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The Isolation and XRD, LC-MS Profiling of Modified Pectin from *Bambusa tulda*

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Keywords: *Bambusa tulda*, extraction, modified pectin, methionine, LC-MS, XRD crystallinity.

ABSTRACT

Bambusa tulda, also known as Green Gold and is sympodial species. It belongs to the family *Poaceae* which a has multipurpose. Bamboo is a green biofuel for fighting deforestation and climate change. *B. tulda* produces highly nutritive shoots and is consumed. These shots contain saccharine, which has anti-aging elements. Pectin is a soluble complex mixture of polysaccharides that contain methylated ester of poly galacturonic acid. It has been successfully used in the food and beverage industry for many years. The study aims to isolate, modify and characterize the pectin from *B. tulda.* In this work, the isolated pectin was modified with sulfur-containing amino acid (methionine) to increase the efficiency of the drug's specialized application. Then the modified pectin was characterized by LC-MS and XRD spectroscopic studies to confirm its structure modification. In light of these findings, it was assumed that *B. tulda* may produce a significant amount of pectin.

INTRODUCTION

Medicinal plants have long been utilized in traditional medicine. They have always played a pivotal role as sources for new drug lead molecules. According to a WHO report, there's an estimation that almost 65 to 80 percent of the World's population relies on traditional (alternative)medicine as their primary form of healthcare source. The bamboo belongs to the *Poaceae family* and is one of the most versatile multiutility forest tree grasses. The Chinese called bamboos "*Friends of the people",* Vietnamese "*My brother", and* Indians"*Green-Gold*"[1].

Bambusa tulda is an indestructible and one of the fast growing bamboo species up to 49 feet by 26 feet, belonging to the *Poaceae*family[2]. It is also known as Indian timber bamboo and evergreen gregarious bamboo with grey or greyish-green culms[3]. It is a fast-growing medium-sized tropical clumping bamboo native to the Indian subcontinent, Indochina, Tibet, and Yunnan[4]. It is reported that raw bamboo consists of cellulose, hemicellulose, lignin, pectin, proteins, pigments, tannins, fat, and ash constituents[5], and the biological activities of these plants were found to have anti-oxidant[6], anti-bacterial[7], anti-allergic, anti-diabetic[8], anti-cancerproperties[9], etc.,

It is considered to be one of the most valuable multi-purpose bamboo species, which usually grows up to a height of 20 m with culm diameters between 5-10 cm. In India, it is used extensively by the paper pulp industry, but due to its nearly solid culms, it is also an excellent and strong timber that can be used in construction and scaffolding[10]. Tea made from the bamboo leaf is rich in silica that improves the health of bones and other rigid tissues[11]. Bamboo charcoal is multi-functional material pyrolyzed from bamboo under anaerobic conditions[12].

Pectin is a complex mixture of polysaccharides consisting of galacturonic acid chains with different in composition, structure, and molecular weight[13]. It is used as an emulsifier, gelling agent, thickener, stabilizer, and fat or sugar replacer in low concentrations[14]. So, in this regard, an attempt has been made to isolate the pectin constituent from the *Bambusa tulda* plant. Much research and technical work have been carried out on the chemical modification of bamboo fibers to improvise their applications. Chemical modifications may include alkali or acid hydrolysis, coupling reactions, etc[15].,

Bambusa tulda is an important medicinal plant and is used traditionally for various diseases and also as a health tonic[15]. The current study is aimed to isolate and characterization of modified chemical constituents from *Bambusa tulda* using X-ray diffraction and LC-MS methods.

MATERIALS AND METHODS

i. COLLECTION OF *BAMBUSA TULDA* PLANTS

Bambusa tulda plants were collected from the Tissue culture garden, Hosur, Tamil Nadu. Then the plant was dissected and washed with running tap water and finally washed with distilled water to remove the dirt. The plant was sun-dried.

ii. AUTHENTICATION OF *BAMBUSA TULDA* PLANT

The *Bambusa tulda* plant has been authenticated by an expert botanist, a fair specimen from the collected plant material, which is having a stem, leaves, etc., was washed with water, then dried, then again washed with alcohol, and kept for drying by pressing method and prepared the herbarium. Plant material was recognized and authenticated by Dr. V Rama Rao, Research Officer(S-2) botany, Central Ayurveda Research Institute, Kanakapura road, Bengaluru. The herbarium prepared from the plant was preserved in the same institute.

iii. PREPARATION OF PLANT MATERIAL

The plant's stem and leaves were cut and washed with tap water. The cleaned parts of the plant were allowed for drying under the sun for 2 to 4 weeks (pic no). The stem and leaves were cut into fine pieces and powdered[16]. Then the residue was weighed and stored in the airtight container for further pharmacognostic analysis.

EXPERIMENTAL PROCEDURE

The experimental procedure was divided into two parts. In the first part, pectin which is one of the active constituents of the *Bambusa tulda* was extracted using respective solvents. In the second part, extracted active constituents were modified to explore their specialized potential.

Then finally characterization using LC-MS, and XRD was carried out to confirm the structure of modified constituents from *Bambusa tulda*.

PREPARATION OF PLANT EXTRACTS

The successive solvent extraction procedure was adopted for the preparation of plant extracts of *Bambusa tulda*[17]. The powdered plant materials were subjected to successive extraction of pectin constituent using acidified distilled water and organic solvents as buffer reagents. The extract was precipitated by an equal volume of ethanol and then filtered using Whatman filter paper no.1 and dried[18]. After drying, the sample was reserved in an air-tight container and used for further studies.

PROCEDURE FOR EXTRACTION OF PECTIN CONSTITUENT

The 150 grams of *Bambusa tulda* plant powder were taken in a 500 ml beaker and 450 ml of distilled water which has to be acidified by using Sulfuric acid. This mixture was divided into two parts, parts A and B. To part A, 8 ml of conc. Sulfuric acid and to part B, 2 ml of conc. Sulfuric acid was added. To maintain the pH levels citrate buffer with different compositions was added. To part A, 10 ml of citrate buffer (82 ml of Citric acid + 18 ml of Trisodium citrate) and to part B, 10ml of citrate buffer (82 ml of Trisodium citrate + 18 ml of Citric acid) was added to maintain the different pH-3 &1 respectively[19&20]. I have tried this technique to check which form is giving more yield. The mixture was taken in a Conical flask and placed at Heating Mandle. The mixture was heated at different pH mediums at 60, 80 º C for different times 30 and 60 minutes. Then hot acid extraction was filtered through Whatman filter paper No. 1 and cooled to room temperature.

PURIFICATION

Isolated pectin was precipitated by adding an equal volume (1:1) ratio of 99% ethyl alcohol at 4º C. Then it was left overnight and filtered using a Vacuum filter. Again, it has been washed with 55% ethyl alcohol and dried in a hotplate, and stored in an air-tight container for further analysis[21]. From this part of extraction, I have got more yield at pH-1 because more acidified solvent has made pectin extract from fiber in more amount. So, for further studies, I have used products obtained from pH-1 i.e part A.

IDENTIFICATION TESTS FOR PECTIN: QUALITATIVE TEST

Pectin extracted was qualitatively for color and solubility which has been determined by the succeeding tests given in USP monographs[22].

a. **Colour** - cream / yellowish powder, this was done by visual observation with naked eyes.

b. **Odour** - less

c. **Taste** -mucilaginous

d. **Solubility**- Soluble in alcohol and other organic solvents

e. **Solubility of dry pectin in cold and hot water:** weighed 1gm of dried pectin samples were taken in a conical flask which contains 10ml of 95% ethanol and a further 50ml of distilled water was added. The mixture was shaken vigorously to form a suspension which was heated at 60-90 C for 30 minutes.

f. **Solubility of pectin solution in cold and hot alkali:** weighed 1.5 ml of pectin solution was added and the second flask was heated at 60-90 C for 15 minutes.

g. Chemical tests[23]:

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

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

3. Test with potassium hydroxide (KOH): To 5ml of 1% w/v solution of pectin sample +1ml of a 2%w/v potassium hydroxide solution at room temperature for 15 minutes will form transparent gel +dilute hydrochloric acid will form a gelatinous precipitate. When it is boiled, it will form a white and flocculent precipitate.

4. Iodine test: To 5ml of recently boiled and cooled 2%w/v solution sample, 0.15 ml of iodine solution was added. No blue color presence was taken as an indicator of a positive test.

5. Test for acidity: An aqueous solution of the pectin sample was acidic to blue litmus paper.

QUANTITATIVE TEST

The dried pectin powder samples obtained from *Bambusa tulda* were characterized by the following quantitative tests[24].

PERCENTAGE YIELD OF PECTIN

$$\textbf{Pectin \%} = \frac{\textbf{weight of dried isolated pectin (g) * 100}}{\textbf{Amount of bamboo leaves used (g)}}$$

The pectin yield was calculated using the above equation. The pectin is stored in an airtight beaker with an Aluminium foil covering and kept in desiccators for further analysis.

CHARACTERIZATION OF PECTIN[24]

Moisture content:

1g of pectin sample was weighed in a China dish and was then dried in an oven for 3 hours at 100-110°C. Then cooled in a desiccator and weighed. The procedure was repeated four 4 times until a constant weight was obtained. The pectin is very hygroscopic, for this reason, it must be preserved in a closed dry atmosphere.

$$M\% = \frac{100\ (w1 - w2)}{w1\text{-}w}$$

Where, W2 - Final weight of porcelain dish and ash,

W1- Weight of porcelain dish,

W- Weight of pectin sample

Ash content:

The Ash content of pectin was determined by Rangana's method (1995). Weighed 1g of pectin sample. The sample was ignited slowly, and heated for 4 hrs at 600 ºC. Then cooled the crucible to room temperature in a desiccator and weighed it properly. The process will be weighted till constant weight come and the final weight will be noticed. The upper limit of ash content for good quality pectin is considered to be 10% from the viewpoint of gel formation.

$$\text{Ash\%} = \frac{\text{weight of ash(g)} * 100}{\text{Weight of pectin(g)}}$$

After the concerning result, the pectin isolated in this study may be considered satisfactorily good in quality.

Equivalent Weight:

Equivalent weight is used to find out the Anhydrouronic acid content and degree of esterification of isolated pectin. It is determined by titration with sodium hydroxide to pH 7.5 using a phenol red indicator. Equivalent weight was determined by Rangana's method (1995).

1 g of pectin sample was taken in a 250 ml conical flask and 5 ml ethanol was added. 1 g of sodium chloride to sharpen the endpoint and 100 ml of distilled water were added. Finally, 6 drops of phenol red were added and titrated against 0.1 N NaOH. The titration point was indicated by the purple color. This neutralized solution was stored for the determination of methoxy content. A high equivalent weight would have a higher gel-forming effect. The lower equivalent weight could be the higher partial degradation of pectin. The increase or decrease of the equivalent weight might be also dependent upon the amount of free acid.

$$\text{Equivalent weight} = \frac{\text{weight of sample(g)} * 100}{\text{ml of alkali} * \text{normality of alkali}}$$

Methoxyl Content (MeO); The methoxyl content or degree of esterification is an important factor in controlling the setting time of pectin. It was examined by saponification of the pectin and titration of the liberated carboxyl groups. Determination of MeO was done by using Rangana's method (1995).

The neutral solution was collected from the determination of equivalent weight, and 25 ml of sodium hydroxide (0.25 N) was added. The mixed solution was stirred entirely and kept at room temperature for35 minutes. After that,25 ml of 0.25 N hydrochloric acids was added and titrated against 0.1 N NaOH to the same endpoint as before like in equivalent weight titration. The spreading quality and sugar-binding capacity of pectin are increased with increased methoxyl content. Based on methoxyl content value, pectin was categorized as high and low methoxyl pectin depending on the reagent used.

$$MeO\% = \frac{\text{Milli equivalent weight of sodium hydroxide} * 31 * 100}{\text{Weight of sample (mg)}}$$

Total Anhydrouronic Acid Content (AUA);

Estimation of Anhydrouronic acid content is essential to determine the purity and degree of esterification, and to evaluate the physical properties. Pectin, which is a partly esterified polygalacturonase, contains 10% or more organic material composed of arabinose, galactose, and perhaps sugars. From the obtained equivalent weight and methoxyl content, the value of titer was calculated and used. The total AUA of pectin was obtained by the following formula (Mohamed & Hasan, 1995).

$$AUA\% = 176 * 100 / /c$$

Where molecular unit of AUA (1 unit) = 176 g, c=w/a+b

Where,

a = ml (titre) of NaOH from equivalent weight determination.

b = ml (titre) of NaOH from methoxyl content determination.

w = weight of the sample

Determination of Degree of Esterification (DE)

The DE of pectin was measured based on methoxyl and AUA content (Owens et al., 1952) and calculated by the following formula

$$DE\% = \frac{176 * MeO\% * 100}{31 * AUA\%}$$

Where

% MeO = Methoxyl content,

% AUA=Anhydrouronic Acid Content.

The degree of esterification decreased with the increase of maturity.

Estimation of Total Phenolic Content (TPC) of Pectin: The phenolic content was taken as mg/g gallic acid equivalents. In brief 100 µl aliquots of the sample were added to 2 ml of 0.2

% (w/v) Na2CO3 solution. After 2 minutes of incubation. 100 µl of 500 ml/1 Follin-Ciocalteu reagent was added and the mixture was allowed to stand for 30 minutes at 25 0C. Then observed the absorbance at 750 nm using a UV-VIS spectrophotometer. The blank consists of all reagents and solvents but no sample. The Total Phenolic Content (TPC) was resolved using the standard gallic acid calibration curve[25].

MODIFICATION OF ISOLATED PHYTOCHEMICAL CONSTITUENT

A current trend for pectin modification can be found leading aside from homogeneous transformation reactions to heterogeneous processes, which are usually followed in industrial applications, considering the reaction efficiency, the cost, and the recycling of solvents. Pectin is a natural and non-toxic complex polysaccharide. Pectin is mainly composed of D-galacturonic acid units. Pectin structure plays a major role which determines its functionality and possibility of potential application in the food and pharmaceutical industries[26].

Pectin can be modified by Amidation or hydrolysis of the pectin ester groups, decarboxylation of free carboxyl groups, β-elimination, or hydrolysis of glycosidic bonds are among theoretically expected reactions[27]. Their identification and extent have been studied in model reactions in future studies. Here, pectin was reacted with methionine so the Amidation of aminolysis reaction took place.

The reaction of pectin with amino compounds

To the 500ml beaker, 2g of pectin and 50 ml of distilled water were added. Followed by this 5ml of 95% ethanol was added and stirred vigorously to make a homogeneous solution. While heating at 60 ºC, 1 g of methionine was added. Then the mixture was left for a 15mins at the chosen temperature with occasional shaking. This mixture was coagulated with acidified ethanol (3 ml of concentrated hydrochloric acid dissolved in 200 ml of 96% ethanol). At last, the mixture was left overnight in a refrigerator. The coagulated mass was centrifuged and washed three times with portions of 100 ml 70% of acid ethanol. Then it was dried at 40º C and stored in an airtight container for future analysis[28].

STRUCTURAL ANALYSIS

DETECTION OF MODIFIED COMPONENTS BY LC-MS ANALYSIS

A typical LC-MS system is a combination of HPLC with MS using an interface (ionization source). It is an analytical technique that combined the physical separation of LC and mass

analysis using Mass spectrometry[29]. As a result, a mass spectrum (a plot of the ion signal as a function of the mass-to-charge ratio) is created, which is used for the separation, identification, and quantification of both unknown and known compounds and also to elucidate the structures and chemical properties of molecules[30].LC-MS analysis of modified constituent from *Bambusa tulda*plant extract was done by selecting a standard compound. Pectin was found to be one of the active compounds in *Bambusa tulda*.

DETECTION OF MODIFIED COMPONENTS WITH XRD

X-ray diffraction analysis (XRD) is an approach used in material science to figure out the crystallographic(atomic or molecular) structure of a material. XRD works by irritating a material with X-rays and then measuring the intensities and scattering angles of the X-rays that leave the material. The working principle of XRD follows Bragg's Law which calculates the d-spacing of each peak[31].

$$n\lambda=2d \sin \theta$$

Where d is the spacing between diffracting planes

Ɵ is the incident angle

n is an integer

ƛ is the wavelength of the beam

XRD is one of the best methods for the characterization of the crystalline form of organic and inorganic materials[32]. XRD analysis was carried out to find the degree of crystallinity of modified constituent from *Bambusa tulda* plant extract.

RESULT AND DISCUSSION

IDENTIFICATION TESTS FOR PECTIN: QUALITATIVE TEST

Pectin extracted was qualitatively determined by the following tests as mentioned in USP monographs.

Table No. 1: Qualitative test results of isolated pectin from *B. tulda*

S.NO.	TESTS PERFORMED	RESULTS
1.	Color	Slight yellowish powder
2.	Odor	Less
3.	Taste	Mucilaginous
4.	Solubility in cold water	Dissolved sparingly and formed a suspension after continuous shaking.
5.	Solubility in hot alkali(2N NaOH)	Dissolved and tuned as a milky liquid
6.	Solubility in cold alkali	Pectin suspension dissolved and formed a slight yellow precipitate.
7.	With ruthenium red	It was turned into pink color against a grey background
8.	Stiff gel test	Positive
9.	Ethanol (95%) test	Positive
10.	Potassium Hydroxide test	Positive
11.	Iodine test	Positive
12.	Acidity test	Positive

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QUANTITATIVE TEST

The dried pectin powder samples obtained from *Bambusa tulda* were characterized by the following quantitative tests.

Figure No. 01 yellowish precipitate of isolated pectin

Figure No. 02 pink colour formation of isolated pectin with ruthenium red

Figure No. 03 stiff gel formed from isolated pectin

Figure No. 04 gel formation of isolated pectin with ethanol

Table No.02: Quantitative test results of isolated pectin

S.NO.	CHARACTERISTIC	ISOLATED PECTIN
1.	Moisture content (%)	9.73
2.	Ash content (%)	2.72
3.	Equivalent weight(mg/mol)	290.89mg/mol
4.	Methoxyl content (%)	3.458%
5.	Anhydrouronic acid content (%)	80.340%
6.	Degree esterification (%)	40.83%

The pectin obtained by precipitation with 95% ethanol was used for characterization in this work. The characteristics of the pectin isolated have been summarized in Table 2.

1. **Moisture content**: The pectin was very hygroscopic. The moisture absorbed by isolated pectin in this work was found to be 9.73%. This value seemed too high (for a material, which was easily susceptible to microbial attack) to be preserved in an open atmosphere. For this reason, it must be preserved in a closed dry atmosphere.

2. Ash content: The ash content of the pectin isolated in this work was found to be as low as 2.72%. This parameter is reported in the literature and it may be varied in a wide range depending on the method and the nature of the citrus fruits used for extraction. The upper

limit of ash content for good-quality pectin is considered to be 10% from the viewpoint of gel formation. Therefore, concerning this parameter, the pectin isolated in this study might be considered to be of satisfactorily good quality.

3. Equivalent weight: The equivalent weight of isolated pectin was found to be 290.89mg/ml and in the range of 476-1209 as reported in the literature. A high equivalent weight would have a higher gel-forming effect.

4. Methoxyl content: The methoxyl content for isolated pectin was found at 3.458%. This parameter may be varied depending on the nature and method used. Methoxyl content was an important factor in determining the gel formation capacity.

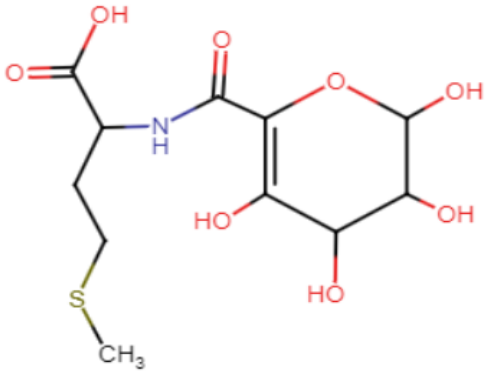
5. Anhydrouronic acid content: Anhydrouronic acid content for Isolated Pectin was found at 80.340%. The content of AUA has been indicating the purity of the extracted pectin and was suggested to be not less than 65% (Food Chemicals Codex 1996). However, the AUA content obtained under this extraction condition was < 65%. The result indicated that the extract may not be sufficiently pure due to the possible presence of proteins, starch, and sugars in the precipitated pectin.

6. Degree of esterification: The degree of esterification for the isolated pectin was found at 40.83%. This result was showing that Bambusa tuldahas a significant amount of pectin.

PURIFICATION AND CHARACTERIZATION OF PECTIN EXTRACTED VIA CHROMATOGRAPHIC AND SPECTROSCOPIC TECHNIQUES:

A. DETECTION OF MODIFIED COMPONENTS BY LC-MS ANALYSIS

LC-MS analysis of modified constituent from *Bambusa tulda*plant extract was done by selecting a standard compound of commercially available pectin. Pectin was found to be one of the active compounds in *Bambusa tulda* and modified pectin with methionine was confirmed by LC-MS data interpretation.



Structure of modified pectin with methionine

From the obtained LC-MS data, after interpretation using Qual browser software. It had been confirmed the structure of modified pectin with methionine with fragmentation peaks. The mass ion peak was obtained at 322.2 and the actual molecular weight of modified pectin with methionine was 323.45mol and the remaining were fragmented ion peaks. Then the resolution time of the peak was obtained at 0.569mV/min. So, it has been confirmed the structure of modified pectin with methionine from the LC-MS spectral image.

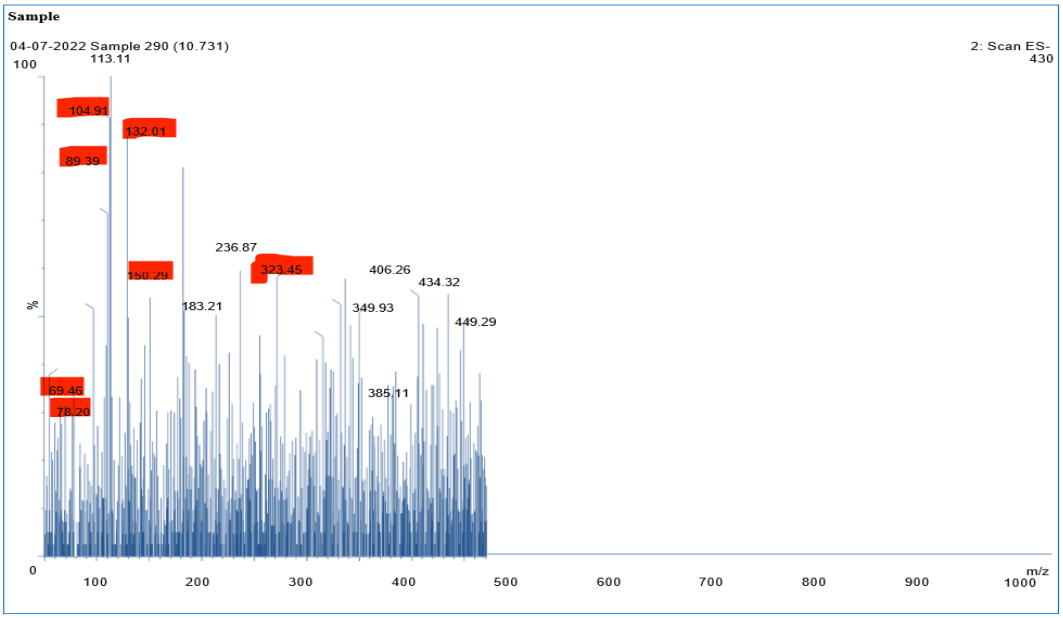


Image no.01 LC-MS spectra of modified pectin with methionine

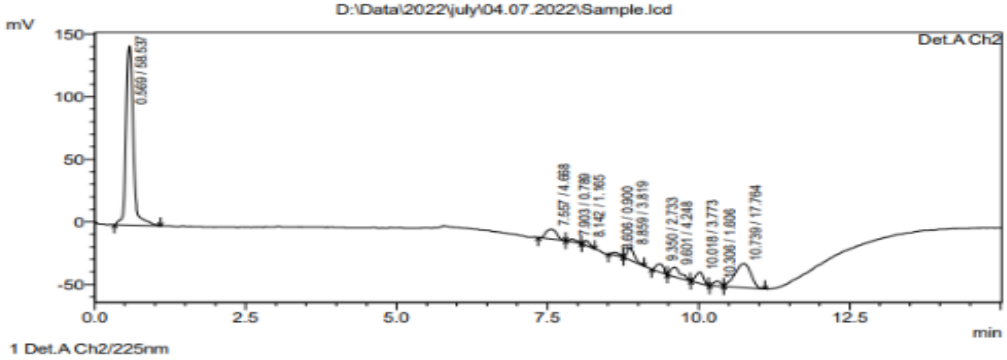
==== Shimadzu LCsolution Analysis Report ====

D:\Data\2022\july\04.07.2022\Sample.lcd

Acquired by	: Admin
Sample Name	: Sample
Sample ID	: Sample
Tray#	: 2
Vail #	: 27
Injection Volume	: 10 uL
Data File Name	: Sample.lcd
Method File Name	: MS Method 15 MIN.lcm
Batch File Name	: 04.07.2022.lcb
Report File Name	: Default.lcr
Data Acquired	: 04-07-2022 19:25:49
Data Processed	: 04-07-2022 19:40:55

<Chromatogram>

D:\Data\2022\july\04.07.2022\Sample.lcd



1 Det.A Ch2/225nm

PeakTable

Detector A Ch2 225nm

Peak#	Ret. Time	Area	Height	Area %
1	0.569	1178150	143071	58.537
2	7.557	93948	7659	4.668
3	7.903	15873	2128	0.789
4	8.142	23437	3926	1.165
5	8.606	18119	1958	0.900
6	8.859	76854	9869	3.819
7	9.350	54997	6377	2.733
8	9.601	85502	7477	4.248
9	10.018	75934	9005	3.773
10	10.306	32315	4005	1.606
11	10.739	357520	19400	17.764
Total		2012649	214874	100.000

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Image no.02 LC-MS spectra of modified pectin with methionine

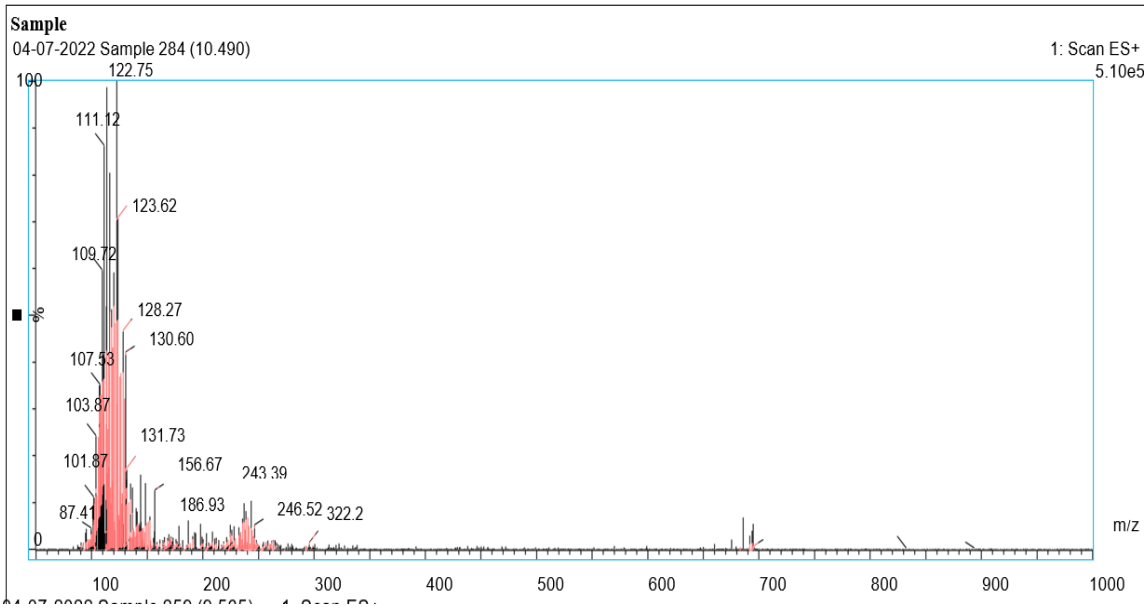

Image no.03 LC-MS spectra of modified pectin with methionine

B. DETECTION OF MODIFIED COMPONENTS WITH XRD

XRD analysis of modified chemical constituent (pectin-methionine) from *Bambusa tulda* was done to know the crystalline structure of constituents and was further demonstrated and confirmed by the characteristic peaks(2Θ values) observed at 19.23, 24.31, 25,27, and 43.52º.

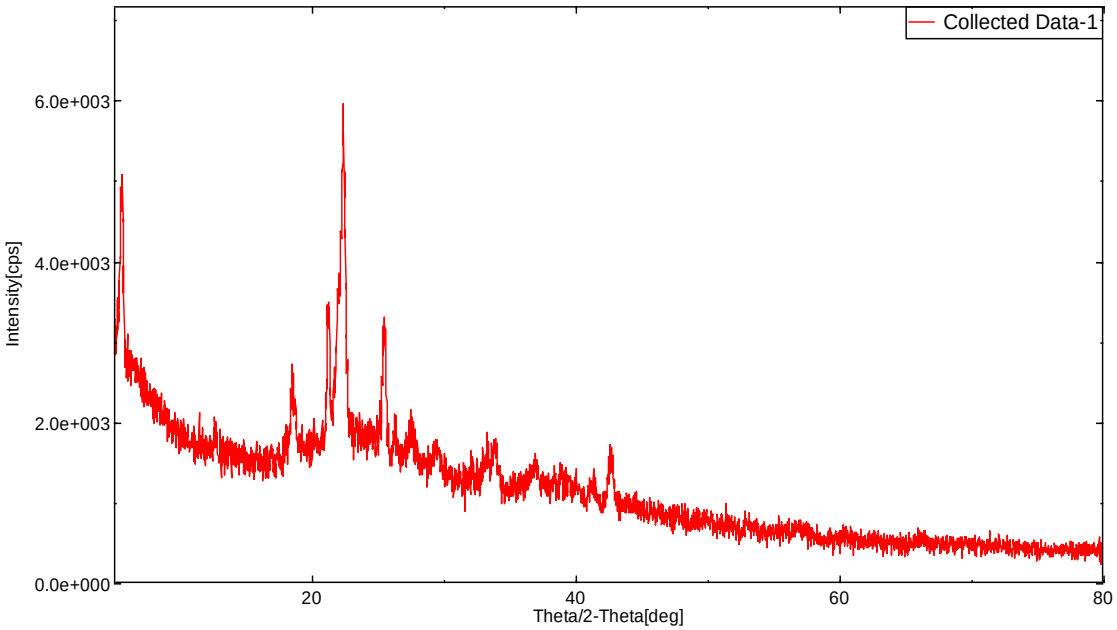

Image no. 04 XRD graph of modified pectin with methionine

Analysis of XRD peak profiles indicated that full-width at half-maximum (FWHM) was sensitive to the variation in microstructure and stress-strain accumulation in the material. The XRD patterns of modified pectin were shown in fig. It had seen that very sharp peaks were present in the spectrum and a narrow range of background patterns indicated that the pectin extracted from *Bambusa tulda* and modified with methionine was a crystalline form. From the XRD data, the crystallinity of modified pectin was calculated using Origin lab software. The result was shown that the degree of crystallinity of modified pectin was 16.35%.

DISCUSSION

The pectin was one of active constituents of *Bambusa tulda* plant. It was isolated by hot acid extraction methods which are a very popular method used by researchers to isolate pectin from various sources like citrus fruits and isolated pectin was modified by aminolysis method a using sulfur-containing amino acids such as methionine to enhance a specialized application of both of them. Their modified structure was characterized by using LC-MS and XRD techniques to confine the structure from obtained data. The data obtained from LC-MS and

XRD peaks, it was assumed the structure and mass, crystallinity of modified pectin with methionine. This could be used for further studies to explore its potential application.

CONCLUSION

This research emphasized pectin extraction and characterization from *B. tulda*. In general, the research had been divided into three parts namely extraction, modification, and characterization of modified pectin. The results indicated that pH, extracting temperature and time affect the extraction yield of pectin from the *B. tulda* plant. After concerning the results obtained, it had been concluded that *B. tuldaalso* gives a significant amount of pectin whereby it can be considered in commercial production of pectin along with other citrus sources and the pectin modification with methionine to enhance its specialized application will be confirmed by docking studies in future research analysis. The structure of modified pectin with methionine had been confirmed by LC-MS and XRD characterization techniques.

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