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IDENTIFICATION OF PLANT EXTRACTS CONTAINING TRYPSIN INHIBITOR

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Abstract: **Plant Protease Inhibitors (PPIs) are widely distributed in plants and plays a role in protecting plants from pest attack. In this study we screened plants to identify protease inhibitors against trypsin. Seeds/leaves were homogenized in bicarbonate buffer pH 9.0 (1ml/g tissue). The homogenate was centrifuged at 9,500 x g for 10 minutes at 4^0 C. The supernatant containing the soluble proteins was used for protease inhibition assay using Nα-Benzoyl-DL-Arginine-P-Nitro Anilide (BAPNA) as substrate. Out of the 20 plants screened 11 plants showed greater than 50% inhibition towards trypsin and out of these for six plants, this is the first report of presence of trypsin inhibitor. The extract from the leaves of *Dalbergia latifolia* showed the highest inhibition of 85.09±0.4%. The other plant extract showed greater than 50% inhibitory activity towards trypsin are *Nephelium lappaceum* (83.45±0.05%), *Cochlospermum religiosum* (79.23±0.04%), *Anacardium occidentae* (79.23±0.04%), *Samanea saman* (78.27±0.08%), *Mucuna pruriens* (71.34±0.06 %), *Psidium gujava* (69.20±0.09%), *Alpinia calcarata* (67.25±0.12%), *Syzigium cumini* (61.10±0.26%), *Gliricidia sepium* (56.90±0.09%) and *Pterocarpus santalinus* (52.49±0.01%). To our knowledge no trypsin inhibitor was reported from *Dalbergia latifolia, Cochlospermum religiosumi, Psidium gujava, Alpinia calcarata, Gliricidia sepium* and *Pterocarpus santalinus*. As lepidopteran larval gut is rich in trypsin-like serine proteases, identification of PPIs against trypsin will be helpful in utilizing them for the controlling the larvae of lepidopteran pests, in addition to other uses of trypsin inhibitors.**

Keywords: **Plant protease inhibitors, Trypsin inhibitors, *Dalbergia latifolia, Cochlospermum religiosum*.**

I. INTRODUCTION

Proteases are protein hydrolyzing enzymes which are abundant in living cells and perform many important biological roles. They are necessary for the normal physiological functions of the body by controlling large number of physiological processes such as cell proliferation, cell cycle progression, cell death, DNA replication, haemostasis, immune response, wound healing and tissue remodeling [1]. Thus appropriate control of the action of these proteases is necessary, and is achieved through many ways such as different compartmentalization of the substrates and enzymes, difference in pH of enzyme action from that of surroundings, and the action of specific inhibitors [2]. Protease inhibitors are molecules that inhibit the protease and are found in animals, plants and microorganisms [3].

Plant protease inhibitors (PPIs) are defense proteins which protect plants from insect attack and mainly found in Leguminosea, Solanaceae, and gramineae [4]. As early as 1947, Mickel and Standish showed that some insect larvae were not able to develop normally on soybean products [5]. Later Lipke et al showed that the trypsin inhibitors present in the soybean were lethal to the larvae of flour beetle, *Tribolium confusum* [6].Following these early studies, several plant species have been reported to contain protease inhibitors in leaves, flowers, seeds and tubers as their defence tools against insect attack [7], [8], [9]. Plant protease inhibitors are grouped primarily as serine, cystein, aspartic or metallo protease inhibitors based on the active amino acid in their reaction center [10]. Out of these, large numbers of PPIs are directed towards serine and cysteine proteases [11], [12] and very few are known for aspartic and metallo-proteases [13], [14]. Proteases present in the Coleopteran and Hemipterans belongs to Cystein proteases, whereas in Lepidopterans, Orthopterans and Dipterans belong to serine proteases [15], [16].

Serine protease inhibitors are classified into 8 families based on the homology in their primary sequence, active site, the enzyme on which they act and their distribution in the plant kingdom [17]. They are Bowman-Birk, Kunitz, PotatoI, PotatoII, Cucurbit, Cereal super-family, Ragi AI. and Thaumatin-PR like families. Out of these the Kunitz type and Bowman-Brik families are best characterized. The major groups of serine proteases include trypsin-like, elastase-like, and chymotrypsin-like proteases. Over the past decade, large number of trypsin inhibitors were isolated and purified from different tissues like seeds, leaves, fruits and tubers of plants of several families [18], [19], [20], [21]. Serine protease inhibitors have anti-nutritional effects against several Lepidopteran insect species [22]. PPIs reduce the availability of amino acids required for the growth and development of the larval pest [23]. From the seeds of *Plathymenia foliolosa*, a 19 kDa trypsin inhibitor (PFTI) was isolated and it exhibits significant inhibitory activity against larval midgut proteases of *Anagasta kuehniella* and *Diatraea saccharalis* [24]. Bowman-Brick type trypsin inhibitor was identified from the mechanically wounded leaves of Alfalfa by Brown and Ryan [25]. Several trypsin and chymotrypsin inhibitors were reported from the seeds of *Vigna unguiculata* (Cowpea) [26]. The complete amino acid sequences of the trypsin and chymotrypsin inhibitor were done from cowpea seeds by Mohry and Ventura [27]. Hilder *et al*. reported the protein and cDNA sequences of Bowman Brick protease inhibitor from the cowpea [28].

Apart from the defensive role in the pest control, there are many applications for protease inhibitors (PIs) in various fields such as health and medicines. Wang and Ng reported the antifungal activity from the 20.5kDa Kunitz trypsin inhibitor from the roots of *Pseudostellaria hetrophylla* [29]. Koo *et al*., purified and characterized three Bowman-Brick trypsin inhibitors from the seeds of *Dolichos lablab* and they observed the preventive effects of these trypsin inhibitors on *Pseudomonal* elastase induced septic hypotension [30]. Kumar and Prasad investigated the antibacterial activity of the isolated trypsin Inhibitor from the seeds of *Abelmoschus moschatus* [31]. The anti-carcinogenic properties of Bowman Brick Proteinase Inhibitor (BBI) from Soybean were clearly demonstrated by Kennedy et al. They reported the significant suppressive effect on MCA (3-methylcholanthrene) induced lung tumors by the BBI derived from Soybean [32]. Clemente et al., studied the influence of BBI from the *Pisum sativum* on human colorectal adenocarcinoma HT29 cells in vitro and a significant decrease in the growth of HT29 cells was observed [33]. Haemagglutinating properties aginst human and animal erythrocytes were reprted from the purified trypsin inhibitors from the seeds of *Abelmoschus moschatus* by Dokka *et al*. [34].

Plant genetic transformation with genes encoding PPIs is a modern and attractive alternative to synthetic chemical insecticides. The Cowpea trypsin inhibitor, CpTi was the first PPI gene to be successfully transferred to the tobacco plant and showed significant resistance against *Manduca sexta* (tobacco hornworm) [35]. In feeding trials under laboratory conditions, the efficiency of transgenic tobacco plants expressing CpTi was tested. Reduction in the biomass up to 50% in armyworm (*Spodoptera litura*) larvae fed on transgenic leaves expressing 3-5 µg of CpTi /g was observed [36]. Since the major proteolytic enzymes of Lepidopteran pest is serine proteases, identifying inhibitors against trypsin, a serine protease, will be useful in utilizing them against the gut proteases of Lepidipteran pests. Thus identification of novel and potent PPIs will be helpful in developing better insect control strategies. In this study we screened plant extracts to identify extract containing trypsin inhibitors.

II. MATERIALS AND METHODS

COLLECTION OF PLANT PARTS AND PREPARATION OF THE EXTRACT

Plant parts (seeds or leaves) were collected from Calicut and Malappuram districts of Kerala, India. They were washed in distilled water and soaked in bicarbonate buffer, pH 9.0(1ml/g tissue). The plant extract were prepared by homogenizing the tissue and the homogenate was centrifuged at 9,500 x g for 10 minutes at 4^0C. The supernatant containing soluble proteins were used directly for protease inhibition assay or stored at -20^0C until use.

PREPARATION OF TRYPSIN

Trypsin for the assay was prepared by dissolving 2.5mg of pure trypsin from bovine pancreas (Sigma Aldrich) in 20ml of cold 0.1mM hydrochloric acid solution.

PROTEASE ASSAY

Protease assay was carried out using 8.4µg trypsin (Bovine), 603µg Sodium chloride, 330µg Nα-Benzoyl-DL-Arginine-P-NitroAnilide (BAPNA, Sigma Aldrich) as substrate in a total volume of 1ml containing 120mM Tris buffer pH 7.8 with 12mM Calcium chloride. A blank containing same volume of 0.1mM HCl instead of trypsin was also done.

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PROTEASE INHIBITION ASSAY

For protease inhibition assay the plant extract was pre-incubated with trypsin for 10 minutes, followed by the addition of 603µg NaCl and 330µg BAPNA in a total volume of 1ml of Tris buffer pH7.8 (120mM Tris, 12mM $CaCl_2$). Proteolytic activity was measured by continuous spectrophotometric rate determination method using UV Spectrophotometer (Shimadzu UV-VIS spectrophotometer) at 405nm for 5minutes. The reaction was started by adding BAPNA.

STATISTICAL ANALYSIS

Statistical analysis was done using R- programme.

III. RESULTS

In this study, leaves or seeds of twenty different plants were screened to identify plant extracts containing inhibitors against trypsin. The list of plants selected for the study is given in table 1. Of the plants tested, eleven plant extracts showed greater than 50%inhibition of trypsin activity. The highest trypsin inhibition was shown by *Dalbergia latifolia* leaves (85.09±0.4%), followed by *Nephelium lappaceum* leaves (83.45±0.05%), *Anacardium occidentale* seeds (79.23±0.04%), *Samanea saman* seeds (78.27±0.08%), *Mucuna pruritia* seeds (71.34±0.06%),*Psidium guajava* leaf (69.2±0.09%),*Alpinea calcaria* leaves (67.25±0.12%),*Syzigium cumini* seeds(61.10±26%), *Gliricidium sepium* seeds (56.90±0.05%), and *Pterocarpus santallium* leaves (52.49±0.01%). Out of this eleven plants showing greater than 50% trypsin inhibition, six of them, to the best of our knowledge are not reported to contain trypsin inhibitors. They include *Dalbergia latifolia* Roxb., *Cochlospermum religiosum* (L) Alston, *Psidium gujava* L., *Alpinia calcarata* (Haw.) Roscoe., *Gliricidia sepium* (Jacq.)Walp. , *Pterocarpus santalinus* L.f. and *Aerva lanata* (L.) Juss.ex Schult .

Table 1.List of plants screened for protease inhibition against trypsin and their percentage inhibition.

Sl.No.	Name of the plant	Plant part used	Mean%inhibition ±SE
1	*Dalbergia latifolia* Roxb.	Leaves	85.09 ±0.4
2	*Nephelium lappaceum* L.	Leaves	83.45±0.05
3	*Cochlospermum religiosum* (L) Alston	Seeds	82.65±0.09
4	*Anacardium occidentale* L	Seeds	79.23±0.04
5	*Samanea saman* (Jacq.Merr.)	Seeds	78.27±0.08
6	*Mucuna pruriens* (L) DC.	Seeds	71.34±0.06
7	*Psidium gujava* L.	Seeds	69.20±0.09
8	*Alpinia calcarata* (Haw.) Roscoe.	Leaves	67.25±0.12
9	*Syzigium cumini* (L) Skeels	Seeds	61.10±0.26
10	*Gliricidia sepium* (Jacq.)Walp.	Seeds	56.90±0.09
11	*Pterocarpus santalinus* L.f.	Leaves	52.49±0.01
12	*Aerva lanata* (L.) Juss.ex Schult	Leaves	43.40±0.40
13	*Theobroma cacao* L.	Leaves	34.31±0.32
14	*Amorphophallus hohenackeri* (Schott) Engl. & Gehrm.	Seeds	20.30±0.08
15	*Bombax ceiba* L.	Seeds	16.20±0.05
16	*Colocasia esculenta* (L.) Schott	Leaves	09.38±0.02
17	*Pouteria campechiana* (Kunth) Beahni	Seeds	05.41±0.13
18	*Lawsonia intermis* L.	Seeds	03.34±0.05
19	*Adathoda vasica* L.Nees	Leaves	0.91±0.01
20	*Clerodendrum thomsoniae* Balf.	Seeds	0.0

IV. DISCUSSION

In the present study, the highest inhibition (85.09 ±0.4%) of trypsin was shown by the leaves of *Dalbergia latifolia* Roxb. There is no report of trypsin inhibitor from this plant. But E-lutein was isolated from the methanolic extract of leaves of

this plant by Niwal etal 2012 and it was identified as the novel inhibitors of lysenin-induced hemolysis of sheep erythrocytes with an effective dose of 0.025–2.5 ng/mL without any toxicity [37]. *D. latifolia* leaves have been used as folk medicine, used against termites [38] as well as for the treatment of diarrhea, indigestion, and spermatorrhea [39].

Leaves of *Nephelium lappacum* showed 83.45±0.05% inhibition against trypsin. A 22.5-kDa trypsin inhibitor (N. lappaceum trypsin inhibitor (NLTI)) was isolated from fresh *Nephelium lappacum* seeds. Purified NTLI showed Anti-HIV-1 Reverse Transcriptase, and Nitric Oxide-Inducing properties [40].

The seeds of *Cochlospermum religiosum* were found to inhibit 82.65±0.09 % of trypsin activity. No trypsin inhibitor was reported from this plant. The gum of *C.religiosum* is traditionally used in treating cough, diarrhea, dysentery, pharyngitis, fistula, gonorrhea, trachoma and syphilis [41]. Goud *et al*., reported antimicrobial activity of the acetone extract from the stem bark of *C.religiosum*[42].

Anacardium occidentale L. tender seed extract showed trypsin inhibition to the extent of 79.23±0.04%. In a crude inhibitor fraction of cashew nut (*Anacardium occidentale* L.) meal, trypsin and chymotrypsin inhibiting activities were repoted by Filho and Ainouz, 1997 [43]. Remya et al., showed that extract from seeds of tender cashew inhibited the larval gut proteases of *Spodoptera mauritia* to the extent of 73.6±0.35% [44].

Samanea saman (Jacq.Merr.) and *Mucuna pruriens* (L) DC. seed extracts inhibited the activity of trypsin to extent of 78.27±0.08% and 71.34±0.06% respectively. A Kunitz type trypsin inhibitor of 17.89 kDa was purified and characterized from the seed of *Samanea saman* [45]. A single trypsin inhibitor was isolated from the seed extracts of *Mucuna Pruriens* by Borde et al. 2012 [46].

The leaf extract of *Psidium gujava* and *Alpinia calcarata* showed e trypsin inhibition of 69.20±0.09 % and 67.25±0.12 % respectively. No trypsin inhibitory activity has been reported from these two plants. Arawwawala et al., demonstrated the anti-inflammatory activity of the extract from the *Alpinia calcarata* in paw oedema induced by the carrageenan in rats [47]. A new source of anti-tumour agent from the ethanolic extract from the rhizome of *Alpinia calcarata* Rosc (EEAC) *alcarata* was identified and showed its in vivo cytotoxicity against Swiss Albino mice bearing Ehrlich ascites carcinoma tumour [48].

Syzigium cumini (L) showed 61.10±0.26 % inhibition towards bovine trypsin. Remya et al reported the protease inhibitor activity from the seed extracts of *S.cumini* against the larval gut protease *Spodoptera mauritia* [44].

The trypsin inhibition of *Gliricidia sepium* and *Pterocarpus santalinus* were 56.90±0.09 and 52.49±0.01 percentage respectively. There are no report of protease inhibitor from *Gliricidia sepium* and *Pterocarpus santalinus*. *Ptercarpus santalinus* (Fabaceae) used as a traditional medicine in Korea, and it showed anti-inflammatory and anticancer effects.

Kwon et al reported the cytotoxic effect of the methanolic extract from the *Ptercarpus santalinus* (MEPS) and its mechanism of cell death against human cervical adenocarcinoma cell line, HeLa [49].

Leaf extract of *Theobroma cocoa* L and the seed extract of *Amorphophallus hohenackeri* inhibit trypsin activity to the extent of 34.31±0.32% and 20.30±0.08% respectively. Tai *et al* reported a 21 kDa protein from the seeds of *cocoa* (*Theobroma cocoa*) and found to be homologous with the SBTI family proteins [50]. No protease inhibitor was reported from the seeds of *Amorphophallus hohenackeri*. The tubers of *Amorphophallus paeoniifolius* are anti-inflammatory in nature and are traditionally used to treat inflammations. The methanolic extracts from the tubers were studied by Shankhajit *etal* and showed that it significantly inhibits paw edema induced cartageenan in the first phase [51].

V. CONCLUSIONS

Among the twenty plant extracts screened for protease inhibitors against trypsin, we identified eleven of them containing protease inhibitor showing inhibition in range of 50 to 86 %. The maximum inhibition shown by the leaves of *Dalbergia latifolia* Roxb.(85.09±0.4%). This is the first report of the presence of trypsin inhibitor from *Dalbergia latifolia* Roxb.(85.09 ±0.4%), *Cochlospermum religiosum* (L) Alston. (82.65± 0.09%), *Psidium gujava* L.(69.20±0.09%),*Alpinia calcarata* (Haw.) Roscoe. (67.25±0.12%), *Gliricidia sepium* (Jacq.) Walp. (56.90±0.09%), *Pterocarpus santalinus* L.f.(52.49±0.01%) and *Aerva lanata* (L.) Juss.ex Schult (43.40±0.40%). These inhibitors can be exploited in the control of lepidopteran pests as the major digestive proteases of Lepidopteran gut is trypsin like proteases. Purification and characterization is necessary for utilization of these protease inhibitors.

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