THE EFFECT OF PLANT EXTRACTS ON AGGREGATION OF PAPAIN

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Chinmay Goyal

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Under the guidance of

Dr. Mili Das

INSTITUTE OF SCIENCE

NIRMA UNIVERSITY OF SCIENCE AND TECHNOLOGY

SARKHEJ-GHANDINAGAR HIGHWAY

AHMEDABAD

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Chinmay Goyal

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1.0 INTRODUCTION

Proteins are heteropolymeric chains of twenty amino acids occurring in nature through the chemically stable peptide bond.

All process in our lives is controlled by proteins. Near about 100,000 proteins are present in our body. For their proper functioning they have to attain the properly folded state in crowded cellular environment which is controlled by molecular chaperones and their native structure is determined by their amino acid sequence. The amino acid sequence alone is sufficient to dictate the native conformation of small proteins; most polypeptides would fail to fold efficiently in the highly concentrated, complex cellular environment without assistance of yet another type of machinery. This type of machinery involves the so-called **molecular chaperones**, i.e., proteins adapted to facilitate protein folding. There is inherent protein folding problem of nascent polypeptide chains. Nascent polypeptide chains emerge in linear manner and hydrophobic stretches and other potentially interactive sites must sometimes wait for downstream sequences to emerge before appropriate folding interactions can be established. Molecular chaperones reversibly interact with nascent chains to minimize off-pathway interactions and increase the yield of native folded protein (Whitesell et al, 1998). The interior of a cell is a highly crowded environment in which proteins and other macromolecules are present at a concentration that can exceed 300 mg/ml (Ellis and Minton, 2003). In such conditions molecular chaperones such as GroEL and GroES bind specifically or non-specifically to protect the aggregation prone regions of nascent polypeptide.

However, in adverse condition these proteins are not able to fold into their native conformation and result in misfolded proteins. Misfolding can broadly be defined as reaching a state that has a significant proportion of nonnative interactions between residues and whose properties differs significantly from those of a similar state having overwhelmingly native-like interactions.

Protein misfolding as a cause of certain diseases was first reported in 1990. Life is co-ordinated activity of proteins. If any imbalance occurs in proteins, it which leads to various diseases and adversely affect the quality of life by production of too less/too much of particular protein or by production of dysfunctional protein/wrong protein in wrong place at

the wrong time.

Misfolded protein may be non-functional or suboptimally functional. These may be degrade by the cellular machinery or may expose epitopes which lead to dysfunctional interactions with other proteins.

There are a number of serious diseases which occur because of inappropriate folding of particular protein. Diseases like cystic fibriosis (misfolded CFTR protein), Marfan syndrome (misfolded fibrillin), Fabry disease (misfolded alpha galactosidase), gaucher's disease (misfolded beta glucocerebrosidase) and retinitis pigmentosa 3 (misfolded rhodopsin) are such examples. In addition, some cancers may be associated with misfolding, and hence ineffective functioning, of tumour suppressor proteins such as von Hippel Lindau protein or p53 (Laurence et al, 2010).

Misfolded proteins which have not been degraded by the cellular machinery form aggregates and cause various diseases like Alzheimer's and Parkinson's diseases, the spongiform encephalopathy and type II diabetes etc. Diseases associated with misfolding and aggregation are socially disruptive and costly in the modern world which affects our life style. About 40 human diseases have been reported which are linked to aggregation of specific proteins (Laurence et al, 2010).

1.1Protein Aggregation

Various types of aggregates are formed and they can be classified into soluble/insoluble, covalent/noncovalent, reversible/irreversible and native/denatured (Cromwell et al, 2006).

Factors that influence the aggregation of proteins are categorized in 3 classes- (Pawar et al, 2008):

- Extrinsic factors
- Physiochemical properties
- Intrinsic factors

Extrinsic factors include interaction with cellular and extracellular components like molecular chaperones that inhibit protein misfolding.

Physiochemical properties include pH, temperature, pressure, ionic strength, protein concentration, concentration of denaturants etc.

Intrinsic factors include net charge on polypeptide, hydrophobicity, patterns of polar and non polar amino acids residues, and tendency to form diverse secondary structures.

Before aggregation, proteins first unfold partially under physiological conditions. Recently there has been a very interesting observation from studies of kinetics of amyloid formation: that polypeptide sequences have local regions that are sensitive to aggregation. Even a single mutation in these regions can change the aggregation rate while similar changes in other regions have little effect on aggregation.

1.2 Five common mechanisms of aggregation **(Philo, 2009):**

Mechanism 1

Native protein monomers have self-complementary. Hence they can readily self associate to form reversible oligomers. As protein concentration increases these form large aggregates which become irreversible. For example- insulin (Pekar, et.al, 1972).

Mechanism 2

The native protein has a low tendency to form reversible aggregates. Because of stress or pressure, native monomers may have altered conformation or may be partially unfolded. These then associate together and form large irreversible aggregates

Mechanism 3

Mechanism 3 is really a variant of Mechanism 2 where the change in protein conformation that precedes aggregation is caused by a difference in the covalent structure. Usually this difference is caused by chemical degradation such as oxidation of methionine, deamidation, or proteolysis. Chemical changes may for example create a new sticky patch on the surface, or change the electric charge in a way that reduces electrostatic repulsion between monomers. Aggregates contain chemically modified protein and native monomer. These aggregates may cause immunogenic reaction.

Mechanism 4

This is nucleation controlled mechanism. Native monomer has a low tendency to form oligomers however if these aggregate attains sufficient size, they rapidly form large aggregates. A characteristic feature of this mechanism is that aggregate are in lag phase for a longer period of time and after that they suddenly become larger visible are seen. This includes "homogeneous nucleation" where nucleus is itself product aggregate and "heterogeneous nucleation" where contaminant act as a nucleation site which form large aggregates.

Mechanism 5

The last mechanism to be discussed here is surface-induced aggregation. This aggregation process starts with binding of the native monomer to a surface.

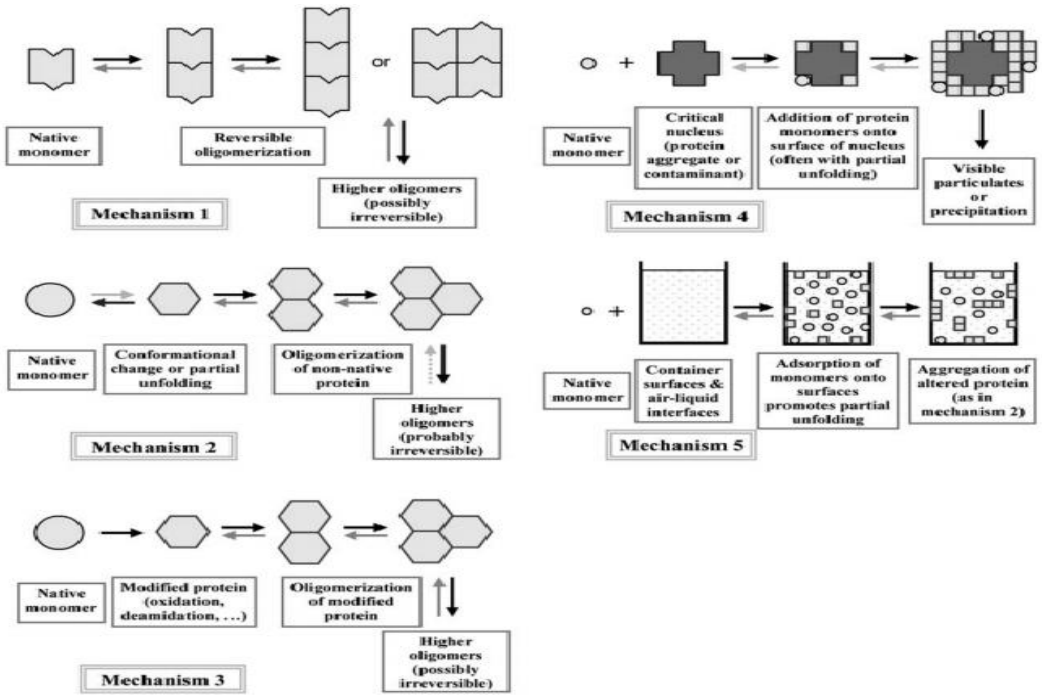


Figure 1: Five common mechanisms of aggregation **(**Philo, 2009**)**

There are reports which state that aggregation of therapeutic proteins leads to health problems. For example, recombinant erythropoietin aggregates cause pure red cell aplasia. (Arakawa, 2008).

Aggregates are a major limiting factor of shelf life of proteins; affect the bioactivity of protein and cause immunogenicity.

The different types of agents which have been shown to prevent aggregation of proteins are listed below.

Table 1 Agents that may promote protein solubility (Bondos et al, 2003)

	Additive	Recommended concentration range
Kosmotropes	$MgSO_4$	0–0.4 M
	$(NH_4)_2SO_4$	0–0.3 M
	Na_2SO_4	0–0.2 M
	Cs_2SO_4	0–0.2 M
Weak kosmotropes	NaCl	0–1 M
	KCl	0–1 M
Chaotropes	$CaCl_2$	0–0.2 M
	$MgCl_2$	0–0.2 M
	LiCl	0–0.8 M
	RbCl	0–0.8 M
	NaSCN	0–0.2 M
	NaI	0–0.4 M
	$NaClO_4$	0–0.4 M
	NaBr	0–0.4 M
	Urea	0–1.5 M
Amino acids	Glycine	0.5–2%
	L-arginine	0–5 M
Sugars and polyhydric alcohols	Sucrose	0–1 M
	Glucose	0–2 M
	Lactose	0.1–0.5 M
	Ethylene glycol	0–60% v/v
	Xylitol	0–30% w/v
	Mannitol	0–15% w/v
	Inositol	0–10% w/v
	Sorbitol	0–40% w/v
	Glycerol	5–40% v/v
Detergents	Tween 80	0–0.2% w/v
	Tween 20	0–120 μM
	Nonidet P-40	0–1%

These additives alter the free energy of protein by binding with water molecules. On increasing the concentration, the additives bind more water. When the additives are removed, the proteins become thermodynamically unfavorable with an increase in free energy. This free energy can be reduced only by forming protein aggregates. But disadvantage of these additives is that they have very low affinity and are required in higher concentration ranging from 100 mM to 2 M and they may cause toxic effects.

1.3 Objective

TO FIND THE BIOACTIVE COMPOUNDS FROM NATURAL PRODUCTS WHICH WILL PREVENT THE AGGREGATION OR PROMOTE DISAGGREGATION OF THE MODEL PROTEIN, PAPAIN

At earlier times, several plant extracts were given to patients to treat certain neurological diseases, even though the etiologies of the disorders were unknown at that time. Later many of these diseases were found to occur because of the aggregation of proteins. Garlic extract has been shown to have antiamyloidogenic activity (Gupta and Rao, 2007). Extracts of ginger, blueberries and cinnamon have been shown to be amyloid-β aggregation blocker (Guo et al, 2010). These plant parts are widely used as medicinal sources, are usually taken in regular diet and are expected to cause very little or no toxicity.

Papain is selected as model protein as it is known to form aggregates which can occur in different ways of aggregation. It is widely used for therapeutic and industrial purposes like in breaking down tough meat fibers, breaking down protein toxin in venom, used in the care of some chronic wounds to clean up dead tissue, used as an ingredient in some toothpastes and teeth whitener etc. It can be easily and cheaply isolated and purified.

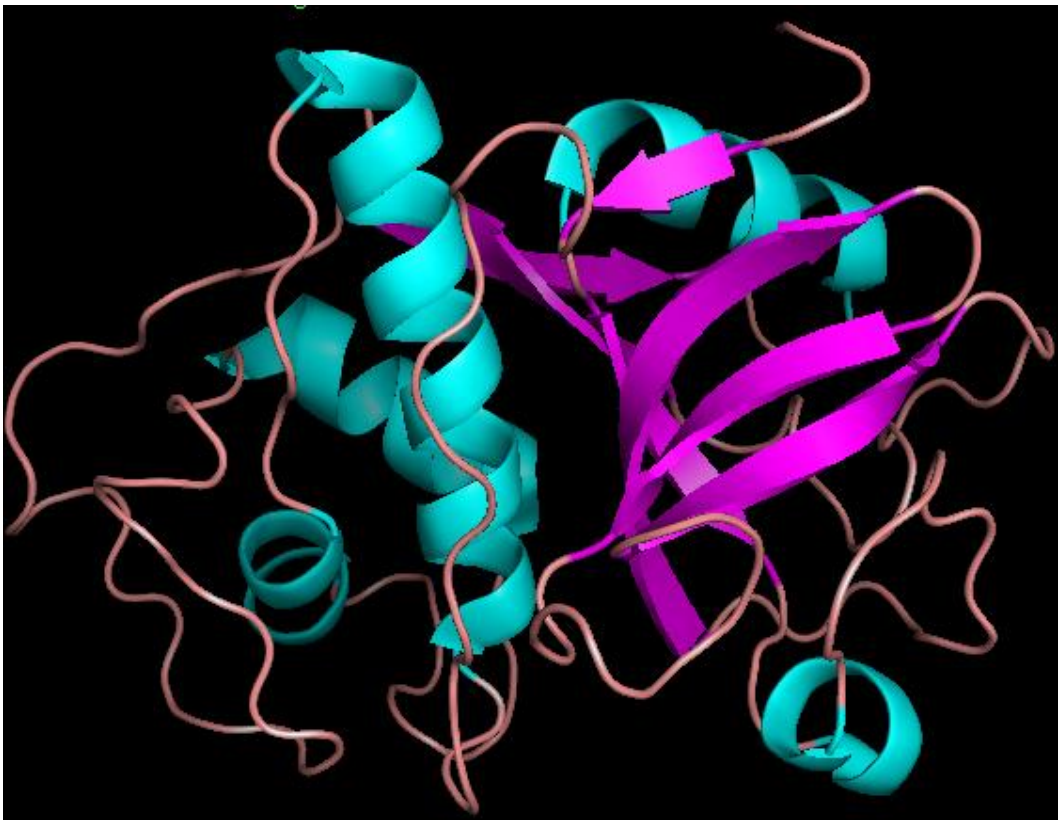
If we get the positive results with selected plant extracts on aggregates of papain, further work will be carried out on more expansive therapeutic proteins, followed by in-vivo studies.

1.4 Papain is a cysteine proteases present in papaya (*Carcica papaya*). The digestant action of juice of papaya has been known from centuries. The results of experimental investigation have been first reported in 1874 (Hwang, 1951)

Papaya latex contain four types of cysteine protease which contribute 69-89% of total protein. These are Papain (less than 10%), chymopapain (26-30%), glycyl endopeptidase (23-28%), caricain (14-26%).These four proteases have approximately similar molecular weight of around 23 kDa (Chaiwut et al, 2006).

Papain belongs to a family of related proteins with a wide variety of activities like endopeptidase, aminopeptidase, dipeptidylpeptidase and enzymes with exo- and endopeptidase activity.

Structure (PDB ID: 9PAP)



Papain is a sulfhydryl protease consisting of a single polypeptide chain of 212 amino acid residues. It is folded into two domains with the active site in the groove between the domains. The protein is stabilized by 3 disulfide bonds. The active site possesses catalytic diad which is likened to the catalytic triad of chymotrypsin. The catalytic diad is made up of amino acid residues cysteine-25 and histidine 159.

Function

The mechanism by which it breaks the peptide bond is by deprotonation of Cys-25 by histidine-159. Aspargine-175 helps to orient the imidazole ring of His-159 to allow this deprotonation to take place. Cys-25 then makes a nucleophillic attack on carbonyl carbon of peptide backbone. This frees the amino terminal of peptide and forms covalent acyl-enzyme intermediate. This enzyme is then deacylated by water molecule to releases the carboxyterminal of the peptide.

The optimum activity of papain occurs at pH 6.2-7.2 (Brenda-enzymes.org) although its pH stability ranges from pH 3-9. The optimum temperature of the protein is 37°C.

MATERIALS AND METHODS

2.0 Materials and methods

2.1 Materials

Raw papaya fruit, ginger, cinnamon and amla were bought from local market in Ahmedabad. Glycine, Acrylamide, Calcium Chloride were purchased from Merck. HEPES, L-cysteine, Guanidine hydrochloride ,beta-mercepto ethanol, Ammonium Persulphate were from SRL. Bis-Acrylamide, Sodium Dodecyl Sulfate (SDS) were bought from HIMEDIA. Hydrochloric acid, ammonium sulphate, coomassie brilliant blue were purchased from S.D. Fine Chem Ltd. Sodium Chloride and was purchased from RANKEM, RFCL, Gelatin, Tritonx100, PMSF (Phenymethanesulfonylfluroide), Isopropanol, Tri-sodium citrate bought from Qualigens Fine Chemicals. Sephadex G-75 from Sigma Aldrich, Sodium carbonate, sodium potassium tartrate, $CuSO_4.5H_20$, Folin Reagent, Whatman Filter Paper No.1.

2.2 Method

2.2.1 Purification of papain from raw fruit of papaya (Kimmel et al, 1953)

Dried papaya latex contains hard particles which must be transformed into fine powder by crushing and grinding. Extraction of activity into aqueous solution was best accomplished at pH 5.5.

a. The dried papaya latex (15 gm) and 4.16 gm. of washed sand were mixed in a mortar and ground thoroughly with 9.16 ml of 0.04 M cysteine. (This solution was made by dissolving the cysteine hydrochloride in 0.054N NaOH. This contained an excess of alkali in order to bring the aqueous extract to pH 5.5).
b. The suspension was allowed to settle and the supernatant fluid was decanted out.
c. The extraction and grinding were repeated with another 9.16 ml portion of the cysteine solution; this was decanted as before and the mortar was washed with the cysteine solution until a total volume of 28ml had been used for extraction and washings.
d. The resulting suspension was stirred well and filtered with Whatman filter paper No.1. The filtrate (Fraction 1) was opalescent, greenish yellow and its pH was near 5.5.

e. Fraction 1 was adjusted to pH 9.0 with a slow addition of 1N NaOH solution. Fine gray precipitate was observed which was removed by centrifugation at 1100 g for 1 hour.
f. The supernatant fluid (Fraction 2) was clear.
g. Fraction 2 was brought to 0.4 saturation with solid ammonium sulfate (250 g/L). A white precipitate appeared and the suspension was allowed to remain for 1 to 2 hours at 4°C.
h. The precipitate (Fraction 3) was then removed by centrifugation at 1000 g for 1 hour and the supernatant solution (Fraction 3a) discarded.
i. The precipitate was washed once with 13.5 ml of cold ammonium sulfate solution (250 g/L) and separated by centrifugation as before.
j. Fraction 3 was redissolved in 16.5 ml. of 0.02 M cysteine (pH 7 to 7.5) and 10% w/v of solid sodium chloride was added slowly.
k. Papain was precipitated as a fine white solid by this procedure. This suspension was allowed to stand for 1 hour at 4°C. It was then centrifuged in the cold for 1 hour at 1000 g. and the supernatant fluid (Fraction 4a) was discarded.
l. The solid material from above (Fraction 4) was suspended in 11 ml of 0.02 M cysteine at pH 6.5. A slight rise in pH generally occurs when the protein was added to the cysteine solution; therefore it was necessary to adjust the suspension to pH 6.5.
m. At room temperature the suspension developed marked crystallization sheet in about 30 minutes which was kept overnight at 4°C.
n. The light crystals (Fraction 5) were removed by centrifugation at 700 to 1000 g for 4 to 5 hours. The supernatant fluid may be discarded (Fraction 5a).
o. The light crystals were redissolved in 7.5 ml of distilled water.

2.2.2 SDS PAGE (Sodium dodecyl sulfate polyacrylamide gel electrophoresis)

Identification of separated and purified Papain was done by Sodium Dodecyl Sulphate (SDS)-PAGE, using Amersham Biosciences SE 245 Dual Gel electrophoresis unit. Estimation of molecular weight was done using BSA, lysozyme and casein as markers. The stacking gel and resolving gel were 5% and 15% respectively. The slab gels (1.0 mm thick) were run at constant current of 100 mA. After electrophoresis, the gel was fixed using methanol (40% v/v) and acetic acid (10% v/v) for about 2 hours. Coomassie Brilliant Blue

R250 (0.1% w/v in fixing solution) was used to stain protein bands. Destaining of the gel was done in acetic acid (7.5% v/v) and methanol (10% v/v) until the background was completely cleared and then the gel was photographed.

2.2.3 Zymography

Zymography is an electrophorectic technique based on SDS PAGE, in which the substrate is copolymerized with the polyacrylamide gel. This technique is used to detect enzymatic activity by gel electrophoresis.

5µl of non-reducing SDS-PAGE sample buffer was added to 20µl of enzyme and incubated at 37°C for 1 hour. The sample was not heated on a boiling water bath. The polyacrylamide gel contained 0.6% w/v of gelatin. Electrophoresis was run as usual. After completion of electrophoresis the gel was removed and soaked in 2.5% Triton X100 for 1 hour at room temperature to wash out SDS. The gel was washed thrice until foam was removed i.e SDS was removed. The gel was then incubated in 1X refolding buffer overnight at 37°C. The gel was rinsed thrice with deionized water and stained with coomassie blue for 1 hour.

2.2.4 Proteolysis

Proteolysis is the directed degradation of proteins by enzymes called proteases.

In this method enzyme was incubated with substrate (casein) for different time intervals and the resultant was run on SDS PAGE. The enzymatic reaction was stopped by protease inhibitor, PMSF after the specified time of digestion.

A solution of casein was prepared in distilled water and used as the substrate. Casein was precipitated from fresh unpasteurized milk by adding HCl up to pH 3-4. After centrifugation, the pellet was dissolved in distilled water of pH 7.0 (Pertzoff, 1927). 10µl of casein in 9 (1.5ml) micro centrifuge tubes were incubated with 5µl of purified papain solution at 37°C for 1min interval. The reaction in consecutive tubes was stopped at 1 minute intervals by addition of 5µl of 20% w/v PMSF solution in isopropanol.

Note: PMSF was added immediately after the end of reaction time.

2.2.5 Quantitation of protein by Folin-Lowry Method

Standard protein solution of volume 0.5 ml of various concentrations was prepared as mentioned in table below. 0.5ml of 2N NaOH solution was added in each tube and hydrolyzed at 100°C for 10 minutes in boiling water bath. Hydrolysate was cooled to room temperature and 5 ml of freshly mixed complex reagent was added to it and the solution was allowed to stand at room temperature for 10 minutes. To this 0.5 ml of Folin reagent was added using vortex mixer and allowed to stand in room temperature again for 30-60 minutes. Absorbance was taken for various concentrations of standard protein solution together with samples of unknown concentration.

Table 2. Standard protein solutions of various known concentrations.

Stock solution (µl)	0	5	12.5	25	50	100	250	500
Water (µl)	500	495	488	475	450	400	250	0
Protein concentration(µg/ml)	0	20	50	100	200	400	1000	2000

2.2.6 Induction of Aggregation of Purified Protein

Various methods used for aggregation of papain:

- Using 2.5M guanidine hydrochloride in 0.2 M sodium acetate buffer at pH 5.6 (Sathwash, 2009)
- Using 1M guanidine hydrochloride at pH 2 (Chamani, 2008)

Best results were obtained with 1M guanidine hydrochloride at pH 2.0. So this method was used for all subsequent experiments

Papain exists in molten globule state at pH 2 and form aggregates at lower concentration of Guanidine Hydrochloride. At 1M GuHCl, papain completely forms insoluble aggregate.

100-300µl of purified protein solution dissolved in CGH-10 buffer of pH 2 make up to 1 ml. 1M Guanidine Hydrochloride was added to the solution.

2.2.7 Monitoring of aggregation:

Aggregation of papain was monitored by:

- Visible inspection of solution
- Gel filtration Chromatography (using Sephadex G-75) for monitoring soluble aggregates. Sephadex G-75 has fractionation range of 3000-80,000 kDa.

2.2.8 Preparation of plant extract

30 gm of dried edible portion of plant was crushed in a mortar and dissolved in 80ml of distilled water. The resultant solution was centrifuged at 15300 g for 45 mins. Clear solution was obtained and 10 ml of it was put in a hot air oven at temperature of 50°C until liquid was completely evaporated. Dried portion was redissolved in the same volume of CGH-10 buffer of pH 2. The solution was centrifuged at 15300 g for 30 mins to get the supernatant as a resultant plant extract solution.

2.2.9 Monitoring the effects of plant extracts on aggregation or disaggregation of papain

- Visible monitoring.
- Spectrometric analysis of scattering light on visible spectrophotometer at 365 nm of wavelength.

2.2.9.1 Visible monitoring for prevention of aggregation

405 µg/ml of purified protein solution was taken in three different test tubes having prepared plant extract of concentration 172 mg/ml of amla, 112.5 mg/ml of ginger or 112.5 mg/ml of cinnamon in CGH-10 buffer of pH 2.0. 1M Guanidine Hydrochloride was then added and mixed in solution to initiate aggregation.

2.2.9.2 Visible monitoring for disaggregation of aggregates

500µl of prepared plant extract was added to preformed aggregates of papain.

2.2.9.3 Monitoring of papain aggregation in presence of plant extracts from light scattering

First a buffer blank was set with CGH-10 buffer at 365nm. Papain (Concentration of 270 µg/ml) was dissolved in CGH-10 buffer of pH 2.0 and 1M Guanidine Hydrochloride was added to the solution to initiate aggregation. Absorbance was taken in Thermo Scientific, Spectronic 20D+ spectrophotometer at intervals of 1 minute maintaining continuous shaking. Prevention of aggregation has been monitored by setting the blank with plant extract solution of concentration 38.4 mg/ml of amla and 25 mg/ml of ginger in CGH-10 buffer separately. The same concentration of papain as above was separatly taken in 38.4 mg/ml of amla solution and 25mg/ml of ginger solution. Light scattering at 365 nm was monitored as a function of time in each case after initiation of aggregation by addition of 1M Guanidine Hydrochloride.

RESULTS AND DISCUSSION

3.0 Results and Discussion

3.1 Purification of papain

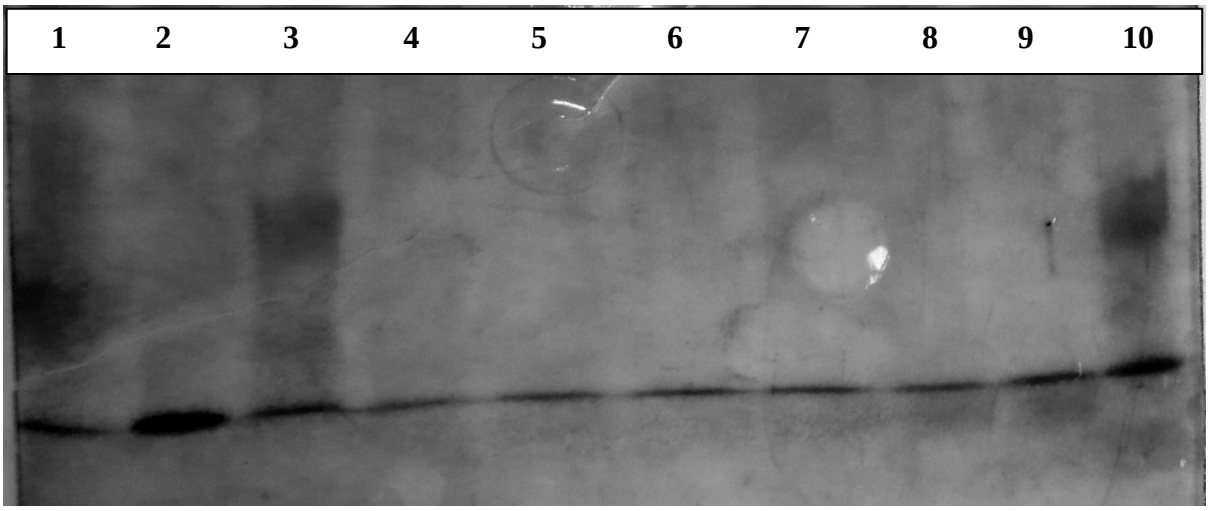


Figure 2 : 15% SDS-PAGE of purified protein sample with BSA, lysozyme and casein as markers. Lane :1 BSA of molecular weight 66 kDa, Lane 2: Lysozyme of molecular weight 14.3 kDa, Lane 3 to Lane 10: Casein of molecular weight of 23kDa, Lane 4 to Lane 9: purified protein sample of volume 5 µl, 6 µl, 7 µl, 8 µl, 9 µl and 10 µl respectively.

After crystallization, white crystals were obtained. This was checked for purity by SDS-PAGE as shown in Figure 2. The molecular weight of papain being 23 kDa, it is expected to migrate at the same level as the 23 kDa casein marker. But there was a single band in the purified protein samples which migrated along with 14 kDa lysozyme. Due to this anomaly in the migration of the purified protein, it could not be confirmed from the SDS-PAGE whether the purified protein was papain or not. So further characterization of the purified protein was done in terms of its proteolytic activity to confirm it as papain.

3.2 Zymography

Figure 3: Zymography of purified protein sample. The polyacylamide gel contained 0.6% w/v of gelatin as a substrate.

To check the proteolytic activity of the purified protein, a zymogram was run. Gelatin is a substrate for the proteolytic enzyme, papain. Although the zymogram was not a very good one, clear zones in the lanes where purified protein samples were loaded (Figure 3) indicate that gelatin has been cleaved by the protein sample. It is very likely that purified protein sample is that of papain.

3.3 Proteolysis

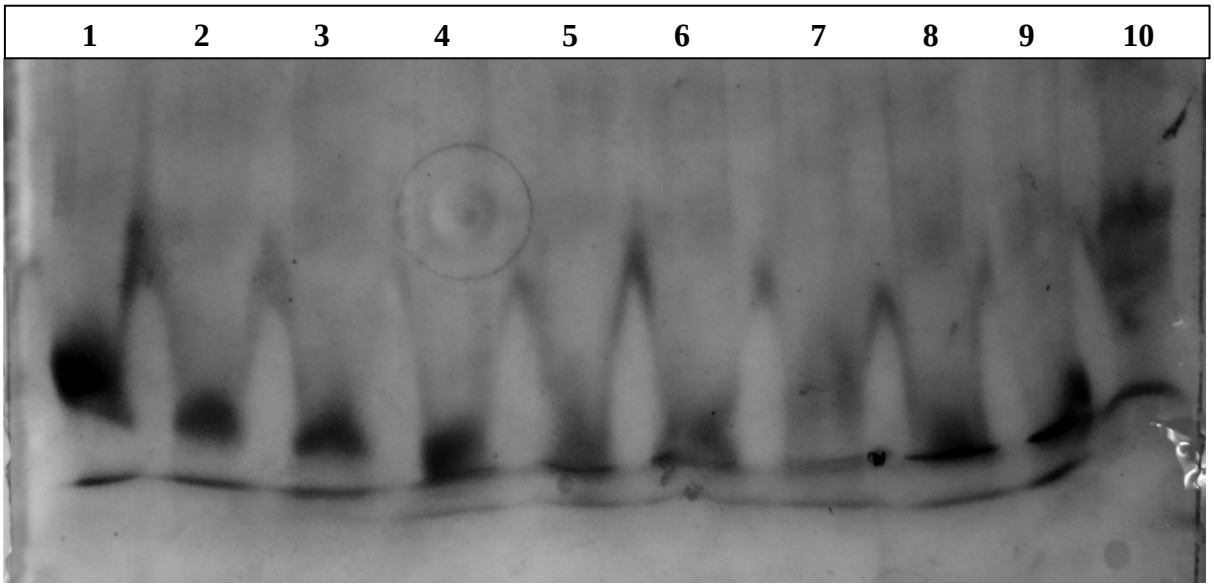


Figure 4: Proteolytic activity of purified protein sample in 15 % polyacrylamide gel. From lane 1-9 decrease in intensity of product band as decrease in duration of reaction time from 9 minutes to 1 minute. Lane 10 has casein as a marker.

To further confirm the identity of the purified protein, its proteolytic activity was checked on casein as a substrate. Figure 4 shows the 15 % SDS-PAGE of the proteolytic cleavage products of casein. The substrate, intact casein was loaded in lane 10. The lanes 9-1 contain a low molecular weight proteolytic cleavage product of casein at 1 minute intervals after addition of the purified protein to casein. Appearance of the low molecular cleavage product from casein upon treatment with the purified protein sample confirms its proteolytic activity. Thus the purified protein is papain and further aggregation experiments were carried out with this protein sample.

3.4 Quantitation of purified protein

The purified papain was quantitated by the Folin-Lowry method. The protein concentration of the papain sample was estimated to be 4.05 mg/ml from standard curve of BSA by this method.

3.5 Aggregation of Papain

3.5.1 Formation of insoluble aggregates

Two different methods were followed for aggregation of papain as described in materials and methods section. When 2.5 M guanidine hydrochloride was added to 405 µg/ml of papain solution in solution in 0.2 M sodium acetate buffer, pH 5.6, no haziness appeared, indicating that no insoluble aggregates were formed under these conditions. The papain solution under these conditions was analyzed by gel filtration chromatography through Sephadex G-75 as described below.

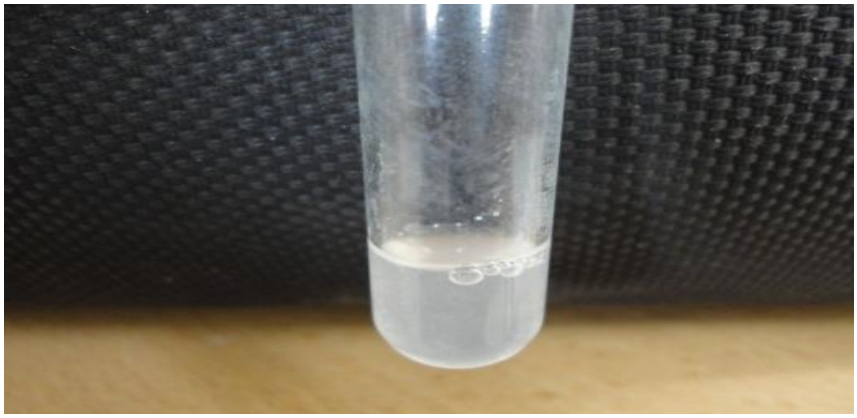


Figure 5: Aggregates of Papain. Tube contained 405 µg/ml of papain in 1 ml CGH-10 buffer, pH 2.0 treated with 1 M GuHCl.

When 405 µg/ml papain in 10 mM citrate-glycine-HEPES buffer, pH 2.0 was treated with 1 M guanidine hydrochloride, there was immediate appearance of insoluble white aggregates. The solution turned hazy as shown in Figure 5. So this method of aggregation was followed for subsequent experiments with plant extracts.

3.5.2 Gel filtration chromatography of soluble aggregates through Sephadex G-75.

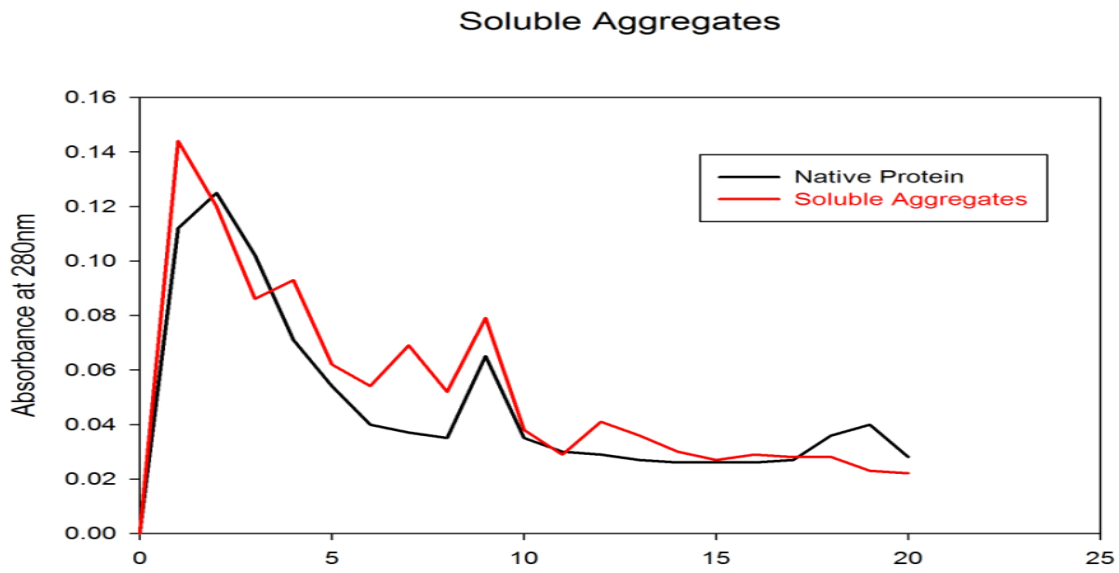


Figure 6 Monitoring soluble aggregates at 280 nm. Black curve is of untreated papain and red curve is of papain treated with 2.5 M GuHCl in 0.02 M sodium acetate buffer, pH 5.6

Figure 6 shows the gel filtration chromatography profile of papain treated with 2.5 M GuHCl at pH 5.6 as well as of the untreated protein. The result from this experiment were inconclusive and was not carried on further.

3.6 Effects of Amla, Ginger and Cinnamon extracts on papain aggregation

3.6.1 Disaggregation of already formed aggregates

When extracts of amla, ginger or cinnamon were added to already formed aggregates of papain, there was no decrease in haziness of the solution, indicating that none of the extracts could promote disaggregation of the aggregates.

3.6.2 Prevention of aggregation

3.6.2.1 Effect of Amla extract

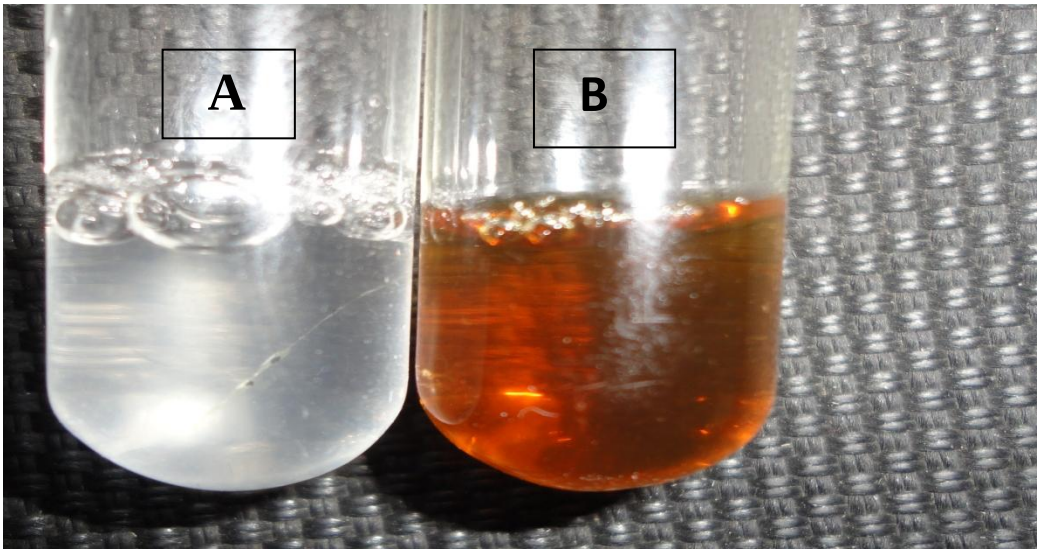


Figure 7: Aggregation analysis of papain with Amla extracts. (Tube A) Aggregates of Papain, (Tube B) Solution of papain of concentration 405 µg/ml in CGH-10 buffer, pH 2.0 containing 172 mg/ml of amla extracts.

Aggregation of papain was initiated by addition of 1 M GuHCl to a solution of papain in CGH-10 buffer, pH 2.0 containing 172 mg/ml amla extract. As seen in Figure 7, the reaction mixture in the presence of amla extract (Tube B) was clear with no haziness, indicating that no aggregates were formed. Hence amla extract could completely prevent papain aggregation.

3.6.2.2 Effect of Ginger extract.

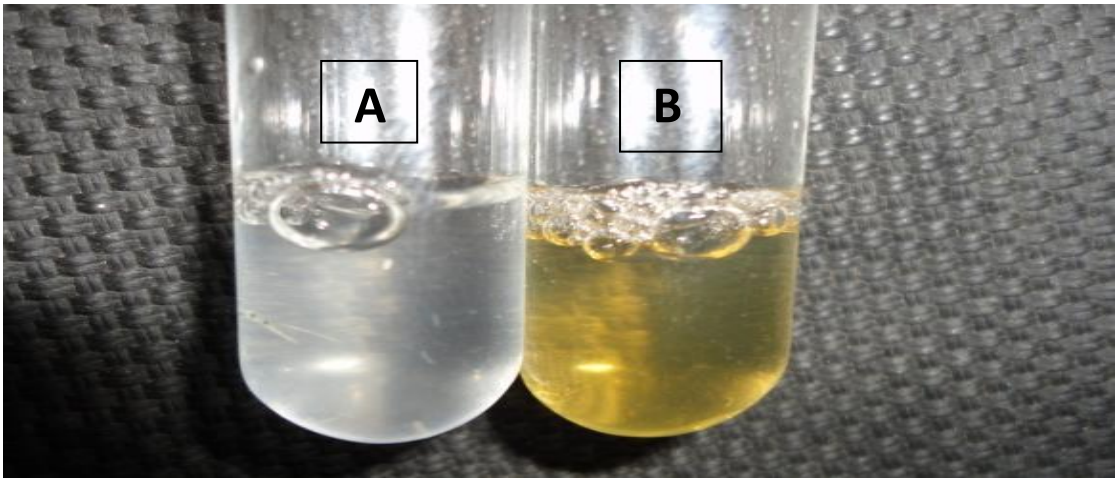


Figure 8: Aggregation analysis of papain with Ginger extracts. (Tube A) Aggregates of papain, (Tube B) Solution of papain of concentration 405 µg/ml in CGH-10 buffer, pH 2.0 containing 112.5 mg/ml of ginger extract.

Similar results were obtained when the papain aggregation reaction was carried out in the presence of 112.5 mg/ml ginger extract. As seen in Figure 8, (Tube B), where aggregation was done in presence of ginger extract, there was very little haziness, indicating that very little aggregates were formed. Hence ginger could also inhibit papain aggregation.

3.6.2.3 Effect of Cinnamon extract

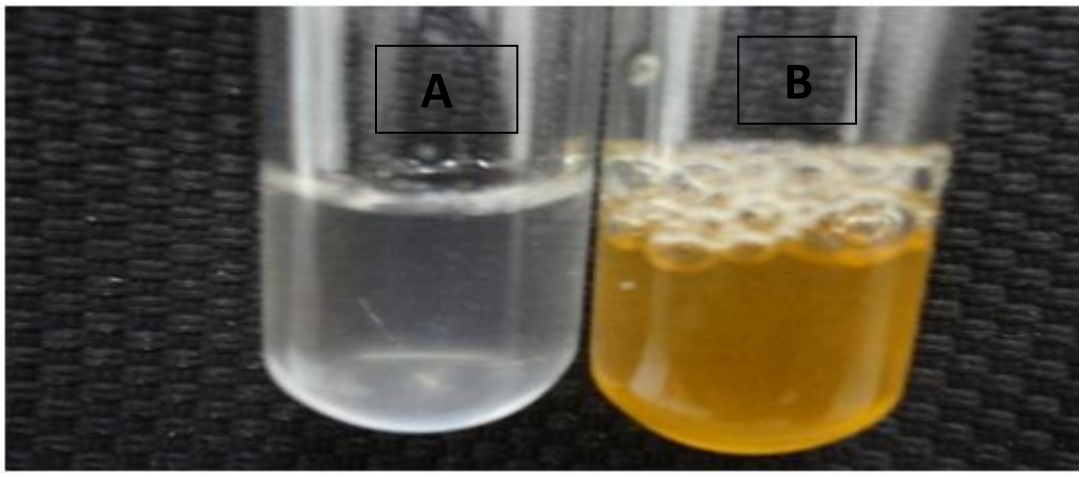


Figure 9: Aggregation analysis of papain with cinnamon extracts. (Tube A) Aggregates of papain, (Tube B) Solution of papain of concentration 405 µg/ml in CGH-10 buffer, pH 2.0 containing 112.5 mg/ml of cinnamon extract.

When the experiment was performed in the presence of 112.5 mg/ml cinnamon extract, there was appearance of haziness in the mixture as in the absence of extract. This indicates that aggregates were formed under these conditions. Hence cinnamon could not prevent papain aggregation at the concentration studied.

3.7 Spectrophotometric analysis of papain aggregation in absence and presence of plant extracts.

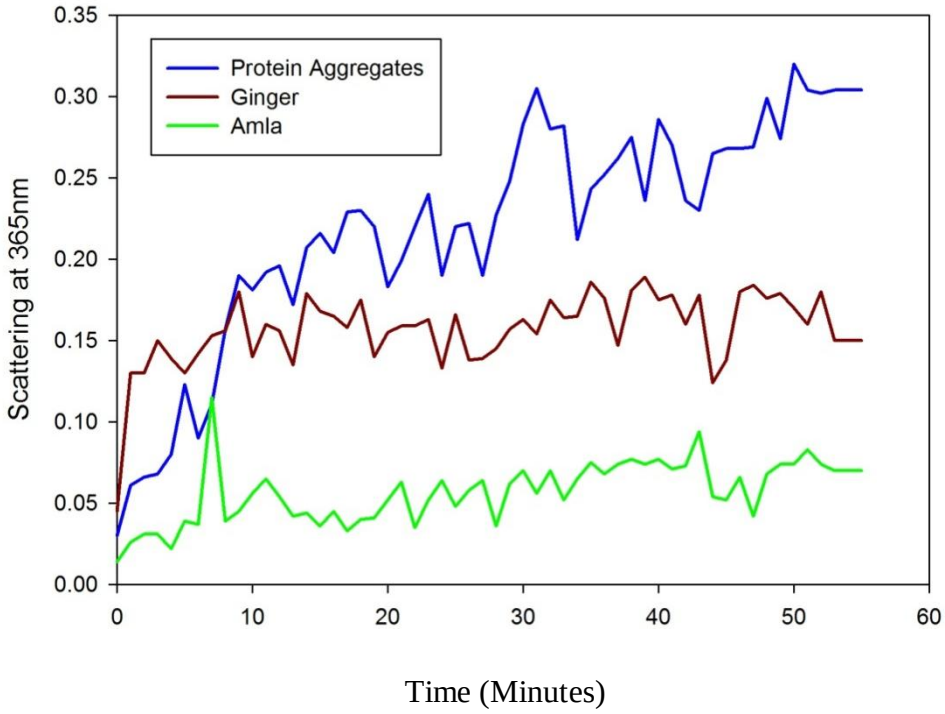


Figure 10: Aggregation analysis of papain in presence and absence of amla (38 mg/ml) and Ginger (25 mg/ml) in visible spectrophotometer.

The kinetics of aggregation of papain in the absence and presence of plant extracts was monitored spectrophotometrically by light scattering at 365 nm. This is shown on Figure 10. It can be seen that amla extract could completely suppress aggregation and no scattering was detected. Ginger extract also had the same effect, but aggregation was not completely prevented by the ginger extract at the concentration used. A higher concentration of the extract may totally prevent papain aggregation

4.0 Conclusion: We have shown that from the selected plant extracts i.e from amla, ginger and cinnamon, amla and ginger extracts are able to prevent the aggregation of papain and none of these plant extracts promote disaggregation of papain aggregates. There may be certain bioactive compound or combination of bioactive compounds in amla and ginger extracts which were preventing aggregation of papain even in acidic condition i.e at pH 2. These extracts may solve the problem of aggregation of therapeutic proteins or diseases caused due to protein aggregation problem.

SUMMARY

5.0 SUMMARY:

Protein misfolding and aggregation can be a nuisance factor in many in vitro studies of protein or it can cause major economic and technical problems in biotechnology and pharmaceutical industries. Its effects are lethal in patients who suffer from a variety of diseases involving protein aggregation, such as Alzheimer's disease, Prion disease etc. Molecular chaperones guide the correct folding of protein in vivo. There are compounds called chemical chaperones, which help in the right folding of proteins in vitro. Plant extracts with medicinal importance, like ginger, amla and cinnamon etc. were checked for anti-aggregation activity on the purified model protein, papain isolated from latex of raw fruit of papaya. This protein was purified using crystallization method. The aggregation of the papain was initiated by guanidine hydrochloride at lower pH i.e. at pH 2. Disaggregation or prevention of aggregation with plant extracts was monitored spectrophotometrically. None of the plant extracts show any results in disaggregating the aggregated model protein. Ginger and amla seems to be effective for preventing aggregation of papain, whereas cinnamon have shown no results for preventing aggregation of papain aggregates.

6.0 Future prospects

We have identified the plant extracts which have the potential of preventing aggregation of papain. The future plan for the project will be to identify the bioactive compound or the combination of the bioactive compounds which are responsible for their property of preventing aggregation and those compounds or combination of compounds will be tested for their action on aggregation of therapeutic proteins like insulin or proteins, which are responsible for many neurodegenerative diseases and major nuisance factor in bioprocessing and biotechnological and pharmaceutical industries. If these plant extracts are found to be effective in preventing aggregation of the therapeutic proteins then further it will be checked on cell cultures and thereafter on animal models.

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8.0 Appendix

8.1 SDS PAGE

- Acrylamide and N,N' –methylene-bwas-acrylamide- Stock solution of 29% acrylamide and 1% N,N' –methylene-bwas-acrylamide.
- Sodium dodecyl sulfate(SDS)- Stock solution of 10% w/v was used.
- Buffers
    - 1.5M Trwas-Cl, pH 8.8 for resolving gel
    - 0.5M Trwas-Cl, pH 6.8 for stacking gel
- Stock sample buffer

  0.06M Trwas-Cl, pH 6.8, 2% SDS, 10% Gylcerol, 0.025% Bromophenol blue
- Ammonium per sulfate (25%)
- TEMED(N,N,N,N-Tetramethylethylenediamine)
- Electrode buffer

  0.025M Trwas, 0.192M glycine, 0.1% w/v SDS, pH 8.3
- Staining dye

  40% Methanol, 10% Acetic Acid, 0.1% w/v Commasie Blue R-250.

- Destaining solution

  7.5% Acetic Acid, 10% Methanol.

	Resolving gel 15% (5ml)	Stacking gel 5% (2ml)
H20	1.1ml	1.150ml
30% Acrylamide	2.5ml	0.330ml
1.5M Trwas (pH 8.8)	1.3ml	0.500ml
10% SDS	0.05ml	0.02ml
25% APS	0.05ml	0.015ml
TEMED	0.002ml	0.002ml

8.2 Zymography:

- Same as SDS PAGE
- Stock gelatin solution (gelatin as substrate): 1.2% w/v in water. Store at 4°C and microwave gently on the deforest setting to melt before use.
- Triton X100: 2.5% w/v in water
- 10X refolding buffer: 0.5M Trwas-HCl, 2M NaCl, 5.5% $CaCl_2$, pH 7.6

8.3 Proteolyswas

- Same as SDS PAGE
- 20%w/v PMSF (Phenymethanesulfonylfluroide) was serine protease inhibitor.
- Wasopropanol- It was used to prepare stock solution of PMSF.

8.4 Folin-Lowry Method

1. Complex forming reagent: Prepare immediately before use by mixing the following stock solutions in thr proportion of 100:1:1 (by vol),respectively
   Solution A: 2% (w/v) Na_2CO_3 in dwastill water.
   Solution B: 1% (w/v) $CuSO_4.5H_20$
   Solution C: 2% (w/v) sodium potassium tartrate
2. 2N NaOH
3. Folin reagent: use at 1N concentration
4. Standards: use a stock solution of standard protein (BSA) containing 2mg/ml protein in dwastill water

8.5 CGH-10 buffer

- 10 mM each of citrate, glycine and HEPES
- pH 2