



## RRST-Biochemistry

# Antimicrobial, Antioxidative and Antilymphoproliferative Activity of a New Protein (INDIN-SAA) Isolated from Roots of *Boerhaavia diffusa* (Punarnava) against *Scrofula adenitis* (Anjeerbal)

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## Abstract

The aim of this present study was to investigate the antimicrobial, antioxidative and antilymphoproliferative activity of the isolated new INDIN-SAA protein from the roots of *B. diffusa* against the disease scrofula adenitis caused by *Mycobacterium scrofulaceum*. Total antioxidant capacity assay showed that purified protein fraction (PPF) show stronger antioxidant activity (3.15 fold vs CPE-1.34 fold vs PPPE), than partially purified protein fraction (PPPF) and crude protein extract (CPE), respectively. The antimicrobial activity of the isolated CPEs and its PPFs on the pathogen *M. scrofulaceum* was evaluated on the basis of inhibition zone (IZ) using hole diffusion method, the minimal inhibitory concentration (MIC) and bacterial growth curve inhibition. The data showed that significant antimicrobial and antioxidative effect of the crude protein and its purified fractions against microorganism (*M. scrofulaceum*). Thus the result of this study could provide efficacious and cost effective treatment of the disease (*Scrofula adenitis*) without any cytotoxic effects.

**Key Words:** Antilymphoproliferative activity, Antioxidant and Antimicrobial activity, *Scrofula adenitis*

## Introduction

*Scrofula adenitis* (Anjeerbal, Scrophula or Struma) is the term used for tuberculosis of the neck, or, more precisely, a cervical tuberculous lymphadenopathy [1]. Scrofula is usually a result of an infection in the lymph nodes, known as lymphadenitis and is most often observed in immunocompromised patients (about 50% of cervical tuberculous lymphadenopathy). About 95% of the scrofula cases in adults are caused by *Mycobacterium tuberculosis*, but only 8% of cases in children. The rest are caused by atypical mycobacterium (*Mycobacterium scrofulaceum*) or nontuberculous mycobacterium (NTM). With the stark decrease of tuberculosis in the second half of the 20th century, scrofula became a very rare disease. With the appearance of AIDS, however, it has shown resurgence and presently affects about 5% of severely immunocompromised patients [2]. The most usual signs and symptoms are the appearance of a chronic, painless mass in the neck, which is persistent and usually grows with time. The mass is referred to as a "cold abscess", because there is no accompanying local colour or warmth and the overlying skin acquires a violaceous (bluish-purple) colour. NTM infections do not show other notable constitutional symptoms, but scrofula caused by tuberculosis is usually accompanied by other symptoms of the disease, such

as fever, chills, malaise and weight loss in about 43% of the patients. As the lesion progresses, skin becomes adhered to the mass and may rupture, forming a sinus and an open wound. *Boerhaavia diffusa* (Punarnava) belonging to the Nyctaginaceae family, is mainly diffused perennial herbaceous creeping weed of India and Brazil and used extensively in liver and kidney disorders. The roots of *B. diffusa* contains alkaloids (punarnavine), rotenoids (boeravinones A-F), flavonoids, amino acids, lignins (liriodendrons),  $\beta$ -sitosterols and tetracosanoic, esacosanoic, searic and ursolic acids. The roots of *B. diffusa* is used for the treatment of many diseases, such as anti-inflammatory [3, 4], diuretic [5], laxative [6], anti-urethritis [7], anticonvulsant [8], antinematodal, antifibrinolytic [9], antibacterial [10], antitheatotoxy [11, 12, 13], anthelmintic, febrifuge, antileprotic, anti-ashmatic, blood impurities, anaemia jaundice, enlargement of spleen, abdominal pain, anticarcinogenic, antiscabby and antistress activities. The aqueous extract of *Boerhaavia diffusa* has protected mice against *E. coli* induced peritonitis based on its immunomodulatory activity. Singh and Udupa [14] reported that the dried root powder showed curative efficiency when administered orally for one month to the children or adults suffering from the helminth infection. An aqueous extract of

thinner roots of *Boerhaavia diffusa* at a dose of 2 mg/kg exhibited the remarkable protection of various enzymes such as serum glutamate oxaloacetate transaminase, serum glutamate pyruvate transaminase and bilirubin in serum against hepatic injury in rats [13]. Maximum diuretic and anti-inflammatory activities of Punarnava have been observed in samples collected during the rainy seasons. Due to the combination of these two activities, Punarnava is regarded therapeutically highly efficacious for the treatment of renal inflammatory disease and common clinical problems such as nephritic syndrome, oedema, and ascites developing at the early onsets of the liver cirrhosis and chronic peritonitis. The treatment with the watery extract from the root of *Boerhaavia diffusa* induced leucocytosis with predominant neutrophils, associated to the phagocytosis ability and it was bactericidal to the neutrophils and the macrophages [15]. The recent study [16] demonstrated that the levels of *Boerhaavia diffusa* reduced the levels of glucose in the blood increasing the insulin release from  $\beta$ -cells of pancreas. The watery extract of *Boerhaavia diffusa* was proved to possess protective abilities to the rodents suffering from the peritonitis induced by *Escherichia coli* [17]. It was evidenced that the leaves and root possessed antifibrinolytic and anti-inflammatory activities [17]. The ethanolic extract of *Boerhaavia diffusa* showed a significant immunosuppressive activity on human cells and on murine cells as well [18]. The world wide use of *Boerhaavia diffusa* for various liver disorders, hypolipidemic, hypoglycaemic and antilymphoproliferative activity has been reported [18, 16].

#### Material and Methods

**Plant Material:** The samples of the roots of *Boerhaavia diffusa* (Nyctaginaceae), collected in Aligarh (UP) were kindly provided by Prof. Siddiqui (Taxonomist), Department of Botany, Aligarh Muslim University, Aligarh, India and the voucher specimen (AMUBT8977) has been preserved in our research laboratory (Dept. of Biochemistry, J.N. Medical College, AMU, Aligarh) for future reference in research.

**Preparation of Plant Extract:** Dried roots of *Boerhaavia diffusa* (377 g) were chopped into small pieces and sterilized. Roots were crushed in 500 ml of extraction buffer containing 1M NaCl, 1M Sucrose, 0.2 M Acetate Buffer pH 5.0, 0.1 M EDTA pH 8.0,  $\beta$ -mercaptoethanol 200  $\mu$ l and 50 ml of distilled water mixed together. The tissue homogenate was filtered through four layer of cheese cloth and centrifuged at 9500 rpm for 30 min. The clear supernatant was referred as crude protein, unless stated otherwise the supernatant was stored at 4°C.

**Characterization and estimation of isolated crude protein:** Protein (INDIN-SAA) present in the crude extract of roots was fractionated by ammonium sulphate precipitation [19]. The protein precipitating at 0-80% saturation of ammonium sulphate were separated from the supernatant by centrifugation at temperature 4°C, 9000 rpm for 1 hour. The pellet thus obtained were dissolved in 2 ml of sodium acetate buffer, pH 5.0 and partially purified protein were stored at 4°C until used. Further protein assay was done [20], using Bovine Serum Albumin (BSA) as a standard.

**Microorganism:** The pure culture of *Mycobacterium scrofulaceum* which was a generous gift from Dr. M. S. Khan, Integral University, Lucknow, UP, was maintained at 4°C until use. The *Mycobacterium scrofulaceum* culture was activated at 37°C for 24 hours on Nutrient Agar media (NAM) prior to screening. The Muller Hinton Agar (MHA) and the nutrient broth were used for antimicrobial assay. The plates inoculated with *Mycobacterium scrofulaceum* were than incubated at 37°C for 24 hours. Antimicrobial activity was evaluated by measuring the diameters of hole and zone of growth inhibition around the hole. The assay was repeated on three plates and mean diameter was recorded as given below

Area of growth zone inhibition =  $\pi (r_2^2 - r_1^2)$

Where  $r_2$  = radius from centre to periphery of growth zone

$r_1$  = radius from centre to periphery of hole,  $\pi = 3.14$

#### Minimal Inhibitory Concentration (MIC) determination by hole diffusion method

Further 10 fold serially decreasing dilutions of all the three fractions were done. So the curve should be in the range of 5-1000  $\mu$ g/ml. Each dilution was tested by hole diffusion method as described earlier and MIC determined as the lowest concentration of proteins that can show a visible inhibition zone.

#### Statistical Evaluation

Statistical analysis of data was done by employing two tailed student t-test as described [21]. P values less than 0.05 were considered significant.

#### Antioxidant Activity

##### Measurement of plasma "total antioxidant power" (FRAP)

The method [22] was used for measuring the ferric reducing ability, the FRAP assay, which estimate the "total antioxidant power", with minor modification. Ferric to ferrous ion reduction at low pH results in the formation of a colored ferrous-tripyridyl triazine complex. The assay was carried out in a total volume of 1.0 ml containing a suitable aliquot of plasma in 0.1 ml and 900  $\mu$ l of freshly prepared FRAP reagent, prepared by mixing 10.0 ml of 22.78 mM sodium acetate buffer, pH 3.6, 1.0 ml of 20 mM ferric chloride and 1.0 ml of 10 mM 2, 4, 6-tripyridyl-s-triazine solution prepared in 40 mM HCl. Before starting the reaction, both FRAP reagent and plasma samples were pre incubated for 5 min at 30°C. Incubation was done for 5 min at 30°C and absorbance was recorded at 593 nm against a reagent blank in a Beckman DU 640 spectrophotometer. Ferrous sulphate was used as a standard for calculating the "total antioxidant power".

**Antimicrobial activity:** The reference antibiotic (RA) Streptomycin, Crude protein extract (CPE), Partially purified protein fraction (PPPF), Purified protein fraction (PPF) was dissolved in dimethyl sulphoxide 10% v/v (DMSO) at 2 mg/ml. Whereas CPE, PPPF, and PPF were used at a concentration of 10 mg/ml and further serial dilution was done in 0.1 M sodium acetate buffer pH 5.0 each.

**Hole diffusion method:** Screening test for antimicrobial activity were carried out by the hole diffusion method [23] by using a cell suspension from the pure culture of about  $1.0 \times 10^4$  colony forming unit (CFU)/ml obtained by McFarland turbidity standard number 0.5. The concentration of

suspension was standardized by taking the OD to 0.1 at 600 nm (Beckman DV 640 Spectrophotometer). Holes of about 3 mm diameter were made on the MHA plate and tilted with the 100 µl (20 mg/ml, 10 mg/ml, 1 mg/ml, 0.1 mg/ml) of CPE, PPPF, PPF and RA.

**Results**

As seen in Figure 1 and Table 1 depicts the antioxidant impact of Crude protein extract (CPE), partially purified protein fraction (PPPF) and Purified protein fraction (PPF) at different concentration range 5-1000 µg/ml protein.

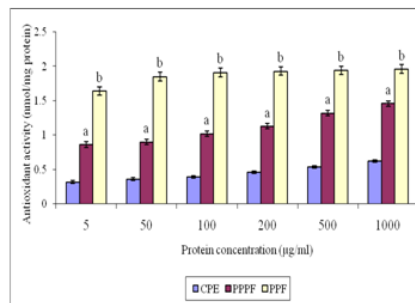


Figure 1: Antioxidant activity (nmol/mg protein) of Crude protein extract (CPE), Partially purified protein fraction (PPPF) and Purified protein fraction (PPF) isolated from the roots of *Boerhaavia diffusa*. All values are mean ± Standard deviation of triplicate values, Significant for CPE at <sup>a</sup> P<0.05 and <sup>b</sup> P<0.01

Table 1: Antioxidant activity (nmol/mg protein) of Crude protein extract (CPE), Partially purified protein fraction (PPPF) and Purified protein fraction (PPF) isolated from the roots of *Boerhaavia diffusa*

Test Sample	Protein (5 µg/ml)	Protein (50 µg/ml)	Protein (100µg/ml)	Protein (200µg/ml)	Protein (500µg/ml)	Protein (1000µg/ml)
CPE (nmol/mg)	0.321±.003	0.362±.004	0.392±.003	0.462±.023	0.536±.032	0.622±.042
PPPF (nmol/mg)	0.862±.004 (62.76%)	0.901±.062 (59.82%)	1.02±.039 (61.56%)	1.13±.062 (59.11%)	1.32±.023 (59.39%)	1.46±.052 (57.39%)
PPF (nmol/mg)	1.64±.031 (80.42%)	1.85±.053 (80.43%)	1.91±.065 (79.47%)	1.93±.072 (76.06%)	1.94±.059 (72.37%)	1.96±.092 (68.26%)

The results obtained, Purified protein fraction (PPF) was more efficient scavengers of peroxy radicals. It was observed that the antioxidant power (Scavengers of peroxy radicals) of PPF (100 µg/ml), PPPF (100 µg/ml) and CPE (100 µg/ml), there is a decrement of 1.96 nmol/mg, 1.46 nmol/mg, respectively. Purified protein fractions (PPF) provide a high antioxidant activity than compared to PPPF and CPE as shown in Table 1. The hole diffusion method results are presented in Figure 2, shows the antioxidant activity as in the form of visible bacterial zone formation. There is no bacterial inhibition zone formation, without the application of either crude extract or its purified fractions. It was found that on application of 100 µg CPE (20 mg/ml), on inoculated MH media plate, the inhibition area correspond of 39.62 mm<sup>2</sup> in CPE, whereas an application of PPP and PP Fractions it was 176.56 and 329.25 mm<sup>2</sup> respectively. It is clear from the data that inhibition zone area, obtained from PPPF, was increased by 77.56 % than CPE applied plates, whereas 87.97% increment was observed when compared with PPF applied plates. On further dilution of the same CPE, PPPF and PPF fractions the same pattern was obtained. The inhibition zone area was increased by 89.90%, 112.04%, 95.56% and 96.38%, than CPE compared to reference antibiotics (*Streptomycin*) applied plates as shown in Table 2. Thus the CPE, PPPF and PPF exhibited both antibacterial and antimicrobial effect, since the CPE, Protein fraction and RA were tested at different concentrations, the

real extend of their inhibitory activities against the test micro organism (*Mycobacterium scrofulaceum*) can be well established only by comparing the minimal inhibitory concentration (MIC) values obtained.

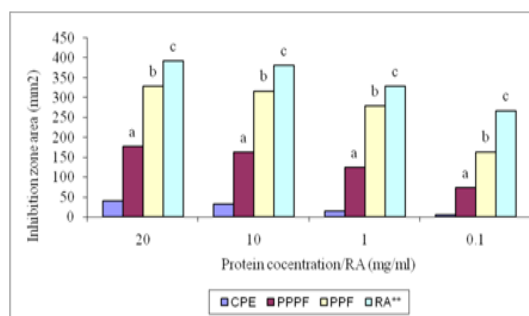


Figure 2: Inhibition zone area (mm<sup>2</sup>) for the Crude protein extract (CPE), partially purified protein fraction (PPPF) and Purified protein fraction (PPF) isolated from the roots of *Boerhaavia diffusa* and Reference antibiotic (RA) against *Mycobacterium scrofulaceum*. Inhibition zone area are mean (mm<sup>2</sup>) ± Standard deviation of triplicate values, \*Inhibition zone area are calculated from the difference of radial of inhibition zone and hole (3 mm), \*\*Reference antibiotics (*Streptomycin*), significantly different from CPE at <sup>a</sup> P<0.05, <sup>b</sup> P<0.01 and <sup>c</sup> P<0.05

Table 2: Inhibition zone area<sup>2</sup>(mm<sup>2</sup>) for the Crude protein extract (CPE), Partially purified protein fraction (PPPF) and Purified protein fraction (PPF) isolated from the roots of *Boerhaavia diffusa* and Reference antibiotic (RA) against *Mycobacterium scrofulaceum*

Test Sample	Protein Conc./RA (mg/ml)	(20 mg/ml)	Protein Conc./RA (10 mg/ml)	Protein Conc./RA (1 mg/ml)	Protein Conc./RA (0.1 mg/ml)
CPE	39.62±0.912		31.26±0.731	14.62±0.531	4.66±.625
PPPF	176.56±2.11 (77.56%)		162.53±2.63 (80.76%)	124.36±2.11 (88.24%)	73.12±1.22 (93.62%)
PPF	329.25±3.63 (87.97%)		316.75±4.12 (90.13%)	279.53±3.61 (94.77%)	163.53±2.11 (97.15%)
RA <sup>2</sup>	391.76±4.32 (89.90%)		381.51±3.13 (112.04%)	329.12±4.22 (95.56%)	267.19±2.69 (96.38%)

The Table 3 indicates the respective MIC values from 50-1000 µg/ml for CPE and 5-1000 µg/ml for PPPF and PPF. The MICs of the CPE and its other functions on test pathogen was either greater or equal to that of RA. The results thus obtained,

confirmed that *Mycobacterium scrofulaceum* was most sensitive organism to the isolated new INDIN-SAA crude protein extract and its purified protein fractions obtained from the roots of *Boerhaavia diffusa*.

Table 3: Minimal inhibitory concentration (MIC) µg/ml, Crude protein extract (CPE), Partially purified protein fraction (PPPF), Purified protein fraction (PPF), Inhibition zone area (- Absent, + Present, ++ Abundant, +++ Very abundant, NA- Not available)

Test Sample	Protein (5µg/ml)	Protein (10µg/ml)	Protein (50µg/ml)	Protein (100µg/ml)	Protein (200µg/ml)	Protein (400µg/ml)	Protein (800µg/ml)	Protein (1000µg/ml)	Without Protein
CPE	-	-	+	++	++	++	+++	+++	---
PPPF	+	+	+	++	++	++	+++	+++	---
PPF	+	+	+	++	+++	+++	+++	+++	---
RA	+	NA	+	++	+++	+++	+++	+++	

The bacterial growth curve in term of optical density (OD) at 600 nm for different time interval (0-90 h) and two concentrations of CPE, PPPF and PPF (10 µg/ml and 20 µg/ml) is shown in figure 3 and 4. It is observed that without the application of INDIN-SAA protein either CPE or PPF, the increase in O.D was observed, whereas on application of CPE and its purified fractions the maximal bacterial growth inhibition in term of decrement in O.D was noted. The maximal bacterial

growth inhibition was found in PPF followed by PPPF and CPE in both concentrations (10 µg/ml and 20µg/ml) as shown in Table no 4, there is a decrement of 0.433 0.314 0.102 and 0.371 (CPE), 0.280 (PPPF), 0.086 (PPF), fold in both concentrations (10 µg/ml and 20 µg/ml) of CPE, PPPF and PPF, respectively.

Table 4: Bacterial growth in term of Absorbance (600 nm), Crude protein extract (CPE) (10 µg/ml and 20 µg/ml), Partially purified protein fraction (PPPF) (10 µg/ml and 20 µg/ml), Purified protein fraction (PPF) (10 µg/ml and µg/ml)

Time (h)	INDIN-SAA Absent	CPE		PPPF		PPF	
		(10µg/ml)	(20µg/ml)	(10µg/ml)	(20µg/ml)	(10µg/ml)	(20µg/ml)
0	0.102±.002	0.103±.002	0.102±.003	0.103±.004	0.101±.007	0.102±.003	0.103±.004
6	0.156±0.011	1.21±.003	1.29±.004	1.43±.005	1.51±.005	1.69±.005	1.73±.005
12	0.193±0.005	1.13±.007	1.23±.005	1.29±.006	1.36±.002	1.43±.007	1.53±.004
18	0.212±0.004	0.951±.005	0.926±.006	1.10±.008	0.931±.004	1.26±.005	1.10±.005

24	0.231±0.007	0.911±.009	0.851±.007	0.936±.007	0.851±.005	1.07±.004	0.961±.003
36	0.352±0.004	0.863±.008	0.718±.007	0.729±.005	0.711±.007	0.862±.007	0.779±.004
48	0.395±0.006	0.713±.007	0.675±.009	0.630±.007	0.581±.005	0.579±.008	0.511±.007
60	0.412±0.002	0.618±.009	0.561±.007	0.536±.008	0.511±.007	0.370±.005	0.362±.008
72	0.439±0.008	0.593±.007	0.537±.008	0.479±.007	0.445±.005	0.258±.021	0.211±.029
90	0.477±0.005	0.524±.009	0.479±.009	0.450±.008	0.423±.007	0.172±.008	0.149±.007
Fold	3.06 fold	0.433 fold	0.371 fold	0.314 fold	0.280 fold	0.102 fold	0.086 fold

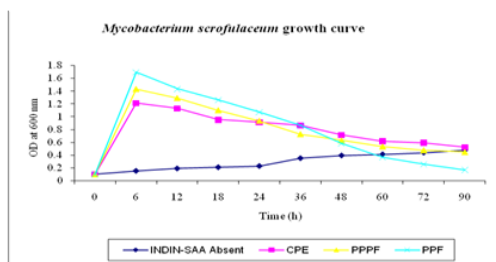


Figure 3: *Mycobacterium scrofulaceum* growth curve in term of absorbance at a different time intervals in absence of INDIN-SAA and presence of CPE (10 µg/ml), PPPF (10 µg/ml), PPF (10 µg/ml), Values are mean (OD) ± Standard deviation for bacterial growth for triplicate assay.

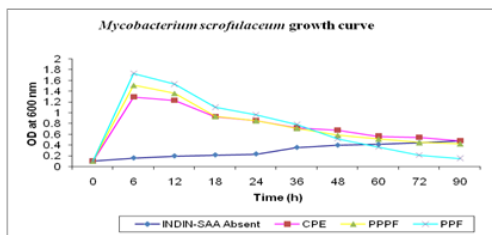


Figure 4: *Mycobacterium scrofulaceum* growth curve in term of absorbance at a different time intervals in absence of INDIN-SAA and presence of CPE (20 µg/ml), PPPF (20 µg/ml), PPF (20 µg/ml), Values are mean (OD) ± Standard deviation for bacterial growth for triplicate assay.

## Discussion

*Boerhaavia diffusa* is one of the most popular herbal remedies in India. Ayurvedic medicine that prescribes roots of Punarnava as diuretic, expectorant, vermifuge, analgesic used to improve the functions of kidney and liver [24]. In this study, it was observed that antimicrobial and anti-amyloproliferative activity of purified INDIN-SAA protein and its *in-vitro* on *Mycobacterium scrofulaceum*. We have made an attempt to provide basic approach for the evaluation of its traditional preparation in order to verify the therapeutic effect of the root

extract in the form of isolated purified INDIN-SAA protein against the bacterium *Mycobacterium scrofulaceum*, as well as the disease *Scrofula adenitis* (Anjeerbal). *Scrofula adenitis* is a disease in which swelling of lymph nodes in neck or throughout body is seen in patients and disease is characterized by progressive painless enlargement of lymphoid tissue throughout the body. The increased frequency of the tuberculosis adenitis reflects a high incidence of the disease in India. The main causative agent of *Scrofula adenitis* is *Mycobacterium scrofulaceum*. To the best of our knowledge there are reports showing the efficacy of *Boerhaavia diffusa* products [25]. The PPF was more efficient scavengers of peroxy radicals. It was observed that the antioxidant power of PPF, PPPF and CPE, there is a decrement of 1.96 1.46 0.622 nmol/mg protein, respectively. Thus, Purified protein fraction (PPF) show high antioxidant activity than compared to PPPF and PPF. On the other hand, the MICs of the extracts and its fractions observed against the sensitive strain of *Mycobacterium scrofulaceum*, ranged from 5-1000 µg/ml, the values were equally effective to R.A. It was further observed that the antimicrobial activity was increased gradually as on application with CPE followed by PPPF and PPF. This antimicrobial activity may be due to alkaloids, phenols, polyphenols, tannins, triterpenes and steroids which were found in crude INDIN-SAA protein and its purified fractions. This phytochemical growth was known to cause antibacterial activities [26]. It was observed that the bacterial growth in term of Optical Density (at 600 nm) was increased by 3.06 fold without application of INDIN-SAA protein whereas on addition of Crude protein extract (CPE), Partially purified protein fraction (PPPF) and Purified protein fraction (PPE) (10 µg/ml and 20 µg/ml), there is a decrement of 0.443 0.314 0.102 fold and 0.371 0.280 0.086 fold, respectively. Further purification and characterization of the active principal compounds from CPE and its purified fractions will provide a better antioxidant, antimicrobial and antilymphoproliferative mechanism. In conclusion, the crude INDIN-SAA protein extract and its purified fraction obtained from the roots of *Boerhaavia diffusa* were found to be active enough for the antimicrobial and antilymphoproliferative activity against the pathogen *Mycobacterium scrofulaceum*. Thus the result of this study could provide efficacious and cost effective treatment of

the *Scrofula adenitis* without any harmful effects on human body.

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