a translucent, gelatinous precipitate produced (distinction from most gums) was taken as positive test [7].

Test with Potassium Hydroxide (KOH): To 5 mL of a 1% w/v solution of pectin sample, 1mL of a 2% w/v solution of KOH was added and set aside for 15 min. A transparent semi gel will be produced. When the above gel is acidified with dilute HCl and shaken well, a voluminous, colorless gelatinous precipitate is formed. This upon boiling will become white and flocculent [7].

Iodine test: To 5 mL of recently boiled and cooled 2% w/v solution of sample, 0.15 mL of Iodine solution was added. No blue color presence was taken as an indicator of positive test [7].

Test for Acidity: An aqueous solution of pectin sample was acidic to blue litmus paper [7].

Qualitative analysis

Solubility

Cold water: To the 5 mL of distilled water added 0.5 g pectin to form a suspension.

Hot water: To 5 mL of hot water added 0.5 g pectin to form a clear solution.

pH : 4.3

Phytochemical screening

The portion of the dry extracts was subjected to the phytochemical screening. Phytochemical screening was performed to test for alkaloids, saponin, tannins, flavanoids, steroids, sugars, cardiac glycosides and anthraquinones [7].

Test for alkaloids

The 0.5 g of the extract was dissolved in 5 mL of 1% HCl and kept in water bath for about 2 min. 1 mL of the filtrate was treated with Dragendroff's reagent. Turbidity or precipitation was taken as indicator for the presence of alkaloids.

Test for Tannins

About 0.5 g of the sample was dissolved in 10 mL of boiling water and was filtered. Few mL of 6% $FeCl_3$ was added to the filtrate. Deep green colour appeared confirmed the presence of Tannins (Trease and Evans, 1983).

Test for Flavanoids

About 0.2 g of the extract was dissolved in methanol and heated for some time. A chip of Mg metal was introduced followed by the addition of few drops of conc. HCl. Appearance of red or orange color was taken as indicator of the flavanoids.

Test for Saponin:

About 0.5 g of the extract was stirred with water in the test tube. Frothing persists on warming was taken as an evidence for the presence of saponin.

Test for Steroids

Salkowski's method was adopted for the detection of steroids. About 0.5 g of extract was dissolved in 3 mL of chloroform and filtered. To the filtrate, conc. H_2SO_4 was added to form a lower layer. Reddish brown color was taken as positive for the presence of steroids.

Test for Cardiac glycosides

About 0.5 g of the extract was dissolved in 2 mL of glacial acetic acid containing 1 drop of 1% $FeCl_3$. This was under laid with conc. H_2SO_4. A brown ring obtained at the interphase indicates the presence of deoxy sugar. A violet ring appeared below the ring while in the acetic acid layer a greenish ring appeared just above ring and gradually spread throughout this layer [8].

Test for reducing Sugars

1 mL each of Fehling's solutions, I and II was added to 2 mL of the aqueous solution of the extract. The mixture was heated in a boiling water bath for about 2-5 min. The production of a brick red precipitate indicated the presence of reducing sugars.

Test for Anthraquinones

5 mL of the extract solution was hydrolyzed with dil/conc. H_2SO_4. 1 mL of dilute ammonia was then after be added to it. Rose pink colour confirmed the presence of anthraquinones.

Quantitative analyisis

Moisture content

The moisture content was calculated by the following formula (Ranganna.1995) 5 gm of sample in previously dried and petri dish was Weighed and placed in hot air oven for 2 h at 130°C [3]. The dish was removed and cooled in a desiccators and then weight [8].

$$\text{Moisture content \%} = \text{weight of residue } (W_1\text{-}W_2) / \text{weight of sample}(W_1\text{-}W_2)$$

Where,
W is weight of petridish (g), W_1 is weight of petridish withsample (g), W_2 is weigh of petridish with dried sample (g)

Ash content

It was determined as per reference of (Ranganna.1995) [3]. Weighed 1.2 g of pectin substance (sample).The sample was ignited slowly and then heat for 3-4 h at 600ºC. Then cooled the crucible to room temperature in a desiccators and weighted properly. The process will be weighted till constant weight come and final weight will be noticed [8].

$$\text{Ash content \%} = \text{weight of ash } (W_2\text{-}W_1) / \text{weight of sample } (W)$$

W_2 is Final weight of dish with Ash, W_1 is Weight of dish. W is Weight of sample

Equivalent weight

Equivalent weight is used for calculating the anhydrouronic acid content and degree of esterification. It is determined by titration with sodium hydroxide to pH 7.5 using either phenol red or Hinton's red indicator. It was determined by Ranganna's method (1995) [3]. Pectin sample (0.5 g) was weighed into a 250 mL conical flask and moistened with 5 mL ethanol; 1.0 g sodium chloride was added to the mixture followed by 100 mL distilled and few drops of phenol red indicator. Care was taken at this point to ensure that all the pectin had dissolved and that no clumping occurred at the sides of the flask before the solution was then slowly titrated (to avoid possible de-esterification) with 0.1 M NaOH to pink color at the endpoint.[8].

$$\text{Equivalent weight} = \text{weight of sample} \times 1000 / \text{mL of alkali} \times \text{normality of alkali}$$

Methoxyl content

Determination of MeO content was done by using the Ranganna's method (1995) [3]. The neutral solution was collected from determination of equivalent weight, and 25 mL of sodium hydroxide (0.25 N) was added. The mixed solution was stirred thoroughly and kept at room temperature for 30 min. After 30 min 25 mL of 0.25 N hydrochloric acid was added and titrated against 0.1 N NaOH to the same end point as before like in equivalent weight titration. The methoxyl content or degree of esterification is an important factor in controlling the setting time of pectin, the sensitivity to polyvalent conations, and their usefulness in the preparation of low solid gels, fibers and film. It is determined by saponification of the pectin and titration of the liberated carboxyl group [8].

$$\text{Methoxylcontent \%} = \text{mL of alkali} \times \text{normality of alkali} \times \text{weight of pecin} \times 100 / \text{weight of sample}$$

Chromatographic and spectroscopic techniques

TLC Profile

TLC Separation was performed for the pectin extracted from apples and oranges. Standard pectin was also taken as reference. In TLC, detection system used was Butanol: Water: Acetic acid (5: 4: 1). Iodine chamber was also used for detection. R_f values were determined safter the appearance of spots.

Fourier Transform Infrared (FTIR) study

The IR spectrum of extracted pectin, purified pectin, modified pectin extracted from orange peel was recorded in Jamia Millia Islamia university (New Delhi), India using a computerized FTIR spectrometer (Bruker Pvt. Ltd. Japan) in the range of 4000-400 cm^{-1} by the KBr pallet techniques.

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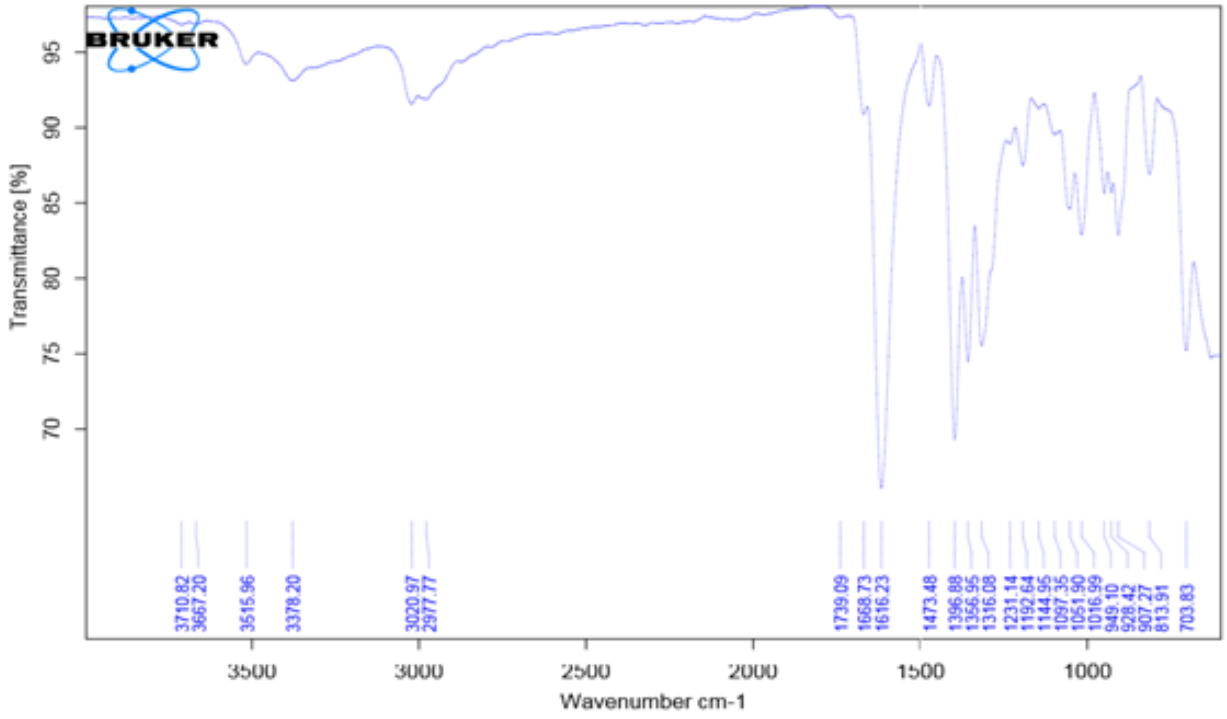
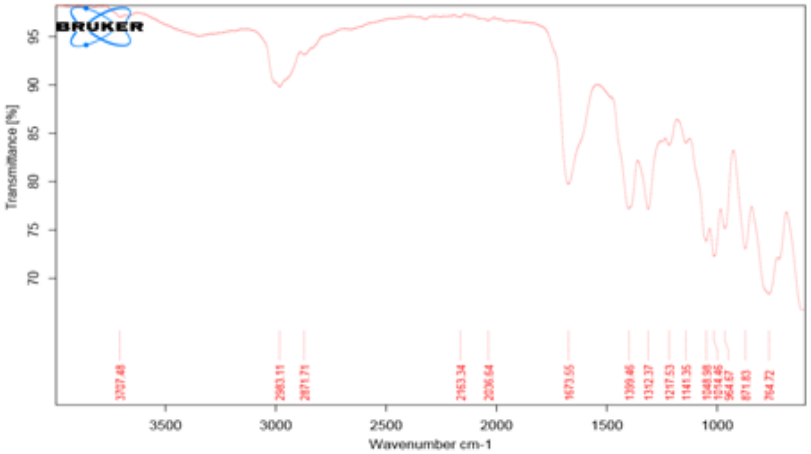
FTIR data shows that extracted pectin gives C=C aromatic & aliphatic stretching frequency and N-H amine stretching frequency but purified pectin & modified pectin does not give.



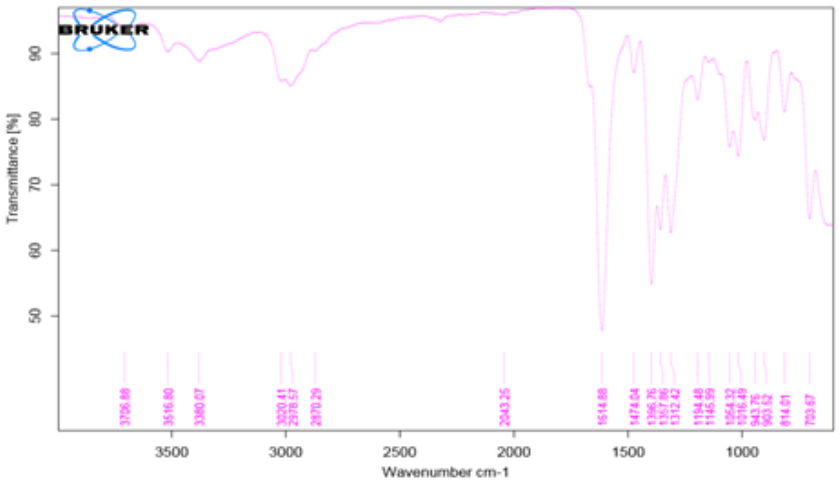
S. No.	Stretching frequency(cm-1)	Functional group
1.	3710	-O-H alcohol
2.	3515	N-H amine
3.	1668	C=O carbonyl
4.	3020	C-C alkane
5.	2977	C-H stretching
6.	1016	C-O-R ester
7.	1616	C=C aliphatic
8.	1668	C=C aromatic
9.	1144	-C-O alcohol

Figure 3: FTIR of extracted pectin



S. No.	Stretching frequency (cm-1)	Functional group
1.	3707	-O-H alcohol
2.	2983	-C-H sp3
3.	1673	C=O ester
4.	1141	C-O alcohol
5.	1014	-C-O-R ester

Figure 4: **FTIR of purified pectin**



S. No.	Stretching frequency (cm-1)	Functional group
1.	3706	-O-H alcohol
2.	3516	-O-H carboxylic
3.	3020	C-H stretching
4.	2870	C-H aldehyde
5.	1145	-C-O alcohol
6.	1016	-C-O-R ester
7.	1614	C=O carbonyl

Figure 5: FTIR of modified pectin

Antioxidant activity

Estimation of Total Phenolic Content (TPC) of Pectin

The Total phenolic content of each pectin sample was determined by the method of Singleton and Rossi (1965). The phenolic content was expressed as mg/g gallic acid equivalents. The method and calculation is same for all extracted pectin, purified pectin and modified pectin.

Preparation of Reagent

(1) 7.5% Sodium carbonate: To 7.5 g of sodium carbonate was dissolved into 100 mL of distilled water.

(2) 10% Folin: To 90 mL distilled water was mixed with 10 mL Folin reagent. Folin reagent is a mixture of hetro polyphospho-molybdate and polyphospho-tungstate.

Preparation of blank: 5 mL folin + 4 ml sodium carbonate solution+1ml distilled water.

Preparation of standard solution of Gallic acid: To 0.025 g Gallic acid was dissolved into 5 mL of distilled water. The concentration of solution was 5 µg/mL.

S. No.	Concentration (µg/ml)	volume taken from std. Gallic acid solution (ml)	Volume of water added (ml)	Final volume (ml)
1	250	0.25	4.75	5
2	200	0.2	4.8	5
3	150	0.15	4.85	5
4	100	0.1	4.9	5
5	50	0.05	4.95	5

Table 1: Standard Gallic acid solution preparation for different concentration

Preparation of stock solution of pectin: To 0.025 g pectin was dissolved into 5 ml of distilled water. The concentration of solution was 5 µg/ml.

S. No	Concentration (µg/ml)	Volume taken from stock solution of pectin (ml)	Volume of water added (ml)	Final volume (ml)
1	250	0.25	4.75	5
2	200	0.2	4.8	5
3	150	0.15	4.85	5
4	100	0.1	4.9	5
5	50	0.05	4.95	5

Table 2: Pectin solution preparation for different concentration.

Method

1 ml aliquots of each sample (standard Gallic acid) was added to 4 ml of 7.5% (w/v) Na2CO3 solution into testtubes. After 2 minutes of the incubation. 5 ml Follin-Ciocalteu reagent was added and the mixture was allowed to stand for 30 min at 25°C. The absorbance was measured at 750 nm using a UV-VIS. spectrophotometer. Same procedure is done with pectin solution. The blank consist of all reagents and solvents but no sample. The Total Phenolic Content (TPC) was determined using the standard gallic acid calibration curve [9].

Scavenging activity of pectin with DPPH

The scavenging activity of extracted pectin, purified pectin and modified pectin was done with DPPH. The calculations are same for extracted pectin, purified pectin and modified pectin. DPPH (1,1-Diphenyl-2-picrylhydrazyl) is a stable free radical with purple color (absorbed at 517 nm). If free radicals have been scavenged, DPPH will generate its color to yellow. This assay uses this character to show herbs free radical scavenging activity.

Preparation of DPPH solution: 0.004 g (4mg) DPPH was taken into 100 ml volumetric flask and filled with methanol up to 100 ml. the concentration of DPPH solution was 0.04 µg/ml.

Preparation of standard L-Ascorbic acid solution: To 0.025 g Ascorbic acid was dissolved into 5 ml of distilled water. The concentration of solution was 5µg/ml.

S. No.	Concentration (µg/ml)	volume taken from std. L-Ascorbic acid solution (ml)	Volume of water added (ml)	Final volume (ml)
1	250	0.25	4.75	5
2	200	0.2	4.8	5
3	150	0.15	4.85	5
4	100	0.1	4.9	5
5	50	0.05	4.95	5

Table 3: Standard L-Ascorbic acid solution preparation for different concentration.

Preparation of stock solution of pectin: To 0.025 g pectin was dissolved into 5 ml of distilled water. The concentration of solution was 5 µg/ml.

S. No	Concentration (µg/ml)	Volume taken from stock solution of pectin (ml)	Volume of water added (ml)	Final volume (ml)
1	250	0.25	4.75	5
2	200	0.2	4.8	5
3	150	0.15	4.85	5
4	100	0.1	4.9	5
5	50	0.05	4.95	5

Table 5: Pectin solution preparation for different concentration.

Preparation of blank: 3 ml methanol+2ml distilled water.

Method

Taken 2 ml of different concentration pectin sample in to separate test tube and added 3 ml of DPPH reagent and the mixture was allowed to stand for 30 minutes at 25°C. The absorbance was measured at 750 nm using a UV-VIS. spectrophotometer. Same procedure is done with pectin solution. The blank consist of all reagents and solvents but no sample. The DPPH radical scavenging effect was calculated by the equation below.

DPPH radical scavenging activity %= [1– (absorbance of sample/absorbance of control)] ×100

Antimicrobial activity

Agar well diffusion method: Agar well diffusion method is widely used to evaluate the antimicrobial activity of plant extract. The pectin extracted from oranges peel was dissolved in (1 mg/ml) and evaluated for in vitro antimicrobial activity.

Culture Media: The media used for antibacterial test is Nutrient L.B. Agar (2.5 g/100 ml water) & L.B. Broth(4 g/100ml water) of Hi media Pvt. Bombay, India.

Inoculum: The bacterial pathogen was inoculated into nutrient broth (4 g/100 ml of water) and incubated at 37°C for 18 h and the suspension will be checked. The inoculated bacteria was stored at 4°C.

Microorganisms used: Pure cultures of pathogenic bacterial *E. coli* taken from biotechnology laboratory of Gautam Buddha University Greater Noida.

Determination of diameter of zone of inhibition by well diffusion method: The agar well diffusion method (Perez *et al*., 1993) was modified. Nutrient agar medium (NAM) was used for growth of pathogenic bacteria cultures [10]. The culture medium was inoculated with the bacterial pathogen separately suspended in nutrient broth. A Total of 8 mm diameter wells were punched into the agar and filled with separate endophytic fractions and solvent blanks. Standard Pectin solution (1 mg/ml) was simultaneously used as the positive control and a volume (20-100 µl) of the pectin extract is introduced into well. Then plates were incubated at 37°C for 18 h. The antibacterial activity was evaluated by measuring the diameter of zone of inhibition observed.

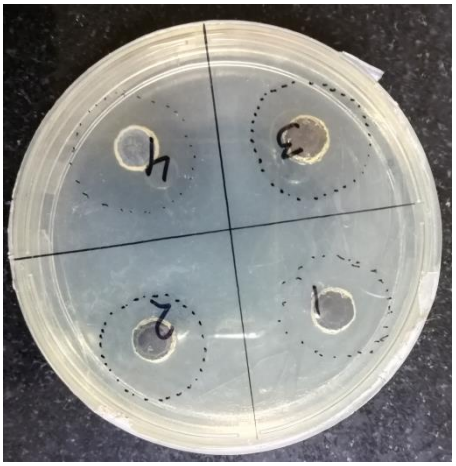


Figure 6: 1) Extracted pectin 2) Modified pectin, 3) Purified pectin and 4) Modified pectin+purified pectin.

RESULTS

Pectin was extracted from orange peel. It was purified and modified. A detailed characterization of the extracted pectin was done which included its phytochemical screening, FTIR analysis, UV analysis and TLC. Extracted pectin has shown good anti-oxidant property. All the extracted, purified and modified pectin has shown good anti-bacterial property against *E.coli*.

CONCLUSION

A very cost effective and novel biopolymer pectin was extacted from orange peel. Extracted, purified and modified pectin all have very strong present and future applications in food industry, in pharma industry, in drug delivery process. A detailed study of physical property, antioxidant property, scavenging property of pectin is done in this paper. Pectin is a promising canditate for further research in pharmacology and medicine application, because of its considerable availability from renewable sources and non-toxic effect.

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