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Phosphorylation of Alkali Extracted Mandua Starch by STPP/STMP for Improving Digestion Resistibility

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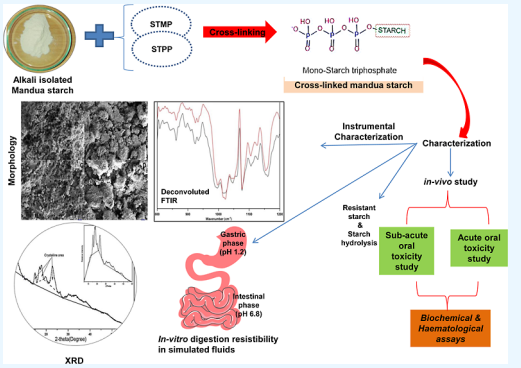
ABSTRACT: The chemical modifications of starch granules have been adopted to improve the characteristics, viz., paste clarity, resistant starch content, thermal stability, and so forth. The modified starch has been applied as a biopolymer in developing various preparations of food, nutraceutical, and pharmaceutical importance. The present work is focused on phosphorylation of alkali extracted mandua starch for improving digestion resistibility. The phosphorylation of mandua starch extracted from grains of *Eleusine coracana* (family *Poaceae*) was carried out by sodium tripolyphosphate/sodium trimetaphosphate at alkaline pH. After chemical treatment of mandua starch, the resistant starch (RS) content was increased significantly. The digestibility of chemically modified starch (CMS) was decreased down after treating by the phosphorylation process. The digestibility of CMS and alkali extracted mandua starch (AMS) in simulated intestinal fluid was found to be 32.64 ± 1.98% w/w and 61.12 ± 2.54% w/w, respectively. After chemical modification of mandua starch, a decrement was observed in amylose content, water-binding capacity, and swelling power. In the three-stage decomposition pattern of CMS studied by thermal gravimetric analysis, the significant changes in decomposition behavior also affirmed the impact of cross-linking in the improvement of stability of internal structure and resistibility of starch. In Fourier transform infrared (FTIR), the formation of the P═O bond was observed in CMS at 1250 cm^{-1}. The acute and sub-acute toxicity studies in terms of behavioral, haematological, and enzymological parameters for CMS were not different significantly from AMS and control ($p > 0.05$). The cellular architecture of the liver and the kidney were found normal after consumption of CMS. The results revealed that significant increment in RS fraction occurred after cross-linking of mandua starch. The prepared starch may be applied in developing various formulations of food and pharmaceutical importance.



1. INTRODUCTION

The starch procured from different bio-resources has been applied in different preparations as excipient, for example binding, gelling, thickening, stabilizing, etc. However, the native starch is associated with some characteristics such as weak shear, higher viscosity, high retrogradation and syneresis, low process tolerance, and so forth.[1] Besides this, the native starches during cooking processes result in the weak and rubbery paste with cohesive nature. All the above properties comparatively limit the acceptability of native starches for broad range applicability in different manufacturing processes of food, pharmaceutical, and nutraceutical industries. Physical, chemical, and enzymatic methods may perform significant modifications in inherent properties of the native starches. The desirable changes can be performed by chemical modifications conveniently and efficiently in starches for improving the functionality and versatility. In chemical modifications, new functional groups are introduced that are responsible for altering the physicochemical properties of the native starches.

The higher amount of resistant starch imparts the dietary fibres to the diet, and it has preventive and therapeutic impact on health. Based on digestion resistibility in the gastrointestinal tract, the resistant starch has been categorized into four types: (1) physically inaccessible starch (RS1), (2) native granular starch (RS2), (3) retrograded starch (RS3), and (4) chemically modified starch (RS4).[2] The stable chemical structure of resistant starch imparts the digestion resistibility, and due to this, it reaches up to the colon by bypassing the digestion processes of the stomach and small intestine. The probiotic bacteria residing in colon ferment the resistant starch and convert it into short-chain fatty acids. Various beneficial effects, viz. lowering of serum cholesterol, reduced risk of diabetes, colon cancer prevention, improvement in laxation, nurturing the growth of beneficial microflora of the colon etc., are associated with these small chain fatty components.[3]

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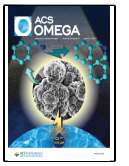

The non-digestible contents in starch can be improved by various treatments such as freeze−thaw cycle, chemical modifications, and so forth. The stability of the chemical structure of starch can also be improved by cross-linking techniques that may expand various functional properties and cold storage stabilities.[4] In the cross-linking process, the granular starch is treated with multifunctional reagents, and these chemical treatments create ether or ester linkages between hydroxyl groups of starch molecules.[1] The formation of intra- and inter-molecular linkages in granular starch structure occurs randomly, and the number of bonds formed imparts the cross-linking density to the chemical structure. In various studies, the cross-linked starch has been termed as "resistant starch type 4" (RS4).[5,6]

Different reagents such as STMP (sodium trimetaphosphate) or a mixture of STMP−STPP (sodium trimetaphosphate-sodium tripolyphosphate) has been applied for phosphorylation process that creates the resistibility toward digestion, and the treated starches have been categorized as "RS4".[7] Various variables, for example, source of the starch, reaction time, pH, temperature, presence and nature of the catalyst, type/concentration of reactant, etc., have significance influence on physicochemical and functional characteristics of the treated starches.[1] In a reported study, a 3 h cross-linking reaction has been performed with an STMP:STPP mixture (99:1) at a ratio of 12% starch basis (sb) to wheat, potato, and rice starches. The temperature during the treatment was maintained at 45 °C and pH was set at 11.5. The results of the study had reflected that the resistant starch content of wheat, potato, and rice starches was increased up to 72.8, 57.8, and 5.4% w/w respectively.[5] On cross-linking the wheat starch with 4, 8, and 12% (sb, starch basis) STMP:STPP (99:1) at 45 °C, pH 11.5 for 3 h, the resistant starch contents in starch samples were improved and reported as 24.5, 60.0, and 81.6% w/w, respectively.[8]

Uses of chemicals in tailoring starch pose threat to human life. Prior to promoting the items, it became imperative for all researchers and entrepreneurs to examine the toxicity profile of the modified starches in dosage forms. Thus, the modified starch needs to be evaluated according to OECD guidelines for the oral acute toxicity study (14 days), sub-acute toxicity study (45 days), and short-term and/or sub-chronic (90 day) studies before promotion or development of pharmaceutical formulations. SinceSince different starches underwent intensive processing to become resistant starch, a number of studies related to toxicities of modified starches have been conducted.[6,9] In the past, a number of studies related to toxicities of modified starches have been conducted. The toxicities of various forms of modified starches such as OS (E 1404), monostarch phosphate (E 1410), distarch phosphate (E 1412), phosphated distarch phosphate (E 1413), acetylated distarch phosphate (E 1414), acetylated starch (E 1420), acetylated distarch adipate (E 1422), hydroxypropyl starch (E 1440), hydroxypropyl distarch phosphate (E 1442), starch sodium octenyl succinate (E 1450), acetylated oxidized starch (E 1451), and starch aluminum octenyl succinate (E 1452) have been investigated. The treatment-related effects relevant for human risk assessment have not been detected in rats fed at very high level of these modified starches (upto 31 000 mg/kg body weight per day).[9] Besides this, the dietary reproductive toxicity studies have also been performed in rats for phosphate distarch phosphate, acetylated distarch phosphate, acetylated starch, and acetylated distarch adipate. Results of three generation reproductive toxicity tests at dietary levels up to 62% (equal to 31000 mg/kg body weight per day) have found no effects on reproductive performance or maternal and developmental toxicities.[9] Two chronic toxicities studies (52 weeks) have also been performed, one with acetylated distarch phosphate and another one for acetylated distarch adipate. Except caecal enlargement, the relative organ weights have shown no differences between the groups. The presence of treatment-related pelvic nephrocalcinosis has been observed in histopathological examinations of kidney sections. Also, an apparent correlation between increased occurrence of pelvic nephrocalcinosis, comparatively more accumulation of calcium in kidneys, and increased urinary excretion of calcium has been observed during the study.[9]

Mandua (*Eleusine coracana*, Family *Poaceae*) has been mentioned in ancient Indian texts as "nrttakondaka (Dancing grain)" and is a source of dietary fiber and calcium.[10] Mandua is a good source of carbohydrates, and the total amount content range is 72−79% w/w. In carbohydrates, free sugars (1.04%), starch (65.5%), and the non-starchy polysaccharides (11.5%) are present. The starch comprises amylose and amylopectin, and about 80 to 85% of starch is amylopectin and remaining part is amylose. A linear alpha-1, 4-D-glucan structure with characteristics of B-and V-type starches is present in mandua starch. It is thermally stable and has enzyme resistant fraction[11,12] and is most difficult to be hydrolyzed in vitro by fungal alpha-amylase. It also has a higher degree of crystallinity and comparatively slower digestibility by the digestive enzymes in vitro.[2] The total dietary fiber content of finger millet is about 11.5%, and it includes insoluble dietary fibers as the major component (15−17%). The soluble fibers are also available as a minor component (1−2%). The water soluble fibers in mandua grains consist of non-starchy polysaccharides, mostly α-glucan and arabinoxylan. However, the water-insoluble fiber (insoluble dietary fibers) contains lignin, hemicelluloses, and cellulose.[13,15] The dietary fibers are digested by fermentation in colon by the micro-organisms, and the fermentation of soluble dietary fiber produces short-chain fatty acids. These byproducts have very important nutritional value because of physiological reward in conditions of hypoglycemia and hypo-cholesterolemia.[14] The dietary fibers containing complex carbohydrates are slowly absorbed and digested than those present in other cereals.[14] The slow digestion process of dietary fibers brings a reduction in postprandial glucose control and is also beneficial in the treatment of diabetes.[13]

However, mandua starch is less utilized by global pharmaceutical, nutraceutical, and food industries. The presence of a marked amount of amylose in mandua starch may be applied for modification by various treatments, and the creation of enzymatic resistant starch may be helpful for various purposes. Also, the extraction processes have an impact on physicochemical properties of starch. Hence, in the present research work, the starch was isolated by the alkali-steeping method and modified by the phosphorylation process to enhance the digestion resistibility. The chemical modification of extracted mandua starch was performed by STMP/STPP in alkaline media. *In vitro* digestibility of chemically modified starch was assessed in simulated conditions of gastric and intestinal fluids. The changes occurred in phosphorylated starch were examined by phosphorus content, water binding capacity (WBC), swelling capacity, degree of cross-linking, paste clarity, oil absorption capacity, and amylose content. The

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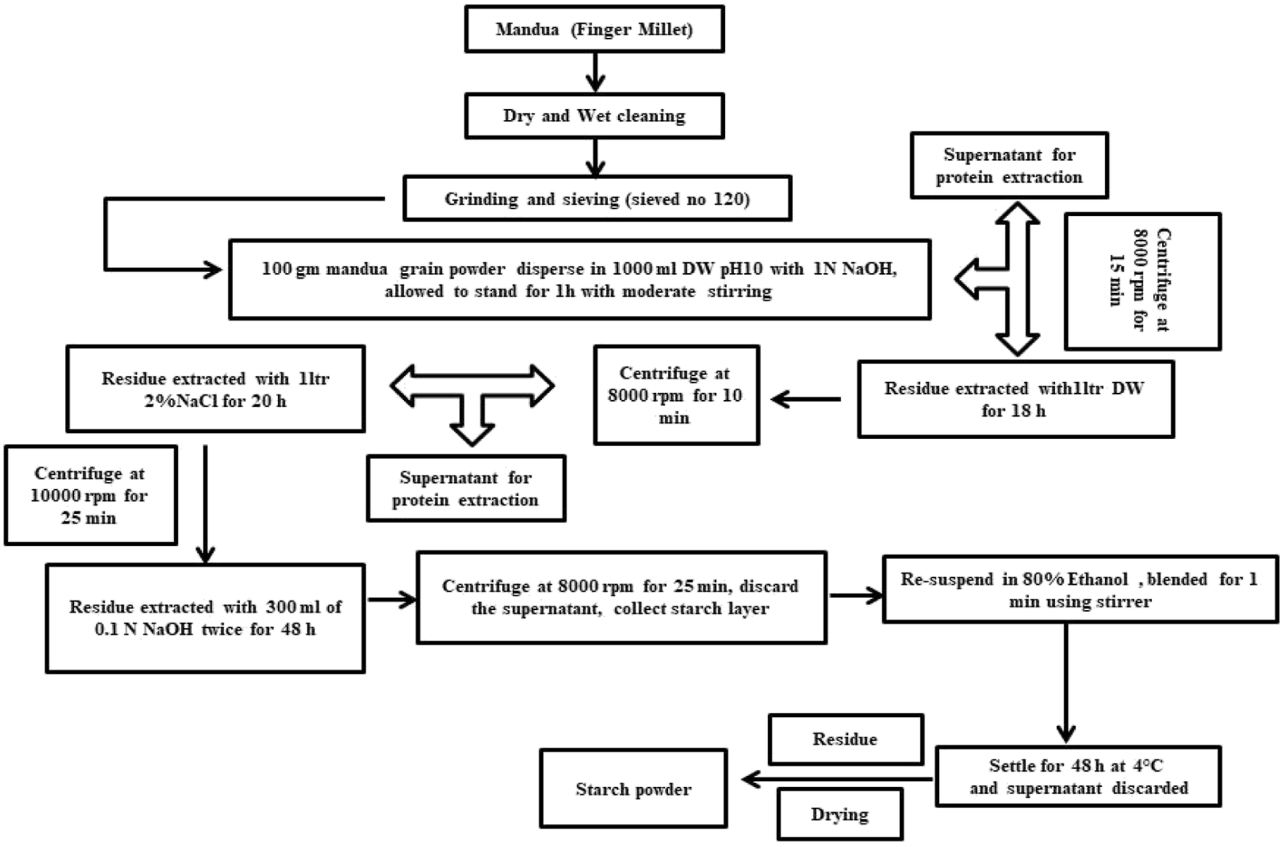

Figure 1. Isolation of starch from mandua grains by the alkaline-steeping method.

phosphorylated starch was characterized by powder X-ray diffractometry, FTIR, scanning electron microscopy (SEM), differential scanning calorimetry (DSC), and thermal gravimetric analysis (TGA). The phosphorylated mandua starch was studied for acute and sub-acute toxicities studies to assess any abnormal effect on physiological, biochemical, and histopathological parameters.

All the characteristics assessed for cross-linked mandua starch may indicate the feasibility of chemical treatment for modification of mandua starch and exploration of extracted starch from mandua grains in developing various preparations of pharmaceutical and nutraceutical importance. The minimal digestion of this starch in the gastrointestinal tract may provide an extra potential to treated mandua starch to be applied as a polymeric carrier for drug delivery devices and for protecting the drug from GIT environment. The resistibility after chemical modification may also assist in modulating the releasing and targeting of an incorporated drug.

2. MATERIALS AND METHODS

Mandua grains (Variety: VL-352) were procured from Vivekananda Parvartiya Krishi Anushandhan Sansthan, Almora (Uttarakhand). Pancreatin (from the porcine pancreas) was purchased from HiMedia, India. The Glucose Estimation Kit was purchased from Arbro Pvt. Ltd., India. All other chemicals and reagents used in this study were procured from CDH, Loba, Sigma-Aldrich, and Fisher Scientific Company.

2.1. Extraction of Starch from Mandua Grains. The alkaline-steeping method with some modifications was applied to isolate the starch.[16−18]

Briefly, the starch enriched powder of dried and de-husked mandua grains was suspended in double distilled water. The pH of the slurry was adjusted to pH 10 using sodium hydroxide. The thoroughly mixed slurry was centrifuged (Remi R-24, India) for 15 min at 8000 rpm. The recovered solid portion was suspended in water for 18 h and then centrifuged for 10 min at 8000 rpm. The suspension and sedimentation steps were repeated once with sodium chloride (2% w/v) and twice with 0.1 N NaOH for 20 and 48 h, respectively. The white starchy layer was collected and treated with 80% ethanol. Finally, alkali isolated starch was collected, dried, and stored in an airtight container. Figure 1 represents the flow sheet diagram for isolation process of starch from mandua grains.

2.2. Estimation of Amylose Content in the Extracted Starch Powder. A 0.1 g (dry basis) of the extracted starch was dispersed in a mixture of ethyl alcohol and 1 M sodium hydroxide solution (1:9 v/v). The mixture was warmed in boiling water for 10 min and then cooled down. Afterward, the volume of the dispersion was made up to 100 ml with distilled water. From this stock solution, an aliquot of 2.5 mL was mixed with diluting and complexing agents: 25 mL of water and 0.50 mL of acetic acid solution (1 M), 0.50 mL of I_2/KI solution (0.0025 M I_2, 0.0065 M KI). The resultant samples were analyzed spectrophotometrically (UV-1800, Shimadzu, Japan) at 620 nm. The amylose from potato (amylose 97%) was used for preparing the calibration curve.[19]

2.3. Production of Cross-Linked Starch by Treatment of Starch with STPP/STMP. The starches can be cross-linked by phosphorylation using phosphorylating agent STMP and STPP in alone and/or in combination.[6] The processes of chemical cross-linking of alkali-extracted mandua starch

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(AMS) for maximum digestion resistibility was optimized by 2-level factorial design (data not shown). The process factors adopted were pH of the process, temperature, and the ratio of STPP/STMP. The responses assessed during optimization of the process were swelling index, cross-linking density, digestion resistibility in simulated gastric fluid (SGF) (pH 1.2), and digestion resistibility in simulated intestinal fluid (SIF) (pH 6.8). In the present study, mandua starch was cross-linked for maximum digestion resistibility. Briefly, the cross-linking of mandua starch was conducted by a reported method with slight modification.[5,20] In this process, the native mandua starch was processed at 70 °C by slurring the starch [35 g, dry basis (db) with 19:1 (w/w) mixture of STMP and STPP for 3 h with sodium sulfate at 10% (starch basis)]. The resultant mixture was kept at pH 12 for 3 h. Then, the pH of the dispersion was maintained at 6.5 with 1M hydrochloric acid. The suspension was centrifuged (Remi R-24, India) at 5000 rpm for 10 min with successive washing of the residue with water (5 × 100 mL) for the recovery of the starch, and then, the recovered treated starch was dried in a tray dryer at 50 °C overnight.

2.4. Characterization of Cross-Linked Starch and Alkali Isolated Starch. *2.4.1. Determination of Percent Yield and Total Phosphorous Content.* The percent yield was calculated as per the earlier described method.[22] The phosphorus content of cross-linked and alkali isolated starch was determined as per the procedure with slight modifications.[21] A 100 mg of starch powder was taken in a porcelain crucible and kept inside for 4 h in a muffle furnace for ignition at 600 °C. Afterward, it was cooled down to room temperature, and the ignited residue of the crucible was admixed with 10 mL of 2.5 N hydrochloric acid and 1 mL of HNO_3. The resultant mixture was boiled and transferred into the volumetric flask (1000 mL). The resultant dispersion was cooled down to room temperature, and the volume was made up to with double distilled water. A 1 mL of this mixture was added to 1 mL of vanado-molybdo reagent solution and 3 mL of double-distilled water. The resultant mixture was kept aside in the dark for 10 min at room temperature and analyzed spectrophotometrically at 420 nm (UV-1800 Shimadzu, Kyoto, Japan). The phosphorus content in phosphorylated mandua starch samples was calculated by the calibration curve prepared by using a standard solution of KH_2PO_4. The estimation of phosphorus content in mandua starch samples was done by the expression

$$P = \text{absorbance}/16.76 \tag{1}$$

The calculated amount of phosphorus was applied to determine the degree of substitution (DS) by the expression given in eq 2.

$$DS = 162xP\%/3100 - 102xP\% \tag{2}$$

In the above expression, 162 denotes to the molecular weight of the monomeric starch unit, *P*% to the phosphorus content in mandua starch samples, number 3100 to the molecular weight of phosphorus multiplied by 100, and 102 represents the molecular mass of monophosphate substituent group.[6] In this expression, the monomeric starch unit is alpha D-glucosyl unit.

2.4.2. Determination of Degree of Cross-linking. The cross-linking density of phosphorylated starch was also determined by the dye adsorption method.[22] This estimation method provides an indirect albeit convenient way to estimate the cross-linking degree.[6,8] Briefly, cross-linked mandua starch (CMS) and AMS were mixed with 10 ml of methylene blue solution. The mixture of starch sample and dye solution was kept aside for 1 h in a dark place and then centrifuged for 10 min at 5000 rpm. The absorbance of the supernatant was measured at 665 nm. The cross-linking density of the treated mandua starch was expressed in terms of the relative amount of adsorbed methylene blue to CMS. The RMB was calculated as follows

$$\text{relative amount of adsorbed methylene blue (RMB)} = \frac{(C_0 - C_1)}{(C_0 - C_2)} \tag{3}$$

where C_0 represents the initial concentration of methylene blue, and C_1 and C_2 denote the concentrations of methylene blue in dispersions having CMS and AMS, respectively. The higher value of the RMB represents the higher cross-linking density.

2.4.3. Estimation of WBC and Oil Absorption Capacity. The WBC of phosphorylated mandua starch was analyzed by a reported method with slight modification.[24] A 1 gm (db) of the starch sample was mixed with 10 ml of pre-heated distilled water at 37 °C in a centrifugation tube, and the suspension was stirred by a magnetic stirrer for 5 min. The dispersion was centrifuged (Remi R-24, India) at 3000 rpm for 15 min, and the supernatant was collected in a 10 ml measuring cylinder. The difference between the initial volume of the water added for forming the starch dispersion and the volume of the supernatant reflected the WBC. Similarly, the oil absorption capacity of mandua starch samples was analyzed by using castor oil in place of water in the above procedure.

2.4.4. Swelling Power and Solubility Estimation. These properties were analyzed by the reported method elsewhere.[25] Briefly, a 500 mg of the starch powder (dry basis, db) was taken in a centrifuged tube and mixed with 20 mL of distilled water. The dispersion was heated at 37 °C for 30 min and centrifuged at 3000 rpm for 15 min. The watery layer of the dispersion was separated out and transferred to a Petri plate and kept in a tray dryer at 105 °C for estimation of solubility of starch. The centrifuge tube containing starch as the sediment was weighed for swelling power estimation.

2.4.5. Paste Clarity. The paste clarity of starches was determined by measuring their light transmittance (% T) as stated in a previously reported method.[26] Briefly, a 10 mg/mL (db) suspension of cross-linked starch and alkali isolated starch were prepared separately in distilled water in screw-cap tubes. The dispersion was heated at 95 °C for 30 min with continuous mixing at 5 min intervals using a vertex mixer and then cooled down to room temperature. The transmittance of starch dispersion was determined at 650 nm (UV-1800, Shimadzu, Japan), and the water was used as blank.

2.4.6. Moisture Content. The moisture content of CMS and AMS was estimated according to the reported method.[27] Briefly, a 200 mg starch sample (db) was dried in a hot air oven at 105 °C until constant weight. The following expression determined the moisture content of starch samples

$$\text{moisture content \%} = 100 \times \frac{(w_1 - w_2)}{w_2} \tag{4}$$

where W_1 represents the initial weight of the starch sample and W_2 denotes the weight of the dried sample.

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2.4.7. In Vitro Gastrointestinal Digestion Resistibility. The resistibility of AMS and CMS for digestion in SGF (pH 1.2) and SIF (pH 6.8) was analyzed by a reported method with minor modification.[28] Briefly, a 100 mg (db) starch powder was dispersed in 2 mL of SGF (pH 1.2) and it was filled immediately in a dialysis bag for dialyzing against 50 ml of SGF maintained at 37 °C for 2 h.[29] During dialysis, the starch dispersion containing dialysis bag was shaken frequently to avoid the starch sedimentation. After 2 h of dialysis treatment, the starch sample remained in the dialysis bag was centrifuged and dried. These samples obtained after this study were labeled as CMS-SGF and AMS-SGF, respectively. Also, a 1 mL of the dialysate samples was withdrawn and studied for sugar release by the GOD−POD method. The duplicate sample of the starch of the above study was centrifuged, and then, it was dispersed in 2 mL of SIF (pH 6.8). The similar procedure for dialysis was followed except that simulated intestinal fluid (SIF, pH 6.8) was taken in place of SGF. The dialysis time was extended to 6 h in SIF. Afterward, the enzymatic activity of SGF and SIF was quenched by mixing the ethanol to the starch samples. The resultant dispersions were centrifuged at 3000 rpm for 10 min, and the sediments were labeled as CMS-SIF and AMS-SIF, respectively. These samples of treated starch were centrifuged and dried. During this process, the dialysate samples were analyzed for sugar release.[29]

2.4.8. Fourier Transform Infrared Spectroscopy. The AMS and CMS were powdered in a glass mortar, screened, and dried. The sample was diluted with KBr (1:100 w/w) before acquisition. The background value was acquired from pure KBr before the samples were scanned. These samples were studied by Fourier transform infrared spectrometer at a resolution of 8 cm^{-1} in the scanning range at 400−4000 cm^{-1}. Further, spectra were deconvoluted after baseline-correction in the region from 1200−800 cm^{-1} using OriginPro 2023 (OriginLab Northampton, MA, USA). A gamma factor of 16 and a smoothing factor of 0.14 were applied to improve the peak resolution. The absorbance intensities of the bands at about 1047, 1035, and 1022 cm^{-1} were used to examine the crystalline structures of alkali isolated mandua starch and cross-linked mandua starch.

2.4.9. Powder X-Ray Diffractometry. The X-ray diffractograms of the starch samples were acquired by a X-ray diffractometer (D8 Discover; Bruker, Billerica, MA) under operating conditions: Operating voltage: 40 kV and 30 mA, scanning angle: 2θ, scanning range: 5−60°, and scanning rate: 0.02°/min. The starch samples were previously dried in a hot air oven at 80 °C for 6 h for removal of moisture and screened through sieve no. 120.

Beside this, any alteration in crystallinity was also estimated from the X-ray diffractogram. The crystallinity was calculated by dividing the area of the crystalline region observed in the diffractogram by the sum of amorphous and crystalline area observed in diffractograms of AMS and CMS.

2.4.10. TGA and DSC. TGA of AMS and phosphorylated mandua starch (CMS) was performed by the Perkin Elmer (TGA 4000, USA) apparatus in air and nitrogen environment (200 mL/min) by maintaining the heating rate at 5 °C/min. The thermal gravimetric analysis was conducted in the temperature range from ambient to 900 °C.

The AMS and CMS samples were also analyzed by the Differential Scanning Calorimeter (Star^C SW 8.10, Model DSC 822e, Mettler Toledo, Japan). In this study, the required amount of starch samples (AMS and CMS) was taken in aluminum pans. The pans were hermetically sealed, and then, analysis was conducted in a nitrogen environment by heating the samples at 5 °C/min from 50 to 300 °C.

2.4.11. Surface Morphological Characterization. The powder starch sample was sprinkled on double-sided adhesive tape attached to a circular specimen stub. The samples were made electro-conductive by coating with gold using a sputter coater. The starch powder samples were observed at different magnifications under a scanning electron microscope. The accelerating voltage during the study was maintained at 15 kV.

2.5. Acute and Sub-acute Toxicities of Phosphorylated Starch. *2.5.1. Acute Toxicity Study.* The acute toxicity study of mandua starch was executed in healthy Swiss albino mice by the "fixed dose" method of OECD (Organization for Economic Cooperation and Development) guideline no. 423. Previously, the protocol for the toxicity study was duly approved by IAEC (Reg. no. BMRL/AD/CPCSEA/IAEC/2019/10/2-I). The animals were procured from an approved breeder with an average body weight of 25 ± 5 g. The standard environmental conditions were provided to the experimental animals and before experimentations, they were acclimatized at laboratory conditions for seven days. During acclimatization, the temperature was maintained at 25 ± 3 °C and relative humidity at 60 ± 5% with a dark−light cycle of 12 h. The animals were supervised to assess their suitability for experimentation, and the non-suitable animals were eliminated in the initial stage. A standard diet was given to the animals with free access to water before and during the experimentation. All the animals were weighed individually and divided into three groups as A, B, and C having both sexes (male and female) in each group. The animals of Group-A received phosphorylated starch (CMS, test), Group-B received alkali extracted mandua starch (AMS, standard) and Group-C was used as the control.

A 2000 mg/kg body weight of the starch samples was administered to the animals orally, and before the study; the animals were fasted overnight *ad libitum*. For oral administration, mandua starch powder was suspended in water. The behavioral, neurological, and autonomic profiles of the animal were continuously observed for 3 h. Observation continued for 4 h at every 30 min and supervised for any mortality after 24 h, 48 h, 7 days, and till 14 days. The behavioral, autonomic, and physical changes of the animals were assessed by observing the mice for any changes in the skin, fur, eyes, mucous membrane, salivation, lethargy, sleep, coma, convulsions, tremors, diarrhea, oral activity, abdominal, and external genitalia. At pre-set time intervals, the animals were removed from their cages to observe any mortality, morbidity, and general conditions.

After due course of the study, the animals were fasted for 12 h and anaesthetized. The blood samples were taken from the tail vein and collected in two tubes. In one tube, sodium EDTA was previously added to prevent the coagulation of blood, and it was used for immediate analysis of haematological parameters such as blood glucose, haemoglobin, white blood cells count (thin-layer chromatography), total red blood corpuscles (RBCs), neutrophils, lymphocytes, eosinophils, monocytes, basophils, and neutrophils. The blood collected in another tube was centrifuged at 1000 rpm for 10 min, and the serum was separated. It was used for the estimation of biochemical parameters like serum urea, serum creatinine, serum glutamate oxaloacetate transaminase (SGOT), and serum glutamate pyruvate transaminase (SGPT).

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The histopathological examinations were conducted by observing the changes in internal structures of vital organs such as liver and kidneys. The mice of various groups were sacrificed by cervical dislocation after anesthesia, and desired organs were excised. Any extraneous tissues adhered to the vital organs were trimmed off, and the vital organs were placed on the gauze pad (soaked in saline solution) to retard desiccation. In histopathological studies of kidney and liver, the fixation of tissues was performed after excision from the body to prevent the post-mortem changes. The tissues were cleaned with normal saline immediately after sacrificing and cut into pieces of appropriate thickness such that the fixative could penetrate readily into the tissues. After washing, the tissues were passed through 70% v/v alcohol for dehydration. The tissues were embedded in paraffin to prepare blocks. Chloroform and xylene were used as the cleaning agent, and isopropyl and acetone as the dehydrating agent. The tissue sections of 5 μm thickness were cut with the help of Spence type rotating microtome. The sections were placed on a slide having a smear of 5% w/v Mayer's egg albumin. The slides with sections were floated in warm water (55−60 °C). The water was drained off, and the slides were dried on a hot plate at about 50 °C for 30 min to allow the fixation of sections on the slides. After fixing the section on slide, the sections were stained by serially placing them in xylol, acetone, 95% v/v alcohol, running water, hematoxylin to stain the cytoplasm of the cells and eosin to stain the nuclei, then changed thrice in alcohol, twice in acetone, and twice in xylol and mounted using diphenyl pthalein xylene. A cover slip was placed on the specimen, and it was studied under the binocular research microscope at 100× to note down the changes in the microscopic features of the tissues.

2.5.2. Sub-acute Toxicity Study. This study was also performed as per OECD guideline no. 407. Three groups of Swiss albino mice of either sex were used in the study. The animals of group 1 (control) received 1 ml of normal saline (vehicle) for 28 days and group 2 (test) and 3 (standard) received the CMS and AMS, respectively, at a dose of 1000 mg/kg per oral, respectively, once daily for 28 days. During this study, the mice of all groups were observed for behavioral changes. Afterward, the animals were fasted for 12 h and anaesthetized with ether. The blood was collected from the tail vein to determine the enzymological and haematological parameters. The histopathological changes were studied in the experimental animals similar to the acute toxicity study.

2.6. Statistical Analysis. During the study, the sample analysis was performed in triplicate and the values were expressed as means ± S.D. The Excel 2016 (Microsoft, U.S.A.) was used for statistical analysis, and significant differences among means were estimated at a probability level (p) of 0.05 using One-way ANOVA and student's t-test.

3. RESULTS AND DISCUSSION

3.1. Starch Isolation by Alkaline-Steeping Method. The alkaline steeping method and acid isolation method are frequently used for starch extraction. In mandua starch extracted by the alkaline steeping method, the amylose content was found to be 35.46 ± 0.42% w/v, as shown in Table 1. According to the amylose content, the starches have been classified in three categories: normal starch (18−30% amylose), waxy starch (about 1% amylose), and high amylose starch (about 70% amylose).[30] In accordance to this classification, the extracted mandua starch may be categorized as regular starch. The alkali used in the starch extraction process softened the starch-protein matrix and due to steeping, continuous successive treatments with alkali, the adhered proteins were separated. The results of mandua starch extracted by alkali treatment are coherent with the study in which the corn starch was extracted by the alkaline steeping method with high amylose content (30.81 ± 0.28%) with low fat and proteins.[30] The starch extraction up to 95% from broken rice has been carried out at pH 9.5, 40 °C by adopting the alkaline steeping method.[31] Hence, the starch extraction from mandua grains was conducted at optimized pH 10 for maximum yield. In the applied alkaline steeping method, maximum protein content from mandua starch was washed out at alkaline pH. The schematic presentation of starch isolation by the alkaline steeping method from mandua grains is shown in Figure 1.

Table 1. Physico-chemical Properties of Chemically Modified Mandua Starch (CMS, Phosphorylated) and AMS

S. no	property	AMS (mean ± SD)[a]	CMS (mean ± SD)
1	yield (% w/w)	35.74 ± 1.88a^a	96.2 ± 0.854ab^a
2	total ash (db**, %w/w)	0.185 ± 0.018b^a	0.0792 ± 0.0076bc^a
3	pH of hydro-dispersion	11 ± 1.0c^a	6.5 ± 0.05cb^a
4	phosphorous content (% w/w)	0.018 ± 0.00074d^a	0.1025 ± 0.0027da^a
5	paste clarity (%T)	6.566 ± 0.321e^a	0.604 ± 0.0089ea^a
6	swelling power (% w/w) in distilled water	226.51 ± 2.174 f^a	155.106 ± 1.998fa^a
7	moisture content (%w/w)	16.767 ± 0.642g^a	10.54 ± 0.670ga^a
8	degree of substitution		0.00491
9	water binding capacity (%w/w)	1.080 ± 0.011h^a	0.962 ± 0.014ha^a
10	oil absorption capacity (%w/w)	0.797 ± 0.051i^a	1.375 ± 0.053ia^a
11	amylose content (% w/w)	35.46 ± 0.423j^a	32.706 ± 0.892ja^a

[a]SD: standard deviation of three successive determination, mean: average value of three determinations (n = 3), db**: dry basis, Different letters in groups indicated the significant difference $p < 0.05$, two tailed t-test (a* & ab*, $p < 0.05$, $p = 0.013$; b* & bc*, $p < 0.05$, $p = 0.011$; c* & cb*, $p < 0.05$, $p = 0.011$; d* & da*, $p < 0.05$, $p = 0.014$; e* & ea*, $p < 0.05$, $p = 0.013$; f* & fa*, $p < 0.05$, $p = 0.016$; g* & ga*, $p < 0.05$, $p = 0.014$; h* & ha*, $p < 0.05$, $p = 0.015$; i & ia*, $p < 0.05$, $p = 0.012$; j & ja*, $p < 0.05$, $p = 0.015$).

3.2. Amylose Content Estimation. The amylose content in alkali extracted mandua starch was found to be 35.46 ± 0.42%, and in phosphorylated starch, it was found to be 32.70 ± 0.89% ($p < 0.05$, $p = 0.015$). The results are shown in Table 1. During the phosphorylation process, the leaching of amylose from mandua starch chains could occur at alkaline pH and it could lead to a lowering of the amylose content.

Besides this, the lower content of amylose after phosphorylation could be due to attachments of phosphate groups in intermolecular bonding of amylose−amylose and amylose-amylopectin. The decrement in the amylose content has been reported in phosphorylated rice starch.[3] The interactions of phosphate groups of STPP/STMP with starch granules could result into formation of di-starch monophosphate and starch di-phosphate during esterification in alkaline pH, and it could

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facilitate the leaching of amylose causing a decrement in total amylose content in cross-linked phosphorylated starch.[3]

3.3. Phosphorylation of Mandua Starch and Estimation of the Degree of Substitution. In cross-linking of mandua starch, the amount of phosphorylating agent (STPP and STMP) and other experimental conditions were screened and maintained during the cross-linking process. As the recommended amount of phosphorus in the form of residual phosphorus in consumable substances is <0.4%, the process parameters and the number of reagents were adopted in accordance to maintain this level of phosphorus in cross-linked mandua starch.

The phosphorylation process of extracted mandua starch was optimized for maximum degree of cross-linking, and it is related to maximum degree of phosphorylation carried out by sodium tripolyphosphate and sodium trimetaphosphate. During optimization of the cross-linking phosphorylation process, the process variables were pH of the reaction mixture, process temperature (°C), and STPP/STMP ratio. The pH varied from 9.5−12, temperature ranged from 45−70 °C, and the ratio of STPP/STMP ranged from 12:08 to 19:01. The degree of cross-linking in different formulations varied from 39.44 to 51.71%. The process variables of different preparations for phosphorylation have been shown in Table 2. The responses of all formulations in terms of degree of cross-linking have been shown in Figure 2. The best optimization conditions for maximum degree of cross-linking were as follows: temperature 70 °C, pH 9.5, and STPP/STMP ratio as 19:01 (w/w). In all sets of phosphorylation exterminations, the reaction time was 3 h with sodium sulfate at 10% (starch basis).

Table 2. Process Variables for Phosphorylation of Alkali Extracted Mandua Starch

	variables			process variables for phosphorylation
F. code	pH	temp (°C)	STPP/STMP ratio	degree of cross-linking
1	9.5	70	19:01	51.71
2	12	70	19:01	50.69
3	9.5	70	12:08	45.24
4	12	70	12:08	42.73
5	9.5	45	12:08	46.29
6	12	45	19:01	40.55
7	9.5	45	19:01	39.44
8	12	45	12:08	45.35

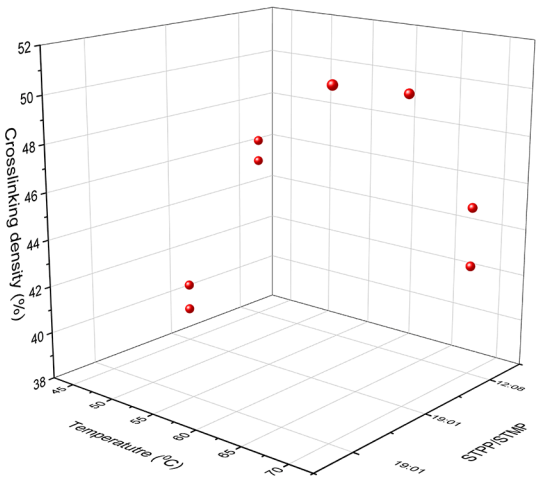

Figure 2. Effect of process variables on degree of cross-linking (cross-linking density %) of phosphorylated mandua starch.

In the phosphorylation process with STPP/STMP, sodium sulfate was applied to prevent the gelatinization of starch granules and to accelerate the phosphorylation reaction. In the modification of mandua starch by phosphorylation, sodium sulfate at 10% (starch basis) was applied and the process was continued for 3 h at alkaline pH. The maintenance of alkalinity of the reaction mixture during phosphorylation was also facilitated by sodium sulfate. The added amount of sodium hydroxide remained in the form of hydroxide and sodium ions, and these were incorporated in re-constituted starch granules. The concentration of STPP/STMP governed the rate of starch phosphorylation, and comparatively higher concentration accelerated the process. The best-optimized conditions of wheat starch have been reported for developing cross-linked RS4 starches efficiently as follows: an alkaline (sodium hydroxide) pH 11−12, a temperature of 35−45 °C, 5−20% (sb) sodium sulfate or chloride, and 5−19% (sb) STMP/STPP (99:1, w/w).[5,32]

In starch, the internal phosphorylation can lead to three positions of a glucosyl residue and it can be at C-6, C-3, and C-2. In potato tuber starch, approximately 70−80 and 20−30% of the phosphate is bound to the C6- and C3-positions, respectively.[33,34] Conversely, 3-O-phosphate groups destabilize the conformational equilibrium of α (1 → 4) linkages, likely promoting amylopectin amorphization.[34,35] The di-phosphate starch formation may be followed without adding sodium sulfate/sodium chloride just by reducing the pH and temperature to <50 °C.[5] In the present process, the substitution in mandua starch by phosphate was conducted by maintaining alkaline pH for 3h.

It has been found that the phosphorous content was comparatively higher in optimized formulation in comparison to other formulations. The total phosphorous content of AMS and CMS are shown in Table 1. The alkali isolated mandua starch contained 0.018 ± 0.00074% w/w of phosphorus. However, the amount of phosphorus in modified starch was found to be 0.1025 ± 0.0027% w/w ($p < 0.05$). Increment in the total phosphorus content in cross-linked starch could be due to phosphorylation process, and the excess phosphorus might be responsible for formation of cross-linked starch phosphate. The phosphorylation would occur at the most prominent positions as C-6 and C-3 in D-glucosyl unit to form the di-starch phosphate. Muhrbeck and Tellier[36] measured the ^{31}P nuclear magnetic resonance spectra of eight samples of potato starch dissolved in methyl sulfoxide and inferred that as the total-P level was increased from 0.0153 to 0.0221% of amylopectin, the level of 3 P remained relatively constant (from 0.0044 to 0.0063%). Whereas, the 6 P level was increased (from 0.0 104 to 0.0 165%).

3.4. WBC and Oil Absorption Capacity. The WBC of AMS and CMS are depicted in Table 1. The results reveal that chemical cross-linking of starch decreased water holding capacity of starch. It has been found that enzymatic modification of finger millet starch decreased the water-binding capacity.[37] The water-binding capacity of cross-linked mandua starch was significantly ($p < 0.05$) less than that of alkali extracted mandua starch. In a previously reported study,

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the water-binding capacity of cross-linked starches prepared by applying 2 g/kg. $POCl_3$ has also been found considerably lower than their native counterparts.[38] The cross-linking of starch with STPP/STMP imparted the tight junctions in starch chains, and these would be impervious to water resulting in comparatively low water holding capacity. At lower level cross-linking, an increment in swelling and solubility is observed due to porous structure formation. However, at higher level cross-linking, the impervious structure is formed resulting in decreased swelling and solubility.[23] Moreover, the lower water holding capacity of phosphorylated starch than alkali extracted starch indicated the formation of tight junctions and consequently, a high degree of cross-linking. The use of sodium sulfate may hinder the gelatinization process and may cause the lower water holding capacity. The decrement in swelling and solubility has also been reported for chemically cross-linked rice and corn starches.[3]

The oil absorption capacity of alkali extracted mandua starch was found to be 0.797 ± 0.051%, and in phosphorylated mandua starch, it was found 1.375 ± 0.053% ($p < 0.05$), as shown in Table 1. The absorption of oil in starch granules may be due to entrapment of oil in the helical structure of starch molecules for developing starch−lipid complex. The attachment of phosphate groups may create the stearic effect in starch chains in granules, and it may create more space for entry of more oil droplets through fine capillaries and consequently resulting in more intake capacity of phosphorylated starch for oil. These results were similar to the previously reported studies for phosphorylated rice starch in which the oil absorption capacity was more than native starch.[3]

3.5. Swelling Power. The swelling power and solubility of AMS and CMS were calculated in phosphate buffer pH 6.8 and in double distilled water at 37 °C. The significant decrement ($p < 0.05$) in solubility by cross-linking may be attributed due to increased cross-linking density in starch structure.[39] Swelling power of AMS was 226.51 ± 2.17%, while for CMS, it was 155.10 ± 1.9%. The results are shown in Table 1. Similar trends of decrement in swelling power of cross-linked starch were observed in phosphate buffer pH 6.8. The lower level of swelling of CMS in pH 6.8 as compared to AMS might be responsible for better digestion resistibility. The phosphorylation of mandua starch may facilitate the bond formation between amylose and amylopectin. The decrement in solubility and swelling power has also been observed in corn starch and rice starch after the cross-linking process.[40−42]

3.6. Paste Clarity. The paste clarity of unmodified starch (AMS) was exhibited as 6.566 ± 0.321% *T*, as shown in Table 1. However, the use of STPP/STMP as cross-linking reagents in phosphorylation of mandua starch leads to a substantial and significant ($p < 0.05$) decrement in the paste clarity. Mandua starch cross-linked with STPP/STMP showed 0.6% *T* past clarity. The paste clarity is generally a measure of starch granules swelling and is expressed by light transmittance.[43] These results of paste clarity of mandua starch were similar to the other reported studies[26,38,44] in which the cross-linked starches reflected lower paste clarity than their respective native starches. Generally, the phosphorylation process by STMP and STPP in neutral or acidic conditions increases the light transmittance of starch paste. In this process, the attachment of phosphate groups occurs on the starch structure and it creates the repulsion between adjacent starch molecules. The hydration of starch molecules also occurs as the increment in inter-chain repulsion facilitates the entry of water molecules. The gradual increment in highly swollen starch molecules after phosphorylation improves the light transmittance. However, the light transmittance of phosphorylated starch was decreased down with increment in pH. The decrement in paste clarity was also observed after phosphorylation of mandua starch. At alkaline pH, the paste clarity was dropped down from 6.5 to 0.6% light transmittance (%*T*). In phosphorylation of starch with STMP, the change in paste clarity starts to accelerate at a pH greater than 8. As the cross-linking is directly linked with paste clarity, the decrement in paste clarity above pH 8 indicates the initiation of effective cross-linking. In phosphorylation of sago starch with STPP alone carried out under the influence of pH from 6 to 9, a slight decrement in clarity of starch paste has been observed, and at a pH of 9−11 of the reaction, the paste clarity has been decreased down by 14−17% *T* respectively. However, in modified sago starch developed by maintaining the pH above 8 of the phosphorylation process, the paste clarity was decreased down drastically from 60 to 3% *T*. On applying a mixture of STPP and STMP for phosphorylation of sago starch in the pH range of 6−10, the paste clarity was decreased down drastically from 85% *T* to 6.5% *T*.[44]

3.7. Moisture and Ash Content. The process adopted for starch preparation and the extent of drying generally affect the moisture content. The ash content of AMS and CMS was observed lower than 1% w/w, and it is consistent to the previous studies conducted for the analysis of millet starch and other cereal starches.[45] Ash and moisture content of AMS and CMS are represented in Table 1. The presence of alkali allows the penetration of sodium ions into the starch structure, and in ash content determination, these remain in the form of ash as residue. In the cross-linked starch sample, the ash content was comparatively lower due to the removal of sodium ion which were available in alkali isolated starch. Comparatively, more ash value of corn starch extracted by the alkaline steeping method has been reported in a comparative assessment of alkali and acid extraction processes.[30]

3.8. In vitro Digestion Resistibility in the Upper GIT. The results of in vitro digestion resistibility of AMS and CMS are depicted in Figure 3. On the basis of starch digestion rate in stomach and small intestine, starch can be divided into rapidly digestible, slowly digestible (digested within 120 min in small intestine), and resistant starch. It has been found that

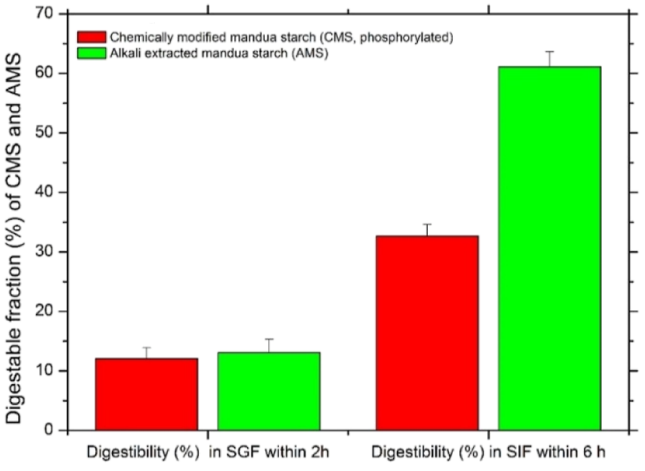

Figure 3. Digestibility of AMS and CMS in SGF (pH 1.2) and SIF (pH 6.8).

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alkali isolated mandua starch contains slightly higher fraction of slowly digestible starch. The results indicate pronounced decrement in digestibility, that is, an increase in digestion resistibility.

The improvement in digestion resistibility may be due to the increased resistant starch (RS, Type RS4) content in CMS, and it may be possible due to the higher degree of cross-linking. Comparatively lower swelling of modified mandua starch may be resulted due to significant increment in degree of cross-linking. The increment up to 10 times in resistant starch has been observed by phosphorylation of sago starch by the mixture of STMP/STPP.[44]

The entry of SGF and SIF through channels and pores in mandua starch granules may be inhibited after phosphorylation. The phosphorylation process creates the attachments of phosphate groups on starch chains, and these linkages obstruct the formation of the starch−enzyme complex. The close attachments of starch chains linked with phosphate groups may reduce the flexibility of starch chains, and this phenomenon may decrease the water retention capacity of phosphorylated starch. The steric hindrance created by phosphate groups in cross-linked starch chains may also improve the resistibility toward digestion by enzymes and consequently results in increment in resistant starch. The increment in RS content with a decrease in digestibility of STPP/STMP cross-linked potato starch has also been studied.[46] The cross-linking causes hindrance for penetration of enzyme inside cereal starch granules leading to decreased enzymatic digestibility.[47] The finger millet starch (VL Mandua-352) has been modified with acetic anhydride and studied that digestibility of native starch and chemically modified starches were 57.32 and 36.02%, respectively.[48] Figure 4 represents the

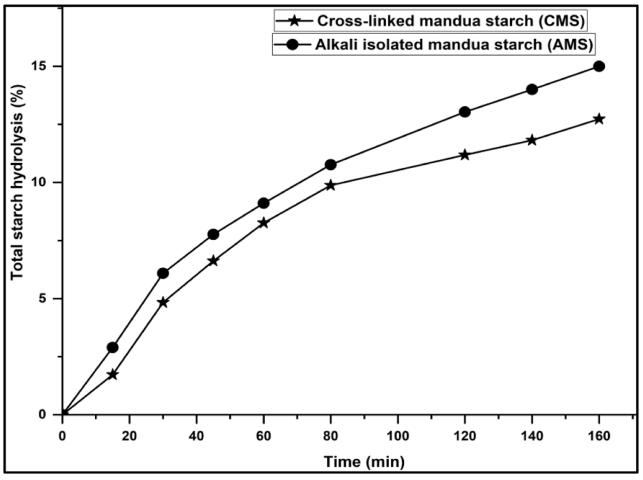

Figure 4. Starch hydrolysis patterns of the CMS and AMS.

hydrolysis patterns of the cross-linked mandua starch and alkali isolated mandua starch. It has been clear from the hydrolysis curve that the alkali isolated starch is much susceptible to the enzymatic attack and much rapidly hydrolyzed than the cross-linked starch. The glycemic index of the starch is directly related to the hydrolysis index. The results of the starch hydrolysis indicated that the glycemic index was significantly changed after cross-linking of starch. The glycemic index was decreased for CMS. The findings of the starch hydrolysis and glycemic index supported the increment in enzymatic resistant fraction that is decrement in rapidly digestible starch fraction in CMS.

3.9. FTIR Characterization. The FTIR spectra of AMS and CMS are shown in Figure 5. The deconvoluted FTIR spectra of native and cross-linked starches are presented in Figure 6. The appearance of an extremely broad band at 3401 cm^{-1} was observed reflecting the presence of stretching vibration of O−H. The interlinking of hydroxyl groups with hydrogen bonds may be responsible for this broadening of the IR band.

The stretching of C−OH was observed at 1020 cm^{-1} which may be due to the amorphous component present in amylose form. The vibrations of O−H were also observed in the form of IR band at 1273 cm^{-1}. The alkali isolated and phosphorylated cross-linked starches reflected the peaks at 3436 cm^{-1} and 3401^{-1} which revealed the vibrations of −OH deformation. The presence of IR bands at 2926 and 2930 cm^{-1} ascribed to C−H bond stretching. In the spectrogram, the symmetrical deformation vibration of CH_3 was observed at 1368 cm^{-1}, and its appearance reflected the presence of water adsorbed in amorphous regions of mandua starch.[45] The appearance of bands at 1155, 1131, and 1020 cm^{-1} may be due to C−O stretching of α-1, 4 glycosidic linkages of the starch skeleton structure. The changes in wavenumbers and positions have been observed in IR spectrograms of chemically modified starch forms. The infrared spectrograms of modified starches prepared by malonic, glutaric, and valeric acid treatments have revealed a slight widening of the band at 1650 cm^{-1} indicating the involvement of the carbonyl group in chemical modifications.[49]

The spectrograms of alkali extracted mandua starch and phosphorylated starch indicated that vibration bands of C−H, O−H, and C−O in native and phosphorylated mandua starch were present without distinctive differences. The cross-linking of starch with STPP/STMP actively involves the −OH groups and creates new bonds with the attachment of phosphate groups. The reduction in broadening of −OH stretching band at 3401 cm^{-1} and presence of peak of P═O bonds at 1244−1266 cm^{-1} affirm that mandua starch reacted efficiently with a mixture of STPP/STMP during the phosphorylation process. In phosphorylated *Canna edulis* starch, the presence of band near 1400 cm^{-1} in the FT-IR absorption spectrum has ascertained the presence of P═O stretching vibration.[23,50] Also, a very strong absorption spectrum has also been observed at about 1000 cm^{-1} that indicated the presence of C_6P−OH. In the FTIR spectrum of phosphorylated mandua starch, the presence of prominent bands at around 1400 and 1000 cm^{-1} has been observed that indicated the effective phosphorylation of alkali extracted mandua starch.

3.9.1. Effect of Modification on Short Range Molecular Characteristics. The FTIR spectrum of the starch granules has shown to be sensitive to alterations in the double helical structure, the crystal form of the starch, and the chain conformation. The deconvoluted FTIR spectra in the range of 1200−800 cm^{-1} of alkali isolated mandua starch and modified mandua starches are shown in Figure 6. The absorbance ratios at 1047/1022, 1047/1035, and 1022/995 were used to measure any change in the ordered starch to amorphous starch, the amount of ordered starch, and to assess the state of organization of the double helices inside crystallite. The absorbance ratio 1047/1022 for the AMS and CMS were 0.9147 and 0.8738, respectively. The ratio 1047/1035 had a similar trend (0.9798 for AMS, 0.9544 for CMS) as that of

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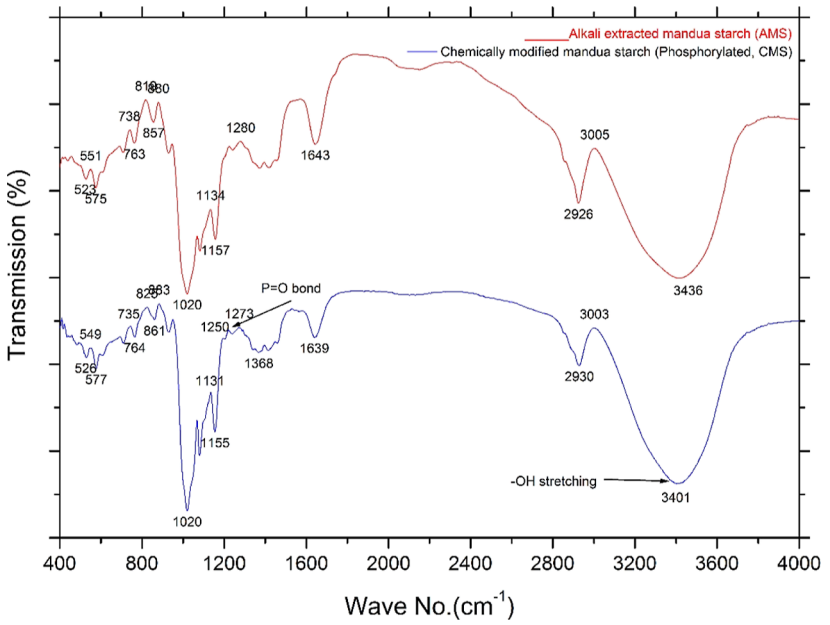

Figure 5. FTIR analysis of AMS and CMS.

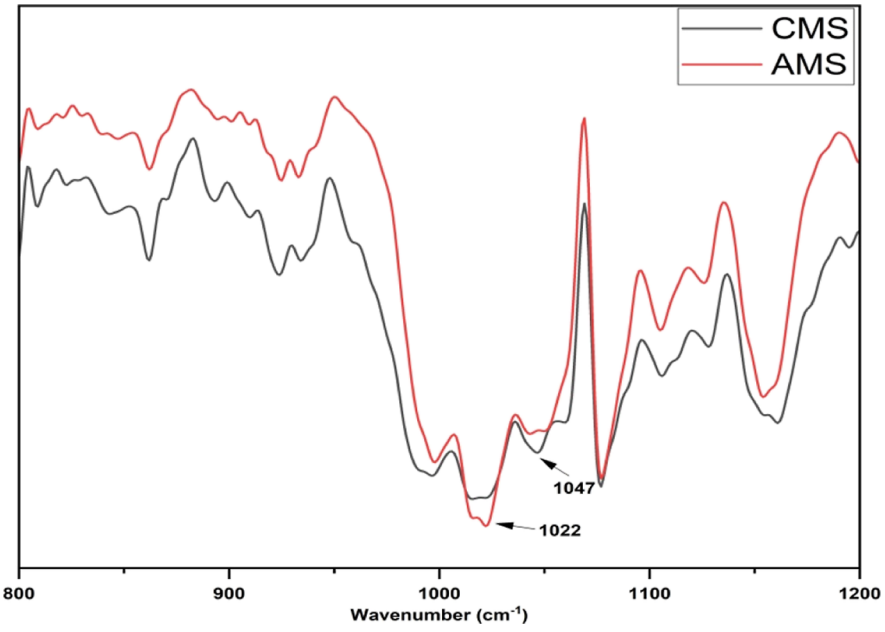

Figure 6. Deconvoluted FTIR spectrum in the range of 1200−800 cm^{-1} of AMS and CMS.

1047/1022, suggesting that cross-linked mandua starch represents a comparatively less amount of short-range order than alkali isolated mandua starch. Regarding the IR ratio 1022/995, a reverse trend was noticed. The increment in ratio for cross-linked starch confirmed that cross-linking of starch with STMP/STPP decreases the degree of order of starch granules. The findings of the present study were in agreement with the earlier reported studies.

3.10. Thermal Gravimetric Analysis and Differential Scanning Calorimetry. The attachment of phosphate groups on mandua starch chains may change the integrated structure and cause the formation of new bonds. The formation of phosphate linkages results in an enhancement in cross-linking density in treated mandua starch. The behavioral changes of chemically modified starch impacted by cross-linking treatment were studied by thermal gravimetric analysis and are shown in Figure 7.

In the decomposition pattern of AMS and phosphorylated mandua starch (CMS), three distinct stages of decomposition were observed. In the initial stage, the decomposition started from 44.5 to 88.97 °C with a weight loss of about 8.5%. During this phase; desorption and evaporation of the water may occur as the moisture may be present in the form of bond and unbond form. A similar pattern of decomposition was followed by chemically modified form (CMS) of mandua starch in which the evaporation of lodged moisture occurred from 36.17 to 51.44 °C with a loss of 13.807%. The removal of water from all surfaces of samples may lead to developing the porous structure of starch. When the heating temperature was gradually increased with a controlled rate of heating, the

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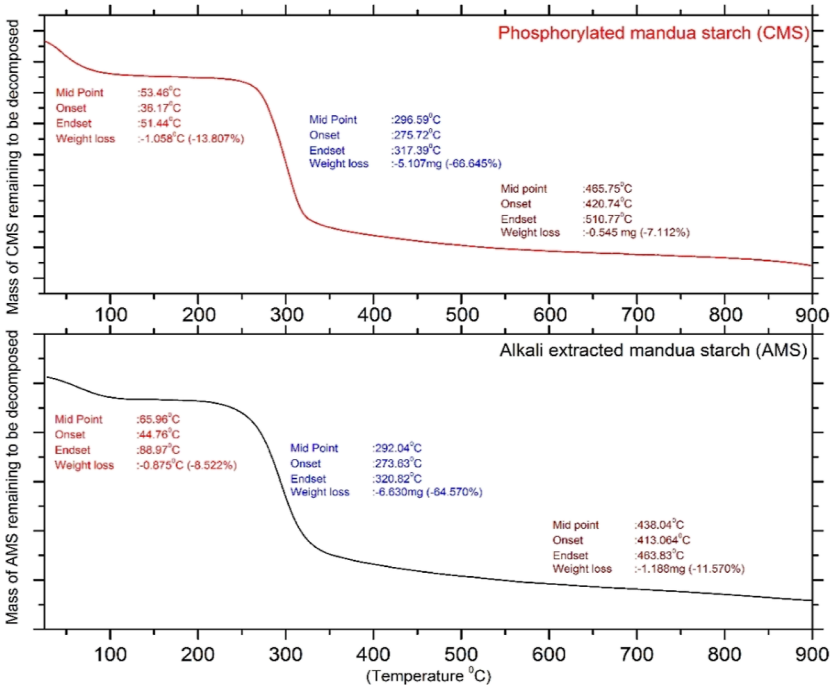

Figure 7. Thermogravimetric analysis of AMS and phosphorylated mandua starch (CMS).

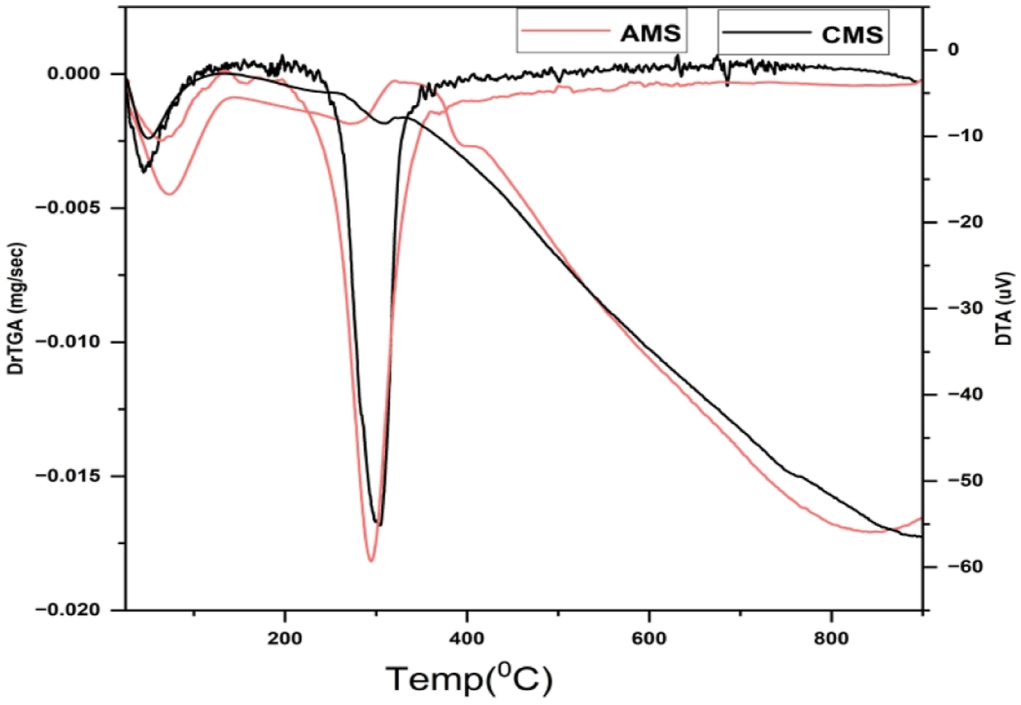

Figure 8. DTA and DrTGA of AMS and phosphorylated mandua starch (CMS).

prominent decomposition in both AMS and CMS was observed. The second phase of decomposition was observed from 273.63 to 320.82 °C with a major weight loss (64.57%). The major decomposition has occurred during this phase in which all porous starch samples were decomposed due to breaking of the starch structure. The mid-point of the second phase decomposition in AMS was observed at 292.04 °C. The peak of the second decomposition was observed at 296.59 °C. 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 like CO, CO_2, and H_2O. The change in the decomposition pattern of chemically modified mandua starch with the significantly higher requirement of thermal energy for decomposition was considered due to the formation of tight junctions in phosphorylated starch by phosphate groups of cross-linking agents, and these might be responsible for the formation of the tight, compact structure of starch as well as thermal stability toward decomposition.

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The third stage decomposition of AMS was observed in the range of 413 to 463.8 °C, and in chemically modified form (CMS), this phase was observed from 420.74 to 510.77 °C with a weight loss of 7.112%. In this phase, the weight loss was less significant ($p < 0.05$) than phase two and this phase consumed more energy and occurred at a higher temperature in chemically modified starch. It may be due to the major decomposition of AMS and CMS in the second phase. In the third phase, the pyrolysis process may occur predominantly and result in the generation of polynuclear aromatic and graphitic carbon structures. The weight loss of CMS was lower in this phase than AMS. It reveals the formation of significant linkages in alkylated manuda starch during the phosphorylation process, and it required more energy for decomposition. It was also observed in the second phase, where the decomposition peak was observed at a comparatively higher temperature in CMS. The results of TGA analysis inferred that the formation of the compact structure would occur after cross-linking by STPP/STMP, and it may lead to resistance for decomposition. It also restricts the movement of cross-linked chains leading to thermal stability. In a thermal analysis of porous wheat starch, the maximum decomposition has also been studied in the second phase of TGA analysis and after cross-linking with sodium trimetaphosphate, the decomposition pattern was changed significantly than native starch.[51] The differential thermal analysis (DTA) curves of AMS and CMS showed better thermal stability of CMS than AMS (Figure 8). It has been clear from the DTG and DTA thermograms of CMS and AMS that modification significantly impacted the thermal stability of the mandua starch. The first derivative thermograms of both the starches showed similar phase of degradation (two phase degradation). However, the change in peak intensity as well as shifting of the peak to higher temperature for cross-linked starch in the range of 200−400 °C showed that the thermal stability of CMS was higher than AMS.

The thermal behavior of AMS and CMS samples in DSC has been depicted in Figure 9. The thermal transition range for AMS was observed from 51.26 to 110.57 °C, and the gelatinization enthalpy (ΔH_{gel}) for this thermal transition was found at 284.56 J/g. The peak for the thermal transition (Tp) was revealed at 79.97 °C. However, in chemically modified mandua starch (CMS), the results for the above parameters were different significantly ($p < 0.05$). The enthalpy for thermal transition for CMS was higher than AMS. The gelatinization enthalpy for CMS was 388.73 J/g, and the thermal transition continued in the range of 54.45 to 139.39 °C. The peak for thermal transition (Tp) was found at 96.48 °C. These results represent the better thermal stability of phosphorylated mandua starch than alkali extracted mandua starch. The effective phosphorylation of starch with a mixture of STPP/STMP may increase the molecular mass and improve the chemical bonding strength occurring in the starch molecular structure during phosphorylation. These tight junctions require comparatively more energy for decomposition, and consequently, phase transition temperature (Tp) is also increased after cross-linking. The significant improvement in transition temperatures of modified starch has been observed on increasing the degree of cross-linking by the phosphorylation process carried out by applying the STMP/STPP mixture.[52,53] The similar pattern of changes in the thermal behavior of rice starch after phosphorylation has also been reported.[3]

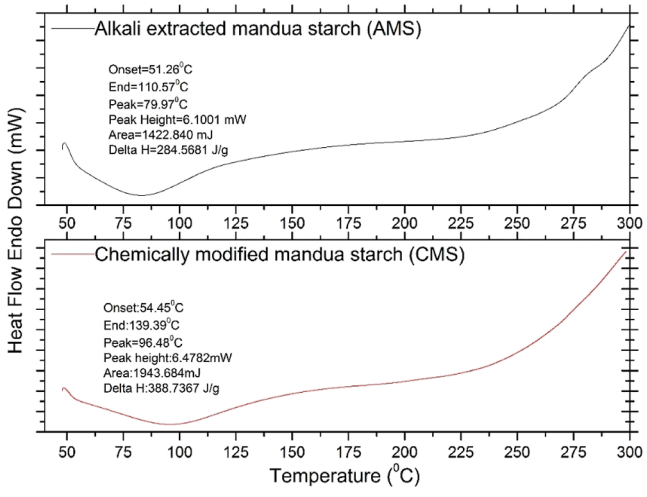

Figure 9. DSC thermograms of AMS and phosphorylated mandua starch (CMS).

3.11. Powder-X Ray Diffractometry. According to XRD patterns, the native starches procured from different plant sources can be categorized into three groups: A-, B-, and C-type. C-type starches have both A- and B-type crystallinity, and these can be divided further into three types: CA- (closer to A-type), CC- (typical C-type), and CB-type (closer to B type). These combinations of C-type starches with A- and B-type are classified according to the proportion of A- and B-type crystallinity from higher to lower, respectively. The strong diffraction peaks at about 17 and 23° 2θ and small peaks at 5.6 and 15° 2θ are present in the X-ray diffractogram of CC-type starch. In CB-type starches, the presence of a peak at 5.6° 2θ is the characteristic of B-type crystallinity. The characteristic peak of A-type crystallinity is presented at about 18° 2θ in the form of a shoulder peak in CA-type starch.[26] The same starch particle may have both type of crystallinity (A- and B-type), and it depends upon various factors, for example, cultivation, storage, processing, etc. Seven cultivars of sweet potato (Shinyulmi, Sinjami, Hogammi, Jeonmi, Jinyulmi, Juhwangmi, and Pungwonmi) grown at the National Institute of Crop Science, Rural Development Administration, Muan, Korea, have shown a C_A XRD pattern.[54] However, in another study, some white and purple sweet potatoes had shown type C_B starches.[55] Genkina et al.[56] reported that orange sweet potato has a type A starch when grown in soil at 33 °C and a type CC-type starch when grown in soil at 15 °C, indicating that the growing temperature has a significant effect on crystalline structure. Generally, cereal starches generate A-type diffraction patterns; tubers and high amylose starches generate B-type patterns; and legume, roots, some fruits, and stem starches show C-type patterns (a mixture of A- and B-type).[57] Various treatments can also induce A-type, B-Type, and both types of crystallinity in starch. Acid modified starch of lotus rhizome has indicated B-type allomorph mainly arranged in the distal region of eccentric hilum. A-type allomorph was present mainly in periphery of hilum, and the center of the granule was a mixed distribution of A- and B-type allomorphs.[58]

In the diffractogram of phosphorylated mandua starch, the prominent peaks were observed at 14.85, 17.92, 18.20, 19.91, 22.97, and 29.52° 2θ. The presence of 17.92 and 18.20° peaks at about 18° 2θ in starch revealed the presence of CA-type crystallinity of starch. Here, it can be speculated that the attachment of phosphate groups may occur in amorphous

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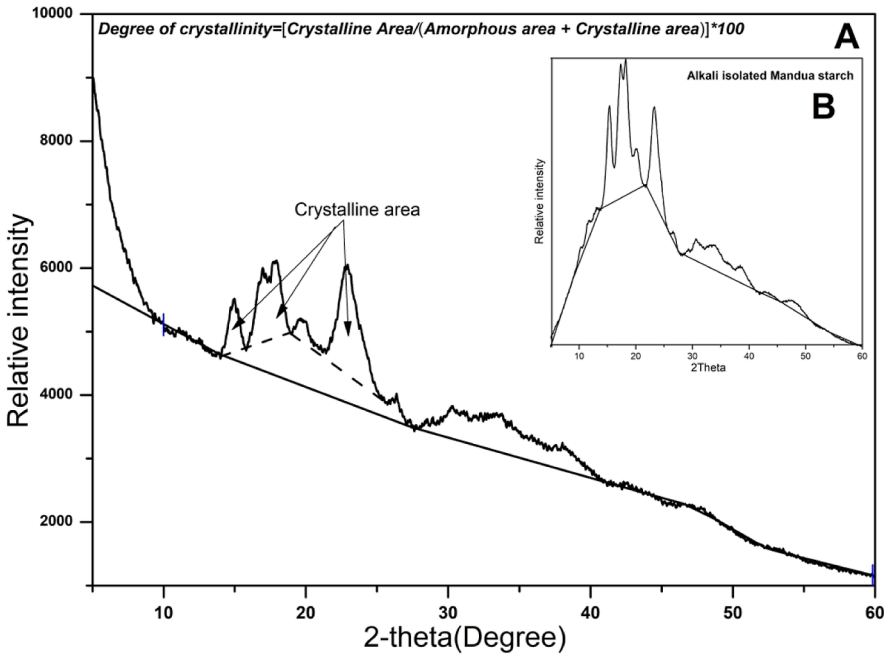

Figure 10. Powder X-ray diffractogram of cross-linked mandua starch (A) and alkali isolated mandua starch (B).

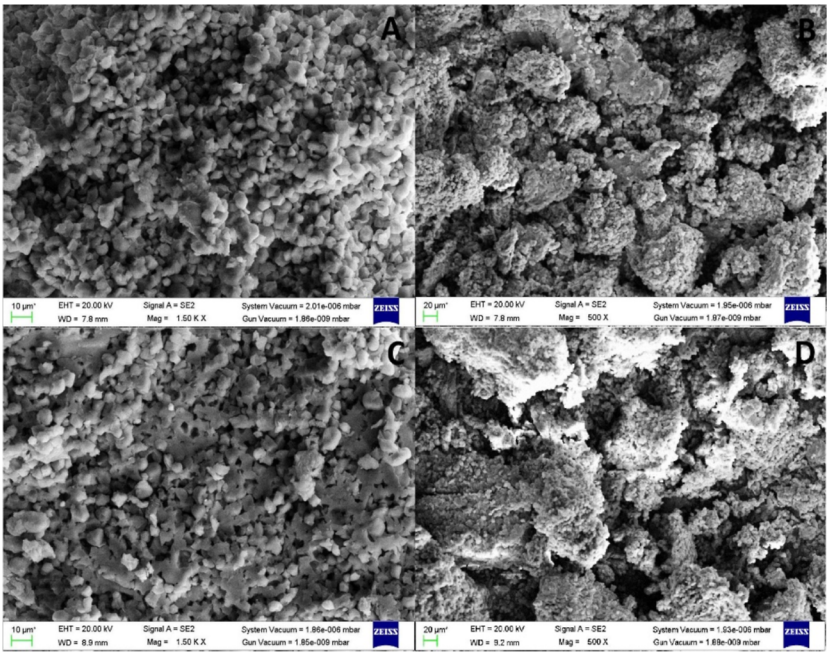

Figure 11. SEM photomicrographs of alkali extracted mandua starch (A,B) and chemically modified mandua starch (C,D).

regions of mandua starch granules resulting in a change of peaks patterns of X-ray diffraction. The presence of specific peaks also reveals the amorphization process undergone in mandua starch, and it may be associated with the inclusion of new groups at random positions along with starch chains. The attachment of new functional groups and changes in the granular starch skeleton may result in random spatial distribution. All of these structural rearrangements occurred due to the phosphorylation process, and the partial gelation might have occurred in alkaline pH. Any change in the crystalline pattern remarkably alters the content of resistant starch via alteration of the interaction among starch granules with the enzyme. It was observed that the degree of crystallinity was 43% for AMS, and it was decreased up to 31% after cross-linking in CMS. The decrement in crystallinity of native mandua starch by the phosphorylation process may result due to destruction of the crystalline structure of starch granules.

The presence of new peaks at regions 27.22, 29.52, and 30.22° 2θ were related to the changes in three-dimensional arrangements of the chains, and it could be speculated due to the formation of di-starch monophosphate. Muhrbeck and Tellier[36] also studied that the crystallinity and enthalpy of gelatinization were decreased with the level of 6-phosphorylation, but not significantly with the level of 3-phosphorylation in starch. The diffractogram of phosphorylated starch

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has been depicted in Figure 10. It has been reported that no significant changes in the XRD pattern of maize starch after cross-linking were observed.[40]

3.12. Morphological Characterization. Scanning electron microscopy was used to perceive the shape and surface characteristics of the starch, and the photomicrographs acquired at 500x and 1500x magnifications are represented in Figure 11. The surface morphology of native mandua starch indicated that the granular structure was irregular and polygonal in shape with a diameter between 5 and 15 mm. However, some of the granules revealed the fissures and smooth indentation. The irregular surface topography of native starch granules may be due to the effect of alkali used for the extraction of starch from mandua grains. After treating the starch by the phosphorylation process, the shape and surface appearance of CMS remained intact. The surface morphology of phosphorylated mandua starch was coherent to the results of starch of *C. edulis* in which the phosphorylation process did not disrupt the starch structure and the degree of crystallinity of *C. edulis* starch phosphate monoester did not nearly change.[50] Similarly, the native starches of rice and oat have not revealed any major differences in the morphological structure after cross-linking processes.[42]

3.13. Toxicity Studies of Alkali Extracted and Phosphorylated Starch. *3.13.1. Behavioral Analysis.* In chemical derivatization of extracted starch of mandua by the phosphorylation process, different chemicals were applied and the residues of these reagents in modified starch may be toxic in consumption. The toxicity studies were conducted to characterize cross-linked mandua starch as "generally regarded as safe (GRAS)" excipient. Besides this, it becomes utmost important when the excipient is to be explored for applications in public health in different forms as exposure to the chemical substances may have deleterious impacts on consumption. The sign of changes in behavioral parameters of animals after consumption of the test substance is the indicator of toxicity.[6] When experimental animals consumed phosphorylated mandua starch at a dose level of 2000 mg/kg. body weight, no toxic symptoms were observed. The behavioral, neurological, and autonomic profiles of the animals were closely supervised continuously for 3 h and then every 30 min, the observation was continued for next 4 h, and finally, the mice were monitored after 24 h, 48 h, 7 days, and till 14 days for any cause of mortality. The animals of control, test, and standard groups were normal, and no major changes were perceived in observations of fur, eyes, mucous membrane, salivation, lethargy, sleep, coma, convulsions, tremors, diarrhoea, oral activity, abdominal, and external genitalia.

The body weight is also a significant parameter for dose calculation of the substances in pre-clinical and clinical studies.[9] In toxicity studies, the alterations in bodyweight gain reveal the toxicity of the substance, and it is more important when the loss in body weight is more than 10% of the initial body weight. In the toxicity studies of phosphorylated mandua starch, the changes in body weight of mice were not significant than control and standard after consumption of CMS at a dose of 2000 mg/kg bodyweight ($p > 0.05$) (The data of body weight has not been shown here). This observation of non-significant alterations in body weight of animals reflected that oral consumption of phosphorylated mandua starch had no sign of toxicity. Besides this, the weight of body organs such as liver and kidney was also measured, and no major changes in each organ weight were observed after consumption of phosphorylated mandua starch (CMS) and AMS ($p > 0.05$).

3.13.2. Histopathological Studies. The consumption of toxic substances affects the physiology of the vital organs, and it reflects in terms of changes in distorted structures. The autopsy of the animals was performed after the experimentation, and no apparent changes in the internal structure of the liver and kidney were observed in histopathological analysis after consumption of treated mandua starch. In photomicrograph of liver-histology, the hepatic parenchyma was in average shape, and no necrosis and hyperemia were observed in the test and control group of animals. The proximity of the centrilobular vein was distinct, and the vasculature to all the tissues was prominent and normal. No distortions in cellular architecture of the organs were observed, and binucleation was found normal. The cellular structure of the liver of test group animals consuming phosphorylated mandua starch was found normal, and no signs of injury and necrosis were found. The congestions, fatty acid accumulation, and hemorrhagic regions were not observed around the central vein or sinusoids of the liver. The hepatic cells in the cross-section study reflected no lyses in the blood cells. The infiltration of the neutrophil, lymphocyte, or macrophage in intercellular spaces and the peripheral area of the central vein was also absent.

The apparent changes in cellular architecture were not observed in histopathological studies of kidneys of mice treated with phosphorylated starch. The glomerular architecture of the treated animal groups was typical and similar to the control and standard groups. The appearance and cellular arrangements of glomeruli and parts of a typical renal filtration unit such as distal, proximal, and collecting tubules in the kidney were normal in animals of all the groups. Besides these, no signs of interstitial and intra-glomerular congestion or tubular atrophies were found in the renal cross-sectional study. The nephron cells were normal in appearance reflecting visible nucleoli. The signs of any infiltration with lymphocytes, degeneration, bleeding, and necrosis were absent in nephron cells. Moreover, the histopathological observations of liver and kidney did not reveal any morphological abnormalities after oral consumption of treated and native mandua starch, and it reveals the safety of phosphorylated mandua starch for oral administration. The photomicrographs of kidney and liver after toxicity studies of phosphorylated mandua starch (CMS) are shown in Figure 12.

3.13.3. Biochemical and Enzymological Studies. The metabolic and excretory functions in the body are performed mainly by liver and kidney, respectively. The metabolism, detoxification, storage, and excretion of xenobiotics and their metabolites are the functions executed by these vital organs, and sometimes, these external substances and their metabolites may cause injury to soft tissues and affect the functioning of these organs.[59] The liver consists of a versatile cellular structure and functioning with remarkable regeneration power. The rejuvenating system acts prominently for wear and tear of the hepatic cells. The intra-cellular enzymes are also released into intercellular spaces after rupturing the hepatic cell membranes and through hepatic vasculature; these enter into the central bloodstream. The enzymes such as aspartate aminotransferase or serum glutamic-oxaloacetic transaminase (AST or SGOT), alanine aminotransferase or serum glutamic pyruvic transaminase (ALT or SGPT), and alkaline phosphatase (ALP) are the critical factors of hepatic metabolic

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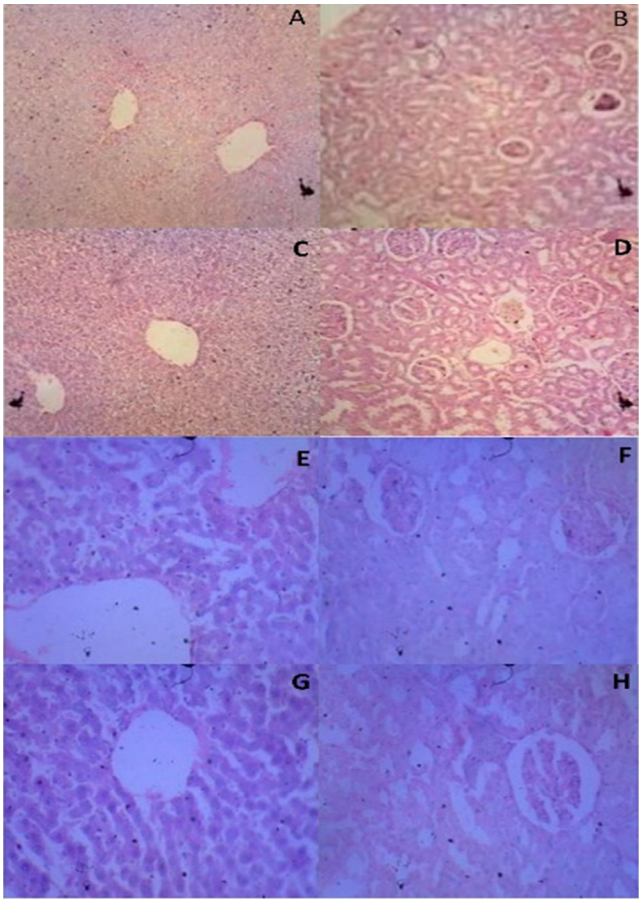

Figure 12. Photographs of liver in acute toxicity (A: AMS, C: CMS, 100×), kidney in acute toxicity (B: AMS, D: CMS, 100×), liver in sub-acute toxicity (E: AMS, G: CMS, 100×), and kidney in sub-acute toxicity studies (F: AMS, H: CMS, 100x).

pathways. The appearance of intracellular enzymes as AST and ALT is indicative of cellular damage.[60]

Consequently, the estimation of these above enzymes in serum can be used to assess any incidental damage of the organ and the fluctuations from the normal range of these enzymes are a universal marker for the detection of liver damage/non-functioning.[61] The findings of acute and sub-acute studies indicated non-significant differences in serum levels of AST and ALT amongst the animals of test, standard, and control groups. In acute and sub-acute toxicity studies, the histological sections of the liver of all mice indicated regular architectures without any lesion. The hepatic cells undergo regeneration after recovery from a disease, and it is rather difficult to differentiate between the cellular damage caused either by drugs or produced spontaneously by a disease. In histo-pathological cross-section study of the liver, no lesions of any injuries were present that indicated the safety of phosphorylated mandua starch.

Kidneys perform the three primary function such as elimination of toxic substances produced during metabolism, regulation of internal liquid medium hemostasis, and production of hormones.[60] In acute and chronic injuries of the kidney, the functioning of the renal system is imbalanced and the end products of nitrogen metabolism build up. The elevation of blood urea nitrogen and serum creatinine occurs due to the accumulation of non-protein nitrogen in the body.[60,62] Besides this, the end product of protein metabolism is urea and is formed from ammonia in the liver. Later on, urea is excreted from the body through the renal system. The alterations in the urea level in the blood from the normal range are indicative of renal functioning. The spontaneous non-enzymatic cleavage of phosphocreatine in the muscles generates creatinine as a byproduct, and the serum creatinine is excreted unchanged through the kidney. Changes in the creatinine level from the normal range are also applied as a marker for assessing the wellness of the renal system. In most of the cases, the creatinine is excreted mainly by filtration and plasma creatinine level is indicative for assessing the glomerular filtration rate. Hitherto, in toxicity studies of phosphorylated mandua starch, the evaluation of the renal functions was performed by assessing the urea and creatinine levels in blood

Table 3. Haematological and Enzymological Parameters after Acute and Sub-acute Toxicity Studies of AMS and Phosphorylated Mandua Starch (CMS)

		mean ± SD[a]				
		acute toxicity study (dose: 2000 mg/kg body wt of animal)		sub-acute toxicity study (dose: 1000 mg/kg body wt of animal)		
S. no	parameters	AMS[a]	CMS[b]	AMS[a]	CMS[b]	Control[a,b]
1	haemoglobin (g/dl)	14.45 ± 0.50	14.42 ± 0.12	14.18 ± 0.21	13.89 ± 0.25	13.50 ± 0.68
2	WBC ($\times 10^3$/mm^3)	8.11 ± 0.32	8.97 ± 0.28	8.81 ± 0.42	8.93 ± 0.34	8.89 ± 0.30
3	RBC ($\times 10^6$/mm^3)	8.08 ± 0.44	8.20 ± 0.14	8.39 ± 0.29	7.77 ± 0.24	7.61 ± 0.45
4	neutrophils ($\times 10^3$/mm^3)	2.60 ± 0.29	2.74 ± 0.14	2.66 ± 0.17	2.53 ± 0.13	2.56 ± 0.55
5	lymphocytes ($\times$ 10^3/mm^3)	6.83 ± 0.87	7.26 ± 0.65	7.49 ± 0.33	7.71 ± 0.39	7.70 ± 0.81
6	eosinophils ($\times 10^3$/mm^3)	0.05 ± 0.01	0.06 ± 0.00	0.05 ± 0.00	0.05 ± 0.00	0.05 ± 0.01
7	monocytes ($\times 10^3$/mm^3)	0.02 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.02 ± 0.00	0.023 ± 0.00
8	basophils ($\times 10^3$/mm^3)	0 ± 0.00	0 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.0 ± 0.00
9	SGOT (IU/L)	42.47 ± 2.13	41.60 ± 1.99	38.07 ± 1.80	36.16 ± 1.68	36.40 ± 1.21
10	SGPT (IU/L)	25.91 ± 3.74	27.70 ± 3.77	29.00 ± 0.99	31.58 ± 1.01	31.41 ± 1.08
11	serum creatinine (mg/dl)[&]	0.41 ± 0.06	0.41 ± 0.02	0.39 ± 0.01	0.38 ± 0.01	0.38 ± 0.01
12	serum urea (mg/dl)[$]	40.98 ± 2.90	37.10 ± 1.60	39.74 ± 0.98	39.97 ± 1.11	39.52 ± 0.58

[a]S.D.: standard deviation of successive determinations (n = 3); Mean: Average value of successive determinations (n = 3).Different letters in columns indicated significant difference ($p < 0.05$) (a and b, $p > 0.05$, $p = 1.00$, $f = 0.0023$; & $p > 0.05$, $p = 0.067$, $f = 3.097$; $ $p > 0.05$, $p = 0.225$, $f = 5.429$).

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plasma. The results of these biochemical parameters reflected that serum urea and creatinine levels were within the normal range during the due course of the study and no statistically significant difference were observed after oral consumption at the dose of 2000 mg/kg body weight in the acute toxicity study and oral administration of 1000 mg/kg body weight in the sub-acute toxicity study as compared to the results of the control group and standard group, respectively ($p > 0.05$). However, plasma creatinine concentration can be used as a sensitive indicator of renal dysfunction when the flow rate of this marker in glomerular filtrate has been dropped down below 50%.[60]

In haematological investigation, there were no significant differences in haemoglobin and white blood cells count, neutrophils percent, lymphocytes percent, monocytes percent, eosinophils percent, and basophils percent ($p > 0.05$) amongst animal groups of test, standard, and control groups. The results of the different parameters are shown in Table 3. A non-significant alteration has also been observed in the serum creatinine, urea, alanine aminotransferase, and aspartate aminotransferase of the test, standard, and control animal groups ($p > 0.05$). Non-significant changes in all biochemical, haematological, and histopathological studies after the oral consumption of phosphorylated mandua starch reflected the safety of phosphorylated mandua starch to be applied as the GRAS excipient in pharmaceutical and other formulations.

4. CONCLUSIONS

The results of the study revealed that the effective modification of alkali extracted mandua starch by the phosphorylation process improved the content of resistant starch significantly in phosphorylated starch. The phosphorus content in modified starch was found within the permissible limit of consumption. The results of the physicochemical properties of modified starch indicated the effective cross-linking of mandua starch by STPP/STMP in alkaline pH. The attachment of phosphate groups on starch chains was affirmed by FTIR study and the formation of monophosphate/diphosphate linkages with the presence of resistant starch was ascertained by powder X-ray diffractometry. This study confirmed the significance of short range molecular order (R1047/1022), crystallinity, and amylose content on tailoring the resistant starch fraction. The significant changes in the thermal behavior of chemically modified mandua starch also reinforced the effective cross-linking and formation of a stable integrated structure. The toxicity studies of modified mandua starch in terms of acute and sub-acute toxicities in animals indicated its safety for consumption and the phosphorylated mandua starch might be categorized as "Generally Regarded as Safe (GRAS)". The significant resistant ability of modified starch toward digestion in SGF and SIF may be useful similar to other forms of polysaccharides in developing targeted drug delivery system for the colon.[63,64] On applying conventional pharmaceutical excipients in such delivery devices, most of the incorporated active pharmaceutical ingredients are released in the stomach and small intestine due to disintegration followed by digestion of drug-carriers. Also, the modified mandua starch may be applied as a novel carrier for developing modified release drug delivery systems. Besides this, the improvement in resistant starch content in phosphorylated mandua starch with a low glycemic index may help in developing food and nutraceutical preparations.

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Author Contributions

M.K.M.: conceptualization, methodology, software, formal analysis, investigation, data curation, and writing—original draft. V.K.: conceptualization, methodology, resources, visualization, writing—review and editing, supervision, and project administration. J.S.: conceptualization, visualization, and supervision. S.K.: conceptualization and visualization. R.D.: visualization. P.B.: visualization.

Notes

The authors declare no competing financial interest.

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