SUMMARY AND CONCLUSION

Twenty-two plants belonging to different families and from different localities of Kerala were screened for their protease inhibitory activity. Many leguminous plants producing protease inhibitor was reported earlier. Plant species with more than 60% protease inhibitor was further selected on the basis of proteinaceous nature of protease inhibitor. *Moringa oleifera*, the panotropical tree with high biomass yield which can withstand many unfavorable environmental conditions was selected as the potential source for protease inhibitor.

Protease inhibitor content was maximum in the leaves (77 %) in mature *Moringa oleifera* plant followed by the seed extract (63 %). The plant parts like flowers, bark and roots were having negligible amount of trypsin inhibitor.

Phosphate buffer was the efficient extracting medium for the complete extraction of the protease inhibitor with a protease inhibitory activity of 79% from mature leaves with maximum protein concentration. Distilled water as extractant showed maximum specific inhibitory activity. The trypsin inhibition was very less in the extract prepared in sodium chloride and sodium hydroxide compared to that prepared in phosphate buffer and distilled water.
Protease inhibitor from *Moringa oleifera* was purified using standard protein purification methods, which included ammonium sulphate precipitation, followed by dialysis, ion exchange chromatography and preparative polyacrylamide gel electrophoresis (PAGE). It was observed that 30-90% saturation of ammonium sulphate is needed for the complete precipitation of plant protease inhibitor. On further purification by ion exchange chromatography, a single peak with maximum protease inhibitory activity was obtained with a 2.5 fold of purification. The purity of the fraction was further analyzed by electrophoresis, which furnished a single peptide band with 41.4 fold of purification.

The purity and homogeneity was confirmed by polyacrylamide gel electrophoresis. The protein eluted from 0.2M NaCl yielded a single protein band under Native-PAGE. In SDS-PAGE under non-reducing conditions, the protein yielded a single polypeptide band with a molecular weight of 23.6 kDa. But under reducing conditions, it yielded two bands with molecular weight of 22 kDa and 14 kDa. This signifies the dimeric nature of the polypeptide. The protease inhibitory activity of the protein band was confirmed by reverse zymography on gelatin incorporated gel and also by doing the *in vitro* inhibitor assay. It was observed that the protease inhibitory activity was highest in the larger fragment compared to the smaller one.

The molecular weight of the protease inhibitor was also confirmed by gel filtration chromatography on Sephadex G75. From the graph obtained for $K_{av}$ versus log molecular weight of standard proteins, it was
found that the molecular weight of protease inhibitor was approximately 29 kDa.

Based on the amino acid composition, it was inferred that the purified protease inhibitor contained highest amount of glycine (27.29 %/g sample) and the lowest one was lysine (0.22 %/g sample).

*Moringa oleifera* protease inhibitor had an optimum pH 7.0 for its activity and they were active over a pH range of 6.0-10.0. The stability of the protease inhibitor at different pH testifies that it is stable over a pH range of 5.0-11.0. The protease inhibitor was active over a temperature up to 50°C and the maximum activity was obtained in a temperature range of 30-40°C. The protease inhibitor was inactive at temperatures above 50°C. It showed stability at 30 & 40°C for two hours with more than 50% protease inhibitory activity.

All the stabilizers promoted the thermal stability and inhibitory activity compared to control at 50°C. 10mM calcium chloride, 1% BSA and 1% sucrose offered complete protection for thermal denaturation of protease inhibitors at 50°C. Two polyols, glycerol and sorbitol enhanced the protease inhibitory activity compared to control. Whereas at 60°C, BSA followed by calcium chloride, casein, cysteine hydrochloride and glycine alone supported stability compared to control. Sucrose and starch, which supported stability at 50°C, did not promote stability at 60°C.

Higher concentration of Zn$^{2+}$ and Hg$^{2+}$ enhanced the protease inhibitory activity to 31% and 64% respectively compared to control. Ca$^{2+}$ and Mg$^{2+}$ at a concentration of 10mM enhanced the protease inhibitory
activity to a marginal level. Presence of \( \text{Na}^{2+}, \text{Ba}^{2+}, \text{Ni}^{2+}, \text{Cd}^{2+}, \text{Mo}^{6+} \) and \( \text{Al}^{3+} \) did not support protease inhibitory activity when compared to control and instead they had a negative effect of protease inhibitory activity. The metal chelation of *Moringa oleifera* protease inhibitor led to a loss in protease inhibitory activity to 53\% compared to control. The presence of 4.26, 0.31 and 0.33 ppm of \( \text{Ca}^{2+}, \text{Mg}^{2+} \) and \( \text{Zn}^{2+} \) was detected in the native protease inhibitor. The additional supplementation of \( \text{Mg}^{2+} \) and \( \text{Zn}^{2+} \) on the demetallized protease inhibitor enhanced the protease inhibitory activity to 27 \& 47\% respectively compared to demetalIized protease inhibitor. The additional supplementation of \( \text{Ca}^{2+} \) enhanced the protease inhibitory activity to a marginal increase at 10mM concentration.

The protease inhibitor retained 62\% inhibitory activity in the presence of SDS compared to control. All the other detergents like Tween 80, Tween 20, Triton X 100 and Brij 20 had negative effect on protease inhibitory activity.

In the presence of oxidizing agents, protease inhibitory activity decreased on increasing the concentrations of oxidizing agents. But the reducing agents had a positive effect on the protease inhibitory activity. The residual activity of protease inhibitor increased on increasing the concentrations of oxidizing agents. DTT and \( \beta \)-mercaptoethanol up to a concentration of 1\% enhanced the protease inhibitory activity to 49 \& 41\% respectively.

The modification of tryptophan residue by N-bromosuccinamide resulted in the activation of protease inhibitor to a great extent. Modification of serine residue by PMSF also enhanced the protease inhibitory activity.
with a residual inhibitory activity of 280% at 25mM compared to control. Modification of cysteine by N-ethylmaleimide resulted an enhancement in protease inhibitory activity only to a marginal level. The higher concentration of DEPC, which resulted in the modification of histidine residue, also enhanced the protease inhibitory activity to a 10% compared to control. Modification of lysine by succinic anhydride resulted in a loss of protease inhibitory activity on increasing the concentration. Lysine modification resulted in a 91% residual inhibitory activity compared to control.

The impact of HCl on protease inhibitor up to 0.02M did not decrease the protease inhibitor activity. But there was a gradual decrease on increasing the concentration of HCl. The pretreatment of protease inhibitor with digestive protease also reduced the protease inhibitory activity on increasing the concentration of trypsin.

The complete inactivation of trypsin by protease inhibitor is in the ratio 1:1.5. At 1.5 nM concentration of protease inhibitor, there was complete inactivation of 1nM trypsin. It is calculated that 35.4 μg of protease inhibitor was necessary to completely inactivate 23.8μg of trypsin. The concentration of protease inhibitor required for the 50% trypsin inhibition was 0.6nM. The kinetics of trypsin inhibition by protease inhibitor from *Moringa oleifera* revealed a reversible mode of uncompetitive protease inhibition. The Kᵢ value was found to be 1.5 X 10⁻⁹ M and the very less Kᵢ value reports the high affinity of *Moringa oleifera* protease inhibitor to trypsin.
The protease inhibitor showed high affinity towards most of the serine proteases such as trypsin, chymotrypsin, elastase and thrombin. This illustrates the use of protease inhibitor in both pharmaceutical and agricultural industries. The protease inhibitor also had affinity towards cysteine proteases like Cathepsin B and papain and the inhibitor did not show any affinity towards collagenase.

*Moringa oleifera* protease inhibitor possessed a high activity spectrum towards many commercially available industrially important proteases. The protease inhibitor completely inactivated proteases isolated from *Bacillus* sps. and *Aspergillus oryzae*. It also showed 76% inhibitory activity towards the protease isolated from *Engyodontium album*. The activity towards subtilisin was very less (3.7%) compared to the other proteases.

The use of protease inhibitor towards seafood preservation was also demonstrated. The *Moringa oleifera* protease inhibitor could be able to regulate the microbial growth present on the shrimp *Peneaus monodon* during the preservation at various conditions compared to the control. The protease inhibitor could prevent the protein degradation to a greater extent. They retained the total protein of the sample throughout their storage time on comparing with control. Thus protease inhibitor could be used as preservative to prevent proteolytic degradation of shrimp during storage.
Conclusion

Protease inhibitors are one of the most important tools of nature for regulating the proteolytic activity of their target proteases. They are synthesized in biological systems and they play a critical role in controlling a number of diverse physiological functions. The current investigation focused on the isolation, purification and characterization of a novel protease inhibitor from *Moringa oleifera*. The results obtained during the course of study opens new perspectives for the utilization of protease inhibitor from *Moringa oleifera* for various pharmaceutical, agricultural and food industries. The biological and physicochemical properties exhibited by the novel protease inhibitor from *Moringa oleifera* clearly testify its suitability for the development as a drug for application in pharmaceutical industries such as anticoagulant agent or biocontrol agent in agriculture and even as a food preservant. There is a scope for further research on the structure elucidation and protein engineering towards a wide range of further applications. Detailed structure/function analysis of these proteins is important to facilitate their use in genetic engineering for various applications.