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Original article

Iridoid glucosides from *Nyctanthes arbortristis* result in increased reactive oxygen species and cellular redox homeostasis imbalance in Leishmania parasite

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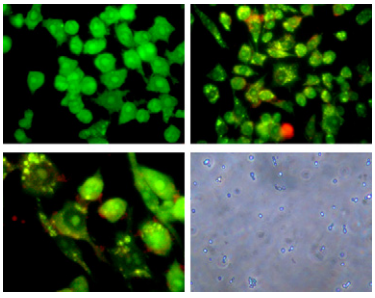
HIGHLIGHTS

- Iridoid glucosides from *Nyctanthes arbortristis* result in increased reactive oxygen species.
- These compounds show significant effect on redox homeostasis and high leishmanicidal activity.
- Parasitic death follows apoptotic mechanism.
- These compounds are safe on human cell lines.

GRAPHICAL ABSTRACT



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ABSTRACT

We report here the effect of iridoid glucosides, isolated from *Nyctanthes arbortristis*, on redox homeostasis of *Leishmania* parasite. These compounds led to an increase in reactive oxygen species by inhibiting a crucial enzyme of redox metabolism of the parasite. Our experiments clearly showed that these compounds are highly active as antileishmanial agents. The *in vitro* experiments on intra-macrophageal amastigotes showed significant killing of parasite even at very low concentration. Determination of mechanism of action of iridoid glucosides showed that increased ROS level leads to oxidative stress, cell membrane damage and apoptosis of *Leishmania* sp. Our cellular toxicity assays on Human embryonic kidney (HEK 293) and mouse macrophage (J774A.1) cell lines showed these compounds to be very safe for therapeutics application.

1. Introduction

Nyctanthes arbortristis L. (Oleaceae) is a plant of significant medicinal value and its medicinal properties have already been proven experimentally [1–3]. Three iridoid glucosides, arbortristoside a, b and c (structures submitted as Supplementary materials 1), used in the current study were tested to be biologically active for antileishmanial activity on hamster [4]. However, the molecular mechanism underlying antileishmanial activity remains elusive.

Leishmaniasis is a neglected tropical disease caused by more than 30 species of protozoan parasite *Leishmania* and transmitted to humans by female sand fly Phlebotomine [5,6]. Visceral Leishmaniasis (also called as "Kala-azar") caused by *Leishmania infantum* and *Leishmania donovani* is a fatal form and the most common in

Abbreviations: TR, trypanothione reductase; NADPH, reduced nicotinamide adenine dinucleotide phosphate; DTNB, 5-5′-dithio-*bis*(2-nitrobenzoic acid).

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India. Currently there is no vaccine for disease and available drugs are considerably toxic, costly and lead to parasitic resistance [7,8]. The emerging resistance of *Leishmania* parasite to these drugs might pose a big threat to the treatment of this deadly disease. Metabolic pathways essential for parasite's survival which are absent in mammalian host are attractive targets for drug discovery. Trypanothione/Trypanothione reductase (TR) metabolism is unique in the parasite and TR is the key enzyme of the pathway. The TR is involved in homeostasis of redox metabolism [9,10]. We have already reported these compounds as good inhibitor of TR [11]. TR inhibition is likely to change parasite's homeostasis unless trypanothione is reduced by alternate stress induced pathways. In current study, we have clearly evaluated the effect of iridoid glucosides from *N. arbortristis* on redox homeostasis of *Leishmania* using several techniques. We have also estimated sustainability of parasite to increased ROS and redox imbalance. Finally, safety measurements of these compounds have also been evaluated on cell lines for their use as potential antiplasmonials.

2. Materials and methods

2.1. Chemicals

Plant (*N. arbortristis*) is also known as night-flowering jasmine and is very common in India. Iridoid glucosides were isolated from *Nyctanthes* seed-kernel by column chromatography of different solvent fractions using silica gel (60–120), followed by elution with acetate–methanol and chloroform–methanol as described earlier [11–13]. Cell permeant probe CM-H_2DCFDA (5-(and -6)-chloromethyl-2,7-dichlorodihydro-fluorescein diacetate acetyl ester), DMEM culture medium, FBS and gentamicin were procured from molecular probe (Invitrogen). Trypanothione was purchased from Bachem. DTNB and all other chemicals of high grade were purchased from Sigma Aldrich, USA.

2.2. Leishmania parasites and cultures

L. donovani (DD8) promastigotes were routinely sub-cultured and maintained in Dulbecco's minimum essential medium (DMEM) with 10% heat inactivated fetal bovine serum, 40 μg/ml gentamicin antibiotic and 25 mM Hepes buffer at 26 °C. Axenic amastigotes were generated from promastigote stage by changing the pH of growth medium to 6.0, temperature 37 °C and providing 5% CO_2 [14,15]. Mouse macrophage (J774A.1) and HEK 293 were maintained in DMEM, 10% FBS, $NaHCO_3$ and 1% penicillin–streptomycin antibiotics.

2.3. Estimation of decrease in reduced thiol level

Inhibition of TR leads to a decrease in reduced trypanothione and it was estimated via microtitre plate assay using DTNB. *Leishmania* promastigotes and axenic amastigotes were grown in 1 ml culture medium for 48 h in presence of various concentrations of iridoid glucosides (1.562, 3.125, 6.25, 12.5, 25, 50 and 100 μM). Cells were centrifuged, dissolved in 10 mM Tris–HCl buffer pH-2.5 and sonicated. Acidic pH was used during sonication to prevent oxidation of free thiol groups. Cell debris was removed by centrifugation. 100 μl of supernatant and 100 μl of 500 mM Phosphate buffer pH-7.5 were taken in each microtitre well followed by addition of 20 μl of DTNB (1 mM) to each well. Absorbance was measured at 412 nm.

2.4. Measurement of ROS elevation in Leishmania promastigotes/axenic amastigotes

Intracellular ROS levels were measured in presence of iridoid glucosides using cell permeant probe CM-H_2DCFDA (5-(and -6)-chloromethyl-2,7-dichlorodihydro-fluorescein diacetate acetyl ester) [16]. *Leishmania* cells were incubated with different concentrations of compounds a, b and c (0, 5, 10 and 20 μM) for different time intervals (3, 12, 24 and 48 h), centrifuged, washed and resuspended in 10 mM Phosphate Buffer Saline pH-7.4. These cells were loaded with 10 μM CM-H_2DCFDA probe in dark for 45 min. ROS levels were measured as an increase in fluorescence due to conversion of non-florescent dye to highly florescent 2′,7′-dichlorofluorescein with an excitation at 488 nm and emission at 530 nm. Morphological changes of *Leishmania* cells as well as ROS levels were further investigated using Flow cytometry (BD FACS caliber). After treatment of *Leishmania* promastigotes and axenic amastigotes with 20 μM of compounds a, b and c, cells were stained with 10 μM CM-H_2DCFDA for 45 min and flow cytometric analysis was done for both treated and untreated cells using laser of 488 nm excitation and 530 nm emission filter.

2.5. Flow cytometric determination of apoptosis using Annexin V-FITC PI kit

Apoptosis causes rapid alterations in phospholipids of cell membrane leading to exposure of phosphatidyl serine on cell surface (early apoptosis) which can be detected by using fluorescein isothiocyanate (FITC) conjugated Annexin V. Necrotic cells also bind with Annexin V-FITC but also get stained with Propidium Iodide (PI). Flow cytometric detection of apoptosis was performed in promastigotes using Annexin V-FITC apoptosis detection kit (Calbiochem) according to manufacturer's protocol [17–19]. Briefly, Promastigotes (1×10^6/ml) were incubated with 20 μM of compounds a, b and c for 24 h. Cells were centrifuged at 1000*g* for 5 min, washed twice with PBS, suspended in 0.5 ml binding buffer followed by addition of 1.25 μl of Annexin V FITC for 15 min at room temperature. Again centrifuged and resuspended in binding buffer followed by addition of 10 μl Propidium Iodide. Flow cytometer (BDFACS caliber) was used with an argon laser (excitation wavelength 488 nm) for detection of Annexin V-FITC stained (early apoptotic) cells in FL1 filter (emission wavelength 518 nm) and PI stained (late apoptotic or necrotic) cells in FL2 filter (emission wavelength 620 nm).

2.6. Estimation of macrophage infectivity in presence of iridoid glucosides

A mouse macrophage (J774A.1) suspension of density 10^6 cells/ml in DMEM with 20% FBS was prepared. Macrophage suspension and promastigotes were mixed (1:10), transferred to 24-well cell culture plate and incubated at 35 °C. Promastigotes gained entry into macrophage in about 8 h; the cells were washed thoroughly to remove non-entered promastigotes [20]. These infected macrophages were incubated with increasing concentration of iridoid glucosides (0–100 μM) for 48 h. Fluorescent images were taken after staining with Ethidium bromide (EB) and acridine orange (AO) double stain (1:1) to differentiate between infected and non-infected macrophage (amastigotes appears as spot in infected macrophage cells). After 48 h, amastigotes were isolated as reported earlier [20]. Briefly, infected macrophages (in PBS with 2 mM EDTA) were passed through a 27-gauge, 0.5 inch syringe thrice followed by

centrifugation at 3500*g* for 5 min to rupture macrophages and remove intracellular amastigotes. The material was suspended in 45% Percoll (in PBS) layered over a cushion of 1 ml 100% Percoll and then centrifuged at 3500*g* for 30 min. The amastigotes were isolated from the interface between 45% and 100% Percoll and counted under microscope. Accordingly, percent infectivity was calculated for all the three iridoid glucosides according to the following formula:

$$\%\ \text{Infectivity} = \frac{\text{Number of intracellular amastigotes in presence of drug}}{\text{Number of intracellular amastigotes in control macrophages}} \times 100$$

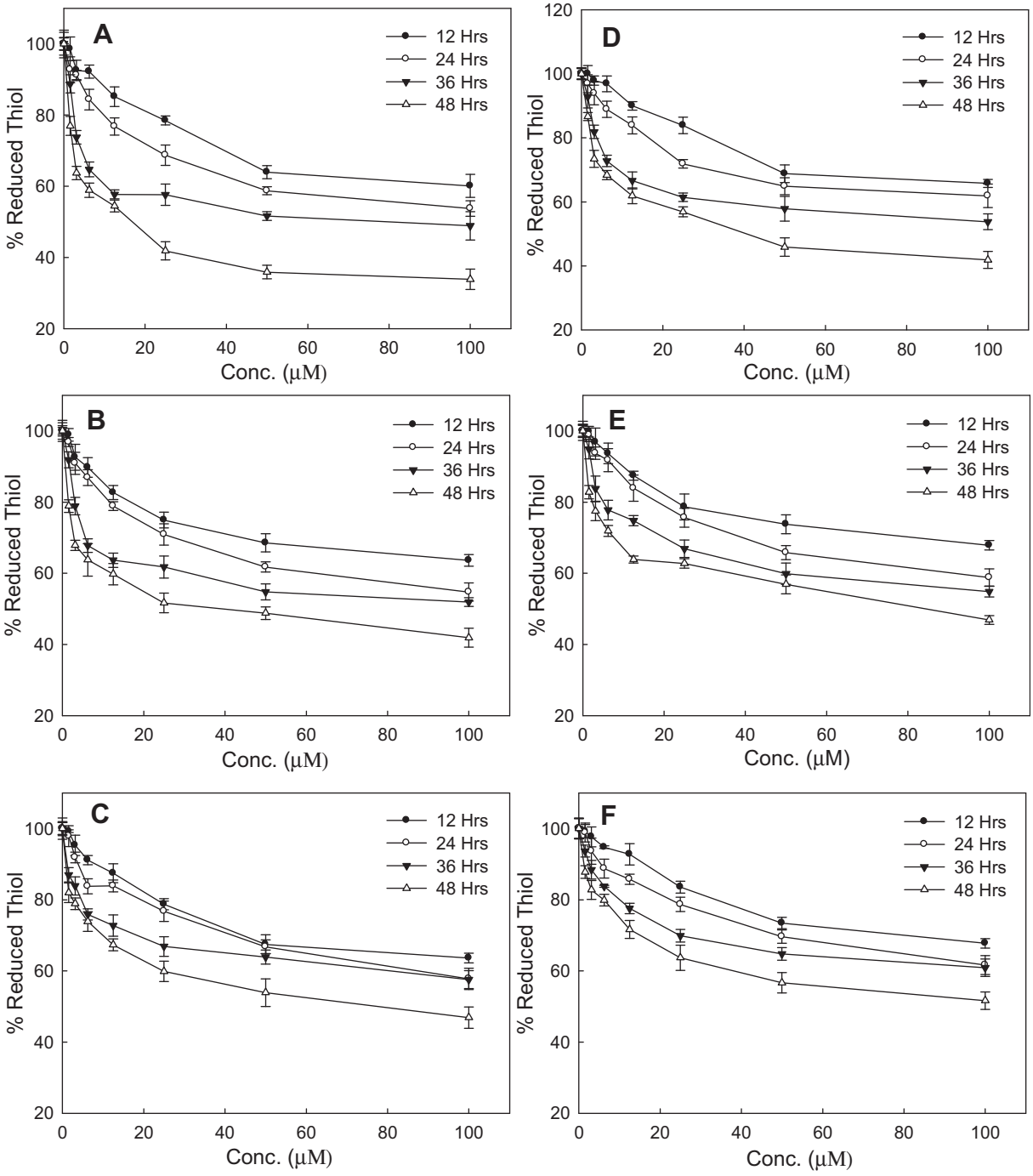

Fig. 1. Estimation of decreased thiol levels: (A), (B) and (C) represent graphs showing decreased thiol levels inside *Leishmania* promastigotes and (D), (E) and (F) inside amastigotes after 12, 24, 36 and 48 h with different concentrations of compounds a, b and c respectively.

2.7. Calculation of IC_{50} values against Leishmania promastigotes/ axenic amastigotes

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) assay [21] was performed for the estimation of viability of promastigotes and axenic amastigotes in presence of iridoid glucosides. Both promastigotes (2×10^6 cells/ml) and axenic amastigotes (1×10^6 cells/ml) were incubated in presence of different concentrations of iridoid glucosides (1 μM-100 μM) for 48 h. The parasite without iridoid glucosides was considered as control. After addition of MTT for 4 h, purple colored formazan complex was formed which was dissolved in DMSO and absorbance was measured at 570 nm to calculate percent viability of the parasite. Viability of control cells was considered as 100%. The absorbance is a measure of living cells and IC_{50} is the concentration of iridoid glucosides which causes death of 50% cells.

2.8. Differentiation of live and apoptotic cells by fluorescence microscopy

Leishmania promastigotes were incubated in 24 well plate with 20 μM compounds a, b and c. The cells were taken out at 0, 12, 24, 36 and 48 h, centrifuged and dissolved in 25 μl PBS. 10 μl EB/AO mixture (100 μg/ml each) was added to the cells and examined by epi-fluorescent microscope in blue filter (470 nm/40×). Live cells get stained with acridine orange and fluoresce green whereas necrotic cells with ethidium bromide and fluoresce red [22].

2.9. Cytotoxicity measurement of iridoid glucosides on HEK 293 cells and mouse macrophage

HEK 293 and mouse macrophage (J774A.1) cells were procured from National Centre for Cell Science, Pune, India. The cells were grown in DMEM containing 1.5 g/l $NaHCO_3$, 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 U/ml) at 37 °C in 5% CO_2. The percent cell viability was estimated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [21] in presence of iridoid glucosides (0, 1, 2, 4, 10, 20, 50 and 100 μM) as described above.

3. Results

Plants are like chemical factories synthesizing diverse set of highly complex compounds whose structures could escape from the imagination of even the most intelligent scientist. Iridoid glucosides from *N. arbortristis* appear to be such compounds. Reports about antileishmanial activity of these compounds on animal model [4] prompted us to understand molecular basis of their antileishmanial effect. In this direction, we have earlier shown these compounds as inhibitors of TR of the parasite [11]. Our current results clearly indicate that these compounds cause imbalance of redox homeostasis and increase in ROS level leading to parasite's death.

3.1. Iridoid glucosides induce decrease in total thiol pool

When promastigotes and axenic amastigotes were incubated with different concentrations of compounds a, b and c for 12, 24, 36 and 48 h, the lowest concentration of 3.125 μM showed a decrease in reduced thiol level up to 92.54 ± 2.87%, 92.58 ± 3.65% and 95.30 ± 2.87% respectively after 12 h. After 48 h, the decrease was 63.72 ± 1.87%, 67.87 ± 1.43% and 78.87 ± 1.65% for the same concentration of each compound. The highest concentration of 100 μM showed a decrease up to 60.09 ± 3.21%, 63.63 ± 1.65%, 62.67 ± 1.33% after 12 h, and 33.82 ± 2.87%, 41.87 ± 2.66%, and 46.87 ± 3.00% after 48 h. Similarly, in case of axenic amastigotes, 3.125 μM concentration showed a decrease in reduced thiol level up to 97.87 ± 1.43%, 96.88 ± 3.87% and 97.66 ± 2.76% after 12 h, and 73.43 ± 2.65%, 77.53 ± 2.1% and 82.76 ± 3.12% after 48 h, 100 μM showed a decrease up to 65.77 ± 1.84%, 67.87 ± 2.76% and 67.76 ± 2.62% after 12 h,

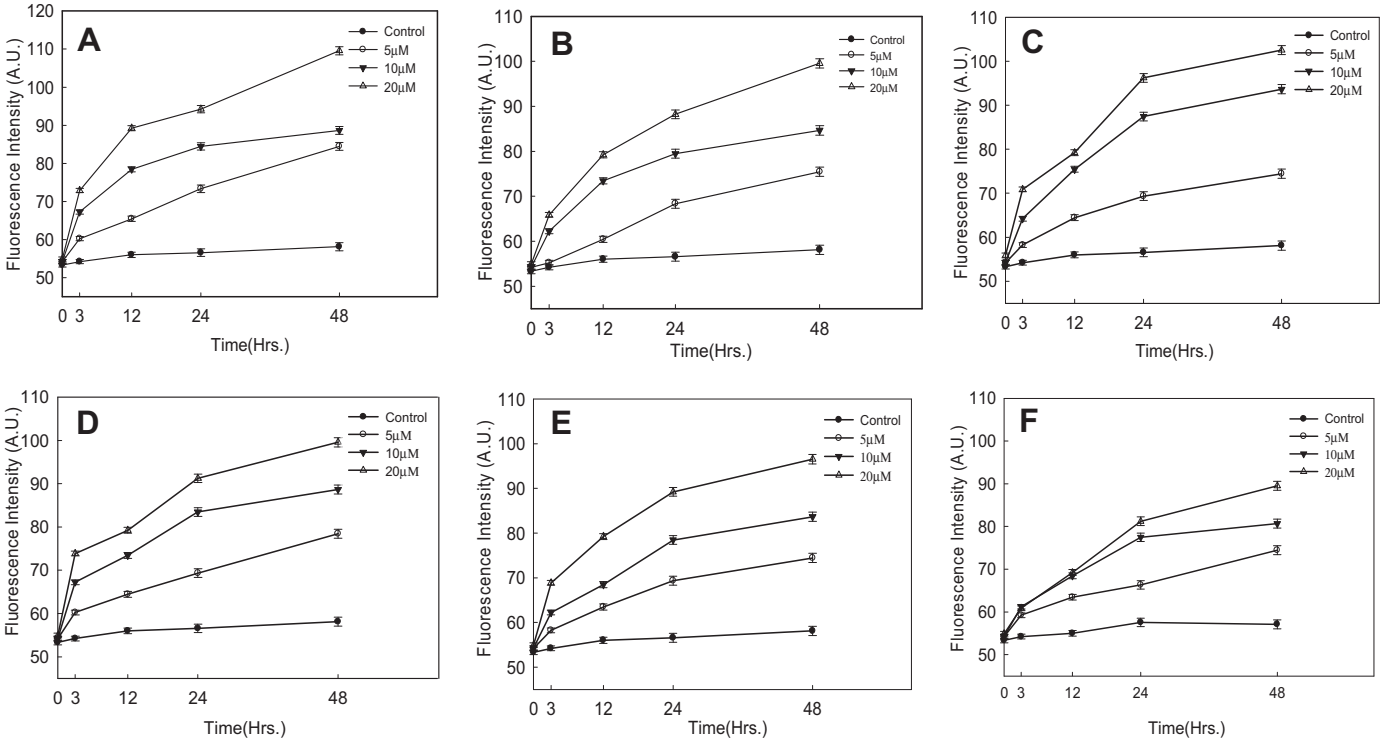

Fig. 2. Measurements of intracellular ROS levels: (A), (B) and (C) fluorescence intensity measurement at 488/530 nm in promastigotes in presence of compounds a, b and c respectively. (D) (E) and (F) fluorescence intensity measurements at 488/530 nm in amastigotes in presence of compounds a, b and c respectively.

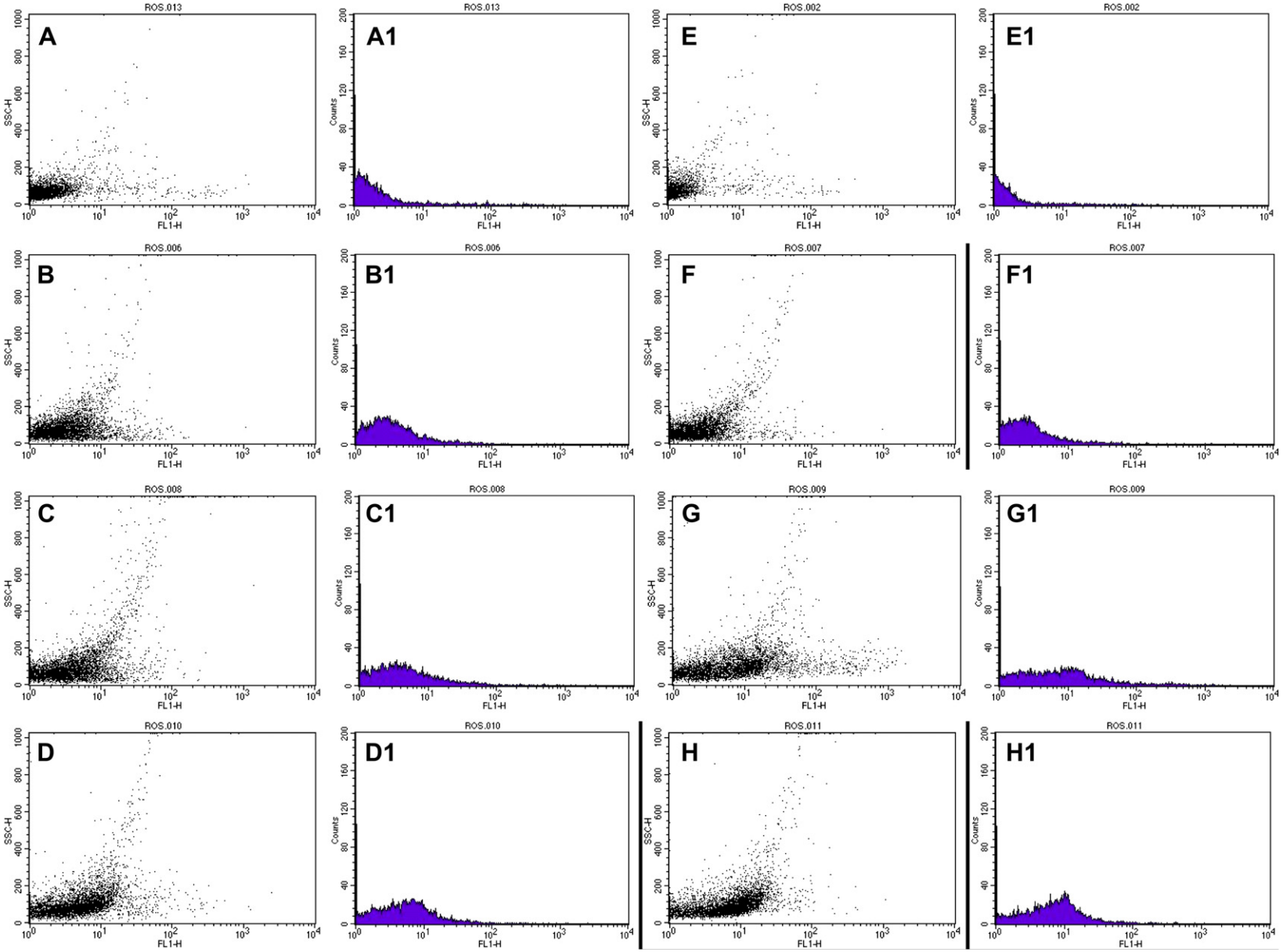

Fig. 3. Flow cytometric analysis in *Leishmania* promastigotes and axenic amastigotes: (A), (B), (C) and (D) represent acquisition dot plots (SSC-H vs. FL1-H) of control promastigotes and promastigotes after treatment with 20 μM of compounds a, b and c respectively, whereas (A1), (B1), (C1) and (D1) represent histogram plot (Counts vs. FL1-H) of control *Leishmania* promastigote and after treatment with compounds a, b and c. Similarly (E), (F), (G) and (H) represent acquisition dot plots and (E1), (F1), (G1) and (H1) represent histogram plot of untreated and treated amastigotes. Both control and treated cells were loaded with 10 μM of CM-H_2DCFDA dye for 45 min in dark and then flow cytometry was performed.

41.87 ± 1.52%, 46.87 ± 2.76% and 51.66 ± 2.43% after 48 h (Fig. 1). These results indicated imbalance in redox homeostasis which leads to a decrease in total reduced thiol levels.

3.2. Iridoid glucosides cause elevation in reactive oxygen species level

Intracellular ROS measurements using CM-H_2DCFDA probe showed that ROS levels were significantly increased with increasing concentration of every compound (Fig. 2). The effect was maximum after 48 h of incubation. Moreover there was no prominent difference in effect on promastigote or amastigotes forms of parasite and both forms appeared to be equally responsive.

Flow cytometric analysis using 488 nm laser and 530 nm emission filter (FL1) showed high population of cells compared to control on density plot, which further confirmed an increase in ROS (Fig. 3). Oxidative stress in the parasite led to morphological changes in the cells. SSC-H is a measure of cell surface topology i.e. when cell is under stress condition, its surface becomes rough and SSC-H increases. FL1 parameter indicates the intensity of fluorescence of probe due to ROS. SSC-H was found to increase substantially in treated promastigotes and amastigotes compared to control, which indicated that cell surface became rough due to oxidative stress. The FL1 intensity, on the other hand, was observed to be greatly increased in treated promastigotes and axenic amastigotes compared to control cells, which in turn confirms significant increase in ROS levels inside the parasite on treatment with iridoid glucosides. These results indicate that *Leishmania* cells are prone to apoptotic death due to increased level of ROS which was further proved by apoptotic assay.

3.3. Iridoid glucosides treated Leishmania promastigotes exhibit apoptosis-like death mechanism

Annexin V, a calcium dependent phospholipid binding protein interacts with externalized PS (Phosphatidyl Serine) by apoptotic cells. Cell impermeable dye PI (propidium iodide) was used to differentiate between necrotic cells and apoptotic cells as Annexin V also labels necrotic cells but PI does not permeate cells until membrane integrity is lost i.e. necrosis of cell occurs. Acquisition dot plot shows quadrant {LL = live cells (both Annexin V and PI Negative), LR = apoptotic cells (Annexin V positive only), UR = necrotic cells (both Annexin V and PI positive) and UL = normally dead cells}. Fig. 4 clearly demonstrates that % of apoptotic and necrotic cells are quite higher in treated cells compared to control cells which may be due to increased ROS level. This data is further substantiated by the fluorescent imaging of *Leishmania* promastigotes stained with double dye (EB/AO). Images showed the progressive death of treated *Leishmania* parasite (200 μM of compounds a, b and c) compared to untreated with increasing time. Compound a is the most effective in killing promastigotes (Supplementary material 2).

3.4. Iridoid glucosides cause hindered growth of intracellular amastigotes

Mouse macrophages (J774A.1) were successfully infected by *L. donovani* DD8 in 8–10 h. Microscopic images clearly showed that in absence of the drug, infected macrophages were damaged and ruptured releasing amastigotes, whereas in presence of 10 μM of

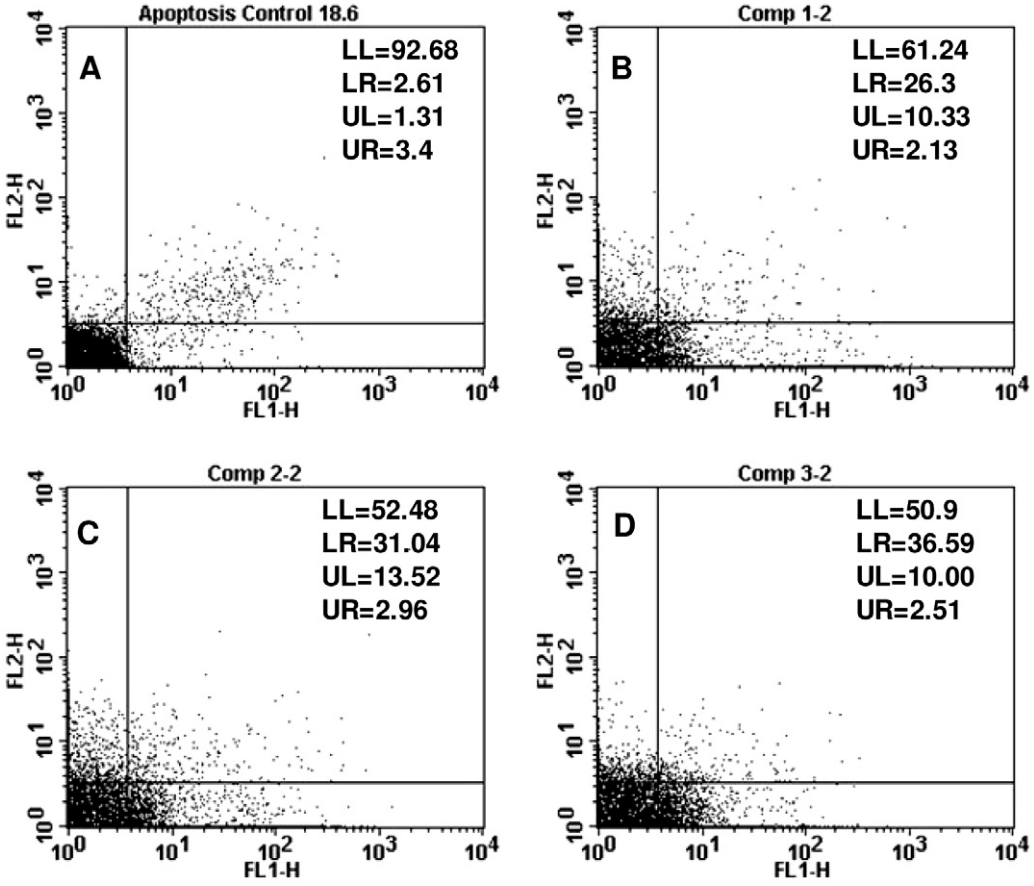

Fig. 4. Flow cytometric determination of apoptosis using Annexin V-FITC, PI kit: acquisition dot plot of (A) control promastigotes, (B) promastigotes in presence of comp a, (C) in presence of comp b and (D) in presence of comp c. Lower left square = live cells, Lower right = early apoptotic cells, Upper right = late apoptotic cells and Upper left = dead cells.

each compound, macrophages were non-ruptured and confluent. Also, macrophages were highly confluent and healthy when 100 μM of compounds a, b and c were present (Fig. 5). It depicts that the growth of intracellular amastigotes is hindered inside the macrophage along with maintaining viability of macrophages in presence of these iridoid glucosides. Epifluorescent images indicated the presence of intracellular amastigotes inside the macrophage as dark-green spots (Fig. 6). Percentage (%) Infectivity was significantly decreased with increasing conc. of each iridoid glucoside as measured by counting the number of isolated amastigotes (Fig. 6E). This indicates that iridoid glucosides inhibit the multiplication of intracellular amastigotes leading to their death.

3.5. Iridoid glucosides lead to death of promastigote and axenic amastigote stages

Compounds a, b and c have shown significant inhibition of growth when tested *in vitro* against *L. donovani* promastigotes and amastigotes via MTT cell viability assay. IC_{50} values of compounds a, b and c against promastigotes and axenic amastigotes were found to be 3.264 ± 0.05 μM, 3.504 ± 0.04 μM, 5.016 ± 0.05 μM and 7.26 ± 0.05 μM, 7.63 ± 0.05 μM, 9.00 ± 0.03 μM respectively (Fig. 7). All these values are very small which signifies these compounds to be very potent inhibitor of cell growth via interfering with the trypanothione metabolism which leads to oxidative stress and death of parasite.

3.6. Iridoid glucosides are not toxic

The cytotoxicity experiments on Human embryonic kidney cells (HEK 293) and macrophage (J774A.1) showed that these compounds have acceptable toxicity if given in limited doses (Fig. 8). 4 μM of compounds a, b and c showed 95.46 ± 1.95%, 93.62 ± 1.50% and 89.63 ± 2.46% cell viability while very high conc. i.e. 100 μM showed 71.25 ± 97%, 54.83 ± 1.60% and 48.62 ± 2.35% cell viability. All the three iridoids have shown negligible toxicity on macrophage. A high concentration of iridoid glucosides (compared to their IC_{50} values) showed slight toxicity on HEK 293 cells. The drug reaches to kidney cells after complete absorption and assimilation process. Thus, even if high initial concentration of these drugs is taken, only a small fraction will reach to kidney cells which will be well within its toxic threshold. Thus, these compounds are safe with respect to human health and have high potential to be developed as antileishmanial drugs. Furthermore, there is no adverse effect of these compounds on macrophage, the host of the parasite, which will lead to decrease in infectivity by *Leishmania*. Taken altogether, these observations pointed out the importance of thiol redox stage for the survival of *Leishmania* parasite and potential application of these compounds as antileishmanials.

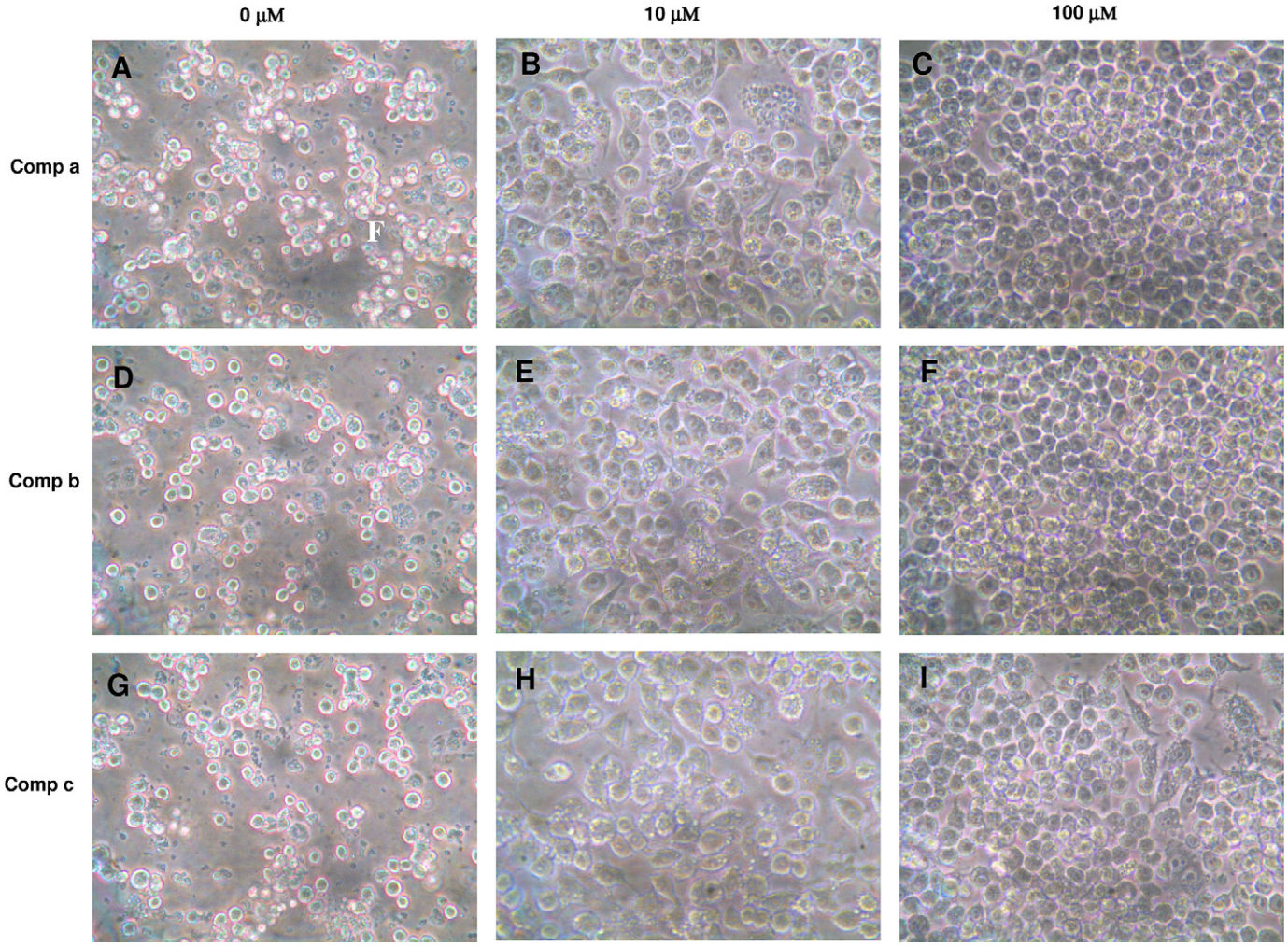

Fig. 5. Microscopic images of infected macrophage at different concentration of iridoids: (A), (B) and (C) at 0, 10 and 100 μM of compound a respectively. (D), (E) and (F) at 0, 10 and 100 μM of compound b respectively. (G), (H) and (I) at 0, 10 and 100 μM of compound c respectively (magnification 100×).

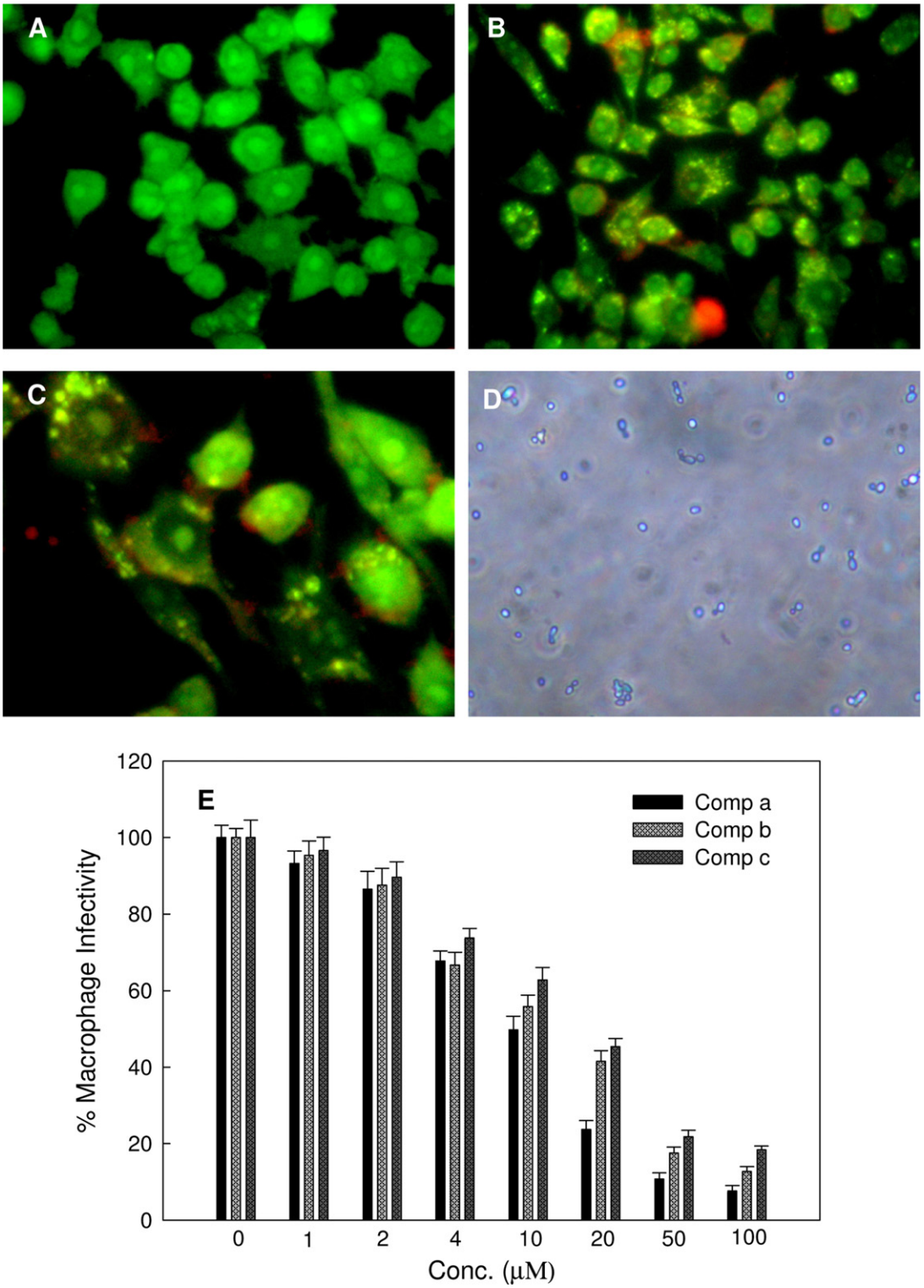

Fig. 6. Optimization of macrophage infection by *Leishmania donovani*: (A) Epifluorescent image of normal EB/AO stained macrophage (Magnification 100×), (B) Epifluorescent image of infected EB/AO stained macrophage (amastigotes are visible as dark-green particles), (C) Epifluorescent image of infected EB/AO stained macrophage at high magnification, (D) Microscopic image of amastigote isolated after infection. (E) Percent infectivity in correlation with the number of isolated amastigotes from infected macrophage at different conc. (0, 1, 2, 4, 10, 20, 50 and 100 μM) of compounds a, b and c. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

Redox homeostasis is crucial for numerous biological events to occur such as enzyme activation, DNA synthesis and cell cycle regulation. As redox imbalance is correlated with increase in ROS, it is likely that the compounds are interfering with some enzyme (s) of trypanothione metabolism involved in removal of oxidative stress. Our preliminary studies established these compounds as

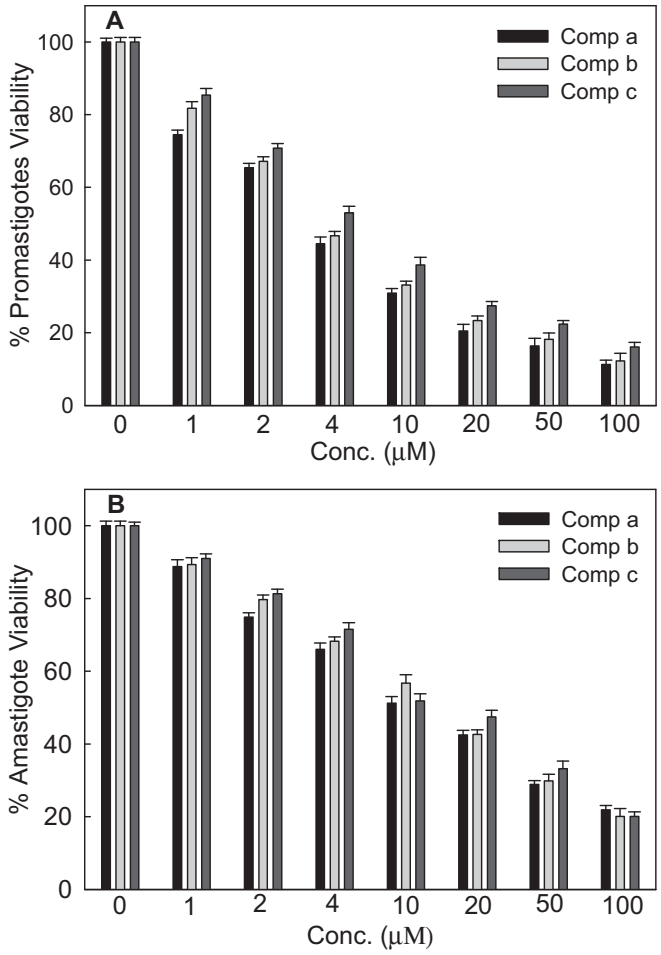

Fig. 7. MTT assay of iridoid glucosides: (A) on *Leishmania* promastigotes and (B) on *Leishmania* axenic amastigotes with different conc. (0, 1, 2, 4, 10, 20, 50 and 100 μM) of compounds a, b and c respectively. Control cells were considered as 100% viable.

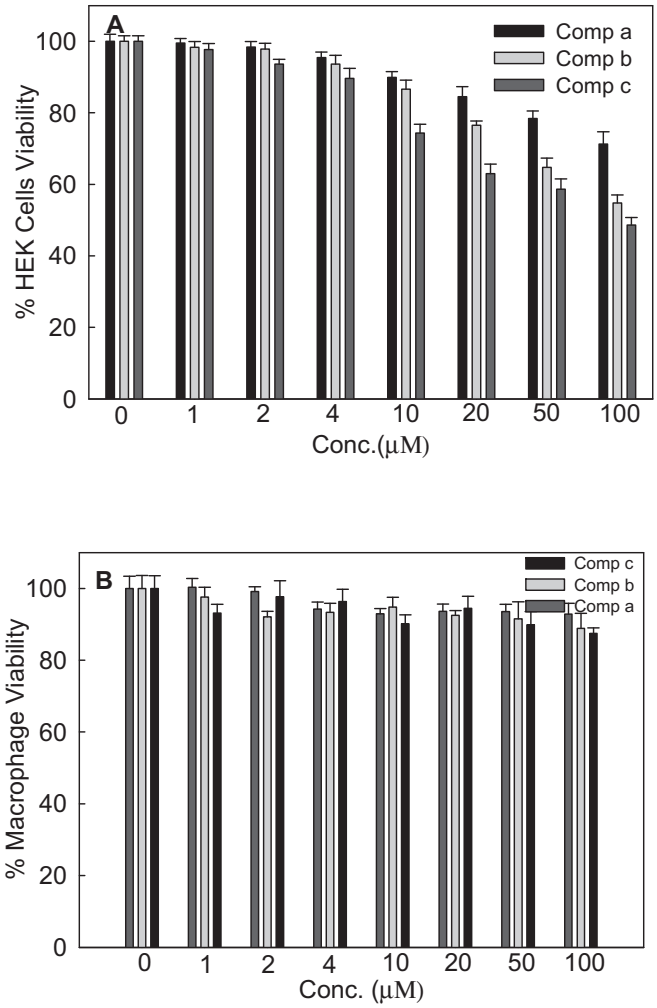

Fig. 8. Safety evaluation of iridoid glucosides: cytotoxicity assay on (A) Human embryonic kidney cells (HEK-293) and (B) mouse macrophages (J774.1A). The cells were incubated with different conc. of compounds a, b and c (0, 1, 2, 4, 10, 20, 50, 100 μM).

inhibitors of TR [11]. However, their effect on other enzymes/proteins involved in transfer of reducing equivalents to Glutathione peroxidase type enzyme (Px) cannot be ruled out. All aerobic organisms, which derive their energy from reduction of oxygen, are quite sensitive to intracellular ROS levels. Small variation in the basal level of intracellular ROS modulates the cell metabolism, gene expression, as well as post-translational modification of proteins [22]. Mammalian cells possess well defined defense mechanisms to detoxify radicals. Catalase or Glutathione peroxidase (GPx) is involved in reduction of hydrogen peroxide into water. *Leishmania* parasite does not have catalase as well as Glutathione peroxidase (GPx) system. However, they have an analogous enzyme called Glutathione peroxidase type enzyme (Px) which carries out the same function [23–25]. Reducing equivalence is provided to Px through cascade of reactions involving several proteins (like TR, thioredoxin and tryparedoxin). Our results so far, pointed out that these iridoid glucosides inhibit TR [11], the crucial enzyme for redox homeostasis of the parasite, and thus stop flow of reducing equivalence to Px. There is a clear correlation between all the results mentioned, such as inhibition of TR causes decrease in total reduced thiol (Fig. 1). Due to lack of reduced thiol, transfer of reducing equivalents to Px is blocked which leads to elevated ROS levels (Figs. 2 and 3). Oxidative stress initiates the apoptosis like death inside *Leishmania* parasite which is proved via apoptosis assay and MTT assay (Figs. 4 and 7). The infection of macrophages is also decreased with increasing conc. of these iridoid glucosides (Figs. 5 and 6). Safety evaluation studies have shown that these iridoid glucosides are quite safe for human administration as indicated by cytotoxicity studies on HEK 293 and mouse macrophages (Fig. 8).

5. Conclusion

Leishmaniasis is a neglected tropical disease categorized as "uncontrolled and spreading" by World Health Organization. There are no vaccines on hand for the disease and the available drugs (such as pentavalent antimonials) have considerable drawbacks i.e. high cost, toxicity and drug resistance. Thus, there is an urgent need for safe and effective novel drug for its cure. Our current reports pointed out toward potential application of iridoid glucosides from *N. arbortristis* as antileishmanials. The compounds seem to have significant antileishmanial effect and adverse effects on parasite redox homeostatic.

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Appendix A. Supplementary data

Supplementary data related to this article can be found online at doi:10.1016/j.ejmech.2012.04.034.

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