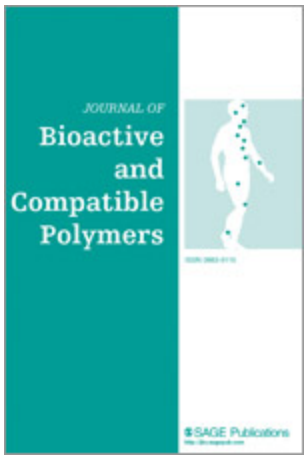


Extraction and Characterisation of Bioactive Proteins from Pongamia pinnata and their Conversion into Bioproducts for Food Packaging Applications

Journal:	*Journal of Bioactive and Compatible Polymers*
Manuscript ID	JBC-20-0123.R2
Manuscript Type:	Original Article
Date Submitted by the Author:	17-Apr-2021
Complete List of Authors:	Nataraj, Divya; Center for Incubation, Innovation, Research and Consultancy Guna, Vijaykumar; Center for Incubation, Innovation, Research and Consultancy Battampara, Prajwal; Center for Incubation, Innovation, Research and Consultancy S, Nagananda; Center for Incubation, Innovation, Research and Consultancy Rizzarelli, Paola; Istituto per i Polimeri, Compositi e Biomateriali Reddy, Narenda; Center for Incubation, Innovation, Research and Consultancy,
Keywords:	Pongamia pinnata, Proteins, Citric acid, crosslinking, films, bioproducts
Abstract:	Proteins extracted from Pongamia pinnata oil meal have been converted into completely biodegradable films, transparent packaging boxes, and containers with inherent antimicrobial and antioxidant properties required for medical, food, biotechnology, and other applications. A simple crosslinking using citric acid can provide the necessary strength and stability to the protein bioproducts. Proteins are preferred over other biopolymers for various end-uses and are ideally suited for direct consumption, also to develop bioproducts for packaging applications. In this research, proteins are obtained from pongamia oil meal and subsequently converted into films and compression molded into various packaging products. Films with a maximum tensile strength of 1.9 MPa were obtained when 10% citric acid was used as the crosslinker. The pongamia protein-based bioproducts showed good activity against

	Bacillus cereus and Aspergillus niger. Unique properties, low cost, and large availability make pongamia proteins an ideal biopolymer for the development of green and sustainable materials and bioproducts.

Extraction and Characterisation of Bioactive Proteins from *Pongamia pinnata* and their Conversion into Bioproducts for Food Packaging Applications

Abstract

In this research, proteins were obtained from *Pongamia pinnata* oil meal and subsequently converted into films and compression moulded into various packaging products. Films with a maximum tensile strength of 1.9 MPa were obtained when 15% citric acid was used as the crosslinker. Minimum swelling of 120% was seen in 20% citric acid crosslinked film whereas the uncrosslinked films readily disintegrated in water. The protein films had excellent antioxidant properties with an IC50 value of 14.6 µg/mL compared to 26.9 µg/mL for the standard ascorbic acid. The pongamia protein-based bioproducts showed good activity against *Bacillus cereus* and *Aspergillus niger*. Unique properties, low cost, and large availability make pongamia proteins an ideal biopolymer for the development of green and sustainable materials and bioproducts.

Keywords: *Pongamia pinnata*, proteins, citric acid (CA), crosslinking, films, packaging, bioproducts

1. INTRODUCTION

Substantial efforts are being pursued towards developing greener materials using renewable resources as substitutes for petroleum-based materials. Among the various renewable resources, the by-products generated during the cultivation of crops are economical and widely available with rather restricted applications. Similarly, plant proteins like pongamia, soy, and zein are produced as coproducts of oil extraction but are mainly used for low-value applications such as fertilizer or animal feed [1, 2].

Plant proteins have several advantages including easy biodegradability, biocompatibility, cytocompatibility, availability of amino and carboxylic groups for chemical modifications, etc [3-5]. Hence, a variety of plant proteins have been converted into films, hydrogels, fibers, micro and nanoparticles, and scaffolds for a range of applications [6-9]. However, compared to many other synthetic materials, the properties of protein films are inferior. Specifically, plant protein-based biomaterials have inferior mechanical characteristics along with inadequate water resistance due to the considerably high hydrophilicity of the proteins [10]. These drawbacks limit the use of plant proteins for various applications.

Proteins contain several functional groups including amines, alcohols thiols, etc. Chemical modification of these functional groups changes the physical properties and reactivities of the proteins making them suitable for specific applications. Researchers have implemented numerous types of chemical modification techniques including crosslinking, esterification, acetylation, etc to improve the performance properties and usability of proteins [7, 11, 12].

Crosslinking using chemical, physical, and/or enzymatic approaches are commonly employed to enhance the properties of protein and other biopolymer-based materials. When proteins react with specific molecules called crosslinkers, interlinks are formed between the

crosslinker and the protein functional groups. Such interconnections improve the mechanical strength of the proteins as a result of the formation of tough and resilient intermolecular covalent bonds between the crosslinks, whereas a decrease in mobility of the polymers and hence elongation of the fibers is noticed. For example, polymers such as soy proteins were crosslinked with transglutaminase for the enhancement of hardness, transparency, and strength for drug release and tissue engineering applications [13, 14]. Similarly, proteins obtained from soybeans and cotton seeds were crosslinked using various aldehydes, glyoxal, and other chemicals for different applications [15-18].

Although many crosslinkers have been used to modify the properties of films, most of the crosslinkers reported are toxic to cells and hence not preferable for health-related applications. Carboxylic acids such as citric acid (CA) helps in cross-linking the polymer without compromising the cytocompatibility [19]. CA is a weak acid with three carboxylic groups, organic and non-toxic as certified by the Food and Drug Administration of the United States of America [20]. The carboxylic functional groups of CA are greatly responsive and proliferate the active sites on the proteins. Enhanced functionality along with ester bond formation can be achieved by CA crosslinking. Also, several reactive groups (-COOH and –OH) existing in CA allow the adhesion of multiple biomolecules and aids in achieving the desired extent of crosslinking [21].

Pongamia pinnata is a non-GMO crop and nitrogen-fixing tree that grows in a varied range of geographical regions. The seed yields non-edible oil which is of medicinal value. The seeds contain lipids (33%), proteins (43%), sugar (20%), and free amino acids (10%)[22, 23].

Pongamia produces oilseeds that are used for various applications like oil for biofuel applications, biogas, for electricity production. Once the oil is removed from the seeds, the deoiled seed cake contains a high amount of crude proteins (30%) which can be

utilized as poultry and cattle feed . This paper for the first time reports the extraction of crude proteins from *Pongamia pinnnata* oil meal and conversion of the proteins into value-added bioproducts.

2. EXPERIMENTAL SECTION

2.1 Materials

Pongamia oil meal was procured from Gandhi Krishi Vignan Kendra, Bangalore, India. Chemicals sodium hydroxide (NaOH) manufactured by Thomas Baker private limited, sodium hypophosphite (SHP) produced by Rolex chemicals private limited, citric acid (CA) from HiMedia laboratories private limited. Acetic acid and hydrochloric acid from Nice chemicals private limited, L-proline, and ninhydrin reagent (2% solution) were purchased from Sigma Aldrich private limited.

Methods

2.2. Extraction of pongamia proteins from the meal

Unfractionated pongamia meal was added into NaOH solution (1% w/v) under continuous stirring for an h at 70 °C. The meal to alkali solution was at a ratio of 1:4 (w/v). Later, the solution was centrifuged at 8000 rpm for 15 minutes. Around 30 ml of 50% v/v acetic acid was added to 400 ml of the supernatant for the protein precipitation to take place. Proteins were obtained after the centrifugation of the precipitated solution. Further, this protein was dried for 24 h at 60 °C [7, 11]. These proteins were used for the preparation of the films. The procedure followed for the protein extraction is schematically shown in Scheme 1.

[insert Scheme 1]

Scheme 1: Schematic representation of the method employed for protein extraction

2.3. Amino acid analysis

The proteins extracted were examined for the various amino acids present in them. A Hitachi L-8900 automatic amino acid analyzer was used for the analysis. Different weights of the

protein (174 mg, 260 mg, and 369 mg) were taken, hydrolyzed at 110±1 °C in 6 mol/L HCl for around 22 h, and later was cooled to room temperature. Nitrogen atmosphere was used to dry the samples for the removal of HCl and was further the amino acids were reconstituted in 5 ml distilled water. Next, the solution was allowed to pass through a 0.22 µm pore size membrane. During the analysis the column temperature was 57 °C, detection wavelength was 570 nm (proline @ 440 nm); buffer flow rate (0.35 ml/minute), and the unit temperature during testing was controlled at 135 °C. The final concentration of amino acids was taken as 100% and the proportion of each type of amino acid was reported.

2.4. Preparation of crosslinked protein films

Pongamia protein (6%w/v) was added into NaOH (0.2 N) solution preheated to 70 °C under continuous stirring for 30 minutes. Different percentages of CA (0 to 20% to the weight of the protein) and catalyst, SHP (50% to the weight of CA) was added to the above solution. Heating along with stirring was sustained for the next 20 minutes. This solution was then poured onto the metal plates and dried at ambient temperatures until the film was formed. For the crosslinking reaction to take place, the dried films were treated at 150 °C for 15 minutes in a vacuum oven [7]. The length and width of the film were multiplied with each other to obtain the area of the film. The dimensions of the developed films was 100mm x 100mm.

2.5. Water sorption studies

The protein films whose initial weights were designated as WI were made into smaller parts (1*1 cm). These smaller portions of the film were immersed in distilled water (around 15 minutes) for the absorption to occur and centrifuged for 10 minutes at 5000 rpm to remove excess water. The final weights (W_f) were calculated. The % water sorption was calculated through the below formula (Equation 1):

$$\% \text{ Water sorption} = \frac{Wf - WI}{WI} \times 100 \quad \text{---------- (1)}$$

2.6. Determining mechanical properties

The tensile characteristics of the pongamia protein films were estimated in the dry state (ASTM standards D 882-02) utilizing a universal tensile tester (Model UTM- G-312C, Shantha engineering, Mumbai). Protein films measuring 10 x 1 cm were prepared. The samples were maintained at room temperature before measurement. The crosshead speed was 30 mm/minute and a load cell with a capacity of 100N was used throughout the analysis. Fifteen specimens for each condition were considered and three replications were done.

2.7. Swelling studies

The pongamia protein films (of uniform thickness) were made into smaller strips having dimensions of 2*2.5 cm and treated in distilled water for 10 minutes. Subsequent changes in the dimension of the films were analyzed. The % increase in the area of the film after swelling (A_f) was calculated using the formula in equation 2:

$$\% \text{ Increase in area} = \frac{Af - AI}{AI} \times 100 \quad \text{---------- (2)}$$

2.8. Fourier transform infrared studies

The FTIR spectrum of the films was determined on a Shimadzu IRAffinity-1S FTIR spectrophotometer. The spectrum was recorded in total attenuated reflectance mode with the aid of a diamond cell. The range of scanning was 400 - 4000 cm^{-1} and 64 scans were done for each sample. Lab solutions series software was employed to examine the obtained spectra.

2.9. Scanning electron microscope images

The surface features of the films were captured using a scanning electron microscope (Hitachi SU3500). The protein samples were coated with gold particles using an ion sputter (Hitachi Ion Sputter MC1000) for 60 seconds since they were nonconducting. The current maintained was 1.8 mA and the images were captured at an operating voltage of 5kV.

2.10. Thermo Gravimetric Studies

Changes in the thermal behavior of pongamia protein powder and the crosslinked films were studied with the help of a Thermo Gravimetric Analyser (Mettler Toledo, Model 822e). Sealed aluminium pans were used to place the samples and heating was carried out at a rate of 10 °C/min starting from 25 °C to 600 °C under nitrogen atmosphere.

2.11. Antioxidant studies

The antioxidant properties in terms of the scavenging activity of the films were obtained using ABTS+ (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) radical decolorization assay. The ABTS+ cation radicals were produced by reaction between 7 mM ABTS and 2.45 mM ammonium persulfate by incubating in dark for 16h to obtain ABTS+. The standard antioxidant compound used was ascorbic acid (1mg/mL).A stock solution of pongamia protein (1mg/mL) was prepared by dissolving in DMSO (Di Methyl Sulphoxide). Different aliquots (10µg/ml to 50µg/ml) of this stock was taken and the volume was adjusted to 500 µL using DMSO. To all the tubes 2500 µL of ethanol was added followed by 300 µL of ABTS+ reagent and incubated for 30 minutes at room temperature in dark, the absorbance was measured at 745nm[24]. The blank comprised 500 µL of DMSO, 500 µL of ethanol, and 150 µL of ABTS+. All the test-tubes displayed decolorization and this was expressed as the

inhibition percentage of the cation available in the sample. The percentage of scavenging activity was determined using equation 3. Next, scavenging activity was plotted against protein concentration in an excel sheet. The linear trendline was added to the plotted curve, further R^2 and a regression equation were obtained in the form of y=mx+c. X is IC50 which needs to be calculated, y is 50 since we are calculating IC50 and a simple substitution of the values provides us IC50. Ascorbic acid was used as positive control and tests were carried out in triplicates.

$$\textit{ABTS radical scavenging Activity (\%)} = \frac{\textit{Control OD} - \textit{Sample OD}}{\textit{Control OD}} \times 100 \quad \text{------ (3)}$$

2.12. Antimicrobial studies

The antimicrobial studies were conducted using the agar well diffusion method [25].

A starter culture of bacteria (*Bacillus cereus* and *E coli*) was prepared by taking a loop full of bacteria from the master culture. This was inoculated in Mueller Hinton broth separately and incubated at 37± 1 °C for 2h on a shaker at 120 rpm. 100 µL of the active bacterial cultures were taken from the broth and spread on Mueller Hinton agar medium using sterile swabs. Wells were punched using a sterile steel cork borer (6mm internal diameter) and 100 µL of the samples were loaded. The standard antibacterial compound used was ampicillin (1mg/mL). Post incubation (at 37 °C for 24 h), the zone of inhibition was measured in millimeters. For antifungal activity, a loop full of fungal spores was suspended in Mueller Hinton broth and a method similar to antibacterial study was followed to know the activity against *Aspergillus niger*. The standard antifungal compound used was fluconazole (1mg/mL). The zone of inhibition was measured in millimetres after 48 h of incubation at 28 °C. The test was conducted in triplicates.

2.13. Developing bioproducts

The proteins extracted from *Pongamia pinnata* oil meal were verified for their likeliness to be shaped into diverse forms for various applications. Protein films were obtained by adding 15% CA and 40% glycerol to the weight of pongamia protein. The obtained films were compressed at 140°C for five minutes into 3D cups and boxes utilizing specific moulds. Pictures of the developed bioproducts are incorporated in the manuscript.

2.14. Statistical analysis

For all the above-stated experiments three trials were performed. The average for all three trials along with standard deviations was measured and reported. ± indicate one standard deviation in the error bars.

3. Results and Discussion

3.1. Crosslinking reaction mechanism

The crosslinking mechanism between CA and protein amine groups is demonstrated in figure 1. Initially, SN2 nucleophilic substitution reaction occurred between protein amine groups and carboxyl groups of citric acid[10]. Since three carboxyl groups are present in citric acid, each citric acid molecule interacted with more than one amine group leading to the formation of intra and intermolecular crosslinks. As depicted in reaction (1), COOH and NH_3 were protonated into COO- and NH_2 in an alkaline medium. COO^- which carries a partial positive charge was attacked by amine nitrogen leading to the formation of an amide as demonstrated in reaction (2). Further, the proteins interacted with the remaining carboxyl group of citric acid as shown in reaction (3).

[insert Figure 1]

Figure1: Mechanism of crosslinking reaction between citric acid and amine group of pongamia protein. P1, P2, and P3 represent pongamia proteins.

3.2. Amino acid analysis

Crude protein was extracted from *Pongamia pinnata* oil meal and the percentage weight fraction of the protein obtained after extraction was around 24. The amino acid analysis was performed to understand the protein content of *Pongamia pinnata* in general. A comparison of the relative % of amino acids present in extracted pongamia proteins with zein and soy proteins is provided in table 1. The amino acid content of pongamia protein is comparable to that found in other plant sources. Glutamic acid was the primary amino acid (12.2%) present in pongamia. Methionine with 1.8% was the lowest amino acid in pongamia unlike in soy and zein which was cysteine [26].

Table 1: Relative proportion of amino acids extracted from *Pongamia pinnata* oil meal, zein, and soy[26].

Amino acid (%)	Type of protein		
	Pongamia	Zein	Soy
Threonine	2.9	1.8	2.3
Methionine	1.8	1.1	0.3
Phenyl alanine	5.4	3.4	3.2
Histidine	3.7	1.1	1.5
Lysine	3.0	1.0	3.4
valine	8.3	2.1	2.2
Isoleucine	4.7	1.7	1.9
Leucine	8.2	8.8	5.0
serine	5.6	2.9	3.4
Glycine	7.2	1.6	2.7
Glutamic acid	12.2	13.1	12.4
Proline	5.6	5.2	3.3
Cysteine	4.4	0.3	0.2
Alanine	6.5	4.8	2.8
Tyrosine	6.2	2.7	2.2
Arginine	7.7	1.7	4.8

3.3. Water sorption studies

Approximately, 24 hrs was required to dry the films at ambient temperature. The thickness of the films was around 0.2mm. The ability of different pongamia protein films to sorb water is depicted in figure 2. protein The water absorption abilities of the film were dependent on the percentage of CA added. Uncrosslinked film absorbed 438% of water whereas maximum sorption of 788% was seen in the case of 5% CA crosslinked pongamia protein films. Minimum sorption of 346% was seen in 20% CA crosslinked films. The uncrosslinked and 5% CA crosslinked films dispersed/disintegrated after water sorption for 15 minutes. However, 10, 15 and 20% crosslinked films were stable and intact even after centrifugation. This relationship between CA % and water sorption % can be ascribed to the formation of crosslinks between the amine/hydroxyl groups of proteins and carboxyl acid groups of CA [27, 28]. In 5% CA films, the amount of CA added was not sufficient enough to crosslink all the freely available functional groups of proteins. Hence, there were many free functional groups present in the protein film in addition to those present in CA available for H- bonding with water molecules. The number of these free amino and hydroxyl groups decreases with increasing CA concentration. Therefore, the least water sorption was seen with 20% CA crosslinked film owing to the unavailability of free amino/ hydroxyl groups [29].

[insert Figure 2]

Figure 2: Effect of citric acid percentage on % water absorption of pongamia protein films.

3.4. Mechanical properties

The tensile strength of pongamia protein films when crosslinked with varying % of CA is shown in figure 3. Maximum strength of 1.9 MPa was exhibited by 15% CA crosslinked pongamia protein film. The crosslinked films (except 20% CA) displayed higher strength in comparison to the uncrosslinked films [7]. The formation of crosslinks between the amino/

hydroxyl groups of proteins and carboxylic groups of CA takes place leading to the establishment of interconnected molecules. This network is responsible for the increased strength of crosslinked films. However, 20% CA crosslinked film displayed considerably low strength in comparison to the uncrosslinked film. This low strength of 0.230 MPa in 20 % CA film is due to the protein precipitation as the addition of CA reduces the pH. Protein dissolution for film formation requires alkaline conditions. Both CA and SHP are considered acidic and when added at higher levels leads to precipitation or partial dissolution of the proteins. Besides, excess CA leads to over crosslinking which limits the mobility of molecules and also decreases strength. Hence films formed at high concentrations of CA have poor tensile properties.

[insert Figure 3]

Figure 3: Effect of citric acid percentage on tensile strength of pongamia protein films. The films were made using a protein concentration of 6% and a catalyst (SHP) equivalent to 50% weight of the crosslinker.

3.5. Swelling studies

The swelling % of various CA crosslinked pongamia protein films upon immersion in water is shown in Figure 4. Within 10 minutes the uncrosslinked film disintegrated and started dissolving. protein Among the CA crosslinked films, 10% CA pongamia protein film exhibited a maximum swelling of 208% and the least swelling of 120% was seen in 20% CA crosslinked film. The swelling properties of the film can be attributed to the existence of free amino/ hydroxyl groups in proteins as discussed earlier for water sorption. The amino/hydroxyl groups present in the proteins crosslink with the carboxylic groups of CA to form a network. These crosslinks formed during the process of crosslinking make the functional groups in proteins unavailable for H- bonding with water molecules. Hence the %

increase in the area of the pongamia protein films decreases with an increase in the percentage of CA. However, initially, when 0 to 10% CA was added, swelling of the film was seen although no apparent dissolution or weight loss was observed. This suggests that there were many hydroxyl/amino groups left uncrosslinked and have led to the formation of hydrogen bonding with water molecules. When CA concentration was increased from 10 to 20%, a decrease in % swelling area was noticed as discussed above.

[insert Figure 4]

Figure 4: Effect of citric acid % on the swelling abilities of pongamia protein films.

3.6. FTIR studies

The FTIR spectra of pongamia protein films without crosslinking and after crosslinking with 15% CA is shown in figure 5. The broad peak at 3400 cm^{-1} in the crosslinked film is due to the presence of OH peaks both from protein and CA. The intensity of this peak is less in uncrosslinked film since the contribution of the OH groups is only from the proteins. A peak at 2900 cm^{-1} was detected as a result of the C-H stretching vibration. This peak is prominent in crosslinked films due to the presence of C-H groups from CA in addition to those present in the proteins. A large, broad peak at 1600 cm^{-1} is because of the C-O ester groups formed as a result of the crosslinking reaction. The peak seen around 1538 cm-1 is due to amide group vibrations. The peaks at 1200-1400 cm^{-1} can be attributed to the COC groups formed due to the interaction between proteins and CA. All the above peaks in addition to those present in uncrosslinked films along with increased intensity in the crosslinked film confirm the process of crosslinking. However, no trend was observed in the intensities of the peaks with increasing CA concentration [30].

[insert Figure 5]

Figure 5: FTIR spectra of the pongamia protein film before and after 15% citric acid crosslinking.

3.7. Morphology analysis

Images captured for the uncrosslinked and 15% CA crosslinked pongamia protein films using a scanning electron microscope (SEM) are provided in Figure 6. The uncrosslinked films were non-uniform with large cracks and holes compared to the 15% CA crosslinked films. The addition of CA was found to minimize the degree of non-uniformity in the crosslinked films by decreasing the number of cracks and holes. As an evidence to this observation, the extent of holes and cracks has reduced substantially in 15% CA crosslinked pongamia protein films [7]. Such change in morphology can be credited to the crosslinking process which binds the individual polymer chains together leading to the creation of a network. The formation of this network probably would have led to a change in the external morphology of the film by reducing the number of holes and making the surface even. Also, in the process of crosslinking, the protein film shrinks due to high temperature which may also be the reason for the uniform morphology of the crosslinked film.

[insert Figure 6]

Figure 6: Scanning electron microscope images of the uncrosslinked (A), 15% citric acid crosslinked pongamia protein film (B).

3.8. Thermogravimetric analysis

The thermal behavior of pongamia protein and 15% CA crosslinked film is shown in Figure 7. As seen from the TGA curve, the degradation patterns of both the powder and crosslinked films are similar except that the latter degrades in three steps compared to the protein powder. The exact degradation temperature is noticeable in the DTA curves. Both the protein powder and crosslinked film displayed a pronounced endothermic effect around 150-470 ^{o}C during

the degradation process. Most of the uncrosslinked protein powder degraded at around 303 °C in one single step. In the case of crosslinked film, endothermic peaks were observed at 3 different temperatures. This variation in degradation temperature can be explained based on the extent of the crosslinking of pongamia proteins. Initially, the uncrosslinked/low molecular weight proteins degraded around 160°C, the majority of the remaining proteins which had not crosslinked effectively degraded around 257 °C. The remaining proteins which had crosslinked effectively and had a higher molecular weight required a higher temperature around 470 °C to degrade. This is because of the formation of strong covalent crosslinks between amino/hydroxyl of proteins with carboxylic groups of CA. However, these crosslinks are absent in uncrosslinked proteins and hence lower temperature is sufficient for complete degradation of pongamia proteins.

A similar observation was reported by Reddy, 2012, wherein higher temperatures were required to melt crosslinked peanut proteins using CA in comparison to the uncrosslinked proteins [7]. In another work by Nataraj et al 2018, banana fiber and wheat gluten were crosslinked using CA and glutaraldehyde. It was reported that CA crosslinked films were more resistant to thermal degradation compared to the glutaraldehyde crosslinked films. [27]

[insert Figure 7]

Figure 7: Images showing the Thermogravimetric (left) and Differential thermogravimetric curves (right) of pongamia protein powder and 15% citric acid crosslinked protein film.

3.9. Antioxidant studies

Scavenging activity is expressed as the ability of the sample to help shield the cells from harm by free radicals. Pongamia protein has intrinsic scavenging potential [30, 31]. However, crosslinking using CA further boosted this potential. As a sign of this observation, the highest

scavenging was displayed by 20 % CA crosslinked protein film compared to others. IC_{50} is the half-maximal inhibitory concentration of the sample that is required to inhibit a biological process by half. This potency varied between the crosslinked and uncrosslinked films. Amongst all the CA crosslinked films, 20% crosslinked film showed excellent antioxidant potential (IC_{50} value of 14.6µg/mL) in comparison to ascorbic acid (IC_{50}-26.9 µg/mL). Thus, it can be inferred that CA crosslinking enhances the antioxidant properties of pongamia protein films. Also, it can be observed that the IC_{50} value obtained is inversely related to the concentration of CA. The scavenging activity of the protein films was plotted against varying concentrations of proteins (figure 8). Similar work carried out by Chopade showed that *Pongamia pinnata* has antioxidant activity in ammonium chloride-induced hyperammonium rats[31].

In another study by Sajid, the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity % of *Pongamia pinnata* seed extract was found to be 15.7-38.0 µg/ml which was quite high in comparison to the values obtained in our studies[32].

[insert Figure 8]

Figure 8: Radical scavenging activity of the pongamia protein films at various concentrations.

3.10. Antimicrobial studies

Antimicrobial studies were performed to assess the antibacterial and antifungal potential of pongamia proteins against test organisms. Table 2 provides the width of the inhibition zone for various samples. In the case of *Bacillus cereus,* the uncrosslinked protein film showed a zone width of 10mm, whereas the 20% CA crosslinked film showed a width of 11.75mm which is close to the value of 11.66 seen for the standard antibacterial compound ampicillin. For E coli, 20% CA crosslinked film showed a maximum zone of inhibition (12.33 mm) which is not comparable to the standard antibacterial compound ampicillin (37mm). In a similar study, it was reported that *Ricinus communis* and *Periploca aphylla* both showed

zones of inhibition of 0.3 mm for *E-coli* which is significantly less in comparison to *Pongamia pinnata* [33]. Hence, the crosslinked films were additionally active against *Bacillus cereus* rather than against *E-coli* [29]. However, for both the bacterial strains it can be observed that the zones of inhibition increased with increasing % of CA, and the inherent antimicrobial property of the protein was retained even after the addition of CA.

The effects of the antifungal activity in the films were evident based on the changes in the zones of inhibition of the uncrosslinked and the standard compound fluconazole. The zones of inhibition were almost similar and no significant difference was observed. However, it was realized that the addition of CA decreased the width of zone of inhibition implying that CA has a negative consequence on the antifungal activity of pongamia proteins because of the acidic nature of CA impeding the development of the fungi [34-37].

.

Table 2: Inhibition zones of pongamia protein films against different microbes in comparison to the standard drug.

Citric acid %	Width of Inhibition zone (mm)		
	Bacillus cereus	***E-coli***	***Aspergillus niger***
0	10 ± 0	10.33± 0.57	14.66± 0.57
10	9 ± 0	9.66±0.57	12.66±0.57
15	9.75 ± 0.35	11.66± 0.57	13± 0
20	11.75± 0.35	12.33± 0.57	10.33± 1.15
Standard drug	11.66± 0.57 (Ampicillin)	37±1 (Ampicillin)	14± 0 (Fluconazole)

3.11. Potential applications of the extracted proteins

A two-step process of obtaining protein films and later molding the films into various shapes enables developing bioproducts from pongamia proteins using biocompatible and food grade chemicals. Glycerol was not removed after moulding. Some glycerol did come out when higher concentrations or pressure was used. Browning of the films due to Maillard reactions was observed after moulding. There was a noticeable but pleasant odour after compression.. Packaging boxes (Figure 9) suitable for food and non-food uses have been prepared using the extracted pongamia proteins. More importantly, the products developed are chemical-free, non-hazardous, and completely biodegradable. They contribute towards a clean environment and also sustainable development. Since pongamia proteins have inherent antimicrobial, antioxidant and thermal stability, these bioproducts can be ideal for food and non-food packaging applications. Similar bioproducts were developed from castor oil cake, wheat gluten/ banana fiber, chitosan by citric acid crosslinking[27, 30, 38].

[insert Figure 9]

Figure 9: Three-dimensional cups and boxes suitable for food and non-food packaging have been developed from pongamia protein, demonstrating the feasibility of preparing bioproducts for commercial applications.

4. CONCLUSIONS

Pongamia proteins can be made into films and other bioproducts with good mechanical properties and water stability required for food packaging. Under optimized conditions, crosslinking with 10% CA increased the strength of the films by about 28% in comparison to the uncrosslinked films. As an evidence to increased water stability of the crosslinked films, minimum swelling of 120% was seen in 20% CA crosslinked film whereas

the uncrosslinked films readily disintegrated in water. Bioproducts made from pongamia proteins show excellent antioxidant properties with an IC_{50} value of 14.6 compared to 26.9 for the ascorbic acid standard. In addition to the above properties, substantial antibacterial and antifungal activity was also exhibited by the pongamia proteins and films against *Bacillus cereus* and *Aspergillus niger,* respectively. This method of producing biodegradable, plant protein-based bioproducts can be used for a wide variety of food and medical applications where antifungal, antibacterial activity and antioxidant properties are highly desirable. Additional studies are being conducted to regulate the shelf-life, effects of temperature, humidity, and gas barrier properties on the contents stored in these packaging materials.

ACKNOWLEDGMENTS

The authors would like to acknowledge CIIRC, Jyothy Institute of technology, Thathaguni for their monetary funding. Narendra Reddy appreciates the Department of Biotechnology, Ministry of Science and Technology, Government of India for funding through the Ramalingaswami Re-entry Fellowship.

Declaration of interest

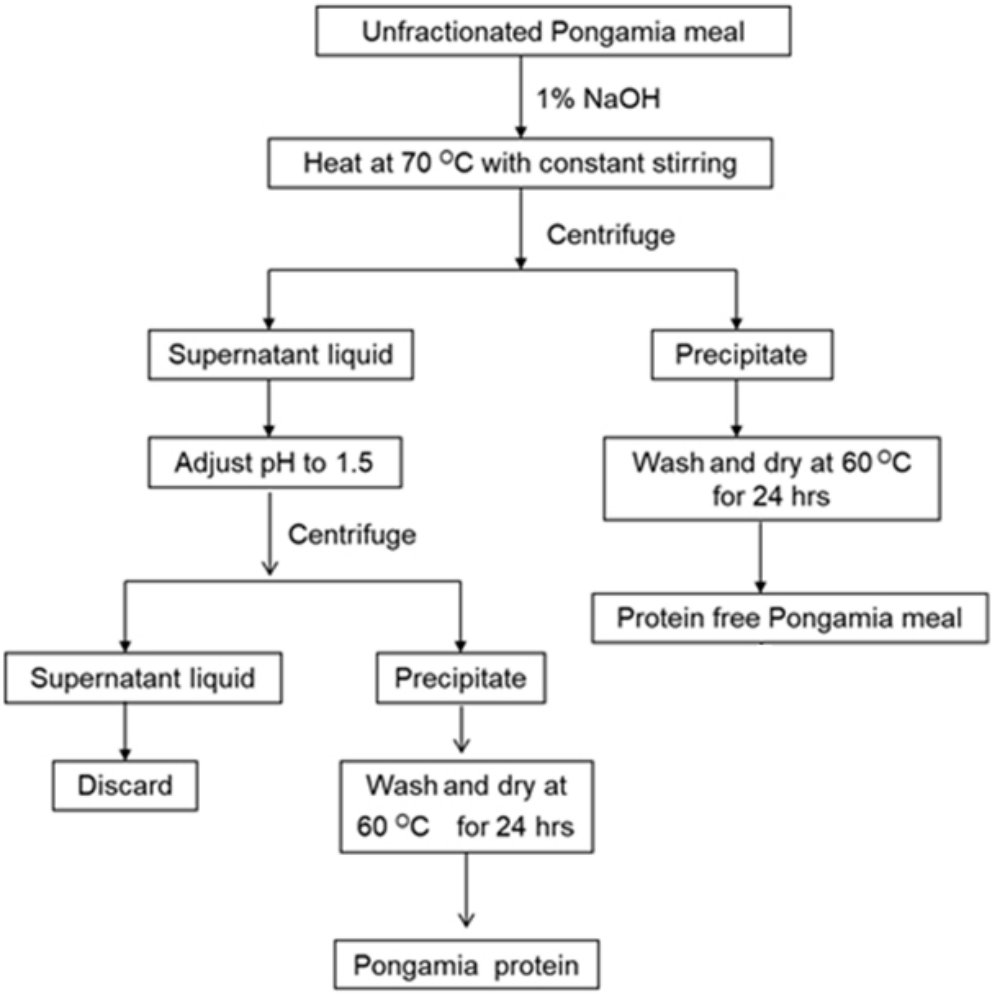
Authors state that they do not have any conflict of interest

REFERENCES

1. Naik M, Meher L, Naik S, et al. Production of biodiesel from high free fatty acid Karanja (*Pongamia pinnata*) oil. *Biomass Bioenerg* 2008; 32(4): 354-357.
2. Taheripour F, Hertel TW, Tyner WE, et al. Biofuels and their by-products: Global economic and environmental implications. *Biomass Bioenerg* 2010; 34(3): 278-289.
3. Chan G and Mooney DJ. New materials for tissue engineering: towards greater control over the biological response. *Trends Biotechnol* 2008; 26(7): 382-392.
4. Chen L, Remondetto GE and Subirade M. Food protein-based materials as nutraceutical delivery systems. *Trends Food Sci Technol* 2006; 17(5): 272-283.
5. Stegemann JP, Kaszuba SN and Rowe SL. Advances in vascular tissue engineering using protein-based biomaterials. *Tissue Eng* 2007; 13(11): 2601-2613.

6. Liu X, Sun Q, Wang H, et al. Microspheres of corn protein, zein, for an ivermectin drug delivery system. *Biomaterials* 2005; 26(1): 109-115.
7. Reddy N, Jiang Q and Yang Y. Preparation and properties of peanut protein films crosslinked with citric acid. *Ind. Crops Prod* 2012; 39(2012): 26-30.
8. Reddy N, Chen L and Yang Y. Biothermoplastics from hydrolyzed and citric acid crosslinked chicken feathers. *Mater. Sci. Eng: C* 2013; 33(3): 1203-1208.
9. Reddy N and Yang Y. Potential of plant proteins for medical applications. *Trends Biotechnol* 2011; 29(10): 490-498.
10. Xu H, Shen L, Xu L et al. Low-temperature crosslinking of proteins using non-toxic citric acid in neutral aqueous medium: Mechanism and kinetic study. *Ind. Crops Prod* 2015; 74 (2015): 234-240.
11. Hu C, Nataraj D and Reddy N. Acetylation of Castor Meal and Castor Proteins for Thermoplastic Applications. *J Polym Environ* 2018; 26(4): 1371-1377.
12. Vananuvat P and Kinsella JE. Preparation of succinylated yeast protein: composition and solubility. *Biotechnol Bioeng* 1978; 20(9): 1329-1344.
13. Gan CY, Cheng LH and Easa A. Assessment of Cross-Linking in Combined Cross-Linked Soy Protein Isolate Gels by Microbial Transglutaminase and Maillard Reaction. *J. Food Sci* 2009; 74(2): C141-C146.
14. Reddy N, Jiang Q and Yang Y. Novel wheat protein films as substrates for tissue engineering. *Biomater. Sci. Polymer Edition* 2011; 22(15): 2063-2077.
15. Essandoh M, Garcia RA and Nieman CM. Chemical and Enzymatic Protein Cross-Linking To Improve Flocculant Properties. *ACS Sustain Chem Eng* 2018; 6(10): 12946-12952.
16. Wang Y, Mo X, Sun XS, et al. Soy protein adhesion enhanced by glutaraldehyde crosslink. *J. Appl. Polym. Sci* 2007; 104(1): 130-136.
17. Caillard R, Remondetto G, Mateescu M, et al. Characterization of Amino Cross-Linked Soy Protein Hydrogels. *J. Food Sci* 2008; 73(5): C283-C291.
18. Marquié C. Chemical reactions in cottonseed protein cross-linking by formaldehyde, glutaraldehyde, and glyoxal for the formation of protein films with enhanced mechanical properties. *J. Agric. Food Chem* 2001; 49(10): 4676-4681.
19. Reddy N, Li Y and Yang Y. Alkali-catalyzed low temperature wet crosslinking of plant proteins using carboxylic acids. *Biotechnol Progr* 2009; 25(1): 139-146.
20. Gyawali D, Nair P, Zhang Y, et al. Citric acid-derived in situ crosslinkable biodegradable polymers for cell delivery. *Biomaterials* 2010; 31(34): 9092-9105.
21. Tran RT, Thevenot P, Gyawali D, et al. Synthesis and characterization of a biodegradable elastomer featuring a dual crosslinking mechanism. *Soft matter* 2010; 6(11): 2449-2461.
22. Usharani K, Naik D and Manjunatha R. *Pongamia pinnata* (L.): composition and advantages in agriculture: a review. *J Pharmacogn Phytochem* 2019; 8(3): 2181-2187.
23. Orwa C, Mutua A, Kindt R, et al. Agroforestree Database: a tree reference and selection guide. Version 4. 2009.
24. Re R, Pellegrini N, Proteggente A, et al. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radic. Biol. Med* 1999; 26(9-10): 1231-1237.
25. Bauer A, Kirby W, Sherris JC et al. Antibiotic susceptibility testing by a standardized single disk method. *Am J Clin Pathol* 1966; 45(4): 149-158.
26. Gorissen SH, Crombag JJ, Senden JM, et al. Protein content and amino acid composition of commercially available plant-based protein isolates. *Amino acids* 2018; 50(12): 1685-1695.
27. Nataraj D, Sakkara S, Meenakshi H, et al. Properties and applications of citric acid crosslinked banana fibre-wheat gluten films. *Ind. Crops Prod* 2018; 124(6): 265-272.

28. Ifebajo AO. Synthesis and characterisation of chemically crosslinked chitosan citrate gels. Eastern Mediterranean University (EMU)-Doğu Akdeniz Üniversitesi (DAÜ), 2014.
29. Sagnella S and Mai-Ngam K. Chitosan based surfactant polymers designed to improve blood compatibility on biomaterials. *Colloids Surf. B* 2005; 42(2): 147-155.
30. Nataraj D, Sakkara S, Meghwal M, et al. Crosslinked chitosan films with controllable properties for commercial applications. *Int. J. Biol. Macromol* 2018; 120(A): 1256-1264.
31. Chopade V, Tankar A, Pande V, et al. *Pongamia pinnata*: Phytochemical constituents, traditional uses and pharmacological properties: A review. *Int. J. Green Pharm* 2008; 2(2): 72-75.
32. Sajid ZI, Anwar F, Shabir G, et al. Antioxidant, antimicrobial properties and phenolics of different solvent extracts from bark, leaves and seeds of *Pongamia pinnata* (L.) Pierre. *Molecules* 2012; 17(4): 3917-3932.
33. Iqbal J, Zaib S, Farooq U, et al. Antioxidant, Antimicrobial, and free radical scavenging potential of aerial parts of *Periploca aphylla* and *Ricinus communis*. *ISRN pharmacology* 2012; 2012:1-6.
34. Wagh P, Rai M, Deshmukh S, et al. Bio-activity of oils of *Trigonella foenum-graecum* and *Pongamia pinnata*. *Afr. J. Biotechnol* 2007; 6(13):1592-1596.
35. Shokri H. Evaluation of inhibitory effects of citric and tartaric acids and their combination on the growth of *Trichophyton mentagrophytes*, *Aspergillus fumigatus*, *Candida albicans*, and *Malassezia furfur*. *Comp Clin Path* 2011; 20(5): 543-545.
36. Jay JM, Loessner MJ and Golden DA. *Modern food microbiology*. Springer Science & Business Media, 2008.
37. Beuchat LR. Surface decontamination of fruits and vegetables eaten raw. 1998. http://www.who.int/foodsafety/publications/fs_management/surfac_decon/en/surface_decon.pdf
38. Silva RV, Gonçalves AD, Vinhal JO, et al. Bioproducts from the pyrolysis of castor seed cake: Basic dye adsorption capacity of biochar and antifungal activity of the aqueous phase. *J. Environ. Chem. Eng* 2021; 9(1): 104825-104839.



Schematic representation of the method employed for protein extraction

161x166mm (96 x 96 DPI)

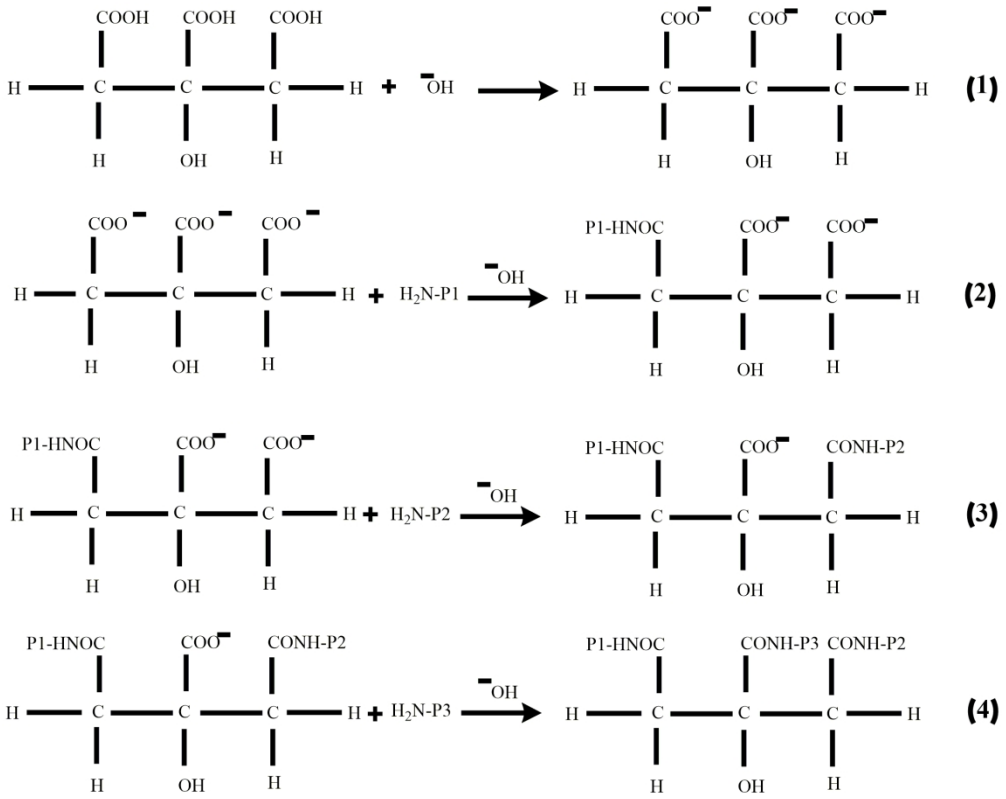


Figure 1 Mechanism of crosslinking reaction between citric acid and amine group of pongamia protein. P1, P2, and P3 represent pongamia proteins

187x148mm (300 x 300 DPI)

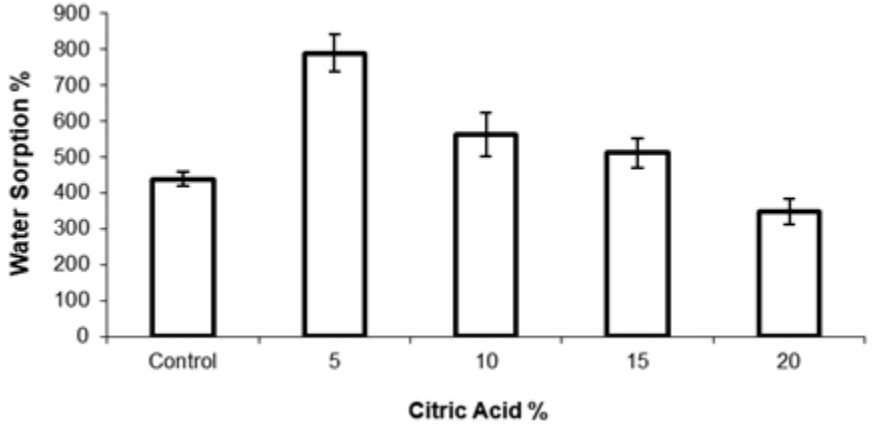


Figure 2: Effect of citric acid percentage on % water absorption of pongamia protein films.

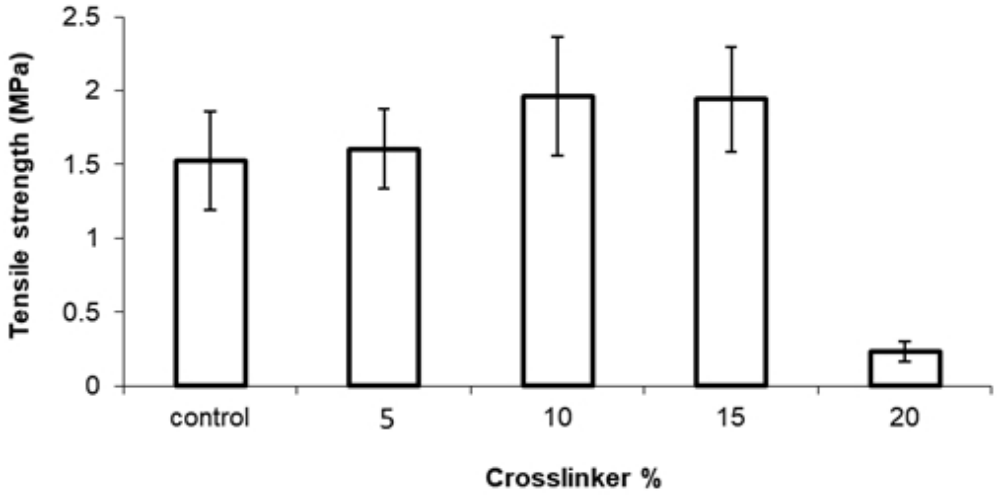


Figure 3: Effect of citric acid percentage on tensile strength of pongamia protein films. The films were made using a protein concentration of 6%(w/v) and a catalyst (SHP) equivalent to 50% to the weight of the crosslinker.

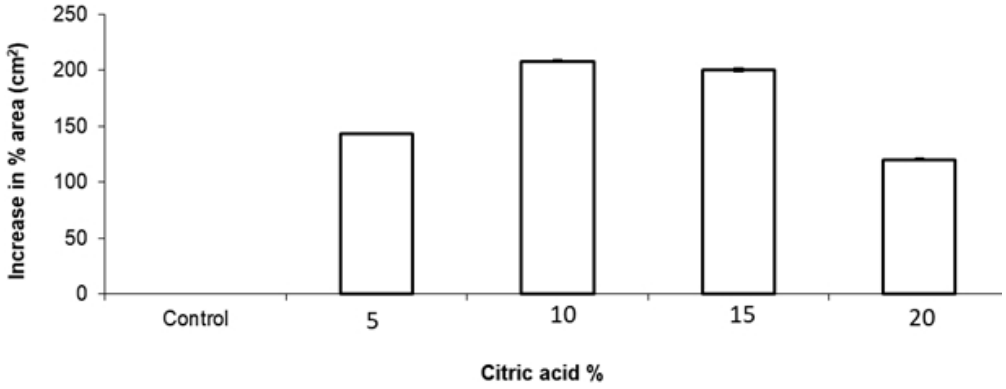


Figure 4: Effect of citric acid percentage on water absorption of pongamia protein films.

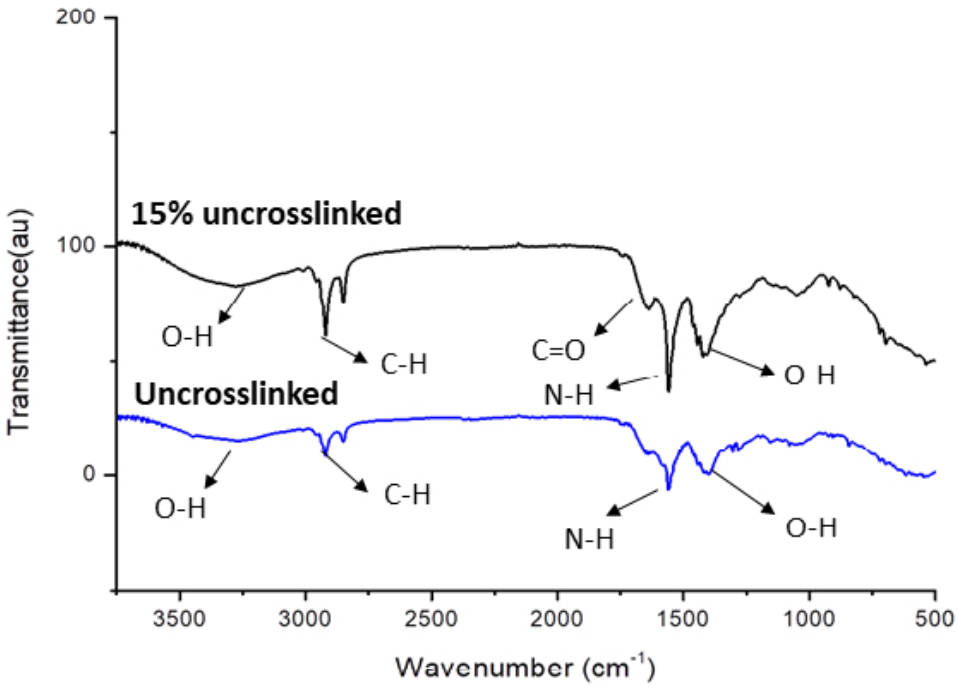


Figure 5: FTIR spectra of the pongamia protein film before and after crosslinking with 15% CA.

154x111mm (96 x 96 DPI)

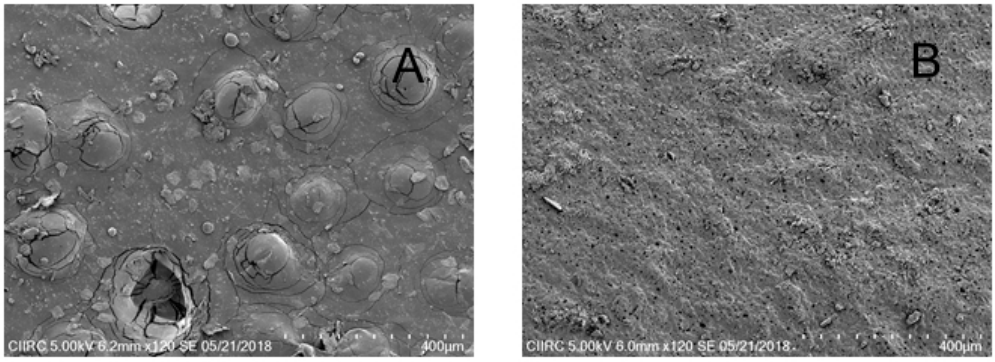


Figure 6: Scanning electron microscope images of the uncrosslinked (A), 15% CA crosslinked pongamia protein film (B).

175x67mm (96 x 96 DPI)

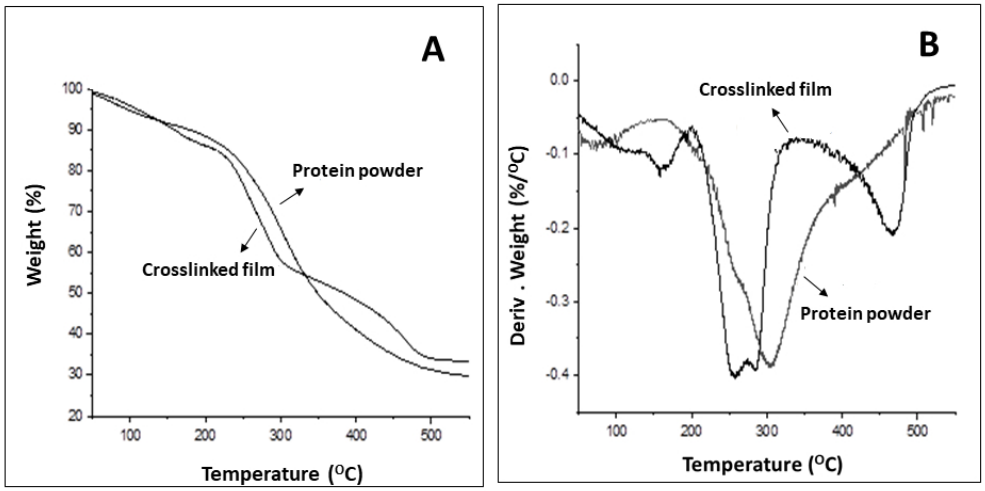


Figure 7 Images showing the Thermogravimetric (left) and Differential thermogravimetric curves (right) of pongamia protein powder and 15% citric acid crosslinked protein film.

165x84mm (144 x 144 DPI)

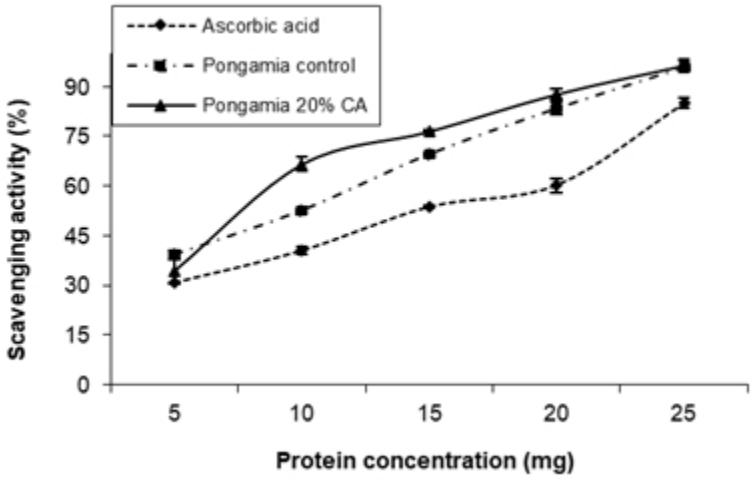


Figure 8: Radical scavenging activity of the protein films at various concentrations.

105x73mm (96 x 96 DPI)

Figure 9: Three-dimensional cups and boxes suitable for food and non-food packaging have been developed from pongamia protein, demonstrating the feasibility of preparing bioproducts for commercial applications.

184x72mm (96 x 96 DPI)

JOURNAL: Journal of Bioactive and Compatible Polymers

Submission no: JBC-20-0123

Response to reviewer comments

We thank the reviewers for his/her time to review our manuscript and finding our results useful and publishable. We also thank the reviewers for providing the valuable comments which have helped us to make our paper better.

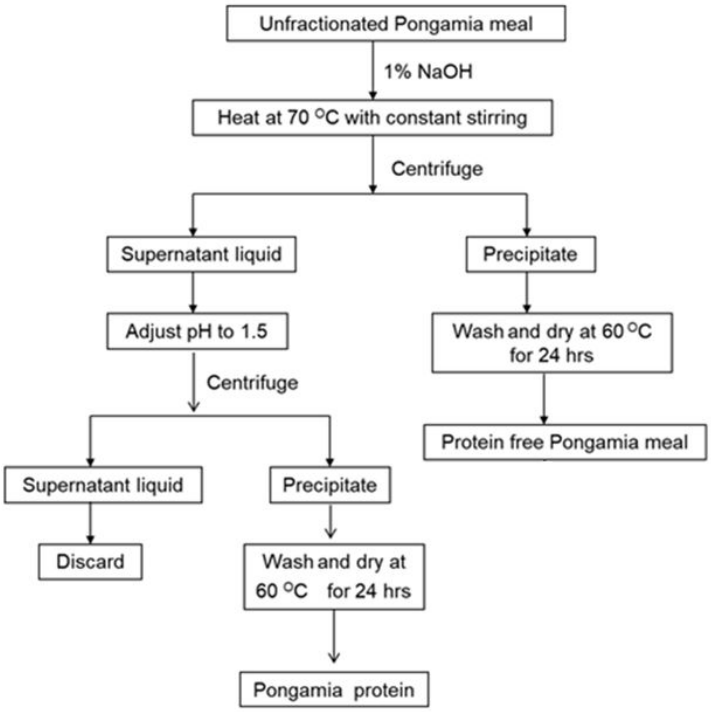
Editor's Comments to Author

Comment 1: From "Scheme 1: Schematic representation of the method employed for protein extraction and preparation of films from the extracted proteins." to Scheme 1: Schematic representation of the method employed for protein extraction.

Response: The caption of scheme 1 is changed into "Schematic representation of the method employed for protein extraction" as per the editor's suggestion.

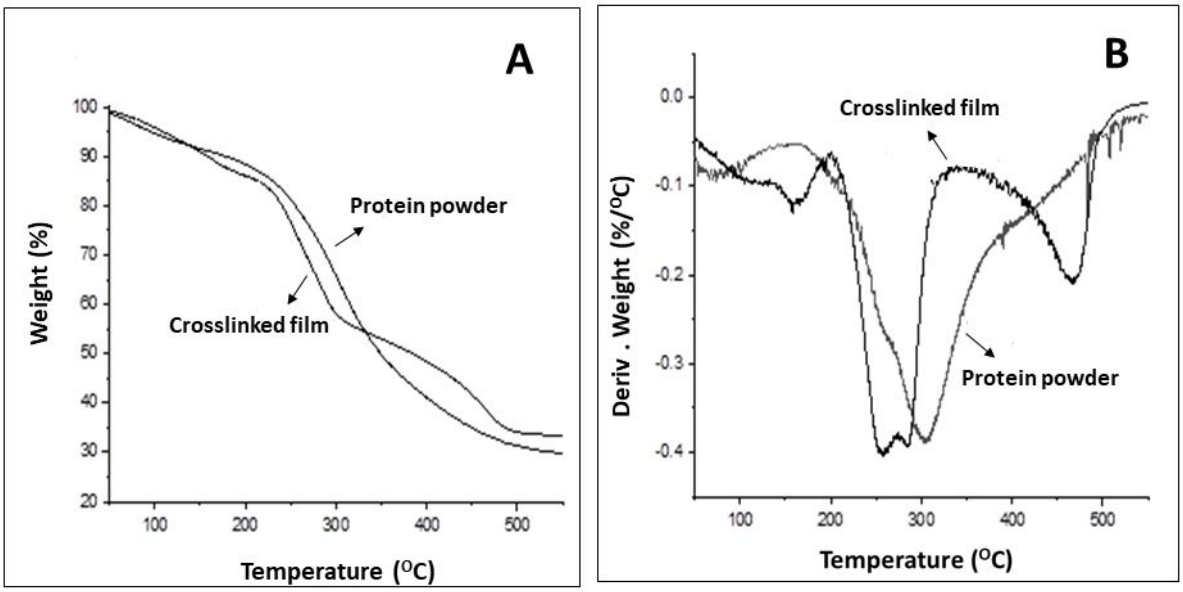
Comment 2: And delete the box for 'bioproducts'.
Or include processes for the preparation of films and moulded products from the extracted proteins and change the title accordingly.

Response: We have deleted the box for bioproducts as per the suggestion. The revised scheme is included in the revised manuscript.



Comment 3: Figure 7: Y-axis to stop at 100%, since greater than 100% wt is not possible.

Response: We apologise to the editor and figure 7 is modified as per the suggestion and is included in the revised manuscript.



Reviewer(s)' Comments to Author:
Reviewer: 2

Comment 1: In table for amino acid add the units.

Response: We apologise to the reviewer for the error and the units (%) has been mentioned in the revised manuscript:

Amino acid (%)	Type of protein		
	Pongamia	Zein	Soy
Threonine	2.9	1.8	2.3
Methionine	1.8	1.1	0.3
Phenyl alanine	5.4	3.4	3.2
Histidine	3.7	1.1	1.5
Lysine	3.0	1.0	3.4
valine	8.3	2.1	2.2
Isoleucine	4.7	1.7	1.9
Leucine	8.2	8.8	5.0
serine	5.6	2.9	3.4
Glycine	7.2	1.6	2.7
Glutamic acid	12.2	13.1	12.4
Proline	5.6	5.2	3.3
Cysteine	4.4	0.3	0.2
Alanine	6.5	4.8	2.8
Tyrosine	6.2	2.7	2.2
Arginine	7.7	1.7	4.8

Comment 2: Zein and soy amino acids were estimated or are taken from literature, if from literature add references.

Response: The following reference has been added in the revised manuscript:

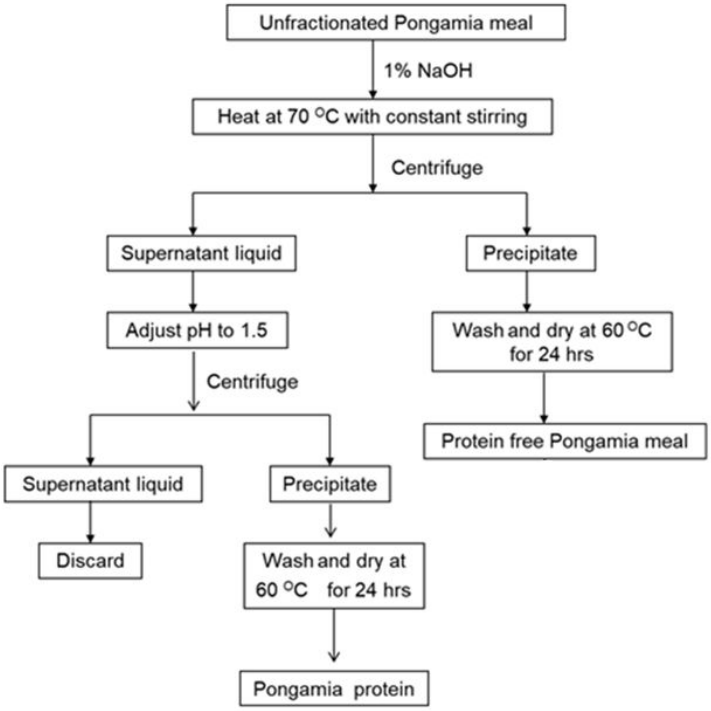
Gorissen SH, Crombag JJ, Senden JM, et al. Protein content and amino acid composition of commercially available plant-based protein isolates. *Amino acids* 2018; 50(12): 1685-1695.

Comment 3: Use h for hour or hours.

Response: We agree with the reviewer and the suggested modification has been incorporated in the revised manuscript.

Comment 4: In scheme 1 title may be changed film is missing in scheme bioproducts is shown in title film word is written.

Response: The caption of scheme 1 is changed into "Schematic representation of the method employed for protein extraction" as per the reviewer's suggestion. We have deleted the box for bioproducts as per the suggestion. The revised scheme is included in the revised manuscript.



Extraction and Characterisation of Bioactive Proteins from *Pongamia pinnata* and their Conversion into Bioproducts for Food Packaging Applications

~~Bioproducts from *Pongamia pinnata* Proteins for Food Packaging Applications~~

Divya Nataraj, Vijaykumar Guna, Prajwal Battampara, Nagananda G.S, Narendra Reddy*

Center for Incubation, Innovation, Research and Consultancy

Jyothy Institute of Technology, Thathaguni Post, Bengaluru 560082

Email: narendra.r@ciirc.jyothyit.ac.in

*Corresponding author

In this research, proteins were obtained from *Pongamia pinnata* oil meal and subsequently converted into films and compression moulded into various packaging products. Films with a maximum tensile strength of 1.9 MPa were obtained when 15% citric acid was used as the crosslinker. Minimum swelling of 120% was seen in 20% citric acid crosslinked film whereas the uncrosslinked films readily disintegrated in water. The protein films had excellent antioxidant properties with an IC50 value of 14.6 µg/mL compared to 26.9 µg/mL for the standard ascorbic acid. The pongamia protein-based bioproducts showed good activity against *Bacillus cereus* and *Aspergillus niger*. Unique properties, low cost, and large availability make pongamia proteins an ideal biopolymer for the development of green and sustainable materials and bioproducts.

~~Proteins extracted from *Pongamia pinnata* oil meal have been converted into completely biodegradable films, transparent packaging boxes, and containers with inherent antimicrobial~~

and antioxidant properties required for food, biotechnology, and other applications. A simple crosslinking using citric acid can provide the necessary strength and stability to the protein bioproducts. Proteins are preferred over other biopolymers for various end-uses and are ideally suited for direct consumption, also to develop bioproducts for packaging applications. In this research, proteins are obtained from pongamia oil meal and subsequently converted into films and compression molded into various packaging products. Films with a maximum tensile strength of 1.9 MPa were obtained when 10% citric acid was used as the crosslinker. The pongamia protein-based bioproducts showed good activity against *Bacillus cereus* and *Aspergillus niger*. Unique properties, low cost, and large availability make pongamia proteins an ideal biopolymer for the development of green and sustainable materials and bioproducts.

Keywords: *Pongamia pinnata*, proteins, citric acid (CA), crosslinking, films, packaging, bioproducts

1. INTRODUCTION

Substantial efforts are being pursued towards developing greener materials using renewable resources as substitutes for petroleum-based materials. Among the various renewable resources, the by-products generated during the cultivation of crops are economical and widely available with rather restricted applications. Similarly, plant proteins like pongamia, soy, and zein are produced as coproducts of oil extraction but are mainly used for low-value applications such as fertilizer or animal feed [1, 2].

Plant proteins have several advantages including easy biodegradability, biocompatibility, cytocompatibility, availability of amino and carboxylic groups for chemical modifications, etc [3-5]. Hence, a variety of plant proteins have been converted into films, hydrogels, fibers, micro and nanoparticles, and scaffolds for a range of applications [6-9]. However, compared to many other synthetic materials, the properties of protein films are inferior. Specifically, plant protein-based biomaterials have inferior mechanical characteristics along with inadequate water resistance due to the considerably high hydrophilicity of the proteins [10]. These drawbacks limit the use of plant proteins for various applications.

Proteins contain several functional groups including amines, alcohols thiols, etc. Chemical modification of these functional groups changes the physical properties and reactivities of the proteins making them suitable for specific applications. Researchers have implemented numerous types of chemical modification techniques including crosslinking, esterification, acetylation, etc to improve the performance properties and usability of proteins [7, 11, 12].

Crosslinking using chemical, physical, and/or enzymatic approaches are commonly employed to enhance the properties of protein and other biopolymer-based materials. When proteins react with specific molecules called crosslinkers, interlinks are formed between the

crosslinker and the protein functional groups. Such interconnections improve the mechanical strength of the proteins as a result of the formation of tough and resilient intermolecular covalent bonds between the crosslinks, whereas a decrease in mobility of the polymers and hence elongation of the ~~fibres~~ fibers is noticed. For example, polymers such as soy proteins were crosslinked with transglutaminase for the enhancement of hardness, transparency, and strength for drug release and tissue engineering applications [13, 14]. Similarly, proteins obtained from soybeans and cotton seeds were crosslinked using various aldehydes, glyoxal, and other chemicals for different applications [15-18].

Although many crosslinkers have been used to modify the properties of films, most of the crosslinkers reported are toxic to cells and hence not preferable for health-related applications. Carboxylic acids such as citric acid (CA) helps in cross-linking the polymer without compromising the cytocompatibility [19]. CA is a weak acid with three carboxylic groups, organic and non-toxic as certified by the Food and Drug Administration of the United States of America [20]. The carboxylic functional groups of CA are greatly responsive and proliferate the active sites on the proteins. Enhanced functionality along with ester bond formation can be achieved by CA crosslinking. Also, several reactive groups (-COOH and –OH) existing in CA allow~~s~~ the adhesion of multiple biomolecules and aids in achieving the desired extent of crosslinking [21].

Pongamia pinnata is a non-GMO crop and nitrogen-fixing tree that grows in a varied range of geographical regions. The seed yields non-edible oil which is of medicinal value. The seeds contain lipids (33%), proteins (43%), sugar (20%), and free amino acids (10%)[22, 23].

Pongamia produces oilseeds that are used for various applications like oil for biofuel applications, biogas, for electricity production. Once the oil is removed from the seeds, the deoiled seed cake contains a high amount of crude proteins (30%) which can be

utilized as poultry and cattle feed ~~for many applications~~. This paper for the first time reports the extraction of crude proteins from *Pongamia pinnata* oil meal and conversion of the proteins into value-added bioproducts.

2. EXPERIMENTAL SECTION

2.1 Materials

Pongamia oil meal was procured from Gandhi Krishi Vignan Kendra, Bangalore, India. Chemicals sodium hydroxide (NaOH) manufactured by Thomas Baker private~~(P)~~ limited, sodium hypophosphite (SHP) produced by Rolex chemicals private~~(P)~~ limited, citric acid (CA) from HiMedia laboratories private ~~(P)~~ limited. Acetic acid and hydrochloric acid from Nice chemicals private~~(P)~~ limited, L-proline, and ninhydrin reagent (2% solution) were purchased from Sigma Aldrich private~~(P)~~ limited.

Methods

2.2. Extraction of pongamia proteins from the meal

Unfractionated pongamia meal was added into ~~sodium hydroxide~~NaOH solution (1% w/v) under continuous stirring for an hour at 70 °C. The meal to alkali solution was at a ratio of 1:4 (w/v). Later, the solution was centrifuged at 8000 rpm for 15 minutes. Around 30 ml of 50% v/v acetic acid was added to 400 ml of the supernatant for the protein precipitation to take place. ~~Acetic acid was added into the supernatant for the protein precipitation to take place.~~ Proteins were obtained after the centrifugation of the precipitated solution. Further, this protein was dried for 24 hours at 60 ℃ [7, 11]. These proteins were used for the preparation of the films. The procedure followed for the protein extraction is schematically shown in Scheme 1.

[insert Scheme 1]

Scheme 1: Schematic representation of the method employed for protein extraction and preparation of films from the extracted proteins.

2.3. Amino acid analysis

The proteins extracted were examined for the various amino acids present in them. A Hitachi L-8900 automatic amino acid analyzer was used for the analysis. Different weights of the protein (174 mg, 260 mg, and 369 mg) were taken, hydrolyzed at 110±1 °C in 6 mol/L HCl for around 22 hours, and later was cooled to room temperature. Nitrogen atmosphere was used to dry the samples for the removal of HCl and was further the amino acids were reconstituted in 5 ml ~~using~~ distilled water. Next, the solution was allowed to pass through a 0.22 µm pore size membrane. During the analysis the column temperature was 57 °C, ~~injection volume was 20 µL,~~ detection wavelength was 570 nm (proline @ 440 nm); ~~injection volume was 20 µL:~~ buffer flow rate (0.35 ml/minute), and the unit temperature during testing was controlled at 135 °C. The final concentration of amino acids was taken as 100% and the proportion of each type of amino acid was reported.

2.4. Preparation of crosslinked protein films

Pongamia protein (6%w/v) was added into NaOH (0.2 N) solution preheated to 70 °C under continuous stirring for 30 minutes. Different percentages of CA (0 to 20% to the weight of the protein) and catalyst, ~~sodium hypophosphite~~SHP (50% to the weight of CA) was added to the above solution. Heating along with stirring was sustained for the next 20 minutes. This solution was then poured on ~~-~~to the metal plates and dried at ambient temperatures until the film was formed. For the crosslinking reaction to take place, the dried films were treated at 150 °C for 15 minutes in a vacuum oven [7]. The length and width of the film were multiplied

with each other to obtain the area of the film. The dimensions of the developed films was 100mm x 100mm.

2.5. Water sorption studies

The protein films whose initial weights were designated as WI were made into smaller parts (1*1 cm). These smaller portions of the film were immersed in distilled water (around 15 minutes) for the absorption to occur and centrifuged for 10 minutes at 5000 rpm to remove excess water. The final weights (W_f) were calculated. The % water sorption was calculated through the below formula (Equation 1):

$$\%\ Water\ sorption = \frac{Wf - WI}{WI} * 100$$

$$\%\ \text{Water sorption} = \frac{\text{Wf - WI}}{\text{WI}} \times 100 \quad \text{---------- (1)}$$

2.6. Determining mechanical properties

The tensile characteristics of the pongamia protein films were estimated in the dry state (ASTM standards D 882-02) utilizing a universal tensile tester (Model UTM- G-312C, Shantha engineering, Mumbai). Protein films measuring 10 x 1 cm were prepared. The samples were maintained at room temperature before measurement. The crosshead speed was 30 mm/minute and a load cell with a capacity of 100N was used throughout the analysis. Fifteen specimens for each condition were considered and three replications were done.

2.7. Swelling studies

The pongamia protein films (of uniform thickness) were made into smaller strips having dimensions of 2*2.5 cm and treated in distilled water for 10 minutes. Subsequent changes in

the dimension of the films were analyzed. The % increase in the area of the film after swelling (A_f) was calculated using the formula in equation 2:

$$\% \text{ Increase in area} = \frac{\text{Af-AI}}{\text{AI}} \text{ x } 100 \quad \% \, increase \, in \, area = \frac{Af - AI}{AI} * 100 \quad \text{----------} \quad (2)$$

2.8. Fourier transform infrared studies

The FTIR spectrum of the films was determined on a Shimadzu IRAffinity-1S FTIR spectrophotometer. The spectrum was recorded in total attenuated reflectance mode with the aid of a diamond cell. The range of scanning was 400 - 4000 cm^{-1} and 64 scans were done for each sample. Lab solutions series software was employed to examine the obtained spectra.

2.9. Scanning electron microscope images

The surface features of the films were captured using a scanning electron microscope (Hitachi SU3500). The protein samples were coated with gold particles using an ion sputter (Hitachi Ion Sputter MC1000) for 60 seconds since they were nonconducting. The current maintained was 1.8 mA and the images were captured at an operating voltage of 5kV.

2.10. Thermo Gravimetric Studies

Changes in the thermal behavior of pongamia protein powder and the crosslinked films were studied with the help of a Thermo Gravimetric Analyser (Mettler Toledo, Model 822e). Sealed aluminium pans were used to place the samples and heating was carried out at a rate of 10 °C/min starting from 25 °C to 600 °C under nitrogen atmosphere.

2.11. Antioxidant studies

The antioxidant properties in terms of the scavenging activity of the films were obtained using ABTS+ (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)~~ABTS~~ radical decolorization assay. The ABTS+ cation radicals were produced by reaction between 7 mM ABTS and 2.45 mM ammonium persulfate by incubating in dark for 16h to obtain ABTS+. The standard antioxidant compound used was ascorbic acid (1mg/mL).~~The standard drug used was ascorbic acid.~~ A stock solution of pongamia protein (1mg/mL) was prepared by dissolving in DMSO (Di Methyl Sulphoxide). Different aliquots (10µg/ml to 50µg/ml) of this stock was taken and the volume was adjusted to 500 µL using DMSO. To all the tubes 2500 µL of ethanol was added followed by 300 µL of ABTS+ reagent and incubated for 30 minutes at room temperature in dark, the absorbance was measured at 745nm[24]. The blank comprised 500 µL of DMSO, 500 µL of ethanol, and 150 µL of ABTS+. All the test-tubes displayed decolorization and this was expressed as the inhibition percentage of the cation available in the sample. The percentage of scavenging activity was determined using equation 3. Next, scavenging activity was plotted against protein concentration in an excel sheet. The linear trendline was added to the plotted curve, further R^2 and a regression equation were obtained in the form of y=mx+c. X is IC50 which needs to be calculated, y is 50 since we are calculating IC50 and a simple substitution of the values provides us IC50. Ascorbic acid was used as positive control and tests were carried out in triplicates.

~~The reaction between 7 mM ABTS and 2.45 mM ammonium persulfate gives rise to ABTS cations (ABTS.$^+$). Various aliquots of the pongamia protein were taken in DMSO and 500 µL of ethanol was added followed by 300 µL of ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) reagent and 2 ml of ethanol. After 30 minutes of incubation, the absorbance was measured at 745nm [24]. The blank comprised of 500 µL of ethanol along with 150 µL of ABTS. All the test-tubes displayed decolorization and this was~~

~~expressed as inhibition percentage of the cation available in the sample. The scavenging activity was determined using equation 3~~

$$ABTS\ radical\ scavenging\ Activity\ (\%) = \frac{Control\ OD - Sample\ OD}{Control\ OD} \times 100 \quad \text{------ (3)}$$

2.12. Antimicrobial studies

The antimicrobial studies were conducted using the agar well diffusion method [25].

A starter culture of bacteria (*Bacillus cereus* and *E coli*) was prepared by taking a loop full of bacteria from the master culture. This was inoculated in Mueller Hinton broth separately and incubated at 37± 1 °C for 2h on a shaker at 120 rpm. 100 µL of the active bacterial cultures were taken from the broth and spread on Mueller Hinton agar medium using sterile swabs. Wells were punched using a sterile steel cork borer (6mm internal diameter) and 100 µL of the samples were loaded. The standard antibacterial compound used was ampicillin (1mg/mL). Post incubation (at 37 °C for 24 hours), the zone of inhibition was measured in millimeters. For antifungal activity, a loop full of fungal spores was suspended in Mueller Hinton broth and a method similar to antibacterial study was followed to know the activity against *Aspergillus niger*. The standard antifungal compound used was fluconazole (1mg/mL). The zone of inhibition was measured in millimetres after 48 hours of incubation at 28 °C. The test was conducted in triplicates.

~~A loop of bacteria was transferred from nutrient agar into the Mueller Hinton broth which was the test culture and this was incubated at 37± 1 °C for 2 hours. The internal diameter of the wells was around 6mm made using a sterile cork borer. The standard drug used was ampicillin. Post incubation (at 37 °C for 24 hours), the diameter of the inhibitory zone was measured. Antifungal studies were also done using a similar approach to know the activity~~

~~against *Aspergillus Niger*. The standard drug used was fluconazole. The width of the inhibition zone was measured after 48 hours at 23 °C.~~

2.13. Developing bioproducts

The proteins extracted from *Pongamia pinnata* oil meal were verified for their likeliness to be shaped into diverse forms for various applications. Protein films were obtained by adding 15% CA and 40% glycerol to the weight of pongamia protein. The obtained films were compressed at 140°C for five minutes into 3D cups and boxes utilizing specific moulds. ~~The films were compressed at 90°C to form a film by adding (40% glycerol was added to the weight of proteins. Subsequently, the film was compression-molded under 90°C for five minutes into 3D cups and boxes utilizing specific molds.~~ Pictures of the developed bioproducts are incorporated in the manuscript.

2.14. Statistical analysis

For all the above-stated experiments three trials were performed. The average for all three trials along with standard deviations was measured and reported. ± indicate one standard deviation in the error bars.

3. Results and Discussion

3.1. Crosslinking reaction mechanism

The crosslinking mechanism between CA and protein amine groups is demonstrated in figure 1. Initially, SN2 nucleophilic substitution reaction occurred between protein amine groups and carboxyl groups of citric acid[10]. Since three carboxyl groups are present in citric acid, each citric acid molecule interacted with more than one amine group leading to the formation of intra and intermolecular crosslinks. As depicted in reaction (1), COOH and NH_3 were

protonated into COO- and NH_2 in an alkaline medium. COO^- which carries a partial positive charge was attacked by amine nitrogen leading to the formation of an amide as demonstrated in reaction (2). Further, the proteins interacted with the remaining carboxyl group of citric acid as shown in reaction (3).

[insert Figure 1]

Figure1: Mechanism of crosslinking reaction between citric acid and amine group of pongamia protein. P1, P2, and P3 represent pongamia proteins.

3.~~1~~2. Amino acid analysis

Crude protein was extracted from *Pongamia pinnata* oil meal and the percentage weight fraction of the protein obtained after extraction was around 24. The amino acid analysis was performed to understand the protein content of *Pongamia pinnata* in general. A comparison of the relative % of amino acids present in extracted pongamia proteins with zein and soy proteins is provided in table 1. The amino acid content of pongamia protein is comparable to that found in other plant sources. Glutamic acid was the primary amino acid (12.2%) present in pongamia. Methionine with 1.8% was the lowest amino acid in pongamia unlike in soy and zein which was cysteine ~~The relative % of amino acids present in pongamia proteins is shown in table 1. As seen from the table, the proteins contained both essential and non-essential amino acids. But, the percentage of non-essential amino acids was higher compared to essential acids. Glutamic acid was the primary non-essential amino acid (12.2%) followed by arginine (7.7%) and glycine (7.2%). Amongst the essential amino acids, valine was the major amino acid (8.3%) followed by leucine (8.2%) and phenylalanine (5.4%). The amino acid content of pongamia proteins is comparable to that found in other plant sources. Generally, the essential amino acid content is relatively reduced in plant-based proteins compared to animal-based proteins which was also observed in this study~~ [26].

~~Table 1: Relative proportion of amino acids extracted from *Pongamia pinnata* oil meal.~~

~~Essential amino acids (%)~~		~~Non-essential amino acids (%)~~	
~~Valine~~	~~8.3~~	~~Arginine~~	~~7.7~~
~~Methionine~~	~~1.8~~	~~Alanine~~	~~6.5~~
~~Threonine~~	~~2.9~~	~~Aspartic acid~~	~~5.6~~
~~Isoleucine~~	~~4.7~~	~~Glutamic acid~~	~~12.2~~
~~Lysine~~	~~3.0~~	~~Cystine~~	~~4.4~~
~~Leucine~~	~~8.2~~	~~Proline~~	~~5.6~~
~~Histidine~~	~~3.7~~	~~Glycine~~	~~7.2~~
~~Phenylalanine~~	~~5.4~~	~~Tyrosine~~	~~6.2~~
		~~Serine~~	~~5.6~~

Table 1: Relative proportion of amino acids extracted from *Pongamia pinnata* oil meal, zein, and soy.

Amino acid	Type of protein		
	Pongamia	Zein	Soy
Threonine	2.9	1.8	2.3
Methionine	1.8	1.1	0.3
Phenyl alanine	5.4	3.4	3.2
Histidine	3.7	1.1	1.5
Lysine	3.0	1.0	3.4
valine	8.3	2.1	2.2
Isoleucine	4.7	1.7	1.9
Leucine	8.2	8.8	5.0
serine	5.6	2.9	3.4
Glycine	7.2	1.6	2.7
Glutamic acid	12.2	13.1	12.4
Proline	5.6	5.2	3.3
Cysteine	4.4	0.3	0.2
Alanine	6.5	4.8	2.8
Tyrosine	6.2	2.7	2.2
Arginine	7.7	1.7	4.8

3.~~2~~3. Water sorption studies

Approximately, 24 hrs was required to dry the films at ambient temperature. The thickness of the films was around 0.2mm. The ability of different pongamia protein films to sorb water is depicted in figure 2. ~~Figure 1 shows the ability of pongamia~~ protein ~~films to sorb water.~~ The water absorption abilities of the film were dependent on the percentage of CA added.

Uncrosslinked film ~~Control film (without CA)~~ absorbed 438% of water whereas maximum sorption of 788% was seen in the case of 5% CA crosslinked pongamia protein films. Minimum sorption of 346% was seen in 20% CA crosslinked films. The uncrosslinked and 5% CA crosslinked films dispersed/disintegrated after water sorption for 15 minutes. However, 10, 15 and 20% crosslinked films were stable and intact even after centrifugation. ~~Thus, from the figure, it can be inferred that the sorption abilities of the films are inversely related to CA percentages.~~

This ~~inverse~~ relationship between CA % and water sorption % can be ascribed to the formation of crosslinks between the amine/hydroxyl groups of proteins and carboxyl acid groups of CA [27, 28]. In 5% CA films, the amount of CA added was not sufficient enough to crosslink all the freely available functional groups of proteins. Hence, there were many free functional groups present in the protein film in addition to those present in CA available for H- bonding with water molecules. The number of these free amino and hydroxyl groups decreases with increasing CA concentration. Therefore, the least water sorption was seen with 20% CA crosslinked film owing to the unavailability of free amino/ hydroxyl groups [29].

[insert Figure ~~1~~2]

Figure ~~1~~2: Effect of citric acid~~CA~~ percentage on % water absorption of pongamia protein films.

3.~~3~~4. Mechanical properties

The tensile strength of pongamia protein films when crosslinked with varying % of CA is shown in figure ~~2~~3. Maximum strength of 1.9 MPa was exhibited by ~~10~~15% CA crosslinked pongamia protein film. The crosslinked films (except 20% CA) displayed higher strength in comparison to the ~~control (~~uncrosslinked~~)~~ films [7]. The formation of crosslinks between the amino/ hydroxyl groups of proteins and carboxylic groups of CA takes place leading to the

establishment of interconnected molecules. This network is responsible for the increased strength of crosslinked films. However, 20% CA crosslinked film displayed considerably low strength in comparison to the uncrosslinked film~~control~~. This low strength of 0.230 MPa in 20 % CA film is due to the protein precipitation as the addition of CA reduces the pH. Protein dissolution for film formation requires alkaline conditions. Both CA and SHP are considered acidic and when added at higher levels leads to precipitation or partial dissolution of the proteins. Besides, excess CA leads to over crosslinking which limits the mobility of molecules and also decreases strength. Hence films formed at high concentrations of CA have poor tensile properties.

[insert Figure ~~2~~3]

Figure ~~2~~3: Effect of citric acid ~~CA~~ percentage on tensile strength of pongamia protein films. The films were made using a protein concentration of 6% and a catalyst (SHP) equivalent to 50% weight of the crosslinker.

3.~~4~~5. Swelling studies

The swelling % of various CA crosslinked pongamia protein films upon immersion in water is shown in Figure 4. Within 10 minutes the uncrosslinked film disintegrated and started dissolving. ~~An increase in the swelling % of various CA crosslinked pongamia~~ protein ~~films are shown in Figure 3. The control film when immersed in water was completely swollen and eventually disintegrated.~~ Among the CA crosslinked films, 10% CA pongamia protein film exhibited a maximum swelling of 208% and the least swelling of 120% was seen in 20% CA crosslinked film. The swelling properties of the film can be attributed to the existence of free amino/ hydroxyl groups in proteins as discussed earlier for water sorption. The amino/hydroxyl groups present in the proteins crosslink with the carboxylic groups of CA to form a network. These crosslinks formed during the process of crosslinking make~~s~~ the

functional groups in proteins unavailable for H- bonding with water molecules. Hence the % increase in the area of the pongamia protein films decreases with an increase in the percentage of CA. However, initially, when 0 to 10% CA was added, swelling of the film was seen although no apparent dissolution or weight loss was observed. ~~growth in the % area of the film was seen.~~ This suggests that there were many hydroxyl/amino groups left uncrosslinked and have led to the formation of hydrogen bonding with water molecules. When CA concentration was increased from 10 to 20%, a decrease in % swelling area was noticed as discussed above.

[insert Figure ~~3~~4]

Figure ~~3~~4: Effect of citric acid~~CA~~ % on the swelling abilities of ~~the~~ pongamia protein films.

3.~~6~~5. FTIR studies

T~~Figure 4 shows t~~he FTIR spectra of pongamia protein films without crosslinking and after crosslinking with 15% CA is shown in figure 5. The broad peak at 3400 cm^{-1} in the crosslinked film is due to the presence of OH peaks both from protein and CA. The intensity of this peak is less in uncrosslinked ~~control~~ film since the contribution of the OH groups is only from the proteins. A peak at 2900 cm^{-1} was detected as a result of the C-H stretching vibration. This peak is prominent in crosslinked films due to the presence of C-H groups from CA in addition to those present in the proteins. A large, broad peak at 1600 cm^{-1} is because of the C-O ester groups formed as a result of the crosslinking reaction. The peak seen around 1538 cm-1 is due to amide group vibrations. The peaks at 1200-1400 cm^{-1} can be attributed to the COC groups formed due to the interaction between proteins and CA. All the above peaks in addition to those present in uncrosslinked~~control~~ films along with increased intensity in the crosslinked film confirm the process of crosslinking. However, no trend was observed in the intensities of the peaks with increasing CA concentration [30].

[insert Figure 45]

Figure 45: FTIR spectra of the pongamia protein film before and after 15% ~~CA~~ citric acid crosslinking.

3.76. Morphology analysis

Images ~~of the films~~ captured for the uncrosslinked~~control~~ and 15% CA crosslinked pongamia protein films using a scanning electron microscope (SEM) are provided in Figure ~~5~~6. The uncrosslinked~~control~~ films were non-uniform with large cracks and holes compared to the 15% CA crosslinked films. The addition of CA was found to minimize the degree of non-uniformity in the crosslinked films by decreasing the number of cracks and holes. As an evidence to this observation, the extent of holes and cracks has reduced substantially in 15% CA crosslinked pongamia protein films [7]. Such change in morphology can be credited to the crosslinking process which binds the individual polymer chains together leading to the creation of a network. The formation of this network probably would have led to a change in the external morphology of the film by reducing the number of holes and making the surface even. Also, in the process of crosslinking, the protein film shrinks due to high temperature which may also be the reason for the uniform morphology of the crosslinked film.

[insert Figure ~~5~~6]

Figure 6: Scanning electron microscope images of the uncrosslinked (A), 15% citric acid crosslinked pongamia protein film (B).

~~Figure 5: Scanning electron microscope images of the control (left), 15% CA crosslinked pongamia film (right).~~

3.78. Thermogravimetric analysis

The thermal behavior of pongamia protein and 15% CA crosslinked film is shown in Figure 7. As seen from the TGA curve, the degradation patterns of both the powder and crosslinked

films are similar except that the latter degrades in three steps compared to the protein powder. The exact degradation temperature is noticeable in the DTA curves. Both the protein powder and crosslinked film displayed a pronounced endothermic effect around 150-470 ^{o}C during the degradation process. ~~The thermal behavior of pongamia protein and 15% CA crosslinked film is shown in Figure 6. As seen from the TGA curve, the degradation patterns of both the powder and crosslinked films are similar except that the latter degrades in three steps compared to the protein powder. The exact degradation temperature is noticeable in the DTA curves.~~ Most of the uncrosslinked protein powder degraded at around 303 °C in one single step. In the case of crosslinked film, endothermic peaks were observed~~the degradation occurs~~ at 3 different temperatures. This variation in degradation temperature can be explained based on the extent of the crosslinking of pongamia proteins. Initially, the uncrosslinked/low molecular weight proteins degraded around 160°C, the majority of the remaining proteins which had not crosslinked effectively degraded around 257 °C. The remaining proteins which had crosslinked effectively and had a higher molecular weight required a higher temperature around 470 °C to degrade. This is because of the formation of strong covalent crosslinks between amino/hydroxyl of proteins with carboxylic groups of CA. However, these crosslinks are absent in uncrosslinked proteins and hence lower temperature is sufficient for complete degradation of pongamia proteins.

A similar observation was reported by Reddy, 2012, wherein higher temperatures were required to melt crosslinked peanut proteins using CA in comparison to the uncrosslinked proteins [7]. In another work by Nataraj et al 2018, banana fiber and wheat gluten were crosslinked using CA and glutaraldehyde. It was reported that CA crosslinked films were more resistant to thermal degradation compared to the glutaraldehyde crosslinked films. [27]

[insert Figure ~~6~~7]

Figure ~~6~~7: Images showing the Thermogravimetric (left) and Differential thermogravimetric curves (right) of pongamia protein powder and 15% citric acid~~CA~~ crosslinked protein film.

3.~~8~~9. Antioxidant studies

Scavenging activity is expressed as the ability of the sample to help shield the cells from harm by free radicals. Pongamia protein has intrinsic scavenging potential [30, 31]. However, crosslinking using CA further boosted this potential. As a sign of this observation, the highest scavenging was displayed by 20 % CA crosslinked protein film compared to others. IC50 is the half-maximal inhibitory concentration of the sample that is required to inhibit a biological process by half. This potency varied between the crosslinked and uncrosslinked films. ~~IC50 which is the minimal inhibitory concentration essential to impede half of the biological function varied between the crosslinked and non-crosslinked films.~~ Amongst all the CA crosslinked films, 20% crosslinked film showed excellent antioxidant potential (IC50 value of 14.6µg/mL) in comparison to ascorbic acid (IC50-26.9 µg/mL). ~~Amongst all the CA crosslinked films, 20% crosslinked film showed excellent antioxidant properties (IC_{50} value of 14.6) in comparison to ascorbic acid (IC_{50} 26.9).~~ Thus, it can be inferred that CA crosslinking enhances the antioxidant properties of pongamia protein films. Also, it can be observed that the IC_{50} value obtained is inversely related to the concentration of CA. The scavenging activity of the protein films was plotted against varying concentrations of proteins (figure 8). ~~Figure 7 shows the scavenging activity of the protein films when varying concentrations of proteins were taken.~~ Similar work carried out by Chopade showed that *Pongamia pinnata* has antioxidant activity in ammonium chloride-induced hyperammonium rats[31].

In another study by Sajid, the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity % of *Pongamia pinnata* seed extract was found to be 15.7-38.0 µg/ml which was quite high in comparison to the values obtained in our studies[32].

~~In another study by Shokeen, the scavenging activity % of methanol-based *Ricinus communis* fractions were found to be 26 [32]. Also, the DPPH assay for *Ricinus communis* of n butanol extract was performed and the IC_{50} values were found to be 140±0.19 µg/ml which is quite high compared to *Pongamia pinnata* [33].~~

[insert Figure ~~7~~8]

Figure ~~7~~8: Radical s~~S~~cavenging activity of the pongamia protein films at various concentrations.

3.10~~9~~. Antimicrobial studies

Antimicrobial studies were performed to assess the antibacterial and antifungal potential of pongamia proteins against test organisms. ~~Antimicrobial studies were performed to assess the potential of pongamia proteins to resist the growth of bacteria (microbe or fungi).~~ Table 2 provides the width of the inhibition zone for various samples. In the case of *Bacillus cereus,* the ~~control~~ uncrosslinked protein film showed a zone width of 10mm, whereas the 20% CA crosslinked film showed a width of 11.75mm which is close to the value of 11.66 seen for the standard antibacterial compound ampicillin~~drug~~. For E coli, 20% CA crosslinked film showed a maximum ~~width of~~zone of inhibition (12.33 mm) which is not comparable to the standard antibacterial compound ampicillin (37mm)~~value of 37mm~~. In a similar study, it was reported that *Ricinus communis* and *Periploca aphylla* both showed ~~a~~ zones of inhibition of 0.3 mm for *E-coli* which is significantly less in comparison to *Pongamia pinnata* [33]. Hence, the crosslinked films were additionally active against *Bacillus cereus* rather than against *E-coli* [29]. However, for both the bacterial strains it can be observed that the ~~width of the~~

~~inhibition~~ zones of inhibition increased with increasing % of CA, and the inherent antimicrobial property of the protein was retained even after the addition of CA.

The effects of the antifungal activity in the films were evident based on the changes in the zones of inhibition of the uncrosslinked and the standard compound fluconazole. The zones of inhibition were almost similar and no significant difference was observed. However, it was realized that the addition of CA decreased the width of zone of inhibition implying that CA has a negative consequence on the antifungal activity of pongamia proteins because of the acidic nature of CA impeding the development of the fungi [34-37].

~~The effects of the antifungal activity in the films were evident based on the changes in the zone width of the control and the standard drug. The zone of inhibitions was almost similar and no significant difference was observed. However, it was realized that the addition of CA decreased the width of the inhibition zone implying that CA has a negative consequence on the antimicrobial activity of pongamia proteins [34] because of the acidic nature of CA impeding the development of the fungi~~.

Table 2: Inhibition zones of ~~the~~ pongamia protein films against ~~the~~ different microbes in comparison to the standard drug.

Citric acid~~A~~ % ~~of the films~~	Width of Inhibition zone (mm)		
	Bacillus cereus	***E-coli***	***Aspergillus niger***
0 ~~(control film)~~	10 ± 0	10.33± 0.57	14.66± 0.57
10	9 ± 0	9.66±0.57	12.66±0.57
15	9.75 ± 0.35	11.66± 0.57	13± 0
20	11.75± 0.35	12.33± 0.57	10.33± 1.15
Standard drug	11.66± 0.57 (Ampicillin)	37±1 (Ampicillin)	14± 0 (Fluconazole)

3.110. Potential applications of the extracted proteins

A two-step process of ~~compressing the proteins to form a~~obtaining protein films and later molding the films into various shapes enables developing bioproducts from pongamia proteins ~~without~~ using biocompatible and food grade chemicals. Glycerol was not removed after moulding. Some glycerol did come out when higher concentrations or pressure was used. Browning of the films due to Maillard reactions was observed after moulding. There was a noticeable but pleasant odour after compression.~~any additional chemicals or polymers~~. Packaging boxes (Figure ~~8~~9) suitable for food and non-food uses have been prepared using the extracted pongamia proteins. More importantly, the products developed are chemical-free, non-hazardous, and completely biodegradable. They contribute towards a clean environment and also sustainable development. Since pongamia proteins have inherent antimicrobial, antioxidant and thermal stability, these bioproducts can be ideal for food and non-food packaging applications. Similar bioproducts were developed from castor oil cake, wheat gluten/ banana fiber, chitosan by citric acid crosslinking[27, 30, 38].

~~, and thermal stability, these bioproducts can be ideal for food and non-food packaging applications.~~

[insert Figure ~~8~~9]

Figure ~~8~~9: Three-dimensional cups and boxes suitable for food and non-food packaging have been developed from pongamia protein, demonstrating the feasibility of preparing bioproducts for commercial applications.

4. CONCLUSIONS

Pongamia proteins can be made into films and other bioproducts with good mechanical properties and water stability required for food packaging. Under optimized

conditions, crosslinking with 10% CA increased the strength of the films by about 28% in comparison to the uncrosslinked~~control~~ films. As an evidence to increased water stability of the crosslinked films, minimum swelling of 120% was seen in 20% CA crosslinked film whereas the uncrosslinked films readily disintegrated in water. Bioproducts made from pongamia proteins show excellent antioxidant properties with an IC_{50} value of 14.6 compared to 26.9 for the ascorbic acid standard. In addition to the above properties, substantial antibacterial and antifungal activity was also exhibited by the pongamia proteins and films against *Bacillus cereus* and *Aspergillus niger,* respectively. This method of producing biodegradable, plant protein-based bioproducts can be used for a wide variety of food and medical applications where antifungal, antibacterial activity and antioxidant properties are highly desirable. Additional studies are being conducted to regulate the shelf-life, ~~and~~ effects of temperature, ~~and~~ humidity, and gas barrier properties on the contents stored in these packaging materials.

ACKNOWLEDGMENTS

The authors would like to acknowledge CIIRC, Jyothy Institute of technology, Thathaguni for their monetary funding. Narendra Reddy appreciates the Department of Biotechnology, Ministry of Science and Technology, Government of India for funding through the Ramalingaswami Re-entry Fellowship.

Declaration of interest

Authors state that they do not have any conflict of interest

REFERENCES

1. Naik M, Meher L, Naik S, et al. Production of biodiesel from high free fatty acid Karanja (*Pongamia pinnata*) oil. *Biomass Bioenerg* 2008; 32(4): 354-357.
2. Taheripour F, Hertel TW, Tyner WE, et al. Biofuels and their by-products: Global economic and environmental implications. *Biomass Bioenerg* 2010; 34(3): 278-289.

3. Chan G and Mooney DJ. New materials for tissue engineering: towards greater control over the biological response. *Trends Biotechnol* 2008; 26(7): 382-392.
4. Chen L, Remondetto GE and Subirade M. Food protein-based materials as nutraceutical delivery systems. *Trends Food Sci Technol* 2006; 17(5): 272-283.
5. Stegemann JP, Kaszuba SN and Rowe SL. Advances in vascular tissue engineering using protein-based biomaterials. *Tissue Eng* 2007; 13(11): 2601-2613.
6. Liu X, Sun Q, Wang H, et al. Microspheres of corn protein, zein, for an ivermectin drug delivery system. *Biomaterials* 2005; 26(1): 109-115.
7. Reddy N, Jiang Q and Yang Y. Preparation and properties of peanut protein films crosslinked with citric acid. *Ind. Crops Prod* 2012; 39(2012): 26-30.
8. Reddy N, Chen L and Yang Y. Biothermoplastics from hydrolyzed and citric acid crosslinked chicken feathers. *Mater. Sci. Eng: C* 2013; 33(3): 1203-1208.
9. Reddy N and Yang Y. Potential of plant proteins for medical applications. *Trends Biotechnol* 2011; 29(10): 490-498.
10. Xu H, Shen L, Xu L et al. Low-temperature crosslinking of proteins using non-toxic citric acid in neutral aqueous medium: Mechanism and kinetic study. *Ind. Crops Prod* 2015; 74 (2015): 234-240.
11. Hu C, Nataraj D and Reddy N. Acetylation of Castor Meal and Castor Proteins for Thermoplastic Applications. *J Polym Environ* 2018; 26(4): 1371-1377.
12. Vananuvat P and Kinsella JE. Preparation of succinylated yeast protein: composition and solubility. *Biotechnol Bioeng* 1978; 20(9): 1329-1344.
13. Gan CY, Cheng LH and Easa A. Assessment of Cross-Linking in Combined Cross-Linked Soy Protein Isolate Gels by Microbial Transglutaminase and Maillard Reaction. *J. Food Sci* 2009; 74(2): C141-C146.
14. Reddy N, Jiang Q and Yang Y. Novel wheat protein films as substrates for tissue engineering. *Biomater. Sci. Polymer Edition* 2011; 22(15): 2063-2077.
15. Essandoh M, Garcia RA and Nieman CM. Chemical and Enzymatic Protein Cross-Linking To Improve Flocculant Properties. *ACS Sustain Chem Eng* 2018; 6(10): 12946-12952.
16. Wang Y, Mo X, Sun XS, et al. Soy protein adhesion enhanced by glutaraldehyde crosslink. *J. Appl. Polym. Sci* 2007; 104(1): 130-136.
17. Caillard R, Remondetto G, Mateescu M, et al. Characterization of Amino Cross-Linked Soy Protein Hydrogels. *J. Food Sci* 2008; 73(5): C283-C291.
18. Marquié C. Chemical reactions in cottonseed protein cross-linking by formaldehyde, glutaraldehyde, and glyoxal for the formation of protein films with enhanced mechanical properties. *J. Agric. Food Chem* 2001; 49(10): 4676-4681.
19. Reddy N, Li Y and Yang Y. Alkali-catalyzed low temperature wet crosslinking of plant proteins using carboxylic acids. *Biotechnol Progr* 2009; 25(1): 139-146.
20. Gyawali D, Nair P, Zhang Y, et al. Citric acid-derived in situ crosslinkable biodegradable polymers for cell delivery. *Biomaterials* 2010; 31(34): 9092-9105.
21. Tran RT, Thevenot P, Gyawali D, et al. Synthesis and characterization of a biodegradable elastomer featuring a dual crosslinking mechanism. *Soft matter* 2010; 6(11): 2449-2461.
22. Usharani K, Naik D and Manjunatha R. *Pongamia pinnata* (L.): composition and advantages in agriculture: a review. *J Pharmacogn Phytochem* 2019; 8(3): 2181-2187.
23. Orwa C, Mutua A, Kindt R, et al. Agroforestree Database: a tree reference and selection guide. Version 4. 2009.
24. Re R, Pellegrini N, Proteggente A, et al. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radic. Biol. Med* 1999; 26(9-10): 1231-1237.

25. Bauer A, Kirby W, Sherris JC et al. Antibiotic susceptibility testing by a standardized single disk method. *Am J Clin Pathol* 1966; 45(4): 149-158.
26. Gorissen SH, Crombag JJ, Senden JM, et al. Protein content and amino acid composition of commercially available plant-based protein isolates. *Amino acids* 2018; 50(12): 1685-1695.
27. Nataraj D, Sakkara S, Meenakshi H, et al. Properties and applications of citric acid crosslinked banana fibre-wheat gluten films. *Ind. Crops Prod* 2018; 124(6): 265-272.
28. Ifebajo AO. Synthesis and characterisation of chemically crosslinked chitosan citrate gels. Eastern Mediterranean University (EMU)-Doğu Akdeniz Üniversitesi (DAÜ), 2014.
29. Sagnella S and Mai-Ngam K. Chitosan based surfactant polymers designed to improve blood compatibility on biomaterials. *Colloids Surf. B* 2005; 42(2): 147-155.
30. Nataraj D, Sakkara S, Meghwal M, et al. Crosslinked chitosan films with controllable properties for commercial applications. *Int. J. Biol. Macromol* 2018; 120(A): 1256-1264.
31. Chopade V, Tankar A, Pande V, et al. *Pongamia pinnata*: Phytochemical constituents, traditional uses and pharmacological properties: A review. *Int. J. Green Pharm* 2008; 2(2): 72-75.
32. Sajid ZI, Anwar F, Shabir G, et al. Antioxidant, antimicrobial properties and phenolics of different solvent extracts from bark, leaves and seeds of *Pongamia pinnata* (L.) Pierre. *Molecules* 2012; 17(4): 3917-3932.
33. Iqbal J, Zaib S, Farooq U, et al. Antioxidant, Antimicrobial, and free radical scavenging potential of aerial parts of *Periploca aphylla* and *Ricinus communis*. *ISRN pharmacology* 2012; 2012:1-6.
34. Wagh P, Rai M, Deshmukh S, et al. Bio-activity of oils of *Trigonella foenum-graecum* and *Pongamia pinnata*. *Afr. J. Biotechnol* 2007; 6(13):1592-1596.
35. Shokri H. Evaluation of inhibitory effects of citric and tartaric acids and their combination on the growth of *Trichophyton mentagrophytes*, *Aspergillus fumigatus*, *Candida albicans*, and *Malassezia furfur*. *Comp Clin Path* 2011; 20(5): 543-545.
36. Jay JM, Loessner MJ and Golden DA. *Modern food microbiology*. Springer Science & Business Media, 2008.
37. Beuchat LR. Surface decontamination of fruits and vegetables eaten raw. 1998. http://www.who.int/foodsafety/publications/fs_management/surfac_decon/en/surface_decon.pdf
38. Silva RV, Gonçalves AD, Vinhal JO, et al. Bioproducts from the pyrolysis of castor seed cake: Basic dye adsorption capacity of biochar and antifungal activity of the aqueous phase. *J. Environ. Chem. Eng* 2021; 9(1): 104825-104839.

REFERENCES

1. Naik M, Meher L, Naik S and Das L. Production of biodiesel from high free fatty acid Karanja (Pongamia pinnata) oil. *Biomass and Bioenergy*. 2008; 32: 354-7.
2. Taheripour F, Hertel TW, Tyner WE, Beckman JF and Birur DK. Biofuels and their by-products: Global economic and environmental implications. *Biomass and bioenergy*. 2010; 34: 278-89.
3. Chan G and Mooney DJ. New materials for tissue engineering: towards greater control over the biological response. *Trends in biotechnology*. 2008; 26: 382-92.
4. Chen L, Remondetto GE and Subirade M. Food protein-based materials as nutraceutical delivery systems. *Trends in Food Science & Technology*. 2006; 17: 272-83.
5. Stegemann JP, Kaszuba SN and Rowe SL. Advances in vascular tissue engineering using protein-based biomaterials. *Tissue engineering*. 2007; 13: 2601-13.

6. Liu X, Sun Q, Wang H, Zhang L and Wang J-Y. Microspheres of corn protein, zein, for an ivermectin drug delivery system. *Biomaterials*. 2005; 26: 109-15.
7. Reddy N, Jiang Q and Yang Y. Preparation and properties of peanut protein films crosslinked with citric acid. *Industrial Crops and Products*. 2012; 39: 26-30.
8. Reddy N, Chen L and Yang Y. Biothermoplastics from hydrolyzed and citric acid crosslinked chicken feathers. *Materials Science and Engineering: C*. 2013; 33: 1203-8.
9. Reddy N and Yang Y. Potential of plant proteins for medical applications. *Trends in biotechnology*. 2011; 29: 490-8.
10. Xu H, Shen L, Xu L and Yang Y. Low-temperature crosslinking of proteins using non-toxic citric acid in neutral aqueous medium: Mechanism and kinetic study. *Industrial Crops and Products*. 2015; 74: 234-40.
11. Hu C, Nataraj D and Reddy N. Acetylation of Castor Meal and Castor Proteins for Thermoplastic Applications. *Journal of Polymers and the Environment*. 2018; 26: 1371-7.
12. Vananuvat P and Kinsella JE. Preparation of succinylated yeast protein: composition and solubility. *Biotechnology and Bioengineering*. 1978; 20: 1329-44.
13. Gan CY, Cheng LH and Easa A. Assessment of Cross-Linking in Combined Cross-Linked Soy Protein Isolate Gels by Microbial Transglutaminase and Maillard Reaction. *Journal of food science*. 2009; 74: C141-C6.
14. Reddy N, Jiang Q and Yang Y. Novel wheat protein films as substrates for tissue engineering. *Journal of Biomaterials Science, Polymer Edition*. 2011; 22: 2063-77.
15. Essandoh M, Garcia RA and Nieman CM. Chemical and Enzymatic Protein Cross-Linking To Improve Flocculant Properties. *ACS Sustainable Chemistry & Engineering*. 2018; 6: 12946-52.
16. Wang Y, Mo X, Sun XS and Wang D. Soy protein adhesion enhanced by glutaraldehyde crosslink. *Journal of Applied Polymer Science*. 2007; 104: 130-6.
17. Caillard R, Remondetto G, Mateescu M and Subirade M. Characterization of Amino Cross-Linked Soy Protein Hydrogels. *Journal of food science*. 2008; 73: C283-C91.
18. Marquié C. Chemical reactions in cottonseed protein cross-linking by formaldehyde, glutaraldehyde, and glyoxal for the formation of protein films with enhanced mechanical properties. *Journal of Agricultural and Food Chemistry*. 2001; 49: 4676-81.
19. Reddy N, Li Y and Yang Y. Alkali-catalyzed low-temperature wet crosslinking of plant proteins using carboxylic acids. *Biotechnology progress*. 2009; 25: 139-46.
20. Gyawali D, Nair P, Zhang Y, et al. Citric acid-derived in situ crosslinkable biodegradable polymers for cell delivery. *Biomaterials*. 2010; 31: 9092-105.
21. Tran RT, Thevenot P, Gyawali D, Chiao J-C, Tang L and Yang J. Synthesis and characterization of a biodegradable elastomer featuring a dual crosslinking mechanism. *Soft matter*. 2010; 6: 2449-61.
22. Usharani K, Naik D and Manjunatha R. Pongamia pinnata (L.): composition and advantages in agriculture: a review. *J Pharmacogn Phytochem*. 2019; 8: 2181-7.
23. Orwa C, Mutua A, Kindt R, Jamnadass R and Simons A. Agroforestree Database: a tree reference and selection guide. Version 4. *Agroforestree Database: a tree reference and selection guide Version 4*. 2009.
24. Re R, Pellegrini N, Proteggente A, Pannala A, Yang M and Rice-Evans C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free radical biology and medicine*. 1999; 26: 1231-7.
25. Bauer A, Kirby W, Sherris JC and Turck M. Antibiotic susceptibility testing by a standardized single disk method. *American journal of clinical pathology*. 1966; 45: 493-6.
26. Gorissen SH, Crombag JJ, Senden JM, et al. Protein content and amino acid composition of commercially available plant-based protein isolates. *Amino acids*. 2018; 50: 1685-95.

27. Nataraj D, Sakkara S, Meenakshi H and Reddy N. Properties and applications of citric acid crosslinked banana fibre-wheat gluten films. *Industrial Crops and Products*. 2018; 124: 265-72.
28. Ifebajo AO. Synthesis and characterisation of chemically crosslinked chitosan citrate gels. Eastern Mediterranean University (EMU)-Doğu Akdeniz Üniversitesi (DAÜ), 2014.
29. Sagnella S and Mai-Ngam K. Chitosan based surfactant polymers designed to improve blood compatibility on biomaterials. *Colloids and Surfaces B: Biointerfaces*. 2005; 42: 147-55.
30. Nataraj D, Sakkara S, Meghwal M and Reddy N. Crosslinked chitosan films with controllable properties for commercial applications. *International journal of biological macromolecules*. 2018; 120: 1256-64.
31. Chopade V, Tankar A, Pande V, et al. Pongamia pinnata: Phytochemical constituents, traditional uses and pharmacological properties: A review. *International Journal of Green Pharmacy (IJGP)*. 2008; 2.
32. Sajid ZI, Anwar F, Shabir G, Rasul G, Alkharfy KM and Gilani A-H. Antioxidant, antimicrobial properties and phenolics of different solvent extracts from bark, leaves and seeds of Pongamia pinnata (L.) Pierre. *Molecules*. 2012; 17: 3917-32.
33. Iqbal J, Zaib S, Farooq U, Khan A, Bibi I and Suleman S. Antioxidant, Antimicrobial, and free radical scavenging potential of aerial parts of Periploca aphylla and Ricinus communis. *ISRN pharmacology*. 2012; 2012.
34. Wagh P, Rai M, Deshmukh S and Durate MCT. Bio-activity of oils of Trigonella foenum-graecum and Pongamia pinnata. *African journal of Biotechnology*. 2007; 6.
35. Shokri H. Evaluation of inhibitory effects of citric and tartaric acids and their combination on the growth of Trichophyton mentagrophytes, Aspergillus fumigatus, Candida albicans, and Malassezia furfur. *Comparative Clinical Pathology*. 2011; 20: 543-5.
36. Jay JM, Loessner MJ and Golden DA. *Modern food microbiology*. Springer Science & Business Media, 2008.
37. Beuchat LR. Surface decontamination of fruits and vegetables eaten raw. 1998.
38. Silva RV, Gonçalves AD, Vinhal JO, et al. Bioproducts from the pyrolysis of castor seed cake: Basic dye adsorption capacity of biochar and antifungal activity of the aqueous phase. *Journal of Environmental Chemical Engineering*. 2021; 9: 104825.