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# *Vallaris solanacea:* A rich source for biopharmaceuticals

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**Abstract**
Plant based medications had served from the earliest period of the human civilization as the most important therapeutic weapon available to man to fight various human and animal diseases.
*Vallaris solanacea* (Roth) Kuntze (Family: Apocyanacea) locally named as Agarmoni, bread flower is a tall climbing shrub. It is distributed throughout India and cultivated as an ornamental plant for its white fragrant flowers traditionally used against ring worms and skin infections. The plant extract contains reducing sugars, tannins, saponins, gums, steroids, alkaloids and glycosides and is medicinally important with its cytotoxic, antioxidant, antinociceptive, antimicrobial, analgesic, anti-inflammatory and antidiarrhoel activities. As an accumulators of pollutants it has an an important role in phytoremediation.
Fascinated by the fact that this plant is a natural source of pharmaceuticals, in the present study a proteolytic enzyme has been extracted from the latex of the plant *Vallaris solanacea.* Preliminary investigations were carried out on this protease which included the effect of time, enzyme concentration, pH, temperature, activators and inhibitors on the caseinolytic activity of crude protease. Stability towards temperature and pH were also checked. Further a cysteine protease was purified from crude latex of *Vallaris solanacea* by fractionation with ammonium sulphate, ion exchange and gel chromatography. The enzyme was purified to a state of near homogeneity as checked by polyacylamide gel electrophoresis at pH 8.3. The proteolytic enzyme present in the latex of *Vallaris solanacea* is referred to as solanain. Its antimicrobial properties were studied. Thus this information emphasizes that *Vallaris* can be explored for new drugs.

**Keywords:** *solanacea*, glycosides, antinociceptive, pharmaceuticals, phytoremediation caseinolytic activity

## Introduction
Nature holds a precious gift of vegetation. Plants are a goldmine of novel chemicals; many modern drugs have been developed from them. There are more than 2, 70,000 higher plants existing on this planet. But so far less than 10% of recorded flora has been explored phytochemically and evaluated for various biological activities [1]. While vast majority of the plant resource is waiting for discovery, these plant based traditional medical systems continue to play an essential role in health care, with about 80% of the world's inhabitants relying mainly on traditional medicines for their primary health care [2]. The world is now looking towards India for new drugs to manage various challenging diseases due to its rich biodiversity of medicinal plants and abundance of traditional knowledge such as Siddha, Ayurveda and Unani [3]. All plant parts contain moderate amount of bioactive ingredients with massive pharmacological properties. Among these, plant latex occupies an important place. Latex has been reported to occur in 12000 plant species belonging to 900 genera.
Latex is an inducible defense system, mobilized and transported to the site of damage immediately after the damage. It is a protein rich multi-component milky sap secreted by the specialized plant cells called laticifers. Plant latex is a good source of secondary metabolites such as alkaloids, tannins, saponins, phenolic components with potent antibacterial, antiviral, antifungal bioactivities. These bioactive constituents of latex also exhibits potent pharmacological activities like anti-tumors, anti-angiogenic, anti-diabetic, antiproliferative, anti-arthritis, anti-inflammatory, antioxidant, antiasthmatic, anti-fertility, analgesic, immune-modulation, wound healing, cytotoxicity, vasodilatory activities.
Proteases are important component of latex. Proteases have a first place in the world market of enzymes, estimated at ~US$3 billion [4]. Proteases are enzymes able to hydrolyze peptide bonds. They can act near the ends of polypeptide chains (exopeptidases) or within them (endopeptidases) [5]. Proteases have been identified and studied from the latex of several plant families such as Asteraceae, Caricaceae, Moraceae, Asclepiadaceae, Apocynaceae and Euphorbiaceae [6].

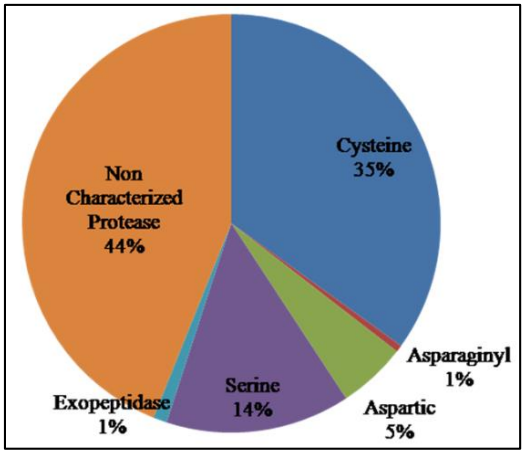


**Fig 1:** Plant proteases.

Most plant-derived proteases have been classified as cysteine proteases and more rarely as aspartic proteases [7]. They have been used for the treatment of cancer [8, 9], as antitumorals [10, 11], for digestion disorders [12, 13] and immune-modulation problems [14, 15]. A good example is bromelain, derived from pineapple, which has been shown to be capable of preventing edema, platelet aggregation and metastasis due to its capacity of modifying cell surface structures by peptide cleavage [16].

*Vallaris solanacea* is commonly called bread flower. It belongs to family Apocyanaceae (Oleander family). Apocynaceae, commonly called the dogbane family includes herbs, shrubs, stem succulents, trees and vines. They are natively found in America, Asia, Europe and Australia tropical or subtropical regions. Bread flower is a hairy, climbing shrub. Stems are covered with a gray, spotted bark, emitting white latex when wounded. Leaves are elliptic-oblong (5 x 2 inches) and oppositely arranged. White, bowl-shaped flowers, fragrant, 1 inch across, occur in 3-6 flowered cymes in leaf axils. The five sepals (2.5 mm long) are narrow and pointed. The five petals are circular in shape. Recently, the antiulcer and antioxidant activity of methanolic extract from stem of the plant *Vallaris solanacea* was evaluated using various experimental models in Wister albino rats. Phytochemical analysis of the dried root of *Vallaris solanacea* indicated the presence of reducing sugars, tannins, saponins, gums, steroids, alkaloids and glycosides.

Taking into consideration, the increasing demand for proteases and the need for economical production of commercially useful industrial proteases from novel sources, the present study was carried with an objective to perform preliminary investigations on proteolytic activity of *Vallaris solanacea.*

## Materials and methods

### Latex collection and enzyme isolation

The present work started with latex collection from the plant *Vallaris solanacea*. The latex was a white thick fluid with pungent odour. The freshly collected latex was diluted with 5 volumes of ice cold distilled water, centrifuged at 10000 rpm for 20 minutes in high speed refrigerated centrifuge and the clear, colourless supernatant was used in the experiment.

### Protease activity

The assay of protease activity was according to the method described by Kunitz [17]. The TCA soluble peptides were estimated by the method of Lowry [18] with a modification using iodate method for elimination of interference by thiol compounds [19].

### Protein determination

Protein content of the latex was determined by the method of Lowry *et al*. (1951) [13] using crystalline bovine serum albumin as the standard.

### Preliminary investigations on crude solanain

Preliminary investigations on crude solanain were analyzed. The investigations included the effect of time, enzyme concentration, pH, temperature, activators and inhibitors on the caseinolytic activity of crude protease. Stability towards temperature and pH were also checked.

### 2.6 Purification of Protease

#### 2.6.1 Ammonium sulphate precipitation

To 25 ml of freshly collected latex, 5 volumes of 0.1M glycine-NaOH buffer (pH 9.0) was added and centrifuged to remove the gums. The supernatant was subjected to 30% ammonium sulphate precipitation and kept overnight at 4°C. After centrifugation at 10000 rpm for 15 min the pellet without any activity was discarded and supernatant was subjected to 50% saturation with ammonium sulphate precipitation and kept overnight at 4°C, centrifuged at 10000 rpm for 15 min. The pellet obtained on centrifugation of 30-50% saturation with ammonium sulphate was dissolved in 0.1 M tris-HCl buffer, pH 8.6 and 1mM PCMB. The enzyme solution was dialyzed overnight against 1 litre 0.1M tris-HCl buffer (pH 8.6) with 3 changes for every 8 hrs using dialysis membrane of 12-14 kDa cut off limits. The dialyzed enzyme solution was centrifuged at 10000 rpm and supernatant was lyophilized and fractionated on DEAE cellulose.

#### 2.6.2 Chromatography of protease on DEAE cellulose

The starting buffer used for equilibration is 0.4M NaCl in 0.1M tris-HCl buffer (pH 8.6). Ammonium sulphate precipitated protein in 5.0 ml of the starting buffer was applied to the DEAE cellulose column (1.2 cm x 30 cm) with a gel bed volume of approximately 75 ml. After washing with 120 ml of the starting buffer the elution was performed with 120 ml of 0.4M NaCl in 0.1M tris HCl buffer (pH 8.6). A flow rate of 30 ml/hr was monitored using peristaltic pump. 5 ml fractions were collected. The fractions obtained were assayed for their caseinolytic activity and protein content.

#### 2.6.3 Gel filtration chromatography

The lyophilized sample obtained from DEAE-cellulose chromatography was dissolved in 2.0 ml of 0.1M Tris-HCl buffer (pH 8.6) and applied on Sephadex G-100 column (1.4 x 40 cm) equilibrated with the same buffer. The elution was carried out with a flow rate of 20 ml/hr and fractions of 3.0 ml were collected using the same buffer. Each fraction was analyzed for protein at 280 nm and assayed for caseinolytic activity. The caseinolytic activity containing fractions were pooled and lyophilized.

#### 2.6.4 Native-Polyacrylamide Gel Electrophoresis

The procedure used was basically as that described by Reisfield [20] and followed by Gabriel [21]. Genei slab gel electrophoresis apparatus was used for polyacrylamide gel electrophoresis.

### Statistical analysis

All assays in this study were carried out in triplicates and the results are presented as the mean of three replicates ± SD. The data of estimates of protease activity was subjected to one way analysis of variance (ANOVA) using Microsoft Excel 2007 to access the significance of the various effects.

## Results and discussion

### Effect of time on the caseinolytic activity

The results show that the activity is not only higher but also enzyme retained activity through a longer period of incubation when β-mercaptoethanol was added to the reaction mixture. Addition of EDTA alone has also brought about slight activation

of the enzyme. Significant differences were observed in the activity (*SS*-11093.52, *df*-4, *MS*-2773.38, *F*-10.564, *P*<0.001, *F crit*-3.055568) of crude solanain with time.

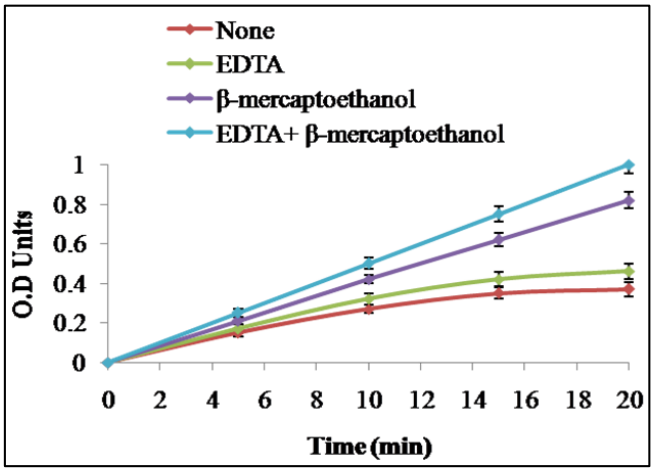


**Fig 2:** Caseinolytic activity of crude Solanain with varying time intervals (The values represent the mean of three replicates ± SD; P<0.001).

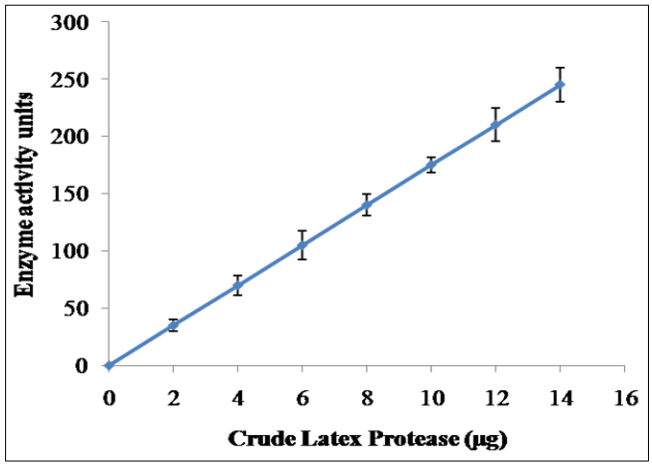


**Fig 3:** Effect of varying concentrations of crude Solanain on casein **(**The values represent the mean of three replicates ± SD; (P<0.001).

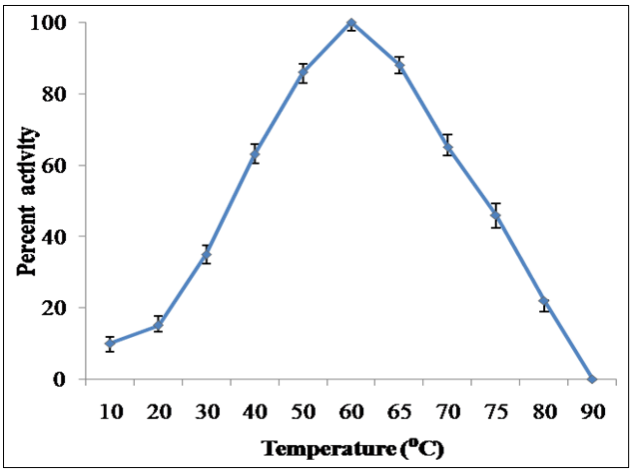


**Fig 4:** Effect of temperature on the caseinolytic activity of crude Solanain (The values represent the mean of three replicates ± SD; P<0.001).

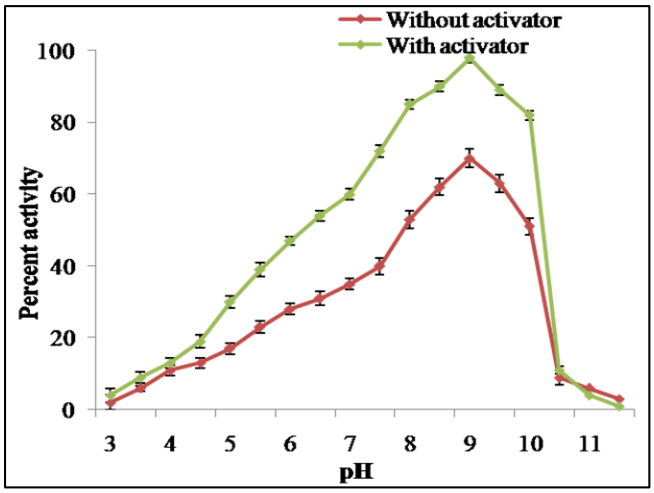


**Fig 5:** Effect of pH on caseinolytic activity of crude Solanain (The values represent the mean of three replicates ± SD (P<0.001).

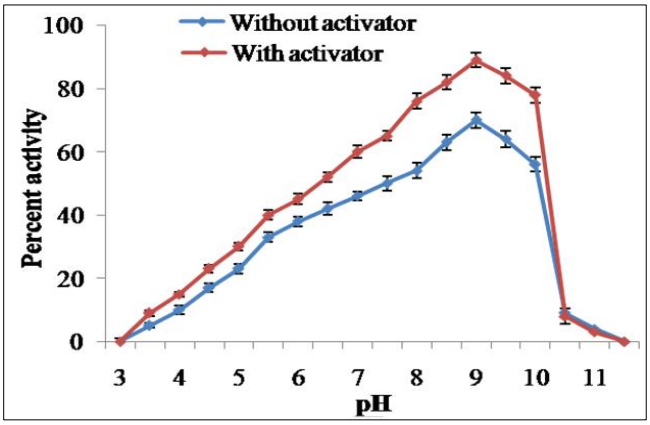


**Fig 6:** Effect of pH on Hemoglobin activity of crude Solanain (The values represent the mean of three replicates ± SD (P<0.001).

**Effect of enzyme concentration**

The effect of varying amounts of crude solanain on the caseinolytic activity was tested. The results show that the activity was linear over a considerable range of enzyme concentration (2-14 µg).

**Effect of temperature**

The activity was maximum at 60°C and the decrease in the activity with increase in temperature beyond 65°C was rather slow with complete inactivation taking place at as high as 90°C temperature. At 75°C the activity was 46% of the optimal (60°C). Significant differences were observed in the activity (*SS*-35183.57, *df*-10, *MS*-3518.357, *F*-362.9128, *P*<0.001, *F crit*-2.296696) of crude solanain with varying temperatures.

**Effect of pH on the activity of crude Solanain**

With both casein and haemoglobin as substrates, the optimum pH was found to be 9.0. In the presence of β-mercaptoethanol and EDTA with casein and hemoglobin as substrates a broad optimal pH range was noticed between 8.0 and 10.0 with maximum activity at pH 9.0. The curve is a typical bell shaped. With casein as the substrate, significant differences were observed in the activity (*SS*-24824.97, *df*-15, *MS*-1654.998 *F*-5.357606, *P*<0.001, *Fcrit*-2.352223) and with haemoglobin as the substrate,

significant differences were observed in the activity (*SS*-23342.47, *df*-15, *MS*-1556.165 *F*-21.45509, *P*<0.001, *F crit*-2.352223) of crude enzyme.

**Effect of activators**

Of the thiol compounds tested, cysteine produced maximum activation followed by β-mercaptoethanol. Glutathione, thioglycolic acid and dithiothreitol produced 85%, 79% and 42% activation respectively. Significant differences were observed in the activity (*SS*-6802.991, *df*-4, *MS*-1700.748, *F*-97.4566, *P*<0.001, *F crit*-3.47805) of crude solanain with various activators.

**Table 1:** Effect of thiol compounds on the activity of crude Solanain.

| SH compound | Final concentration (mM) | Activation (%) |
|---|---|---|
| Cysteine | 10 | 100.0 |
| β-mercaptoethanol | 10 | 99.8 |
| Glutathione | 10 | 85.5 |
| Thiglycolic acid | 10 | 79.2 |
| Dithiothreitol | 10 | 41.6 |

**Effect of inhibitors**

Significant differences were observed in the activity (*SS*-402.3947, *df*-10, *MS*-402.7394 *F*-7.581517, *P*<0.001, *F crit*-2.29669) of crude solanain with various inhibitors during inhibition. Significant differences were observed in the activity (*SS*-44507.12, *df*-10, *MS*-4450.712, *F*-268.8218, *P*<0.001, *F crit*-2.296696) of crude solanain with various inhibitors during reactivation. Among the SH reagents tested, iodoacetate and N-bromosuccinimide caused complete and irreversible inactivation. PCMB and DTNB produced total inhibition of the enzyme activity but the inhibition caused by them could be reversed by β-mercaptoethanol. Phenyl methyl sulphonyl fluoride and N-ethylmaleimide caused partial inhibition of the enzyme activity. Heavy metal ions such as $Hg^{2+}$, $Zn^{2+}$, $Fe^{2+}$, $Cd^{2+}$ and $Cu^{2+}$ produced complete inhibition. The inhibition caused by $Hg^{2+}$ and $Zn^{2+}$ could be completely reversed by the addition of both β-mercaptoethanol and EDTA. However, the inhibition caused by $Cu^{+2}$, $Fe^{+2}$ and $Cd^{+2}$ were only partially reversible by β-mercaptoethanol and EDTA.

**Table 2:** Effect of inhibitors on the activity of crude Solanain

| Inhibitor added | Final Concentration | Inhibition (%) | Reactivation (%) |
|---|---|---|---|
| **SH – agents** | | | |
| 5, 5'-dithio-bis- | $10^{-3}$M | 100 | 98.6 |
| (2-nitrobenzoic acid) Iodoacetate | $10^{-3}$M | 100 | 0.0 |
| N-bromosuccinimide | $10^{-3}$M | 100 | 0.0 |
| N-Ethylmaleimide | $10^{-3}$M | 62 | 80.0 |
| p-chloromercury benzoate | $10^{-5}$M | 100 | 100.0 |
| Phenylmethyl sulfonyl fluoride | $10^{-3}$M | 68 | 100.0 |
| Metallic agents | | | |
| Cadmium sulphate | $10^{-3}$M | 100 | 72.3 |
| Copper sulphate | $10^{-3}$M | 100 | 52.5 |
| Ferrous sulphate | $10^{-3}$M | 100 | 86.2 |
| Mercuric chloride | $10^{-5}$M | 100 | 100.0 |
| Zinc sulphate | $10^{-3}$M | 100 | 100.0 |

The values represent the mean of three replicates ± SD (P<0.001, ANOVA)

**Stability towards pH**

The pH stability of PCMB uninhibited enzyme was also tested at 4°C. PCMB uninhibited enzyme retained full activity against casein when kept at 4°C for 24 hours in the pH range from 4.0 to 10.0 with as much as 35% activity remaining even at pH 11.9. However, when the PCMB uninhibited enzyme was kept at 30°C, maximal stability was seen only over a narrow pH range of 7.0 to 8.0. At alkaline pH the activity loss was maximal with only 25 to 40% activity remaining after 24 hours in the pH range of 10.0 to 11.5. Nevertheless, the PCMB inhibited enzyme was quite stable in the pH range of 5.0 to 11.9 for 30 minutes at 30°C and 6.0 to 10.0 for 24 hours. The loss of activity of PCMB inhibited enzyme at pH 11.0 to 11.5 over a period of 24 hours at 30°C was only 20-25%, which was far less than that of PCMB uninhibited enzyme. Significant differences were observed in the activity (*SS*-25906.35, *df*-11, *MS*-2355.123, *F*-166.6524, *P*<0.001, F *crit*-2.216309) of crude solanain over a pH range of 2.0 to 11.9 at 30ºC for 30 minutes and (*SS*-33748.98, *df*-11, *MS*-3068.089, *F*-375.3397, *P*<0.001, *F crit*-2.216309) for 24 hours with PCMB uninhibited enzyme. Significant differences were observed in the activity (*SS*-20168.9, *df*-10, *MS*-1833.536, *F*-87.78502, *P*<0.001, *F crit*-2.216309) of crude solanain over a pH range of 2.0 to 11.9 at 30ºC for 30 minutes and (*SS*-32555.22, *df*-11, *MS*-2959.565, *F*-186.9199, *P*<0.001, *F crit*-2.216309) for 24 hours with PCMB inhibited enzyme.

**Table 3:** Effect of pH on the stability of crude Solanain

| pH | Activity (%) | | | |
|---|---|---|---|---|
| | PCMB uninhibited | | PCMB inhibited | |
| | 30 min | 24 hrs | 30 min | 24 hrs |
| 2.0 | 10.5 | 0.0 | 16.1 | 0.0 |
| 3.0 | 39.2 | 28.6 | 69.3 | 42.2 |
| 4.0 | 61.4 | 34.2 | 82.2 | 67.0 |
| 5.0 | 79.8 | 55.8 | 100.0 | 81.4 |
| 6.0 | 100.0 | 76.0 | 100.0 | 96.5 |
| 7.0 | 100.0 | 98.0 | 100.0 | 100.0 |
| 8.0 | 100.0 | 96.0 | 100.0 | 100.0 |
| 9.0 | 100.0 | 65.3 | 100.0 | 100.0 |
| 10.0 | 80.4 | 41.8 | 100.0 | 100.0 |
| 11.0 | 65.6 | 32.0 | 100.0 | 80.3 |
| 11.5 | 63.0 | 25.1 | 100.0 | 74.8 |
| 11.9 | 55.0 | 7.6 | 92.4 | 42.2 |

The values represent the mean of three replicates ± SD (P<0.001, ANOVA)

**Stability towards temperature**

At 70°C the stability of the crude protease with and without the inhibitor was studied. The PCMB uninhibited enzyme retained full activity against casein for 2 hours at 50°C and even at 24 hours, 45% of the activity remained. At 70°C, however, 80% of the activity was lost in 20 minutes with complete inactivation taking place in 2 hours whereas only 10% of the activity of PCMB treated enzyme was lost in 20 minutes at 70°C and the loss of activity thereafter was only 5% in the next 100 minutes. Significant differences were observed in the activity (*SS*-27102.38, *df*-2, *MS*-13551.19, *F*-29.59288, *P*<0.001, *F crit*-3.554557) of crude solanain when studied at pH 9.0 with 0.05M glycine-NaOH buffer at 50°C and 70°C.

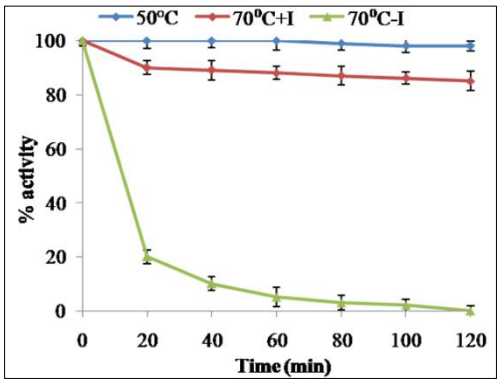


**Fig 7:** Effect of pre-incubation time on the activity of crude Solanain at temperature 50ºC and 70ºC. The values represent the mean of three replicates ± SD (P<0.001, ANOVA)

**Stability towards denaturants**

The crude enzyme was exposed to various denaturants for 30 minutes at the concentrations indicated and their caseinolytic activities were tested after dilution with assay mixture. Exposure of the enzyme to 70% methanol and 15% dimethyl sulfoxide caused little or no inactivation of the enzyme. Exposure to 5% TCA and 1% SDS produced total irreversible inactivation of the enzyme. The enzyme retained full activity in 8M urea. 70% alcohol, 40% ethylene glycol and 30% dioxane produced 28%, 13% and 65% loss in the enzyme activity respectively. Significant differences were observed in the activity (*SS*-40011.87, *df*-7, *MS*-411.9626, *F*-411.9626, *P*<0.001, *F crit*-2.657197) of crude solanain with various denaturants.

**Table 4:** Effect of various denaturants on the activity of crude Solanain

| Denaturant | Final concentration | Activity (%) |
|---|---|---|
| Alcohol (90% ethanol) | 70% | 72.4 |
| Dimethyl sulfoxide | 15% | 100.0 |
| Dioxane | 30% | 35.2 |
| Ethylene glycol | 40% | 87.3 |
| Methanol | 70% | 98.0 |
| Sodium dodecyl sulphate | 1% | 0.0 |
| Trichloro acetic acid | 5% | 0.0 |
| Urea | 8 M | 100.0 |

The values represent the mean of three replicates ± SD (P<0.001, ANOVA)

**Discussion**

The latex of *Vallaris solanacea* has high proteolytic activity, the activity being comparable to those of other known latex proteases such as papain, ficin, bromelain, asclepain and calotropain etc [20, 21]. The specific activity of the crude latex towards casein at pH 9.0 is 87.5. A study of time course of activity revealed that the enzyme is dependent on the presence of a free SH group for the activity. The pH optima observed with crude enzyme towards casein and haemoglobin as substrates was 9.0. This observation suggests the possibility of existence of one component with proteolytic activity. The temperature for optimum activity was as high as 60°C and is comparable to other plant proteases. Stability studies showed that proteases may be stored in reversibly inhibited state. Resistance towards heat denaturation of the enzyme is remarkable and resembles other plant proteases. Preliminary studies on the protease activity of the crude latex revealed that the enzyme resemble other plant proteases in their a) requirement of a reducing agent for maximal activity; b) Inhibition by several conventional agents such as PCMB; and c) presence of an active SH group. The pH and temperature activity curves and other stability studies suggest the possible presence of one proteolytic enzyme in the latex of *V. solanacea*. Among the thiol compounds studied on the activation of PCMB inhibited solanain; maximum activation was observed with cysteine, followed by β-mercapethanol. Glutathione, thioglycolic acid and dithiothreitol produced 87%, 85% and 60% activation respectively. A study of the effect of various sulfhydryl reagent and metal ions revealed that the activity of solanain was completely inhibited by sulfhydryl reagents such as PCMB, DTNB and iodoacetate and also heavy metal ions like $Hg^{2+}$, $Zn^{2+}$, $Fe^{2+}$, $Cu^{2+}$ and $Cd^{2+}$. The inhibition caused by PCMB and DTNB was completely reversible by the addition of β-mercaptoethanol and EDTA. However, iodoacetate and N-bromosuccimide produced irreversible inactivation of the enzyme. The inhibition by $Hg^{2+}$ and $Zn^{2+}$ was completely reversible with β-mercaptoethanol and EDTA. The inhibition caused by $Cu^{2+}$, $Fe^{2+}$ and $Cd^{2+}$ was reversed by 89%, 84% and 80% respectively with β-mercaptoethanol and EDTA.

**Purification of Protease**

Fractionation of the proteolytic components of solanain was done in January, April and June months. Essentially, similar chromatographic patterns were obtained without any seasonal variations. PCMB inhibited proteases are more stable than the uninhibited enzymes and the inhibition of the enzyme activity by PCMB was completely reversible. For this reason, purification of protease was carried out as its PCMB-inhibited derivative, a method that was used by several workers [22, 23, 24]. For fractionation of the enzyme protein, PCMB treated protease was adsorbed at pH 8.6 (0.1 M tris-HCl buffer, pH 8.6) on DEAE cellulose in a column and elution of the enzyme from the column was carried out using a linear gradient of sodium chloride (0.4M).

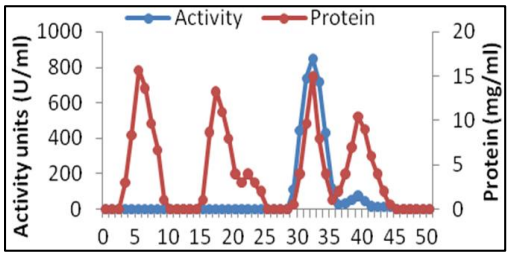


**Fig 8:** Elution Profile of Solanain on DEAE Cellulose

Among the four peaks appeared, peaks III showed maximum protease activity and IV showed very little protease activity. Peak

I and II has no caseinolytic activity. Peak III showing caseinolytic activity appeared at 0.34 M NaCl. Peak IV with low protease activity was ignored and peak III with high activity was selected for further purification.

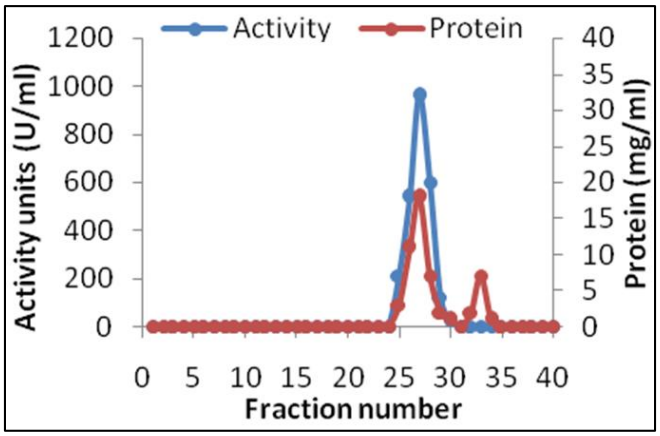


**Fig 9:** Elution Profile of Solanain on Sephadex G-100 Gel Chromatography

**Table 5:** Purification of Solanain from Latex of *V.solanacea*

| Purification step | Total activity (U) | Total protein (mg) | Specific activity (U/mg) | Yield of activity (%) | Purification (Fold) |
|---|---|---|---|---|---|
| Crude extract | 9850 | 112.5 | 87.5 | 100 | 1.00 |
| Ammonium sulfate precipitation | 8630 | 51.2 | 168.6 | 87.6 | 1.93 |
| Ion exchange chromatography (DEAE cellulose) | 7960 | 21.3 | 373.7 | 80.8 | 4.27 |
| Gel filtration chromatography (Sephadex G-100) | 7552 | 17.4 | 434.0 | 76.6 | 4.96 |

From the electrophoretic pattern, it is evident that the crude and ammonium sulphate fractionation contain many protein bands and were not properly resolved. Ion exchange chromatographic fraction showed 2 distinct protein bands, whereas gel filtration distinct chromatographic fraction showed a single protein band corresponding to single peak of enzyme activity observed in gel filtration elution profile. It indicates that protein was purified to apparent homogeneity.

## Conclusion

The latex of *Vallaris solanacea* has high proteolytic activity, the activity being comparable to those of other known plant latex proteases. Preliminary investigations on crude solanain were conducted. The investigations included the effect of time, enzyme concentration, pH, temperature, activators and inhibitors on the caseinolytic activity of crude protease. Stability towards temperature and pH were also checked.

## Acknowledgement

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