Chapter- 5

Materials and Methods

Table 5.3.1.1. Composition of modified Ray's blood agar medium

Modified Ray's blood agar medium	
Composition	**Quantity**
NaCl	2.5g
Glucose	7.0g
Peptone	4.0g
Agar	7.0g
Panmede	2.5g
Rabbit blood	10.0ml

All the above-mentioned ingredients, except glucose and rabbit blood, were taken in a 500 ml conical flask. The volume was made upto 350 ml with double distilled water. Then the materials were melted on water bath and pH 7.5 was adjusted by using 20% w/v sodium hydroxide solution. The 7 gm glucose was taken in a separate culture test tube and 10 ml of double distilled water added. Then the medium, glucose solutions and culture tubes were sterilized in an autoclave at 15 lbs pressure for 15 – 20 minutes. Then the medium was allowed to cool to 50 – 60°C and sterilized culture tubes, glucose solution and required amount of rabbit blood were kept in UV chamber. The blood, glucose solution and 10 – 15 mg of ampicillin–cloxacillin were added to the tubes aseptically and mixed thoroughly. About 10 ml of media was poured into each sterilized tubes, allowed to cool in UV–chamber and then kept at 24°C in B.O.D incubator for 3 days and finally stored at 4°C in a refrigerator. Subcultures were made on these slants with *L. donovani* strain. The logarithmic growth phase was reached within 72 hours after subculture.

The peptone serves an amino acid source and glucose as carbohydrate and energy source. They need at least 10 amino acids for their growth [96, 104]. Haemin of rabbit blood acts as growth promoter [97]. Protozoa fails to synthesize it [98, 99]. Other compenents of blood, other than haemin, are also responsible for growth of haemoflagellates [100]. Sodium chloride acts as the osmotic pressure stabilizer. Agar is used as solidifying agent.

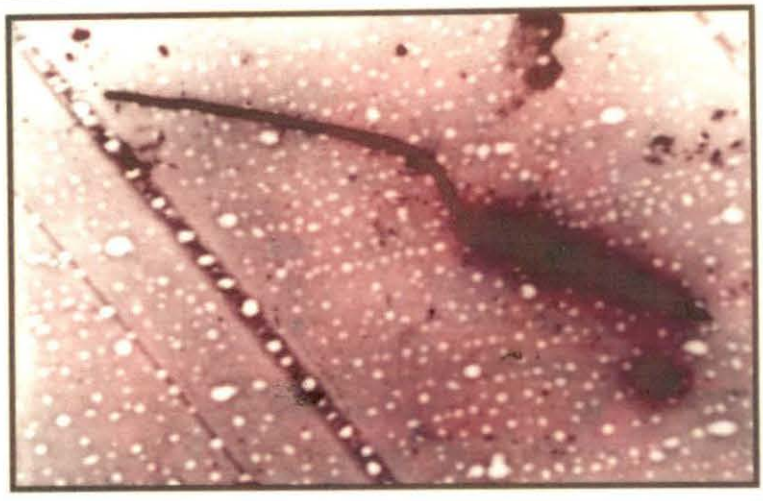


Fig. 5.3.A. 1% Uranylacetate stained micrograph of a single *Leishmania donovani* promastigote cell (magnification X 15000)

Fig. 5.3.B. 1% Uranylacetate stained micrograph of a field containing *Leishmania donovani* promastigote cells (magnification X 15000)

5.3.2. Preparation of Buffers

Table 5.3.2.1. Composition of major buffers

Composition ↓	Different Major Buffers				
	Washing buffer	**Phosphate buffer saline (PBS)**	**EDTA–PBS buffer**	**Acetate buffer**	**Digitonin permeabilization buffer**
NaCl	125mM	135mM	135mM	120 mM	–
KCl	5mM	2.75mM	2.75mM	–	50mM
Na_2HPO_4	–	0.08mM	0.08mM	–	–
KH_2PO_4	–	0.15mm	0.15mM	–	–
$MgSO_4$	–	–	–	5 mM	–
EDTA Na_2	1mM	–	1mM	–	–
Sucrose	–	–	–	–	300 mM
Glucose	5mM				
Tris- HCl	–	–	–	–	50 mM
Hepes	20mM				
Potassium acetate	–	–	–	20 mM	–
EGTA	–	–	–	–	2 mM
pH ⇨	**7.5**	**7.2**	**7.2**	**6.4**	**7.0**

Table 5.3.2.2. Composition of minor buffers

Composition ⇩	Different Minor Buffers				
	Standard buffer	**Choline buffer**	**Sodium free buffer**	**Potassium free buffer**	**Chloride free buffer**
NaCl	135 mM	–	–	135 mM	–
KCl	5 mM	–	5 mM	–	–
Gluconate - Na	–	–	–	–	135 mM
Potassum acetate	–	–	–	–	5 mM
$MgSO_4$	10 mM	1 mM	1 mM	1 mM	1 mM
Hepis	10 mM	10 mM	10 mM	10 mM	10 mM
Choline chloride	–	140mM	140mM	5 mM	–
pH ⇨	**6.4**	**6.4**	**6.4**	**6.4**	**6.4**

5.3.3. Preparation of cell suspension

The subcultured colonies on modified Ray's blood agar medium after 72 hours were scrapped off carefully with a sterilized loop and suspended in isolation and washing buffer of pH 7.5. After centrifugation of the homogeneous suspension at 3000x g for 10 minutes, the supernatant was discarded and the pellets were again suspended in isolation and washing buffer (pH 7.5). Homogeneous suspension was made by cyclomixer and absorbance was measured at 660nm with the help of spectrophotometer. The amount of protein of *Leishmania donovani* was calculated from a standard graph. The suspension was again centrifuged at 3000 x g for 10 minutes and pellets were again suspended by vortexing either in acetate buffer, pH 6.4 or phosphate buffer saline (PBS), pH 7.2 according to the requirement.

5.3.4. Protein estimation

The amount of protein was determined by the biuret method in the presence of 0.2% deoxycholate [402]. Bovine serum albumin (BSA) was used as standard. 1 mg whole–cell protein corresponds to 1.4 x 10^8 cells.

5.3.5. Cell surface iodination of intact cells

Leishmania cell surface proteins were labeled with ^{125}I by a modification of the lactoperoxidase-glucose oxidase method of Hubbard and Cohn [403]. Briefly, cells were harvested, washed twice in PBS buffer, pH 7.2 and resuspended in 5 ml ice-cold PBS buffer, pH 7.2 that contained 50 mM glucose and 3 μMNaI. 5 mCi of carrier free Na (^{125}I) was added to this mixture. Lactoperoxidase (700 odianisidine units) were added to initiate the labeling reaction. Cells were agitated every 2 min. After 15 min, cells were washed with PBS, spun through 20 ml of bovine serum, and washed twice with PBS, all by centrifugation at 2000×g for 5 min. Samples of cells from controls (incubated without enzymes) and from the full incubation mixture were saved for determination of cell viability and ^{125}I incorporation. Cell viability, as monitored by trypan blue exclusion, remained N90%. The amount of ^{125}I into protein of controls was b1% of the enzyme mediated incorporation.

5.3.6. Plasma membrane preparation

Parasites were harvested at a concentration of 108 cells/ml (5000×g, 10min, 4°C). The cell pellet was suspended to 2×107 cells/ml in PBS buffer, pH 7.2 that contained 10 mM $MgCl_2$ and rapidly mixed with equal volume of 1mg/ml concanavalin A in the same buffer. Cell aggregation was apparent within 1min. After 5min, cells were gently spined at 1000×g for 1min to remove excess concanavalin A. The supernate was discarded, and the cell pellet was re-suspended in 12ml of 10mMTris–HCl buffer pH 7.5, that contained 10μg leupeptin/ml and 1mM $MgCl_2$. After swelling for 10min in that hypotonic buffer, cells were homogenized by 18–20 strokes in tight fitting Dounce-type homogenizer. Cell lyses and formation of membrane sheets were verified by phase-contrast microscopy. The homogenate was layered over a two- step gradient consisting of 8ml of 0.5 M mannitol over 4ml 0.58 M sucrose, both in Tris buffer, and spin at 1000×g for 20min. For analysis, material remaining at the top of the 0.5 M mannitol (supernate I) was saved. Large crude plasma membrane fragments were separated as a tight pellet at the bottom of the gradient (pellet I). This pellet was resuspended in Tris buffer that contained 1M α-methylmannoside and left on ice for 40 min with occasional mixing. This plasma membrane, free from bulk concanavalin A, was diluted into three volumes of Tris buffer and homogenized by 80 strokes with a glass Dounce-type homogenizer. This second homogenate was layered on a single-step gradient that consisted of 20% sucrose in Tris buffer and spun for 30 min at 500×g. Scrolls and large plasma membrane sheets above the 20%(w/v) sucrose layer (supernate III) was collected by centrifugation at 40,000×g for 1 h. The pellet (pellet IV) containing the enriched plasma membrane was resuspended in Tris buffer. All samples were either assayed immediately or frozen at −20 °C for further use.

5.3.7. Marker enzymes

The following enzymes were used as markers of the subcellular fractions: tartarate-resistant acid phosphatase [404] for plasma membrane, cytochrome oxidase [405] for the inner mitochondrial membrane and NADPH-cytochrome c reductase [406] for microsomes.

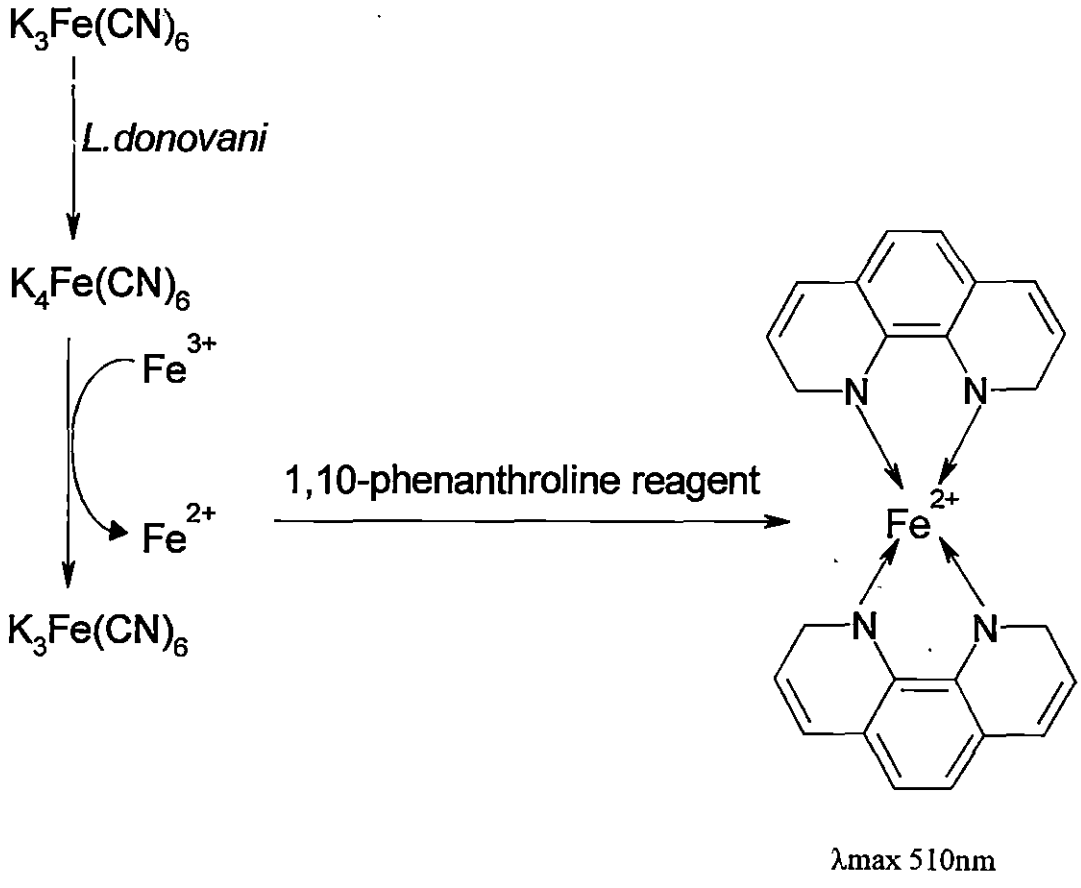
5.3.8. Measurement of ferricyanide reduction by *L. donovani* cells (LDC)

Ferrocyanide quantitation was performed using 1, 10–phenanthroline complex (Scheme 5.1) as described by Avron and Shavit [407]. The incubation mixture contained acetate buffer (potassium acetate 20 mM, sodium chloride 120 mM, magnesium sulphate 5 mM), pH 6.4, 3 mg LDC, 5 µmoles D–glucose and 3 µmoles $K_3Fe(CN)_6$ in a final volume of 1 ml. The incubation was carried out at 25°C. The reaction was terminated by the addition of 0.1 ml 30% (w/v) trichloroacetic acid (TCA) followed by centrifugation at 10,000 xg for 15 mins. Ferrocyanide in supernatant was measured by 1.5 ml of 1,10–phenanthroline reagent containing 1.5 mmoles sodium acetate, 0.1 mmoles citric acid, 0.75 µmoles ferric chloride and 12.6 µmoles 1,10–phenanthroline at 510 nm. The blanks were carried out with all reagents except LDC.

5.3.9. ALA, DTNS and NQSA reduction assay

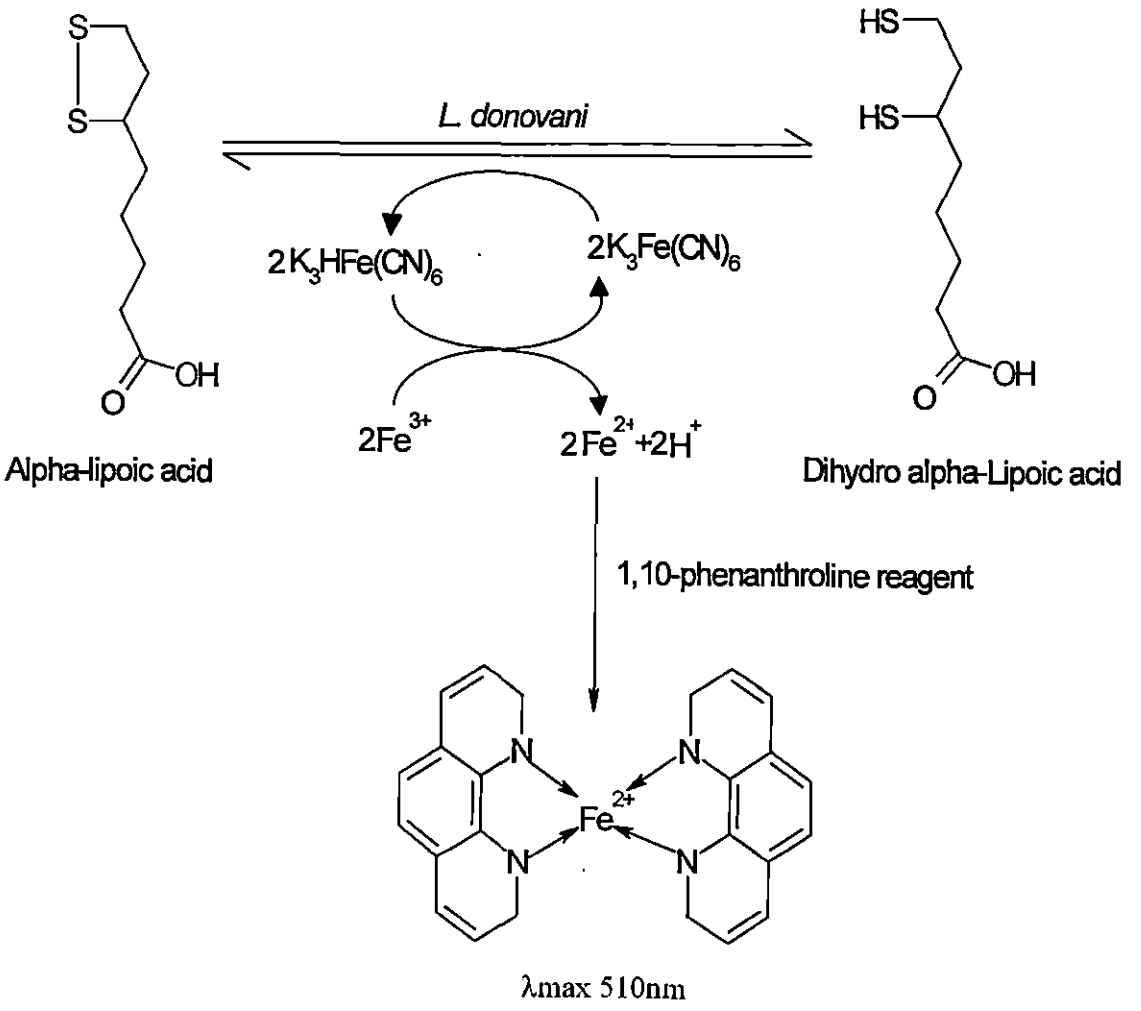
ALA, DTNS and NQSA reduction by *L. donovani* promastigote cells was assayed as the formation of ferrocyanide [$K_3HFe(CN)_6$]as a result of the reduction of ferricyanide by dihydrolipoic acid, 5-mercapto-2–nitroaniline-N-sulfonic acid and hydroquinone of NQSA (Scheme 5.2, 5.3 and 5.4).

Ferrocyanide was estimated according to the method of Avron and Shavit [407]. The reaction mixture consisted of 3 mg LDC, acetate buffer (potassium acetate 20 mM, sodium chloride 120 mM, magnesium sulphate 5 mM), pH 6.4, 5 mM D-glucose and 1 mM ALA or 0.05 mM DTNS or 0.4 mM NQSA in a final volume of 3 ml. The reaction mixtures were incubated for 10 min at 25°C. After incubation, the reaction mixtures were kept in ice and then centrifuged at 4°C at 10000xg for 10 min. 1.4 ml of the supernatant was removed and added to 0.1 ml of 75mM $K_3Fe(CN)_6$, followed by 1.5 ml 1,10–phenanthroline reagent. The absorbance was recorded at 510 nm. The blanks were carried out with all reagents except LDC. Two molecules of ferricyanide have been considered to react with one molecule of dihydrolipoic acid or one molecule of hydroquinone of NQSA or two molecule of 5–mercapto–2–nitroaniline–N–sulfonic acid.

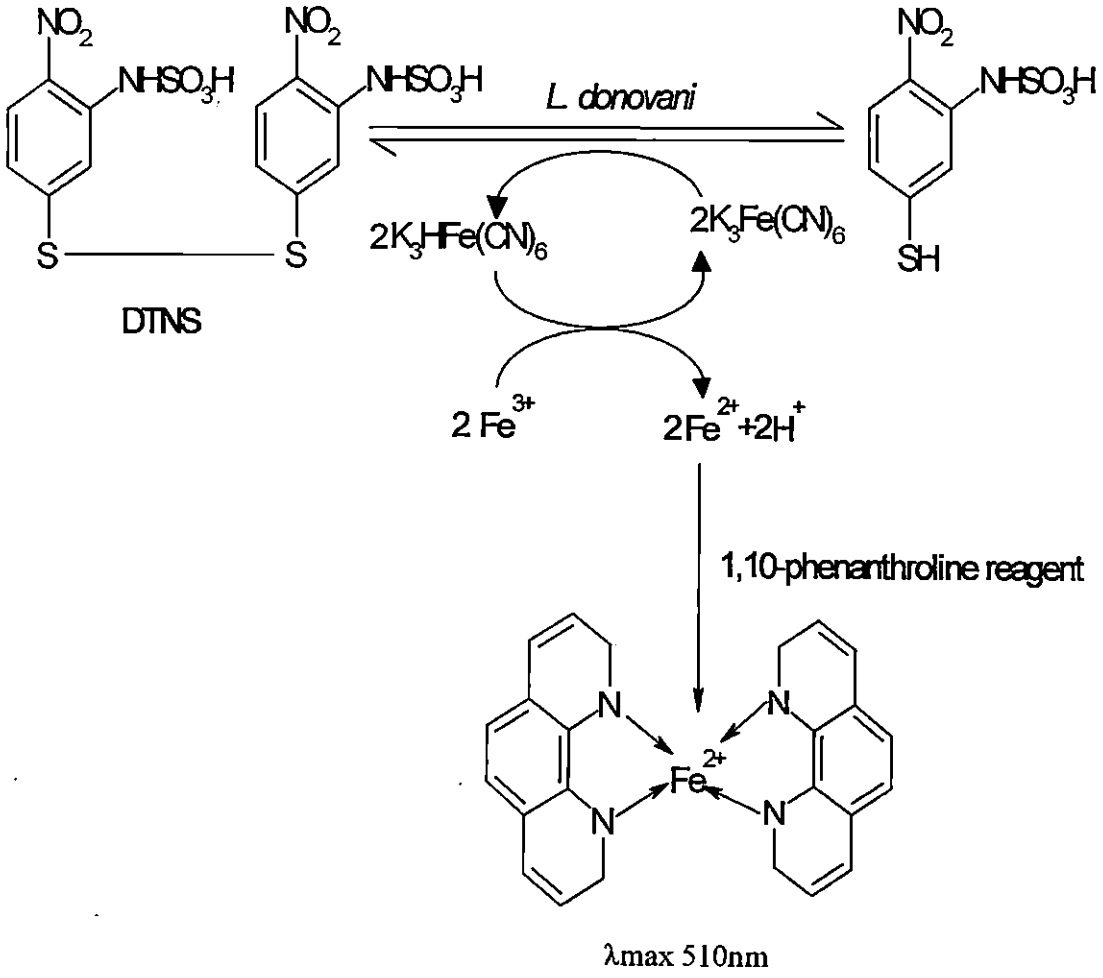


λmax 510nm

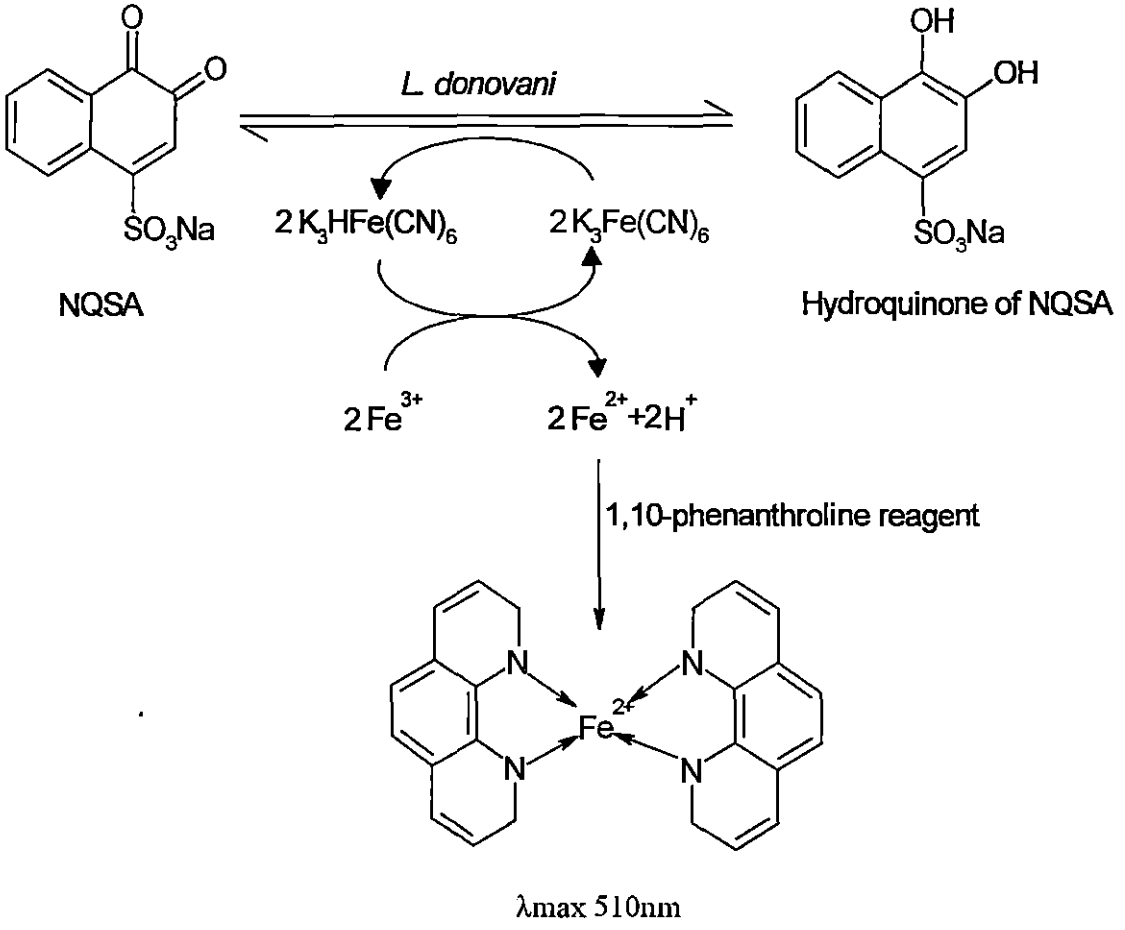
Scheme 5.1. Ferricyanide reduction by *L.donovani* promastigote cell.



Scheme 5.2. ALA reduction by *L.donovani* promastigote cell.



Scheme 5.3. DTNS reduction by *L.donovani* promastigote cell.



Scheme 5.4. NQSA reduction by *L.donovani* promastigote cell.

5.3.10. Exposure of *L. donovani* promastigotes to Capsaicin, TTFA, Trifluoperazine and Amiloride

The liquid media used for growth experiments was a semisynthetic medium developed by Kar et al [408]. Cultures of promastigotes in 100 ml conical flask where incubated for 96 h with added compound supplied as a solution in DMF (5 µl/ml medium) with proper vehicle control (DMF). After incubation, the numbers of elongated motile promastigotes were counted.

5.3.11. Measurement of Oxygen Uptake

Oxygen uptake of the *Leishmania donovani* promastigote cells was determined with late log-growth-phase cells (72h) from blood agar slants. After washing in sterile PBS (pH 7.2) and protein estimation, cells were suspended in PBS (pH 6.4) containing 10mM glucose to give a cell density of 4mg/ml. Oxygen consumption was measured at 25°C with a DW1 Hansatech oxygraph plus (Hansatech Instruments Ltd., Norfolk,UK [409]. Respiration of 2mg cells in a 1-ml final volume was measured, and effect of the addition of effectors was evaluated. Water insoluble compounds were given as a solution in dimethyl formamide (DMF; 5µl/ml PBS). Appropriate vehicle controls (DMF) were used for each experiment. Respiration rates are expressed as nanomoles of oxygen consumed per milliliter per minute.

5.3.12. Measurement of ALA, DTNS, NQSA and $K_3Fe(CN)_6$ reduction by LDC under anaerobic condition

Anaerobiosis was carried out in glass centrifuge tube in presence of oxygen scavenging enzymic system [410]. The tube contained 5 mg LDC, 120 mM NaCl, 20 mM potassium acetate, 20 mM Hepes, pH 6.4, 5 mM $MgCl_2$, 5 mM D–glucose, 10 units of glucose oxidase, 200 units of catalase in a volume of 2.6 ml. Open surface of liquid was covered by 1 ml heavy liquid paraffin. After 10 minute incubation, ALA, DTNS, NQSA or $K_3Fe(CN)_6$ was injected through the paraffin layer in a final concentration of 1 mM, 0.05 mM, 0.4 mM or 3 mM respectively. The assay system was incubated for 10 min at 25°C. The reaction was terminated by immersing the tubes in ice and centrifuged at 3000xg for 10 min at 4°C. 1.3 ml of supernatant was removed by syringe and added to 0.1 ml of 70

mM $K_3Fe(CN)_6$, followed by 1.6 ml 1,10–phenanthroline reagent. The absorbance was recorded at 510 nm. The blanks were carried out with all reagents except LDC.

5.3.13. UV irradiation of *L.donovani* promastigote cells

L. donovani promastigote cells were UV irradiated by modifying the procedures as described by Brodie et al. [411]. This treatment was performed as follows: LDC at a protein concentration of 10 mg/ml of PBS, 140 mM, pH 6.4, was placed in Petri dishes at 4 °C. A 30-cm long Eye G8T5 lamp (maximum emission 360 nm) was placed at a distance of 3 cm from the petridishes and they received 36 W/m2. Control (UV-unexposed) cells were treated similarly without UV irradiation.

5.3.14. Preparation of digitonin permeabilized *L.donovani* cells

After protein estimation, the suspension was centrifuged at 3000xg for 10 minutes to obtain the pellets. Digitonin (50µg/mg protein) and buffer for digitonin permeabilization (sucrose 300mM, Tris-Hcl 50mM, KCl 50mM, EGTA 2mM), pH 7 were added to the resulting pellets and vortexed for 10 minutes followed by incubation in ice for 5 minutes. Then the suspension was again centrifuged at 3000xg for 10 minutes and pellets were suspended by vortexing with same buffer according to the requirement.

5.3.15. Measurement of ATPase activity in digitonin permeabilized LDC

ATPase is an enzyme, which converts ATP to ADP and P_i. Both exoplasmic and endoplasmic ATPase activity was measured by phosphate release method [412]. After protein estimation and washing with PBS-EDTA buffer, pH 7.5, cells were suspended in assay buffer. The incubation mixture contained buffer for digitonin permeabilization, pH 7, $ATP.Na_2$, magnesium acetate and digitonin permeabelized LDC in a final volume of 0.86 ml. The reaction mixture was incubated for 10 min at RT and the reaction was stopped by the addition of 0.43ml of 12% SDS followed by centrifugation at 31,304xg (20,000 rpm for 5min). To the supernatant solution 0.86ml of 6% ascorbic acid in 1N HCl and 1% ammonium molybdate solution was added followed by 0.86ml of 2% sodium citrate, 2% sodium meta-arsenite and 2% acetic acid mixture. The absorbance was recorded at 850 nm. The blanks were carried out with all reagents except LDC.

5.3.16. Measurement of PPase activity in digitonin permeabilized *L.donovani* cell

PPase activity in terms of phosphate release was assayed as described in ATPase measurement [412]. After protein estimation and washing with PBS-EDTA buffer, pH 7.5, cells were suspended in assay buffer. The incubation mixture contained buffer for digitonin permeabilization (sucrose 300mM, Tris-Hcl 50mM, KCl 50mM, EGTA 2mM), pH 7, pyrophosphate 0.25mM, magnesium acetate 0.5mM and 0.1mg of digitonin permeabelized LDC (50µg digitonin/mg protein), in a final volume of 0.86 ml. The reaction mixture was incubated for 10 min at RT°C and the reaction was stopped by the addition of 0.43ml of 12% SDS followed by centrifugation at 31,304xg (20,000 rpm for 5min). To the supernatant solution 0.86 ml of 6% ascorbic acid in 1N HCl and 1% ammonium molybdate solution was added followed by 0.86 ml of 2% sodium citrate, 2% sodium meta-arsenite and 2% acetic acid mixture. The absorbance was recorded at 850 nm. The blanks were carried out with all reagents except LDC.

5.4. Synthesis of 5, 5′-dithiobis (2-nitroaniline-N-sulfonic acid) [DTNS]

The various steps in our synthesis are shown on the Scheme 5.5.

- **Synthesis of 5-chloro-2-nitroaniline-N-sulfonic acid (II)**

17g (0.098mol) of 5–chloro–2–nitroaniline was dissolved in a mixture of 80ml of anhydrous acetone and 12 ml dry pyridine in a conical flask cooled to 4°C. 7 ml (0.05mol) of chlorosulfonic acid was added slowly with stirring. Water was added to the reaction product and the resultant solid was separated. The product 5–chloro–2–nitroaniline–N–sulfonic acid was recrystalized from ethanol (23% yields).

- **5,5′-dithiobis (2-nitroaniline-N-sulfonic acid) (III)**

5.4g (0.22 mol) of 5–chloro–2–nitroaniline–N–sulfonic acid (II) was suspended in 150 ml of water and concentrated NaOH was added to bring the pH 7.2. To this solution 2.4 g (0.03 mol) of Na_2S (anhydrous) in 30 ml water was added. This solution was stirred and heated to 50–60°C for 2 hours. Reaction mixture was oxidised with iodine in potassium iodide solution (4g I_2 in 100 ml of 5% KI). Iodine solution was added until the orange colour of the thiol anion disappeared and was replaced by pale reddish yellow colour of the disulfides. When the solution was acidified with acetic acid, oil, which crystallizes on further stirring, was formed. The product was recrystallized from glacial acetic acid

(0.79g, 15% yield) : mp 145–147°C; TLC (2–butanone/n–butanol/chloroform 30 : 40 : 30) R_f = 0.87; IR (KBr pellet) 3380, 3339, 1621, 1562, 1371, 1244, 1162, 802, 434 cm^{-1}; ^1H NMR (dimethylsulfoxide – d_6) δ 8.01 (2H, d, aromatic proton), 7.59 (2H, s, NH), 7.13 (2H, d, aromatic proton), 6.70 (2H, d, aromatic proton). Anal. ($C_{12}H_{10}N_4O_{10}S_4$) C, H, N.

5.5. Synthesis of 2-methyl-3-(1'-oxooctadecyl)-1,4-napthoquinone [MOON]

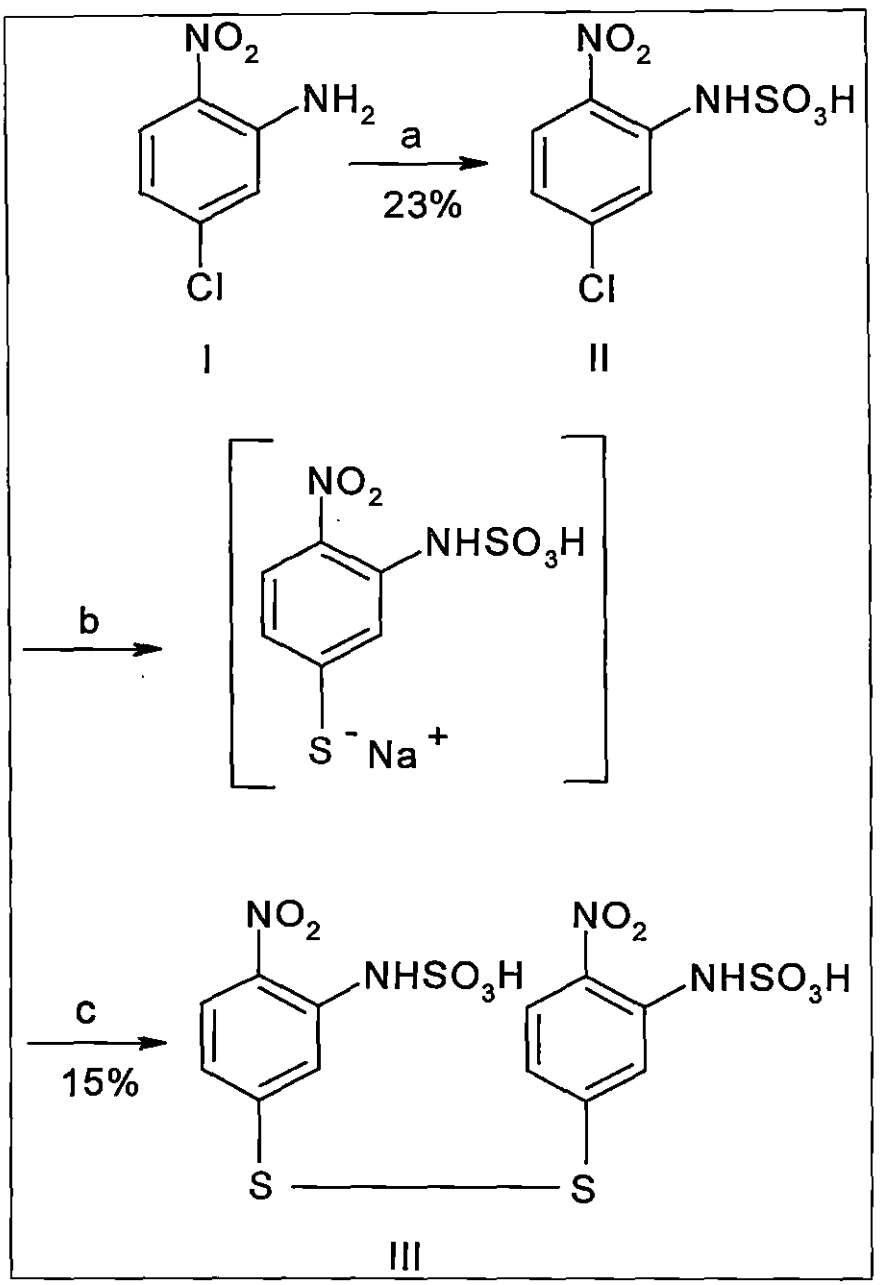
Synthesis of MOON is mentioned in scheme 5.6.

7.74 g (0.045 mol) of dihydromenaquinone and 2.5 g (0. 0045 mol) of stearic anhydride was dissolved in 100 ml of dry diethyl ether. To this solution, 1.2 g (0. 0045 mol) of anhydrous AlCl3 was added and stirred for 30 min at room temperature under the atmosphere of nitrogen. This solution was stirred and heated to 60°–70 °C for further 1 h. Solution was separated from $AlCl_3$ by filtration and ether was removed in vacuum. Solid mass was extracted with 40°–60°C petroleum ether. Petroleumether of the extract was removed in vacuo. Light yellowsolidmass was recrystallized from methanol (1.5 g, 75% yield): mp 42°–45 °C; TLC (2-butanone/n-butanol/chloroform 30:40:30) Rf=0.92; 1H NMR (CDCl3) δ7.56 (2H, m, aromatic proton), 7.47 (2H, d, aromatic proton), 2.2 (3H, s, ring methyl), 2.33–2.36 (2H, t, methylene adjacent to keto), 1.25(26H, m, chain alkane methylene), 0.86–0.89 (3H, t, terminal methyl of alkane chain). (M+23)+,461.

5.6. Synthesis of 4-aminophenyl arsineoxide-N-sulphonic acid (APAOS)

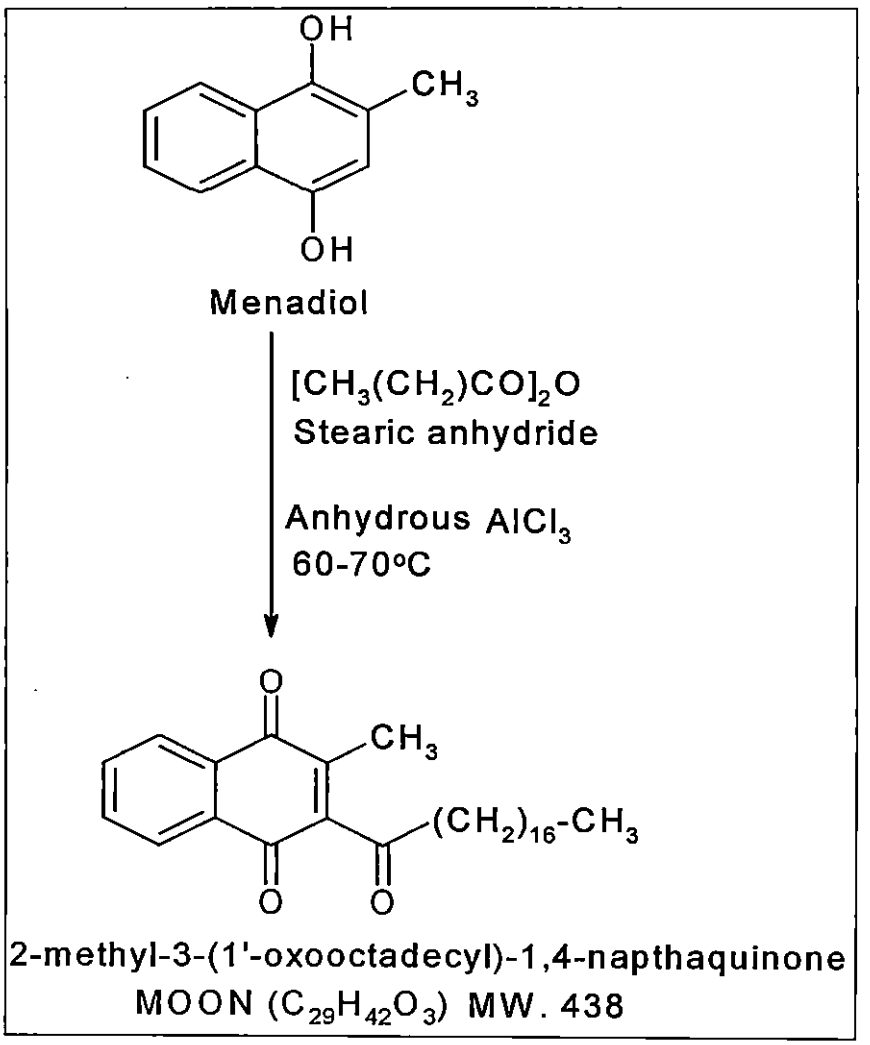
4-aminophenyl arsineoxide-N-sulphonic acid (APAOS) was synthesized in our laboratory by one of our senior scholar, which has mentioned in scheme 5.7.

Scheme 5.5.

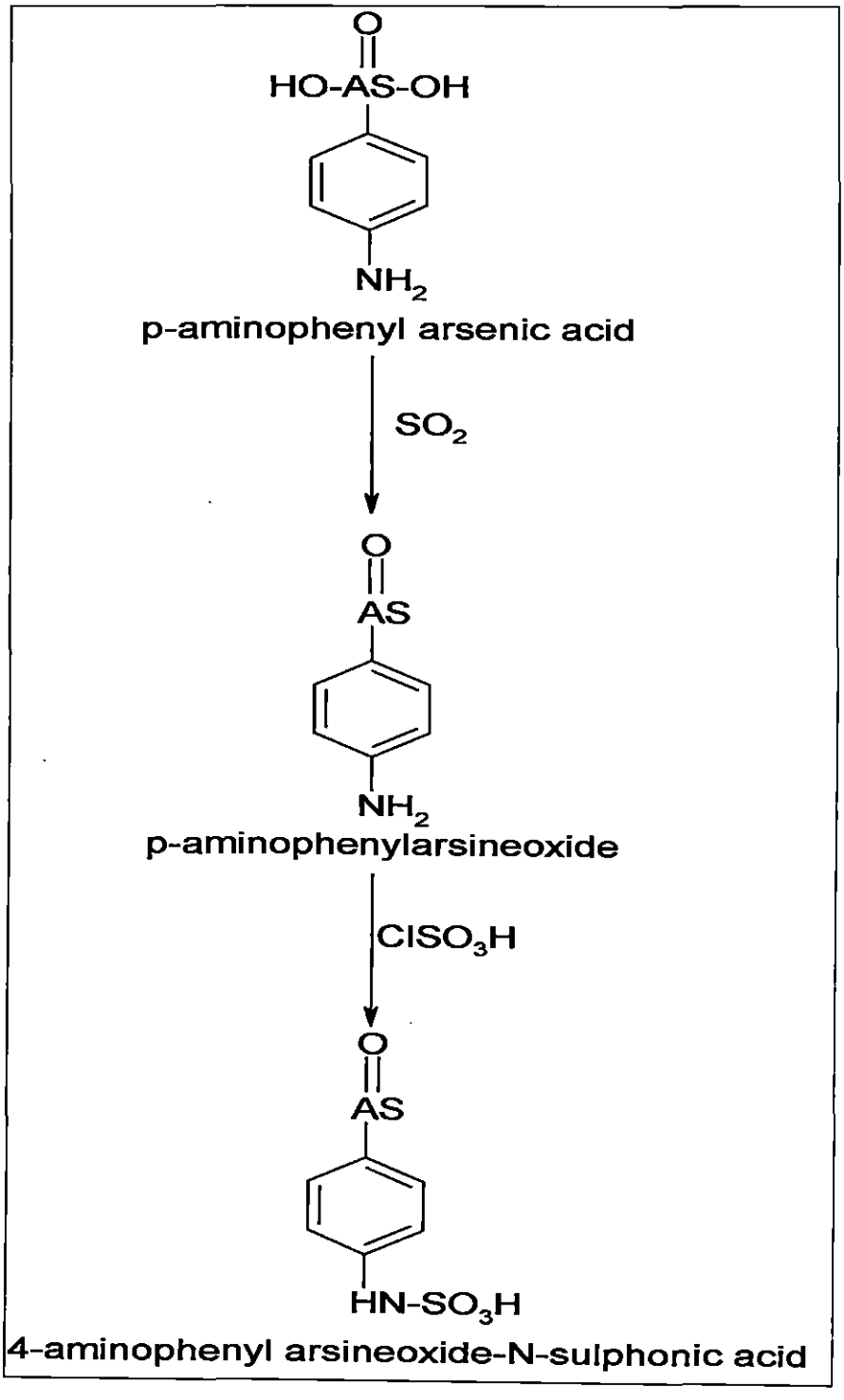


[a] Reagents and conditions: (a) acetone, pyridine, $ClSO_3H$, 4°C, 30 min (23% for II); (b) Na_2S, NaOH, 55°C, 2h; (c) iodine in potassium iodide solution, RT (15% for III).

Scheme 5.6.


OH
CH3
OH
Menadiol
[CH3(CH2)CO]2O
Stearic anhydride
Anhydrous AlCl3
60-70°C
O
CH3
(CH2)16-CH3
O
O
2-methyl-3-(1'-oxooctadecyl)-1,4-napthaquinone
MOON (C29H42O3) MW. 438

Scheme 5.7.


HO-AS-OH
O
NH2
p-aminophenyl arsenic acid
SO2
AS
NH2
p-aminophenylarsineoxide
ClSO3H
AS
HN-SO3H
4-aminophenyl arsineoxide-N-sulphonic acid