1. **Chemicals and Reagents**

Sodium para nitrophenyl phosphate (pNPP), Orthophospho-L-Tyrosine, Orthophospho-L-Threonine, Orthophospho-L-Serine, fructose-6-phosphate, phosphoenolpyruvate, Adenosine di phosphate, Sodium-β-naphthyl phosphate, Sodium orthovanadate, Phenylarsine oxide, Sodium Fluoride, Ammonium molybdate, Okadaic acid, Cantharidin, p-HMB, CM-Sephadex C50 beads, Trysin (proteomic grade), Ortho phenylene diamine (OPD), H₂O₂, 3,3’-diaminobenzidine (DAB), Peroxidase conjugated anti human IgG, Biotin conjugated anti human IgG1, Biotin conjugated anti human IgG4, ALP-conjugated anti human IgE, MTT, DEPC, EDAC were purchased from Sigma-Aldrich chemical company, USA.

Concavalin 4B Sepharose beads were purchased from GE Healthcare Biosciences. Sephadex G-200 and Sephadex G-25 were purchased from Pharmacia Biotech, Uppsala, Sweden. Prestained molecular weight markers for SDSPAGE were purchased from Biotech, India. Iodoacetic acid (IAA), EDTA, NaN₃, Sodium deoxycholate, DTT, 2-ME, DMF, DMSO, n-Butanol, EtOH, ammonium bicarbonate, Trifluoroacetic acid, Acrylamide, bis-Acrylamide, SDS, APS, Triton X-100, Tween-20 were purchased from Hi-Media Laboratories, Mumbai, India.

All other chemicals used were of analytical grade.

2. **Preparation of KRB buffer**

Kreb’s Ringer bicarbonate (KRB) buffer was prepared essentially according to **Deluca and Cohen (1964)**. NaCl (9g), KCl (0.42g), MgSO₄(0.038g), glucose (0.5g), NaHCO₃ (0.25g), CaCl₂ (0.42g), KH₂PO₄ (0.021g), streptomycin (0.1g) and penicillin (0.1g) were added to sterilized distilled water, made up to 1L and sterilized through Millipore filters (0.22 mm pore size). KRB was stored at 4°C until used.
3. Collection of human sera

_W. bancrofti_ infected human sera were collected by carrying out field survey in known filarial endemic area (Sreegovardhanpur) of Varanasi city. Non-endemic normal sera were kindly gifted by the volunteers who had recently been to Banaras Hindu University, Varanasi from the regions declared as nonendemic zones for lymphatic filariasis. Nocturnal blood smears were examined for the presence of microfilariae by means of night blood examination between 9 pm and 12 pm. Thick smears with 20 mm$^3$ measured blood by finger prick were examined (Narayandas, 1958) after dehaemoglobinisation and staining with Leishman stain for the detection of microfilariae (mf). The mf count per 20 mm$^3$ of blood was estimated wherever the parasite was present. The blood from patients having 100-200 mf/mm$^3$ of blood was collected. Acute and chronic manifestations of filariasis were also recorded in the schedule after physical verification of all persons. Sera were separated by keeping the blood at 4ºC for a few hours, centrifuged at 10,000 rpm for 20 min in a cooling centrifuge and was stored at -20ºC with protease inhibitors till further use.

4. Collection of parasitic material and preparation of homogenate

Adult, motile _Setaria cervi_ parasites were procured from the peritoneal folds of freshly slaughtered Indian water buffaloes and brought to the laboratory from local abattoir in Kreb’s Ringer bicarbonate (KRB) maintenance medium supplemented with streptomycin, penicillin, glutamine and 1% glucose. The parasites were washed with phosphate buffered saline (PBS), pH 7.4 and maintained in supplemented ringer solution or freezed at -20ºC till further use.

**Adult female parasites** were dried with clean filter paper folds, weighed and homogenized in 100mM Tris-HCl, pH 7.0, 1mM PMSF, 1mM EDTA, 10% glycerol and
0.02% NaN₃ using motor driven Remi homogenizer type RQ127A. All steps were carried out in ice-bath. The crude homogenate was then centrifuged at 12,000 x g for 1 h at 4°C. The resulting supernatant was treated as crude soluble extract.

**Microfilariae** of *S. cervi* were obtained by dissecting the uterus of gravid females and were maintained in similar supplemented ringer solution for 4 h at 37°C under sterilized condition. The supernatant of maintenance medium was treated as excretory-secretory (ES) product of adult/mf. The soluble extract was prepared by sonicating the mf using a MSE 150W ultrasonicator MK2 at 20 KHZ for 10 min (30 sec sonication cycle followed by 30 sec interval) in cold 100 mM Tris-HCl pH 7.0 containing 1mM PMSF, 1mM EDTA and 0.02% NaN3. The extract was centrifuged at 16,000 x g for 30min at 4°C. The clear supernatant was treated as mf extract and stored at -20°C until used.

5. **Preparation of detergent soluble extract**

The detergent soluble extract of adult worms was prepared according to the method described by Acosta et al., 1998. The sedimented pellets obtained after centrifugation at 12,000 x g were washed several times (at least five times) with homogenization buffer and finally incubated in 0.1 M Tris-HCl, pH 8.0 containing 0.15M NaCl, 1% sodium deoxycholate, 1 mM PMSF and 0.02% NaN₃ for 1 h at room temperature with intermittent shaking. Next, the mixture was incubated at 37°C and 4°C for 30 min and 1 h respectively. The crude extract was then centrifuged at 12,000 x g for 30 min at 4°C and the supernatant was collected. The detergent was removed by passing the extract through a Sephadex G-25 column equilibrated with homogenization buffer containing no sodium deoxycholate.
6. Preparation of surface antigen

Surface antigens were extracted by the method of Phillip et al., 1984 with some modifications. The intact adult female worm were incubated with 0.2% NP-40 in PBS vortexed at full speed for 30 sec at every 10 min for 30 min at 37°C. It was then incubated for 4°C for 2 h and again centrifuged at 5000 rpm for 15 min at 4°C. Supernatant was dialyzed overnight at 4°C against PBS.

7. Protein Estimation

Protein was estimated routinely using the protein dye binding method of Bradford, (1976) with bovine serum albumin as a protein standard. The protein content in the eluted fractions during enzyme purification process was monitored by measuring the absorbance at 280 nm using a double beam UV/VIS spectrophotometer (Shimhadzu, Japan).

8. Phosphatase Assay

8.1 Using general substrate, Para nitro phenyl phosphate (pNPP)

The general acid phosphatase activity was measured by the method of Chen et al., (1999) using pNPP as substrate. Briefly, crude extract or purified acid phosphatase was incubated in 0.1 M sodium acetate buffer pH 4.8 containing 50% glycerol, 10 mM NaCl and 10 mM pNPP in a final reaction volume of 0.5 ml for 30 min at 37°C. Reaction was terminated by addition of equal volume of 1M NaOH and para-nitrophenol liberated was measured spectrophotometrically at 405 nm. The activity was calculated using extinction coefficient of 17.5 x 10^3 M^-1 cm^-1. One unit is defined as the amount of acid phosphatase that produces one micro mole of para-nitrophenol per minute.
8.2 Using specific substrates

Method described by Taga et al., (1982) was followed to assay hydrolysis of other substrates (O-P-L-Tyrosine, O-P-L-Serine, O-P-L-Threonine, Adenosine di phosphate, Fructose-6-phosphate, Sodium-α- naphthyl phosphate, Phosphoenol pyruvate) by measuring free phosphate liberated. This was measured by the quantitation of reduced phosphomolybdic acid at 700nm using a molar extinction coefficient of $4 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$. The enzyme was incubated at 40°C in 1 ml of a reaction mixture containing 10 mM substrate, 100 mM NaCl in 50 mM sodium acetate pH 5.0. The reaction was stopped after 20 min by addition of 1 ml of 3% ammonium molybdate in 200 mM sodium acetate pH 4.0. After addition, 0.1 ml of 1% ascorbic acid in 200 mM sodium acetate pH 4.0 was added and the color was allowed to develop for 30 min.

9. Ecto-PTP activity measurement

The PTP activity was measured according to Lemos et al. (2002) with slight modifications. Briefly, the intact active two adult females/ml or $2 \times 10^6$ microfilariae/ml were incubated for one hour at 37°C in 1.0 ml of a mixture containing 50.0 mM Tris-HCl, pH 7.2, 5.4 mM KCl, 116.0 mM NaCl, 5.5 mM D-glucose and 10 mM p-NPP. The tubes were then centrifuged at 1,200 rpm for 10 min at 4°C and clear supernatant was collected. The reaction was terminated by the addition of 1.0 ml of 1.0 M NaOH to this supernatant. The optical density of the supernatant was measured spectrophotometrically at 405 nm. The phosphatase activity was calculated by using an extinction coefficient of $17.5 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ and subtracting the nonspecific p-NPP hydrolysis measured in the absence of parasites. To restrain out the possibility that the p-NP produced was because of the secreted soluble enzymes, the parasites were incubated in the same reaction buffer (without the substrate). After incubation for 60 min, the supernatant (which contains the excretory secretory products released by the parasites) was centrifuged to remove
released microfilariae and checked for phosphatase activity. It showed less than 2% of the total phosphatase activity as compared to that observed in the parasites. We have subtracted this activity from the total phosphatase activity. The hydrolysis of PTP using O-phospho-L-Tyrosine, O-phospho-L-Serine and O-phospho-L-Threonine was also performed in a similar way using ammonium molybdate and ascorbic acid as quenching agents instead of NaOH. The O.D. was measured at 700 nm (molar extinction coefficient: $4 \times 10^3 \text{M}^{-1}\text{cm}^{-1}$ (Rathaur et al. 2009).

10. Column chromatography techniques used for purification of protein tyrosine phosphatases

Different protein tyrosine phosphatases were purified from adult female *S. cervi* cytosolic and detergent soluble membrane bound fractions using combination of different column chromatography techniques.

10.1. Affinity chromatography

10% Crude soluble extract/ Detergent soluble extract was loaded on to Concavalin Sepharose affinity matrix equilibrated with 20mM Tris-Hcl pH7.5 (containing 0.5M NaCl, 1 mM CaCl$_2$, 1 mM MgCl$_2$ and 1 mM MnCl$_2$) and partially purified protein phosphatase was eluted in the (0-1M) mannose gradient in the same buffer. The homogeneity of enzymatically active fractions was checked by SDS PAGE followed by silver/ commassie staining depending on the protein content of the fraction.

10.2. Cation exchange chromatography

The partially purified fraction derived from detergent soluble extract of *S.cervi* after affinity chromatography was further loaded on the CM-sephadex C-50 cation exchange column (bed volume = 15 ml) equilibrated with 50 mM sodium acetate buffer pH 6.0 and protein
was eluted with 0-0.3 M NaCl gradient. Gradients having acid phosphatase activity were pooled separately, concentrated and stored at -20°C for further use.

10.3. Gel filtration chromatography

Partially purified fractions derived from crude soluble extract of *S. cervi* after affinity chromatography were further loaded on the G-200 column (bed volume = 30 ml) equilibrated with 0.1 M Tris buffer pH 7.0. 1.5 ml fractions were collected and protein tyrosine phosphatases was eluted with same buffer.

11. Electrophoretic Techniques

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS) was carried out according to the method of Laemmli (1970). Stacking gel containing 5.5% acrylamide and resolving gel containing 10 % acrylamide were prepared. The Sample buffer constitutes 2.5 ml 0.625M Tris Hcl buffer pH 6.8, 2.5 ml of 20% SDS, 2.0 ml glycerol, 1.0 ml 0.01% bromophenol blue, made upto 18.5 ml with water. Equal volume of test sample buffer boiled for 90 sec at 100°C and applied to current, 10 mA for stack and 15 mA for the resolving gel. Molecular weight markers ranging from 11-235 kDa were used for the determination of subunit molecular mass of the purified phosphatases.

12. Staining techniques

12.1 Silver staining

A highly sensitive silver staining method of Allen et al., (1983) was used for detection of protein bands in after SDS-PAGE. Briefly, fixed gel was incubated in solution A (0.5M sodium acetate, 30% ethanol, 0.5% glutaraldehyde and 0.2% sodium thiosulphate) for overnight at 4°C.
Following day gel was washed thrice with distilled water and stained in solution B (0.1% silver nitrate and 0.02% formaldehyde) for 1 hr and developed with solution C (2.5% Sodium carbonate and 0.01% formaldehyde) until brown bands appeared.

12.2 Colloidal commassie staining

A very sensitive colloidal commassie staining protocol of Dyballa and Metzger was (2009) was used for staining the gels. Briefly, the gels were fixed for 2-3 h (if freshly prepared) to overnight at room temperature in a mixture of 0.02% (w/v) CBB G-250, 5 % (w/v) aluminium sulfate, 10 % (v/v) ethanol, 2 % (v/v) 85 % orthophosphoric acid. The stain was removed and rinsed twice with distilled water. It was then incubated in the detach solution containing 10 % ethanol and 2% orthophosphoric acid (85 %).

13 Protein sequencing by MALDI MS/MS

13.1 Trypsin digestion and preparation of the sample

MALDI mass sequencing of different protein/ was done following the standard trypsin digestion protocol (Shevchenko et al.,2006). Briefly, the bands obtained were extracted from the gel and crushed in a 1.5 ml microcentrifuge tube followed by treatment with washing solution [1:1 100 mM ammonium bicarbonate: Acetonitrile ( ACN, HPLC grade)] to wash off CBB stain. The gel pieces were then dehydrated with 100 % ACN and air dried followed by treatment with a reduction solution (10 mM DTT in water). Equal volume of 100 mM ammonium bicarbonate was added and the setup was incubated at 50°C for 30 mins. The reduction solution was discarded and gel pieces were treated with alkylation solution (50 mM lodoacetamide in water). Equal volume of 100mM ammonium bicarbonate was added to the mixture following incubation for 30 mins in dark at room temperature. The alkylating solution was discarded and gel pieces were thoroughly washed with the washing solution followed by dehydration with 100% ACN. Trypsin (in
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25mM ammonium bicarbonate solution) was added to a final concentration of 25 ng/µl and the gel pieces were incubated overnight at 37°C. The gel extract (trypsin solution) was transferred into a fresh microcentrifuge tube leaving the gel pieces in the same tube for further extraction with extraction solution (1:1 100% ACN: 1% Trifluoroacetic acid (TFA) in water). The pooled samples were concentrated by speed-vac (EPPEDORF concentrator plus) and analysed with Bruker Daltonics MALDI "FLEX" series system at the Interdisciplinary School of Life Sciences, DBT-Banaras Hindu University.

13.2 Bioinformatics Analysis

The MASCOT database search engine was used for the identification of the obtained peptides. BLAST and CLUSTALW softwares were used for the homology and multiple sequence alignment studies.

14. Kinetic parameters

14.1. pH optima of purified PTP

The pH optima was determined at pH range of 3.5-8.0 using O-P-L-Tyrosine as substrate. The different buffers used were Citrate phosphate buffer, 0.1M (pH 3.5); Sodium acetate buffer, 0.1M (pH 4.0-6.0); Sodium phosphate buffer, 0.1M (pH 6.5-7.5), Tris-HCl, 0.1M (pH 8.0).

14.2. Temperature optimum of Purified PTP

The optimum temperature of the purified enzyme was determined by incubating protein tyrosine phosphatase routine assay mixture in 0.1 Sodium acetate buffer pH 5.0 at 0°, 10°, 20°, 30°, 40°, 50° and 60° C, respectively.

14.3. Km and Vmax of the purified PTP

Km and Vmax values for purified PTP were determined with pNPP, O-Phospho-L-Tyrosine and O-Phospho-L-threonine at concentration range of 2.0 mM to 20.0mM. The
values were obtained using Michaelis menton curve and Lineweaverburk plot drawn using by GraphPad Prism 5.0 software. IC\(_{50}\) and K\(_i\) (Inhibition constant) of the purified PTP were determined by using sodium orthovanadate and Phenylarsine oxide as inhibitor at concentration range of 10 µM to 50 µM and 1-10 nm respectively.

15. Inhibition/Activation Studies

The effects of a range of general acid phosphatase, specific protein tyrosine phosphatase inhibitors, divalent cations and DTT on PTP activity were assessed by incorporating them, at a range of concentrations, into the assay buffer (0.1M Sodium acetate pH 5.0). Crude/Purified enzyme/ intact parasites was pre-incubated with the activators or inhibitors for 15 min at 40°C prior to the addition of O-P-L-Tyrosine substrate. Rest assay procedure was similar to that described above in sections 8.1, 8.2 and 9.

16. Active site determination

To determine the active site residues on PTP various chemical modifiers of amino acids were used. The enzyme activity without any treatment was taken as control.

Cysteine modified with Iodoacetamide in 100mM sodium acetate buffer, pH 5.5; concentration 0-1 mM; incubation time 15 min.

Arginine modified with 1, 2-cyclohexendione in 50mM Borate buffer; pH 8.0; concentration 0- 1.0 mM; incubation time 30 min.

Aspartate modified with 1-ethyl-3dimethylaminopropyl carbodiimide (EDAC) at 0- 25 mM in presence of 30 mM Alanine ethyl ester at pH 6.0, 100mM tris-Hcl buffer; incubation time 60 min. The reaction was quenched by addition of equal volume of 1 M Na-acetate buffer pH 5.5 and residual activity was determined under standard assay conditions using O-P-L-Tyrosine as phosphatase substrate.

Histidine modified with 0- 1 mM Diethylpyrocarbonate in 100mM sodium acetate buffer, pH 5.5; incubation time 30 min.
17. Tolerance of acid phosphatase to oxidative stress

Oxidative stress was generated by Fe$^{2+}$/H$_2$O$_2$/Ascorbate system using method of Fernandes et al., (2003). The purified enzyme was preincubated with 0.2 M Fe$^{2+}$ and 10 mM Ascorbate, in 0.1 M acetate buffer pH 4.8 at 37°C. Oxidation was initiated by adding 14.5 mM Hydrogen Peroxide. At different intervals the reaction was stopped by adding 1 mM EDTA. Residual activity was determined by using O-P-L-Tyrosine.

18. 2D gel electrophoresis

18.1 Sample preparation

The homogenate (prepared in Tris buffer pH 7.5) was treated with 10 volume ice chilled acetone and kept at −20 °C for 3 h for protein precipitation followed by centrifugation at 7500 rpm for 10 min at 4 °C. Pellet was collected and air dried for 5 min and dissolved in 150 μl freshly prepared lysis buffer (20 mM Tris (pH 7.5), 7 M Urea, 2 M Thiourea, 4% CHAPS, 10 mM DTT, 1 mM EDTA, protease inhibitors cocktail (20 μg/μl) and 1 mM PMSF).

18.2. Isoelectric focusing (IEF)

The IEF was conducted according to our standard laboratory protocol (Rathaur et al., 2011). The pre-cast Biorad immobilin Dry strips (IPGstrip; 11 cm, pH range 4–7) were first rehydrated with rehydration stock solution (8 M urea, 0.5% (w/v) CHAPS, 0.2% (w/v) DTT 0.5% (v/v) IPG buffer and 0.002% bromophenol blue) containing 1 mg protein. Further, the strips were dipped in cover dry solution containing pharmalyte and left for 10 h at 25 °C. For IEF, rehydrated strips were processed in IPGphor IEF unit (BioRad) for 8–10 h. The program was set initially for 0.01 h at 300 V, followed by 1.30 h at 3500 V, 2 h at 5000 V, 7 h at 8000–13,000 V; and terminated after reaching up to 14, 000 V.
**18.3. Second dimension electrophoresis**

The focused strips were equilibrated in 10 ml equilibration solution [50 mM Tris–HCl, pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (v/v) SDS, 0.002% Bromophenol blue and 1% DTT] for 20 min at room temperature and washed twice with 1×SDS gel running buffer. The strips were then loaded on 10% SDS-PAGE for second dimension separation gel system and run at 150 volts. The gels were stained with colloidal commassie stain.

**18.4. Image analysis**

The PDQUEST image analysis software was used for quantitative analysis of spots. Experiment was conducted twice and resulted spots were normalized by using mean spot volume of three unchanging spots in two conditions. A Scatter plot-pairs signed-ranks test was performed to analyze whether the observed differences were statistically significant. Spot intensity/volume detected above and below 1.5 fold change was considered as significantly up-regulated or down-regulated respectively.

**18.5. MALDI-MS/MS analysis**

The spots significantly regulated were excised from the commassie stained gel, trysin digested and processed for MALDI-MS/MS analysis as described earlier.

**19. Ex vivo effect of antifilarial drugs/ PTP inhibitors on S. cervi**

**19.1 Exposure of adult parasites/mf to drugs/compounds**

For *ex vivo* experiments, equal number of adult female *S. cervi* (15 parasites in 30 ml)/ mf (2 x 10^6 mf/ 0.5 ml) were incubated in maintenance medium containing antifilarial drugs/inhibitors for 6 h at 37°C and 5% CO_2_. Parasites incubated only in maintenance medium served as control. After the desired period of incubation, parasites were recovered, washed with fresh PBS and either stored at -20°C, fixed in paraformaldehyde...
or, homogenized and centrifuged for determination of different oxidative stress, apoptotic and metabolic parameters.

19.2. Effect of drugs on parasite motility

The motility of parasites was performed by visual inspection or through Motic B1 series microscope (in case of mf) at different time intervals of exposure to antifilarial drugs/inhibitors. The parasites incubated without inhibitors served as Control. The parasite motility was scored as -, no movement; +, least active; ++, less active; +++, moderately active; and ++++, highly active. The treated parasites were transferred into fresh medium (without inhibitor) after 4 h and the recovery in their motility was compared to control group. Parasites incubated in compound containing medium were assessed visually till 4 h and scored either positive or negative (+/-) depending on their motility. The recovery of motility was recorded by keeping the parasites in fresh medium.

19.3 Effect on parasite viability

The MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] reduction assay was carried out to determine the parasite viability according to the method of Mosmann et al (1988) with slight modifications. The parasites were incubated in PBS medium containing 1.0 ml of 0.5 mg/ml MTT for 2 h at 37 °C. The parasites were then transferred to a 200 µl of dimethyl sulphoxide (DMSO) to solubilize the formazan crystals. After 1 h the medium was then carefully removed without disturbing the dark blue formazan crystals. The optical density of the resulting formazan solution was determined on a microplate reader (BioRad) at a wavelength of 540 nm.
19.4. Effect on oxidative stress parameters

19.4.1 Estimation of total Reactive Oxygen Species (ROS)

The ROS production was measured using colorimetric assay. The method is based on the conversion of Nitro blue tetrazolium (NBT) into blue formazan crystals in the presence of the superoxide anion. The assay was performed in the control and treated worms by the method described by Choi et al, 2006 with minor modifications. The *S. cervi* worms were incubated with 2% NBT solution for 1 h at RT. After the incubation, worms were washed with 10 mM phosphate buffer saline followed by a second wash with methanol. Subsequently, the worms were suspended in 2 M KOH to disrupt the cell membrane and further DMSO was used to dissolve the formazan crystals with gentle shaking for 10 min at RT. The absorbance was recorded at 620 nm.

19.4.2 Measurement of Glutathione level

The GSH level was measured according to the method of Ellman, 1959 with minor modifications [23]. Equal volumes of 5% meta-phosphoric acid and the adult worm extract were mixed, centrifuged at 3000 rpm for 10 min at 4ºC and supernatant was collected. A total of 2 ml reaction mixture containing 100 µl of supernatant, 1.88 ml 0.1 M potassium phosphate buffer, pH 8.0 and 0.02 ml of 4% DTNB [5,5'-dithiobis-(2-nitrobenzoic acid)] was incubated at room temperature (RT) for 15 min in dark. The absorbance of coloured product was recorded at 412 nm. The level of GSH was calculated from the standard graph prepared using GSH.

19.4.3 Thioredoxin reductase assay

The TrxR activity in *S.cervi* extract (mitochondrial and cytosolic) was spectrophotometrically measured by the reduction of DTNB in the presence of NADPH according to the method of Holmgren and Bjorsnstedt (1995). The assay mixture (1 ml)
contained 0.1 M potassium phosphate buffer (pH 7.0) containing 2 mM EDTA with final concentration of 10µM NADPH and 50µM DTNB. The reaction was started by the addition of NADPH and the increase in absorbance at 412 nm was monitored every minute for 3 min at 25°C. One unit of enzyme activity is defined as the NADPH-dependent production of 2 µmol of 2-nitro-5-thiobenzoate (ε 412 nm 13.6 mM⁻¹ cm⁻¹) per minute.

19.4.4 Glutathione reductase (GR) activity

The GR activity was assayed according to the method described by Carlberg and Mannervik (1981). The reaction was initiated by the addition of 0.1 mM NADPH to the mixture of enzyme in 50 mM potassium phosphate buffer (pH 7.0) containing 2 mM EDTA and 0.5 mM GSSG. The change in optical density was monitored at 340 nm for 3 min by a UV–Vis spectrophotometer. One unit of GR activity is defined as the amount of enzyme that catalyses the reduction of 1µmole of NADPH per minute (ε 340 nm for NADPH 6.22 mM⁻¹ cm⁻¹).

19.4.5. Glutathione-S-transferase (GST) activity

The GST activity in crude extract was estimated according to the method of Habig et al., 1974 using 2 mM GSH and 1 mM CDNB (1-chloro-2,4-dinitrobenzene) as substrate. The assay was initiated by mixing 50 µl worm extract in 0.5 ml 0.1 M phosphate buffer, pH 6.5 at 25°C. One unit of enzyme activity is defined as the amount of enzyme catalysing the oxidation of 1 millimole of substrate (CDNB)/ ml/ min at 25°C.

19.4.6. Estimation of protein carbonyl (PC) content

The protein oxidation was monitored by measuring protein carbonyl content in the parasites by using 2, 4–dinitrophenyl hydrazine (DNPH) (Levine et al., 1994. Briefly, 10% cold trichloroacetic acid (TCA) was added to the extract in 1:1 ratio followed by centrifugation at 6000 g for 5 min at 4°C. 10 mM DNPH dissolved in 2 N HCl was added
to the pellet and was allowed to stand in dark at RT for 1 h with intermittent vortexing. It was then centrifuged at 6000 g for 5 min at 4°C. The supernatant was discarded and 20% TCA was added to the pellet followed by centrifugation at 6000 g for 5 min at 4°C. The pellet was washed 2-3 times with ethanol: ethyl acetate mixture (1:1) till the pellet became clean. The final pellet was suspended in 800 µl of 6M guanidium hydrochloride and the absorbance was measured at 370 nm. The molar extinction of 22,000 x 10⁶ µM⁻¹cm⁻¹ was used for calculations.

19.4.7 Estimation of lipid peroxidation

The lipid peroxidation was estimated by the method of Ohkawa et al., 1979 by measuring the malondialdehyde (MDA) level. 100 µl of 10% SDS was added to 300 µl of extract and incubated for 5 min at RT. 600 µl of 20% acetic acid was added to the reaction mixture and incubated for 2 min at RT. Further 600 µl, 0.8%, 2-thiobarbituric acid (TBA) was added and the final reaction volume was made up to 3 ml with distilled water. This was boiled for 1 h in water bath. The reaction mixture was allowed to cool at 4°C and then centrifuged at 10000 g for 5 min. The supernatant containing the active TBA reactive substances (TBARS) was collected and the absorbance was measured at 532 nm. The molar extinction coefficient of MDA used in the calculation was 1.53 x 10⁵ M⁻¹cm⁻¹.

19.4.8 NADPH oxidase activity

For NADPH oxidase activity, treated worms were homogenized in 50 mM phosphate buffer at pH 7.2 and 0.25% SDS. The homogenate was centrifuged at 2000 rpm for 10 min at 4°C in 10 mM phosphate buffer at pH 7.2 containing 1 mM MgCl₂, 80 µM cytochrome c and 2 mM sodium azide were added to 100 µl of supernatant obtained after centrifugation to make the final reaction volume to 1 ml. NADPH was added finally to
initiate the reaction. The absorbance was read at 550 nm (Heyneman and Vercauteren, 1984).

19.5 Effect on apoptotic markers

19.5.1 Caspase-3 activity assay

The activity of caspase-3-like proteases in the extract was determined using a colorimetric caspase-3 (Sigma) assay with minor modifications ((Schroeter et al., 2001)). The assay was carried out in a 96 well plate. Briefly, a 100 µl reaction mixture containing 30 µl of adult worm extract and 10 µl of the caspase-3 substrate acetyl-Asp-Glu-Val-Asp-p-nitroanilide (final concentration, 200 µM) in assay buffer was incubated for 90 min at 37°C. The absorbance of the yellow colored product formed was taken at 405 nm using a microtiter plate reader. One unit of the caspase-3 activity was defined as the µmols of p-nitroanilide (Extinction coefficient 9.96 mM⁻¹cm⁻¹sec⁻¹) released per min per ml at 37°C.

19.5.2 Calpain activity

The calpain activity was assayed using 35µL (150-200µg) of adult parasite extract from cytosol or mitochondria, 7 µL fluorogenic substrate, N-Succinyl-Leu-Leu-Val-Tyr 7-Amido-4-Methylcoumarin (from 5mM stock in DMSO) diluted in 315 µL with 50 mM Tris HCl pH 7.5 and make up the reaction volume to 700 µL with reaction buffer (50mM Tris–HCl, pH 7.5, 5mM MgCl₂, 1mM DTT, and 1mM ATP) at 37 °C for 90 min. The hydrolyzing activity of the enzyme was determined by fluorimetric quantification of the substrates Suc-LLVY-AMC at 380 nm excitation/440nm emission, using a Nis –Elements F 3.0 fluorescence spectrophotometer.

19.5.3 Tyrosine phosphatases assay The Tyrosine phosphatase activity was assayed as described in earlier sections of this chapter.
19.5.4 Cytochrome c oxidase (COX) Assay

The Cytochrome c oxidase assay is based on the oxidation of ferrocytochrome c to ferricytochrome c in the presence of COX and observing the decrease in absorbance at 550 nm (Trounce et al., 1996). For enzyme assay, the mitochondria were isolated from the parasites according to the method of Ericson et al. (2012) with minor modifications. Briefly, 10% adult female extract was prepared in homogenization buffer containing 0.32 M sucrose, 1 mM EDTA and 10 mM Tris-HCl (pH 7.8). The homogenate was then centrifuged at 1000 g for 10 min at 4°C to pellet down the nuclei and cell debris. The supernatant was collected and again centrifuged at 1000 g for 10 min at 4°C to remove the remaining nuclear material. The supernatant was taken and centrifuged at 13000 g for 20 min to obtain the mitochondrial pellet. The Ferrocytochrome c (substrate) was prepared by adding 10 μl of 250 mM DTT solution to cytochrome c solution (10 mM). The solution was mixed well and incubated for 20 minutes at room temperature (RT). The reaction was started by adding 50 μl substrate solution to the reaction mixture containing 50 mM Tris buffer, pH 8.0, 10 μl NP-40, mitochondrial extract (0.5–2 mg) in a final reaction volume of 100 μl. The change in absorbance was read continuously at regular interval of 30 seconds for 2 mins at 550 nm. The activity of COX was calculated using the extinction coefficient of the reduced cytochrome c solution (19.6 mM⁻¹cm⁻¹sec⁻¹) at 550 nm.

19.5.5 Expression analysis of ced-3 and ced-9 genes by RT-PCR

Total RNA was isolated using Trizol reagent according to the laboratory standardized protocol and was reverse-transcribed into single stranded cDNA with Moloney murine leukemia virus reverse transcriptase and oligo (dT) primer. The ced-3 and ced-9 genes were amplified using specific primers according to Saini et al., 2012 The following conditions were used for the PCR reaction: 10X PCR buffer mix, 10 mM dNTP mix, 5
pmol of each primer, and 1 unit of Taq DNA polymerase in a total volume of 25 ml. The cDNA was denatured at 95°C for 2 min, annealed at 47°C for 1 min, and elongated at 72°C for 3 min for 35 cycles. PCR products were separated on a 1% agarose gel, and photographs were taken using gel documentation system (BioRad). β-tubulin was used as the loading control.

19.5.6 DNA fragmentation analysis by agarose gel electrophoresis

Total genomic DNA was isolated from adult parasite according to the method of Smith and Rajan (2000) with slight modifications. Briefly, a small piece of adult female parasite was homogenized in lysis buffer, pH 8.0 containing 20 mM Tris buffer, 50 mM EDTA, 0.5% SDS, 100 mM NaCl, 1% β-mercaptoethanol and 0.1 mg/ml proteinase K and incubated at 55 °C for 3 h. The lysates were then extracted with phenol-chloroform-isoamyl alcohol (25:24:1) and centrifuged at 16000 g for 10 min. Subsequently, the upper layer of supernatant was treated with 3 M sodium acetate and 100% cold ethanol and again centrifuged at 16000 g for 10 min. The pellet obtained was washed twice with 70% ethanol and resuspended in 10 mM Tris-EDTA (TE) buffer, pH 8.0. The extracted total genomic DNA was loaded on 1.0 % agarose gel containing ethidium bromide and bands were visualized under UV trans-illuminator.

20. Western blotting

The purified protein/ surface antigen was electrophoretically separated by a preparative 10% SDS-PAGE gel and electrotransferred onto PVDF membrane as described by Lunde et al., (1988). The monoclonal antibody (clone FG6-1G, Calbiochem, San Diego, CA) generated against the catalytic domain of a recombinant human placental PTPase 1B (1µg/ml) as primary antibody and peroxidase conjugated rabbit anti-mouse IgG (1:5000) as secondary antibody were used. ImageJ 7.0 software was used to quantify the western blot bands.
21. **Tissue fixation and immunostaining**

The adult female parasites were incubated at 37°C, 5 % CO₂ for 4 h in Krebs-Ringer Bicarbonate (KRB) medium in the presence/absence of Aspirin and SK7, methylated chalcone. The parasites were then fixed in 4% paraformaldehyde for 20 mins and dehydrated in graded series of ethanol. The parasite paraffin sections were cut at 6 µm using a Leica microtome. The sections were first rehydrated in different grades of ethanol and incubated in a blocking buffer (5 % skimmed milk in phosphate buffer saline PBS) for 2 hours at RT to avoid nonspecific binding. They were then incubated overnight at 4°C with primary antibody (anti human PTP1B; 1µg/ml) in phosphate buffer saline tween (PBST). After washing with PBST, they were incubated with secondary antibody (goat anti mouse IgG, FITC conjugated, 1:2000) in PBST for 2 h in dark to avoid photobleaching. The sections were then washed, incubated with phalloidin-TRITC (1:200) in PBS for 1 h at RT and mounted in DABCO. The images were taken using Zeiss LSM-510 Meta confocal microscope at the National confocal facility, Banaras Hindu University.

22. **ELISA**

NUNC 96 wells ELISA plates were coated with purified RPTP (2μg/ml) diluted in 0.096M carbonate buffer pH 9.6 and incubated overnight at 4°C. A standard protocol of Rathaur et al (1987) was followed for whole IgG detection in filarial patient sera using horseradish peroxidase conjugate (Sigma A8792; 1:5000 in PBS–0.05%) and orthophenyldiamine (OPD; 1mg/ml, Sigma P9187) for color development. For IgG subclasses detection, biotin labeled IgG1 and IgG4 (1:1000 in PBS) secondary antibodies were used. After incubation with streptavidin peroxidase (1:1000 in PBS) reaction was developed as described with IgG peroxidase conjugate. Immunoplates were coated with RPTP (2μg/ml) and incubated overnight at 4°C. After being blocked and washed, the plates were incubated with diluted serum samples in 1:100 dilutions (primary antibody). Antigen-specific IgG1 and IgG4 were
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detected by using horseradish peroxidase-conjugated anti-IgG1 (Sigma B6775) and IgG4 (Sigma B3648). The reaction was developed with OPD and absorbance was read at 492 nm. For IgE detection, alkaline phosphatase-conjugated monoclonal anti-human IgE (Sigma A-3076; 1:5000 in PBS–0.05% Tween-20) was used and color was developed with para nitro phenyl phosphate (pNPP, Sigma N4645, 1 mg/ml in diethanolamine buffer, Sigma N-2765). The optical density was determined spectrophotometrically using ELISA reader (Bio-Rad) at 405 nm.

2.23. Statistical analysis

All experiments were done in triplicate (n=3) and the data were expressed as mean ± SEM. Mean, SEM and statistical significance were calculated using the Graph Pad Prism 5.0. Statistical significance between the treated and the control worms was determined by using a two-tailed Student’s t-test and P < 0.05 was considered significant.