African Journal of Pharmaceutical Research and Development
Available online at https://ajopred.com
Vol. 16 No.2; pp. 60-66 (2024)

Original Research Article

EXTRACTION, PURIFICATION AND CHARACTERIZATION OF PECTIN OBTAINED FROM THE PULP OF *PARKIA BIGLOBOSA* AND THE EVALUATION OF ITS CYTOTOXIC ACTIVITY ON HEPG2 CANCER CELL LINE

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ABSTRACT

Pectin, a naturally occurring polysaccharide that has a wide range of industrial applications especially in the pharmaceutical industries, has in recent times gained a lot of attention by researchers with potential breakthrough as a therapeutic agent. The aim of this research was to extract, purify and characterize pectin obtained from the pulp of *Parkia biglobosa* and to evaluate its cytotoxic assay on HepG2 cancer cell line. Acidified water (using HNO_3 and HCl) was used for the extraction at pH levels 1.5, 3 and 5. Chemical and spectroscopic characterizations were adopted for this rresearch,and it was observed that pectin was best extracted from the source at a pH level of 1.5. The oil holding capacity was 1.41 g/g, while the percentage solubility, ash content and moisture content were 70%, 2.87% and 15.72% respectively. The pectin extract was found to have an equivalent weight of 953.19 $kgmol^{-1}$, whereas the AUA (anhydrouronic acid) content, methoxyl content, and DE (degree of esterification) were found to be 60.48%, 7.40%, and 69.50% respectively. The following functional groups: carboxyl, hydroxyl (3500 - 3300 cm^{-1} indicating -OH stretching vibration of carboxylic group), C-O-C stretching vibration, and an aromatic C-H vibration of methyl group, were found present. The percentage cytotoxicity values obtained at various concentrations (125 µg/cm^3, 250 µg/cm^3, 500 µg/cm^3 and 1000 µg/cm^3) of the pectin extract, were 4.60 %, 13.70 %, 16.8 % and 46.8 % respectively. This shows the cytotoxic activity of pectin extracted from the pulp of *Parkia biglobosa* on HepG2 cancer cells, and its potential of being an anticancer agent. The findings in this research have further placed *Parkia biglobosa* as a good source of commercial pectin for pharmaceutical application.

ARTICLE INFO

Received 03 May, 2024
Accepted 06 July, 2024
Published 13 July, 2024

KEYWORDS

Anticancer,
Cytotoxicity assay,
Human Hepatoma Cancer,
MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide),
Parkia biglobosa,
Pectin

INTRODUCTION

Diseases have had a large impact in science as they pose a serious threat to human life, but so many efforts in research carried out on plants have made plant extracts a reliable means of prevention of diseases, and possible treatment.

About eighty percent (80 %) of the world's population have been reported to rely on traditional medicine. In the last decades, several drugs of plant origin were discovered as

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https://doi.org/10.59493/ajopred/2024.2.6 ISSN: 0794-800X (print); 1596-2431 (online)

anticancer agents, and many of which have achieved both pre-clinical and/or clinical development [1].

Plants also play a vital role in the food chain and as a source of income, as about 90 percent of Africa's poorest population rely on plant [2]. Dietary fibre contains quite a number of non-starch polysaccharides, consisting of pectin, hemicellulose, gums, lignin, cellulose and b-glucans. This is contained mainly in the cell walls of plants of which fruits, pulses, cereals and vegetables are mostly consumed as dietary fibre. The most important source of vegetable fibre is the parenchymatous tissues [3].

Pectin is a naturally occurring polysaccharide that has a wide range of applications in the field of medicine, the pharmaceutical industries and biotechnology industry, amongst others. It also finds application in the food and beverage industry as a colloidal stabilizer, thickening and gelling agents. In the pharmaceutical industry, it is used as a matrix for drugs (drug coating) due to its unique properties. Pectin consists mainly of galacturonic acid (monomeric) units linked by α-(1,4) linkages [4].

Pectin was first isolated in 1825 by Henri Braconnot (a French chemist) who named it from the Greek word 'pektos', which means "solidify" [5]. For a good quality pectin, the DE (degree of esterification) provides information on its solubility, condition necessary for gelation (gel forming ability) and gelling temperature. The degree of esterification is the ratio of the esterified D-galacturonic acid units to the total D-galacturonic acid units of the pectin [6].

Commercial pectins are mostly isolated from apple pomace or citrus peel, which are both by products from juice production. There is about 20-30 % of pectin in citrus peel, while apple pomace contains about 10-15 % of pectin. Other sources include carrots, sugar beets, mango, banana, cabbage, guava, plums, pomelo peel amongst others [7].

The tree of *Parkia biglobosa* is a very good source of food in the arid and semi-arid region of Africa, especially during the period of drought and food shortages, whose importance cuts across both regional and international recognition. This is evident, as seen in its utilization in some societies in African as a therapeutic food, and as well, a source of income, which makes it not only a source of food [8].

The 'locust bean' is the seed (matured) covered by the pulp and are within the pod. The processed seeds are the fermented condiments which is known in Hausa, Igbo and Yoruba languages as 'Dawadawa', 'Ogiri', and 'Iru' respectively [9].

The aim of this research was to extract, purify and characterize pectin obtained from the pulp of African locust (*Parkia biglobosa*) bean tree and to evaluate its cytotoxic assay on liver cancer cells (HepG2 cancer cell line).

MATERIALS AND METHODS

Instruments and Equipment

The instruments and some equipment that were used includes; pH metre (model number; PHS-25 pH meter, China), FTIR (Fourier Transform Infrared) Spectrophotometer (Agilent technology Cary 630 Country of make, USA), Oven (DHG-9053A, China), Furnace (SX-5-D, China), Hot water bath (HH-4, China), Analytical balance (model number; ME204E, Switzerland), Magnetic stirrer-Hot plate (85-2, China), Centrifuge, xMark microplate spectrophotometer (Bio-Rad, India).

Sample Collection

The pods of African locust bean were obtained fresh from *Parkia biglobosa* tree at Nigerian Defence Academy (permanent site), Afaka, Kaduna State, of which the fresh leaves of the plant were taken to the Department of Biological Sciences (herbarium unit) at Nigerian Defence Academy, Kaduna, for identification. The plant was identified and authenticated by Prof. G. A. Ajibade, and the voucher number was assigned: NDA/BioH/2023/30.

Sample Preparation

The pods of African locust bean were dehusked to separate the husk from the fruit, with subsequent dehulling to separate the pulp from the seed. The pulp was then dried in an oven (temperature of 15 °C) before mashing, in which a powdered product of the pulp was obtained.

Extraction and Purification of Pectin from the Pods of *Parkia biglobosa*

Different methods have been used for the extraction of pectin. However, the method of extraction adopted for this experiment was as described by Khamsucharit *et al* [10]. Exactly 50 g of the sample (pulp of African Locust Bean) was weighed and immersed in a 200 ml distilled water, with the pH of the mixture adjusted to 1.5, by adding 0.2 M hydrochloric acid. This was repeated in two separate conical flasks but with pH maintained at 3 and 5 respectively. The mixtures (A, B and C) were heated at 60°C for 2 hours with constant stirring, after which they were passed through two folds of muslin and then cooled to room temperature. Equal volume of 95% ethanol was added to the filtrates to precipitate the pectin, of which the mixtures were then filtered, and the extracted product (pectin) was dried at 35°C in a hot air oven, weighed (separately) and stored in an airtight container.

The above procedure was repeated using 0.2 M HNO_3 in place of 0.2 M HCl earlier used.

The purification of the extracted pectin was carried out as described by Kumar *et al* [11]. Distilled water was added to the extracted pectin and boiled to make a solution. To every 100 cm^3 of the cooled pectin solution, 40 cm^3 of N/10 bromine water was added and allowed to oxidize. To remove the excess bromine water, the mixture was swirled 2 to 3 times, and ether was added until a colourless aqueous layer was obtained. The pectin was then precipitated, filtered and dried as earlier described in the extraction process.

Chemical Characterization of the Extracted Pectin

Oil Holing Capacity: this was determined by adopting the method described by Gan *et al* [12]. Exactly 1 g of the extract was weighed, mixed with 10 cm^3 of coconut oil (density: 0.91 g/cm^3) and kept for 5 hours in a desiccator. The suspension was then centrifuged for 15 minutes. The supernatant solution was weighed and the oil holding capacity was expressed as gram (g) of oil held per gram (g) of sample.

Solubility Test: a suspension of 0.2 g of the pectin extract with 15 cm^3 of buffer solution was made and incubated at room temperature for about 18 hours with constant shaking. The suspension solution was then centrifuged for 15 minutes. The supernatant was dried in an oven at 50 °C, until constant weight was obtained. The percentage solubility was then expressed as shown in equation 1:

$$\% \text{ Solubility } = w_1/w_2 \times 100 \text{----------} \textit{Equation 1}$$

where w_1 is the weight of dried supernatant and w_2 is the initial weight of the sample

Determination Ash Content: exactly 3.5 g of the pectin extract was incinerated in a muffle furnace at 600°C for 4 hours, after which the ash obtained was weighed to determine the percentage ash content, which was calculated using equation 2:

$$Ash\ content\ (\%) = (weight\ of\ ash\ /\ weight\ of\ sample) \times 100$$
……..Equation 2

Determination of Moisture Content: the amount of moisture present in the extract was determined by drying 2 g of the extract at 105°C to a constant weight, the dried extract was then weighed and the percentage moisture content was determined using the formula (equation 3):

$$\text{Moisture Content } (\%) = w_1/w_2 \times 100 \text{-------} \textit{Equation 3}$$

where w_1 is the weight of the residue and w_2 is the weight of the sample.

Effect of pH on percentage yield: the pH of the different acid solutions (nitric acid and hydrochloric acid) used for the extraction were varied (pH 1.5, 3 and 5) for separate extractions, and the percentage yield was determined respectively.

Equivalent weight (Ew): the equivalent weight was determined using standard method as explained by Altaf [13]. Exactly 0.5 g of pectin extract was weighed in a 250 cm^3 conical flask, it was then moistened with 5 cm^3 of ethanol. After which 100 cm^3 of deionised water was added with 5 drops of phenol red indicator. The pectin substance was then stirred rapidly using a magnetic stirrer (to dissolve it) and titrated with 0.1 M sodium hydroxide (slowly) until the colour of the solution changed and stayed for a minimum of 30 seconds. The neutralized solution was saved for the subsequent determination of methoxyl content.

The equivalent weight of pectin was then expressed as (Equation 4).

$$\text{Equivalent weight (Ew)} = (w \times 1000)/v \times M \text{----------} \textit{Equation 4}$$

where w is the weight of sample, v is the volume of alkali and M is the molarity of alkali used.

Methoxyl content (MeO): the methoxyl content was also determined by titration. Exactly 25 cm^3 of 0.25 M sodium hydroxide was added to the neutral solution of pectin, it was then allowed to stand for 30 minutes (after mixing thoroughly) at room temperature in a stopper flask. Exactly 25 cm^3 of 0.25 M HCl was then added and titrated with 0.1 M sodium hydroxide to the same end point as earlier obtained in Ew. The percentage methoxyl content was then calculated using the expression.

$$\% \text{ methoxyl content} = (v \times M \times 3.1)/w \text{-----} \textit{Equation 5}$$

where w is the weight of sample, v is the volume of alkali and M is the molarity of alkali used.

Anhydrouronic Acid (AUA): the percentage anhydrouronic acid content was determined using the formula proposed by Sayed *et al* [14] (Equation 6).

$$\% \text{ Anhydrouronic Acid } = \frac{(176 \times 0.1z \times 100)}{\text{Weight of Sample(g)} \times 1000} + \frac{(176 \times 0.1y \times 100)}{\text{Weight of Sample(g)} \times 1000} \text{ ----------} \textit{Equation 6}$$

where z is the volume (cm^3) of alkali from the equivalent weight determined, and y is the volume (cm^3) of alkali from the methoxyl content.

Degree of Esterification (DE): % degree of esterification was also determined by using the formula in equation 7.

$$\% \text{ Degree of Esterification (DE) } = \left(\frac{176 \times \% \text{ MeO}}{176 \times \% \text{ AUA}}\right) \times 100 \text{----------} \textit{Equation 7}$$

where % MeO is the value obtained for the methoxyl content while % AUA is the value obtained for the percentage anhydrouronic acid content.

Spectroscopic Characterization

FTIR Spectrophotometer was used to identify the respective functional groups present in the pectin extract. This was carried out using an FTIR spectrophotometer by measuring the absorption of infrared radiation made by each bond contained in the molecule.

The FTIR analysis of the pectin extract was carried out using Agilent Technology, CARY 630 FTIR, USA. and the corresponding functional groups present were detected from the FTIR spectra obtained.

Cytotoxicity Assay

MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) Assay: Anticancer Activity of Pectin on Human Hepatoma (HepG2) Cancer Cell Line.

The MTT assay relies on the reduction of 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide, which is a yellow water soluble tetrazolium dye, to purple coloured formazan crystals. The formazan crystals were then analysed with a spectrophotometer after dissolution in dimethyl sulphoxide (DMSO). The obtained absorbance of the treated and untreated cells gave an estimate of the extent of the cytotoxicity of the pectin extract on the HepG2.

HepG2 cancer cells were grown in a 96-well plate at a density of 3 ×10^3 cells per well (100 µL) for 24 hours in a Roswell Park Memorial Institute (RPMI) 1640 medium, supplemented with 10 % Fetal Bovine Serum (FBS), Penicillin (100 U/cm^3) and streptomycin (100 µg/cm^3) and incubated in a humidified atmosphere of 50 µg/cm^3 CO_2 at 37 °C.

The HepG2 cells were treated with different concentrations of the pectin extract (125, 250, 500 and 1000 µg/cm^3) in 100 µL volume for 48 hr. After the treatment, 20 µL of MTT solution (5mg/cm^3 concentration) was added to each well (of which the final concentration of MTT was 0.5 mg/mL) and re-incubated for 2 hr.

To make the formazan crystals soluble, the medium was replaced with DMSO, and the optical densities were measured at 570 nm using an xMark microplate spectrophotometer (Bio-Rad, India).

The percentage cytotoxicity was then calculated as shown in equation 8;

$$\% \text{ Cytotoxicity } = (1 - \frac{\text{Optical Density of treated cells}}{\text{Optical Density of untreated cells}} X\ 100) \text{----------}$$

Equation 8

RESULTS

Percentage Yield of The Pectin Extract

Table 1 shows the percentage yield of pectin extract from *Parkia biglobosa* at different pH levels; 1.5, 3 and 5 respectively, this indicates that pectin was best extracted at a lower pH level as shown in Tables 1.

Table 2 represents the chemical characterisation of pectin, and it indicates that the oil holding capacity was 1.41g/g, while the percentage solubility, ash content and moisture content were 70%, 2.87 % and 15.72 % respectively. The pectin extract was found to have an equivalent weight of 953.19, whereas, the AUA (anhydrouronic acid) content, methoxyl content, and DE (degree of esterification) were found to be 60.48 %, 7.40 %, and 69.50 % respectively.

The spectrum of pectin extract as obtained from the FTIR analysis (Figure 1) shows that; at 3300 cm^{-1}, the broad and intense peak is an indication of -OH stretching vibration (of carboxylic acids) while at 2900 cm^{-1} (C-H stretching of methyl ester) indicates the main monomeric unit (galacturonic acid) present in pectin. The presence of peak at 1700 cm^{-1} (C=O stretching vibration) further indicates an esterified carboxyl group, while the presence of peaks at 1736.9 cm^{-1} indicates the existence of an unsaturated ester. Peaks at 1600 cm^{-1}, and 1300 cm^{-1} (C=O stretching vibration and aromatic C-H vibration) indicates the presence of ionic carboxyl group and methyl group respectively. The peculiar peak of C-O-C stretching vibration at 1220 cm^{-1} indicate the presence of an ester (-O-CH_3) group, while the presence of glycosides is shown by the peak around 1000 cm^{-1} (slightly below).

Cytotoxic Activity

Table 3 shows the percentage cytotoxicity of the pectin extract, the % absorbance of the two wells (well 1 and well 2) were taken, and the % cytotoxicity values obtained were 4.60 %, 13.70 %, 16.8 % and 46.8 % for treatment concentrations of 125 µg/cm^3, 250 µg/cm^3, 500 µg/cm^3 and 1000 µg/cm^3 respectively.

DISCUSSION

This research was aimed at accessing the functional quality of pectin extract from the pulp of *Parkia biglobosa* and its ability of being a good anticancer agent for liver cancer (HepG2). The findings in this research showed good outcomes as the result was comparable to the findings of [15-16] and those attained by [17] with some significant improvement. At pH level 1.5, the pectin yield obtained from the pulp of *Parkia biglobosa* when HNO_3 and HCl were used for the extraction, were; 19.68 % and 21.72 % respectively, indicating that pectin is best extracted using HCl at a lower pH level. The increase in the yield with respect to pH value was probably due to the presence of more H^+ ions at lower pH, resulting to an increase in hydrolysis of the pectin extract [18]. The range of the percentage yield obtained shows that *Parkia biglobosa* has favourably higher pectin content than commercial pectin. The higher yield of pectin in *Parkia biglobosa* pulp could be due to loosen cell walls and improved pectin quality found in *Parkia biglobosa* as evident in the result obtained from the characterisation of the pectin extract.

The cytotoxic activity of the extracted pectin on HepG2 cancer cell line shows its potential of being an anticancer agent; this is also in line with the findings of [19] and [11]. Though percentage cytotoxicity obtained in this research were at higher concentrations compared to those obtained from mulberry pectin as recorded by [11] which were at a much lower concentration of 10 µg/cm^3.

The contributing factors for a good quality pectin which were also observed in this research (the extracted pectin), are attributed to; lower ash content [20] which is greatly influenced by the composition and amount of mineral

Table 1: Percentage yield of pectin extract from *Parkia biglobosa* at different pH levels of HNO_3 and HCl used for the acidified water.

Acids Used	Percentage Yield of pectin at different pH levels (%)		
	pH 1.5	pH 3	pH 5
HNO_3	19.68	18.50	17.34
HCl	21.72	19.88	17.96

Table 2: Chemical characterization of the extracted pectin

S/N	Characterization	Pectin
1	Oil Holding Capacity per gram of sample(g^{-1})	1.41 g/g
2	Percentage Solubility (%)	70 %
3	Ash Content (%)	2.87
4	Moisture Content (%)	15.72
5	Effect of pH on percentage yield	Higher at pH 1.5
6	Equivalent Weight	953.19
7	Methoxyl Content (%)	7.40
8	Anhydrouronic Acid (%)	60.48
9	Degree of Esterification (%)	69.50

Spectroscopic Characterisation of Pectin

FTIR Analysis

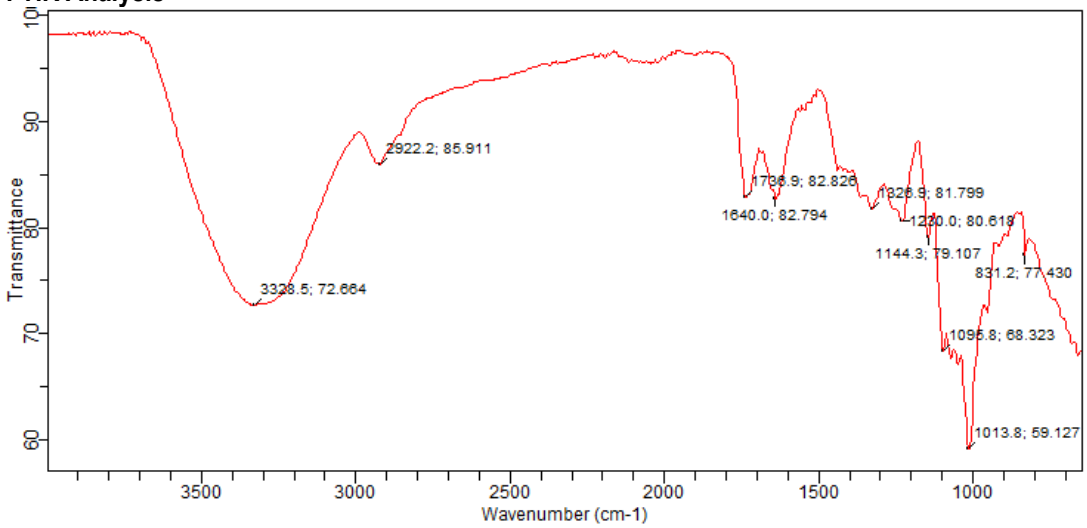

Figure 1: FTIR Spectrum of the pectin extract obtained from the pulp of *Parkia biglobosa*

Table 3: MTT Assay: Anticancer Activity of Pectin on Liver (HepG2) Cancer Cell Line

Pectin Concentration (µg/mL)	Absorbance			% Cytotoxicity
	Treat well_1[a]	Treat well_2[b]	Mean Absorbance ±SD	
1000	0.288	0.364	0.326±0.038	46.80
500	0.545	0.475	0.510±0.035	16.80
250	0.513	0.545	0.529±0.016	13.70
125	0.506	0.664	0.585±0.079	4.60
Untreated cells	0.637*	0.588*	0.613±0.024	

Key: 'a': Absorbance of Well_1 containing treated cells, 'b': Absorbance of Well_2 containing treated cells, '': Absorbance of Well_1 and well_2 containing untreated cells*

materials available in the extracted source; the higher the ash content, the lower the purity of the pectin [21]. Low moisture content is also needed for safer storage and to maintain good quality by inhibiting the growth of micro-organism; this pectin is however, having a higher moisture content but closely comparable to that of commercial citrus pectin [22]. The AUA is also important in verifying how pure the extracted pectin is, the higher the anhydrouronic acid value, the higher the purity of the pectin [23]. However, the AUA value obtained here is slightly lower than that of commercial pectin as reported by [13]. Other factors are the methoxyl content and the degree of esterification which helped to classify pectin into high methoxyl pectin (DE>50%) and low methoxyl pectin (DE<50%) [24]. The findings in this research indicates that the pectin obtained from the pulp of *Parkia biglobosa* can therefore, be classified as a high methoxyl pectin with methoxyl content and DE values of 7.40% and 69.50% respectively.

CONCLUSION

Pectin has various applications due to its gelling ability in the food industry. But its application has gone beyond the food industry and has extended to the pharmaceutical industry as it has recently attracted quite a number of medicinal applications. The pulp of African Locust bean (*Parkia biglobosa)* can be a good source of pectin due to its significant amount of pectin, the pectin yield was higher than that in citrus (a commercial source of pectin), it can therefore, be used as a commercial source of pectin.

The pectin from *Parkia biglobosa* has been proven to have medicinal applications as its cytotoxicity value on HepG2 cancer cell line in this research makes it a potential therapeutic agent for the treatment of liver cancer.

AUTHORS' CONTRIBUTION

ARJ conducted the experiment, analyzed and interpreted the data, contributed to manuscript write-up and wrote the original draft of the manuscript; SG formal analysis(FTIR), interpreted the data and supervision; KA did formal analysis, contributed to manuscript write-up, analyses and interpreted the data, supervised and proofread the manuscript. Approval of the edited and final version of the manuscript was given by all the authors.

CONFLICT OF INTEREST

The authors declare no conflict of interest

ACKNOWLEDGMENT

The authors would like to acknowledge Mr. Mutiu Aliyu (Chief Laboratory Technologist at Chemistry Department, Air force Institute of Technology), Mr. Kabiru Dauda, Hajiya Rabi (KASU) and Mr. Abdulrahman Yahaya Gwio-kura for their technical support. The authors would also like to acknowledge Prof. A. O. Aliyu for her professional advice and as well Dr. M. O. Shoge for proof-reading the manuscript. Finally, the authors would like to acknowledge the effort of PTDF (Petroleum Technology Development Fund) for its contribution to the success of this research, by way of funding.

FUNDING

This research was funded by the Petroleum Technology Development Fund (PTDF).

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