RESEARCH PAPER | Biochemistry | Volume : 3 | Issue : 3 | March 2013 | ISSN - 2249-555X

A trypsin inhibitor- SNTI with antidandruff activity from Sapindus trifoliatus

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

K. Vijaya Rachel	Y. Vimala	D. Apta Chaitanya
Department of Biochemistry, GITAM University, Visakhapatnam - 530 045, AP, India	Department of Microbiology, GITAM University,Visakhapatnam - 530 045, AP, India.	Department of Biotechnology, Majhighariani Institute of Technology and Science, Rayagada-765017, Orissa, India.

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 which are implicated in causing dandruff. SNTI exhibited antibacterial activity against 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. Dermatophytoses are among the most prevalent infections in the world. Species of Malassezia and Trichophyton have been implicated as contributors to seborrheic 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, Pandirimamidi, Rampachodavaram, AP (INDIA). Fruits were collected at ripened stage. The endosperm is 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, thermolysin, 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 4oC. 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 anti microbial activities.

Volume : 3 | Issue : 3 | March 2013 | ISSN - 2249-555X

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 1210C 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 is adjusted to 5.6, autoclaved and then actidione (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.

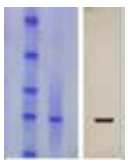
Assay for antibacterial and antidandruff activity
The bacteria were grown on Nutrient agar and fungi in Sabouraud dextrose agar/malt agar at 370C and 250C respectively. Inoculum of test bacterial species was prepared by growing pure isolate in nutrient broth at 370C for overnight and fungi in sabouraud 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 450C 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 370C for 24h while Sabouraud dextrose agar plates at 250C 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) (Shaoyun 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 which closely correlated to that obtained by SDS-PAGE. The putative sequence of SNTI predicted was composed of single polypeptide chain that is about 278 amino acids long. Phylogenetic analysis showed that SNTI could be classified into Barley trypsin inhibitor family (Rachel KV 2012).

Figure – 1 SDS – PAGE



1 2 3

(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 pneumonia, Proteus vulgaris and Staphylococcus aureus where 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
Antibacterial activities of different extracts of Sapindus trifoliatus pericarp and SNTI

Pericarp extract	Escherichia coli	Bacillus subtilis	Klebsiella pneumonia	Proteus vulgaris	Staphylococcus aureus	
Aqueous						
Extract	5µl	8mm	10mm	8mm	10mm	12mm
	25µl	10mm	12mm	9mm	12mm	13mm
	50µl	12mm	15mm	11mm	15mm	16mm
Methanol						
Extract	5µl	7mm	10mm	7mm	4mm	5mm
	25µl	8mm	10mm	8mm	6mm	7mm
	50µl	10mm	12mm	10mm	10mm	10mm
Ethanol						
Extract	5µl	7mm	4mm	5mm	6mm	12mm
	25µl	8mm	8mm	6mm	8mm	13mm
	50µl	10mm	15mm	10mm	10mm	15mm
Hexane						
Extract	50µl	-ve	-ve	-ve	-ve	-ve
Chloroform extract	50µl	-ve	-ve	-ve	-ve	-ve
SNTI	5µg	10mm	16mm	10mm	12mm	15mm
	25µg	14mm	18mm	12mm	14mm	18mm
	50µg	16mm	20mm	15mm	17mm	21mm

Antidandruff/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 and 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.

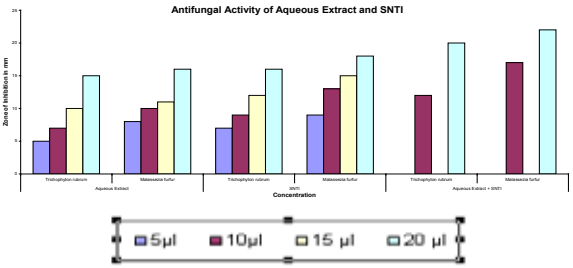
Volume : 3 | Issue : 3 | March 2013 | ISSN - 2249-555X

TABLE – 2
Zones of inhibition of different samples

Sample	Diameter of zone of inhibition	
	Trichophyton	Malassezia
(NH4)2SO4dialysate	10 mm	12mm
CM-Cellulose elute	14mm	15mm
Sephadex G-100 elute	16mm	18mm
SNTI		
5 µg	7 mm	9 mm
10 µg	9mm	13mm
15 µg	12mm	15mm
20 µg	16mm	18mm
Pericarp extract		
5 µl	5 mm	8 mm
10µl	9 mm	10 mm
15 µl	10 mm	11 mm
20 µl	15 mm	16 mm
SNTI and Pericarp Extract		
10 µl (5 µg +5 µl)	12 mm	17 mm
20 µl(10 µg +10 µl)	20mm	22mm
Fluconazole (20 µg)	10mm	9mm
Ketoconazole (20 µg)	8mm	6mm
Selenium oxide (20 µg)	18mm	19mm

Malassezia and Trichophyton rubrum are implicated as causative agents for seborrheic dermatitis or dandruff. SNTI strongly inhibited dandruff causative fungi - Malassezia fur fur and Trichophyton sps 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 ofthe 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., Solmon, K.S., Kiranmayi, P., Reddy, I.B., & Prasad, D.S. (2012) In silico modelling and docking studies of Soap Nut Trypsin Inhibitor, Process Biochemistry. 47, 453–459.