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The Trypsin Inhibitory Activity of *Vigna unguiculata* ssp. Sesquipedalis (Hawari Mae) Seeds Grown in Sri Lanka was Evaluated

YN Wickramaratne [a], SAADSD Peiris [a], DMHSK Doranegoda [a], AALT Ampemohotti [b], FASS Pillay [b], KDKP Kumari [c] and ARN Silva [c*]

[a] Department of Pharmacy, Faculty of Allied Health Sciences, General Sir John Kotelawala Defence University, Sri Lanka.
[b] Faculty of Graduate Sciences, General Sir John Kotelawala Defence University, Sri Lanka.
[c] Department of Basic Sciences, Faculty of Allied Health Sciences, General Sir John Kotelawala Defence University, Sri Lanka.

Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Aims: This study aimed to screen the entire seed sample of Vigna unguiculata ssp sesquipedalis (Hawari mae) for serine protease inhibitory activity. Additionally, the study explored the impact of various factors such as temperature, pH, metal ions, detergent, oxidizing and reducing agents on the inhibitory activity.

**Corresponding author: E-mail: nsrajith2005@yahoo.com;*

Methodology: A batch of Hawari Mae seeds was obtained from the Plant Genetic Resources Center in Sri Lanka. A concentration gradient of aqueous seed extracts was tested for Serine Inhibitory Activity (SIA). To carry out the SIA assay, seed extracts were mixed with trypsin in phosphoric acid buffer (pH 7.6) and casein was added as the substrate. The samples were then incubated at 37°C and trichloroacetic acid was used to stop the reaction. The supernatants were checked for absorbance at 280 nm and the percentage of serine inhibitory activity (SIA%) was calculated. All experiments were conducted three times to ensure accuracy. The inhibitory activities were tested under various conditions, including different temperatures, pH levels, metal ions, detergents, and oxidizing and reducing agents.

Results: The crude extract containing 10% of the substance showed the highest level of trypsin inhibitory activity at 96.03±0.005%. This particular extract was selected for further analysis. The study revealed that the enzyme inhibition activity was highest at a temperature of 37°C (95.42±0.006%) and a pH of 7.6 (95.71±0.003%). It was observed that all tested metal ions, except Cu^{2+}, significantly reduced ($P<0.05$) the trypsin inhibitory activity in Hawari mae. The presence of the detergent Triton X-100 did not significantly affect ($P>0.05$) the trypsin inhibitory activity of Hawari mae. However, the presence of DMSO and dithiothreitol significantly ($P<0.05$) reduced its activity.

Conclusion: Significant trypsin inhibitory activity was observed in the seed extract of Hawari mae. Further studies on the effects of various physio-chemical parameters can aid in the purification and isolation of trypsin inhibitors.

Keywords: Proteases; trypsin inhibitors; legumes; Vigna unguiculate; Hawari mae.

1. INTRODUCTION

Proteases are enzymes that break down proteins. They belong to the class of enzymes called hydrolases and are also known as proteinases or proteolytic enzymes. Proteases can be found in plants, animals, and microorganisms. They are the largest family of enzymes in humans, with approximately 1.7% of the human genome encoding different proteases [1]. Plant proteases regulate biological processes such as defense responses and can activate protease-activated receptors. Examples include papain, bromelain, keratinases, and ficin [2]. Proteases are a large family of enzymes in the human proteome, with five types based on catalytic mechanisms: serine, cysteine, aspartic, threonine, and metalloproteases. Their specificity is controlled by the substrate's amino acid sequence and 3D structure [3].

Protease inhibitors are classified based on the type of proteases they inhibit (serine, cysteine, aspartic acid, and metallocarboxy), as well as their mechanism of inhibition. They can also be grouped into families based on sequence homology and clans based on protein tertiary structure. Plants contain protease inhibitors which protect them from herbivores and inhibit degradation of storage proteins. These inhibitors have potential health benefits against various [4] (Srikanth and Chen, 2016). Trypsin inhibitors are used to treat chronic diseases associated to overweight, metabolic syndrome and obesity by controlling the energy balance and appetite. The trypsin inhibitors also have anti-carcinogenic and radio-protective activity thus reduce risks of colon, prostate, breast and skin cancers [5].

Legumes are a rich source of protease inhibitors. Hawari Mae seed, a local variety of *Vigna unguiculata* ssp sesquipedalis, was screened for trypsin inhibitory activity to isolate protease inhibitors for drug development. Yard long bean is a climbing annual plant that belongs to the Fabaceae or Leguminosae family. It's also known as long-podded cowpea, string bean, snake bean, and Chinese long bean. This plant can fix atmospheric nitrogen through the symbiosis process. It has very long pods, which are about 30-90cm in length and can reach up to 9-12 feet in height [6]. Yard long beans (Figs. 1 & 2) are highly nutritious and can be used to reduce blood cholesterol and improve kidney functions. Hawari mae is a local cultivar with light green pods and black seeds.

The present study was designed to screen the trypsin inhibitory activity in seeds of Hawari mae (*Vigna unguiculata* ssp sesquipedalis (Yardlong bean)) with the purpose of isolation of protease inhibitors for protease targeted drug development in future.

Fig. 1. Hawari mae cultivar of *Vigna unguiculata* ssp sesquipedalis

Fig. 2. Diagram of a whole plant of *Vigna unguiculate*

2. METHODOLOGY

2.1 Collection and Drying of Samples

Vigna unguiculata ssp sesquipedalis (Hawari mae) seeds were collected from the Plant Genetic Resources Center in Sri Lanka. The seeds were cleaned and dried, and their weight was measured using an electric balance (SARTORIUS TE64 23350109).

2.2 Preparation of 20% Seed Extract

The Hawari mae seed sample was crushed and ground into a dry powder mass. Then the seed sample was weighed using an electric balance after being powdered (SARTORIUS TE64 23350109). To extract 20%, the powdered seed samples were weighed and mixed with distilled water in a mortar and pestle. The solution was then filtered and its total volume was measured. The filtrate was centrifuged at 10000rpm for 15 minutes using the Orto alresa (REF CE146 SN 200379/04) machine. The supernatant was collected and its final volume was measured in a 250ml beaker.

2.3 Preparation of Reagents

2.3.1 Phosphate buffer (0.5M, pH 7.6 and 1M, pH 2)

44.4g $Na_2HPO_4.2H_2O$ was dissolved in 250ml distilled water in a 500ml conical flask. The pH was adjusted to 7.6 using 6M NaH_2PO_4 solution, and the volume was adjusted to 500 ml using distilled water. The solution was stored at room temperature. Phosphoric acid (H_3PO_4) was weighed and mixed with 160ml distilled water in a 200ml conical flask. The solution was stirred continuously and pH was adjusted to 2 using a saturated sodium hydroxide solution. The final volume was adjusted to 200ml with distilled water and stored at room temperature.

2.3.2 Casein solution (1%), trypsin (1%) solution, and trichloroacetic acid (TCA) (5%)

Casein 1.5g was dissolved in 150ml of 0.5M Phosphate buffer (pH 7.6) by warming on a steam bath at 50-55°C for 1-2 hours and then stored in Eppendorf tubes at -20°C. Trypsin 0.5g was dissolved in 50 ml of 0.001M of HCL and stored in Eppendorf tubes at -20°C. TCA 12.5g was dissolved in 250ml of distilled water and stored at room temperature in an amber color bottle.

2.3.3 Triton X-100 (1% w/v), dithiothreitol (1M) and dimethyl sulfoxide (DMSO) (5% v/v)

0.1g Triton X-100 was dissolved in 10ml distilled water and incubated at 37°C for 30min in a Stuart orbital incubator SI500 while covered with Aluminum foil. 1.54g Dithiothreitol was dissolved in 10ml water, incubated for 30min at 37°C, covered with foil. DMSO (1ml) was dissolved in 200ml distilled water and incubated in Stuart orbital incubator for 30min at 37°C, covered with aluminum foil.

2.4 Determination of Trypsin Inhibitory Activity of Seed Extracts

From the 20% supernatant, a 1:1 dilution series of the seed extracts (20% -1.25%) was prepared by using distilled water as the diluent.

A dilution series was used to determine the maximum inhibitory activity of trypsin with different concentration percentages of seed extract. Tubes containing phosphate buffer, trypsin and plant extract were incubated and casein was added. After incubation, TCA was added and the supernatants were used to create a 1:4 dilution series to determine the absorbance of UV radiation of the diluted seed extracts. The inhibitory activity percentage was calculated using an equation.

$$\text{Inhibitory activity} = \frac{[\text{NC (maximum)} - \text{TS (minimum)}]}{[\text{NC (maximum)}]} \times 100\%$$

NC - Negative Control
TS – Test Sample

2.5 Determination of the Effect of Temperature and pH on the Trypsin Inhibitory Activity

The maximum inhibitory percentage of seed extract concentration was determined by carrying out a trypsin assay with different pre-incubation temperatures (0, 20, 37, 60, 80, and 100^0C) for 30 minutes. The experiment involved adding reagents to Eppendorf tubes labeled T1, T2, T3, NC1, NC2, and NC3, with varying amounts of plant extract and trypsin. The tubes were then incubated at different temperatures for 1 hour. After incubation, casein was added to all tubes and incubated again. The supernatant was collected, and diluted, and the absorbance was measured using a UV Spectrophotometer (Thermo scientific Genesys 50). The inhibitory activity percentage was calculated using the same equation.

For the determination of pH, trypsin assays were carried out by changing the pH ranges of the phosphate buffer to pH 5.8, 6.4, 7.0, 7.4, 7.6, and 8.0. All the other steps were the same as above.

2.6 Determination of the Effect of Metal Ions, Detergents, Oxidizing and Reducing Agents on the Trypsin Inhibitory Activity

For the determination of the effect of metal ions, the solution of plant extract and metal ions ($FeCl_3$, $Cu(CH_3COOH)_2$, $ZnC_4H_6O_4$, $BaCl_2$, and NaCl) was mixed and incubated. For the detergents, a solution of 1:1 plant extract: detergent (Triton x-100) was created and incubated. For the oxidizing agent, a solution of plant extract and oxidizing agent (Dimethyl sulphoxide (DMSO)) was mixed and incubated. For reducing agents, a solution of seed extract was mixed with a reducing agent (Dithiothreitol) and kept in a water bath. Then trypsin assays were carried out with the resulting solution. After incubation, casein was added and incubated again. The supernatant was collected and diluted for UV absorbance measurement. Inhibitory activity percentage was calculated.

2.7 Determination of Enzyme Activity Using the Standard Curve on L-tyrosine Production

L-tyrosine concentrations were measured at 280nm and a standard curve was drawn. Test and negative control samples' absorbance were converted into enzyme activity using the standard curve equation.

The average enzyme activity of seed samples was presented as mg of tyrosine produced per minute.

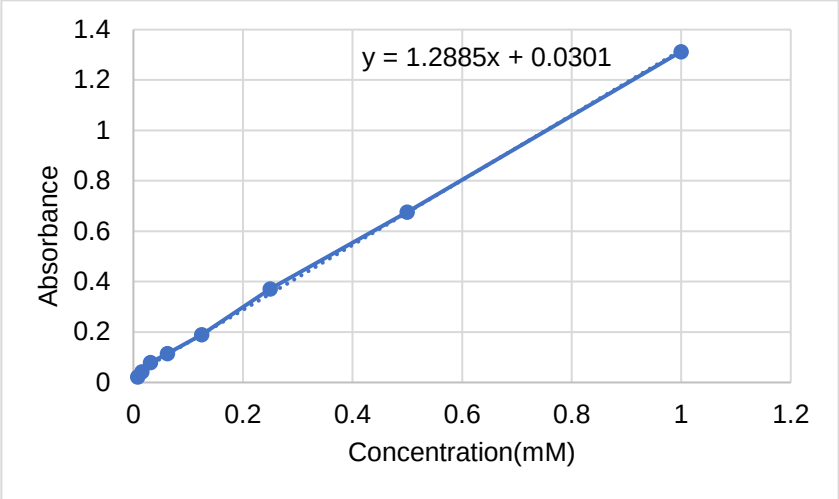

Fig. 3. Standard curve for L-tyrosine production

2.8 Statistical Analysis

Data were analyzed using ANOVA (Analysis of Variance) and student's t-test in SPSS (Statistical Package for the Social Sciences) 26.0. A P-value of less than 0.05 was considered significant. Data are presented as mean +SD (Standard Deviation).

3. RESULTS

3.1 Trypsin Inhibitory Activity Hawari Mae Seed Sample

The 10% extraction of Hawari mae seed extracts of *V. unguiculata* showed the highest trypsin inhibitory activity of 96.03%, while 1.25% concentration showed the least activity of 42.19%. The 10% concentration was chosen for further studies.

3.2 The effect of Temperature on Trypsin Inhibitory Activity

Hawari mae seed extracts of *V. unguiculata* showed the highest trypsin inhibitory activity (95.42±0.006%) at 37°C and the least activity (31.88±0.003%) at 100°C (Table 2).

3.3 The Effect of pH on Trypsin Inhibitory Activity

The Hawari mae seed extract of *V. unguiculata* showed the highest trypsin inhibitory activity (95.71±0.003%) at pH 7.6 and the least activity (53.84±0.003%) at pH 6.4 (Table 3).

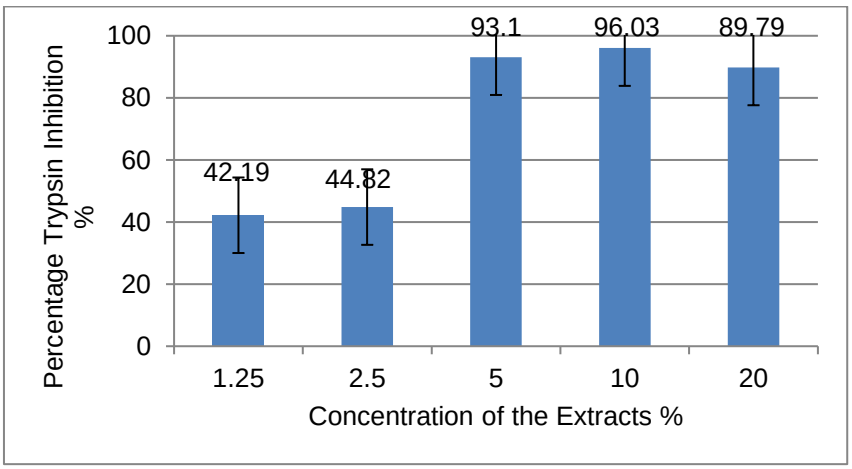

Fig. 4. Percentage of trypsin inhibitory activity of different concentrations of Hawari mae seed extracts of *V. unguiculate*

Table 1. Percentage of trypsin inhibitory activity of Hawari mae seed extracts of *V. unguiculata*

The concentration of the crude extract mg/ml (% w/v)	Average enzyme activity of Hawari mae seed sample (mg of tyrosine/min)	Average enzyme activity of negative control (mg of tyrosine/min)	Percentage of trypsin inhibitory activity (%±SD) of Hawari mae seeds
12.5 (1.25% w/v)	$1.12x10^{-1}$±0.003	$2.13x10^{-1}$±0.01	42.19±0.004
25 (2.5% w/v)	$1.80x10^{-1}$±0.003	$3.43x10^{-1}$±0.004	44.82±0.005
50 (5.0% w/v)	$3.28x10^{-3}$±0.002	$2.68x10^{-1}$±0.005	93.10±0.003
100 (10% w/v)	$5.35x10^{-3}$±0.004	$7.19x10^{-1}$±0.004	96.03±0.005
200 (20% w/v)	$4.32x10^{-3}$±0.003	$2.43x10^{-1}$±0.003	89.79±0.005

Table 2. Percentage of trypsin inhibition activity of Hawari mae seed extract (10%) at different temperatures

Temperature (°C)	Average enzyme activity of Hawari mae seed samples (mg of tyrosine/min)	Average enzyme activity of negative control (mg of tyrosine/min)	Percentage of trypsin inhibitory activity (%±SD) of Hawari mae seeds
0	$1.95x10^{-2}$±0.005	$2.80x10^{-1}$±0.005	85.71± 0.007*
20	$5.36x10^{-3}$±0.006	$3.24x10^{-1}$±0.003	92.65±0.008
37	$6.65x10^{-3}$±0.005	$6.39x10^{-1}$±0.006	95.42±0.006
60	$1.20x10^{-2}$±0.003	$3.55x10^{-1}$±0.005	90.79±0.005
80	$5.87x10^{-3}$±0.002	$3.58x10^{-2}$±0.004	50.00±0.003**
100	$1.23x10^{-2}$±0.002	$2.96x10^{-2}$±0.003	31.88±0.003**
Control	$5.35x10^{-3}$±0.004	$7.19x10^{-1}$±0.004	96.03±0.005

*Significance compared to negative control * P<0.05, ** P<0.001 and *** P<0.0001*

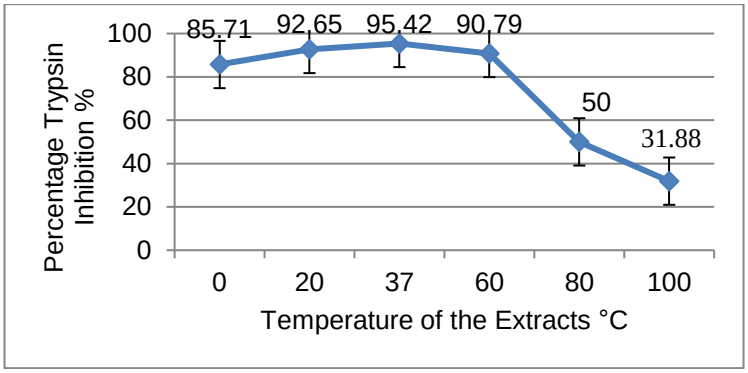

Fig. 5. Percentage of trypsin inhibition activity of Hawari mae seed extract (10%) at different temperatures

Table 3. Percentage of trypsin inhibition activity of Hawari mae seed extract (10%) at different pH values

pH value	Average enzyme activity of Hawari mae seed samples (mg of tyrosine/min)	Average enzyme activity of negative control (mg of tyrosine/min)	Percentage of trypsin inhibitory activity (%±SD) of Hawari mae seeds
5.8	$3.56x10^{-2}$±0.003	$2.07x10^{-1}$±0.002	74.49±0.005*
6.4	$1.03x10^{-1}$±0.002	2.49 $x10^{-1}$±0.004	53.84±0.003*
7	$4.32x10^{-3}$±0.002	3.31 $x10^{-1}$±0.027	92.17±0.003
7.4	$3.54x10^{-3}$±0.001	2.54 $x10^{-1}$±0.019	90.30±0.002
7.6	$6.13x10^{-3}$±0.002	6.10 $x10^{-1}$±0.012	95.71±0.003
8	$1.23x10^{-2}$±0.003	2.94 $x10^{-1}$±0.005	88.72±0.005*
Control	$5.35x10^{-3}$±0.004	$7.19x10^{-1}$±0.004	96.03±0.005

*Significance compared to negative control * P<0.05, ** P<0.001 and *** P<0.0001*

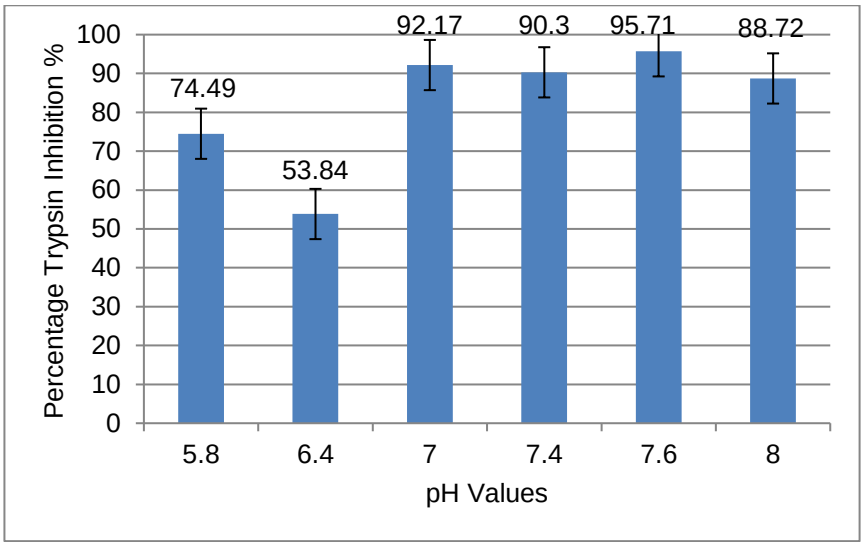

Fig. 6. Percentage of trypsin inhibition activity of Hawari mae seed extract (10%) at different pH values

Table 4. Percentage of trypsin inhibition activity of Hawari mae seed extract (10%) at different metal ion solutions

Metal ion extracts	Average enzyme activity of Hawari mae seed samples (mg of tyrosine/min)	Average enzyme activity of negative control (mg of tyrosine/min)	Percentage of trypsin inhibitory activity (%±SD) of Hawari mae seeds
$FeCl_3$	$2.37x10^{-2}$±0.001	$2.13x10^{-1}$±0.005	79.80±0.001*
$Cu(CH_3COO)_2$	$5.09x10^{-3}$±0.001	$3.64x10^{-1}$±0.003	92.58±0.002
$ZnC_4H_6O_4$	$2.94x10^{-2}$±0.007	$1.43x10^{-1}$±0.002	68.51±0.001**
$BaCl_2$	$6.38x10^{-3}$±0.001	$2.33x10^{-1}$±0.003	88.48±0.002*
NaCl	$1.23x10^{-2}$±0.001	$3.33x10^{-1}$±0.002	89.97±0.002*
Control	$5.35x10^{-3}$±0.004	$7.19x10^{-1}$±0.004	96.03±0.005

*Significance compared to negative control * P<0.05, ** P<0.001 and *** P<0.0001*

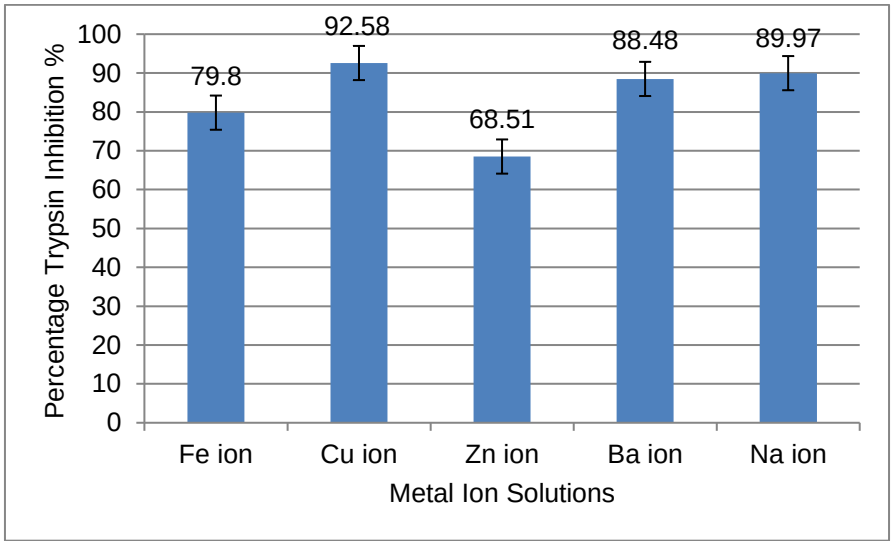

Fig. 7. Percentage of trypsin inhibition activity of Hawari mae seed extract (10%) at different metal ion solutions

3.4 The Effect of Metal ion on Trypsin Inhibitory Activity

Hawari mae seed extract of *V. unguiculata* showed trypsin inhibitory activity ranging from 68.51-92.58%. Cu $(CH_3COO)_2$ ion extract exhibited the highest activity, while $ZnC_4H_60_4$ metal ion extract showed the least (Table 4).

3.5 The Effect of Detergents, Oxidizing Agents, and Reducing Agents on Trypsin Inhibitory Activity

Table 5. Percentage of trypsin inhibition activity of Hawari mae seed extract (10%) with detergents, oxidizing, and reducing agents

Detergent/ Oxidizing agent/Reducing agent	Average enzyme activity of Hawari mae seed samples (mg of tyrosine/min)	Average enzyme activity of negative control (mg of tyrosine/min)	Percentage of trypsin inhibitory activity (%±SD) of Hawari mae seeds
Detergent-Triton X-100	$2.50x10^{-3}$±0.001	$2.47x10^{-1}$±0.002	90.51±0.001
Oxidizing agent-DMSO	$4.32x10^{-3}$±0.001	$2.18x10^{-1}$±0.001	88.74±0.002*
Reducing agent-Dithiotretol	$4.31x10^{-2}$±0.003	$2.04x10^{-1}$±0.001	70.40±0.004**
Control	$5.35x10^{-3}$±0.004	$7.19x10^{-1}$±0.004	96.03±0.005

*Significance compared to negative control * P<0.05, ** P<0.001 and ***P<0.0001*

4. DISCUSSION

The study aimed to discover serine protease inhibitory activity in *Vigna unguiculata* ssp sesquipedalis (Hawari mae) seed extract. Screening assays were conducted to determine trypsin inhibitory activity and identify the concentration with the highest activity. The extract was then characterized, and the effect of physio-chemical parameters on the trypsin inhibitors was evaluated. The study utilized the trypsin assay, which is a procedure that involves the use of trypsin, a proteolytic enzyme that breaks down proteins into smaller fragments such as peptides and amino acids [7]. Casein is a milk protein that is broken down by the enzyme trypsin. It was used in the study as the substrate for trypsin activity.

In Hawari mae seeds of *V. unguiculata*, the highest trypsin inhibitory activity was found at 10g/ml concentration while the lowest was observed at 1.25g/ml concentration. The protease inhibitors in seed extract become low when the concentration of seed extract is low. But when the seed extract concentration is high the activity exhibited was comparatively lower, this may be due to the amount of different bioactive compounds contains in crude seed extract also high, and, therefore trypsin inhibitors may get inhibited by various other bioactive compounds present. It may be the reason for lower enzyme inhibitory activity seen in 20g/ml concentration than in 10g/ml concentration. 20g/ml concentration was found to be the most effective for seeds of black gram (*Vigna mungo*) in Sri Lanka. The present study used distilled water to extract trypsin inhibitors.

The assay to determine the effect of temperature on trypsin inhibitory activity showed that Hawari mae has the highest percentage of trypsin inhibition activity at 37°C. The enzyme inhibition activity was found to be highest at 37°C and gradually decreased from 37°C to 100°C. Therefore, Hawari mae seed samples can be used as sources to isolate therapeutics for the treatment of human diseases due to their high trypsin inhibition activity at 37°C. The study showed that the highest trypsin inhibitory activity was observed in Hawari mae seed extract at pH 7.6. Similar enzyme inhibition was observed at pH 7.0 and 7.4, while the lowest inhibition was seen at pH 6.4. Other studies also suggest that trypsin inhibitors in various seed samples exhibit optimum activity at a wide range of pH values.

The study showed that the presence of metal ions reduced the trypsin inhibition activity on the Hawaii mae seed sample. Fe3+ ions reduced the inhibition activity moderately, while Na+ ions reduced it significantly compared to the control. The impact of different metal ions on the trypsin inhibitory activity in seeds of various *Vigna*

species is different. The study on detergents showed that Triton X-100 significantly decreased the inhibition activity in the Hawari Mae seed variety. Tritons are non-ionic detergents that can denature proteins. Similar observations were made in the impact of different detergents on trypsin inhibitory activity in *Vigna mungo* seeds [8]. Further studies on the interaction between trypsin inhibitors and detergents may be important for drug development from plant trypsin inhibitors. DMSO in Hawari mae seed extract showed increased enzyme inhibition activity in a study on the effect of oxidizing agents on trypsin inhibition. Hydrogen peroxide and dimethyl sulfoxide reduced trypsin inhibitory activity of distilled water extract of *Vigna mungo* seeds [9]. Dithiothreitol increased enzyme inhibition activity in Hawari mae seed extract. Beta-mercaptothion gradually decreased inhibitory activity in *Vigna mungo* [9].

The study suggests that the impact of physio-chemical factors on trypsin inhibitory activity varies between closely related subspecies. Further purification and isolation of active compounds should be preceded by separate characterization studies of seed samples [10-13].

5. CONCLUSION

The results of the present study revealed that the seed of a local variety of *V. unguiculata* exerts remarkable trypsin inhibitory activity. Therefore, it can be considered a promising candidate for the discovery of new serine protease inhibitors with therapeutic value in the future. The information on the effect of different physio-chemical parameters will be useful for further studies on the purification and isolation of trypsin inhibitors from the seed of the tested local cultivar of *V. unguiculata.*

6. FURTHER STUDIES AND RECOMMENDATIONS

Further studies can be carried out on Hawari mae for purification and isolation of active protein fractions from crude protein extract of Hawari mae seed sample should be carried out for further studies to isolate active agents for the development of new medicines.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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