Materials and Methods

Materials
All chemicals were obtained from Sigma-Aldrich (St. Louis, Missouri, USA) except N-1 napthyl ethylene diamine dihydrochloride (Loba Chemie Pvt. Ltd., Mumbai, India), phenazine methosulphate (PMS) and phenylmethylsulfonylfluoride (Sisco Research Laboratories, Mumbai, India), JC-1, Z-Val-Ala-DL-Asp (methoxy)-fluoromethylketone (Z-VAD-FMK) and Fluo 4AM (Invitrogen, California, USA), Caspase-3/ CPP32 Colorimetric assay kit (Biovision, California, USA), Quick apoptotb DNA ladder detection kit, Invitrogen, California, USA (Catalog no. KH01021). Diff-Quicx® (Medion Diagonostics, Duedingen, Switzerland), antibodies against phospho-p38 MAP< (Th180/Tyr182) (3D7), p38 MAPK, ERK and β-actin (Cell Signalling Technology. Frankfurt Am Main, Germany). Sulphanilamide, p38 MAPK inhibitor, SB203580, MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium], inner salt (Promega, Madison, Wisconsin, USA), 4,5 dianminofluorescein -2 diacetate (DAFDA, Cayman Chemicals, Ann Arbor, Michigan, USA), 5,6 chloromethyl, 2',7'- dichloro hydrofluoresceine diacetate (CMH2DCFDA, Invitrogen, California, USA) 16 well _ab-tek chamber slides, (Nalgen Nunc International, Rochester, New York USA), RNAqueous® Kit (Ambion, Austin, Texas, USA), One Step RT-PCR kit from Qiagen (Hilden, Germany), primers (Sigma Genosys, India), antibodies against phosphorylated p38 (pp38) MAPK. extracellular stress-related kinase-1/2 (p-ERK), anti GAPDH and alkaline phosphatase-conjugated anti-mouse immunoglobulin G (Santa Cruz Biotechnology Inc, Santa Cruz, California, USA), anti-mouse IL-12 and biotinylated anti-mouse IL-12 (eBioscience, Kensington South Australia, Australia). A 50 mM stock solution of Berberine chloride was prepared in methanol and stored at -20°C.

Parasite culture
Promastigotes of Indian Leishmania donovani strains (Ganguly et al., 2006), previously isolated from patients with VL were routinely cultured at 24°C in Medium 199 (M199) supplemented with 10% fetal bovine serum (FBS), penicillin G (50 IU/ml) and streptomycin (50 µg/ml). Log phase promastigotes were subcultured every 72 - 96 h, inoculum being 1 x 10⁵/ml.

For infection of macrophages, stationary phase promastigotes i.e. 4-5 days old culture were centrifuged and the pellet resuspended in Schneider's medium (pH 5.5, da Luz et al., 2009) 24 h prior to the experiment.
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Cell culture
A human non adherent leukemic monocyte lymphoma cell line (U937) was subcultured in RPMI 1640 medium supplemented with 10% FBS, penicillin G (50 IU/ml) and streptomycin (50 μg/ml), at 37°C in a humidified atmosphere containing 5% CO2, log phase cells were subcultured every 72 h, inoculum being 5 x 10^5/ml.
(These cell lines were obtained from National Centre for Cell Science, Pune, India).

Animals
BALB/c mice (average wt, 25-30 gm) were maintained at standard temperature (25 ± 5°C), 12 h day/night cycle, fed a standard pellet diet and provided water ad libitum. All experimental protocols received prior approval from the Institutional Animal Ethical Committee.

In vitro evaluation of anti-promastigote activity
The anti-leishmanial activity of Berberine chloride was established in promastigotes, and cell viability was measured using the modified MTS- PMS assay (Ganguly et al., 2006). Briefly, log phase promastigotes (2 x 10^5 cells/200μl of M199 medium/well) were incubated with Berberine chloride (0 – 50 μM) for 48 h. MTS [3-(4, 5 dimethylthiazol-2-yl) 5-(3-carboxymethoxyphenyl)-2-(4-sulfonyl)-2H-tetrazolium, inner salt] (2.0 mg/ml) and PMS (Phenazine methosulphate) (0.92 mg/ml) were added in a ratio of 5:1 (20 μl per well) and plates incubated for 3 h at 37°C; resultant absorbances were measured at 490 nm in an ELISA reader. Accordingly, the specific absorbance that represented formazan production was calculated by subtraction of background absorbance from total absorbance. The mean % viability was calculated as follows:

\[
\text{Mean specific absorbance of treated parasites} \times 100
\]
\[
\text{Mean specific absorbance of untreated parasites}
\]
where specific absorbances were determined by subtracting the background absorbance of medium. The results were expressed as the IC_{50} i.e. the concentration that inhibited 50% cell growth, which was enumerated by graphical extrapolation using Graph Pad Prism software (version 5). Accordingly, the IC_{50} for each drug, i.e., the concentration of drug that decreased the percent viability by 50% was graphically extrapolated by plotting percent viability against the respective drug concentration.
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Ex vivo anti amastigote activity in macrophages
Peritoneal macrophages lavaged from BALB/c mice (Sen et al., 2010) were seeded in 16 chambered slides (1.5 x 10^6/200 µl/well of complete RPMI-1640 PR' medium) and after a 2-4 h incubation at 37°C, 5% CO₂, the supernatants (containing lymphocytes) were gently removed and kept aside at 37°C, 5% CO₂. Adherent macrophages were then infected with stationary phase L. donovani promastigotes (previously preconditioned at 25°C for 24 h in Schneider's insect medium, pH 5.5, da Luz et al., 2009) at a macrophage: parasite ratio being 1:10 and incubated for 5 h at 37°C, 5% CO₂. After removal of non-internalized parasites, macrophages were then co-cultured with supernatants in the presence or absence of Berberine chloride (0 - 25 µM) for 72 h., after which cells were fixed, Giemsa stained (1:7 dilution in deionized water, pH 6.8) and examined microscopically for intracellular amastigotes. At least 100 macrophages per well were counted to calculate the percentage of infected macrophages. The infection rate of treated macrophages was normalized to 100% for further analysis of results.

Safety index of Berberine chloride
To evaluate the cytotoxic activity of Berberine chloride, murine macrophages (2 x 10^5 /200µl/well) were incubated with Berberine chloride (0 - 100 µM) at 37°C, 5% CO₂ for 48 h and cytotoxic effects enumerated by the MTS assay (Ganguly et al., 2006). The safety index was calculated as follows:

\[ \frac{IC_{50} \text{ of compound in macrophages}}{IC_{50} \text{ of compound in amastigotes}} \]

Generation of reactive oxygen species (ROS) in promastigotes
Generation of ROS in promastigotes were measured using 2, 7 dichlorodihydrofluorescein diacetate (H2DCFDA), which within the cell, is cleaved by non-specific esterases into impermeable non-fluorescent H2DCF, that subsequently gets oxidized by ROS into a highly fluorescent compound DCF. Therefore, the intensity of fluorescence is directly proportional to the amount of ROS present. To study the effect of Berberine chloride on generation of ROS, log phase promastigotes (1x 10^6/ml) following incubation with Berberine chloride (0 - 50 µM, 3 h) were washed with phosphate buffered saline (0.02 M, pH 7.2, PBS) and incubated with H2DCFDA (50 µM) for 45 min at 37°C and fluorescence acquired on a flow cytometer (FACS Calibur, Becton Dickinson, USA) using forward vs. side scatter to gate the parasite population and a FL1 histogram to quantify fluorescence of viable parasites.
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(Mandal et al., 2007; Saha et al., 2009). The subsequent analyses were done using BD CellQuest Pro software.

**Measurement of non protein thiols in promastigotes**

To examine the effect of Berberine chloride on levels of non protein thiols, mercury orange (MO) was used as it reacts with all sulfhydryl (-SH) groups generating a non permeable fluorescent product (a mercurial compound that binds stoichiometrically to sulfhydryl groups) which is retained within cells. However, as the reaction rate of MO with non protein thiols is much faster than with protein thiols, incubation for 5 min on ice allowed it to react selectively with non protein –SH groups; accordingly, the fluorescence represented the level of cellular non protein thiols (O’Connor et al., 1988). Briefly, promastigotes (1x10^6 cells /ml) in serum free M199 were incubated with Berberine chloride (0 - 50 μM) at 37°C for 3 h. Cells were then washed with ice cold PBS, resuspended in MO (500 μM in acetone) and incubated precisely for 5 min on ice. Cells were immediately washed thoroughly with chilled PBS and acquired on a flow cytometer (FACS Calibur, Becton Dickinson, USA) using forward vs. side scatter to gate the parasite population and a FL3 histogram to quantify fluorescence of viable parasites and analyzed using BD CellQuest Pro software (Saha et al., 2009).

**Analysis of phosphatidylserine externalization in L. donovani promastigotes**

Translocation of phosphatidylserine from the inner aspect to the outer leaflet of the plasma membrane occurs during apoptosis of metazoan and unicellular parasites (Sudhandiran and Shaha, 2003; Mehta and Shaha, 2004; Debrabant et al., 2003; Koonin and Aravind, 2002). The high binding affinity of Annexin V, a Ca^{++} dependent phospholipid binding protein towards phosphatidylserine helps ascertain whether parasite death is mediated via apoptosis or necrosis, the latter being identified by P, a non permeable stain having affinity towards nucleic acids, and selectively entering necrotic cells. Therefore, co-staining of Annexin V and PI helps discriminate between live parasites (PI and Annexin V negative), cells in early apoptosis (Annexin V positive, PI negative), cells undergoing late apoptosis (both Annexin V and PI positive) or necrotic cells (PI positive, Annexin V negative).

Double staining for annexin V–FITC and propidium iodide (PI) was performed (Dutta et al., 2007a; Saha et al., 2009). Briefly, promastigotes incubated with an IC_{90} conc. of Berberine chloride (50 μM) for 0 - 6 h at 24°C were centrifuged (1000 g x 10 min), washed twice in
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PBS and resuspended in annexin V binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂). Annexin V-FITC was added according to the manufacturers' instructions and cells kept for 30 min in the dark at 24°C. Prior to acquisition, PI (0.1 μg/ml) was added and the % positive cells determined on a flow cytometer (FACSCalibur, BD Biosciences, USA) using Cell Quest Pro software.

Measurement of intracellular Ca²⁺ in L. donovani promastigotes

Changes in intracellular Ca²⁺ were monitored using the fluorescent probe Fluo-4 AM, a membrane-soluble dye. Upon entering the cells, the AM or acetoxymethyl ester is cleaved off by the cellular esterases and Fluo-4 binds with intracellular Ca²⁺ and fluorescence is a measure of intracellular Ca²⁺ (Nagamune et al., 2007; Saha et al., 2009). Briefly, log phase promastigotes (2 X 10⁶ cells) were equilibrated with loading medium (Fluo-4 AM, 2.5 μM, pluronic acid F127, 0.02% and sulfipyrinzone, 0.25 mM in M199 medium) at 24°C for 30 min. Cells were then washed with medium containing 0.25 mM sulfipyrinzone (Medium A) and incubated at 24°C for 30 - 60 min to allow for de-esterification of Fluo-4AM. Subsequently, cells were incubated with an IC₉₀ conc. of Berberine chloride (50 μM, 0 - 3 h); in parallel, cells were incubated with a Ca²⁺ ionophore (lomycin 3 μM) and specificity confirmed by addition of a chelating agent, EGTA (5 mM). Cells were washed, resuspended in Medium A and fluorescence measured in a spectrofluorimeter (excitation, 485 nm and emission, 520 nm). To convert fluorescence values into absolute [Ca²⁺]ᵳ, calibration was performed at the end of each experiment and calculated as follows (Mehta and Shaha, 2004):

\[ [\text{Ca}^{2+}]_\text{ᵳ} = \frac{K_d(F - F_{\text{min}})}{F_{\text{max}} - F} \]

where \( K_d \) is the dissociation constant of the Ca²⁺ Fluo 4 complex (345 nM), \( F \) represents fluorescence intensity of Berberine chloride treated cells, \( F_{\text{max}} \) is maximal fluorescence i.e. cells treated with lomycin (3 μM) while \( F_{\text{min}} \) corresponded to minimum fluorescence, i.e. cells treated with lomycin (3 μM) and EGTA (5 mM).

Analysis of mitochondrial transmembrane potential

The mitochondrial transmembrane electrochemical gradient (Δψᵳ) was measured using JC-1 (5, 5', 6, 6'-tetrachloro-1, 1', 3, 3'-tetraethylbenzimidazolylcarbocyanine iodide), a cell permeable, cationic and lipophilic dye as previously described (Sen et al., 2007; Saha et al., 2009). The loss of mitochondrial membrane potential is a characteristic feature of metazoan apoptosis, also evident in protozoans (Sen et al., 2007; Saha et al., 2009). To measure the
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Mitochondrial membrane potential, JC-1 was used which freely permeates the mitochondrial membrane and forms J aggregates that fluoresce red; accordingly, viable cells stained with JC-1 exhibit a pronounced red fluorescence. An apoptotic stimulus induces a decrease in the mitochondrial membrane potential; JC-1 fails to enter the mitochondria remaining as cytosolic monomers and emits a green fluorescence. Therefore the ratio of J-aggregates/monomers serves as an effective indicator of the mitochondrial energy state of the parasite allowing apoptotic cells to be easily distinguished from their non-apoptotic counterparts (Verma et al., 2007). Briefly, promastigotes incubated with an IC₉₀ conc. of Berberine chloride (50 μM, 0 - 6 h at 24°C) were stained with JC-1 (2 μM in PBS) for 10 min at 37°C. Cells were acquired on a FACS Calibur on the basis of quadrant dot plot to determine monomers and J aggregates and analyzed using Cell Quest Pro software. To set quadrants, promastigotes were treated with H₂O₂ (15 mM, 10 min), representing cells with depolarized mitochondrial membrane potential.

**Cell cycle analysis**

Flow cytometric analysis helped to quantify the percentage of cells in different phases of the cell cycle, the amount of bound dye, Propidium iodide (PI) representing DNA content. Accordingly, DNA fragmentation that occurs in apoptotic cells is known to inhibit cells not to enter onto the next phase of cell division and causing an increased sub G₀/G₁ peak. Promastigotes (1 x 10⁶) were treated at 24°C with an IC₉₀ conc. of Berberine chloride (50 μM, 0 - 12 h), fixed in chilled ethanol (70%) and kept at 4°C until analysis. Prior to analysis, cells were washed in PBS and the resultant pellet resuspended in DNase-free RNase (200 μg/ml, 0.5 ml) for 1 h at 37°C; cells were then stained with PI (40 μg/ml), acquired on a Flow cytometer (FACS Calibur) and analyzed using Cell Quest Pro software (Sen et al., 2007; Saha et al., 2009).

**DNA fragmentation assay by agarose gel electrophoresis**

To determine DNA fragmentation, total cellular DNA was isolated from *Leishmania donovani* promastigotes treated with Berberine chloride (50 μM, 48 h) according to manufacturer’s instructions and analysed by 1.5% agarose gel electrophoresis containing ethidium bromide (0.5 μg/ml) in TBE buffer and visualized on a Molecular Imager Chemi Doc XRS System (Bio Rad, California, USA) (Verma and Dey, 2004).
Determination of caspase activity in Berberine chloride treated promastigotes

Caspase activity was measured using a commercially available kit as per the manufacturer’s instructions. The assay is based on the spectrophotometric detection of the chromophore p-nitroanilide (pNA) following cleavage from the labeled CED3/CPP32 group substrate DEVD-pNA. The pNA light emission can be quantified using a spectrophotometer or a microtiter plate reader at 405 nm.

Berberine chloride (50 μM, 12h) treated promastigotes were washed with ice cold PBS, cell lysates were prepared and protein conc. estimated. To detect activity of CED3/CPP32 group of proteases, cell lysates (100 μg protein in 50 μl lysis buffer) were combined with 50 μl of 2X reaction buffer (containing 10 mM DTT), substrate DEVD-pNA (4 mM, 5 μl) and incubated at 37°C up to 4h; the emission of pNA was quantified by measuring absorbances at 405 nm. In parallel, *Leishmania* parasites and U937 cells were treated with H₂O₂ (4 mM, 30 min) and Miltefosine (40 μM, 12 h).

To study the biological role of caspases in Berberine chloride induced death, log phase promastigotes (2 x 10⁵ in 200 μl/well) were seeded in 96 well tissue culture plates. Following a 48 h incubation at 24°C with Berberine chloride (0 - 50 μM) in the presence of a pan caspase inhibitor, Z-Val-Ala-DL-Asp (methoxy)-fluoromethylketone (Z-VAD-FMK, 100 μM), cell viability and IC₅₀ values were evaluated by the MTS-PMS assay (Ganguly et al., 2006).

Isolation of neutrophils

Neutrophils were isolated from peripheral blood as previously described (van Zandbergen et al., 2002). Briefly, 3 ml of lymphocyte separation medium 1077 was layered onto 5 ml of Histopaque® 1119 to which 4 ml of blood was layered. Following centrifugation (800 x g, 25 min., room temperature), the first interphase layer, rich in lymphocytes and monocytes was discarded; the two lower granulocyte rich interphase layers were collected and washed with phosphate buffered saline (PBS, 0.02 M, pH 7.2) at 250 x g for 10 min. The pellet was resuspended in RPMI 1640 medium containing 10% FCS and layered on a discontinuous Percoll® gradient consisting of multiple densities, namely 1105 g/ml (85%), 1100 g/ml (80%), 1093 g/ml (75%), 1087 g/ml (70 %), 1081 g/ml (65%) from bottom to top, respectively. The gradient column was centrifuged (800 x g for 25 min) followed by collection of the interphase between Percoll layers (80% and 85%); cells were collected, washed with PBS and resuspended in
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medium (RPMI 1640 medium supplemented with 20% heat-inactivated fetal calf serum). Cell viability and purity was checked by trypan blue exclusion and Diff-Quick® staining of cytocentrifuged slides respectively and was >99%.

Morphological evaluation for apoptosis in neutrophils
Nuclear morphology was assessed on Diff-quick stained cytocentrifuged slides; briefly, neutrophils (1 x 10^5 cells/100 μl medium) were centrifuged (400 x g for 5 min) in a cytocentrifuge, Cytospin 3 (Shandon, Frankfurt, Germany). Slides were air dried, fixed and stained in Diff-Quick® staining set as per the manufacturer’s instructions. Cell morphology was examined under oil immersion light microscopy and >200 cells/slide were graded as apoptotic or non apoptotic based on morphological changes emphasizing separation of nuclear lobes and presence of dark stained pyknotic nuclei considered as characteristic feature of apoptosis (Payne et al., 1994; Squier et al., 1995). Each sample was studied in duplicate and independently counted by two individuals to minimize inter and intra individual variance.

Determination of parasite load in neutrophils
Stationary phase L. donovani promastigotes were incubated with neutrophils (parasite: neutrophil ratio being 10:1) at 37°C, 5% CO₂ for 3 h following which Berberine chloride (0 - 50 μM) was added for 18 h; cells were then fixed, stained with Diff-Quick® and examined microscopically for intracellular parasites.

Measurement of oxidative stress in neutrophils
To compare the degree of oxidative stress present in uninfected vs. Leishmania infected neutrophils, cells (5 x 10^5/ml) were incubated with stationary phase promastigotes, (parasite: neutrophil ratio being 10:1) for 3 h followed by addition of Berberine chloride (0 - 50 μM) up to 18 h. Following a 3 or 18 h incubation, cells were washed twice with PBS, stained with 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CMH₂DCFDA) (50 μM) and incubated at 37°C for 30 min. Cells were finally washed twice with PBS, resuspended in sheath fluid and acquired on a Flow Cytometer.

Annexin-V binding assay
Non-infected or Leishmania infected neutrophils, (5 x 10^5/ml), incubated with Berberine chloride (0 - 50 μM) for 18 h at 37°C, 5% CO₂ were washed twice with PBS,
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resuspended in Annexin V binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 5 mM CaCl_2 (5 x 10^5 / 100 μl) and stained with FITC labelled Annexin V for 30 min. Cells were washed, resuspended in binding buffer and incubated with Propidium Iodide (PI, 1 μg/ml) before analysing on a Flow cytometer.

**Immunoblotting**

Whole cell lysates of neutrophils (3 x 10^6) were prepared from uninfected and *Leishmania* infected neutrophils. Briefly, cells were centrifuged (216 x g, 10 min), the resultant pellet resuspended in 10 % trichloroacetic acid (500 μl), kept on ice for 10 minutes and after centrifugation (14,000 x g, 5 min, 4°C), the resultant precipitate was washed twice with 100% acetone (500 μl, 14,000 x g for 5 min at 4°C), resuspended in 1X sample buffer and boiled for 5-7 minutes at 100 °C.

Lysates (25 μl) were electrophoresed, transferred on to nitrocellulose membranes which were blocked in Tris buffered saline (TBS, pH 7.2 containing 0.1% Tween-20 and 5% BSA, TBST) for 1 h. Following an overnight incubation at 4°C with the appropriate primary antibody (anti phospho-p38 MAPK, anti p38 MAPK or anti-phospho ERK diluted 1:1000 in 5% BSA in TBST), membranes were extensively washed with TBS containing 0.1% Tween-20 for 45 min., and binding detected using horse radish peroxidase-conjugated polyclonal anti-rabbit IgG (diluted 1:5000 in TBST) followed by ECL western blotting detection system (Immobilion™ western).

**Determination of intracellular NO in L. donovani infected macrophages**

Intracellular generation of NO was measured in *L. donovani* infected macrophages using 4,5 diaminofluorescein -2 diacetate (DAF-2DA), which has the propensity to enter the cell, release the diacetate groups of DAF-DA following hydrolysis by cytosolic esterases and the resultant DAF-2 in the presence of NO gets converted into an impermeable and importantly, highly fluorescent triazolofluorescein (DAF-2T) (Chatton and Broillet, 2002, Rathel et al., 2003).

Murine peritoneal macrophages (1 x 10^6/ ml of complete RPMI-1640 PR medium) were seeded in 6-well plates and infected with stationary phase *L. donovani* promastigotes as described above. Cells were then incubated with Berberine chloride (0 - 10 μM) for 24 - 48 h, adherent cells were scraped, washed and resuspended in PBS containing DAF-2
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DA (2.0 μM, Tiscornia et al., 2009, Schachnik et al., 2009) for 30 min at 37°C and the fluorescence was acquired on a FACS Calibur (Becton Dickinson) in terms of the geometric mean fluorescence channel (GMFC) of 10,000 macrophages (as defined by forward and side scatter) and analyzed by BD CellQuest Pro software (BD Biosciences, USA).

Determination of nitric oxide (NO) production in macrophages

Levels of nitrite, a stabilized oxidized product of NO that accumulated in the culture medium and therefore an indicator of NO production, were measured using the Griess assay (Sarkar et al., 2008). The Griess Reagent System is based on the chemical reaction which uses sulfanilamide and NED under acidic (phosphoric acid) conditions in the presence of nitrite to yield an azo compound that can be measured spectrophotometrically. This system detects NO₂ in a variety of biological and experimental liquid matrices such as plasma, serum, urine and tissue culture medium.

Briefly, mouse peritoneal macrophages (1 x 10⁶/ml of complete RPMI-1640 PR⁻ medium) were seeded in 6-well plates and incubated for 2-4 h to allow for adherence of macrophages. The supernatants (containing lymphocytes) were gently removed and kept aside. The macrophages were then infected with L. donovani promastigotes, the macrophage: parasite ratio being 1:10 for 5 h at 37°C, 5% CO₂. Non-internalized parasites were removed and infected macrophages then co-cultured with supernatants containing lymphocytes and treated with Berberine chloride (0-10 μM) for 48 h. Supernatants were collected at 48 h and NO measured using Griess reagents, NED (0.1% in distilled water) and Sulphanilamide (1% in 5% H₃PO₄). A standard curve was generated using NaNO₂ (0 - 100 μM) (Sarkar et al., 2008).

Reverse transcriptase polymerase chain reaction (RT-PCR) in macrophages

Total RNA was isolated from normal and Leishmania infected mouse peritoneal macrophages obtained from BALB/c mice that were treated with Berberine chloride (2.5 - 10 μM) for 18 h, using the RNAqueous® Kit (Ambion, Inc.). Subsequently, RT-PCR was carried out with the one-step RT-PCR kit (Qiagen, Inc.) using RNA (200 ng/reaction) that was reverse-transcribed into cDNA and amplified, using gene-specific primers for β-actin (Sense: 5'-TGGAATCCTGTGCGATCCCATGAAAC-3', Anti-sense: 5'-
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TAAAACGCAGCTCAGTAACAGTCCG-3'), IL-12 p40 (Sense: 5'
CAGAAGCTAACCATCTCCTGGTTT-3', Anti-sense: 5'-
TCCGGAGTAATTTGGTGCTTCACAC), IL-10 (Sense: 5'
CTGGGAAGACCAAGGTGTCTAC-3', Anti-sense: 5'-
GAGCTGCTGCAGGAATGATGA-3') and iNOS (Sense: 5'-CATGGCTTGCCCCTGGAAAGTTTCCTCATCG-3' Anti-sense:
5'-GCAGCATCCCTCTGATGGTGCATCCG-3'). For reverse transcription, all samples
were subjected to an initial incubation at 50°C for 30 min followed by an initial PCR
activation (95°C for 15 min) as per the manufacturer's instructions. Samples underwent
35 cycles of denaturing (94°C for 30 s), annealing (58°C for 45 s) and extension (72°C
for 30 s) (Ganguly et al., 2007). After a terminal extension step at 72°C for 10 min, RT-
PCR products were resolved by electrophoresis on agarose gels (1.5 %) containing
ethidium bromide (0.5 μg/ml) and visualized with the Molecular Imager Chemi Doc XRS
System (Bio Rad, California, USA). The extent of IL-12 p40, IL-10, iNOS expression and
β-actin was quantified by densitometric analysis using Versa-doc, USA, software being
Quantity one - 4.6.2 (basic).

Measurement of cytokines by sandwich ELISA

Enzyme-linked immunosorbent assay (ELISA), also known as an enzyme immunoassay
(EIA), is a biochemical technique used to detect the presence of an antibody or an
antigen in a sample. The sandwich ELISA measures the amount of antigen sandwiched
between two layers of antibodies (i.e. capture and detection antibody). The antigen to be
measured must contain at least two antigenic sites capable of binding to both the
capture and detection antibody. Both monoclonal and polyclonal antibodies can be used
as the capture and detection antibodies in sandwich ELISA systems.

Levels of mouse cytokines present in supernatants of uninfected and L. donovani
infected macrophages treated with Berberine chloride (0-10 μM, 24 h) were measured
using an ELISA kit as per the manufacturer's instructions. Briefly, capture antibody for
IL-12p40 in coating buffer (PBS, 0.02M, pH 7.2) was added to wells (2 μg/ml, 100μl/well)
and incubated overnight at 4°C, wells were washed thrice with wash buffer and to
prevent non-specific binding, 200 μl/ well blocking buffer (PBS+ 2% FCS) was added
and incubated for at least 1 h at room temperature. After incubation, wells were washed
twice with wash buffer and cytokine standard of IL-12p40 (31.25 pg/ml – 2000 pg/ml,
diluted in dilution buffer) as also supernatants of uninfected and L. donovani infected
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Macrophages treated with Berberine chloride were added (100 µl/well). Following an overnight incubation at 4°C, wells were rinsed five times with wash buffer, and biotin-conjugated detection antibody (1 µg/ml, 100 µl/well in assay diluent) was added and incubated for at least 1 h at room temperature. After that, wells were rinsed five times with wash buffer and binding detected using streptavidin-HRP (horseradish peroxidase conjugated, diluted 1:10,000 in assay diluent, 100 µl/well) and incubated for 30 min at 37°C. The wells were rinsed five times with washing buffer, leaving the wash buffer (in the wells) for five min. during each wash. Finally, binding was detected with ABTS (100 µl/well) for 2 h., after which stop solution was added (100 µl/well) and absorbances were measured at 405 nm in an ELISA reader. From the standard curve, the amount of IL-12p40 present in the test samples were calculated.

Western blotting

Peritoneal macrophages isolated from BALB/c mice and infected with stationary phase promastigotes (1:10) were treated with Berberine chloride (10 µM) for 15 min to 6 h. The adherent cell population was scraped, centrifuged (400 x g x 15 min, 4°C) and resuspended in ice-cold extraction buffer containing Tris-HCl (50 mM, pH-7.5), EGTA (50 mM), β-mercaptoethanol (50 mM) and protease inhibitors, leupeptin (0.33 mM), phenylmethylsulfonyl fluoride (0.2 mM), antipain (0.35 mM), chymostatin (0.24 mg/ml), pepstatin (0.35 mM) and aprotinin (4.8 units/ml). The cell suspensions were sonicated at 4°C and the resultant lysate centrifuged (4,250 x g for 10 min at 4°C) to remove the nuclear fraction. Equal amounts of total cellular proteins (40 µg) were resolved on SDS-polyacrylamide mini gels (SDS-PAGE 10%) and transferred to nitrocellulose membranes and blocked overnight with blocking buffer (TBS+3% BSA). Following blocking of non-specific binding sites, membranes were incubated overnight with anti-phosphorylated p38 or anti- phosphorylated ERK 1/2 (1:500 dilution in 0.5% BSA in TBS) and binding was detected using alkaline phosphatase conjugated anti-rabbit IgG wherein the immuno reactive bands were visualized using nitroblue tetrazolium-5-bromo-4-chloro-3-indolylphosphate, BCIP (Bhattachryya et al., 2001) and quantified densitometrically by Versa doc Imaging system, Bio Rad (USA), software being Quantity one - 4.6.2 (basic).
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Statistical Analysis

Results were expressed as mean ± SEM as indicated. Statistical analysis was evaluated by one way ANOVA (Tukey’s Multiple Comparison Test) or Students t test using GraphPad Prism software, version 4, p<0.05 was considered as statistically significant.
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Preparation of buffers and solutions:
Unless mentioned, reagents were prepared in deionised water or tissue culture grade dimethyl sulfoxide (DMSO).

Berberine chloride (MW = 371.81, 50 mM stock in methanol)
18.6 mg Berberine chloride was weighed and dissolved in 1 ml methanol and vortex. Aliquoted in small volumes, stored at -20°C.

Miltefosine (MW = 497.0, 2 mM stock) 1 mg was weighed and added to 1 ml stock medium (without FBS), mixed carefully, didn't vortex or should not be pipetted vigorously.

SAG (Sodium antimony gluconate, SbV, 1 mg/ml, in stock medium): 1 mg SAG was weighed and added to 10 μl 1(N) HCl, and vortexed vigorously, volume was then adjusted to 1ml with stock medium and vortexed.

Phosphate buffered saline (PBS, 0.2 M or 10X):

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Weigh/addition</th>
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<tbody>
<tr>
<td>Na₂HPO₄ (MW= 178)</td>
<td>35.6 g</td>
</tr>
<tr>
<td>NaH₂PO₄ (MW= 156, 0.2 M)</td>
<td>31.2 g in 1L</td>
</tr>
<tr>
<td>NaCl (0.85 %)</td>
<td>85.0 g</td>
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</tbody>
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Preparation:
Na₂HPO₄ was dissolved in approx. 500 ml of water, NaCl was added to it and the pH was adjusted with NaH₂PO₄ (0.2 M) to pH 6.8; volume was then made up to 1L (it was observed that addition of NaCl changes the pH slightly, so it was added before); the solution was diluted to 1X, checked for pH (7.2- 7.4) and stored at 4°C.

MTS (2 mg/ml): 2.0 mg MTS was dissolved in 1 ml PBS (0.02M, kept in dark), stored at -20°C.

PMS (9.2 mg/ml, 10x): 9.2 mg was dissolved in 1 ml PBS (0.02M, kept in dark); then diluted ten times in PBS (1x, 0.92 mg/ml), aliquoted and stored at -20°C.

N-acetyl L-cysteine (NAC, MW = 163.19, 50 mM): 4.07 mg NAC dissolved in 0.5 ml medium.

Determination of H₂O₂ conc.:
Using Beer's law, the conc. of H₂O₂ was determined; H₂O₂ was initially diluted in 50 mM PBS (1:5) and then further diluted (1:20) and absorbances measured at 240 nm keeping PBS as blank.

Conc. of H₂O₂ in mM = Sp. ab of H₂O₂ at 240 nm/ 0.0436
(Where 0.0436 = ε₃₃₂, molar extinction coefficient of H₂O₂)

H₂DCFDA stock solution (MW = 487.29, 2 mM in PBS containing 3% ethanol)
• 20.0 mg H₂DCFDA was weighed in a beaker (50 ml) placed on ice; dissolved in 600 μl absolute ethanol (kept protected from light).
• Gradually 19.4 ml cold PBS was added, stirred for at least 30 min. Aliquoted in small volumes, stored at -20°C and freeze thawing avoided.

Mercury Orange (MO, MW = 483.31) 2.41 mg MO was weighed and dissolved in 10 ml acetone (500 μM solution). Vortexed and kept in -20°C, to be prepared fresh before experiment.

Griess reagent preparation:
• NED (0.1%): 0.01 g NED was dissolved in 10 ml water.
• H₃PO₄ (5%): 588 μl H₃PO₄ (88%) was added to 9.412 ml water.
Materials and Methods

- **Sulphanilamide (1%)**: 0.1 g sulphanilamide was dissolved in 5 ml 5% H₃PO₄ solution, vortexed vigorously and volume made up to 10 ml. To dissolve bigger particles, the solution was kept at 37°C for a short period (kept on checking). Griess reagent was always prepared fresh NED (0.1%) and Sulphanilamide (1%) in 1:1 ratio.

**NaNO₂ (MW = 69, 10 mM)**: 0.69 mg NaNO₂ was dissolved in 1 ml de-ionised water. A working stock of 100 µM was freshly prepared.

**Solutions for measurement of intracellular Calcium**
- **Fluo4-AM stock (MW = 1096.94, 5 mM)**: 1 mg Fluo4-AM was dissolved in 182.3 µl DMSO in dark. Stored as small aliquots at -20°C, freeze-thawing was avoided.
- **Pluronic acid F127 (20% w/v)**: 2 mg of pluronic acid was dissolved in 10 ml water.
- **Sulfinpyrazone (MW= 404.48, 250 mM)**: 101 mg of sulfinpyrazone was dissolved in 1 ml DMSO.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Addition (final conc.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluo-4 AM (5 mM)</td>
<td>2.5 µl (2.5 µM)</td>
</tr>
<tr>
<td>Pluronic acid F127 (20%)</td>
<td>5 µl (0.02%)</td>
</tr>
<tr>
<td>Sulfinpyrazone (250 mM)</td>
<td>5 µl (0.25 mM)</td>
</tr>
<tr>
<td>RPMI PR+ medium</td>
<td>4987.5 µl</td>
</tr>
</tbody>
</table>

**Medium A**: Washing medium containing 50 µl of sulfinpyrazone (250 mM) was added to 50 ml RPMI PR+ medium.

**EGTA (MW= 380.35, 250 mM)**: 95 mg EGTA was dissolved in 1 ml water.

**JC-1 (MW= 652, 10 mM)**:
- 3.28 mg JC-1 was weighed and dissolved in 0.5 ml of DMSO, in dark. Stored in small aliquots at -20°C, freeze-thawing was avoided.
- A working stock of JC-1 (2 µM) was freshly prepared, in the dark, initially diluted 1:10 in DMSO, and further diluted 1:500 in cold PBS; vortexed (till the dye was properly dissolved, solution was light pink in colour) and kept at 4°C.

**DNase free RNase solution (10 mg/ml)**: 5 mg of RNase was dissolved in 0.5 ml de-ionized water, heated to 65 -70°C for 15 min and allowed to cool and stored at -20°C in small aliquots; freeze thawing was avoided.

**EDTA-Na₂ (MW= 372.24, 0.5 M, pH = 8.0)**: 18.6 g of EDTA was added to 80 ml of ddH₂O and after pH was adjusted to 8.0 with NaOH (MW = 40, 1M), the final volume was made up to 100 ml.

**Tris-Borate-EDTA Buffer (TBE, 1X, 1L)**:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Weigh/addition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris base (MW = 121.14)</td>
<td>5.4 g</td>
</tr>
<tr>
<td>Boric acid (MW = 61.83)</td>
<td>2.8 g</td>
</tr>
<tr>
<td>EDTA-Na₂-salt (0.5 mM)</td>
<td>2.0 ml</td>
</tr>
</tbody>
</table>

**Preparation**:
Tris-base and boric acid were added to 800 ml of ddH₂O, stirred, and after EDTA (0.5 M) was added and the pH adjusted to 8.2-8.4, the volume was made up to 1L.
Materials and Methods

Gel loading dye (Specific for DNA laddering):

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Weight/addition</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDS (0.1%)</td>
<td>0.1 g</td>
</tr>
<tr>
<td>Bromophenol blue (0.25%)</td>
<td>0.25 g</td>
</tr>
<tr>
<td>Glycerol (30%)</td>
<td>3 ml</td>
</tr>
<tr>
<td>ddH2O</td>
<td>to make up 10 ml</td>
</tr>
</tbody>
</table>

Preparation:
SDS, bromophenol blue were mixed in 7 ml of ddH2O, thereafter 3 ml of glycerol was added.

DEPC (0.1%, v/v): 500 µl of DEPC was added to 500 ml of autoclaved water.

Tris-HCl (Tris MW= 121.14, pH. 7.4, 200 mM): 2.42 mg Tris was dissolved in 80 ml of water, thereafter pH was adjusted with HCl (MW = 36.5, 1N) to 7.4, volume made up to 100 ml.

NaCl (MW = 58.44, 400 mM): 233.76 mg was dissolved in 10 ml water.

Preparation of lysis buffer (1 ml):

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Addition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl (pH. 7.4, 0.2 M)</td>
<td>100 µl</td>
</tr>
<tr>
<td>NaCl (0.4M)</td>
<td>100 µl</td>
</tr>
<tr>
<td>EDTA (0.5M, pH 8.0)</td>
<td>10 µl</td>
</tr>
<tr>
<td>Protease Inhibitor Cocktail (20X)</td>
<td>20 µl</td>
</tr>
<tr>
<td>ddH2O</td>
<td>680 µl</td>
</tr>
</tbody>
</table>

Phosphate buffer (PB, 0.1 M, 200 ml):

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Addition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na2HPO4 (MW = 178)</td>
<td>3.56 g</td>
</tr>
<tr>
<td>NaH2PO4 (MW = 156, 0.1M)</td>
<td>3.12 g in 200 ml</td>
</tr>
</tbody>
</table>

Preparation:
Na2HPO4 was dissolved in 50 ml; to this solution was added NaH2PO4 (0.1M) to make the pH 7.2; volume made up to 200 ml. The working stock was made by diluting the solution 10 times and check the pH, which should be 7.8.

Wash buffer (ELISA): PBS (pH 7.2) containing 0.05% v/v Tween 20.

Blocking solution (ELISA): 2 ml FCS (2% v/v) was added to 100 ml PBS (0.02 M, pH 7.2).

Dilution solution (ELISA): 1 ml FCS (1% v/v) and 50 µl Tween 20 (0.05% v/v) was added to 100 ml PBS (0.02 M, pH 7.2).

Diffquick stain: The Diff-Quick stain consists of 3 solutions:

1. Diff-Quick fixative reagent
   - Triarylmethane dye
   - Methanol

2. Diff-Quick solution I (eosinophilic)
   - Xanthene dye
   - pH buffer
   - Sodium azide

3. Diff-Quick solution II (basophilic)
   - Thiazine dye
   - pH buffer

The smear was air dried, then fixed in "Diff Quick" Fixative reagent for 30 secs/drain. It was stained with "DiffQuick" solution II for 30 secs/drain. Counter stained with "Diff Quick" solution I for 30 secs/drain. Smear was rinsed in tap water to remove excess stain then checked under microscope and cell morphology was examined under oil immersion light microscopy.
**Materials and Methods**

CMH$_2$DCFDA [5-(and-6)-chloromethyl-2', 7'-dichlorodihydrofluorescein diacetate] (MW= 577.80, 1 mM stock in DMSO): 1.16 mg CMH$_2$DCFDA was weighed and dissolved in 2 ml DMSO to get 1 mM stock. Stored at -20°C in small aliquots; freeze thawing was avoided.

**Preparation of stacking gel (3.9%)**

<table>
<thead>
<tr>
<th>Stock solution</th>
<th>Addition</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% acrylamide/ 0.8% bisacrylamide</td>
<td>0.65 ml</td>
</tr>
<tr>
<td>4X Tris-Cl/ SDS (pH 6.8)</td>
<td>1.25 ml</td>
</tr>
<tr>
<td>H$_2$O</td>
<td>3.05 ml</td>
</tr>
<tr>
<td>10% (v/v) ammonium persulfate</td>
<td>0.025 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.005 ml</td>
</tr>
</tbody>
</table>

In a 25 ml flask, mix 0.65 ml of 30% acrylamide/ 0.8% bisacrylamide were mixed, 1.25 ml of 4X Tris-Cl/ SDS (pH 6.8) and 3.05 ml H$_2$O was added. Then 0.025 ml of 10% (v/v) ammonium persulfate and 0.005 ml TEMED was added, swirled gently to mix. To be used immediately (Failure to form a firm gel usually indicates a problem with the persulfate, TEMED or both).

**Preparation of separating gel (10%)**

<table>
<thead>
<tr>
<th>Stock solution</th>
<th>Addition</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% acrylamide/ 0.8% bisacrylamide</td>
<td>5.0 ml</td>
</tr>
<tr>
<td>4X Tris-Cl/ SDS (pH 8.8)</td>
<td>3.75 ml</td>
</tr>
<tr>
<td>H$_2$O</td>
<td>6.25 ml</td>
</tr>
<tr>
<td>10% (v/v) ammonium persulfate</td>
<td>0.05 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.01 ml</td>
</tr>
</tbody>
</table>

In a 25 ml flask, mix 5 ml of 30% acrylamide/ 0.8% bisacrylamide were mixed, 3.75 ml of 4X Tris-Cl/ SDS (pH 8.8) and 6.25 ml H$_2$O was added. Then 0.05 ml of 10% (v/v) ammonium persulfate and 0.01 ml TEMED was added, swirled gently to mix. To be used immediately (Failure to form a firm gel usually indicates a problem with the persulfate, TEMED or both).

**Reagents used in Gel:**

**30% acrylamide/ 0.8% bisacrylamide**

Mix 30.0 g acrylamide and 0.8 g N, N' methylene bisacrylamide were mixed with H$_2$O in total volume of 100 ml. The solution was filtered through a 0.45 μm filter and stored at 4°C in the dark (should be discarded after 30 days, as acrylamide gradually hydrolyze to acrylic acid and ammonia). (Acrylamide monomer is neurotoxic, gloves should be worn while handling the solution and the solution should not be pipetted by mouth).

**4X Tris-Cl/ SDS, pH 6.8 (0.5 M Tris-Cl containing 0.4 % SDS)**

6.05 g Tris base was dissolved in 40 ml H$_2$O, pH was adjusted to 6.8 with 1(N) HCl. H$_2$O was added to 100 ml total volume. The solution was filtered through 0.45 μm filter, 0.4 gm SDS was added, and stored at 4°C up to 1 month.

**4X Tris-Cl/ SDS, pH 8.8 (1.5 M Tris-Cl containing 0.4 % SDS)**

91 g Tris base was dissolved in 300 ml H$_2$O, pH was adjusted to 8.8 with 1(N) HCl. H$_2$O was added to 500 ml total volume. The solution was filtered through 0.45 μm filter, 2.0 g SDS was added, and stored at 4°C up to 1 month.

**Running Buffer/ SDS electrophoresis buffer (10X)**

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Addition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-base (0.125 M final) (MW= 121.14)</td>
<td>15.14 g</td>
</tr>
<tr>
<td>Glycine (0.98 M final) (MW= 75.07)</td>
<td>72.06 g</td>
</tr>
<tr>
<td>SDS (0.5% final)</td>
<td>5.0 g</td>
</tr>
<tr>
<td>H$_2$O (volume make up to)</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>
**Materials and Methods**

**SDS Sample buffer, 6X**

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Addition</th>
</tr>
</thead>
<tbody>
<tr>
<td>4X Tris-Cl/ SDS, pH 6.8</td>
<td>7.0 ml</td>
</tr>
<tr>
<td>Glycerol</td>
<td>3.0 ml (30 % v/v final)</td>
</tr>
<tr>
<td>SDS</td>
<td>1.0 g (10% final)</td>
</tr>
<tr>
<td>DTT (MW= 154.25)</td>
<td>0.93 g (0.6 M final)</td>
</tr>
<tr>
<td>Bromophenol blue</td>
<td>1.2 mg (0.012% final)</td>
</tr>
</tbody>
</table>

H₂O was added to make up volume 10 ml, stored in 0.5 ml aliquot at -70°C

**Transfer buffer:**

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Addition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris Base (25 mM) (MW= 121.14)</td>
<td>3.028 g</td>
</tr>
<tr>
<td>Glycine (192 mM) (MW= 75.07)</td>
<td>14.41 g</td>
</tr>
<tr>
<td>Methanol</td>
<td>200 ml</td>
</tr>
</tbody>
</table>

H₂O was added to make up volume 1000 ml, kept at 4°C

**Ponceau S solution**

0.5 g Ponceau S was dissolved in 1 ml glacial acetic acid; brought to 100 ml with water. Prepared just before use.

**TBS (Tris Buffered saline) (10X) (0.5 M, pH 7.4)**

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Addition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-Cl (0.5 M) (MW= 121.14)</td>
<td>60.57 g</td>
</tr>
<tr>
<td>NaCl (0.5 M) (MW=58.5)</td>
<td>29.25 g</td>
</tr>
</tbody>
</table>

60.57 g Tris base and 29.25 g NaCl was dissolved in 800 ml H₂O, pH was adjusted to 7.4 with 1(N) HCl. Volume made up to 1L.

**Wash buffer (TBS-T) (Western Blotting): TBS (1X) (0.05 M, pH 7.4) containing 0.1% v/v Tween 20.**

**Blocking solution (Western Blotting): 3.0 g BSA was dissolved to 100 ml TBS (1X) (0.05 M, pH 7.4)**

**NBT/BCIP substrate:**

NBT: 7.5 mg dissolve in 175 μl DMF (Dimethyl formamide) + 75 μl H₂O

BCIP (5-Bromo-4-chloro-3-indolyl phosphate toluidine): 3.75 mg dissolved in 250 μl DMF.

Make a buffer of

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Addition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-Cl</td>
<td>1.21 g</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>1.02 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.584 g</td>
</tr>
</tbody>
</table>

Above salts were dissolved in 80 ml H₂O and adjusted pH to 9.0-9.5 with 1(N) HCl. H₂O was added to make up 100 ml total volume.

The above NBT/BCIP solution was added to 25 ml of the above buffer (60 μl NBT + 60 μl BCIP + 24.88 ml buffer).