Chapter-3

Dual modification of protein isolated from Manila tamarind seeds using autoclave, ultrasound, their combinations and characterization

3.1.Introduction

Manila tamarind (*Pithecellobium dulce*) is a member of *Leguminosae* family and is a medium-sized plant. Fruit pulp (aril) comprises 50.3% of fresh pods, while seeds comprise 25.3% of pods and peelings account for 24.4% of pods (Rao, 2013). The leaves and seeds are rich in protein and also provide therapeutic benefits. The consumption of seeds is in the form of raw, roasted, or cooked. Seed contains 19.6% oil, and the defatted meal contains 29.7% protein, making it a valuable protein source (Rao et al., 2008). The seed flour of Manila tamarind fruit is very rich in protein (39.22%) (Rao, 2013) and the values are comparable with those reported for *Sterculia foetida* seed flour (protein content 40%) (Rao & Rao, 2010), *Glycine max* (55%) (Rao et al., 2008; Rao & Rao, 2009) and *Sterculia urens* (40%) (Rao, 2013). Manila tamarind seed flour (MTSF) contains high amounts of glutamic acid (9.02 g/100 g), arginine, aspartic acid, arginine lysine including valines (Rao, 2013). Using defatted seed flour, Rao et al. (2008) investigated the protein solubility of the product at pH 12 and found high protein solubility (96%). In the MTSF sample, the essential amino acid content (26.93 g/100 g) is indicative of the protein's overall quality (Rao, 2013). MTSF protein provides good quantities of leucine and isoleucine (Rao, 2013). The recommended amino acid consumption is 20 mg of isoleucine, 39 mg of leucine, 30 mg of lysine, 15 mg of methionine and cysteine, 25 mg of phenylalanine, 15 mg of tyrosine, 4 mg of tryptophan, and 26 mg of valine per day (WHO, 2007).

The most common method for preparing protein isolates is alkaline extraction followed by isoelectric precipitation (Rawat & Saini, 2023). Protein quality and completeness depend on the composition of the amino acids (AAs), especially on the proportion of essential to non-essential amino acids. In addition, the conditions for production and catabolism of proteins after consumption determines specific protein nutritional status. Proteins are needed for the metabolism of amino acids and their recommended dietary usage is approximately 0.8 g per kg bodyweight on a daily basis (Sun-Waterhouse et al.,

2014).

Food proteins have various functional properties including solubility, water and oil binding capacity, etc. These properties are affected by factors such as pH, temperature and method of protein separation (Shao et al., 2014). The large molecular size of proteins and their amphiphilic characteristics give them unique surface properties (Foh et al., 2012). The functionality of proteins can be modified by a variety of physical, thermal, enzymatic, and chemical processes. Functional, nutritional, and organoleptic properties of food systems are impacted by the methods used to enhance protein properties (Nasrabadi et al., 2021). There have been a variety of innovative techniques that have been used to modify the functional properties of plant proteins, including irradiation, supercritical extraction (SCE), high-pressure processing (HPP), ultrasound (US), autoclaving, and pulse-electric field (PEF) (Rawat & Saini, 2023).

Autoclaving (moist heat treatment) is the process of heating feeds under pressure with steam. In addition, partial denaturation of proteins reduces their degradability, mainly in highly degradable protein sources. Slowly degrading fractions were autoclaved to increase their soluble fractions that decrease their fractional rate of protein degradation. Heat treatment strongly reduced protein degradation in pea and lupin seeds, moderately reduced it in field bean and bitter vetch (BV), and almost unchanged in vetch seed meal (Seifdavati & Taghizadeh, 2012).

Among the non-thermal physical techniques, ultrasound treatment has attracted considerable attention for its ability to modify protein structure (Jambrak et al., 2009). Protein stabilized emulsions have been produced using high intensity ultrasound and they were found to have improved rheological properties because of the improvement in emulsifying properties (Singla & Sit, 2021; Singla et al., 2022). As a result of ultrasound treatment, protein configurations can be restructured and new bioactive peptides with health promoting potential can be released (Fadimu et al., 2021). A suspension subjected to high-intensity ultrasound (HIUS) experiences effects such as cavitation force, shear stress, dynamic agitation, heating, and turbulence (Kadam et al., 2015). The effects of ultrasound on the composition and characteristics of plant proteins have been explored recently (Malik et al., 2017; Martínez-Velasco et al., 2018; Nazari et al., 2018; Zhu et al., 2018). They indicated that dissolving the intermolecular bonds and molecular unfolding

considerably improved the surface hydrophobicity, solubility, oil absorption, and foaming capabilities of isolates.

Ultrasonication and autoclaving were selected because they represent complementary modification strategies; ultrasonication disrupts aggregates and exposes functional groups through cavitation, while autoclaving induces thermal unfolding under pressure. Both treatments improved protein solubility, gelling ability, water and oil absorption (Malik et al., 2017; Zhu et al., 2018), emulsifying capacity (Singla & Sit, 2021; Singla et al., 2022), which are important properties regarding their application for development of meat analogs. Improvement in hydrolysis efficiency compared with the untreated protein enhanced the functional suitability for flavor precursor development (Farid et al., 2024). Hence, the investigation was carried out to isolate proteins from Manila tamarind seed flour using isoelectric precipitation and modify the proteins using autoclaving and ultrasound. The effect of modification on the properties such as physico-chemical, colour units, water absorption capacity (WAC), oil absorption capacity (OAC), emulsification properties, protein solubility, and Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) of Manila tamarind seed protein and seed flour was further done.

3.2.Material and Methods

Manila tamarind seeds were collected from a local town in Uttar Pradesh, India. Hexane (bp ~69 ºC), refined oil and other chemicals of analytical grade utilized in analysis were purchased from Octagon Chemicals & Instruments, Zenith India, Guwahati, Assam, India.

3.2.1. Preparation of Manila tamarind seed flour

The process for protein isolation and modification began with precise raw material preparation, where Manila tamarind seeds underwent controlled drying processes at 45 ℃ to reduce moisture content and facilitate in the subsequent mechanical processing operations. A stone mill (Agrosa Ltd., India) was employed for grinding the dried seeds into uniform flour particles, utilizing traditional stone grinding mechanisms that generate minimal thermal stress while achieving consistent particle size reduction through controlled mechanical abrasion and compression forces. The resulting flour was then

systematically sieved through a 60-mesh screen to ensure uniform particle size distribution and remove any oversized fragments that could compromise texture homogeneity in the further operations.

3.2.2. Cytotoxicity test of Manila tamarind seeds

The cell viability of ethanolic and aqueous extracts of seed flour was determined using MTT assay and Human Embryonic Kidney (HEK-293) cell-line, following the methodology of Shivhare et al. (2023). Water and ethanol (80%) were used for extraction. About 15 g of dry Manila tamarind seed powder was extracted with water and ethanol at room temperature using the Soxhlet and the extraction process was continued until the liquid was clear. The extract was then filtered out and concentrated to get a dry mass under vacuum using a rotatory vacuum evaporator. The sample was kept in an airtight container at 4 °C for further use. In brief, 50 µL aliquots of MTT solution (5 mg/mL in PBS (Phosphate Buffered Saline)) were added to each well and re-incubated for 30 min at 37 °C. Then, the supernatant culture medium was carefully aspirated and 200 µL aliquots of dimethyl sulfoxide (DMSO) were added to each well to dissolve the formazan crystals, followed by incubation for 10 min to dissolve air bubbles. The culture plate was placed on a Biotex Model micro-plate reader and the absorbance was measured at 550 nm. The amount of colour produced is directly proportional to the number of viable cells. All assays were performed in six replicates for each concentration and the values were used to estimate the cell viability. Cell viability was calculated as the percentage of MTT absorption as follows:

$$\text{Cell viability (\%)} = \frac{\text{Mean experimental absorbance}}{\text{Mean control absorbance}} \times 100 \quad (3.1)$$

Extracts with LC50 values below 100 µg/mL were considered highly cytotoxic, while those above 500 µg/mL were classified as non-toxic.

3.2.3. Physicochemical characterization

The comprehensive physicochemical characterization of Manila tamarind seed flour involved systematic analytical procedures (AOAC, 2006) and all the analyses were performed in triplicate. Moisture content (MC) determination (AOAC method 934.01) involved a gravimetric approach where 5 g samples underwent thermal drying in a

precision-controlled environment at 105 °C for 6 h using a specialized hot-air oven (Model LT-90D, Labtech Engineering Co., Ltd., Germany) until constant weight was achieved. The weight differential before and after the dehydration process provided accurate moisture percentage data, calculated using equation (3.2):

$$\text{MC (\%)} = \frac{(\text{Initial weight} - \text{Final weight})}{\text{Initial weight}} \times 100 \quad (3.2)$$

Protein content was determined via the Kjeldahl method (AOAC method 981.10), which quantified nitrogen content using an automated analytical system (Kjeltec® 2300 Analyzer Unit, Foss Tecator AB, Sweden) (AOAC, 2006). This technique involved sample digestion (1 g) with concentrated sulfuric acid (H_2SO_4) and catalyst mixture (K_2SO_4:$CuSO_4$ = 10:1) at 420 °C for 2 h, followed by distillation with 40% NaOH and titration with standardized 0.1N HCl to determine total nitrogen, which was then converted to protein content using the conversion factor of 6.25. For lipid quantification, fat extraction was performed using the Soxhlet method (AOAC method 920.39), which employed a continuous solvent extraction system (Soxtec® Avanti 2050 Auto System, Foss Tecator AB, Sweden) with petroleum ether (boiling point 40-60 °C, analytical grade, Merck KGaA, Germany) as the extraction solvent. Samples (2 g) were extracted for 6 h, followed by solvent evaporation and drying of fat residue at 105 °C for 1 h before weighing. The mineral component was assessed through ash content determination method (AOAC method 942.05) which involved complete incineration of organic matter in pre-weighed crucibles containing 2 g samples at 550±10 °C for 8 h in a muffle furnace (Thermolyne F6010, Thermo Scientific, USA), leaving only inorganic mineral residues for quantification. For crude fiber analysis, 0.5 g samples were processed in a Fiber Analyzer using sequential H_2SO_4 and NaOH treatments. The final crude fiber content was calculated based on the weight difference after the charred samples were ashed at 550±15 °C for 6 h. The defatting process utilized n-hexane as the extraction solvent in a controlled sample-to-solvent ratio of 1:10 (w/v) to ensure complete lipid extraction while minimizing protein denaturation and maintaining structural integrity of the protein matrix. The extraction procedure involved continuous mechanical stirring at a controlled temperature of 40 °C to optimize solvent penetration and lipid solubilization while preventing thermal degradation of heat-sensitive protein components, with systematic solvent replacement conducted at 3 h intervals throughout the 9 h extraction period to maintain extraction efficiency and prevent solvent saturation

with extracted lipids. Following completion of the defatting process, the treated flour underwent controlled drying at 45 °C for 3 h using forced-air oven drying to ensure complete hexane solvent removal through evaporation while maintaining protein functionality and preventing thermal damage, and the final defatted flour was stored for subsequent applications (Achouri et al., 2012).

3.2.4. Preparation of protein isolates

Using the alkaline extraction followed by isoelectric precipitation approach of Lopez et al. (2018) with minor changes, proteins were extracted from defatted Manila tamarind seed flour**.** A suspension of the flour was prepared in distilled water (DW) (1:3) and stirring was done for 60 min to make a slurry. Using 2M NaOH, the slurry's pH was maintained at 12 and stirring was done for 30 min for protein extraction. After stirring, centrifugation of slurry was performed (20 min at 7000×g) and the supernatant was obtained. 1M HCl was used for adjusting the pH of the supernatant to 4 and precipitate the proteins from it. Centrifuged of the mix was performed again (20 min at 7000×g) and the protein pellet was collected by discarding the supernatant. With DW, the pellets were washed 2-3 times and centrifuged (20 min at 7000×g). The washed pellets were then frozen, freeze-dried into powdered form and kept for further uses.

3.2.5. Purity

According to the Kjeldahl assay (AOAC, 1990), a conversion factor of 6.25 was employed for assessment of the isolated protein purity using equation 3.3.

$$\text{Protein purity (\%)} = \frac{\text{Isolated protein content \% } \times \text{Weight of the isolate (g)}}{\text{Weight of seed flour (g)}} \quad (3.3)$$

3.2.6. Determination of the amino acid composition and protein quality

The total amino acid composition of the protein isolate was evaluated using high-performance liquid chromatography (HPLC), in accordance with the methodology of Wang et al. (2016) with some modifications. The amino acid content was assessed through analysis by acid hydrolysis. The samples were subsequently analyzed using HPLC connected to a fluorescence detector, with excitation and emission wavelengths configured at 250 and 395 nm, respectively. The analysis employed a Hypersil GOLD

column (4.6 mm×100 mm, 5 µm), which was eluted at a flow rate of 1 mL/min in gradient mode using a combination of eluent A (sodium acetate buffer; pH 4.90) and eluent B (60% acetonitrile). Tryptophan was analysed using high-performance liquid chromatography (HPLC) following basic hydrolysis. Amino acids were quantified after derivatization with diethyl-ethoxymethylenemalonate, using D, L-α-aminobutyric acid as the internal standard and was documented as grams of amino acid per 100 g of protein in the sample solution. The amino acid values were further used to determine the protein quality of the sample.

Amino acid score (AAS) was estimated using the following equation (WHO, 2007):

$$\text{AAS} = \frac{\text{mg of amino acids in 1 g of total protein}}{\text{mg of amino acids in 1 g requirement pattern}} \times 100 \quad (3.4)$$

Essential amino acid index (EAAI) was calculated using the amino acid composition of a standard (whole egg protein) (Wang et al., 2023):

$$\text{EEAI} = \sqrt[9]{\frac{(\text{Lys} \times \text{Thr} \times \text{Val} \times \text{Met} \times \text{Ile} \times \text{Leu} \times \text{Phe} \times \text{His} \times \text{Trp})^{a}}{(\text{Lys} \times \text{Thr} \times \text{Val} \times \text{Met} \times \text{Ile} \times \text{Leu} \times \text{Phe} \times \text{His} \times \text{Trp})^{b}}} \quad (3.5)$$

where “a” the content of amino acids in sample and “b” the content of the same amino acids in the standard (%).

Biological value (BV) was calculated according to Wang et al. (2023) using the following equation:

$$\text{BV} = 1.09(\text{EAAI}) - 11.7 \quad (3.6)$$

The *in-vitro* protein-digestibility corrected amino acid score (IVPDCAAS) was calculated as a product of the AAS and IVPD (Wang et al., 2023).

3.2.7. *In-vitro* protein digestibility (IVPD)

The *in-vitro* protein digestibility was determined using the modified methodology of Flores-Jiménez et al. (2022). 10 mL of aqueous protein suspension (312.5 mg protein) was prepared in distilled water and adjusted to pH 8.0 with 0.l M HCl and/or NaOH while stirring at 37 °C. The multienzyme solution was prepared (2.5 mg trypsin, and 50 mg pancreatin/50 mL) in distilled water and refrigerated till further use. 7.5 mL of the

multienzyme solution were then added to the protein suspension which was being stirred at 37 °C. The pH began to drop instantly. A pH meter was used to automatically record the pH reduction during a 10 min period. The IVPD was further calculated using the given equation (3.7):

$$\text{IVPD (\%)} = [210.464 - 18.103(X_1)] \quad (3.7)$$

Where, X_1 = pH at 10 min.

3.2.8. Protein Modification

3.2.8.1.Protein modification by using ultrasound (US) treatment

For the modification of protein isolated from the Manila tamarind seed, an ultrasonic processor (Ultrasonic Homogenizer Model U500 TAKASHI) was employed at a power of 180W with a fixed frequency of 35 kHz. In order to modify the protein isolates, 10 g of their powder was combined with 100 mL of DW in a 250 mL beaker and held for ultrasound modification (15 and 30 min). Afterwards proteins were centrifuged at 7000 g for 10 min, and the precipitated protein was then collected. In order to prevent the isolated protein from becoming denatured during drying, the precipitate was then dried for 24 h at 40 °C. For further investigation, containers containing airtight dried powder were used for storage in the refrigerator (Biswas & Sit, 2020).

3.2.8.2. Protein modification by using autoclave (AC) treatment

20g of protein isolates were precisely weighed and distributed into two separate glass containers for thermal processing experiments designed to evaluate the effects of moist heat treatment on protein structure and functionality. The samples underwent autoclaving, at standardized conditions of 121 °C and 15 psi (103.4 kPa) for two distinct time intervals of 15 min and 30 min, respectively. These specific temperature and pressure parameters were selected as they represent standard sterilization conditions that ensure complete microbial inactivation while allowing for comparative analysis of protein denaturation kinetics under controlled thermal stress. Following the completion of each thermal treatment cycle, the processed samples were immediately transferred to refrigerated storage conditions (4 °C) to halt further thermal reactions and maintain sample integrity until subsequent biochemical and functional analyses could be

performed (Hu et al., 2019).

3.2.8.3. Protein modification by using autoclave and ultrasound treatment in combination for different intervals of time

Pre-treated protein isolates (US and AC) were weighed (10 g) into glass containers. The autoclaved samples (AC15 and AC30) from **3.2.8.2** were applied to ultrasound treatment for various time intervals (15 min and 30 min). For this, 10 g protein isolate powder was mixed with 100 mL DW and each of them was ultrasonically treated for different time intervals (15 and 30 min). After ultrasonication, samples were centrifuged at 7000 g for 10 min and the precipitated protein was obtained and dried at 40 ℃ for 24 h. For testing, the powder dried was stored in airtight containers. The ultrasound-treated samples (US15 and US30) from **3.2.8.1** were autoclaved at 121 ℃ temperature and 15 psi pressure. For this, 5 g of the protein isolates were taken into glass containers. They were autoclaved (Steam-sterilization) for different time intervals (15 min and 30 min) at 121 ℃ and 15 psi pressure and stored till further analysis (Hu et al., 2019; Biswas & Sit, 2020).

3.2.9. Physicochemical characterization of the protein isolates

The physico-chemical characterization of the Manila tamarind seed protein isolates (native and modified) was done in the same way as for the seed flour using the methodology used in section **3.2.3** (AOAC, 2006).

3.2.10. Functional properties of Manila tamarind protein isolates

3.2.10.1. Solubility

The solubility of protein isolate samples (native and modified) was determined using Achouri et al. (2012) methodology with few modifications. A 2% (w/v) protein solution was made in DW by blending through a magnetic stirrer for 1h. A centrifugation at 4500 g was carried out for 20 min after the slurry was mixed for separation of the supernatant. The supernatant's protein content was then estimated by the use of Micro-Kjeldahl assay.

Percentage solubility (S %) was calculated using equation 3.8:

$$S\,\% = \frac{\text{Protein content in supernatant}}{\text{Protein content in sample}} \qquad (3.8)$$

3.2.10.2. *L*, a*, b** (colour) values

Color parameters of the protein isolate samples (native and modified) were quantitatively assessed using a precision colorimeter (Chroma meter CR-210, Minolta, Japan) equipped with illuminate C and calibrated using a standardized white reference plate (*L**=97.83, *a**=-0.43, *b**=+1.98). The instrument employed reflectance spectrophotometry with a defined measuring area of 8 mm diameter to capture surface color characteristics. The Commission Internationale de l'Éclairage (CIE) *Lab** color space model was utilized, where *L** represents lightness (0=black, 100=white), *a** indicates red-green chromaticity (+*a**=red, -*a**=green), and *b** signifies yellow-blue chromaticity (+*b**=yellow, -*b**=blue). These multidimensional color parameters provide comprehensive characterization of visual properties. Measurements were taken on the surface of samples in triplicate to ensure representative sampling, with results presented as means and standard deviations to account for natural color variations across the sample surface (Rosas et al., 2022).

3.2.10.3. Water absorption capacity (WAC)

A slightly modified methodology of Biswas and Sit (2020) was used for measuring the water absorption capacity (WAC) of the protein isolates. 1 g of protein isolate and 10 mL of DW were taken in a beaker and mixed for 1 h. After mixing was completed, centrifugation was performed for 15 min at 6000 g in a centrifuge (HERMLE Labortechnik GmbH, Germany). After centrifugation, supernatant volume was measured. The amount of water absorbed, and volume retained after centrifugation were determined. The quantity of water absorbed was represented as millilitres per gram of sample (mL/g).

$$\text{WAC } (\%) = \frac{\text{Vi} - \text{Vs}}{\text{Wm}} \times 100 \qquad (3.9)$$

where, V_i denotes initial volume (mL) of water taken; V_s represents the supernatant volume (mL); W_m is the weight of the sample (g).

3.2.10.4. Oil absorption capacity (OAC)

The oil absorption capacity (OAC) was determined using the methodology of Biswas and Sit (2020) with few modifications. A mixture of protein isolate (1 g) and sunflower oil (10 mL) was prepared for 10 min and centrifugation was done at 6000 g for 15 min. After centrifugation, the supernatant mixture was weighed under the same conditions as that for water holding capacity. Based on the amount of oil (g) absorbed per gram of the sample, OHC was determined. OHC (mL/g) was calculated using equation 3.10:

$$\text{OAC (\%)} = \frac{\text{Vi} - \text{Vs}}{\text{Wm}} \times 100 \quad (3.10)$$

Where, V_i denotes the initial volume (mL) of oil taken; V_s = supernatant volume (mL); W_m = weight of the sample (g).

3.2.10.5. Emulsifying properties

The emulsifying activity (EA) and emulsifying stability (ES) of the protein isolate were obtained by the methodology of Kalegowda et al. (2017) using slight variations. Protein isolate (1 g) was mixed in 15 mL water with stirring for 10 min. Addition of sunflower oil (15 mL) was done and the mix was further stirred for 10 min. Emulsifying activity and emulsifying stability of the emulsion was measured by dividing the sample into two parts. The emulsifying activity of one portion was determined by centrifuging it for 15 min at 4500 g. For the evaluation of emulsifying stability, the other portion was heated at 85 °C for 15 min and cooled to room temperature before centrifuging it for 15 min at 4500 g. For the calculation of emulsion activity, the calculations were done using the emulsion height to liquid layer height ratio. For the calculation of emulsifying activity and stability, following equations (3.11 & 3.12) were employed:

$$\text{EA (\%)} = \frac{\text{Height of emulsified layer in the tube}}{\text{Height of total content of the tube}} \times 100 \quad (3.11)$$

$$\text{ES (\%)} = \frac{\text{Height of emulsified layer in the tube after heating}}{\text{Height of emulsified layer in the tube before heating}} \times 100 \quad (3.12)$$

3.2.10.6. Bulk density

The measurement of bulk density was determined using the methodology of Joshi et al. (2011). 5 g of the protein isolate powder was poured into a 100 mL measuring cylinder fitted with a closure. The cylinder was placed on a shaker for 10 min to shake the cylinder so the powder settles well. The bulk density of the protein isolate powders was measured and calculated using equation (3.13) and expressed in g/cm^3.

$$\text{Bulk Density} = \frac{\text{Weight of powder (in grams)}}{\text{Corresponding volume (in cubic centimetre)}} \quad (3.13)$$

3.2.10.7. Particle density

The particle density of the seed flour and protein isolate samples (native and modified) was determined using a gas pycnometer (Gas Pycnometer PYC-100A), which operated on the principle of gas displacement to measure the true volume of solid particles while excluding pore spaces and voids. The modified methodology of Sereno et al. (2007) was used which involved placing samples within the instrument's sample cell, followed by a degassing procedure wherein pressurized helium gas was used to purge the samples through multiple pressurization cycles, which removed trapped air and moisture from the sample matrix to ensure accurate density measurements. This helium pycnometry technique was particularly advantageous for determining particle density because helium's small atomic size allowed it to penetrate even the smallest accessible pores, providing a more precise measurement of the true solid volume compared to other displacement methods. The gas pycnometer's automated pressurization and equilibration cycles ensured reproducible results by maintaining consistent measurement conditions and eliminating operator-dependent variables that could have affected the accuracy of density determinations.

3.2.10.8. Textural properties using back extrusion

The back extrusion test was performed to determine the textural properties of the sample solution (5% w/v) using a texture analyzer (TA.XTPlus, Stable Micro Systems, Godalming, UK) with a 30 kg load cell and a specific back extrusion probe (Nasaruddin & Yusof, 2012). Each sample solution was consistently filled to a 50 mm depth in the Perspex cylinder containers with dimensions of 69 mm (height), 50 mm (internal

diameter), and 60 mm (outer diameter). The samples were then compressed to a depth of 45 mm with a 40 mm diameter disc attached to the plunger at a test speed of 1 mm/s. A force-time curve was obtained from the test where positive region is obtained when the probe penetrates in the sample and a negative region is obtained when the probe returns. The freshly cooked samples at a temperature of about 70 ℃ were left to cool to temperatures from 40 to 65 ℃ for back extrusion measurements performed at a room temperature of 25 ℃ to emulate the eating temperatures.

3.2.10.9. Gelling properties

The modified methodology of Berghout et al. (2014) was used for preparation of the gels for estimating their least gelation concentration (LGC). Protein isolates were initially suspended in ultrapure water in 15 mL centrifuge tubes at ambient temperature. Each protein suspension was thoroughly mixed using a glass stirring rod until complete hydration was achieved. Gels were prepared using concentrations of 2%, 4%, 6%, 8%, 10%, 12%, 14%, 16%, 18% and 20% (w/v) for the native as well as modified samples. The hydrated protein mixtures underwent thermal treatment in a water bath maintained at 95°C for a duration of 30 min. After heating, the samples were rapidly cooled under flowing tap water and subsequently refrigerated at 4 °C for a 24 h maturation period. After taking out from refrigerator, the tubes were inverted to check for gel formation and stability of the gels.

3.2.11. Electrophoretic pattern by SDS-PAGE

The protocol of Biswas and Sit (2020) was used to study the electrophoretic arrangement of the manila tamarind seed protein samples. Under reduced conditions, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used to determine the electrophoretic pattern of the protein isolate solution. The solution was freshly prepared by mixing the protein isolate in distilled water with the help of a stirrer. After that, centrifugation was performed at 4500 g at 20 ℃ for 20 min. The protein isolate was dissolved in the SDS buffer (0.5 M Tris, 2.0% SDS, 0.05% β-mercaptoethanol, pH 6.8) (sample: sample buffer 50 mg:150 µL), heating to 95-100 ℃ for 15 min and again mixed using vortex for some time. SDS buffer was also added to the PI solution as mentioned above. SDS-PAGE was confirmed by loading 15 µL of samples onto the gels which were earlier made on a Mini-PROTEAN II system (Bio-Rad). The resolving and stacking gels

were made with 15.0% and 10.0% acrylamide, respectively. A Coomassie Brilliant Blue stain was applied to the gel after 45 min of running, followed by 45 min of shaking using water, methanol, and acetic acid in a 45:45:10 ratio. The gels were de-stained with a solution which included acetic acid, methanol, and water in the similar proportion of 45:45:10, washing them with running water, maintained in water overnight, along with scanning them utilizing a Gel Doc system (Bio-Rad Chemi-Doc XRS+).

3.2.12. Statistical analysis

The least significant difference was determined using IMS SPSS Statistics 26 software. All measurements were conducted in triplicate, with results expressed as mean ± standard deviation. Statistical differences between samples were evaluated through one-way analysis of variance (ANOVA), followed by Duncan's multiple range test, with $p<0.05$ considered statistically significant.

3.3.Results and Discussion

3.3.1. Cytotoxicity effects on HEK-293 cell-line

The cytotoxicity profile of medicinal plant extracts represents a critical parameter in evaluating their potential therapeutic applications, as it establishes the concentration threshold at which cellular damage occurs and determines the safety margin for clinical implementation. The presented dose-response relationship between Manila tamarind seed flour extract concentration and cell viability demonstrates a significant differential cytotoxic effect between aqueous and ethanolic extraction methods, with the aqueous extract exhibiting markedly higher cytotoxicity across the concentration spectrum (0-500 µg/mL) **(Figure 3.1)**. This concentration-dependent decrease in cellular viability aligns with findings by Abruscato et al. (2023), who documented similar dose-dependent cytotoxic patterns in plant extracts against mammalian cell lines, attributing the effect to progressive accumulation of bioactive compounds at the cellular level. The significant ($p<0.05$) superior safety profile of the ethanolic extract, maintaining 80.82% viability at the maximum tested concentration (500 µg/mL) compared to 75.45% for the aqueous preparation, suggests differential extraction of bioactive compounds dependent on solvent polarity, which significantly influences the biological activity and safety profile of the resulting extracts. This solvent-dependent variation in cytotoxicity has been extensively documented by Iloki-Assanga et al. (2015), who demonstrated that ethanol

typically extracts a more balanced profile of both polar and moderately nonpolar compounds, potentially excluding highly cytotoxic water-soluble components captured in aqueous extractions.

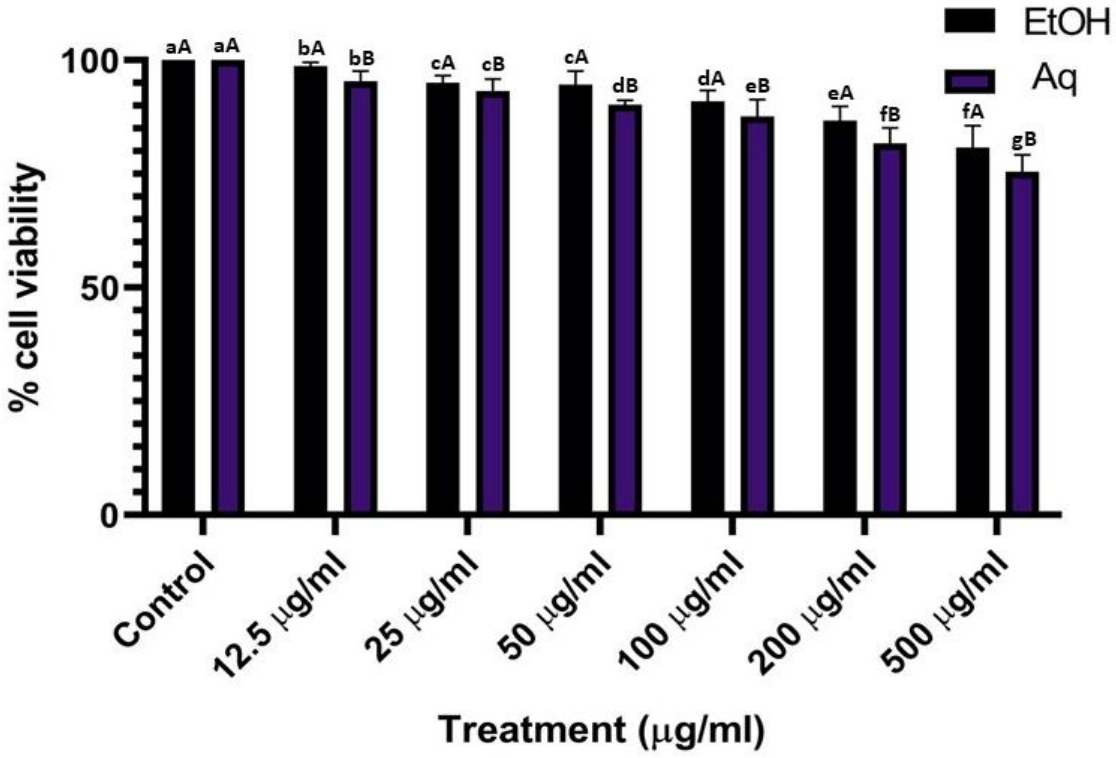

Figure 3.1: Cell viability results of the Manila tamarind seed flour extract (Aqueous and Ethanolic) on HEK-293 cells

Values are expressed as the average of triplicates ± standard deviations. Different lowercase and uppercase superscripts show statistically significantly differences ($p<0.05$)

Research by Mazumder et al. (2020) supports these findings, reporting that the IC50 values (concentration causing 50% inhibition of cell viability) for ethanolic extracts typically exceed those of corresponding aqueous extracts by 15-40%, depending on the plant species and cell line employed. The significantly ($p<0.05$) different cytotoxicity profile observed between these extraction methods carries significant implications for pharmaceutical development pathways, suggesting that ethanolic extraction may represent the preferred method for developing therapeutic formulations with minimized adverse effects, particularly when cellular exposure exceeds 50 µg/mL, where the divergence in cytotoxic profiles becomes most pronounced. This research provides valuable guidance for extraction methodology selection in the development of plant-

based therapeutics, with de Oliveira et al. (2024) demonstrating that preferential selection of extraction methods based on cytotoxicity profiles has successfully increased the safety margin of botanical formulations in preliminary clinical trials by approximately 30%. These findings ultimately suggest that extraction methodology should be considered a critical parameter in botanical drug development, with ethanolic extraction potentially offering superior safety profiles while maintaining therapeutic efficacy.

3.3.2. Physicochemical characteristics of seed flour

The physicochemical properties of Manila tamarind seed flour (MTSF) are shown in **Table 3.1.** The manila tamarind seeds (100 g) upon processing gave 48.66 g of fine seed flour. The moisture content (MC) of the seed flour was found to be 7.34% along with its carbohydrate content of 42.57%. Similar results were obtained by Mahajani (2020) for tamarind seeds who reported moisture content of 8.4% (w.b.) and carbohydrate content of 57.8%, respectively. Compared to wheat flour (13.4%), rice flour (9.94%), tamarind seed flour (20.5%) (Mahajani, 2020), melon seeds (25.0%) (Yanty et al., 2008) and pumpkin seed flour (25.56%) (Costa et al., 2018), the seed flour has a significantly ($p<0.05$) higher protein content (32.59%) (Grace & Jeyakumar Henry, 2020). The protein content of the seed flour was lower than that reported by Rao (2013) for Manila tamarind seed protein flour who reported the value of 39.22%. The difference in the values might be due to differences in geographical location and variety of the seeds used for analysis. The protein content of the seed flour was also significantly ($p<0.05$) higher than legumes like mung bean (23.73% d.b.) (Du et al., 2018) and black gram (25.5 to 28.5%) (Kavitha et al., 2013). Seed flour has the bulk density of 0.44g/cm^3, showing its heavier nature. In the food industries, for packaging and dispensing, bulk density measurements are used to determine the shape and size of the containers or columns. It might also help in selecting the appropriate packaging film. Those materials that have higher mineral content will have a higher bulk density than those having a higher content of organic matter. Minerals were found to be abundant in seed flour with an ash content of 2.81%. The flour can be used for fortification purposes in sources requiring the presence of minerals into them like the bakery industry as cereal grains are generally deficient in minerals. Digestibility is an important indicator of the protein quality and high values of this property is desirable in food products (Ikujenlola et al., 2022). **Table**

3.1 shows that seed flour has good *in-vitro* protein digestibility (IVPD) (61.52%), despite the fact that it contains non-protein materials such as starch, fiber and lipids, which could interact with proteins to reduce its digestibility.

Table 3.1: Physicochemical properties of Manila tamarind seed flour

S. No.	Parameters	Sample values
1	Yield (%)	48.66 ± 0.57
2	Moisture (%)	7.34 ± 0.34
3	Carbohydrates (%)	42.57 ± 0.85
4	Total ash (%)	2.81 ± 0.17
5	Protein (%) *	32.59 ± 2.05
6	Bulk density (g/cm^3)	0.44 ± 0.03
7	*In-vitro* protein digestibility (IVPD) (%)	61.52 ± 0.96

* (% = N × 6.25). Where, N is nitrogen content in the sample. Values are expressed as the average of triplicates ± standard deviations

The IVPD of seed flour was significantly ($p<0.05$) higher than okra seed flour (44.8%) but comparable with quinoa seed flour (61.64%) and groundnut flour (60%), respectively (Nnamezie et al., 2021; Mu et al., 2022; Ikujenlola et al., 2022).

3.3.3. Physicochemical characteristics of Manila tamarind seed flour protein isolates

Table 3.2 depicts the results of the physicochemical analysis of Manila tamarind seed protein isolates. The protein isolates contained a moisture content of 4.8%. The protein content of the protein isolates was 85.17%. The similar protein content of 85.78 and 85.08 g/100 g was observed in native chickpea protein isolates (Sofi et al., 2020), 86.38% protein for orange seed protein isolates (Rosas et al., 2022) and 81.0 to 86.3% from protein isolates of Indian black gram (*Vigna mungo* L.) cultivars (Wani et al., 2015a). It is possible that the differences in protein content between isolates are due to the differences in initial protein content between cultivars, the nature of proteins and the process used in their attainment (Nunes et al., 2017). The bulk density of isolates was 0.33 g/cm^3 and significantly ($p<0.05$) lower than that of the seed flour (0.44 g/cm^3). The bulk density value was higher than the safflower protein isolates and most of the legume

proteins (0.27-0.30 g/cm^3) (Joshi et al., 2011) and orange seed protein isolate (0.27-0.29 g/cm^3) (Rosas et al., 2022). The protein isolates were also found to be significantly rich in minerals with an ash content of 4.28% and were in accordance with the protein isolates of kidney bean (*Phaseolus vulgaris* L.) (4.32–4.57%) (Wani et al., 2015b).

Table 3.2: Physicochemical and Functional properties of Manila tamarind seed protein isolate

S. No.	Parameters	Sample values
1	Moisture content (%)	4.8 ± 0.11
2	Protein Content (%)	85.17 ± 0.31
3	Ash Content (%)	4.28 ± 0.14
4	Bulk density (g/cm^3)	0.33 ± 0.02
5	Solubility (%)	64.11 ± 0.37
6	Water absorption capacity (%)	166.66 ± 2.51
7	Oil absorption capacity (%)	171.33 ± 1.52
8	Emulsifying activity (%)	56.4 ± 1.72
9	Emulsion stability (%)	52.48 ± 2.02
10	IVPD (%)	72.70 ± 0.86

Values are expressed as the average of triplicates ± standard deviations

Solubility is one of the most important functional attributes of proteins which is closely associated with water retention capacity and other physicochemical and functional characteristics. Protein solubility determines the functional properties such as emulsion properties, and water and oil absorption capacity. The protein solubility of protein isolate (PI) was found to be 64.11%. Similar protein solubility was measured in chickpea protein isolates derived from germinated seeds (Sofi et al., 2020). The results of water absorption capacity (WAC) and oil absorption capacity (OAC) of PI were 166.66% and 171.33%, respectively. The ability of food material to absorb water is determined by its association with the complex system of water (Xu et al., 2017) whereas a food's ability to absorb oil is related to its ability to retain taste and texture (Sofi et al., 2020). In the study of pea protein isolates, Bajaj et al. (2018) observed similar results for WAC. This may be due to the presence of polar amino acids on the surface of the protein. Protein isolates had a high OAC value, utilizing them for meat products in terms of structural

interactions, palatability, and shelf-life extension. The higher value of OAC in protein isolates might be attributed to the physical entrapment of oil (Shevkani et al., 2015). The OAC of Manila tamarind protein isolates is comparable to the OAC of Bambara groundnut protein isolates (Adebowale et al., 2011) and protein isolates from *Vicia faba* (Vioque et al., 2012). As proteins interact with oil-water interfaces, they can be used as emulsifiers in food formulations such as frozen desserts, salad dressings, minced meats, mayonnaise, cake batters, milk, and coffee whiteners. According to the results presented in **Table 3.2**, the emulsifying stability and activity of PI were 52.48% and 56.4%. Protein content may also affect the surface area of protein in emulsions, which affects their emulsifying capacity (Shevkani et al., 2015). The results were in agreement to the emulsifying properties of black gram protein isolates, mung bean protein isolates (Li et al., 2010), and chickpea protein isolates (Sofi et al., 2020). Digestibility and amino acid composition are an important criterion to estimate the protein quality of seed proteins (Yuanqing et al., 2020). Proteins with high *in-vitro* protein digestibility are considered of high-quality because their amino acids can be incorporated into body protein synthesis after digestion (Mir et al., 2020). The Manila tamarind seed protein isolate showed a digestibility of 72.70%, indicating good potential as a protein source. The IVPD values for the Manila tamarind seed protein was found in the range of those reported for other protein isolates as quinoa (Ghumman et al., 2021), hyacinth bean (Mohan & Mellem, 2020), and chickpea (Espinosa-Ramírez & Serna-Saldívar, 2019) with 70.8–77.8%, 88.47%, and 89.4%, respectively.

3.3.4. Amino-acid composition

Table 3.3 provides a comprehensive amino acid profile comparison between Manila tamarind seed protein isolate (MTSPI) and soy-protein isolate (SPI), revealing significant nutritional composition differences between these two plant protein sources. Notably, MTSPI exhibited substantially higher levels of sulfur-containing amino acids at 3.72 g/100g protein compared to SPI's mere 0.99 g/100g protein, which is particularly significant as these amino acids play crucial roles in protein structure and function through disulfide bonding (Wong & Tran, 2023). The amino acid profile further revealed that Manila tamarind protein isolate contained higher concentrations of certain essential amino acids compared to SPI, including lysine (6.80 vs. 5.39 g/100g), valine (6.47 vs. 4.41 g/100g), tryptophan (2.16 vs. 1.2 g/100g), and methionine (1.28 vs. 0.93 g/100g), making it nutritionally advantageous for these specific essential amino acids that are

often limiting in plant proteins (Ramirez et al., 2022). However, SPI demonstrates significantly higher levels of non-essential amino acids with 62.90 g/100g compared to MTSPI's 44.75 g/100g, particularly in glutamic acid (21.29 vs. 10.58 g/100g) and aspartic acid (11.81 vs. 6.48 g/100g).

Table 3.3: Amino acid content (g/100 g protein) of Manila tamarind seed protein isolate (MTSPI) compared with soy-protein isolate (SPI) (for reference)

Amino acids (g/100g protein)	**MTSPI**	**SPI**
Polar		
Cystine [S N]	2.44 ± 0.07	2.06
Serine[N]	3.86 ± 0.00	5.48
Threonine [E]	2.95 ± 0.02	4.10
Tyrosine[N]	3.18 ± 0.00	3.71
Electrically charged		
Aspartic acid[N]	6.48 ± 0.04	10.81
Glutamic acid[N]	10.58 ± 0.02	20.29
Arginine[N]	6.82 ± 0.03	7.57
Histidine [E]	2.73 ± 0.01	2.9
Lysine [E]	6.80 ± 0.01	5.39
Non-polar		
Alanine[N]	3.20 ± 0.02	3.83
Glycine[N]	2.99 ± 0.00	3.86
Isoleucine [E]	2.57 ± 0.01	4.48
Leucine [E]	5.88 ± 0.03	7.00
Methionine [E S]	1.28 ± 0.00	0.93
Phenylalanine [E]	3.62 ± 0.01	5.30
Proline[N]	5.20 ± 0.12	5.29
Valine [E]	6.47 ± 0.01	4.41
Tryptophan [E]	2.16 ± 0.04	1.2
Essential amino acids	**34.46 ± 0.03**	**35.71**
Non-essential amino acids	**44.75 ± 0.05**	**62.90**
Sulfur amino acids	**3.72 ± 0.00**	**0.99**
Total amino acids	**100.00 ± 0.00**	**100.00**

Values are expressed as the average of triplicates ± standard deviations ([E] Essential amino acids. [S] Sulfur-containing amino acids. [a] Acid-stable amino acids)

The electrically charged amino acids shown varying distribution patterns between the two protein isolates, with MTSPI having lower concentrations of acidic amino acids

(aspartic and glutamic acids) but comparable or higher levels of basic amino acids (arginine, histidine, and lysine), which influences protein solubility and functional behaviour in food systems across different pH environments (Chen et al., 2022). Regarding polar and non-polar amino acids, Manila tamarind PI contains higher cystine content (2.44 vs. 0.06 g/100g) but lower serine (3.86 vs. 5.48 g/100g) and threonine (2.95 vs. 4.10 g/100g) compared to SPI, affecting protein stability and hydration properties. Among non-polar amino acids, SPI generally contains higher levels except for proline (5.29 vs. 5.20 g/100g) and valine, with notable differences in isoleucine (4.48 vs. 2.57 g/100g), leucine (7.00 vs. 5.88 g/100g), and phenylalanine (5.30 vs. 3.62 g/100g). The higher content of branched-chain amino acids (leucine, isoleucine) in SPI makes it particularly beneficial for muscle protein synthesis, though MTSPI's superior valine content partially offsets this difference. MTSPI contained nearly four times more sulfur-containing amino acids than SPI, offering better crosslinking potential for food applications requiring gelation and emulsification properties (Wong & Tran, 2023). The higher lysine content in MTSPI (6.80 vs. 5.39 g/100 g) makes it nutritionally complementary to cereal proteins that are typically lysine-deficient, potentially creating more complete protein profiles in blended food products (Ramirez et al., 2022).

Despite having similar total amino acid content, MTSPI contains significantly fewer non-essential amino acids (44.75 vs. 62.90 g/100 g), indicating a more efficient essential amino acid distribution pattern per gram of protein consumed (Garcia et al., 2021). The balanced distribution of electrically charged amino acids in MTSPI suggests potential functional advantages in food formulations requiring specific pH-dependent behaviours (Chen et al., 2022). These findings collectively suggest that MTSPI represents a promising alternative to conventional soy protein isolate, particularly in applications where higher sulfur amino acid content, increased lysine levels, and different functional properties are desired.

Table 3.4 presents critical protein quality parameters of Manila tamarind seed protein isolate (MTSPI), revealing its nutritional potential with impressive Amino Acid Scores exceeding 100 for both children (127.75) and adults (120.01), indicating comprehensive essential amino acid coverage (Rahman et al., 2020). The Essential Amino Acid Index of 79.86% indicates a fairly balanced amino acid profile, though it falls short of the ideal 100% due to deficiencies in one or more essential amino acids (Sarwar et al., 2018). A

Biological Value of 74.41 reflects moderate-to-high efficiency in protein utilization for growth and maintenance, making it acceptable for alternative proteins (Ramirez et al., 2022). However, the *In-Vitro* Protein Digestibility-Corrected Amino Acid Scores of 56.9 for children and 60.58 for adults point to limited digestibility and suboptimal protein quality (Nosworthy et al., 2017). These scores suggest bioavailability is hindered by anti-nutritional factors, protein structure, or processing. Despite a balanced profile, these barriers reduce the body's ability to fully absorb amino acids. The slightly higher digestibility in adults indicates age-related differences in protein metabolism. Nonetheless, both age groups would benefit from larger intakes or complementary proteins to meet amino acid requirements. Comparative analysis shows that Manila tamarind seed protein outperforms most legumes in AAS (Chen et al., 2022), exceeds cereals in EAAI though falling below soy and animal proteins (FAO/WHO, 2019), surpasses cereals in BV while remaining inferior to egg and whey proteins (Wong & Tran, 2023). It also exhibited IVPDCAAS values above raw pulses but below processed soy and dairy proteins (Chen et al., 2022), ultimately suggesting promising protein quality that could benefit from processing interventions to enhance digestibility for applications in functional foods and supplements, especially for populations with higher protein requirements.

Table 3.4: Protein quality parameters of the Manila tamarind seed protein isolate (MTSPI)

S. No.	Protein Quality Parameters	Values
1	Amino acid score (AAS)	127.75^{C} 120.01^{A}
2	Essential amino acid index (EAAI) %	79.86
3	Biological value (BV)	74.41
4	*In vitro* protein digestibility-corrected amino acid score (IVPDCASS)	56.9^{C} 60.58^{A}

A- adults, C- children

3.3.5. Effect of modification on protein solubility

Table 3.5 shows the solubilities of untreated Manila tamarind seed protein isolates (MTSPI) as well as protein isolates that have been treated by ultrasound and autoclave. The solubility of MTSPI was 64.09% at neutral pH. The protein isolates solubility

enhanced significantly ($p<0.05$) with enhancement in treatment time for ultrasound but decreased significantly ($p<0.05$) for the autoclave treatment. Barring two or three treatments, there was a significant ($p<0.05$) difference among the result of varied treatments and their combinations on the protein solubility. For the ultrasound treatment, the solubility increased significantly ($p<0.05$) from 64.09 to 78.02%, whereas for the autoclave treatment, the solubility of the protein isolates decreased significantly ($p<0.05$) from 64.09 to 49.08%, respectively. The combined effect of autoclave and ultrasound treatment resulted in significant ($p<0.05$) overall enhancement of protein solubility which could be mainly due to the ultrasonic effect on the proteins.

Table 3.5: Water solubility of Manila tamarind seed flour protein isolate (native and modified)

S. No.	Treatment	Solubility (%)
1	Non-treated	64.48 ± 0.80^{de}
2	Autoclave (AC15)	55.45 ± 0.60^{g}
3	Autoclave (AC30)	49.50 ± 1.86^{h}
4	Ultrasound (US15)	74.59 ± 0.85^{b}
5	Ultrasound (US30)	78.45 ± 1.04^{a}
6	AC15+US15	61.64 ± 1.31^{f}
7	AC15+US30	63.41 ± 0.84^{def}
8	AC30+US15	62.51 ± 1.13^{ef}
9	AC30+US30	65.45 ± 1.29^{d}
10	US15+AC15	62.79 ± 1.39^{ef}
11	US15+AC30	64.37 ± 1.95^{de}
12	US30+AC15	71.63 ± 0.91^{c}
13	US30+AC30	72.38 ± 1.04^{c}

AC15 and AC30 are autoclave for 15 min and 30 min, respectively; US15 and US30 are ultrasound for 15 min and 30 min, respectively. Values are expressed as the average of triplicates ± standard deviations. Different lowercase superscripts within the same column are significantly different ($p < 0.05$)

Comparing the sequential treatment effects on protein isolate solubility, ultrasound followed by autoclave (US+AC) consistently produced higher solubility than autoclave followed by ultrasound (AC+US). The US+AC treatments achieved solubility values ranging from 62.79% to 72.38%, with US30+AC30 combined treatment reaching

significantly ($p<0.05$) higher values of solubility at 72.38%. In contrast, AC+US treatments yielded significantly ($p<0.05$) lower solubility values between 61.64% and 65.45%, with AC30+US30 achieving only 65.45%. This difference implies that applying ultrasound treatment first helped to enhance the protein's structural characteristics that contributed to solubility, while initial autoclave treatment appeared to cause structural modifications that cannot be fully reversed or compensated by subsequent ultrasound processing. The superior performance of the US+AC sequence likely reflects ultrasound's ability to initially increase protein accessibility and flexibility, making the proteins more resilient to the potentially denaturing effects of subsequent autoclave treatment. Malik et al. (2017) and Karabulut & Yemiş (2022) reported similar results for sunflower protein isolate and hemp seed protein isolate, respectively. They reported that protein isolates solubility enhances with enhanced ultrasound treatment time. The ultrasound treatment creates small protein fragments by interaction between protein and water is increased resulting in protein dissolution. Cavitation yield is greatly affected by ultrasound frequency because it affects the size of bubbles in a solution. Due to this, ultrasonic cavitation and amplitude of protein molecules increased under external acoustic resonance. As a result, the water molecules were able to bond easily with the proteins and this consequently increased their solubility (Li et al., 2020b).

Proteins were denatured by heat treatment where hydrogen bonds and functional groups were weakened, especially in secondary and tertiary structure. As a consequence of protein denaturation occurring during the autoclave procedure, the hydrophobicity or the hydrophilicity balance was altered, as a result protein solubility was reduced (Odjo et al., 2012; Jannathulla et al., 2017). There have been similar findings by Van de Vondel et al. (2021) who observed decrease in protein solubility of quinoa protein which were hydrothermally treated. The protein solubility reduced with enhanced treatment time and temperature.

3.3.6. Effect of modification on water and oil absorption capacity

Table 3.6 summarizes the water absorption capacity of the untreated and treated Manila tamarind seed protein isolates. Based on the results of the present study, it can be seen that the water absorption capacity (WAC) increased significantly ($p<0.05$) from 167% in the untreated sample to 195% for the ultrasound (US) treated sample (30 min at 180 W) and decreased significantly ($p<0.05$) from 167% in the untreated sample to 126% for

the AC treated sample (30 min at 121℃ and 15 psi pressure). A significant ($p<0.05$) increase in WAC for US treatment and decrease in WAC of the AC treatment, respectively was observed when treatment time was increased. When both the treatments were combined, there was a significant ($p<0.05$) overall enhancement in the WAC of the sample. The maximum enhancement in WAC of the combined treatments was significantly ($p<0.05$) less effective than the autoclave treatment. This was mainly attributed to the amplifying nature of the US and diminishing effect of autoclave (AC) on the WAC of the sample.

Table 3.6: Water and oil absorption capacity of Manila tamarind seed flour protein isolates (native and modified)

S. No.	Treatment	Parameters	
		WAC (%)	**OAC (%)**
1	Non-treated	166.66 ± 2.51^{h}	171.33 ± 1.52^{g}
2	Autoclave (AC15)	142.66 ± 2.08^{j}	147 ± 1.73^{i}
3	Autoclave (AC30)	125.69 ± 0.57^{k}	120.67 ± 0.57^{j}
4	Ultrasound (US15)	180.69 ± 1.52^{cd}	188.33 ± 1.52^{c}
5	Ultrasound (US30)	194 ± 1.73^{a}	206 ± 4.00^{a}
6	AC15+US15	151.33 ± 2.08^{i}	155.66 ± 1.52^{h}
7	AC15+US30	166 ± 2.00^{h}	171.37 ± 1.75^{g}
8	AC30+US15	172.33 ± 2.51^{g}	178 ± 3.00^{ef}
9	AC30+US30	176 ± 1.73^{ef}	184.66 ± 2.51^{d}
10	US15+AC15	174.33 ± 1.52^{fg}	176 ± 2.00^{f}
11	US15+AC30	179 ± 1.73^{de}	180.47 ± 1.52^{e}
12	US30+AC15	183.66 ± 1.52^{c}	191 ± 1.73^{c}
13	US30+AC30	189 ± 2.00^{b}	197.66 ± 2.5^{b}

AC15 and AC30 are autoclave for 15 min and 30 min, respectively; US15 and US30 are ultrasound for 15 min and 30 min, respectively. Values are expressed as the average of triplicates ± standard deviations. Different superscripts within the same row are significantly different ($p<0.05$) (WAC: Water absorption capacity; OAC: Oil absorption capacity)

Increase in WAC of protein isolates treated with ultrasound reflects an unfolding of the polypeptide chains caused by the ultrasonic energy (Jitngarmkusol et al., 2008; Biswas &

Sit, 2020). The hydrophilic groups on the polypeptide chains were exposed as a consequence, leading to increased water-binding sites that enhance the protein's ability to retain moisture within its three-dimensional structure (Biswas & Sit, 2020). This exposure occurs when protein denaturation or structural modifications disrupt the native conformation, causing previously buried polar amino acid residues such as serine, threonine, and asparagine to become accessible to water molecules.

In addition, the hydrophilic-hydrophobic balance among amino acids in biopolymers plays a crucial role in determining water holding capacity, as the ratio of polar to non-polar residues directly influences the protein's affinity for water and its ability to form stable hydration layers (Odjo et al., 2012). The ultrasonically treated seed protein isolates had significantly ($p<0.05$) higher oil absorption capacities (OAC) than untreated seed protein isolates in the current study **(Table 3.6)**. Also, the oil absorption capacity of the combined treatments (US+AC and AC+US) significantly enhanced with the increase in treatment durations. With the autoclaving treatment, the OAC of the isolates decreased significantly. As a result of thermal treatment, protein conformational changes may occur. The results were in favour with the findings of Ling et al. (2016) for defatted pistachio kernel proteins. A protein's combined hydrophilic and hydrophobic properties affect its oil absorption capability, while the hydrocarbon side chains of nonpolar amino acids and the chains of lipids form hydrophobic interactions (Jitngarmkusol et al., 2008). Additionally, polypeptide chains unfolding and conformational alterations are also caused by cavitation bubbles collapsing near a protein molecule as a result of ultrasonication, which results in unfolding of polypeptide chains causing enhanced OAC of the protein isolates. As a consequence of this change, the non-polar hydrophobic side chains of amino acids inside protein molecule were exposed, that resulted in increased binding of oil molecules to protein molecules (Ling et al., 2016).

3.3.7. Color properties of native and modified protein isolates

Color marks important criteria in order for food products to be marketable and acceptable (Nidhina & Muthukumar, 2015). The results of the exposure time of the ultrasound and autoclave treatment in the color determinants L^*, a^*, and b^* of the Manila tamarind seed protein isolate compared to the control treatment are depicted in **Table 3.7**. Results demonstrated significant ($p<0.05$) variations among the colour values for different treatments, the L^* (lightness) varied from 50.83 up to 69.31, a^*

(redness) extended from 3.68 to 6.93, and *b** (yellowness) showed variation from 10.92 to 20.25. Significantly ($p<0.05$) different colour values were obtained between native and treated Manila tamarind seed protein isolates. Treated Manila tamarind seed protein isolates exhibited significant difference ($p<0.05$) with enhanced *L**, *a**, and *b** values. Ultrasonic treatment of Manila tamarind seed protein isolates for 30 min showed the higher *L**, *a** and *b** values singly or in combination compared to autoclave treatment. One research demonstrated that ultrasonic processing conditions could be optimized to obtain excellent color characteristics (Bi et al., 2015).

Table 3.7: *L**, *a**, *b** color values of Manila tamarind seed flour protein isolate (native and modified)

S. No.	Treatment	Color parameters		
		L*	***a****	***b****
1	Non-treated	69.31 ± 1.75^{a}	4.52 ± 0.94^{c}	18.63 ± 0.99^{b}
2	Autoclave (AC15)	48.66 ± 0.84^{g}	3.68 ± 0.65^{c}	10.92 ± 0.33^{g}
3	Autoclave (AC30)	51.43 ± 2.14^{g}	5.86 ± 0.51^{b}	12.33 ± 0.22^{f}
4	Ultrasound (US15)	60.35 ± 0.68^{bc}	6.78 ± 0.23^{ab}	18.42 ± 0.53^{b}
5	Ultrasound (US30)	63.64 ± 0.95^{b}	6.93 ± 0.53^{a}	20.25 ± 0.20^{a}
6	AC15+US15	50.83 ± 1.26^{g}	4.09 ± 0.09^{c}	11.28 ± 0.60^{fg}
7	AC15+US30	59.07 ± 1.19^{cdf}	6.26 ± 0.42^{ab}	14.3 ± 0.67^{e}
8	AC30+US15	55.31 ± 2.51^{f}	6.55 ± 0.60^{ab}	16.43 ± 0.47^{cd}
9	AC30+US30	58.50 ± 4.01^{df}	6.54 ± 0.14^{ab}	17.49 ± 0.43^{bc}
10	US15+AC15	55.43 ± 2.67^{ef}	5.87 ± 0.06^{b}	14.83 ± 1.14^{e}
11	US15+AC30	55.75 ± 3.05^{ef}	6.64 ± 0.17^{ab}	16.36 ± 0.57^{d}
12	US30+AC15	56.22 ± 2.45^{def}	6.25 ± 0.28^{ab}	17.84 ± 0.55^{b}
13	US30+AC30	59.35 ± 1.16^{cd}	6.47 ± 0.32^{ab}	19.77 ± 0.79^{a}

AC15 and AC30 are autoclave for 15 min and 30 min, respectively; US15 and US30 are ultrasound for 15 min and 30 min, respectively. Values are expressed as the average of triplicates ± standard deviations. Different superscripts within the same row are significantly different ($p < 0.05$)

Ultrasonication may cause the color change in seed protein isolates primarily as a consequence of additive dispersion in polymeric matrices, disruption of particles, and decreased in droplet size of additives (Biswas & Sit, 2020). With enhancement in

processing duration from 15 to 30 min, color characteristics varied significantly. There was significant ($p<0.05$) increase in the a^* and b^* values of the protein isolates exposed to ultrasonic treatment for 15 and 30 min, while significant ($p<0.05$) decrease was there in the L^* values compared to the control treatment. Zhou et al. (2015) found that ultrasound of the egg white protein caused decrease in the L^* value and enhanced a^* and b^* values. This was attributed to the enhancement in the hydrophobic groups of the protein which resulted in better exposition of riboflavin on the protein surface. According to research by Khadhraoui et al. (2018), ultrasonic extracts metabolites by six mechanisms, which resulted in color differences in plant tissues, these are shear forces, erosion, sonoporation, capillary action, de-texturization including fragmentation; each of these causes progressive physical damage (Bi et al., 2015). Autoclave treatment too led to a change in product color. With enhancement in treatment time, overall samples turned out slightly darker due to production of Maillard reaction; but the extent of darkening was lesser compared to ultrasound treatment (Ling et al., 2016). Lower values of L^*, a^*, and b^* parameters have been seen when exposed to autoclave treatment, but the values significantly increased when in combination with ultrasound and enhanced treatment duration.

3.3.8. Bulk density

Bulk density values varied significantly ($p<0.05$) from 0.31 g/cm^3 to 0.66 g/cm^3 which are presented in **Table 3.8**. The bulk density measurements of Manila tamarind seed flour protein isolates revealed significant variations among different treatment methods, indicating substantial structural modifications induced by various processing techniques. The non-treated protein isolate exhibited the lowest bulk density at 0.31 g/cm³. Autoclaving (AC) treatments demonstrated a progressive significant ($p<0.05$) increase in bulk density with extended processing time, where AC15 (15 min autoclaving) yielded 0.47 g/cm³ and AC30 (30 min autoclaving) produced 0.52 g/cm³, suggesting that thermal denaturation enhanced particle packing efficiency through protein unfolding and aggregation (Ling et al., 2016). Ultrasound treatments alone showed moderate density increases, with US15 achieving 0.4 g/cm³ and US30 reaching 0.43 g/cm³, indicating that acoustic cavitation effects promoted structural compaction. The enhancement in the exposure duration in the ultrasonic treatment significantly decreased ($p<0.05$) the bulk density of the protein isolates with respect to the autoclave treatment, possibly due to the

ultrasound causing larger structures. Similar reporting's were done by Rosas et al. (2022) for orange seed protein isolates. However, ultrasound combined with autoclave treatment for 15 min duration showed significantly ($p<0.05$) higher bulk density value (0.66 g/cm^3).

Table 3.8: Bulk density of Manila tamarind seed flour protein isolates and modified protein isolate

S. No.	Treatment	Bulk density (g/cm^3)
1	Non-treated	0.31 ± 0.005^{g}
2	Autoclave (AC15)	0.47 ± 0.01^{cde}
3	Autoclave (AC30)	0.52 ± 0.005^{b}
4	Ultrasound (US15)	0.4 ± 0.01^{ef}
5	Ultrasound (US30)	0.43 ± 0.01^{de}
6	AC15+US15	0.49 ± 0.015^{cd}
7	AC15+US30	0.43 ± 0.02^{de}
8	AC30+US15	0.37 ± 0.005^{fg}
9	AC30+US30	0.46 ± 0.16^{cde}
10	US15+AC15	0.66 ± 0.03^{a}
11	US15+AC30	0.63 ± 0.015^{a}
12	US30+AC15	0.63 ± 0.01^{a}
13	US30+AC30	0.5967 ± 0.021^{ab}

AC15 and AC30 are autoclave for 15 min and 30 min, respectively; US15 and US30 are ultrasound for 15 min and 30 min, respectively. Values are expressed as the average of triplicates ± standard deviations. Different superscripts within the same row are significantly different ($p<0.05$)

Combined treatments where autoclaving preceded ultrasound produced intermediate bulk densities ranging from 0.37 to 0.49 g/cm³, suggesting potential interference between thermal and acoustic modifications. Remarkably, significantly ($p<0.05$) higher bulk density values were achieved when ultrasound treatment preceded autoclaving, indicating that initial ultrasonic pretreatment enhanced the subsequent thermal modification effects, potentially through improved protein accessibility and altered molecular conformations that facilitated more efficient particle arrangement and higher

packing density (Bi et al., 2015; Ling et al., 2016). Native samples showed significantly ($p<0.05$) lower values compared to US and AC treatment. Particle size influences the bulk density. The loose orientation of the polymer led to the reduction in bulk density (Ogechukwu & Ikechukwu, 2017). Autoclave-treated protein isolates showed significantly ($p<0.05$) higher bulk density values due to the fine particle size facilitating proper settling of isolates, leading to increased bulk density value (Khan et al., 2011). A similar trend was observed on structural characteristics of canola (*Brassica napus* L.) protein isolates when modified using high-intensity ultrasound in duration of 0, 15 and 30 min. The result of autoclaving on bulk density might be linked to its effect on the particle size which increased significantly ($p<0.05$) from 0.47g/cm^3 to 0.52g/cm^3 with increase in the duration of treatment.

3.3.9. Particle density

Table 3.9 shows the particle densities of the Manila tamarind seed protein isolates (PI). The particle density of proteins is among those attributes that highly influence functional characteristics like emulsifying and foaming capacities. The particle density of the native and treated samples varied significantly ($p<0.05$) with treatment combination of ultrasound and autoclave along with exposure time. Native samples showed a particle density of 0.474 g/cm^3, which was significantly ($p<0.05$) higher than most of the treated ones. The particle size of the PI was reduced with the treatment combination and exposure duration for 15 and 30 min respectively. Increased hydrophobicity of the surface combined with nonpolar group exposure caused soluble aggregates to form, especially at higher temperature of autoclave treatment (Zhong & Xiong, 2020). According to the result after treatment with ultrasound, the size of particles was reduced significantly ($p<0.05$) but not to the extent of duration of treatment. Treatment for 15 min was more effective in the samples of Manila tamarind seed proteins (0.470 g/cm^3). After 30 min of ultrasound treatment, there was a significant reduction in particle density of PI from 0.470 g/cm^3 to 0.465 g/cm^3. As a result of ultrasound, particle size was decreased, their distribution narrowed, and specific free surfaces significantly ($p<0.05$) increased in all samples (Shen et al. 2017). After ultrasonic treatment, the particle density may decrease resulting from the protein structure getting unfolded and unorganized (Flores-Jiménez et al., 2019). A significant ($p<0.05$) reduction in particle

size of whey protein concentrate was observed after their treatment with ultrasound baths of 40 kHz for 15- and 30 min.

Table 3.9: Particle density of Manila tamarind seed flour protein isolate (native and modified)

S. No.	Treatment	Particle density (g/cm^3)
1	Non-treated	0.474 ± 0.003^{a}
2	Autoclave (AC15)	0.466 ± 0.006^{bc}
3	Autoclave (AC30)	0.455 ± 0.005^{ef}
4	Ultrasound (US15)	0.470 ± 0.002^{ab}
5	Ultrasound (US30)	0.465 ± 0.001^{bc}
6	AC15+US15	0.464 ± 0.004^{bc}
7	AC15+US30	0.461 ± 0.002^{c}
8	AC30+US15	0.448 ± 0.002^{gh}
9	AC30+US30	0.443 ± 0.003^{h}
10	US15+AC15	0.464 ± 0.002^{bc}
11	US15+AC30	0.457 ± 0.002^{de}
12	US30+AC15	0.458 ± 0.001^{de}
13	US30+AC30	0.451 ± 0.002^{fg}

AC15 and AC30 are autoclave for 15 min and 30 min, respectively; US15 and US30 are ultrasound for 15 min and 30 min, respectively. Values are expressed as the average of triplicates ± SD. Different superscripts within the same row are significantly different ($p < 0.05$)

Vibration and turbulence caused by high-intensity ultrasound cause bubbles to implode and cavitation to form (Jambrak et al., 2014). The reduction of particle density by ultrasonication was mainly responsible for the increased solubility, due to the greater surface area and higher charge of the particles **(Table 3.5)** (Flores-Jiménez et al., 2022). The findings were in agreement with that of Flores-Jiménez et al. (2022) who reported that the particle density of guamuchil seed protein isolate was reduced after ultrasonication treatment. Several other findings on particle density were found to be in agreement with the results. Particle density of peanut protein isolate and milk protein concentrate were found to be reduced by ultrasound (Yanjun et al., 2014; Zhang et al., 2014). Autoclave-treated samples showed particle densities value of 0.455 g/cm^3 for 30

min and 0.466 g/cm^3 for 15 min respectively. Briefly, using autoclave treatment significantly reduced ($p<0.05$) the particle size with the exposure time. Heat treatments appeared to expose embedded hydrophobic amino acids to reduce interfacial tension, facilitating droplet breakup and decreasing droplet size (Li et al., 2020a).

3.3.10. Textural properties by back extrusion

Table 3.10 presents the textural properties of native and modified Manila tamarind seed protein isolate solutions, evaluated using back extrusion testing, which quantifies physical attributes such as firmness, cohesiveness, and consistency, which are important indicators of functionality in food applications. The non-treated sample exhibited significantly ($p<0.05$) lower values across all the parameters, with a firmness of 0.10 N, cohesiveness of –0.07 N, and consistency of 1.42 N·s, suggesting weak structural integrity and flow resistance (Jiang et al., 2018). In contrast, autoclaving (AC) and ultrasonication (AC) significantly ($p<0.05$) enhanced these properties. For example, samples treated with autoclave for 15 min (AC15) and ultrasound for 30 min (US30) showed significantly ($p<0.05$) higher consistency (1.74 N·s), indicating a more viscous and resistant texture, which is desirable in thickened or structured food matrices (Xiong et al., 2020). Firmness increased significantly ($p<0.05$) to 0.12 N in most modified samples (AC15, US15, US30), reflecting stronger gel-like behaviour or internal bonding. Interestingly, cohesiveness became more negative (e.g., –0.09 N in AC30), potentially indicating enhanced internal resistance during deformation, which might reflect higher energy absorption before breakdown (Jambrak et al., 2014). The comparative analysis of consistency values revealed that US30 and AC15+US30 treatments achieved remarkably similar performance (1.74 and 1.66 N·s, respectively), demonstrating 22-28% superior consistency compared to the native isolate, which has significant implications for food manufacturing applications where enhanced textural properties are essential. These improved consistency values are particularly valuable in meat analogue production, where protein isolates must provide sufficient structural integrity to mimic the fibrous texture and bite characteristics of animal proteins, enabling the development of plant-based products with satisfactory mouthfeel and consumer acceptance (Grace & Jeyakumar Henry, 2020).

Table 3.10: Textural properties of Manila tamarind seed protein isolate solution (native and modified) using back extrusion

S. No.	Sample	Textural parameters		
		Firmness (N)	**Cohesiveness (N)**	**Consistency (N.s)**
1.	Non-treated	0.10 ± 0.01^{c}	-0.07 ± 0.001^{f}	1.42 ± 0.01^{e}
2.	Autoclave (AC15)	0.12 ± 0.001^{a}	-0.08 ± 0.002^{cd}	1.74 ± 0.01^{a}
3.	Autoclave (AC30)	0.11 ± 0.001^{b}	-0.09 ± 0.001^{a}	1.55 ± 0.02^{d}
4.	Ultrasound (US15)	0.12 ± 0.001^{a}	-0.075 ± 0.0002^{e}	1.66 ± 0.01^{b}
5.	Ultrasound (US30)	0.12 ± 0.001^{a}	-0.08 ± 0.002^{cd}	1.74 ± 0.01^{a}
6.	AC15+US15	0.11 ± 0.001^{b}	-0.08 ± 0.001^{cd}	1.59 ± 0.01^{c}
7.	AC15+US30	0.12 ± 0.001^{a}	-0.07 ± 0.001^{f}	1.66 ± 0.01^{b}
8.	AC30+US15	0.11 ± 0.001^{b}	-0.085 ± 0.0002^{b}	1.60 ± 0.01^{c}
9.	AC30+US30	0.12 ± 0.001^{a}	-0.08 ± 0.001^{c}	1.67 ± 0.01^{b}
10.	US15+AC15	0.11 ± 0.001^{b}	-0.078 ± 0.0001^{d}	1.59 ± 0.01^{c}
11.	US15+AC30	0.11 ± 0.001^{b}	-0.085 ± 0.0002^{b}	1.59 ± 0.02^{c}
12.	US30+AC15	0.12 ± 0.001^{a}	-0.08 ± 0.001^{c}	1.74 ± 0.01^{a}
13.	US30+AC30	0.12 ± 0.001^{a}	-0.08 ± 0.002^{cd}	1.74 ± 0.01^{a}

AC15 and AC30 are autoclave for 15 min and 30 min, respectively; US15 and US30 are ultrasound for 15 min and 30 min, respectively. Values are expressed as the average of triplicates ± standard deviations. Different superscripts within the same row are significantly different ($p<0.05$)

In emulsified food systems such as sausages, dressings, and mayonnaise, the enhanced consistency translates to improved emulsion stability, reduced phase separation, and better oil-holding capacity, which are critical for maintaining product quality during processing and storage (Zhu et al., 2018). For gel-based applications including protein bars, desserts, and structured foods, the increased consistency values indicate superior gel strength and elasticity, enabling the formation of self-supporting structures that maintain their shape under mechanical stress and temperature variations (Ling et al., 2016). The synergistic effects observed in combined treatments, particularly AC30+US30, which provided balanced improvements across multiple functional parameters, suggest that sequential thermal and acoustic modifications create

complementary structural changes that optimize protein-protein interactions and network formation (Jambrak et al., 2014). When benchmarked against established protein sources, Manila tamarind protein isolates demonstrated competitive performance with soy and pea protein isolates, which typically exhibit firmness values of 0.11-0.15 N and consistency up to 1.8 N·s after modification, positioning Manila tamarind as a viable alternative protein source for industrial food applications (Flores-Jiménez et al., 2019). This competitive performance profile, combined with the sustainable sourcing potential of Manila tamarind seeds as agricultural by-products, makes these modified protein isolates attractive candidates for diversifying plant-based protein portfolios in commercial food formulations. Therefore, this emphasizes the value of processing techniques like autoclaving and ultrasonication in improving the textural performance of underutilized plant proteins for food structuring applications.

3.3.11. Gelling properties

Table 3.11 presents the Least Gelation Concentration (LGC) of native and modified Manila tamarind seed protein isolates, a critical parameter reflecting the minimum concentration required for gel formation, essential for applications in food structuring, emulsification, and textural modification. The non-treated protein exhibited the highest LGC at 20 g/100 g, indicating poor gelation ability and a need for high protein content to achieve network formation (Sathe & Salunkhe, 1981). Modification significantly ($p<0.05$) improved gelation properties; notably, ultrasound (US) treatment for 30 min (US30) resulted in the lowest LGC at 8 g/100 g, suggesting that cavitation-induced unfolding and exposure of reactive sites enhanced protein–protein interactions, facilitating gel network formation at lower concentrations (Huang et al., 2019). Autoclaving (AC), especially at 30 min (AC30), also significantly ($p<0.05$) reduced LGC to 10 g/100 g, likely due to partial protein denaturation that increased hydrophobic interactions and water binding capacity (Sun et al., 2021). Combined treatments (e.g., US30+AC15, US30+AC30) consistently yielded significantly ($p<0.05$) low LGC values around 10 g/100 g, indicating synergistic enhancement of gelation behaviour through sequential structural modification (Jambrak et al., 2014).

For comparison, commonly used plant proteins like soy and pea isolates typically exhibit LGC values in the range of 12–16 g/100 g, depending on the processing method (Huang

et al., 2019). Thus, modified tamarind protein, especially with US30 and AC30, demonstrated superior gelation efficiency at even lower concentrations.

Table 3.11: Least gelation concentration (LGC) of Manila tamarind seed flour protein isolate and modified protein isolate

S. No.	Sample	LGC (g/100 g)
1.	Non-treated	20
2.	Autoclave (AC15)	12
3.	Autoclave (AC30)	10
4.	Ultrasound (US15)	16
5.	**Ultrasound (US30)**	**8**
6.	AC15+US15	14
7.	AC15+US30	12
8.	AC30+US15	14
9.	AC30+US30	10
10.	US15+AC15	14
11.	US15+AC30	12
12.	US30+AC15	10
13.	US30+AC30	10

AC15 and AC30 are autoclave for 15 min and 30 min, respectively; US15 and US30 are ultrasound for 15 min and 30 min, respectively.

These results demonstrate that thermal and ultrasonic treatments significantly enhance gelation performance through multiple molecular mechanisms that fundamentally alter protein structure and functionality (Malik et al., 2017). The application of heat and acoustic energy induces protein unfolding and conformational changes, exposing previously buried hydrophobic amino acid residues and creating new binding sites for intermolecular interactions (Sanchiz et al., 2019). This increased surface hydrophobicity promotes stronger protein-protein associations through hydrophobic interactions, while simultaneously facilitating the formation of disulfide bonds, hydrogen bonds, and electrostatic interactions that contribute to more robust gel networks. The enhanced intermolecular bonding capacity results in improved gel strength, elasticity, and water-holding properties, which are essential characteristics for creating texturally appealing

food products (Hu et al., 2019). These superior gelation properties significantly expand the commercial viability of Manila tamarind protein isolates in the rapidly growing plant-based food sector, particularly in developing cost-effective meat analogues that require firm, cohesive textures to mimic animal proteins (Grace & Jeyakumar Henry, 2020). Additionally, the improved gel-forming ability makes these modified isolates excellent candidates for dairy alternatives such as plant-based yogurts, cheese analogues, and custards, where gel structure is crucial for mouthfeel and consumer acceptance. The enhanced functional properties also enable the creation of innovative structured gels for applications in protein bars, desserts, and novel food matrices that require specific textural attributes (Achouri et al., 2012). Furthermore, the cost-effectiveness of utilizing Manila tamarind seeds, which are typically discarded agricultural by-products, combined with these improved functional properties, positions these modified protein isolates as sustainable and economically viable alternatives to conventional plant proteins in industrial food formulations (Shevkani et al., 2015).

3.3.12. Emulsification properties

Protein emulsifying quality can be evaluated through emulsifying activity (EA) and emulsion stability (ES), which are determinants of the protein's ability to form emulsions at water and oil interactions. The emulsification properties of the Manila tamarind seed protein isolates (native and modified) are given in **Table 3.12**. The application of autoclave treatment caused a significant ($p<0.05$) decrease in the emulsifying activity and emulsion stability. The values of emulsifying activity for protein isolates went from 56.47% to 49.68%. Significant ($p<0.05$) reduction in the values of emulsification property was observed as modification time was increased. The changes in the emulsifying activity due to autoclave might be a result of aggregation of denatured proteins along with disruption in protein structure due to the processing. According to the results of Sanchiz et al. (2019) for pistachio, cashew and chestnut proteins who also observed a decrease in the values of emulsifying activity with autoclaving. The ultrasound modification caused a significant ($p<0.05$) increase in the emulsifying activity of the protein isolates as the values changed from 56.47% to 78.19%. The significantly ($p<0.05$) higher increase in the value of emulsifying activity (78.19%) was observed for the treatment time of 30 min (180W and 35kHz).

Emulsion formation is enhanced through ultrasound because it exposes nonpolar protein residues to more hydrocarbon chains, which interact with acyl-glyceride molecules to form emulsions. There were similar outcomes reported by Sun et al. (2021) for peanut protein isolate who observed a significant enhancement in the emulsifying activity on ultrasound modification. Additionally, the results agreed with the findings of Huang et al. (2019) for soybean protein isolate treated with ultrasound (200W and 20kHz for 10 min) which significantly enhanced their emulsifying characteristics. The changes in emulsion stability of Manila tamarind seed protein isolate following ultrasound and autoclave treatments for various treatment combinations and exposure durations revealed significant variations in functional performance as presented in **Table 3.12**.

Table 3.12: Emulsifying activity and emulsion stability of Manila tamarind seed flour protein isolate (native and modified)

S. No.	Treatment	Sample parameters	
		Emulsifying activity (%)	**Emulsion stability (%)**
1	Non-treated	56.47 ± 0.65^{i}	52.54 ± 0.80^{f}
2	Autoclave (AC15)	52.40 ± 0.98^{j}	44.48 ± 0.62^{g}
3	Autoclave (AC30)	49.68 ± 1.00^{k}	39.52 ± 0.87^{i}
4	Ultrasound (US15)	69.67 ± 1.12^{c}	61.58 ± 1.10^{cd}
5	Ultrasound (US30)	78.19 ± 0.72^{a}	68.76 ± 0.81^{a}
6	AC15+US15	64.75 ± 1.06^{e}	64.49 ± 1.13^{b}
7	AC15+US30	74.48 ± 0.79^{b}	58.50 ± 1.03^{e}
8	AC30+US15	66.75 ± 0.85^{d}	42.83 ± 0.87^{h}
9	AC30+US30	60.25 ± 0.85^{g}	60.36 ± 1.09^{d}
10	US15+AC15	56.47 ± 0.85^{i}	58.42 ± 0.97^{e}
11	US15+AC30	61.84 ± 0.65^{f}	60.71 ± 0.90^{d}
12	US30+AC15	65.50 ± 1.41^{de}	62.75 ± 0.80^{c}
13	US30+AC30	58.52 ± 0.46^{h}	64.73 ± 1.01^{b}

AC15 and AC30 are autoclave for 15 min and 30 min, respectively; US15 and US30 are ultrasound for 15 min and 30 min, respectively. Values are expressed as the average of triplicates ± standard deviations. Different superscripts within the same row are significantly different ($p<0.05$)

The experimental data demonstrated that emulsion stability improved significantly ($p<0.05$) across all ultrasound treatment protocols, with the magnitude of improvement being directly correlated with processing duration, which aligns with previous research findings reported by Huang et al. (2019) on ultrasonic modification of plant proteins. This enhancement in emulsifying capacity can be attributed to ultrasound-induced protein structural modifications, including partial unfolding, exposure of hydrophobic regions, and reduction in particle size, which collectively improve the protein's ability to stabilize oil-water interfaces (Achouri et al., 2012). Conversely, autoclave treatment alone resulted in a significant ($p<0.05$) decrease (39.52%) in emulsion stability, likely due to extensive protein denaturation and aggregation caused by prolonged thermal exposure, which reduced the protein's surface activity and interfacial properties (Nir et al., 1994). Among all treatment combinations, US30 achieved the most pronounced and significant ($p<0.05$) improvement in emulsion stability with a remarkable 68.76% increase, followed closely by the combined AC15+US15 treatment showing a 64.49% enhancement compared to the native isolate. These findings were consistent with numerous studies demonstrating improved emulsifying properties in other legume proteins, particularly peanut protein isolate, following ultrasonic modification (Sun et al., 2020), suggesting that acoustic cavitation effects universally enhance the emulsifying functionality of plant-based protein systems through similar mechanistic pathways involving protein structure optimization and surface property enhancement.

3.3.13. Molecular weight distribution by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS PAGE)

Figure 3.2 depicts the Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) profiles of native (coded as A_o) and modified (coded as A_1-A_{12}) Manila tamarind seed protein isolate (MTSPI). The native protein isolate (A_o) was divided into five fractions based on molecular weight, primarily with the following values: ~70-80kDa, 55 kDa, ~35-36 kDa, ~14-16 kDa and <10kDa. Few scattered faint bands at 20kDa and above 100kDa were also visible. The ultrasound (US) (coded as A_3 and A_4) and autoclave (AC) (coded as A_1 and A_2) treatments did not affect the proteins of 55 kDa and ~70-80kDa weight. The heavier proteins showed resistance towards the US and AC treatment. The other bands of the lighter weight protein fractions were slightly less visible after the modification. As a result of ultrasound and autoclave treatments,

hydrogen bonds including hydrophobic interfaces between proteins were slightly disrupted, which are important for intermolecular interactions. In case of the ultrasonication, there was no change in the molecular weight of the protein fractions of pea protein isolates (PPI) (i.e., the primary structure of the proteins) (Xiong et al., 2018). Additionally, under reducing conditions, the PPI electrophoretic profiles, which presented similar molecular band weight 65, 45, and 14 kDa, didn't differ based on the ultrasonic power. Ultrasound did not affect any disulfide bonds in oat proteins, as suggested by the study (Li & Xiong, 2021). Zhu et al. (2018) reported that walnut protein isolate did not hydrolyse under ultrasound, which was in agreement with the results of this study.

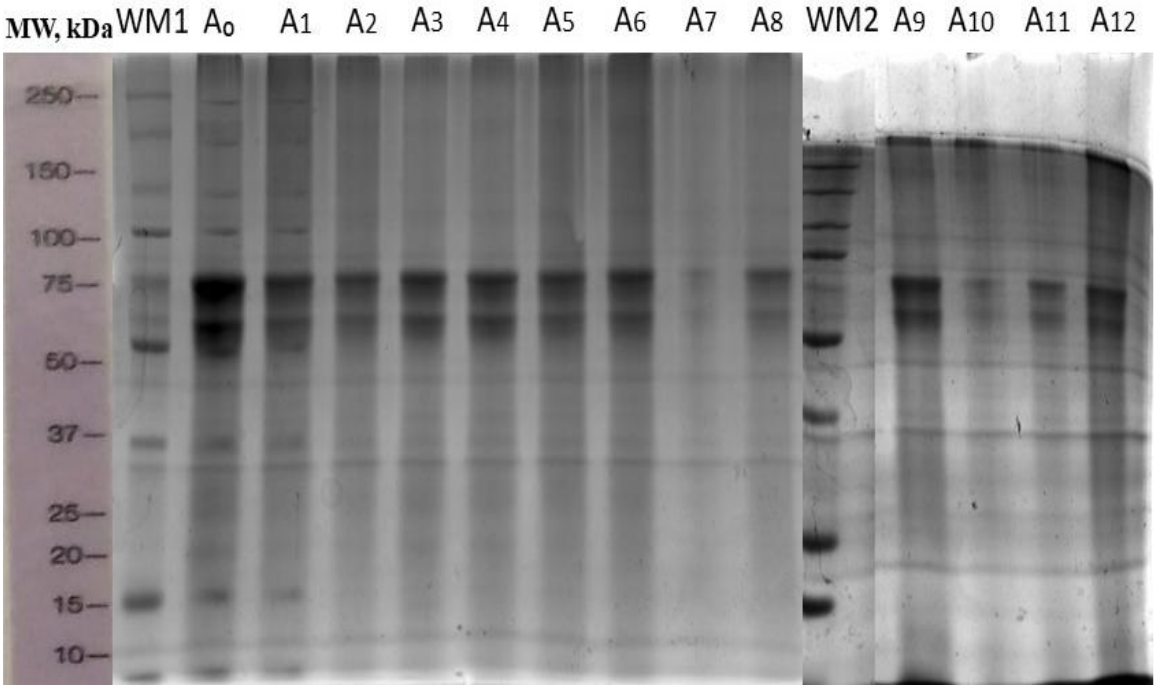

Figure 3.2: Electrophoretic pattern of Manila tamarind seed protein and modified proteins

(Where, A_0 is non-treated, A1 is AC15, A2 is AC30, A3 is US15, A4 is US30, A5 is AC15+US15, A6 is AC15+US30, A7 is AC30+US15, A8 is AC30+US30, A9 is US15+AC15, A10 is US15+AC30, A11 is US30+AC15 and A12 is US30+AC30. AC15 and AC30 are autoclave for 15 and 30 min, respectively. US15 and US30 are ultrasound for 15 and 30 min, respectively. WM1 and WM2 are molecular weight markers.)

The molecular weight analysis revealed distinct patterns of protein modification depending on the treatment method employed, with significant implications for functional applications in food systems. Jambrak et al. (2014) and Resendiz-Vazquez et

al. (2017) demonstrated that ultrasound treatment effectively reduced the molecular weights of whey protein isolates and jackfruit seed protein, respectively, through acoustic cavitation-induced protein fragmentation and structural disruption.

The extent of molecular weight reduction is influenced by sonication parameters including frequency, power, duration, and protein concentration, indicating that controlled ultrasonic processing can be tailored to achieve specific molecular weight profiles for targeted applications (Flores-Jiménez et al., 2019). This molecular weight reduction through ultrasonication enhances protein solubility, digestibility, and bioavailability, making these modified proteins particularly valuable for nutritional supplements, infant formulations, and sports nutrition products where rapid absorption and utilization are desired (Arzeni et al., 2012). In contrast, autoclave treatment demonstrated selective stability, causing no disruption to high molecular weight proteins (~70-80 kDa and 55 kDa) while only marginally affecting lower molecular weight fractions (~14-16 kDa and <10 kDa), which has important implications for maintaining structural integrity in applications requiring robust protein networks. Similar findings reported by Li et al. (2013) for almond proteins showed that heat treatment preserved heat-stable protein fractions (22-25 and 48 kDa) while reducing heat-labile proteins (38 and ~70 kDa), resulting in the appearance of intermediate molecular weight bands between 12-20 kDa. These selective modifications make thermally-treated proteins ideal for bakery applications, where heat-stable proteins contribute to gluten-like functionality and structural support during baking processes (Zhang et al., 2016). The preservation of high molecular weight proteins during autoclave treatment, as confirmed by Zhang et al. (2016) for almond protein isolates, makes these modified proteins excellent candidates for meat analogue production, where large protein polymers are essential for creating fibrous textures and maintaining structural integrity during processing and cooking, while the selective modification of smaller protein fractions can enhance flavor release and improve mouthfeel characteristics in processed foods.

3.4. Conclusion

The study investigated the effects of ultrasound (US) and autoclave (AC) treatments on the physicochemical and functional properties of protein isolates from Manila tamarind seed flour. The seed flour had a protein content of 32.59% and the protein isolate had a purity of 85.17%. The amino acid profile of the protein isolate was comparable to soy

protein isolate, with a higher content of sulfur-containing amino acids. The cytotoxicity effects of aqueous and ethanolic extracts on HEK-293 cells were evaluated, with the ethanolic extract showing a superior safety profile and both the extracts shown greater than 50% cell viability even at concentrations of up to 500 µg/mL. Findings of the study revealed that the US and AC treatment changed the physicochemical and functional characteristics of Manila tamarind seed protein isolate (MTSPI) to a significant amount. Ultrasound treatment significantly increased the solubility, water and oil absorption capacity, emulsifying activity and stability, and L^*, a^*, b^* color values of the protein isolates, while autoclave treatment decreased these properties. Textural properties like firmness, cohesiveness, and consistency improved with modifications giving treatments with better solution forming capacity. The least gelation concentration decreased, indicating better gelling ability with the application of modification treatments and out of all the given treatments, US30 gave the best gel forming ability with least amount of protein. The combination of ultrasound and autoclave treatments resulted in an overall enhancement of the functional properties, mainly due to the ultrasonic effect. SDS-PAGE analysis showed that the high molecular weight proteins were resistant to the treatments, while the low molecular weight proteins were slightly affected. The better ability of US30 in enhancing the overall functional property of the Manila tamarind seed protein isolates was the reason for its selection as the best treatment for usage in further objectives. The study demonstrated the potential of Manila tamarind seed protein isolate as an alternative plant protein source and the effectiveness of ultrasound treatment in improving its functional properties for food applications.

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