

Bio-Based Polymeric Resin from Agricultural Waste, Neem (*Azadirachta indica*) Seed Cake, for Green Composites

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ABSTRACT: Protein-based polymeric resin has been developed from nonconventional and nonedible “neem seed cake (NSC)” that has very limited low-value applications. Neem protein (NP), after extraction from defatted NSC, was used to prepare resin with two common plasticizers (glycerol and sorbitol). Properties of the NP resin sheets were evaluated as a function of plasticizer content. Increase of plasticizer content in NP sheets from 15 to 30% (w/w) enhanced fracture strain with a reduction in tensile strength, modulus, and thermal properties. Sorbitol-plasticized NP sheets showed better mechanical and thermal properties in comparison to glycerol-plasticized sheets. Effect of cross-linking with glyoxal on the mechanical and thermal properties of sorbitol-plasticized NP sheets was also investigated. Properties improved significantly at 10% (w/w) glyoxal content. Overall, with the enhanced properties of NP sheets, NP can be a viable alternative for edible protein-based resin for making green composites. NP resin can also be used to replace some synthetic resins. © 2014 Wiley Periodicals, Inc. *J. Appl. Polym. Sci.* **2015**, *132*, 41291.

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INTRODUCTION

Growing ecological concerns, economic awareness, and worldwide availability of plant sources have fueled an interest in research for the development of environment-friendly sustainable green materials as alternatives to nondegradable petroleum based materials. Numerous studies have shown that agricultural raw materials could be used as a source of various bio-based polymers for the production of biodegradable materials.^{1–10} Among bio-based polymers, plant proteins have been considered as attractive and sustainable sources for biodegradable materials due to their being inexpensive, yearly renewable, and plentiful availability.^{11–16} Plant proteins offer a wide range of potential functional properties as bio-based resins due to the presence of different reactive groups of the amino acids that can be easily accessed to form inter- and intra-molecular bonds. Some agro-seeds such as soybean, peanut, corn, peas, rice, sunflower, wheat, etc., are used as important protein sources for humans and other living stocks as well as biopolymers in food packaging industries. Some other agro-seeds such as neem (*Azadirachta indica*), karanja (*Pongamia pinnata*), and jatropha (*Jatropha curcus*) do not compete with the edible protein sources in foods and packaging industries due to the presence of toxic compounds in them. However, these oil-rich seeds have been the subject of extensive study in the past years as

raw materials for bio-based oil production and biodiesel.^{17–20} After extracting oil from the seeds, these protein-rich defatted seed cakes remain as waste stream for which no known applications beside low-value fertilizer or soil additive or fuel sources, exist at present, without complete detoxification.^{21,22} Additional value may be easily obtained from the seed cakes if the protein in them can be used in nonfood applications such as bio-based “green” resins to produce fiber-reinforced composite structures. In some cases, it may be even advantageous to use such materials if the toxic compounds in the protein can protect the products from microbial attacks or from other sources such as termites or ants.²³

Azadirachta indica, commonly known as neem, grows in almost every country in south Asia where it has been used for medicinal purpose and pest management.²⁴ It has anti-feedant, growth-inhibiting, antioviposition, insecticidal activities, and antimicrobial properties due to the presence of Azadirachtin (C₃₅H₄₄O₁₆), an active chemical compound in it that provides an excellent commercial value to the plant.²⁵ Neem seed contains 40–50% oil and 40–50% protein. However, after extraction of oil, the defatted seed cake has no known applications besides using it as low-value fertilizer or soil enhancer. As a result, it is very inexpensive or essentially free in South Asian countries.

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While soy protein has been investigated extensively,^{3,5,6,12–16} sheet forming abilities of neem protein (NP) from defatted neem seed cake (NSC) or using it as resin has not been explored. Feasible utilization of such abundant and free resource as polymeric resin in bio-based “green” composites can greatly reduce waste and enhance economic efficiency. In the present research mechanical, thermal, optical, and physicochemical properties of NP resin sheets have been characterized from the point of using it as a bio-based resin. Particularly, the effects of increasing ratio of plasticizers and cross-linking agents on the properties of NP resin sheets have been extensively investigated. The results suggest that NP resin properties can be manipulated to obtain desired properties such that it can be used as resin to fabricate sustainable “green” composites using plant based and other biodegradable fibers.

EXPERIMENTAL

Materials

Defatted NSC was purchased from The Ahimsa Alternative, Bloomington, MN. Analytical grade sodium hydroxide (NaOH) pellets and hydrochloric acid (37%), reagent grade glycerol ($\geq 98\%$ purity), D-sorbitol ($\geq 98\%$ purity), and glyoxal (40 wt % in water) were purchased from Sigma-Aldrich Chemical, Allentown, PA. All chemicals for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as mentioned in characterization section were purchased from Life Technologies, Grand Island, NY.

Extraction of Neem Protein

NP was obtained by alkaline extraction and acid precipitation of defatted NSC.²⁶ At first, the NSC was ground into powder using a blender and passed through 300- μm mesh sieve to obtain homogenous and small particles. Sieved powder was then dispersed in deionized (DI) water at a ratio of 1:10 (w/v) and adjusted to pH of 10.7 ± 0.2 using 1M NaOH. The mixture was stirred at 300 rpm and 75°C for 60 min. The insoluble residues were discarded by centrifugation at 5000 rpm for 20 min at room temperature and the supernatant was collected. The pH of the supernatant was adjusted to 4.5 ± 0.1 with 1M HCl and stirred for 20 min at 300 rpm to allow isoelectric precipitation. The suspension was centrifuged again at 5000 rpm for 20 min. The resulting brown suspensions, referred to as NP, were separated, freeze-dried, ground, sieved at mesh 250 μm , and stored at room temperature before characterizing their properties.

Preparation of NP Resin Sheets

NP was mixed with DI water at a ratio of 1 : 10 on a weight basis. Sheets made of pure NP were too brittle to handle. Glycerol or sorbitol was added separately as plasticizer to overcome the brittleness. Plasticizer concentrations were varied to obtain the best loading for optimum mechanical and thermal properties of NP resin sheets based on the current processing method. After the homogenization of mixtures by a magnetic stirrer at 300 rpm for 20 min, the pH of the solution was adjusted to 10.7 ± 0.2 using a 1M NaOH solution and was further homogenized by stirring further for 30 min at 75°C. This step is called “pre-curing” of NP as it absorbs water, swells and the protein molecules are partially cured or cross-linked at this tempera-

ture.³ Similar procedure with some alteration in processing parameters has been followed for soy protein film formation in our laboratory.^{3,12} The pre-cured resin solution was dried by pouring it on Teflon[®]-coated glass plate and kept at room temperature for 20 h. The sheet was further dried in an air circulating oven at 40°C for 4 h. Finally, dried NP sheets were cured using Carver Hydraulic hot press at 120°C for 25 min under a pressure of 2 MPa.

Glyoxal was added to plasticized NP solutions to introduce additional cross-linking and thus to increase the mechanical and thermal properties of the resin sheets as well as to increase the moisture resistance.^{27,28} Similar processing technique was used, as described above for NP resin sheets that contained plasticizers. The predetermined amount of glyoxal was added to NP/plasticizer mixture after 20 min of pre-curing at 75°C and continued to stir at 300 rpm for 10 min. The pre-cured glyoxal cross-linked NP resin was dried and cured using the same cycle as described above. The cured NP sheets were then conditioned at the ASTM conditions of 21°C and 65% RH for 48 h before testing.

Characterization of NP

Moisture, crude protein, crude fat, starch, water and ethanol soluble carbohydrate, simple sugars, crude fibers and ash contents were determined according to standard “Association of Analytical Communities (AOAC)” methods. The crude protein content was obtained by Leco FP-528 Nitrogen/protein analyzer using a conversion factor of 6.25.²⁹ All analyses were conducted at Dairy One, Ithaca, NY, in triplicate, to confirm the reproducibility.

The molecular weight distributions of NP were characterized by SDS-PAGE.³⁰ NP was first solubilized in 100 mM tris-HCl (pH 8.0) and 1.0% SDS. 10 μL protein-buffer mixtures (0.5 μL solubilized protein, 6 μL H₂O, 1 μL DTT, 2.5 μL NuPAGE 4X LDS loading buffer) were injected onto each lane in NuPAGE Novex 10% Bis-Tris precast gels (1 mm thick with 10 lanes) with NuPAGE MOPS SDS running buffer. The electrophoresis was carried out for about 50 min at 200 V constant. After electrophoresis, protein-containing gel was stained with Invitrogen Colloidal Coomassie Blue overnight, destained with water for 3 h, and photographed.

Amino acid composition analysis of NP was conducted by high-performance liquid chromatography (HPLC) according to AOAC Official Method 982.30 E.

Characterization of NP Resin Sheets

Tensile strength, Young’s modulus and fracture strain of NP resin sheets were determined according to ASTM D880-02 with an Instron (Model No. 5566) universal tensile testing machine. Sheets with dimensions of 10 mm \times 60 mm were tested at a strain rate of 1 min^{-1} and a gauge length of 30 mm. The thickness of the sheets required to calculate the tensile properties was measured to the nearest 0.001 mm using a hand-held digital micrometer. A minimum of 10 specimens were tested for each composition.

Thermal degradation behavior of resin sheets was investigated using thermo-gravimetric analysis, TGA, (TGA-2050, TA Instruments,

DE). Specimens weighing (~6 mg) were scanned from 30 to 600°C at a heating rate of 10°C/min under a flow of 60 mL/min nitrogen gas. The degradation onset temperature of NP sheets was determined from this analysis using Universal Analysis software (TA Instruments). Three specimens from each composition were investigated to ensure repeatability.

Differential scanning calorimetric (DSC) analysis was performed using DSC 2920 thermal analyzer (TA Instruments, DE). Specimens (~5 mg) were put individually in hermetically sealed aluminum pans to prevent any mass loss. DSC test was performed by heating from 30 to 110°C at a rate of 10°C/min, held at that temperature for 1 min, then cooled to 30°C at a cooling rate of 20°C/min (first scan) before carrying out the second heating scan to 180°C at a heating rate of 10°C/min. Nitrogen flow (60 mL/min) was maintained throughout the tests. The glass transition temperature (T_g) and denaturation temperature (T_{den}) of NP sheets were determined from the second heating scans using Universal analysis software. T_g of NP sheets was considered to be the midpoint temperature of the shift in the base line from the discontinuity of specific heat and T_{den} was considered as the maximum peak temperature of the endothermic phenomenon of the specimens during heating scans. The first heating scan was meant to discard thermal history, if any, of the specimens. Four specimens from each composition were investigated to ensure repeatability.

Moisture content (MC) of NP sheets was measured according to a method described by Rhim et al.³¹ The specimen strips were weighed (W_1), subsequently dried in an air circulating oven at 105°C for 24 h and reweighed to obtain dry weight (W_2) determine MC values. Water solubility (WS) or percentage of dry matter soluble in water was measured according to a method described by Cuq et al.³² with some minor modification. After getting MC values, specimens were immersed in 30 mL of DI water in a beaker for 24 h at 25°C. Beakers were covered with Parafilm “M” wrap and stirred occasionally. Then, specimens were dried in an air circulating oven at 105°C for 24 h and weighed to obtain redried weight (W_3). Four replicates of analyses were carried out for each category. MC and WS values were calculated using the eqs. (1) and (2), respectively:

$$MC (\%) = \left(1 - \frac{W_2}{W_1}\right) 100 \quad (1)$$

$$WS (\%) = \left(1 - \frac{W_3}{W_2}\right) 100 \quad (2)$$

Water absorption (WA) was determined by a slight modification of the ASTM standard D870-02. Conditioned specimens (~20 × 20 mm) were dried at 105°C for 24 h in an air-circulating oven and weighed to obtain dry weight (W_2). The specimens were then immersed in 30-mL of DI water for specific time at 25°C. The specimens were removed from water medium at every 20 min intervals and reweighed (W_4) after removal of excess water from the specimen surfaces using absorbent paper. WA value was calculated using eq. (3):

$$WA(\%) = \left(\frac{W_4}{W_2} - 1\right) 100 \quad (3)$$

Optical parameters of the sheets were measured using Macbeth Color-eye spectrophotometer. Sheet specimens were placed on a

white plate, and the CIELAB color scale was used to measure color: $L^* = 0$ (black) to $L^* = 100$ (white), $-a^*$ (greenness) to $+a^*$ (redness), and $-b^*$ (blueness) to $+b^*$ (yellowness). Standard values for the white calibration plate were $L^* = 95.99$, $a^* = -0.04$, and $b^* = 1.01$. Considering standard light source D65 and standard observer 10°, color parameters L^* , a^* , b^* , and whiteness index (WI) were calculated. Values were expressed as the averages of 10 measurements on different areas of each Sheet. The WI was calculated using eq. (4):

$$WI = 100 - \sqrt{(100 - L^*)^2 + (a^*)^2 + (b^*)^2} \quad (4)$$

Optical transmittance of resin sheets was measured at selected wavelengths between 400 and 800 nm with a data interval of 1 nm using a UV-Visible spectrophotometer (Lambda 35, Perkin Elmer, Norwalk, CT) according to ASTM D1746-03. The transparency value (TV) at unit light path length of the Sheets was calculated by the eq. (5).³³

$$TV = (-\log T_{660})/d \quad (5)$$

where, T_{660} is the transmittance at 660 nm and d is sheet thickness in mm. The greater TV represents lower transparency. Three specimens from each category were tested to obtain mean values.

Statistical evaluations were carried out by analysis of variance (ANOVA) followed by multiple comparison tests using Tukey-Kramer's HSD at 95% confidence level. All analyses were performed using JMP statistical software (SAS Institute, Cary, NC).

RESULTS AND DISCUSSION

Characteristics of NP

Table I shows proximate composition of the defatted NSC and NP. Included in Table I are also the determination methods used for obtaining the constituents. As shown in Table I, the extraction method presented here was successful in obtaining NP with 80–82% protein content, almost twice that of defatted NSC. This increase is significant since the properties of resin are dependent on the percentage of protein. In addition, it has been shown that the fiber/resin bonding is also dependent on the protein content in the resin in the case of soy protein.¹⁵ Protein recovery was found to be about 63–65% by the extraction process. It is to be mentioned that protein recovery and protein content are not only dependent on the protein extraction process parameters but are also affected by the oil extraction method prior to protein extraction. Lestari et al. showed protein recovery from defatted jatropha seed cake was lower than that of nondefatted jatropha seed cake.³⁴ Heat and organic solvents used during oil extraction might influence negatively by reducing the protein solubility in solvents and hence, total extractability of the protein.

Figure 1 shows SDS-PAGE of extracted NP with molecular weight marker. As can be seen from Figure 1, two major protein bands differing in molecular weight were observed clearly. The top band contained protein molecules between 35 and 45 kDa and the bottom one showed protein molecules between 15 and 25 kDa. There are also two unclear bands between 50 and 80 kDa which might be the proteins having lower content. These

Table I. Proximate Composition of Defatted Neem Seed Cake (NSC) and Neem Protein (NP)

Constituents	Defatted NSC	NP	Determination method
Moisture (%)	10.6 ± 0.9	3.4 ± 1.1	AOAC 930.15
Crude protein (%)	42.9 ± 1.4	81.0 ± 1.8	AOAC 992.23
Crude fat (%)	7.9 ± 0.9	1.8 ± 0.7	AOAC 2003.05
Starch (%)	0.6 ± 0.2	0.3 ± 0.2	YSI 2700 SELECT Analyzer
Water soluble carbohydrate (%)	15.3 ± 2.2	1.3 ± 0.9	UV spectrophotometry ⁶⁷
Simple sugars (%)	7.8 ± 0.9	2.3 ± 0.2	UV spectrophotometry ⁶⁷
Crude fiber (%)	4.2 ± 0.2	-	AOAC 962.09
Ash (%)	7.4 ± 0.6	2.8 ± 0.2	AOAC 942.05

results suggest that the molecular weight of NP subunits is comparable to that of soy protein obtained earlier by others.³⁵

Table II shows composition of amino acids present in NP. As can be seen in Table II, about 50% of total amino acids presented in NP are charged polar amino acids that can participate in various ionic interactions. The presence of arginine (~10%), lysine (~3%), cystine, tyrosine, and histidine (~2%) allows certain chemical modifications of protein through cross-linking and thus obtain desired mechanical and thermal properties.^{36,37}

The composition of amino acids in NP was comparable with that of conventional soy protein, reported in literature.³⁸ Relatively higher content of polar amino acids might facilitate the formation of three-dimensional covalent cross-linking interactions in NP over soy protein.

Physico-Chemical Properties of NP Resin Sheets

NP resin sheets with smooth surfaces were produced without any additives or plasticizers. However, they were too brittle to characterize mechanical properties. NP resin sheets had thicknesses of 0.3 ± 0.02 mm. The difference in sheet thicknesses was not significant (P -value > 0.05). Our observation indicates that the final thickness mainly depends on the shrinking of the sheet during the evaporation of the solvent, water in this case, as it is not a linear function of the dry matter content in the sheet-forming solution.³⁹ Dry matter content in NP sheets was 87.5 ± 1.0 g/100 g, and the remaining material was water. The color of the control NP sheet was brown with the whiteness index of 55.2 ± 0.10 . Optical transmittance of the sheet was

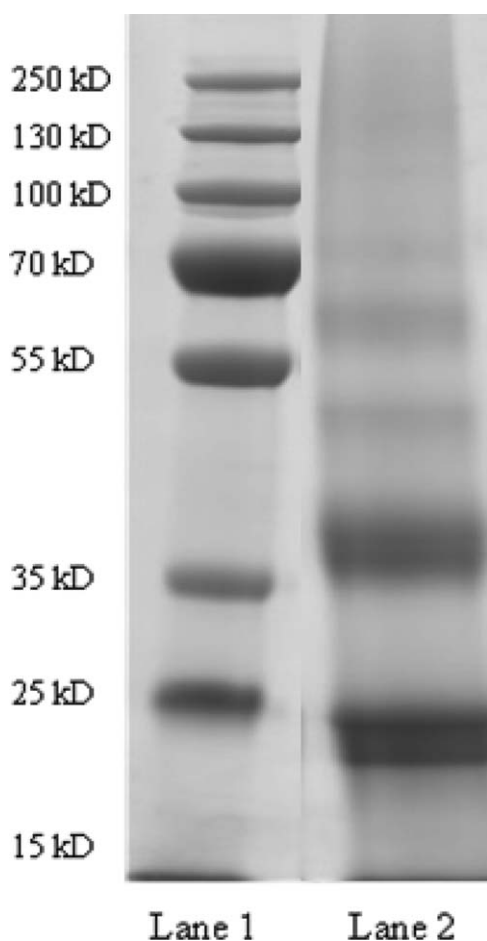


Figure 1. SDS-PAGE of extracted neem protein (NP). Lane 1- molecular weight marker and Lane 2- 16 μ g of total neem proteins.

Table II. Amino Acid Composition of NP

Classification	Amino acids	NP (g/16 g Nitrogen)
Polar charged	Arginine	9.7
	Lysine	2.9
	Histidine	2.0
	Aspartic acid	9.7
	Glutamic acid	25.4
Polar neutral	Cystine	1.8
	Tyrosine	2.3
	Serine	4.2
	Threonine	3.2
Nonpolar	Alanine	4.3
	Glycine	4.1
	Leucine	7.8
	Isoleucine	4.0
	Methionine	1.1
	Proline	4.0
	Valine	6.4
	Phenylalanine	4.0
	Tryptophan	1.2

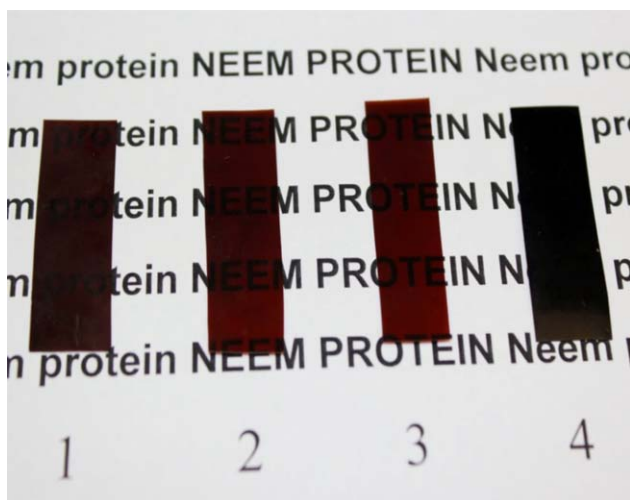


Figure 2. Photographic images of control NP (1), 15% sorbitol-plasticized NP (2), 15% glycerol-plasticized NP (3), and 15% sorbitol-plasticized with 10% glyoxal cross-linked NP sheets (4). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

found to be only $3.0 \pm 0.2\%$ for the current thickness of 0.3 mm. Figure 2 shows photographic images of control (no additive or modifier), plasticized, and cross-linked NP sheets. As can be seen from Figure 2, the specimen cross-linked with glyoxal was the most opaque which has been discussed later in section 3.4.

Effect of Increasing Ratio of Plasticizers

Addition of a plasticizer in the sheet-forming solution was necessary as the NP sheet was too brittle to be handled or tested without it. According to the lubricity theory of the plasticization, the plasticizer acts as a lubricant to facilitate the movements of the protein chains over each other to avoid brittleness.⁴⁰ Also, a plasticizer, being a small molecule, significantly increases the number of ends and thus enhances free volume in the protein systems according to free volume theory.⁴⁰ However, plasticizing effects do vary from one protein to another protein due to differences in amino acid content as well as their sequences.⁴¹ Protein–protein interactions can be varied by varying contents of plasticizer which determine the properties of the sheet.⁴² To investigate the effects on NP, Sorbitol and glycerol, the most common polyol type plasticizers for protein-based polymers, were used to plasticize the resin sheets. It was found that, NP sheets with sorbitol content lower than 15% (g/100 g dry protein) were difficult to handle after drying and was not possible to be hot-pressed due

to brittleness. On the other hand, the sheets with more than 30% glycerol were very flexible. As a result, a range of 15 to 30% (g/100 g dry protein) plasticizer content was selected to seek a compromise between strength and elasticity and to determine optimal plasticizer content. Increased level of plasticizers in the sheet-forming solutions did not exhibit any statistically significant differences in the thickness values of sheets (results not shown) (P -value > 0.05). NP Sheets with plasticizers had an average thickness of 0.3 ± 0.04 mm. From these results, it can be concluded that plasticization with sorbitol or glycerol has no significant effect on the thickness of NP sheet.

Effects of plasticizers (glycerol and sorbitol) on tensile properties of NP sheets are presented in Table III along with the statistical analysis based on the Tukey-Kramer HSD tests. The effect of plasticizer incorporation is clearly evident if the fracture strain data are considered. Fracture strain increased continuously, as expected, with the increase of plasticizer content. However, tensile strength and modulus decreased as the plasticizer content increased. The differences in tensile properties were significant (P -value < 0.05). In case of both glycerol and sorbitol, the Young's modulus and fracture strength were highest at 15% plasticizer content. However, sorbitol performed much better in terms of mechanical properties.

Amino acid profile of NP reveals the presence of both polar and nonpolar groups that have strong inter- and intramolecular interactions such as hydrogen bonding, dipole–dipole, charge–charge, and hydrophobic interactions. Usually, strong polar interactions between groups of protein molecules impede molecular movement and segmental rotations that can result in higher modulus, strength and thermal stability in protein sheets.⁴³ However, these interactions are also responsible for the increased brittleness. While glycerol and sorbitol do not form any covalent linkages with the protein, the hydroxyl groups of these molecules can interact with protein at amino, carboxyl, and hydroxyl sites by hydrogen bonds which results in reduced inter- and intra-molecular interactions between the protein chains. According to the gel theory of plasticization, plasticizers hinder polymer molecules from interacting with each other by breaking the attachments along the chains and reduce the functional properties.⁴⁰ Based on the amino acid composition of NP, it is expected that hydrogen bonding would occur among $-\text{NH}_2$ groups in arginine and lysine, $-\text{NH}-$ groups in proline and histidine, $-\text{OH}$ groups in tyrosine, threonine, and serine, and $-\text{COOH}$ groups in glutamic and aspartic acids.

Table III. Effects of Plasticizers (Glycerol and Sorbitol) on Tensile Properties of NP Sheets

Plasticizer	Concentration	Tensile strength (MPa*)	Young's modulus (MPa*)	Fracture strain (%*)
Glycerol	15%	6.1 ± 0.63^b	168.2 ± 25.94^{ab}	63.8 ± 12.35^{bc}
	22.5%	4.5 ± 0.61^{bc}	81.9 ± 32.21^{bc}	86.5 ± 18.39^{ab}
	30%	3.6 ± 0.49^c	54.9 ± 20.93^c	103.4 ± 12.11^a
Sorbitol	15%	7.9 ± 1.48^a	239.1 ± 48.70^a	56.0 ± 10.48^c
	22.5%	6.1 ± 0.78^{ab}	143.9 ± 34.74^{bc}	71.3 ± 14.55^{bc}
	30%	4.2 ± 0.31^c	114.5 ± 24.83^{bc}	92.3 ± 11.03^{ab}

*Means not connected by same letters are significantly different at 95% confidence level through Tukey-Kramer HSD test.

Table IV. Effect of Plasticizers on Moisture Content (MC) of NP Sheets

Plasticizer concentration	MC of NP sheets* with glycerol (%)	MC of NP sheets* with sorbitol (%)
15%	18.9 ± 0.31 ^{ab}	17.8 ± 0.65 ^b
22.5%	19.9 ± 0.76 ^a	18.0 ± 0.67 ^b
30%	20.1 ± 0.79 ^a	18.8 ± 0.78 ^{ab}

*Means not connected by same letters are significantly different at 95% confidence level through Tukey-Kramer HSD test.

NP sheets plasticized with sorbitol had significantly higher tensile strength and modulus than those plasticized with glycerol. Similar behavior was also observed for other protein-based sheets.^{15,44} Previously, Griffin and Lynch showed that glycerol has infinite solubility in water at 25°C with a medium-high hygroscopicity whereas sorbitol has 71% solubility with medium-low hygroscopicity.⁴⁵ Also, being lower molecular weight plasticizer, glycerol brings more free volume in the protein system for which it is easier for the water to diffuse in. Higher hygroscopicity makes glycerol more effective plasticizer while sacrificing the strength and stiffness as water is also an effective plasticizer.⁴⁶

Table IV shows the effect of plasticizers (sorbitol and glycerol) on moisture content of NP sheets. NP sheets plasticized with sorbitol and glycerol at same loading differs significantly according to Tukey's test. As can be expected, glycerol-plasticized sheets showed higher moisture content compared to sorbitol at the same loading. Although both plasticizers have similar straight-chain molecular structures, the lower molecular weight plasticizer "glycerol" has been shown to have higher water affinity at same RH from sorption and desorption isotherms.⁴⁷ Another study by Mali et al. revealed lower equilibrium moisture for sorbitol-plasticized sheets as compared with glycerol-plasticized sheets.⁴⁸ The difference in hydrophilicity of the plasticizers has also been reflected on the MC values of NP sheets.

Glass transition and denaturation temperature were measured from the DSC thermograms of NP sheets. Figure 3(a,b) shows the effect of plasticizers on the glass transition temperature (T_g) and, denaturation temperature (T_{den}) of NP sheets. As shown in Figure 3(a), T_g of NP sheets decreased with an increase in plasticizer content, which was expected according to the free volume theory of plasticization. Basically, T_g of polymer reflects the tightness of molecular packing or supramolecular structure⁴⁹ and is mainly governed by noncovalent interactions such as hydrogen bonding, van der Waals and hydrophobic interaction in case of protein.⁵⁰ The T_g of the nonplasticized sheet was found at about 102°C and shifted lower to about 93, 90, and 83°C after plasticization by 15, 22.5, and 30% glycerol, respectively. Similar behavior was obtained in sorbitol-plasticized sheets. However, the T_g values were higher for sorbitol-plasticized NP by about 2 to 3°C compared with glycerol-plasticized NP in all cases. A linear regression was applied to compare the efficiency of plasticizers which revealed a higher slope for glycerol-plasticized NP specimens than those plasticized by sorbitol. The slopes of denaturation temperature (T_{den})

versus plasticizer content curves showed similar behavior which indicates a better plasticization action of glycerol as shown in Figure 3(b).

The thermal stability of plasticized NP sheets was characterized using thermogravimetric analyzer (TGA). Figure 3(c) shows typical TGA thermograms of control and plasticized NP sheets and Figure 3(d) shows the effect of plasticizers at various concentrations on degradation onset temperature (T_d). As observed in Figure 3(d), T_d decreased as the plasticizer's content increased. As expected, plasticization reduces thermal stability by interspersing itself around polymer and breaking polymer-polymer interactions according to gel theory.⁴⁰ The results of sorbitol-plasticized NP showed higher T_d than that of glycerol-plasticized specimens indicating the superior performance of sorbitol. However, both plasticizers resulted in lower T_d compared with control specimens as was expected. Among the plasticized specimens the maximum T_d was observed for 15% sorbitol-plasticized NP sheet (244.44°C), a decrease of about 20°C compared with control specimens whereas the lowest was observed in 30% glycerol-plasticized NP sheet (206.27°C), a decrease of over 55°C. The difference between sorbitol-plasticized and glycerol-plasticized NP sheet was found to be significant (P -value < 0.05). The degradation of plasticized resin proceeded in three weight loss steps. Weight loss below 150°C was attributed to the evaporation of water. Plasticizer evaporation occurred between 150 and 250°C (Boiling point of glycerol is 290°C and sorbitol is 296°C), and the final weight loss beyond 250°C was attributed to the thermal decomposition of the NP with complete evaporation of plasticizers. As observed in Figure 3(c), the differences in weight loss for a particular temperature below 150°C in all categories were not significant. However, a significant difference in weight loss for a particular temperature was observed at 150–300°C between control and plasticized specimens. This difference may be attributed to the evaporation of the plasticizers in plasticized specimens. For this reason, control specimens lost less weight in comparison with plasticized ones at same temperatures beyond 150°C. Also, a delay in the onset of degradation temperature in the plasticizers (between 150 and 250°C) is greater in the case of sorbitol-plasticized resin compared with glycerol-plasticized resins. This might be due to stronger interactions between sorbitol and the polypeptide chains of NP.

Considering one of the ideal applications of NP as resin in fiber reinforced composites, sheet made with 15% sorbitol was selected as control for noncross-linked specimens for further investigations as the optimum amount of plasticizer due to its highest tensile strength, Young's modulus, thermal transitions, and degradation with moderate fracture strain.

Effect of Cross-Linker Ratio

In general, proteins exhibit poor mechanical, thermal and moisture resistance properties, especially in humid conditions which limit their use in structural applications. Chemical cross-linking provides a mechanism to improve the mechanical and thermal properties as well as water resistance and solubility of protein-based biopolymers. In chemical cross-linking process, a low molecular weight component reacts with functional groups of

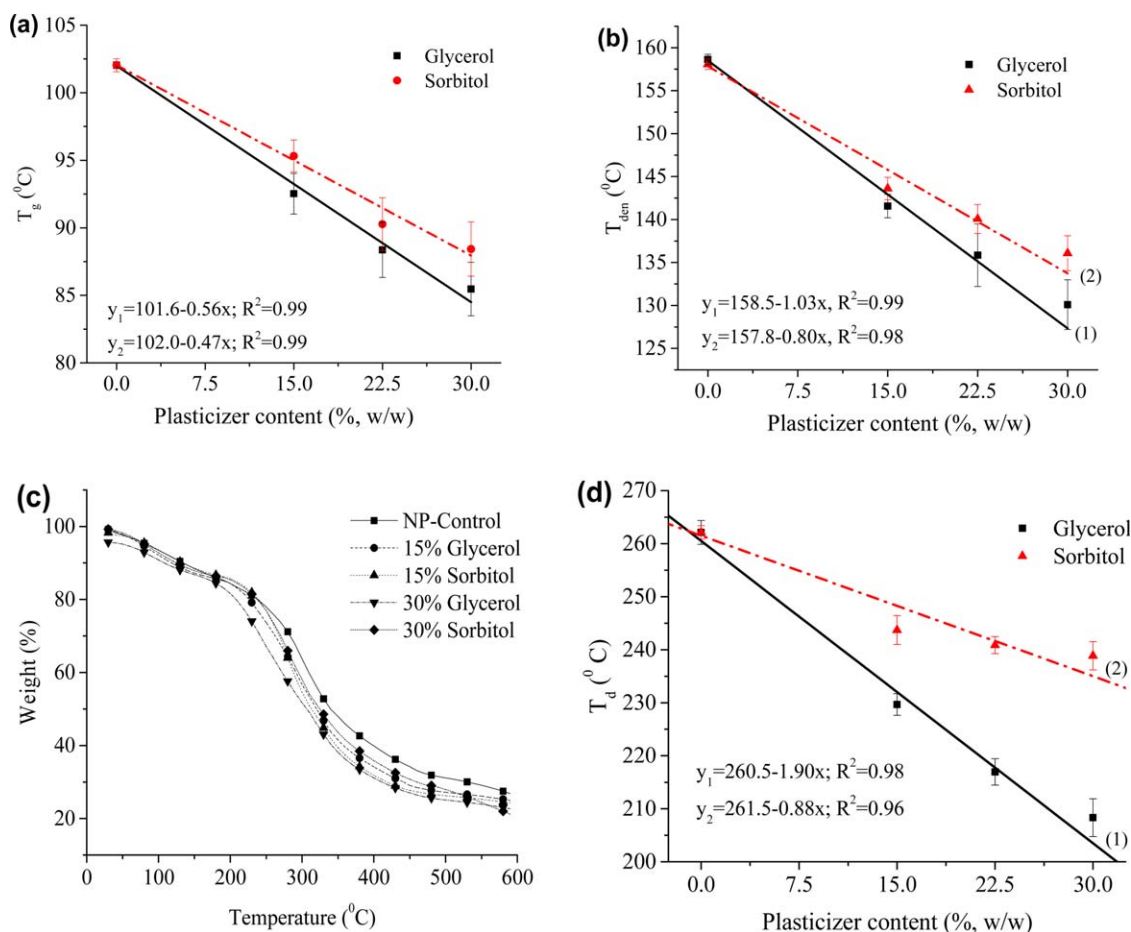


Figure 3. Effect of plasticizers on glass transition temperature, T_g (a), denaturation temperature, T_{den} (b), typical TGA thermograms of control and plasticized NP sheets (c), and degradation onset temperature, T_d (d) of NP sheets. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

polypeptide chains, forming tri-dimensional intermolecular covalent bonds between them. Aldehydes such as glyoxal, glutaraldehyde, and formaldehyde have been most commonly used cross-linking agents for protein-based sheets by solution casting methods.^{36,37,51} In this study, glyoxal was used to crosslink NP due to its lower toxicity and volatility compared with glutaraldehyde and formaldehyde.⁵² Glyoxal has been shown to react with guanidine group of arginine and ϵ -amino group of lysine of protein to form Schiff bases and initiate Maillard reaction.⁵³ Effect of glyoxal content, between 0 and 15% (g/100 g dry protein), was studied in the 15% sorbitol-plasticized NP sheets.

Figure 4 shows the effect of glyoxal addition on moisture content (MC) and water solubility (WS) of 15% sorbitol-plasticized NP sheets. As can be seen from Figure 4, MC of the specimens decreased linearly with cross-linker content ($R^2 = 0.99$). While a significant difference was observed between behaviors of cross-linked and noncross-linked sheets, MC did not vary significantly among the cross-linked sheets. NP sheets cross-linked with 5% glyoxal showed MC of slightly less than 17% while those cross-linked with 15% glyoxal had about 15% MC. WS, however, showed significant decrease after cross-linking. There was an exponential decrease in WS with the increasing of glyoxal (Figure 4). A reduction of about 25% in WS was observed for NP speci-

mens cross-linked with 10% Glyoxal. About 38% solubility in water was observed in control NP sheets while cross-linked sheets with 10% glyoxal showed the values about 28%. Similar reduction in MC and WS has been reported on other protein-based

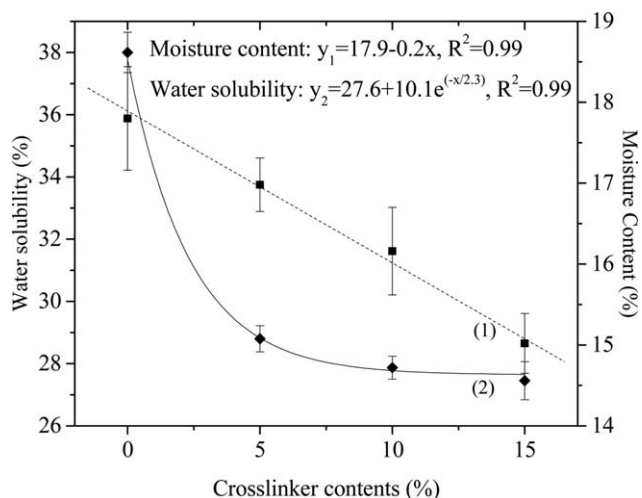


Figure 4. Effect of glyoxal (cross-linker) on moisture content (1) and water solubility (2) of 15% sorbitol-plasticized NP sheets

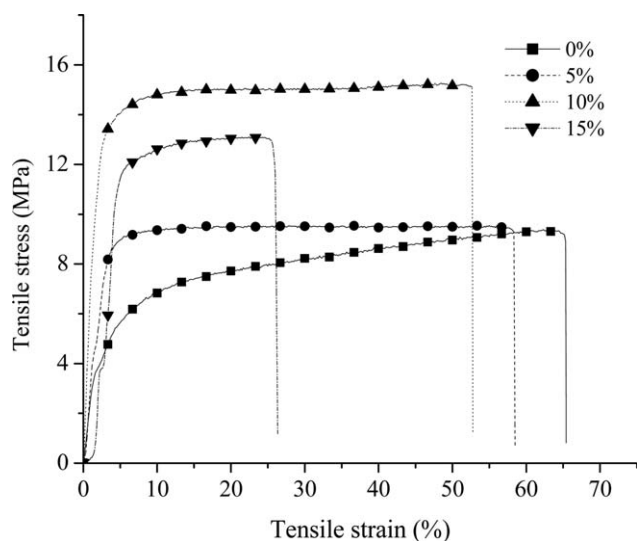


Figure 5. Typical stress–strain plots of 15% sorbitol-plasticized NP sheets for different glyoxal contents.

sheets by cross-linking.⁵⁴ The reduction in MC and WS value is a consequence of the formation of a covalently bonded three-dimensional network resulting from the NP-glyoxal cross-linking. Since some of the hydrophilic amine groups are consumed in cross-linking with aldehyde groups, glyoxal treatment reduction in WS and MC can be expected. Also, formation of tighter and compact network structures due to cross-linking results in higher moisture resistance than the control specimens. However, cross-linker content of higher than 5% did not significantly change the WS. It may be that the inter-molecular cross-linking reaches its limiting value under the experimental conditions at 5% glyoxal in NP.

Glyoxal has been found to be a useful cross-linker to increase the hydrophobicity of proteins by other researchers as well.⁵⁵ This suggests that cross-linking could be one way to reduce the moisture absorption by NP sheets. Figure S1 (Supporting Information) shows the effect of glyoxal cross-linking on water absorption behavior of 15% sorbitol-plasticized NP sheets as a function of immersion time. As shown in Supporting Information Figure S1, cross-linking of NP sharply decreased its water absorption. About 300% of water was absorbed within 20 min by control (noncross-linked) NP sheets while cross-linked sheets with 15% glyoxal had water absorption of about 50%, a reduction of over 80%. Also, absorption of water increased sharply in the first 20 min after which it did not change much. This behavior was observed for all compositions. Because of the hydrophilicity of the control sheets, they initially showed softening and subsequently warped. Higher cross-linking made the structure rigid and less expandable with smaller capacity for softening and apparently did not allow it to warp due to the formation of an isotropic three-dimensional structure, a significant advantage in real life applications. A similar behavior was also observed in biodegradable resins made from soy protein cross-linked with glyoxal.⁵⁵

The effects of glyoxal on mechanical properties were evaluated by tensile testing. Tensile strength, Young's modulus and frac-

ture strain values were obtained from the stress–strain plots of the cross-linked and noncross-linked NP sheets. Figure 5 shows typical stress–strain plots of 15% sorbitol-plasticized NP sheets for different glyoxal contents and Table V shows the effect of cross-linking on tensile properties of sorbitol-plasticized NP sheets. As seen from data presented in Table V, tensile strength and Young's modulus increased with the increasing ratio of glyoxal whereas fracture strain dropped moderately. The addition of 10% glyoxal increased the tensile strength by about 68%. Also, glyoxal at 10% lead to the modulus of NP sheets almost three times higher than that of noncross-linked specimens. However, addition of glyoxal was found to have a negative effect on the fracture strain of the NP sheets. It is known that cross-linking restrains molecular movement and thus reduces the fracture strain. The addition of 10% glyoxal made the network rigid to decrease the fracture strain to 38% from 56% observed for control sheets, a decrease of about 35%. Increasing the glyoxal content to 15%, the fracture strain further decreased to 23%, a decrease of about 60% while modulus also decreased by about 17%. However, tensile strength did not vary significantly at 15% glyoxal contents. The reduction in the fracture strain can be attributed to a fact that the number of possible inter- and intra-molecular bonding between polypeptide chains increased as the cross-linker was increased, causing overstretching in the systems.⁵⁶ On the other hand, 15% glyoxal in NP resin may create a nonstoichiometric ratio for cross-linking with lysine and arginine that causes an excess of glyoxal in the protein system which then acts as plasticizer. As a result, Young's modulus decreased at 15% glyoxal contents.

Typical DSC thermograms of the control, plasticized and cross-linked NP sheets are shown in Figure S2 (Supporting Information) and typical TGA thermograms of 15% sorbitol-plasticized NP sheets for different glyoxal contents has been shown in Figure S3 (Supporting Information). Figure 6 shows the effect of cross-linking on glass transition temperature (T_g), denaturation temperature (T_{den}) and degradation onset temperature (T_d) of 15% sorbitol-plasticized NP sheets. Cross-linking generally induces an increase in thermal stability by an appreciable increase of T_g and T_{den} . As shown in Figure 6, the noncross-linked NP sheets with 15% sorbitol had T_g at $95 \pm 1^\circ\text{C}$ and T_{den} at $141 \pm 2^\circ\text{C}$ whereas a maximum T_g and T_{den} were observed at $102 \pm 1^\circ\text{C}$ and $152 \pm 1^\circ\text{C}$, respectively, for specimens with 10% glyoxal content. Similarly, T_d of control NP

Table V. Effect of Cross-Linking on Tensile Properties of Sorbitol-Plasticized NP Sheets

Specimen*	Tensile strength (MPa**)	Young's modulus (MPa**)	Fracture strain (%**)
Glyoxal 0%	7.9 ± 1.48^a	239.1 ± 48.70^a	56.0 ± 10.48^a
Glyoxal 5%	9.8 ± 0.76^a	296.3 ± 34.65^a	54.7 ± 8.82^a
Glyoxal 10%	13.3 ± 1.28^b	705.1 ± 46.21^b	38.0 ± 13.56^a
Glyoxal 15%	12.4 ± 0.79^b	579.6 ± 51.66^c	22.9 ± 9.52^b

*15% sorbitol is present in all these specimens.

**Means not connected by same letters are significantly different at 95% confidence level through Tukey-Kramer HSD test.

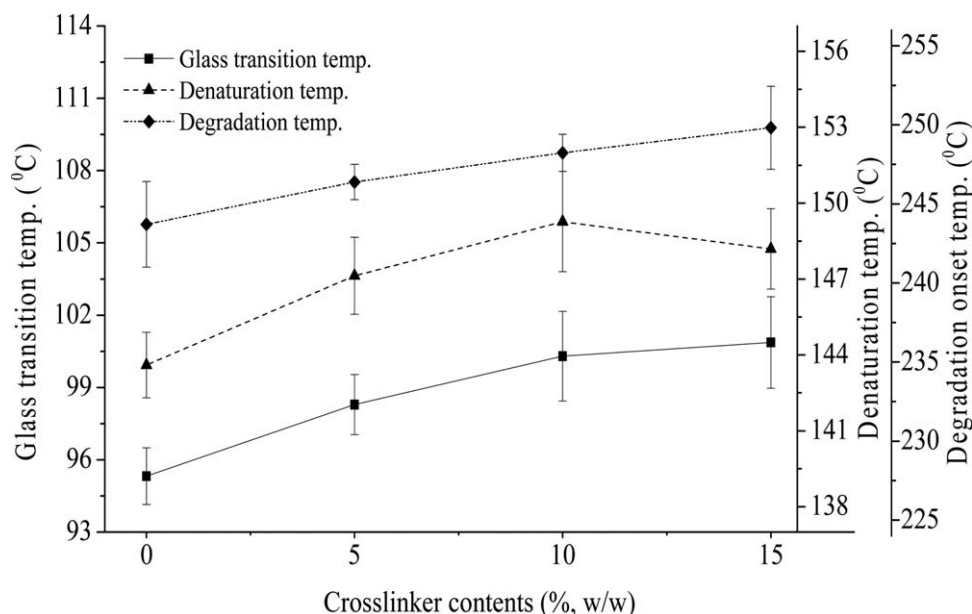


Figure 6. Effect of cross-linking on glass transition temperature (T_g), denaturation temperature (T_{den}), and degradation onset temperature (T_d) of 15% sorbitol-plasticized NP sheets.

sheets was observed at $244 \pm 2^\circ\text{C}$ while cross-linked NP sheets with 10% glyoxal had a T_d value of $249 \pm 1^\circ\text{C}$.

The effect of cross-linking on the optical properties, whiteness and transparency, of 15% sorbitol-plasticized NP sheet, measured using colorimeter and UV-Visible spectrophotometer are shown in Figure 7. As seen in Figure 7, whiteness index decreases with the increase in cross-linking. Earlier, Figure 2 showed the color of plasticized and cross-linked NP which turns from light brown for control specimens to dark brown after cross-linking. It is well-known that Maillard reactions are responsible for the darker colors in the food industry. The Maillard reaction occurs between amine groups in protein (lysine and arginine) and aldehyde groups of the cross-linker in the presence of heat. This reaction produces irreversible adducts on proteins both intra- and inter-molecularly, collectively known as

advanced glycation end (AGE) products, that are identified by the color change.^{57,58} In general, color changes from uncolored through yellow, golden, and cinnamon to reddish brown with the progression of Maillard reaction.⁵⁹ The results, as can be seen in Figure 7, confirm qualitatively that glyoxal reacted with the lysine and arginine residues of denatured NP, changing color from light brown to darker brown due to Maillard reaction. Similar color changes have also been found in aldehyde cross-linked soy protein as well.^{3,13,14,60} Transparency is also another criterion for judging the cross-linking effect on the optical transmittance. As the resin gets darker, the transparency is affected. As observed in Figure 7, the transparency of the sheets decreased as cross-linker content increased. Control NP sheets had a transparency value of 2.5 ± 0.5 whereas cross-linked NP sheets with 15% glyoxal showed a value of 6.0 ± 0.7 , indicating lower transparency. As can be seen from Figure 2, the opaqueness increased in cross-linked sheets as color changed from light brown to darker brown due to Maillard reaction.

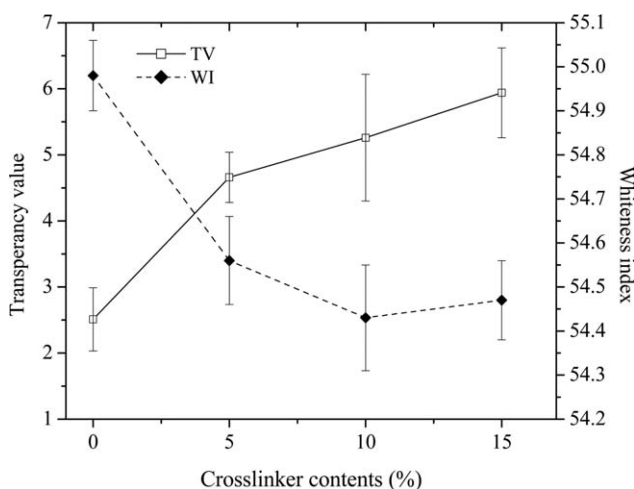


Figure 7. Effect of cross-linking on optical properties of 15% sorbitol-plasticized NP sheets.

Considering mechanical and thermal properties of NP obtained in this study, it can easily replace other edible proteins such as soy and, hence, more sustainable. In fact, NP and modified NP sheets with 15% sorbitol and 10% glyoxal showed comparable and, in some cases, better mechanical properties than some edible protein sheets^{61–66} which reveal a sustainable bio-based resin for fiber-reinforced composite structures. The fracture strain was also found to be acceptable. This new nonedible NP protein resin can be used for packaging and fabricating fiber reinforced composites.

CONCLUSIONS

In summary, this study introduces a new biodegradable protein-based resin from neem seed cake. The study has shown that the mechanical and thermal properties of NP can be fine-tuned by manipulating the use of plasticizers and cross-linkers.

Investigation of plasticization by sorbitol and glycerol on the mechanical and thermal properties showed that incorporation of 15% sorbitol in NP resulted in best mechanical properties, under the current experimental conditions. Cross-linking NP with 10% glyoxal in 15% sorbitol-plasticized NP sheets resulted in even better mechanical and thermal properties as well as higher water resistance. Overall, this paper explores the utilization of waste residues from neem seed as a sustainable and "green" bio-resin to replace conventional petroleum-derived as well as edible protein based bio-resins.

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