A trypsin inhibitor- SNTI with antidandruff activity from Sapindus trifoliatus

**KEYWORDS**

SNTI, Pericarp extract, MALDI-TOF, Antidermatophytic, AntiDandruff

**ABSTRACT**

A trypsin inhibitor, designated Soap Nut Trypsin inhibitor (SNTI) with both antifungal and antibacterial activity exhibiting a molecular mass of 29kDa on SDS–polyacrylamide gel electrophoresis, was isolated from soap nut seeds (Sapindus trifoliatus L) by a combination of ammonium sulfate precipitation, ion exchange chromatography and gel permeation chromatography on Sephadex G-100. It exerted potent antifungal activity against dermatophytic fungi (Trichophyton rubrum and Malassezia furfur) and in causing dandruff which at an equitoxic concentration to Staphylococcus aureus, Bacillus subtilis, Proteus vulgaris and Escherichia coli. The extracts of the pericarp which contained secondary metabolites also exhibited antifungal activity to a lesser extent compared to SNTI. The results obtained herein indicate that SNTI possess exploitable potentials for therapeutic applications.

**Introduction**

Protease inhibitors play an important role in the protection of plant tissues from pest and pathogen attack by virtue of an anti nutritional interaction. Protease inhibitors with antifungal activity have captured the attention of a number of researchers on account of their tremendous potential in protecting crops from invading fungi and thus have important economic implications. Antifungal proteins may contribute to both defense against predators such as insects (Murdock LL et al., 1990) as well as pathogens such as fungi (Wang SY et al., 2005;2006). It is well identified that there is a spectacular diversity of antifungal proteins produced by plants and have been classified into different groups based on their structure and/or functions (Ng TB, 2004). Sometimes, a combination of antifungal proteins is found in a single species of bean. For instance, proteins and peptides such as lysozyme, non-specific lipid transfer protein, and protease inhibitor were isolated from mung bean, and all have fungal inhibition activity (Wang SY et al., 2005;2006;2004).

HIV protease inhibitors and SARS coronavirus protease inhibitors are used to fight against HIV and SARS virus, respectively (Ng TB et al., 1997). Plant protease inhibitors may be involved in the regulation of programmed cell death in plants. One of the common type of protease inhibitors is trypsin inhibitors, which have been isolated from both animal and plant tissues. Kunitz-type trypsin inhibitors also inhibit chymotrypsin, α-amylase and human plasmin, and block the conversion of prothrombin to thrombin (Birk Y, 2003; Zhao M et al., 1996).

Dermatophytes, a group of about 40 related fungi belong to three genera: Microsporum, Trichophyton and Epidermophyton cause cutaneous mycoses infecting only the keratinized tissue like skin, hair and nails. Dermatophytophytes are among the most prevalent infections in the world. Species of Malassezia and Trichophyton have been implicated as contributors to seborrhic dermatitis or dandruff. This condition is partially alleviated by ketoconazole treatment (A Sanfilippo and JC. English III, 2006).

Soap nuts are being considered for commercial use in cosmetics and detergents, among many other products. Reports on the antimicrobial activity of SNTI a trypsin inhibitor are not yet available in the literature. Hence, it is worthwhile to investigate the effect of SNTI on selected bacterial strains and dermatophytic fungi causing dandruff.

**Materials and Methods**

The Sapindus trifoliatus trees bearing soap nuts were selected from Horticulture Research Station, Pandirumamidi, Rampachodavaram, AP (INDIA). Fruits were collected at ripened stage. The endosperm was used for the isolation and purification of SNTI. Various chemicals used in the present investigation were purchased from the following sources.

Bovine pancreatic α-chymotrypsin (3x crystallized, type II), N-acetyl-L-tyrosine ethyl ester (ATEE), dimethyl sulfoxide, elastase, elastin congo red, pronase, papain, pepsin, thrombin, α-amylase, acrylamide, bis-acrylamide, protein markers and sephadex G-100 were obtained from Sigma Chemical company, St. Louis, Missouri, USA.

The bacterial strains Bacillus subtilis (NCIM 2063), Staphylococcus aureus (NCIM 3021), Escherichia coli (NCIM 2066), Klebsiella pneumonia (NCIM 2957), Proteus vulgaris (NCIM 2027) were from NCIM Pune and fungal species Malassezia fur fur (MTCC 1374) was procured from MTCC, Chandigarh. Trichophyton rubrum was collected from Andhra Medical College, Visakhapatnam.

**Sample preparation**

The outer hard seed coat was removed and 25 g of kernel is homogenized with 200 ml of 50 mM phosphate buffer, pH 7.6 and then made up to 250 ml with the same buffer. The clear supernatant obtained after centrifugation at 2500 rpm was treated with 50% ice cold acetone and again centrifuged at 2500 rpm for 15 minutes at 4°C. The precipitate dissolved in 250 ml of 50 mM phosphate buffer, pH 7.6 was used for further investigations.

**Purification of SNTI**

Purification was carried out according to the procedure adopted by (Sai Annapurna et al., 1991). All operations were carried out at 4°C unless otherwise stated.

**SDS-PAGE**

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to the method of (Laemmli UK and Favre M, 1973).

**Preparation of pericarp extract for Antimicrobial activity**

The pericarp from soap nut was removed and air dried and then ground to powder using an electric mill. The powdered material (10g) was subjected to soxhlet extraction for 18h using water, methanol, ethanol, chloroform and hexane separately. The extracts were concentrated and further used for antimicrobial activities.
Preparation of Nutrient Agar medium

Peptone (10g), Yeast extract (5g), Sodium chloride (5g), Agar (1.5g) were mixed and dissolved by heating in 100ml of distilled water. The pH was adjusted to 7.5 and sterilized by autoclaving at 121°C for 15min and poured into sterile petri plates. After setting, the media is preserved in refrigerator for use.

Sabouraud dextrose Agar medium

Dextrose (2g), neopeptone (1g), agar (2g) were mixed and dissolved by heating in 100ml of distilled water. pH adjusted to 5.6, autoclaved and then acetidone (10µg) was added. 10 – 20ml of medium was poured into petri plates under sterile conditions.

Malt Agar medium

Malt extract (30g), peptone (5g), agar (15g) were mixed and dissolved by heating in 100ml of distilled water. pH adjusted to 5.6, autoclaved and then 1ml actidione (50µg), chloramphenical (5µg) were added. Then 10 – 20ml of medium was poured into petri plates under sterile conditions.

Assay for antibacterial and antifungal activity

The bacteria were grown on Nutrient agar and fungi in Sabouraud dextrose agar/malt agar at 37°C, and 25°C respectively. Inoculum of test bacterial species was prepared by growing pure isolate in nutrient broth at 37°C for overnight and fungi in sabouraud dextrose broth/malt broth for 7days. The broth cultures were sub cultured in fresh nutrient broth, sabouraud dextrose broth and malt broth for 3h to obtain log phase culture. The agar plates were prepared by pour plate method using 20ml nutrient medium.

The molten sterile nutrient agar medium was cooled to 45°C and mixed thoroughly with 1ml of growth culture of concerned test organism (1 x 108 cells) and then poured into the sterile petri plates (100x17mm) and allowed to solidify. Malassezia fur fur was grown on Malt Agar and Trichophyton rubrum on Sabouraud dextrose agar plates. Wells of 6mm size were made and 5µl, 25µl, and 50µl of extracts were added. The agar plates were incubated at 37°C for 24h while Sabouraud dextrose agar plates at 25°C for 1 week. The diameters of zones of inhibition were measured in mm using Himedia Zone reader.

Results and discussion

The purified SNTI exhibited a single band on SDS–polyacrylamide gel electrophoresis under non-reducing conditions (Fig.1) signifying the absence of isoforms which are common in many sources (Maria, L.R. Macedo et.al., 2000). The inhibitor showed a molecular weight of 29kDa. The inhibitor showed a sharp band on SDS-PAGE when stained with silver supporting the monomeric nature of the protein. Majority of protease inhibitors have molecular weights ranging from 8 kDa -20 kDa (Hung et.al., 2003). A trypsin-chymotrypsin inhibitor, designated Limenin, with both antifungal and antibacterial activity, and exhibiting a molecular mass of 18.0 kDa on SDS–polyacrylamide gel electrophoresis, was isolated from the large lima bean (Phaseolus limensis) (Shao-yun Wang and Pingfan Rao, 2010). Most of the inhibitors isolated from legumes have a molecular weight around 25 kDa.

The calculated mass from MALDI-TOF was found to be 31kDa attributed to 5µl. The calculated mass from MALDI-TOF was found to be 31kDa attributed to 5µl.

(1) Molecular weight markers: Phosphorylase b (97.4 kDa), Bovine serum albumin (66 kDa), Ovalbumin (43 kDa), Carbonic anhydrase (29 kDa), Lysozyme (14.3 kDa)

(2) Purified SNTI (coomassie brilliant blue stained) (3) Purified SNTI (silver stained)

Antibacterial activity

The effect of pericarp extracts and SNTI on bacterial growth is shown in Table – 1. The aqueous, methanol, ethanol extracts of pericarp were active against Escherichia coli, Bacillus subtilis, Klebsiella pneumoniae, Proteus vulgaris and Staphylococcus aureus whereas as the chloroform and hexane extracts were ineffective towards these microorganisms. Aqueous extract was effective against all bacterial species tested with a maximum zone of inhibition of 16mm against Staphylococcus aureus followed by Proteus vulgaris and Bacillus subtilis with 15mm each.

SNTI (50µg) exhibited maximum zone of inhibition of 21mm against Staphylococcus aureus followed by Bacillus subtilis (20mm), Proteus vulgaris (17mm) and Escherichia coli (16mm). With Ampicillin, Tetracycline and Rifampicin as controls, the inhibition zones observed were in the range of 8-10mm.

TABLE – 1

| Extract                  | SNTI (50µg) | Bacillus subtilis (20µg) | Proteus vulgaris (20µg) | Staphylococcus aureus | E. coli (10µg) |
|--------------------------|------------|-------------------------|------------------------|-----------------------|----------------|
| Aqueous                   | +ve         | +ve                     | +ve                    | -ve                   | -ve            |
| Ethanol                  | +ve         | +ve                     | +ve                    | +ve                   | -ve            |
| Methanol                 | +ve         | +ve                     | +ve                    | -ve                   | +ve            |

Antifungal activity

Aqueous extract of pericarp was further tested for its antifungal activity against dermatophytic fungi Trichophyton rubrum and Malassezia furfur. With 20µl sample, a zone of inhibition of 14mm for Trichophyton and 16mm for Malassezia was observed (Table – 2).

Samples obtained from different stages of purification of SNTI showed varied zones of inhibition for the fungi as shown in Table – 2. Homogenous SNTI from Sephadex G-100 fractions showed maximum zone of inhibition (18mm). The extracts of the pericarp which contained secondary metabolites also exhibited antifungal activity to a lesser extent compared to SNTI. A much higher zone of inhibition was observed when SNTI and aqueous extract of the pericarp together was tested on the fungi (Fig-2). This could possibly be due to an additive effect of SNTI and secondary metabolites in the extract. Fluconazole (20µg) and Ketoconazole (20µg) showed inhibition zones of 10mm and 8mm against Malassezia respectively. Selenium oxide (20µg) was more effective towards the fungi compared to Fluconazole, Ketoconazole and the inhibition exhibited was close to that observed with SNTI. The results obtained support that purified sample of the SNTI is more effective against dandruff causing fungi compared to the antifungal chemical drugs- fluconazole and ketoconazole.

Table 1. Antibacterial activities of different extracts of Sapindus trifoliatus pericarp and SNTI

| Extract | SNTI (50µg) | Bacillus subtilis (20µg) | Proteus vulgaris (20µg) | Staphylococcus aureus | E. coli (10µg) |
|---------|------------|-------------------------|------------------------|-----------------------|----------------|
| Aqueous | +ve         | +ve                     | +ve                    | -ve                   | -ve            |
| Ethanol | +ve         | +ve                     | +ve                    | +ve                   | -ve            |
| Methanol| +ve         | +ve                     | +ve                    | -ve                   | +ve            |

Figure – 1 SDS – PAGE
TABLE – 2
Zones of inhibition of different samples

| Sample | Diameter of zone of inhibition |
|--------|--------------------------------|
| Trichophyton | Malassezia |
| (NH₄)₂SO₄dialysate | 10 mm | 12 mm |
| CM-Cellulose elute | 14 mm | 15 mm |
| Sephadex G-100 elute | 16 mm | 18 mm |
| SNTI | 5 µg | 10 mm | 15 mm | 16 mm |
| Pericarp extract | 5 µl | 9 mm | 10 mm | 15 mm | 18 mm |
| 10 µl | 11 mm |
| 15 µl | 12 mm |
| 20 µl | 13 mm |
| SNTI and Pericarp Extract | 10 µl (5 µg +5 µl) | 12 mm | 17 mm |
| 20 µl (10 µg +10 µl) | 20 mm | 22 mm |
| Fluconazole (20 µg) | 10 mm | 9 mm |
| Ketoconazole (20 µg) | 8 mm | 6 mm |
| Selenium oxide (20 µg) | 18 mm | 19 mm |

Malassezia and Trichophyton rubrum are implicated as causative agents for seborrhoeic dermatitis or dandruff. SNTI strongly inhibited dandruff causative fungi - Malassezia furfur and Trichophyton spp and various bacterial species. SNTI was found to exhibit antibacterial and maximum anti-dermatophytic activity. Ketoconazole which is often used in anti-dandruff shampoos showed negligible inhibition. Though selenium oxide showed inhibition very close to that of SNTI, it is a dangerous chemical causing many health hazards.

Figure – 2
Antifungal Activity of Aqueous Extract and SNTI

SNTI inhibited the growth of a variety of bacteria. A maximum zone of inhibition of 21mm against Staphylococcus aureus followed by Bacillus subtilis, Proteus vulgaris and Escherichia coli were observed. SNTI is devoid of hemagglutinating activity. Since SNTI does not inhibit subtilisin and endogenous α-amylase it falls into the first category of cereal trypsin inhibitors.

The activity of PIs is due to their capacity to form stable complexes with target proteases, blocking, altering or preventing access to the enzyme active site. The versatile biological activities showed by SNTI imply exploitable potentials for the therapeutic applications as antifungal protein.

REFERENCE
Murdock, L.L., Huesing, J.E., Nielsen, S.S., Prat, R.C., & Shade, R.E. (1990) Biological effects of plant lectins on the cowpea weevil, Phytochemistry, 29, 85–89. | Wang, S.Y., Ng, T.B., Chen, T., Lin, D.Y., Rao, P.F., & Ye, X.Y. (2005) First report of a novel plant lysozyme with both antifungal and antibacterial activities, Biochem Biophys Res Commun, 327, 820–827. | Wang, S.Y., Lin, D.Y., Ye, X.Y., Ng, T.B., & Rao, P.F. (2006) Isolation and characterization of a novel mung bean protease inhibitor with antipathogenic and anti-proliferative activities, Peptides, 27, 3129–3136. | Ng, T.B. (2004) Antifungal proteins and peptides of leguminous and non-leguminous origins, Peptides, 25, 1215–1222. | Wang, S.Y., Wu, J.H., Ng, T.B., Ye, X.Y., & Rao, P.F. (2004) A non-specific lipid transfer protein with antifungal and antibacterial activities from the mung bean, Peptides, 25, 1235–1242. | Ng, T.B., Huang, B., Fong, W.P., & Yeung, H.W. (1997) Anti-human immunodeficiency virus (anti-HIV) natural products with special emphasis on HIV reverse transcriptase inhibitors, Life Sci, 61, 933–949. | Birk, Y. (2003) Plant Protease Inhibitors: Significance in nutrition, plant protection, cancer prevention and genetic engineering, Springer-Verlag, Germany. | Zhao, M., Naude, R.J., Muramoto, K., & Oelofsen, W. (1996) Purification and characterization of ostrich pancreatic secretory trypsin inhibitor, Int J Pept Protein Res, 48, 174–181. | Angela, Sanfilippo., & Joseph, C. English III. (2006) An Overview of Medicated Shampoos Used in Dandruff Treatment, P&T®, 31(7), 396-400. | Sai Annapurna, S., Candadai, S. R. & Siva Prasad, D. (1991) Characterisation of a trypsin/chymotrypsin inhibitor from jack fruit (Artocarpus integrifolia) seeds. Journal of the Science of Food and Agriculture, 54 (3), 399–411. | Laemmli, U.K., & Favre, M. (1973) Maturation of the head of Bacteriophage T4, J Mol Biol, 80, 575–599. | Maria, L.R. Macedo., Daniela, G. G. de Matos., Olga, L.T. Machado., Sérgio, M., José, C., & Novello. (2000) Trypsin inhibitor from Dimorphandra mollis seeds: purification and properties, Phytochemistry, 54(6), 553-558. | Hung,C.H., Huang,C.C., Tsai,W.S., Wang,H., & Chen,Y.L. (2003)Purification and Characterization of a Trypsin Inhibitor from Brassica campestris Seeds, J Yuanpei Univ Sci Tech, 10, 13-22. | Shaoyun, Wang, & Pingfan, R. (2010) A leguminous trypsin-chymotrypsin inhibitor Limenin with antifungal activity from Phaseolus limensis, Eur Food Res and Tech, 231, 331-338. | Rachel, K.V., Solomon, K.S., Kiranmaya, P. Reddy, I.B., & Prasad, D.S. (2012) In silico modelling and docking studies of Soap Nut Trypsin Inhibitor, Process Biochemistry. 47, 453-459.