

Improvement in Digestion Resistibility of Mandua Starch (*Eleusine coracana*) after Cross-Linking with Epichlorohydrin

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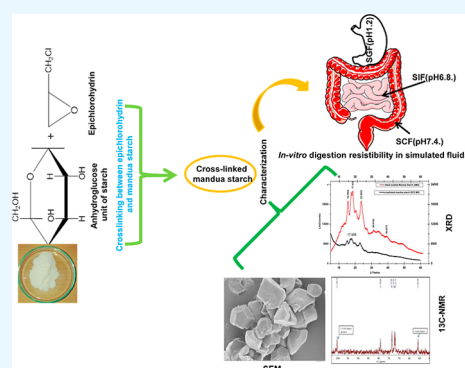
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ABSTRACT: Starch, being a polymer of excessive demand for the development of products of pharmaceutical importance, has been tremendously treated in many ways for improving the desired characteristics such as viscosity, paste clarity, digestibility, swelling, syneresis, and so forth. In the present study, alkali-extracted starch of mandua grains (*Eleusine coracana*; family Poaceae) was treated with epichlorohydrin for cross-linking and the modified starch was assessed for swelling, solubility, water binding capacity, moisture content, and degree of cross-linking. The digestion resistibility of modified starch was analyzed in simulated gastric fluid (pH 1.2), simulated intestinal fluid (pH 6.8), and simulated colonic fluid (pH 7.4). The structural modifications in treated mandua starch were analyzed by Fourier transform infrared (FTIR) spectroscopy, powder X-ray diffraction (XRD), scanning electron microscopy, thermogravimetric analysis, and C^{13} nuclear magnetic resonance (^{13}C NMR). The results of the study reflected the significant modification in mandua starch after treatment with epichlorohydrin (1.0% w/w sdb, solid dry basis). The degree of cross-linking of treated mandua starch was 85.15%, and the swelling capacity of mandua starch changed from 226.51 ± 2.175 to $103.14 \pm 1.998\%$ w/w after cross-linking with epichlorohydrin. A remarkable increment in digestion resistibility was observed in modified mandua starch. The XRD pattern and FTIR spectra revealed the presence of resistant starch after chemical modification. The decomposition pattern of modified mandua starch was also different from extracted mandua starch. All the results reflected the effective modification of mandua starch by epichlorohydrin and the formation of resistant starch to a significant content. The treated mandua starch may have the potential in developing various preparations of food, nutraceuticals, and pharmaceuticals.



1. INTRODUCTION

Mandua (finger millet, *Eleusine coracana*, family Poaceae) is one of the oldest, most common, and most grown cultivars used in the human diet in Uttarakhand state of India. In India, it is referred to as “nrattakondaka” in the ancient Indian Sanskrit literature, which means “dancing grain”. It is also addressed as “rajika” or “markataka”. Its grain is smaller than that of several millet crops. In India, the production of mandua had declined to 2,608,100 and 2,374,600 tons in 1998–1999 and 2001–2002, respectively.¹ In the world, the finger millet ranks fourth in importance among millets after the foxtail millet, pearl millet, and sorghum millet.²

Deskbound lifestyle and high-glycemic-index foods have become a potent risk factor for the community because of increased lifestyle diseases such as hypertension, colon cancer, diabetes, hypercholesterolemia, obesity, and so forth. There is a growing interest in developing foods having resistant starch (RS) and dietary fibers to overcome such diseases.³ RS has several health benefits including positive impacts on bowel health. It is also associated with increased apoptosis of colorectal cancer, tumors, and decrement in body weight.⁴

RS is defined as the total amount of starch and starch degradation products that resist digestion in the small intestine. However, RS is further classified into five categories based on its digestion. RS4 represents a group of starches that have been chemically modified to reduce their digestibility.^{5,6} RS is also present in ragi.⁷ Finger millet starch is slightly resistant to amylolysis.⁷

Starch is used in various industrial applications. However, native starches in the food and pharmaceutical industries are restricted because of their particular physical (retrogradation, high degree of syneresis, low resistance to shear stress, and opaqueness) and chemical properties.⁸ To engender the range of applicability in industries, modification or processing of starch is necessary.^{9–12} India demands around 27,500 tons of

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modified starch per annum with an 8–9% growth rate per annum.^{13,14} The content of RS or slowly digestible starch can be improved by various methodologies such as genetic manipulation, enzymatic debranching, hydrothermal treatments, and chemical modifications including cross-linking and γ -irradiation exposure, as well as lipid complexation.¹⁵ It has been reported that cross-linking of starches using reagents such as sodium trimetaphosphate (STMP) and sodium tripolyphosphate (STPP) cause resistance to digestion, and therefore, these starches fit within the type RS4.¹⁶ It is known that cross-linking changes the starch structure, making it inaccessible to digestive enzymes (α -amylase and amyloglucosidase) and resulting in a high level of nonhydrolyzed starch fractions.¹⁷ The cross-linking of starches adds intra- and intermolecular bonds in random positions in the starch granules, resulting in stabilization of the molecule.¹⁸ Epichlorohydrin (ECHD) is the most commonly used starch cross-linking reagent. The treatment of ECHD with starch in the presence of an alkali yields glycerol mono- and diethers. ECHD cross-linked starch is firmly resistant to pH and shear due to the formation of ether linkages between the cross-links and the hydroxyl groups.¹⁹ Resistance to pH makes it suitable for use in the development of pH-responsive targeted drug-delivery devices.

Currently, there is little information available on the properties of chemically modified mandua starch isolated from the hilly varieties of mandua that can be used further in pharmaceutical, food, and other industrial applications. Hence, the present study is focused on examining the effect of ECHD as a cross-linking agent on *in vitro* gastrointestinal digestion resistibility, physicochemical, and morphological properties of alkali-isolated starch from mandua cultivars grown in hilly areas of Uttarakhand, India. Different parameters were studied to examine the changes that occurred in ECHD-treated starch. The minimal digestion of this starch in the gastrointestinal tract (GIT) may be a critical factor in protecting drug-delivery devices from the GIT environment and may modulate the releasing and targeting of an incorporated drug.

2. MATERIALS AND METHODS

Mandua grains (VL mandua-352) were purchased from Vivekananda Parvatiya Krishi Anushandhan Sansthan, Almora (Uttarakhand). Pancreatin (from the porcine pancreas) was purchased from HiMedia, India. Isoamylase (1000 U/mL), Total Resistant Starch Assay Kit, and Glucose Assay Kit were procured from Megazyme International Ltd. (Co. Wicklow, Ireland) and Arbro Pvt. Ltd., India. All the other chemicals used in the study were of reagent grade and purchased from CDH, Fisher Scientific Company, and Sigma-Aldrich.

2.1. Starch Isolation. The alkaline steeping method with some modification was used to isolate starch from mandua grains.²⁰ Mandua grains were dried, cleaned, dehusked, and powdered. The powdered flour enriched with starch was diluted with distilled water (10 times), and the pH was adjusted to 10 with 1 N sodium hydroxide. The slurry was mixed intermittently for 1 h with a magnetic stirrer at room temperature. The suspension was centrifuged at 8000 rpm for 15 min, and the supernatant was carefully discarded. The sediment was then resuspended in double-distilled water (10 times) and maintained at 4 °C for 18 h. The suspension was centrifuged at 8000 rpm for 10 min, and the supernatant was removed. The sediment was again suspended in a 2% w/v sodium chloride solution for 20 h at 4 °C. The suspension and

centrifugation processes were repeated twice with 0.1 N NaOH for 48 h. The suspension was neutralized and then centrifuged at 10,000 rpm for 15 min. The white starch layer was collected, washed, resuspended in 80% ethanol at 80 °C for 1 h, cooled down to room temperature, and allowed to settle for 4 h at 4 °C. The supernatant was discarded, and the residue was dehydrated. The alkali-isolated mandua starch (AMS) was powdered and dried at 30 °C in a hot-air oven overnight and stored in an airtight container.

2.2. Cross-Linking of Mandua Starch with Epichlorohydrin. The cross-linking reaction of starch with ECHD was carried out by the reported method with slight modifications.¹⁹ In brief, AMS was dispersed in distilled water (1:2) in a three-neck round-bottom flask equipped with a condenser and thermometer. The resultant paste was stirred magnetically for 10 min. The pH of the slurry was adjusted to 10.5 by 0.1 M sodium hydroxide. The cross-linking agent, ECHD (1.0% w/w, solid dry basis, sdb), was added into the starch slurry over 30 min. The flasks were closed tightly, and the slurry was stirred continuously on a magnetic stirrer, warmed up to 50 °C, and maintained at this temperature for 5 h. Afterward, the slurry was neutralized to pH 6.0 with 1.0 M hydrochloric acid to terminate the reaction. The modified starch slurry was collected by centrifugation at 3000 rpm. The product was washed several times with distilled water, and the supernatant was collected for quantification of the unreacted cross-linking reagent. A similar process was carried out for blank to assess the effect of acidic conditions on used ECHD. The resultant mandua starch cross-linked with epichlorohydrin (ECC-MS) was kept in the refrigerator for some time and then dried overnight in a hot-air oven at 40 °C. The yield of the cross-linked mandua starch was calculated by comparing the weight of native starch with the weight of cross-linked starch as follows

$$\text{yield (\%)} = \frac{\text{wt of native starch} - \text{wt of starch after cross-linking}}{\text{wt of native starch}} \times 100 \quad (\text{i})$$

The amount of unreacted ECHD after the cross-linking reaction was quantified by the previously reported method.^{19,21} In brief, the unreacted ECHD was converted to glycerol by hot alkali treatment, which was further converted to formaldehyde by periodate oxidation and estimated colorimetrically at 570 nm using a chromotropic acid reagent reaction. A standard calibration was prepared using a known amount of ECHD.

2.3. Characterization of Cross-Linked Starch and Alkali-Isolated Starch. **2.3.1. Estimation of Water Binding Capacity.** The water binding capacity (WBC) of ECC-MS and AMS was measured by the reported method with slight modification.^{21,22} A 0.1 g of starch (dry basis, db) and 10 mL of distilled water preheated at 37 °C were mixed in preweighed centrifuge tubes (W_1). The tubes were kept at 37.5 ± 0.5 °C in a water bath for 15 min. After cooling to room temperature (25 °C), all tubes were centrifuged at 3000 rpm for 10 min. After centrifugation, the supernatant was decanted and tubes with residue were weighed (W_2). The following formula was used to calculate the WBC²¹

$$\text{WBC (g/g)} = \frac{(W_2 - W_1) - \text{initial weight of starch (db)}}{\text{initial weight of starch}} \times 100 \quad (\text{ii})$$

2.3.2. Swelling Capacity and Solubility. The swelling capacity and solubility of AMS and ECC-MS were calculated with slight modification in the reported method.²³ A 500 mg of starch (db) was taken in a predried and preweighed centrifuge tube. A 20 mL of preheated distilled water (37.5 ± 0.5 °C) was added to the centrifuge tube and kept for 30 min at a preset temperature. The resultant mixture was centrifuged at 3000 rpm for 15 min. The supernatant was transferred to a dried and preweighed petri plate and kept in a hot-air oven at 105 °C for estimation of the solubility of starch. The centrifuge tube containing residue was weighed for determining the swelling capacity.

$$\text{Swelling capacity(\%)} = \frac{\text{wt. of wet starch}}{\text{wt. of initial starch}} \times 100 \quad (\text{iii})$$

$$\begin{aligned} \text{Solubility(\%)} \\ = \frac{\text{weight of starch available in the supernatant}}{\text{initial weight of starch}} \times 100 \end{aligned} \quad (\text{iv})$$

2.3.3. Moisture Content and Ash Content. Moisture contents of ECC-MS and AMS were estimated according to the previously reported method.²⁴ At the same time, the ash content was determined by the reported method.²⁵ A 200 mg of starch (db) was dried in a hot-air oven at 105 °C until it reached a constant weight. The moisture content of ECC-MS and AMS was calculated by measuring the wet weight. The following expression was used to determine the moisture content

$$\text{moisture content(\% w/w)} = 100 \times \frac{(w_1 - w_2)}{w_2} \quad (\text{v})$$

where w_1 is the sample's initial weight and w_2 is the weight of the dried sample.

2.3.4. In Vitro Gastrointestinal Digestion Resistibility. The in vitro digestion resistibility of AMS and ECC-MS was determined by following the reported method with slight modifications.²⁶ Simulated gastric fluid (SGF), simulated intestinal fluid (SIF), and simulated colonic fluid (SCF) were prepared according to the reported method.²⁷ The SGF (pH 1.2) was prepared by dissolving NaCl (0.2 g), 37% HCl (7 mL), and pepsin (3.2 g). The SIF of pH 6.8 consisted of KH_2PO_4 (6.8 g), 0.2 M NaOH (190 mL), and pancreatin (10.0 g). The SCF had a pH of 7.4.

Duplicate 100 mg (dry basis) of AMS and ECC-MS was weighed, suspended in 2 mL of SGF prepared immediately before use (pH 1.2), placed in a dialysis bag (HiMedia), and dialyzed against 50 mL of SGF in a beaker at 37 °C for 2 h. A dialysis bag loaded with starch was shaken every 10 min to avoid starch sedimentation during a 2 h period. After 2 h, 1 mL of the dialysate samples was taken and analyzed for sugar release using a GOD-POD kit. After 2 h, the dialysate was centrifuged at 3000 rpm for 20 min and the supernatant was discarded. Afterward, the solid residue was suspended in 2 mL of SIF (pH 6.8). The previous dialysis procedure was followed except that SGF was replaced by the SIF (pH 6.8) and the dialysis time was extended to 6 h. After that, these starch samples were mixed with ethanol to quench the enzyme activity. The sediments, named AMS-SIF and ECC-MS-SIF, respectively, were dried and ground for further use. The dialysate samples were also used for sugar release analysis. For determining the digestibility of treated mandua starch and

extracted mandua in SCF, the above process was carried out in SCF (pH 7.4) for 6 h.

2.3.5. Paste Clarity. Paste clarity of mandua starch samples was determined according to the previous method.^{20,28} A 10 mg/mL (db) suspension of cross-linked starch and alkali-isolated starch in distilled water was prepared separately in a centrifuge tube and heated at 95 °C for 30 s every 5 min. After cooling to 4 °C, the starch clarity (% T , transmittance) was measured on a spectrophotometer (UV-1800, Shimadzu, Japan) at 650 nm against distilled water as blank.

2.3.6. Surface Morphological Characterization. ECC-MS was sprinkled on a double-sided adhesive tape attached to a circular specimen stub and then coated with gold using a sputter coater. The sample was viewed using SEM (Gemini SEM 300, Carl Zeiss, Germany) at an accelerating voltage of 15 kV.

2.3.7. Fourier Transform Infrared Spectroscopy. AMS and ECC-MS were ground in a glass mortar and dried. The samples were scanned using a Fourier transform infrared spectrometer (Cary 630, Agilent Technologies, USA) at a resolution of 8 cm^{-1} in the scanning range of 450–4000 cm^{-1} .

2.3.8. Powder XRD. Powder X-ray diffractograms were obtained using an X-ray diffractometer (D8 Discover; Bruker, Billerica, MA) under operating conditions as follows: the X-ray generator was run at 40 kV and 30 mA, and the scanning angle 2θ was set from 5 to 60° at a scanning rate of 0.02°/min. Furthermore, any change in crystallinity was observed by dividing the crystalline area. The crystallinity was calculated by dividing the crystalline area by the total area (amorphous and crystalline area) observed in diffractograms of native mandua starch and epichlorohydrin-treated mandua starch.

2.3.9. ^{13}C NMR Spectroscopy of ECC-MS (^{13}C NMR). ^{13}C Nuclear magnetic resonance spectra of ECC-MS were obtained on a Bruker AVANCE 400 MHz spectrometer. The spectra were obtained in dimethyl sulfoxide ($\text{DMSO}-d_6$) as a solvent. The chemical shift was recorded in parts per million. The spectra were obtained from 4000 scans. Before the measurements, the samples were appropriately dried and dissolved in $\text{DMSO}-d_6$ by heating the starch and $\text{DMSO}-d_6$ at 60 °C to get a clear solution; after that, the centrifuge tubes were centrifuged at 4000 rpm to get the precise solution to get a spectrum of good resolution.

2.3.10. Thermogravimetric Analysis. The thermogravimetric analysis (TGA) of AMS and ECC-MS was conducted using a simultaneous thermal analyzer (STA 6000, PerkinElmer, Germany) in an air and nitrogen environments (200 mL/min) at a heating rate of 5 °C/min. The temperature range for the analysis was maintained from ambient to 900 °C.

2.3.11. Quantification of RS. The RS fraction was quantified by using the Megazyme resistant starch assay kit. The method of RS quantification was based on AACC Method 32-40.01, AOAC Method 2002.02, and CODEX Method Type II. Furthermore, the data obtained was processed using a Megazyme mega calculator.

2.3.12. Statistical Analysis. The student “ t ” test was used when significant differences were found at a significance level of $p < 0.05$. Pearson correlation analysis was performed at the $p < 0.01$ level to establish correlations among the different evaluated parameters. The principal component analysis (PCA) was also performed with the aim to understand the interactions among the evaluated parameters. PCA for all numerical results was performed using the XLSTAT Trial version (Addinsoft, New York).

3. RESULTS AND DISCUSSION

3.1. Starch Isolation by the Alkali Extraction Method.

The isolation methods have their impacts on different characteristics of extracted starch.²⁹ Starch isolation steps include grating or milling, fiber separation, centrifugation, washing or purification, freeze dehydration, or oven drying. For starch extraction; alkali isolation method, acid isolation method, oil extraction method, water stepping, and enzymatic isolation method are used, and these are based on the desired properties and purity of starch. However, alkaline treatment with low shear isolation method produces starch with better yield, purity, and pasting properties.^{30,31} Starch contains two types of proteins, granule surface and interior proteins. The surface proteins are easily removed from starch using some mild extraction methods without destroying the granule structure, while the removal of interior proteins requires more disruptive extraction methods. Removal of surface proteins also eliminates surface lipids.^{32–34} In the present study, the starch from mandua grains was isolated by the alkali isolation method due to economic viability and effective surface protein removal capability with high purity. The yield of extracted starch from mandua grains has been shown in Table 1, and the yield was observed as $35.74 \pm 1.880\%$ w/w.

Table 1. Characteristics of ECC-MS and AMS^a

s.no	property	AMS (mean \pm S.D.)	ECC-MS (mean \pm S.D.)
1.	yield (%)	35.740 ± 1.880	90.8 ± 0.854
2.	total ash (db, % w/w)	0.185 ± 0.018	0.248 ± 0.076
3.	pH of aqueous extract	11	6.5
4.	paste clarity (% T)	6.566 ± 0.321	3.5 ± 0.009
5.	swelling capacity (% w/w)	226.510 ± 2.175	103.140 ± 1.998
6.	moisture content (% w/w)	16.767 ± 0.642	3.540 ± 0.671
7.	degree of cross-linking (%)		85.15
8.	water binding capacity (% w/w)	1.080 ± 0.011	1.756 ± 0.015

^aS.D.: standard deviation of three successive determinations, mean: average value of three determinations.

3.2. Water Binding Capacity. WBC is related to the property of starch to bind or connect with water. WBC is influenced by the type of starch, type of modification, and the severity of the treatment. WBC may be lowered by the involvement of hydroxyl groups in the formation of hydrogen and covalent bonds among starch components. The WBCs of the native and cross-linked starches are shown in Table 1. The WBC for native mandua starch was $1.080 \pm 0.011\%$ w/w, and for ECHD-treated mandua starch, the WBC was found to be $1.756 \pm 0.015\%$ w/w, respectively. It reflected the increment in the WBC of ECC-MS after treating it with ECHD. An increase in the WBC of ECHD cross-linked cassava starch (84.0% w/w) has also been reported in comparison to native starch (68.3% w/w).¹⁹ Rice starch citrates have also shown significantly higher WBC than control at 25 °C.²² Comparatively, the lower WBC of alkali-isolated starch may be attributed to the blocking of water binding sites with sodium ions during alkali isolation, making them unavailable for binding of a water molecule. However, a considerable

decrement in the WBC of rice starch has been reported upon cross-linking with ECHD (0.25%).³⁵

3.3. Swelling Capacity and Solubility. The swelling capacities of both AMS and ECC-MS are presented in Table 1. It has been reported that due to cross-linking, starch possesses an abnormal capacity of accommodating water into the network as a cross-linked starch network can be imagined as an ultrahigh molecular weight starch molecule containing pores in its structure.³⁵ The swelling capacity of ECC-MS was found to be $103.14 \pm 1.998\%$ w/w, and for AMS, it was $226.51 \pm 2.175\%$ w/w. Starch granules with more significant crystalline areas and strong covalent interactions in the crystalline regions usually show little swelling power in cold water than in the subsequent heating conditions.^{37,38} It has also been reported that modified starch citrates (starch from basmati rice) at 25 °C had a higher swelling capacity than control.²² However, it has been proposed that due to strong bonding forces within the granules of starch during cross-linking, swelling became restricted.²² However, a decrement in the swelling power of potato starch cross-linked with ECHD has also been noticed but the temperature conditions were different from the present study.^{39,40} The high solubility of the starch can restrict its use as excipients in prolonged drug-delivery devices. The solubility of ECC-MS was found less than the native mandua starch. The decrement in solubility by cross-linking may be attributed due to increased cross-linking density in the starch structure.^{41,42} A similar trend of decreased solubility was reported for onset starch cross-linked with ECHD.⁴³ The decreased solubility also confirmed the strong molecular arrangement.^{38,58}

3.4. Ash and Moisture Content Estimation. The ash content and moisture content of the ECC-MS and AMS are shown in Table 1. The ash contents of ECC-MS and AMS were found as $0.248 \pm 0.076\%$ w/w and $0.185 \pm 0.018\%$ w/w, respectively. The moisture contents of ECC-MS and AMS were found as $3.54 \pm 0.671\%$ w/w and $16.767 \pm 0.642\%$ w/w, respectively. Carmona-Garcia et al. (2009) reported a 1-fold decrement in moisture content in banana starch after cross-linking with ECHD (7.5 mL per 100 g of starch) when compared with its native sample.⁴¹ It was proposed that this pattern is related to the reaction between –OH groups of glucose units of starch and the bi- or polyfunctional chemical reagent used in this chemical modification. This process decreases the possibility of reaction between –OH groups of starch chains and the water molecules and, consequently, affects the joining of water to the polymer. The low moisture content of RS samples could be probably due to removing pentosans and water-binding proteins during alkali extraction.⁴² Previous work reported that ECHD cross-linked onset starch contains less moisture than the unmodified starch.⁴³

3.5. Paste Clarity. This specific property depends on the weight of amylopectin and amylose and final molecular size. The difference in molecular size, weight of amylopectin and amylose of starch significantly affect the paste clarity, functionality, and digestion characteristics. Paste clarity is a property of interest due to the application of starch in food industries to produce turbid pastes (low transmittance) to prepare dressings.⁴⁴ Moorthy (2002) concluded that smaller taro starch granules scattered more amount of light.⁴⁵ Also, B-type crystallinity and B-type starches exhibit better clarity than A-type starches.⁴⁴ Paste clarity is a result of rupture of swollen starch granules, and cross-linking improves the integrity of swollen granules, resulting in reduced paste clarity.⁴⁶ The paste

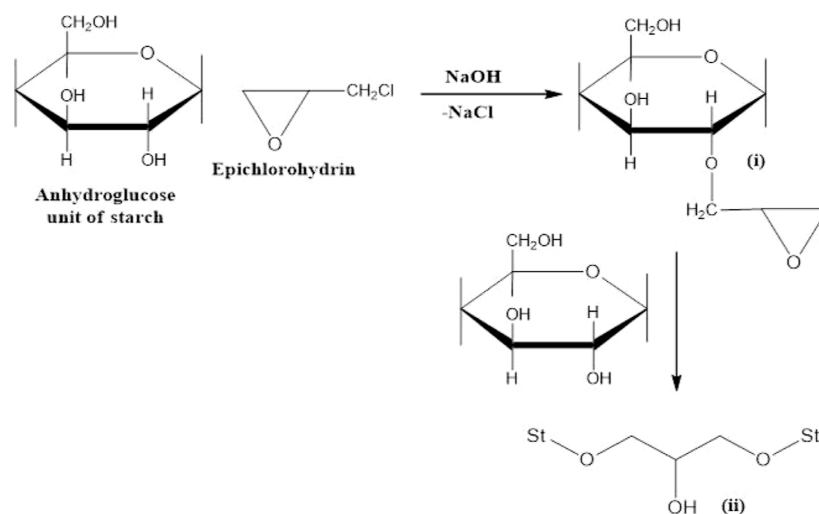


Figure 1. Cross-linking of epichlorohydrin (ECHD) with AMS in an alkaline medium.

clarity of ECC-MS and AMS has been shown in Table 1. The paste clarity of AMS and ECC-MS samples was found to be $6.566 \pm 0.321\%$ T and $3.5 \pm 0.009\%$ T, respectively. The paste clarity of the ECHD-treated mandua starch sample was lower and significantly different from the paste clarity of the alkali-isolated non-modified mandua starch ($p < 0.05$). Low starch paste clarity of ECC-MS could be explained in terms of destruction/cleavage of polymeric chains of starch. The starch granules which were stable to destruction during the application of heat showed lower starch paste clarity compared to the high paste clarity of the native starch. At the same time, AMS showed greater paste clarity because of rupturing of starch molecular chains. Daramola and Bamidele (2015) reported a decrement in the paste clarity of ECHD-treated cassava starch samples compared to the high paste clarity of the native cassava starch.⁴⁷ Núñez-Bretón et al. (2019) reported that the paste clarity showed an inversely proportional relationship with the ECHD concentration for cross-linked Mexican *Oxalis tuberosa* starch.⁴⁴ It has also been observed that thermal treatment increases the % T values for acid-treated cereal starches, but alkali incubation causes a decrease in % T.^{44,48}

3.6. Determination of the Degree of Cross-Linking.

The unreacted ECHD content is a value that indirectly tests the cross-linked degree because when a high amount of cross-linking agent is found as unreacted, a lower cross-linking of the polymer is carried out and the extent of the reaction is comparatively lower.⁴⁹ The molar degree of cross-linking has also been studied on the basis of the amount of unreacted ECHD.⁴⁴ The degree of cross-linking of ECC-MS was found 85.15% as calculated on the basis of the amount of extracted starch. It has been found that at a higher concentration of starch and lower amount of sodium hydroxide; most of the amount of the applied ECHD reacts with the starch; thereby, about 75–92% is bound by the primary cross-linking reaction, forming glycerol diether cross-links, and 8–25% of ECHD is bound by the side reaction as a glycerol mono-ether derivative.⁴⁴ As starch modifications with chemical agents are nucleophilic reactions, a certain amount of alkali can promote a reaction by ionizing the hydroxyl groups in the starch molecules into an alkoxide anion. Therefore, alkali is an efficient catalyst for the chemical modification of starch.^{50–52} Considering these findings, a small quantity of alkali was

utilized to maintain the reaction pH and to catalyze the reaction. It has been found that 85.15% of used ECHD was reacted in the cross-linking reaction of AMS. Kim and Lee (2002) calculated the degree of cross-linking and product yield by determining the amount of unreacted ECHD and found that the average reaction yield between ECHD and starch was about 85%. In the present study, because of the lower ECHD (1.0%, dsb) to starch ratio, most of the ECHD was used by the starch anions.⁵³ Therefore, the reaction rate would be higher. A study conducted elsewhere concluded that the adsorption of ECHD in aqueous reaction conditions was favorable because of the poor affinity of ECHD toward water.⁴¹ In this study, the ratio of starch to water was maintained at 1:2 during cross-linking of mandua starch with ECHD because cross-linking is favored by a high starch/water ratio.⁴¹ These conditions might favor the utilization of most of the amount of ECHD ($\geq 85\%$) in the cross-linked starch reaction. The mechanism of cross-linking of ECHD with AMS in an alkaline medium has been shown in Figure 1.

3.7. In Vitro Digestion Resistibility. This property plays a vital role in the formulation of targeted characteristics. Several factors affect the digestibility of starch, including but not limited to its botanical origin, granule size, content of amylose and amylopectin, crystallinity and the presence of amylose–lipid complexes, and modification methods.^{9,54} Modification of AMS with 1.0% ECHD resulted in an increment in digestion resistibility in the regions of GIT. An increase in in vitro digestion resistibility may be due to increased resistant starch content, that is, the formation of resistant starch type IV (RS4). It has been found that the digestion resistibility of the ECC-MS starch was increased remarkably after treatment with ECHD ($p < 0.05$). Similar results have also been observed in a study conducted on the modification of srpanjka starch, a variety of wheat with ECHD.⁵⁵ Ačkar et al. (2010) observed a 2-fold increase in RS content and reduction of digestibility on modification with 0.3 and 0.5% ECHD.⁵⁵ Woo and Seib (2002) found that RS content in modified wheat starch was increased up to 53 and 76% on treatment with ECHD at concentrations of 1% w/w (dsb) and 2% w/w (dsb), respectively.¹⁶ The increase in digestion resistibility of starch after cross-linking could be associated with highly compact particles that behave as a physical barrier to the digestive enzyme causing a delay in

penetration of the enzyme into starch granules. Findings confirmed that the condition applied for cross-linking of mandua starch affected the molecular structure of starch. Furthermore, similar observations have been reported for heat–moisture-treated rice, maize, and potato starches.⁵⁵ The digestion resistibility pattern of modified (ECC-MS) and AMS are depicted in Figure 2. A change in molecular size affected

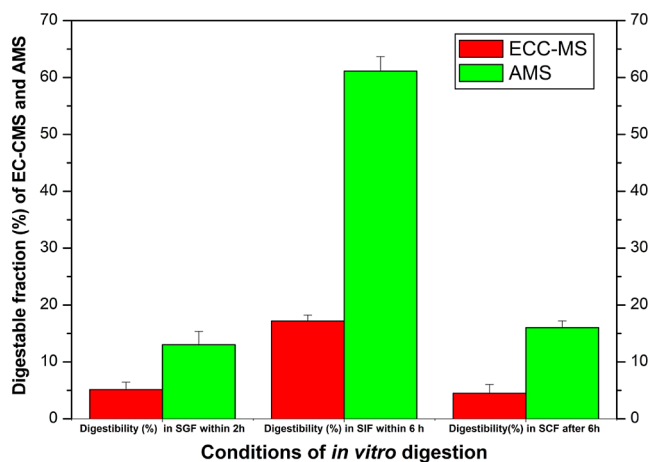


Figure 2. Digestibility of in vitro AMS and ECC-MS in SGF (pH 1.2), SIF (pH 6.8), and SCF (pH 7.4).

the paste clarity, swelling power, and ratio of constituents of starch, causing a change in the enzymatic degradation behavior of starch. The results of the paste clarity of the mandua starch also supported higher digestion resistibility after cross-linking with ECHD.

3.8. Morphological Characterization. The appearance of starch granules after cross-linking (ECC-MS) and AMS was studied by SEM. The surface morphologies of AMS and ECC-MS have been shown in Figures 3 and 4 respectively. SEM

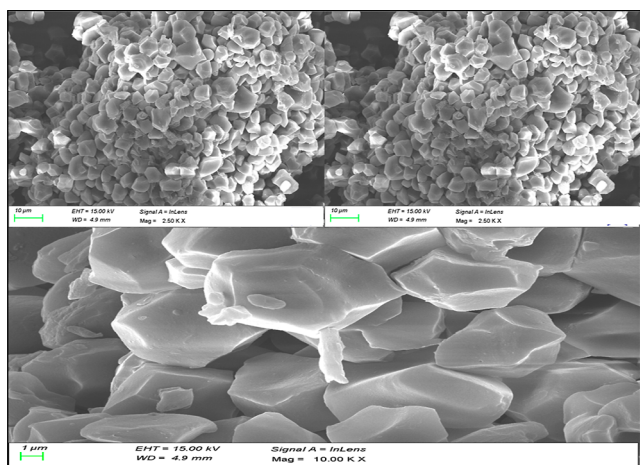


Figure 3. Scanning electron micrographs of AMS at different magnifications.

investigations revealed that the cross-linking caused slight changes in the granular morphology of ECC-MS. Due to cross-linking in starch (ECC-MS), the surface of granules became slightly rough and the formation of pores or grooves was observed on some of the granules that indicated slight fragmentation. A slight increment for water uptake in ECC-

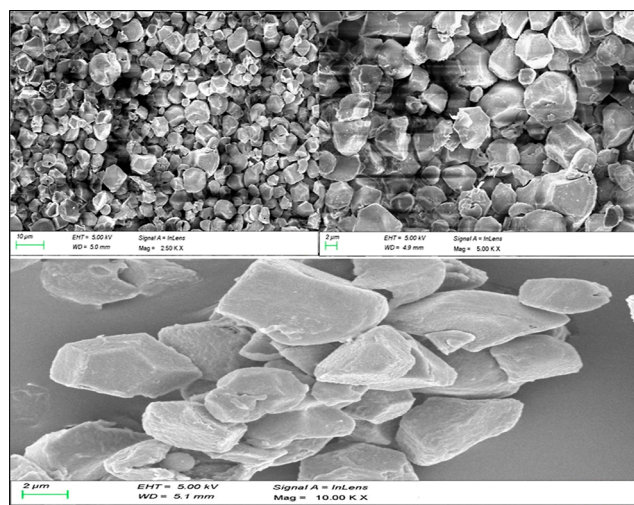


Figure 4. Scanning electron micrographs of ECC-MS at different magnifications.

MS as compared to AMS also confirms that some pores would be developed in cross-linked starch. These results were consistent with previous studies.^{8,28,41} However, scanning electron micrographs of native and modified rice starch samples indicated that chemical modification of cross-linking with ECHD (0.3% w/w, on dry starch basis) had no remarkable effect on rice starch granules' morphological properties.⁴⁰

3.9. FTIR Characterization. The FTIR spectra of modified starch (ECC-MS) and AMS displayed all the typical absorption bands for polysaccharides, as shown in Figure 5. Generally, the IR spectra of modified starches show the typical peaks for the starch backbone. Compared to an absorption peak around 3500–3000 cm^{-1} in alkali-isolated starch, this peak became broadened and less sharp in chemically cross-linked starch spectra, which might be due to hydrogen bond stretching. Additionally, in the IR spectrum of the cross-linked starch, the characteristic absorption bands were observed at 1019 and 1155 cm^{-1} which might be due to C–O stretching vibrations. However, these were highly diminished in intensity. The absorption bands at 1639 cm^{-1} enlightened the existence of C=C stretching in alkali-isolated starch.^{2,19} The peaks at 709 cm^{-1} indicated low content of coordinated water molecules due to wagging (out-of-plane bending) vibrations of the –OH groups in water.^{36,36} Moreover, FT-IR spectra reinforced the existence of cross-linking between AMS and ECHD. The molecular interaction was further confirmed by the FTIR spectra. The FTIR spectra showed a vibration peak in the area at 1018–1242 cm^{-1} corresponding to the C–O–C functional group. The peaks at around 3400 cm^{-1} were broadened after cross-linking.

3.10. Powder XRD. The XRD patterns of ECC-MS and AMS are shown in Figure 6. Powder XRD is the only method that can measure the long-range crystalline order.^{57,58} Generally, the A-type crystalline structure of starch contains diffraction peaks at 2θ of 15, 17, 20, and 23°; for the B-type crystalline structure, it is present at 5, 17, 20, 22, and 24°. For the C-type crystalline structure, a combination of A- and B-types is present. However, for a typical V-type crystalline structure, the diffraction peaks are present at 7, 13, and 20°.^{24,29} In the diffractogram of ECC-MS, the prominent peaks were observed at 15.178, 17.05, 17.958, 18.012, 19.954, and

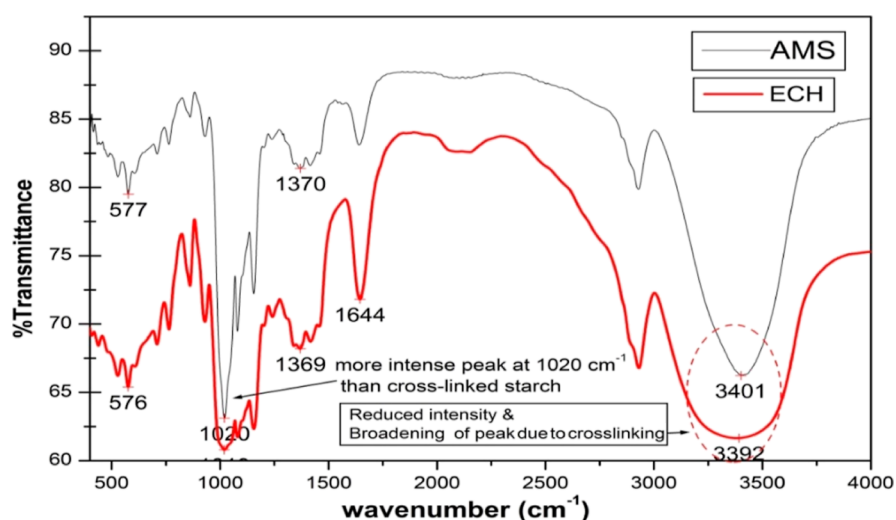


Figure 5. FTIR spectrum of AMS and ECC-MS; explanatory note: ECH-ECC-MS.

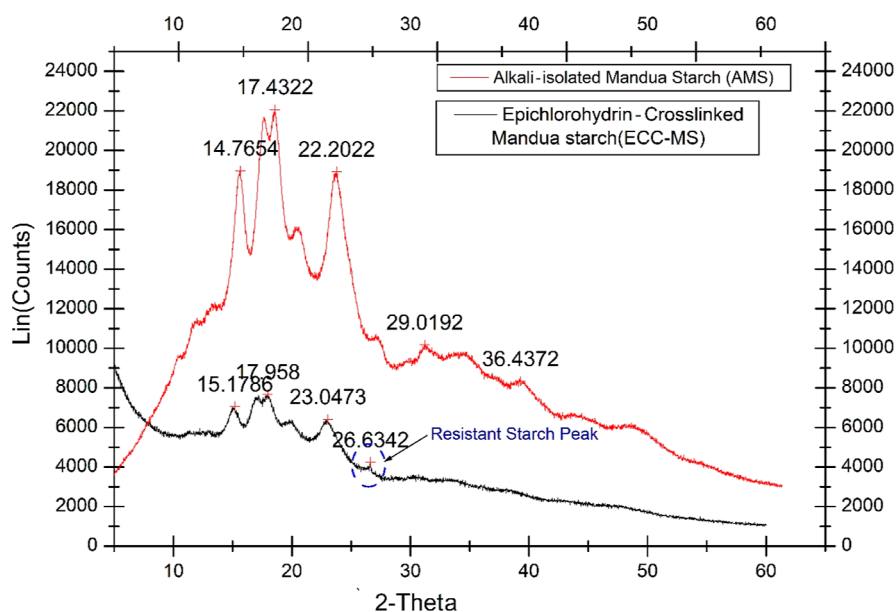


Figure 6. Powder X-ray diffractograms of AMS and ECC-MS.

23.04°. The presence of 17.958 and 18.012° peaks at about 18° in starch revealed the starch with CA-type crystallinity. The peak at a 2θ of 20° is typical of a V-type pattern, and this is commonly observed in case of parboiled cereals.⁵⁹ ECC-MS exhibited a single peak at a 2θ of 19.954°, indicating a typical V-type pattern. Mangala et al. (1999) studied the X-ray diffractogram of RS isolated from ragi (Indaf variety) and found that RS showed significant peaks at 17.2 and 17.6° with characteristics of V- and B-type starches.⁶⁰ In the diffractogram of ECC-MS, the presence of a clear peak at 26.68° reinforced that the cross-linked starch contained a marked RS fraction (RS4). According to Situ et al. (2014), the peak at around $2\theta = 26.0^\circ$ is due to a RS fraction. This peak becomes more pronounced as the RS content increases, which agrees well with the results of this study.⁶¹

Any change in the crystalline pattern and molecular structure significantly affects the RS content through alteration of the reaction between starch granules with the enzyme.⁶² Furthermore, the loss in crystallinity caused by cross-linking is the most conspicuous change in X-ray diffractograms. It was

observed that the degree of crystallinity was 43% for AMS, which decreased to 32% after cross-linking. Similar results related to the decrement of crystallinity of starch after modifications have been reported.⁶³ It was concluded in the study that the decrease in crystallinity was due to the disorder in the chain caused by the phosphates replacing hydroxyl groups during cross-linking process. Furthermore, a decrease in crystallinity of cassava starch and potato starch has also been reported with an increase in cross-linking and RS.⁶⁴

3.11. NMR Characterization. It has been reported earlier that six necessary signals occur at 100, 79, 74, 72, 71, and 61.5 ppm due to the C-1, C-4, C-3, C-2, C-5, and C-6 atoms of the glucopyranose unit, respectively. The resonances at 79 and 70 ppm are due to C-4 involved in the α' -(1,4) linkages and C-4 of the nonreducing terminal units, respectively.⁶ The ¹³C NMR spectra of ECC-MS have been depicted in Figure 7. The ¹³C NMR spectra of cross-linked starch produced strong and intense signals at 78.86, 73.35, 72.07, 71.71, and 60.59 at a lower intensity signal of 100.16 ppm. The signals at 100.16 and 60.59 ppm were due to low-molecular-weight starch

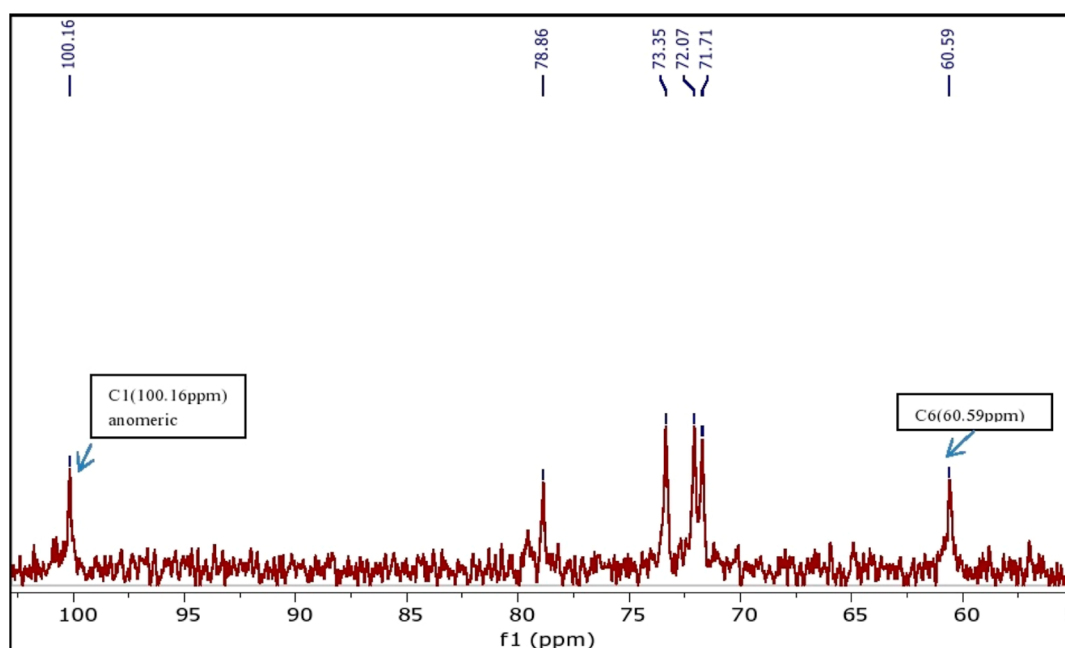


Figure 7. ^{13}C NMR study of ECC-MS.

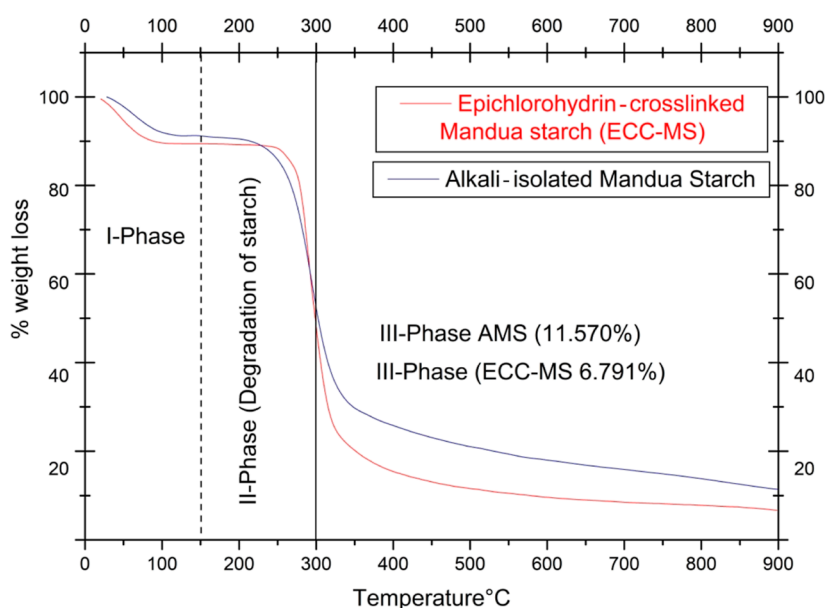


Figure 8. Thermograms of ECC-MS and AMS.

components. These signals are related to $\text{O}-\text{CH}_2$, $\text{CH}(\text{OH})$, C-1 (100.1), and C-6 (60.59 ppm) due to low-molecular-weight components which may change their mobility in the presence of water. The results agreed with the previous study in which ECHD cross-linked potato starch produced strong and intense signals at 72.9, 71.4, 70.0, 63.6, 61.7, 52.3, and 45.6 ppm with a lower intensity signal at 100.5 ppm.^{65,66} The absence of signals at 30, 130, and 180 ppm further confirmed the purity of starch by ^{13}C NMR spectra as these signals occurred in aliphatic, carbonyl, and olefin carbon regions and are attributed to impurities present in industrial byproducts.

The peak at 60.59 ppm in ECC-MS spectra assigned to C-6 could be referenced to the chemical shifts of C-6 of β -D-glucose, which was assigned at 60.59. The large degree of signal overlap in the range of 60–85 ppm may be attributed to

the cross-linking step which induces the formation of a polymer network. A previous report suggested that after cross-linking, the C-1 signal (100–105 ppm) decreased when the cross-linking agent's amount was increased due to a reduction of the glucose quantity and an increase in the disorder introduced by the cross-linking as well as higher rigidity of cross-linked starch.⁶⁷ In this study of ECC-MS, a similar trend was observed as after cross-linking, the signal (100.16 ppm) became less intense.⁶⁵ Interestingly, Mangala et al. (1999) also suggested similar findings for RS derived from ragi and found almost comparable spectra for ragi amylose and RS from processed ragi.⁶⁰

3.12. Thermogravimetric Analysis. The behavioral changes of chemically modified starch impacted by cross-linking treatment were studied by TGA and are shown in

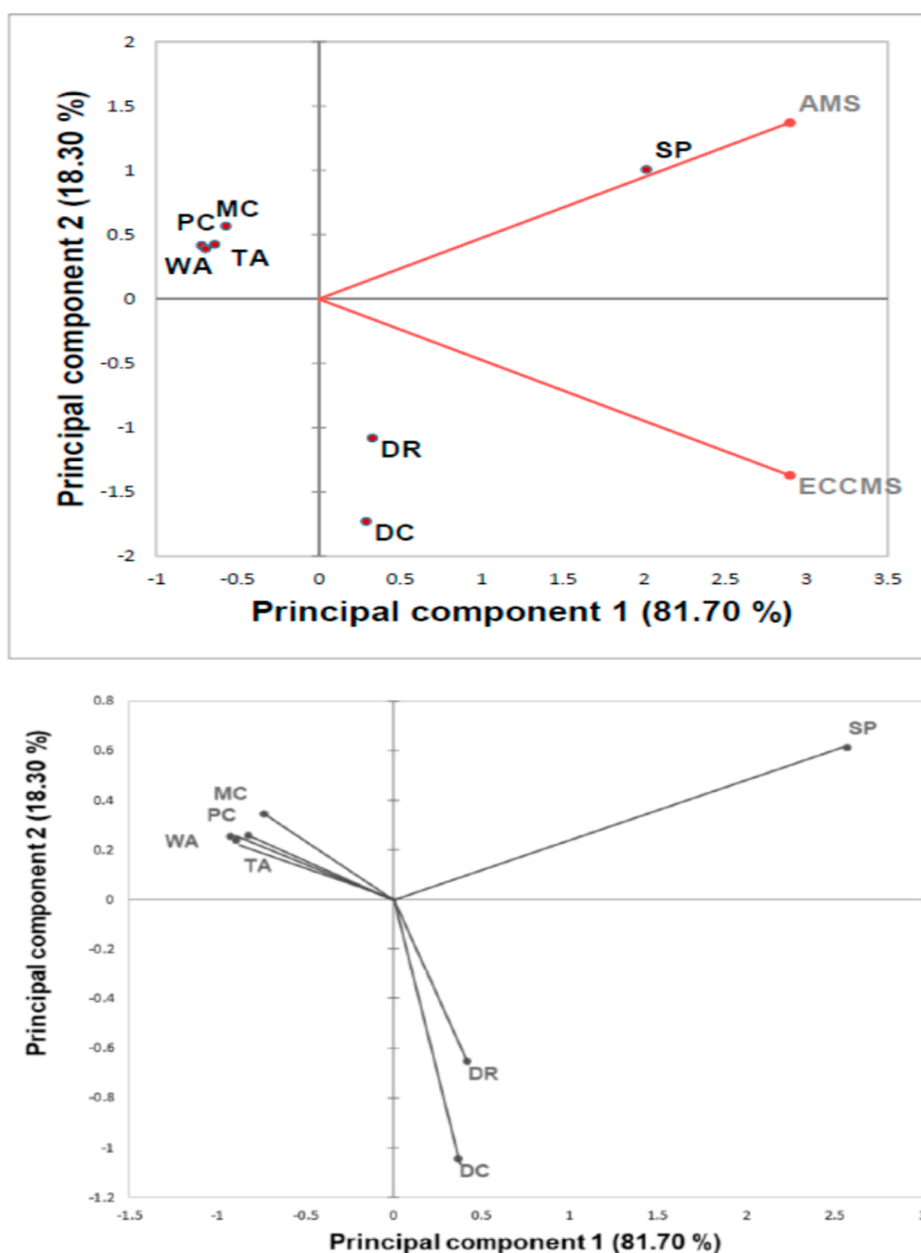


Figure 9. PCA biplot for different variables of AMS and ECC-MS on PC1 (81.70% of total variance) and PC2 (18.30% of total variance). Explanatory notes: WA = water binding capacity, MC = moisture content, PC = paste clarity (% T), SP = swelling power, DR = digestion resistibility, DC = degree of cross-linking, TA = total ash content.

Figure 8. Three different decomposition stages were observed in the decomposition pattern of AMS and ECC-MS. In the initial stage, the decomposition of AMS started from 44.5 to 88.97 °C with a weight loss of about 8.5%. During this phase, the water's dehydration and evaporation may occur as moisture may be present in the form of bonded and unbonded states. A similar decomposition pattern was followed by ECC-MS in which the evaporation of lodged moisture occurred from 31.76 to 68.75 °C with a loss of 9.090%. The % weight loss during this phase is directly proportional to moisture content; that is, the more the moisture present in the sample, the more will be the weight loss. The removal of water from all sample surfaces may lead to the development of the porous structure of starch. When the heating temperature was gradually increased with a controlled heating rate, prominent decomposition in both AMS and ECC-MS was observed. Liu et al. (2016) also

observed a similar pattern (three-phase) of decomposition of sodium trimetaphosphate cross-linked high amylose corn starch, with a considerable increment of the decomposition temperature for modified starch when the phosphorus content was increased from 0 to 0.02%.⁶⁸ In cross-linked starch, the second phase of weight loss occurred actually due to the thermal decomposition of starch. The second decomposition phase was observed from 273.63 to 320.82 °C with a significant weight loss (64.57%). The midpoint of the second phase decomposition in AMS was observed at 292.04 °C, and the peak of second decomposition was observed at 295.45 °C in ECC-MS. The maximum weight loss during the second phase may be due to depolymerization with rupturing of carbon-carbon and carbon-oxygen bonds of the ring structures available in starch, and it may be responsible for the evolution of small molecules such as CO, CO₂, and H₂O. It

is also reported that dehydration of the saccharide ring occurs in this phase.⁶⁹ The change in the decomposition pattern of chemically modified mandua starch with a significantly higher requirement of thermal energy for decomposition was considered due to the formation of tight junctions induced in cross-linked starch by cross-linking agents, and these might be responsible for the formation of the tight, compact structure of porous starch and thermal stability toward decomposition. Carmona-Garcia et al. (2009) concluded that due to the presence of a glycerol bond in ECHD-treated starch, gelatinization of cross-linked starch occurred at a higher temperature. Glycerol bonds tightened the molecular organization of a starch molecule, thereby increasing the gelatinization temperature.⁴¹ The third stage decomposition of AMS was observed in the range of 413–463.8 °C, and in a chemically modified form, this phase was observed from 410.25 to 484.20 °C with a weight loss of 6.791%. In this phase, the weight loss was significantly lower ($p < 0.05$) than in phase two, and this phase consumed more energy and occurred at a higher temperature (410.25–484.20 °C) in chemically modified starch. This may be due to the significant decomposition of AMS and ECC-MS in phase two. However, pyrolysis may be responsible for the weight loss in the third phase. The results of TGA inferred that compact structure formation occurs after cross-linking by ECHD and leads to the resistance to decomposition. It also restricts the movement of cross-linked chains, leading to thermal stability.

Hollinger et al. (1974) reported that in the presence of a diluent, the thermal stability of cross-linked starch increased in the granular, crystalline state.⁴⁸ In a study, the decomposition temperature of cross-linked starch (cross-linked by hexamethylene diisocyanate) has been reported to be higher than that of the native starch.⁷⁰ According to Singh et al. (2007), an increase in gelatinization temperature of cross-linked starch is related to the reduced mobility of amorphous chains in the starch granule due to the formation of intermolecular bridges.⁷¹ Thermal stability of moth bean starch was slightly increased on cross-linking of moth bean starch with POCl_3 in an alkali environment.⁷² The differential thermal analysis curve of AMS showed two endothermic peaks at 51.52 °C (7.62 μV) and 267.74 °C (7.66 μV) and one exothermic peak at 322.11 °C (2.83 μV), while modified starch exhibited endothermic decomposition at the temperature of 71.29 °C (15.42 μV) and exothermic decomposition at 283.43 °C (6.67 μV) (graphs have not been shown). These results represent better thermal stability of ECC-MS than AMS.

3.13. Quantification of RS. The RS content in AMS was 9.5% w/w. However, RS content in cross-linked mandua starch was found to be 27% w/w. It has been reported that any change in swelling behavior significantly influences the penetration of the enzyme in starch granules. This phenomenon causes a change in the digestion behavior and RS fraction. The findings of this study were in agreement with the digestion resistibility of the modified mandua starch calculated in different simulated fluids because of the enzymes used in simulated fluids. However, it was observed that the RS content was slightly lower when quantified using an enzymatic kit. This change in the RS content may be due to the use of amyloglucosidase and α -amylase. A similar trend in the increase in RS content after enzymatic digestion was observed earlier for cross-linked maize starch.⁷³

3.14. Principal Component Analysis. The advantage of performing PCA is to transform the correlated variables into a

new uncorrelated variable. It is performed to recognize the likeness between treatments of dependent variables established on correlations among them. Hence, the data become easier to explain in a chart. In this study, Pearson correlation analysis showed strong correlations ($p < 0.01$) among some starch properties that are known to directly impact functionality. The principal component score and loading plots have been merged into biplots to facilitate better data visualization, as shown in Figure 9. The biplot confirmed that the degree of cross-linking and digestion resistibility is significantly negatively correlated with water absorption capacity and paste clarity (%T) (r close to -1). The swelling power lies almost at an orthogonal angle to digestion resistibility, which is the evidence for a less significant effect on the other properties. The positions of AMS and ECC-MS at different quadrants of the PCA biplot demonstrate the existence of distinguished treatment effects on all the properties of AMS. Analogous pieces of evidence have been reported for the relationship between the digestion property and the structural characteristics.⁷⁴

4. CONCLUSIONS

Cross-linking with ECHD caused the changes in physicochemical and in vitro digestion resistibility properties of AMS. The digestion resistibility of ECC-MS showed significant increment in all simulated fluids of GIT, and it indicated an increment in resistant starch content after ECHD treatment of mandua starch. Cross-linking and purity were confirmed by ^{13}C NMR and by other parameters. Powder XRD results revealed a RS peak in cross-linked starch. The swelling capacity, solubility, and paste clarity decreased after cross-linking. It was found that the level of modification had a strong influence on the thermal properties of the cross-linked starch. Moreover, cross-linking using ECHD could improve the resistant content of mandua starch. The increment of RS content might be responsible for the improvement in digestion resistibility. The cross-linked mandua starch may be used as a replacement for dietary fiber in the food industry and as a sustained-release matrix modifier in pharmaceutical industries.

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Notes

The authors declare no competing financial interest.

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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