Indian Journal of Natural Products and Resources
Vol. 12(2), June 2021, pp. 195-201

Identification of *Artocarpus hirsutus* and *Garcinia gummi-gutta* as the sources of trypsin inhibitory proteins

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Received 20 May 2020; Revised 18 March 2021

Natural trypsin inhibitors from plant tissues are emerging with promising therapeutic uses. They have profound applications in medicine and biotechnology and are extensively used in the food and medicine industry. Their activities are affected by detergents, metal ions, and reducing or chelating agents that are commonly used in these industries. *Artocarpus hirsutus* and *Garcinia gummi-gutta* are two tropical trees wherein most of the plant parts except the seeds were extensively studied and proved to possess medicinal properties. In the present study, the seeds of these tropical plants are proved to possess trypsin inhibitory activity. We report here the partial purification of trypsin inhibitory proteins from mature seed extracts of *A. hirsutus* and *G.gummi-gutta* in 50 mM phosphate buffer (pH 7.6). The partial purification was done by ammonium sulphate precipitation. Modulation of activity of *A. hirsutus* and *G. gummi-gutta* TIs by thermal stabilisers, metal ions and detergents were analysed. There was a significant fold of purification, in both cases. The thermal stabilisers, metal ions and detergents modulate the activities of the two TIs in their way. The study effectively provides choices of optimal additives to be used, where industrial processing of these TIs is required for therapeutic applications.

Keywords: *Artocarpus hirsutus*, *Garcinia gummi-gutta*, Industrial additives, Partial purification, Protease inhibitor, Trypsin inhibitor.

IPC code; Int. cl. (2015.01)- A61K 36/00

Introduction

Proteases are known as “enzymes of digestion” and are present in all living organisms[1,2]. They are linked to every functional aspect of organisms and play a critical role in many physiological and pathological processes[3-5]. However uncontrolled proteolytic pathways lead to diseases[6,7]. These proteases can be inactivated by degradation or interaction with inhibitor molecules. Protease inhibitors (PIs) are widespread in plants, animals and microorganisms, abundant in reproductive and storage organs and vegetative tissues of most plant families[8,9]. PIs are one of the prime candidates with highly proven inhibitory activity against insect pests and also known to improve the nutritional quality of food[10]. They are also used as therapeutic agents[11]. Trypsin inhibitors (TIs) are the most important among the studied PIs, since trypsin activates several digestive proteases which are secreted as proenzymes in the digestive tract, and also regulates their secretion. TIs present in different plants show variable levels of activity towards the proteolytic enzymes of target insects[12]. Various serine proteases including plant proteases have no requirement for any co-factors, hence it is highly desirable to identify and characterize novel PIs for their multipurpose values from plant resources.

Artocarpus hirsutus is an evergreen, tall tree that grows up to 75 meters in height (Fig. 1a). In the Ayurvedic system of medicine, the plant is used to pacify anorexia, burning sensation and sexual weakness. Unripe fruit cause indigestion and impotency. Ripe fruits are used for cooling, and as an aphrodisiac. Bark cures diarrhoea, pimples and ulcers[13,14]. *Garcinia gummi-gutta* is one of the small medium-sized trees in the Guttiferrae family which is emerged in Southeast Asia (Fig. 1b). This tropical fruit is well established as a weight-loss supplement for the reason that it can control the craving and interrupt the fat making capacity of the body[15,16]. TIs from plant sources possess antibacterial and antioxidant activities[17,18].

Herein we report the isolation and partial purification of TIs from mature seeds of *A. hirsutus* and *G. gummi-gutta*. These plants were selected based on a preliminary screening analysis (data not given)

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Fig. 1 — a) *A. hirsutus* plant, b) *G. gummi-gutta* plant, c) *A. hirsutus* seeds, and d) *G. gummi-gutta* seeds.

done in our laboratory. Since TIs are extensively used in the food and medicine industry they are to be treated with metal ions, detergents and reducing or chelating agents that are commonly used in these industries. Hence the objective of the present investigation is to study the effects of different additives on the activity of partially purified TIs from these two plant seeds.

Materials and Methods

Chemicals

N-Benzoyl-DL-arginine-4-nitroanilide hydrochloride (BAPNA) was procured from M/s. Sigma Aldrich Chemical Company, St. Louis, MO, USA. All the other chemicals used in the study were purchased from M/s. Sisco Research Laboratories Pvt. Ltd. (SRL), India. The dialysis membrane (12-14 kDa) was purchased from Himedia.

Collection of plant materials

Mature seeds of *A. hirsutus* (Fig. 1c) and *G. gummi-gutta* (Fig. 1d) were collected from Vallivattam and Chandrapinni respectively, places in Thrissur district of Kerala during the summer season and were authenticated at the Department of Botany, University of Calicut. The accession (Herbarium specimen) numbers of *A. hirsutus* and *G. gummi-gutta* are No. 7043 and No.7042 respectively. The seeds were washed thoroughly with distilled water, shade dried and ground to powder using an electrical blender. The seed flour was defatted and decolourized before extraction.

Extraction and recovery of TIs

A buffer extract was prepared by stirring 10 g each of defatted decolourized samples in 50 mL of 50 mM phosphate buffer, pH 7.6 in a magnetic stirrer. The slurry was centrifuged at 10,000 rpm for 30 minutes at 4 °C for removing any cell debris that remains in the preparation. The clear supernatant obtained represented the crude extract which was assayed for trypsin inhibitory activity and protein content[19].

Assay of trypsin inhibitory activity

The trypsin inhibitory activity was assayed according to the procedure described by Benjakul *et al.*, using the synthetic substrate, BAPNA (N-Benzoyl-DL-arginine-4-nitroanilide hydrochloride)[20]. Crude extracts were incubated with trypsin and BAPNA for 10 minutes at 37 ^{o}C. The reaction was terminated by adding a 30% TCA solution. The absorbance of released P-nitroaniline was measured at 410 nm. An increase of 0.01 absorbance units at 410 nm per 10 mL of the reaction mixture under the assay conditions is equivalent to one trypsin inhibitory unit (TIU).

Protein estimation

Protein content was determined using the method described by Lowry *et al.*[21]. The samples were incubated for 30 minutes in dark after adding alkaline copper sulphate solution (Mixture of 2% sodium carbonate in 0.1 N NaOH and 0.5% copper sulphate solution in 1% sodium potassium tartrate solution) and Folin Ciocalteu reagent. Absorbance was measured at 660 nm. Bovine serum albumin was taken as standard. A standard graph was plotted with the concentration of standard on the x-axis and OD on the y-axis. Total protein content in the sample was calculated.

Ammonium sulphate precipitation

The crude extracts were precipitated by ammonium sulphate and used for further analysis. Ammonium sulphate precipitation of the prepared sample was done according to the method described by Saxena *et al.*[22] The precipitate obtained after adding ammonium sulphate in different concentrations (30, 60, and 90%) was dialysed against 500 mL 50 mM phosphate buffer (pH 7.6), the fraction of each

sample having the highest activity was lyophilised and stored till further analysis.

Effects of various additives

Effect of stabilizers on the thermal stability of TIs

The enhancement of thermal stability of TIs at 50 and 60°C was evaluated by the addition of thermal stabilizers like glycine (1 M), cysteine hydrochloride (10 mM), BSA (1%), calcium chloride (10 mM) and casein (1%) to the partially purified samples. Samples with the additives were incubated for 4 hours at 50 and 60°C. At the end of incubation, the samples were further incubated on ice for 15 minutes and a TI assay was carried out.

Effect of various metal ions on trypsin inhibitory activity

The effect of various metal ions on the activity of TIs was evaluated by incubating them (partially purified fractions) with 1 mM and 10 mM concentrations of various metals ions in the inhibitor solution for 30 minutes at 37 °C followed by measuring the trypsin inhibitory activity. The metals studied included calcium chloride, magnesium sulphate, ferric chloride, mercury chloride and zinc sulphate which contributes to the metal ions, Ca^{2+}, Mg^{2+}, Fe^{3+}, Hg^{2+}, and Zn^{2+} respectively.

Effect of various detergents on trypsin inhibitory activity

Effect of various non-ionic and ionic detergents such as Triton X-100, SDS and 2-mercaptoethanol (1 and 0.5%) on trypsin inhibitory activity was determined by incubating the partially purified TIs in each detergent for 30 minutes, dialyzed against 50 mM phosphate buffer pH 7.6 and estimated trypsin inhibitory activity.

Statistical analysis

All the data were subjected to analysis of variance (ANOVA) and the differences between means were carried out using Duncan's Multiple Range Test. Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS for Windows, version 21; Inc.).

Results

Isolation of TIs

Exactly 27 and 30 mL of crude extract in 50 mM phosphate buffer (pH 7.6) were obtained from 10 g of *A. hirsutus* and *G. gummi-gutta* dried seeds, respectively. The extracts of both the plant seeds selected for this research also show significant trypsin inhibitory activity.

Partial purification of TIs

30-60% and 60-90% ammonium sulphate saturations were efficient for precipitating *A. hirsutus* TI compared to the other fractions while all the fractions of *G. gummi-gutta* were equally efficient. The results are given in Fig. 2. The yield and fold of purification of TI from seeds of *A. hirsutus* and *G. gummi-gutta* are summarized in Table 1. The yield of TI from mature *A. hirsutus* seeds obtained after ammonium sulphate precipitation is 48.85% while the

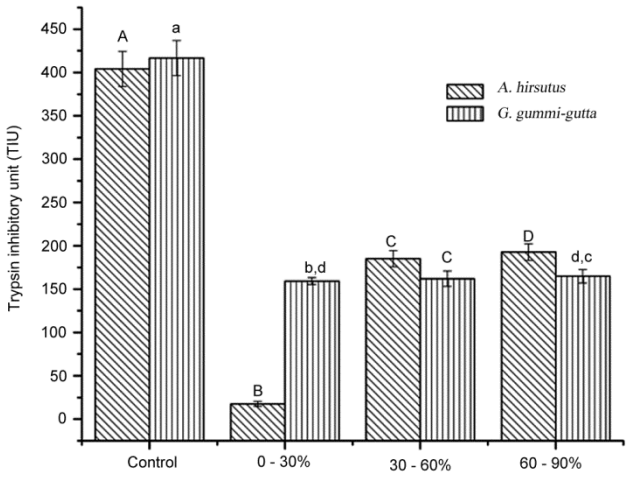

Fig. 2 — Trypsin inhibitory activity in seeds of *A. hirsutus* and *G. gummi-gutta* in different ammonium sulphate saturation. Values are the mean of six estimations. Control represents the trypsin inhibitory activity of crude extracts. Error bars indicate Standard Deviation (SD). Different alphabets indicate significant difference at P <0.05. F value for *A. hirsutus* and *G. gummy-gutta* are 6027 and 4.226 respectively.

Table 1 — The yield and fold of purification of TI from seeds of *A.hirsutus* and *G.gummi-gutta*

Sample	Volume (mL)	Total protein (mg)	TI activity (TIU/g)	Specific inhibitor activity (TIU/g)	Yield of activity %	Fold of purification
Crude extract						
A.hirsutus	27	3.09±0.02	404.06±0.25	132.72±0.14	100	1.00
G.gummi-gutta	30	2.63±0.02	416.60±0.49	158.71±0.30	100	1.00
Ammonium sulphate fraction						
A.hirsutus	5	1.02±0.01	197.40±0.16	195.38±0.17	48.85	1.40
(30-90%)	5	0.75±0.10	174.14±0.32	232.45±0.37	41.85	1.46
G.gummi-gutta (0-90%)						

yield of TI from *G.gummi-gutta* seeds is 41.85%. The fold of purification of TI from *A. hirsutus* and *G. gummi-gutta* has much more similar values 1.4 and 1.46 respectively.

Effects of additives

Effect of stabilizers on the thermal stability of TIs

Effect of additives as thermal stabilizers of TI was studied at 50 and 60 °C using glycine (1 M), cysteine hydrochloride (10 mM), BSA (1%), calcium chloride (10 mM) and casein (1%). Data presented in Fig. 3 indicate that in general, all the stabilizers promoted thermal stability and inhibitory activities of *A. hirsutus* TI at 50 °C compared to that of control (in the absence of stabilizers). It can be seen in Fig. 4 that all the stabilizers promoted thermal stability and inhibitory activities at 50 and 60˚C of TI from *G. gummi-gutta*, although only $CaCl_2$ and BSA act as promoters for TI from *A. hirsutus* seeds.

Effect of various metal ions on trypsin inhibitory activity

From the results presented in Fig. 5, it can be noted that ferric chloride at a concentration of 10 mM enhanced the activity of TI. 1 mM calcium chloride also enhanced the activity. Results indicate that the presence of 10 mM zinc sulphate, calcium chloride, mercuric chloride and magnesium sulphate has a negative effect. As can be seen from Fig. 6 it can be observed that except zinc sulphate all the metal ions enhanced the TI activity. Results indicated that 10 mM and 1 mm zinc sulphate had a negative effect.

Effect of detergents on trypsin inhibitory activity

Results presented in Fig. 7 and 8 indicate that SDS and 2-mercaptoethanol enhance the activity of both TIs. Triton x 100 had a negative effect. 2-mercaptoethanol increases the inhibitory activity at a marginal level.

Discussion

In the present study, the potential of *A. hirsutus* and *G. gummi-gutta* as sources of trypsin inhibitory proteins is indicated. Phosphate buffer with different concentrations and pH were used for the extraction process to fix the condition which gave maximum TI activity (data not shown). The 50 mM phosphate buffer (pH 7.6) was selected for extraction of TI from *A. hirsutus* and *G. gummi-gutta* mature seeds. 10g of dried seeds of *A. hirsutus* and *G. gummi-gutta* respectively produced 27 and 30 mL of crude extract in 50 mM phosphate buffer (pH 7.6). It is reported that seeds, leaves, and tubers are the main location of PIs[23] and several investigations were held on the

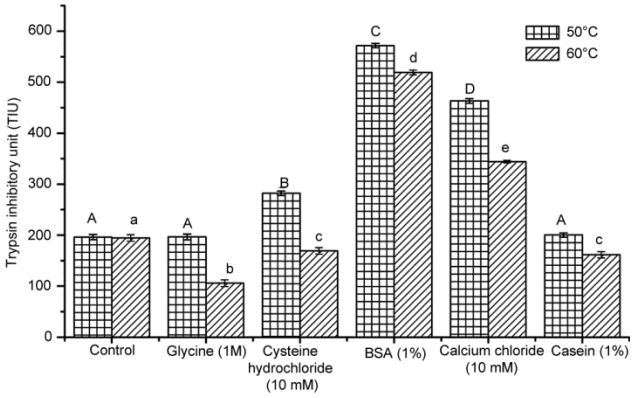

Fig. 3 — Effect of stabilizers on the activity of TI from *A. hirsutus.* Values are the mean of six estimations. Control represents the trypsin inhibitory activity of ammonium sulphate fractions at 50°C and 60°C. Error bars indicate Standard Deviation (SD). Different alphabets indicate significant difference at P <0.05. F value for 50°C and 60°C are 735.309 and 1117.220 respectively.

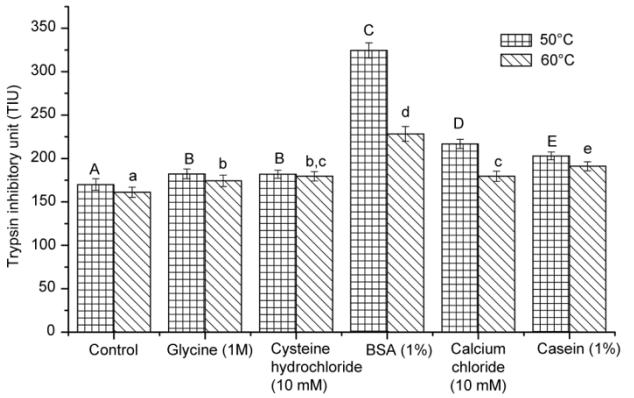

Fig. 4 — Effect of stabilizers on the activity of PI from *G. gummi-gutta.* Values are the mean of six estimations. Control represents the trypsin inhibitory activity of ammonium sulphate fractions at 50°C and 60°C. Error bars indicate Standard Deviation (SD). Different alphabets indicate a significant difference at P <0.005. F value for 50°C and 60°C are 901.815 and 158.561 respectively.

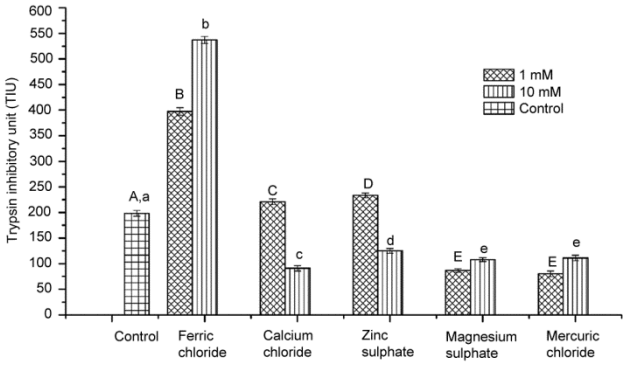

Fig. 5 — Effect of metal ions on trypsin inhibitory activity of *A. hirsutus* TI. Values are the mean of six estimations. Control represents the trypsin inhibitory activity of the ammonium sulphate fraction. Error bars indicate Standard Deviation (SD). Different alphabets indicate significant difference at P <0.05. F value for 10 mM and 1 mM are 1617.94 and 920.157 respectively.

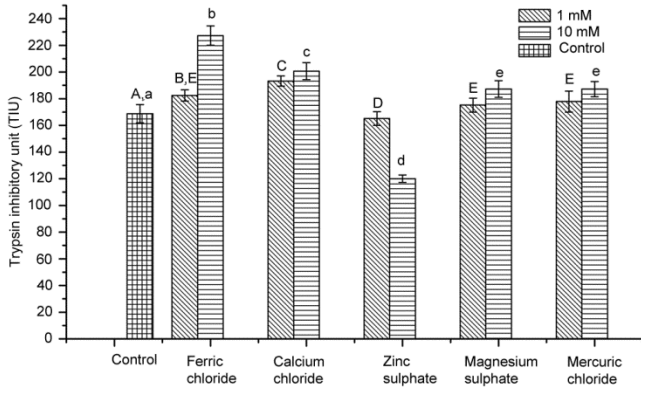

Fig. 6 — Effect of metal ions on protease inhibitory activity of *G. gummi-gutta* TI. Values are the mean of six estimations. Control represents the trypsin inhibitory activity of the ammonium sulphate fraction. Error bars indicate Standard Deviation (SD). Different alphabets indicate a significant difference at P <0.005. F value for 10 mM and 1 mM are 352.430 and 42.775 respectively.

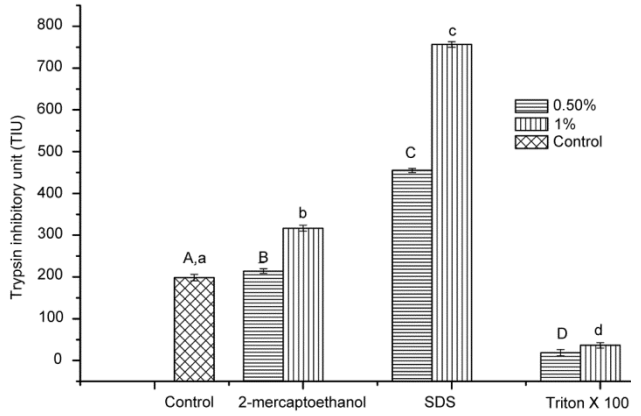

Fig. 7 — Effect of detergents on trypsin inhibitory activity of *A. hirsutus* TI. Values are the mean of six estimations. Error bars indicate Standard Deviation (SD). Control represents the trypsin inhibitory activity of the ammonium sulphate fraction. Different alphabets indicate significant difference at P <0.05. F value for 1% and 0.5% are 1989.97 and 3010.918 respectively.

isolation, purification and characterization of PIs from plant seeds[24-27]. We have reported the antioxidant and microbicidal effects of TI from *G.gummi-gutta*[17]. Due to its harmless effects on most proteins and large solubility in water ammonium sulphate is commonly used for salting out proteins. Partial purification of TI was carried out by ammonium sulphate. During fractionation proteins present in extracts begin to separate into different fractions based on properties such as size or charge. Hence proteins separated in the same fractions may be of similar size or charge. The fold of purification is a measure of how much more pure the protein is after a purification step in comparison to the crude. PIs from *A. hirsutus* and

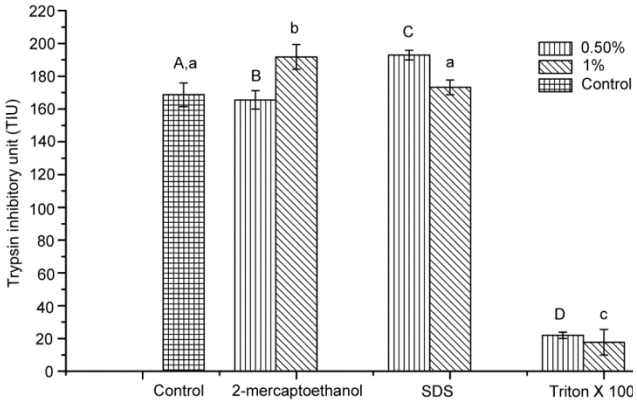

Fig. 8 — Effect of detergents on trypsin inhibitory activity of *G. gummi-gutta* TI. Values are the mean of six estimations. Control represents the trypsin inhibitory activity of the ammonium sulphate fraction. Error bars indicate Standard Deviation (SD). Different alphabets indicate a significant difference at P <0.005. F value for 1% and 0.5% are 3626.024 and 3453.908 respectively.

G. gummi-gutta has almost the same purity when compared to each other after the ammonium sulphate precipitation process, and their fold of purification are 1.4 and 1.46 respectively. The percentage of yield expresses how much of the protein desired was recovered. The yield of TIs from matured *A. hirsutus* and *G. gummi-gutta* seeds obtained is 48.85 and 41.85% respectively.

Glycine, Cysteine hydrochloride and Casein have a negative effect at 60 °C. In the case of *G. gummi-gutta* data presented in Fig. 4 indicate that almost all the stabilizers improved thermal stability and inhibitory activities. At 50 °C, maximal stability was promoted by BSA followed by Calcium chloride and Cysteine hydrochloride. At 60 °C BSA is found to be the best stabilizer followed by Calcium chloride. When PIs are used in the detergent and pharmaceutical industry and in leather processing, they are subjected to high temperatures[28]. Thermal stabilizers can be used for stabilising PIs under extreme temperatures. The efficiency of proteins, which are used in commercial purposes can be improved by their thermal stability[29]. The increase of hydrophobic interactions inside the proteins is related to their stability. Low dielectric constant solvents can strengthen the hydrophobic interactions among nonpolar residues. Hence in the presence of such solvents proteins show greater resistance to thermal denaturation processes. In this study, $CaCl_2$ shows a significant increase in the stability of the TI at higher temperatures. Ca^{2+} ions stabilize the protein through specific and non-specific binding sites and also allow for additional binding within the protein molecule

and prevent the unfolding of the protein at a higher temperature. Similar results were reported by Bijina *et al*[30].

The activity profile of PIs in the presence of different monovalent and divalent metal ions was determined by incubating with different concentrations of metal ions. In the case of TI from *G. gummi-gutta* all the metal ions enhanced the activity except zinc sulphate, however in the case of TI from *A. hirsutus* seeds only the addition of Ferric chloride and Zinc sulphate enhanced inhibitory activity. In a reversible mechanism metal, ions will bind to the protein and retain their conformational stability for their biological activity. Metals have played an important role in medicine for years[31]. PIs contain metal-binding or metal-recognition sites, which can bind or interact with metal ions and potentially influence (increase or decrease) their bioactivities[32]. Sweeney D *et al.*, reported that in the absence of metal ions, phenformin was a weakly competitive PI however, metformin was non-inhibitory. They discussed the results in relation to Zn^{2+} interactive inhibition of insulin degradation in hormone target tissues, and Fe^{3+} interactive inhibition of haemoglobin degradation in parasite food vacuoles[33].

Effect of stabilizers on trypsin inhibitory activity of both TI from *A. hirsutus* seeds and *G.gummi-gutta* seeds shows similar results. SDS and 2-mercaptoethanol enhanced the activity. Triton X 100 had a negative effect. Detergents act as surfactants as they lower the surface tension of water and mimic the native, hydrophobic environment of the phospholipid bilayer *in vivo*. The ability of detergents to solubilise the protein from lipid membranes and other protein-bound membranes is made use in protein extraction processes. To prevent proteolysis, PIs along with detergents are normally employed in cell lysis buffers. This procedure facilitates membrane protein solubilization in protein purification processes. Nonionic detergents do not interact extensively with the protein surface and are generally considered mild detergents. The ionic detergents like SDS, generally bind non-specifically to the protein surface, leading to protein unfolding[34,35].

Conclusion

In the present study, the potential of *A. hirsutus* and *G. gummi-gutta* as fair natural sources of trypsin inhibitory proteins is indicated. Trypsin inhibitory activity can be modulated using different additives which are currently used in different biomedical and biotechnological industries. The trypsin inhibitory protein from *G. gummi-gutta* has already reported possessing antibacterial and antioxidant activities and hence it can be used for drug development. In this scenario, the study proves to be instrumental since it provides choices of optimal additives to be used, where industrial processing of these TIs is needed. We conclude that both *A. hirsutus* and *G. gummi-gutta* seeds are natural sources of potential trypsin inhibitory proteins that may have application in biotechnology and the biomedical field.

Acknowledgement

The authors acknowledge the Science and Engineering Research Board, Department of Science and Technology, Government of India for the financial support as Start-Up Research Grant (Life sciences) scheme No.SB/YS/LS-183/2013, to the corresponding author.

Conflict of interest

The authors declare that there are no conflicts of interest.

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