IJPSR (2023), Volume 14, Issue 12 (Research Article)

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 30 April 2023; received in revised form, 27 June 2023; accepted 04 July 2023; published 01 December 2023

COMPARATIVE STUDY OF ANTI-AGGREGATION PROPERTY OF LEAF PROTEINS OF BRAHMI, FENUGREEK AND TRIDAX ON THE FORMATION OF LYSOZYME AMYLOID FIBRILS

Sayantan Sil [1], Sudip Kumar Nag [1], Labani Hazra [1] and Srabani Karmakar [* 2]

Department of Biotechnology [1], Techno India University, West Bengal, EM4/1, Sector V, Salt Lake, Kolkata - 700064, West Bengal, India.
Kingston College of Science [2], Barasat, Berunanpukuria - 700126, West Bengal, India.

Keywords:

Amyloid, lysozyme, Congo red, ThT, Brahmi, Methi

Correspondence to Author:
Dr. Srabani Karmakar

Principal,
Kingston College of Science,
Barasat, Berunanpukuria - 700126,
West Bengal, India.

E-mail: snat14@gmail.com

ABSTRACT: Lysozyme amyloid formation is studied as model *in-vitro* system to find out natural or synthetic inhibitors of amyloidosis. Protein aggregation and amyloid formation is the basic reason for many neurodegenerative diseases. Indigenous medicinal plants are great source of therapeutic molecules and drugs. Ethnobotanical studies have indicated many plants have neuroprotective functions. We have studied three indigenous medicinal plant namely *Bacopa monnieri* (BM), *Trigonella foenum-graecum* (TFG) and *Tridax procumbens* (TP) showing various pharmacological properties as inhibitors of lysozyme aggregation. We isolated leaf proteins and did the protein aggregation studies. Anti-aggregation properties of leaf extract were carried out by following assays - DTT induced lysozyme aggregation assay, Congo red binding Assay, Thiaflavin binding assay and protein stability assay by Tryptophan Fluorescence. BM leaf protein extract showed maximum inhibition followed by Methi (TFG) and then TP.

INTRODUCTION: The failure of proteins to fold into their native state, or to remain in their native state, can disrupt normal biological functions and cause protein misfolding [1]. This misfolding sometimes generates highly organized fibrillar aggregates showing a cross-beta secondary structure termed "Amyloid." [2]. Amyloid fibrils generates several tissue damage, organ dysfunction, malfunctions of internal organelles and are also characteristic features of different types of neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, Type II diabetes, Huntington's disease, Rheumatoid arthritis *etc*.

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.14(12).5895-01

This article can be accessed online on www.ijpsr.com

DOI link: https://doi.org/10.13040/IJPSR.0975-8232.14(12).5895-01

Till now 28 different proteins have been identified as causative agents of neurodegenerative diseases like amyloid beta, transthyretin and Superoxide dismutase *etc* [3]. Hen Egg-White Lysozyme (HEWL) is a model protein which can form amyloid fibrils in adverse conditions like pH 2.0 and temperature 55°C [4]. We have chosen HEWL as a model protein for anti-aggregation assay to find out natural inhibitors from indigenous medicinal plants.

Lysozyme fibrillation is known to be prevented by ascorbic acid [5]. Several plant protein extract can inhibit these types of protein aggregation and amyloid fibril formation [6]. *Withania somnifera, Curcuma longa, Centella asiatica, Convolvulus pluricaulis, Nardostachys jatamansi, Celastrus paniculatus, Ilex paraguariensis, Ginkgo biloba, Coriandrum sativum, Ficus carica, Nardostachys jatamansi, Crocus sativus, Zingiber officinale,*

Moringa oleifera all these plants have potential anti-aggregation properties [7].

***Bacopa monnieri* (BM):** is a known medicinal plant, commonly termed as Bacopa or 'Brahmi'. It is a herb from the Scrophulariaceae family of plants which has been used since centuries in Ayurvedic medicine [8, 9]. BM has been shown to contain a complex mixture of constituents including alkaloids, saponins and flavonoids. BM leaf extract is reported to lower Aβ 1-40 and 1-42 levels in cortex of mice expressing amyloid proteins by as much as 60%. *Bacopa monnieri* (also known as brahmi, water hyssop), is a creeping perennial with small oblong leaves and purple flowers, found in warm wetlands native to India, Australia, East Asia and the United States [10]. BM plant extracts also showed neuroprotective character in Alzheimer's disease (AD) which happens due to accumulation of amyloid-beta (Aβ) in senile plaques [8-11].

***Trigonella foenum-graecum (TFG)*:** commonly known as'Methi' and in English it is known as 'Fenugreek'. Fenugreek is a herb similar to clover that is native to the Mediterranean region, southern Europe, and western Asia. Fenugreek is taken by mouth for digestive problems such as loss of appetite, upset stomach, constipation, inflammation of the stomach (gastritis), diabetes, obesity *etc* [12, 13].

***Tridax procumbens* (TP):** is commonly known as 'Ghamra' and in English popularly known as 'coat buttons' because of the appearance of its flowers [14]. Tridax plant is present throughout India and is employed as indigenous medicine for variety of ailments. It has been found to possess significant medicinal properties against blood pressure, bronchial catarrh, malaria, dysentery, diarrhea, stomach ache, headache, wound healing [15].

In this study we used BM, TFG and TP leaf aqueous extract and the leaf proteins to check inhibition of lysozyme aggregation and inhibition of lysozyme amyloid fibril formation by using biophysical techniques. BM leaf extract protein showed inhibition of DTT induced lysozyme aggregation and inhibition of HEWL amyloid fibril formation in terms of congo red binding and ThT assay. Stability of lysozyme was enhanced in presence of BM leaf protein as observed by equilibrium denaturation study.

MATERIALS and METHODS:

Chemicals and Reagents: The BM plant is taxonomically verified from the Botanical Survey of India. Majority of the chemicals used in this study like Guanidine-HCl, Ammonium sulphate, Sodium chloride (NaCl), Dithiothreitol (DTT), Disodium hydrogen phosphate, Sodium dihydrogen phosphate, Bradford reagent, Bovine serum albumin (BSA), Glycine, Acetic acid, Methanol & Ethanol, Glycerol, Bromophenol blue, Isopropyl alcohol, Ammonium persulphate (APS).

Sodium dodecyl sulphate (SDS), Acrylamide, Bisacrymamide, Tris were from SRL. N, N, N', N' Tetramethylethylene-1, 2-diamine (TEMED), Prestained protein marker and HEWL used in this study were purchased from SIGMA.

Collection and Extraction of Plant Proteins: Fresh leaves of *BM*, *TFG* and *TP* are grown and collected from home and washed gently in tap water thoroughly and followed by distilled water. Then the leaves are allowed to dry under the air for 30-40 min. Then the dry leaves were crushed in phosphate buffer saline (PBS) with optimum pH - 7.4. The juices are then filtered through Whattman No 1 filter paper to remove suspension. Then the fresh filtered juices are kept in fresh falcon for further use.

The juices extracted and filtered through Whattman filter paper. T the plant samples were taken and the samples were kept in aliquots in 2 ml Eppendorf for further use.

Partial Purification of Samples Extract (60% Ammonium Sulphate Precipitation): The crude extract was taken, and the desired amount of ammonium sulphate was added gradually and stirred for 1 hour in a magnetic stirrer [16]. Then the extract was centrifuged at 10000 rpm for 15 min at 4C. Then the supernatant was discarded, and the pellet was kept in aliquots for future use.

Sodium Dodecyl Sulphate- polyacrylamide Gel Electrophoresis (SDS-PAGE): SDS PAGE was done by following Laemmli's protocol [17]. Running gel (15%) and stacking gel (5%) were prepared to make the matrix for the SDS polyacrylamide gel electrophoresis process.

Gel Staining: Protein bands were visualized by both Coomassie blue staining. The gel was stained with 0.1% Coomassie Brilliant Blue R 250.

Determination of Total Protein Content: Protein concentration was determined by using Bradford assay. I n this assay we have taken a known protein (Bovine Serum Albumin) in different concentration within phosphate buffer (pH-7.4). Bradford reagent was added and absorbance checked at 595nm by a UV-Vis spectrophotometer. Then Protein extracts of the plant *Bacopa monnieri* were taken in different tubes and Bradford reagent is added into it. Absorbance checked at 595 nm by the UV-Vis spectrophotometer.

DTT-Induced Lysozyme Aggregation Assay: DTT-induced lysozyme aggregation assay was performed by observing scattering in terms of absorbance at 400 nm. We have taken 0.5mg/ml as a working solution of lysozyme and 20 mM of DTT and mixed the solution in PBS (pH-7.4). This is taken as control. Another reaction goes as lysozyme, DTT, leaf extract and PBS. Both reactions were allowed to be incubated at 37º for 2-3 hours. We checked through the spectrophotometric method in 15 min.

Congo Red Binding Assay: The lysozyme aggregation and amyloid formation inhibition was confirmed by Congo red binding assay. A red shift in absorbance maxima of Congo red absorption spectrum from 490 nm to 540 nm indicates presence of cross β-sheet rich structure [18]. A fresh solution of 100µM Congo red was prepared in phosphate buffer (From 10mM stock of Congo red in ethanol). Control A is prepared with a mixture containing, lysozyme (2mg/ml), and phosphate buffer (pH-7.4). Control B is prepared with a mixture of lysozyme, sample (plant protein extracts) and phosphate buffer. Thenboth the control and Lysozyme plus plant leaf protein sample were incubated for 4 hours at 55ºC. After 15 minutes time interval aliquots were taken and mixed with CR dye by dilution to measure the absorbance spectra from 400 nm to 600 nm.

ThT Binding Assay: ThT stock 500 µM was prepared in ethanol and concentration was calculated by the molar extinction coefficient [5, 18]. Solutions containing 2 mg/ml lysozyme were incubated for different time points at pH 2.5 and 55 °C in presence and absence of plant leaf extract proteins at two different concentrations. The incubated solutions were diluted in sodium phosphate buffer to measure the fluorescence to make final concentration of ThT as 10 µM. The excitation wavelength is 440 nm and emission was taken in the range of 450 to 600 nm. The measurements were done in a Hitachi flurimeter.

Protein Stability Assay by Tryptophan Fluorescence: The stability of lysozyme in the presence and absence of BM leaf protein was determined by equilibrium chemical denaturation experiments by a Fluorescence Spectrophotometer (JASCO) [19]. Proteins (0.1 mg/ml in 50 mM phosphate buffer, pH 7.4) were incubated at 25 °C in various Gu-HCl concentrations from 0-5 M for 18 hr. Tryptophan fluorescence spectra of all solutions were takening an excitation wavelength of 295 nm and an emission wavelength of 310-400 nn. Both the excitation and emission band pass were set at 4mm/4mm. The ratio of fluorescence intensity at 337 nm and 350 nm were measured and plotted against various Gu-HCl concentrations.

RESULTS AND DISCUSSION:

Isolation and Extraction of BM, TFG and TP Leaf Protein: The plant leaves of *Bacopa monnieri* (BM) **Fig. 1A** were crushed in Phospahte Buffer Saline (pH- 7.4). The proteins were extracted by using 60% ammonium sulfate precipitation [16].

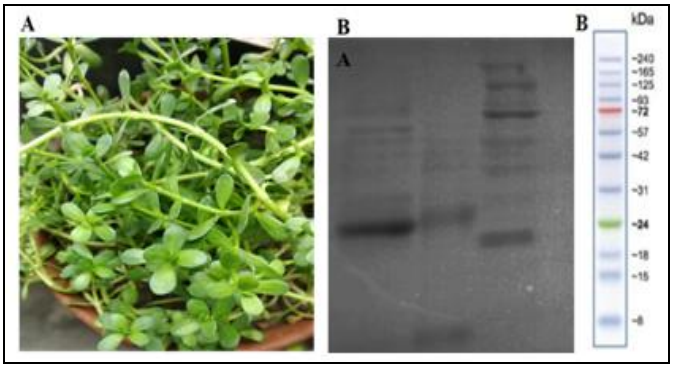

FIG. 1: A. THE BRAHMI PLANT (*BACOPA MONNIERI)* B. SDS PAGE OF BM LEAF PROTEINS [60% AMMONIUM SULPHATE CUT LANE 1 AND CRUDE LANE 2 AND MOLECULAR WEIGHT ACCORDING TO THE LADDER IN KDA LANE 3

Fig. 1B showed SDS-PAGE (15% resolving gel) and revealed a major protein band at 22 kDa. We have calculated the total protein concentration of

BM, TFG and TP leaf extracts. Protein concentration of *BM* leaf protein was found as 0.618 mg/ml. TFG leaf protein concentration was 0.473 mg/ml and TP was found as 0.28 mg/ml.

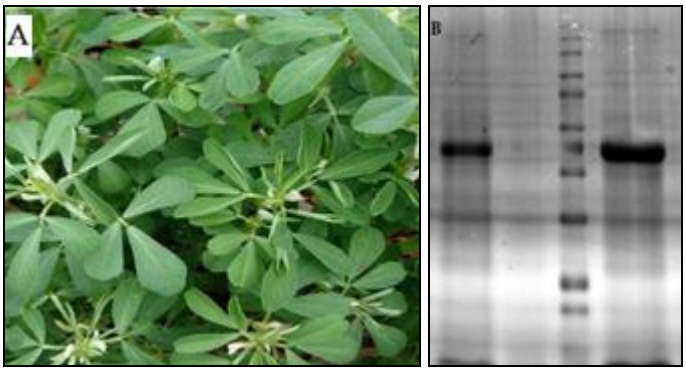

FIG. 2: A. THE FENUGREEK PLANT *TRIGONELLA FOENUM-GRAECUM* (TFG) B. SDS PAGE OF FENUGREEK (TFG) LEAF PROTEINS [60% AMMONIUM SULPHATE CUT LANE 1 AND 4 CRUDE LANE 3 MOLECULAR WEIGHT MARKER

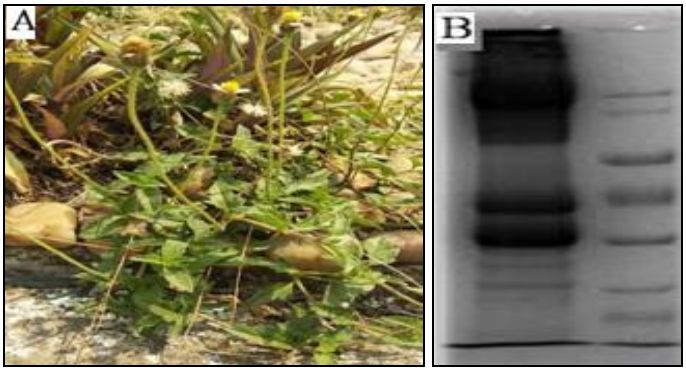

FIG. 3: A. THE TRIDAX PLANT (*TRIDAX PROCUMBENS*) TP B. SDS PAGE OF TP LEAF PROTEINS [60% AMMONIUM SULPHATE CUT LANE 1 CRUDE LANE 2 MOLECULAR WEIGHT MARKER

DTT-Induced Aggregation is Inhibited in the Presence of BM leaf Protein: The results showed **Fig. 4** that DTT-induced aggregation of lysozyme was inhibited by the presence of *Bacopa* leaf protein extract. We have also observed dose dependent protection of aggregation. Light scattering was measured by absorbance at 400 nm in UV-vis spectrophotometer. Scattering of lysozyme aggregation was reduced in the presence of *Bacopa* leaf protein. High concentration (60 microgram) of *Bacopa* protein showed 60 % protection and low concentration (30 microgram) showed 40 % protection. Inhibition of DTT induced aggregation is very less in case of TFG and TP leaf protein extract **Fig. 1** and **2.**

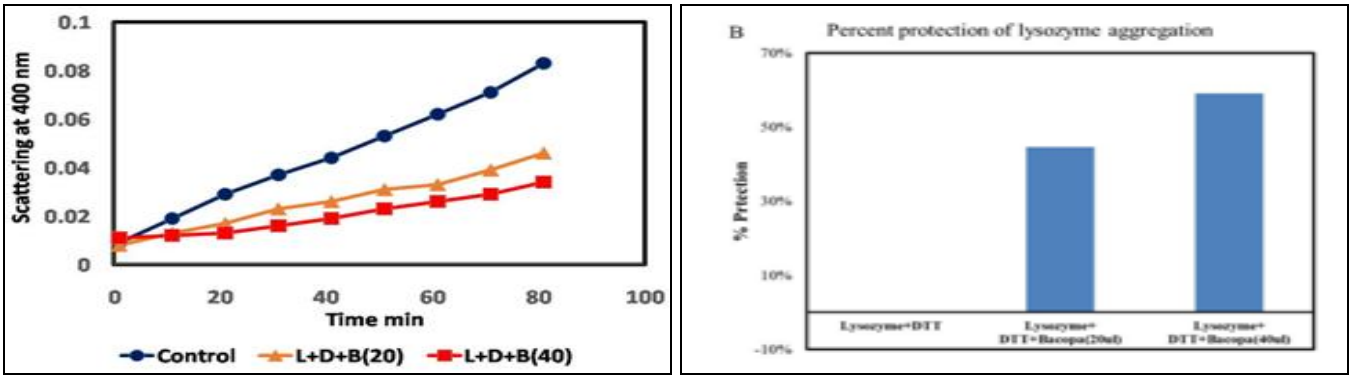

FIG. 4: DTT INDUCED AGGREGATION ASSAY WAS MONITORED BY MEASURING LIGHT SCATTERING IN TERMS OF ABSORBANCE AT 400 nm A. TIME DEPENDENT CHANGE IN THE ABSORBANCE VALUE AT 400 nm OF DTT INDUCED LYSOZYME AGGREGATION IN THE ABSENCE AND PRESENCE OF BM LEAF PROTEINS (TWO CONC 20µL AND 40µL) B. PERCENTAGE PROTECTION VALUES ARE SHOWN FOR THE VALUES AT 80 MINUTES

Congo red Binding to HEWL Fibril is Reduced in the Presence of BM Leaf Protein: The results of the Congo Red (CR) Assay on amyloid fibrillation of HEWL in the presence and absence of the plant protein extracts can be observed in **Fig. 3**. CR binding to the incubated lysozyme results in an enhanced absorption as well as a bathochromic shift in its absorption spectrum (from approximately 480 nm unbound to 540 nm bound). Control experiment of CR assay showed a complete shift of the absorption maxima from 480 nm to 540 nm indicating fibrillation of lysozyme **Fig. 5A**. When the lysozyme is incubated in the presence of *Bacopa* leaf protein extract (60% ammonium sulphate precipitation); shift in the CR absorbance maxima is reduced significantly indicating inhibition of lysozyme fibril formation **Fig. 5B.** Spectral shift is retained in the presence of TFG and TP leaves protein extract **Fig. 3.**

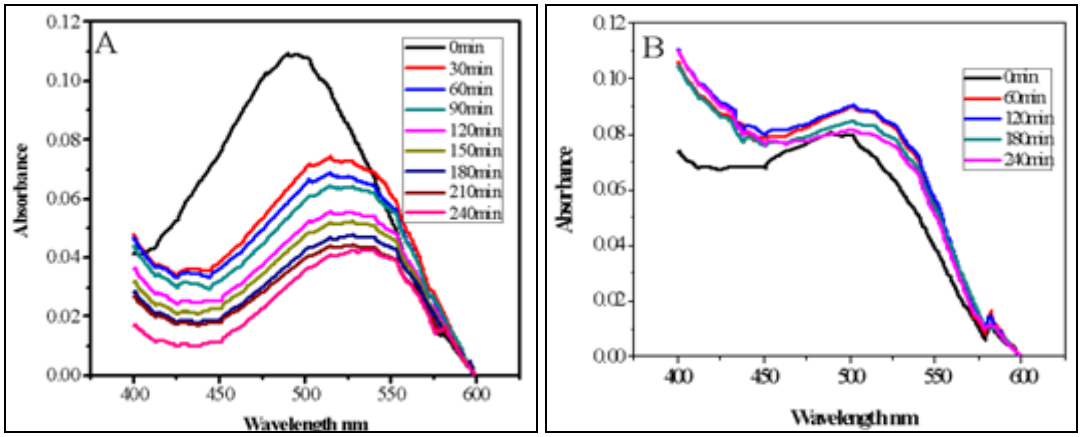

FIG. 5: CONGO RED BINDING OF HEWL FIBRILS SHOWN IN PRESENCE AND ABSENCE OF BM LEAF PROTEIN. HEWL was incubated at 55ºC and pH 2 for 4 hours. Every hour aliquot was taken and mixed with congo red and absorption spectra was taken from 400 nm to 600 nm. The black line indicates the values taken in 0 min, Red line indicates 1 hour, Blue line indicates 2 hours, Green line indicates 3 hours and the pink line indicates the spectra taken after 4 hours of incubation HEWL fibril.

ThT Binding of HEWL Fibrils is Inhibited in the Presence of BM leaf Protein: Thiaflavin T, when bound to amyloid fibrils gives more fluorescence. ThT binding to lysozyme after incubation in amyloid-forming conditions was plotted in **Fig. 6**. It showed the enhancement in ThT fluorescence at 485 nm against days of incubation. It followed ThT binding kinetics pattern similar to the reported amyloid fibril formation by lysozyme. ThT binding kinetics is inhibited for two different concentrations of BM leaf protein extract (30 and 60 micrograms).

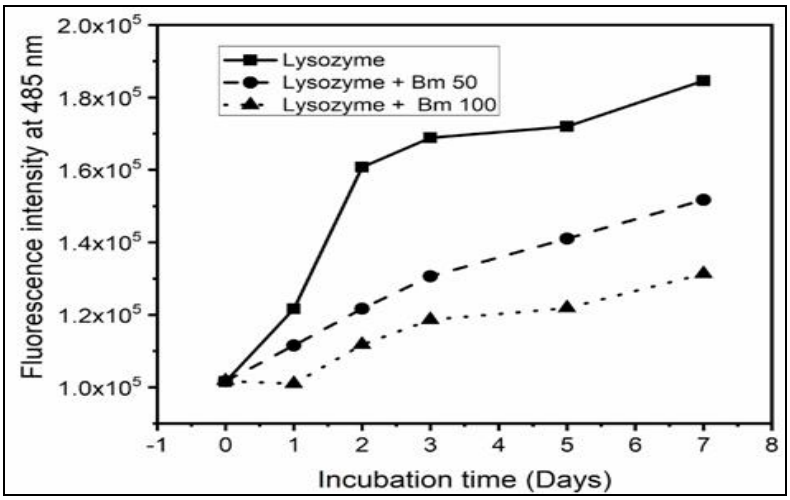

FIG. 6: THIAFLAVIN T BINDING OF HEWL FIBRILS (A) IN ABSENCE AND (B) PRESENCE OF BM LEAF PROTEIN. HEWL was incubated at 55ºC and pH 2 for 4 hours. Every hour aliquot was taken and mixed with ThT and fluorescence spectra was taken. Excitation of ThT was 410 nm and Emission was taken from 450 to 550 nm. Fluorescence emission at 485 nm was plotted against time.

Protein Stability Enhanced in Presence of BM Leaf Protein: The equilibrium unfolding profile was fitted according to sigmoidal fit and $C_{1/2}$ value estimated. In the Protein stability assay by Tryptophan Fluorescence, the estimated chemical denaturation value ($C_{1/2}$) of HEWL in presence of *BM* (1.51 M GuHCl) is higher than when in absence of *BM* (1.02 M GuHCl) **Fig. 7 A** and **B**. That means BM protein extract is increasing the stability of the HEWL to protect it from the chemical denaturation caused by GuHCl as higher concentration of Gu-Hcl is needed to denature half of the HEWL proteins when *BM* protein extract is added.

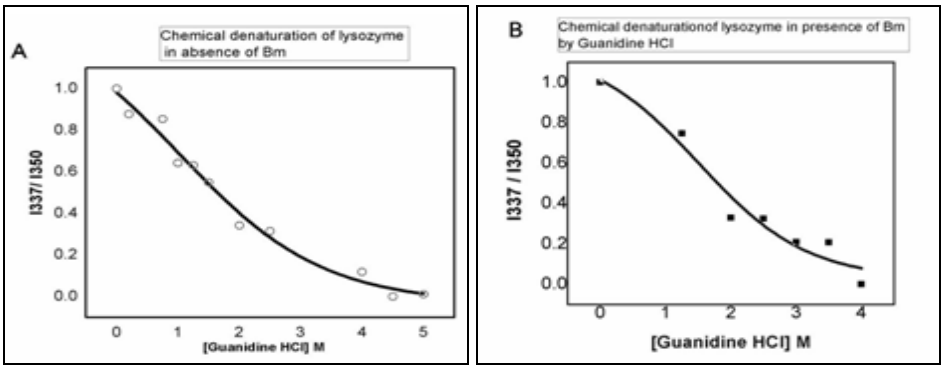

FIG. 7: EQUILIBRIUM GU-HCL UNFOLDING PROFILE OF LYSOZYME IN THE ABSENCE (A) AND PRESENCE (B) OF BM LEAF PROTEIN AT 25 °C. Gu-HCl concentration versus fraction folded (Ratio of intensity at 337 nm and 350 nm) was plotted. The protein concentration was 0.1 mg/ml. The symbols represent the experimental data points and solid line represents the sigmoidal fit.

CONCLUSION: Lysozyme amyloidosis has been studied in different contexts and proven to be a very good model system to identify novel molecules of interest from natural and synthetic sources [5, 20-24]. A comparative study from three medicinal plants showed that BM leaf protein extract can inhibit lysozyme fibrillation. Our results clearly showed the protection of amorphous aggregation of lysozyme as well as inhibition of congo red binding to lysozyme amyloid fibrils at pH 2.5 by BM leaf protein extract. Moreover, enhancement of structural stability is observed in presence of BM leaf protein. Inhibition of ThT fluorescence is also reported in presence of BM leaf protein.

ACKNOWLEDGEMENTS: We are thankful to the lab in charge Dipak. Konar of the Protein Chemistry Laboratory, Department of Chemistry, BOSE INSTITUTE (Main Campus). We also want to thank Dr. Padmaja Prasad Mishra of Saha Institute of Nuclear Physics. We would like to acknowledge the SERB TARE grant TAR/2018/000932 funding.

CONFLICTS OF INTEREST: None

REFERENCES:

1. Dobson CM: Protein folding and misfolding. Nature 2003; 426(6968): 884-90.
2. Pepys MB: Amyloidosis. Annual Review of Medicine 2006; 57(1): 223-41.
3. Kapoor P, Thenappan T, Singh E, Kumar S and Greipp PR: Cardiac amyloidosis: a practical approach to diagnosis and management. The American Journal of Medicine 2011; 124(11): 1006-15.
4. Swaminathan R, Ravi VK, Kumar S, Kumar MV and Chandra N: Lysozyme: a model protein for amyloid research. Advances in Protein Chemistry and Structural Biology 2011; 84: 63-111.
5. Patel P, Parmar K, Patel D, Kumar S, Trivedi M and Das M: Inhibition of amyloid fibril formation of lysozyme by ascorbic acid and a probable mechanism of action. Int J Biol Macromol 2018; 114: 666-78.
6. Dhouafli Z, Cuanalo-Contreras K, Hayouni EA, Mays CE, Soto C and Moreno-Gonzalez I: Inhibition of protein misfolding and aggregation by natural phenolic compounds. Cellular and molecular life sciences. CMLS 2018; 75(19): 3521-38.
7. Akram M and Nawaz A: Effects of medicinal plants on Alzheimer's disease and memory deficits. Neural Regeneration Research 2017; 12(4): 660-70.
8. Tripathi YB, Chaurasia S, Tripathi E, Upadhyay A and Dubey GP: *Bacopa monniera* Linn. as an antioxidant: mechanism of action. Indian Journal of Experimental Biology 1996; 34(6): 523-6.
9. Nemetchek MD, Stierle AA, Stierle DB and Lurie DI: The Ayurvedic plant *Bacopa monnieri* inhibits inflammatory pathways in the brain. Journal of Ethnopharmacology 2017; 197: 92-100.
10. Chaudhari KS, Tiwari NR, Tiwari RR and Sharma RS: Neurocognitive Effect of Nootropic Drug Brahmi (*Bacopa monnieri*) in Alzheimer's disease. Annals of Neurosciences 2017; 24(2): 111-22.
11. Abdul Manap AS, Vijayabalan S and Madhavan P: *Bacopa monnieri*, a Neuroprotective Lead in Alzheimer Disease: A Review on Its Properties, Mechanisms of Action, and Preclinical and Clinical Studies. Drug Target Insights 2019; 13: 1177392819866412.

12. Basu TK and Srichamroen A: Chapter 28 - Health Benefits of Fenugreek (*Trigonella foenum-graecum* leguminosse). In: Watson RR, Preedy VR, editors. Bioactive Foods in Promoting Health. San Diego Academic Press 2010; 425-35.
13. Syed QA, Rashid Z, Ahmad MH, Shukat R, Ishaq A and Muhammad N: Nutritional and therapeutic properties of fenugreek (*Trigonella foenum-graecum*): a review. International Journal of Food Properties 2020; 23(1): 1777-91.
14. Bhalerao SA and Kelkar TS: editors. Phytochemical and Pharmacological Potential of *Tridax procumbens* Linn 2012.
15. Ingole VV, Mhaske PC and Katade SR: Phytochemistry and pharmacological aspects of *Tridax procumbens* (L.): A systematic and comprehensive review. Phytomedicine Plus 2022; 2(1): 100199.
16. Duong-Ly KC and Gabelli SB: Chapter Seven - Salting out of Proteins Using Ammonium Sulfate Precipitation. In: Lorsch J, editor. Methods in Enzymology 541: Academic Press 2014; 85-94.
17. Gallagher SR: One-dimensional SDS gel electrophoresis of proteins. Current Protocols in Immunology 2006; 8: 4-8.
18. Alam P, Chaturvedi SK, Siddiqi MK, Rajpoot RK, Ajmal MR and Zaman M: Vitamin k3 inhibits protein aggregation: Implication in the treatment of amyloid diseases. Sci Rep 2016; 6: 26759.
19. Chang JY and Li L: The unfolding mechanism and the disulfide structures of denatured lysozyme. FEBS Letters 2002; 511(1): 73-8.
20. Brudar S and Hribar-Lee B: The Role of Buffers in Wild-Type HEWL Amyloid Fibril Formation Mechanism: A Methodological Approach. In: Cieplak AS, editor. Protein Aggregation: Methods and Protocols. New York, NY: Springer US 2023; 285-96.
21. Muthu SA, Sharma R, Qureshi A, Parvez S and Ahmad B: Mechanistic insights into monomer level prevention of amyloid aggregation of lysozyme by glycyrrhizic acid. Intern J of Biological Macromolecules 2023; 227: 884-95.
22. Cui L, Wang S, Zhang J, Wang M, Gao Y and Bai L: Effect of curcumin derivatives on hen egg white lysozyme amyloid fibrillation and their interaction study by spectroscopic methods. Spectrochimica Acta Part A: Molecular and Biomolecular Spectro 2019; 223: 117365.
23. Gao W, Jin L, Liu C, Zhang N, Zhang R and Bednarikova Z: Inhibition behavior of Sennoside A and Sennoside C on amyloid fibrillation of human lysozyme and its possible mechanism. International Journal of Biological Macromolecules 2021; 178: 424-33.
24. Makshakova O, Bogdanova L, Faizullin D, Khaibrakhmanova D, Ziganshina S and Ermakova E: The Ability of Some Polysaccharides to Disaggregate Lysozyme Amyloid Fibrils and Renature the Protein Pharmaceutics 2023; 15(2): 624.

How to cite this article:
Sil S, Nag SK, Hazra L and Karmakar S: Comparative study of anti-aggregation property of leaf proteins of Brahmi, fenugreek and Tridax on the formation of lysozyme amyloid fibrils. Int J Pharm Sci & Res 2023; 14(12): 5895-01. doi: 10.13040/IJPSR.0975-8232.14(12).5895-01.