CHAPTER- VI

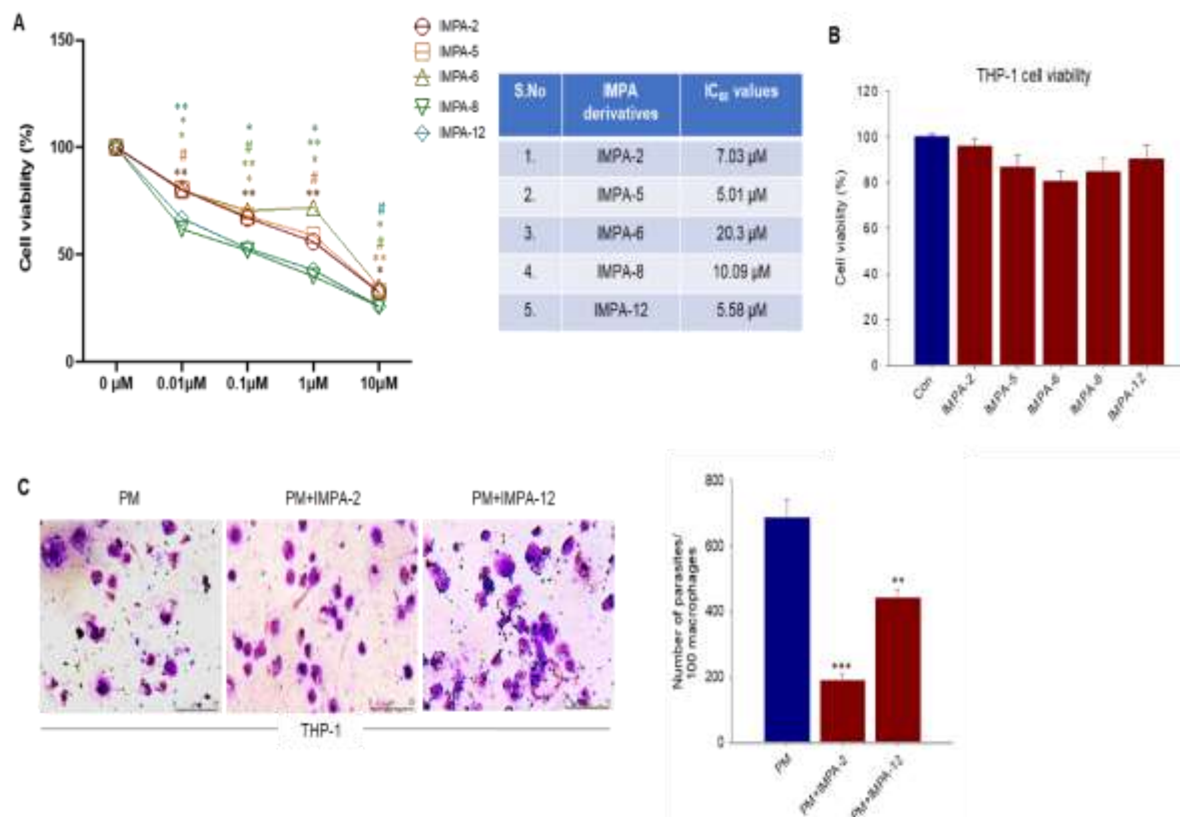
Investigating therapeutic efficacy of Imidazo[1,2-α] pyridine derivatives on the growth inhibition and apoptotic cell death in *L. donovani* promastigotes

6.1 Results

6.1.1 Evaluation of anti-leishmanial activity of the novel imidazo [1,2-α]pyridine-2-amino-4H pyrane derivatives

Synthesis of imidazo[1,2-α] pyridine scaffold and its derivatives by coupling the imidazo[1,2-α] pyridine with 2-amino-4H-pyran was previously described by Dr. Rambabu Gundla and his group for examining their anti-cancer efficacy against non-small cell lung cancer (NSCLC) [2]. Several reports denoted that imidazo[1,2-α] pyridine (IMPA) as a potential core pharmacophore for mitigating leishmaniasis [3-6]. In our study we used five IMPA derivatives (IMPA-2,-5,-6,-8, and-12) for investigating their cytotoxic nature on *L. donovani* promastigotes. For this reason, we treated *L. donovani* promastigotes with these five IMPA derivatives at different concentrations (0.01 µM - 10 µM) for 24 h and cell viability was then assessed by MTT assay. Incubation of *L.donovani* promastigotes with these IMPAs exhibited a significant reduction of their viability in a dose dependent manner **(Fig 6.1A)**. The IC_{50} values of IMPA-2,-5,-6,-8 and -12 against *L. donovani* promastigotes cytotoxicity were found to be7.03 ±0.84 µM, 5.013 ± 0.70 µM, 20.33 ± 1.30 µM, 10.09 ± 1.00 µM, and 5.58 ± 0.74 µM, respectively, suggesting their potential as anti-leishmanial agents. However, when human THP-1 macrophage cells were incubated with these five IMPA derivatives with their respective IC_{50} dosage, we observed that IMPA-2 and IMPA-12 showed minimal cell toxicity against THP1 macrophages **(Fig 6.1B)**. Based on these observations, we considered IMPA-2 and IMPA-12 for further studies. For establishing a successful infection by *L. donovani* the macrophage cells serve as a perfect niche and thereforeit is important to examine the efficacy of IMPA-2 and IMPA-12 whether they could inhibit intra-macrophage *L.donovani* parasite growth and proliferation [9]. We therefore infected THP-1 macrophages with *L. donovani* promastigotes for 24 h and then exposed to IC_{50} concentration of IMPA-2 and IMPA-12 for evaluating their anti-leishmanial activity. A significant reduction of parasite burden in THP-1 macrophages was noticed when treated with IMPA-2 and IMPA-12, however, the effect was more pronounced in IMPA-2 treated cells as

compared to IMPA-12 (**Fig 6.1C**). Collectively, all these results suggest thatIMPA-2 and IMPA-12 possesses profound anti-leishmanial activity against *L.donovani* promastigotes and THP-1 macrophage-infected conditions. Moreover, IMPA-2 and IMPA-12 at their IC_{50} concentrations demonstrated insignificant cytotoxicity against the macrophages.



S.No	IMPA derivatives	IC_{50} values
1.	IMPA-2	7.03 µM
2.	IMPA-5	5.01 µM
3.	IMPA-6	20.3 µM
4.	IMPA-8	10.09 µM
5.	IMPA-12	5.58 µM

Fig 6.1: Imidazo[1,2-α] pyridine-2-amino-4H-pyrane (IMPA) derivatives induces cytotoxicity in *L. donovani* promastigote and inhibits parasite burden in THP1 macrophages. (A) Determination of *L. donovani* promastigote cytotoxicity when incubated with varying concentrations (0.01µM-10µM) of IMPA derivatives (IMPA-2,5,6,8,12) for 24 h (left) and IC_{50} values of each IMPA derivatives were calculated by dose response curve using graph pad prism 8 software (right). Each value is the mean ± SEM of threeindependent experiments, *p < 0.05, **p < 0.01 vs. Control. (B) THP-1 macrophage cell viability was assessed after 24 h incubation of IMPA derivatives. All experiment was performed in triplicate. Each value is the mean ± SEM of three independent experiments.(C) Giemsa-stained micrograph of THP-1 cells incubated with IMPA-2 and IMPA-12 at their respective IC_{50} concentrations (left) and the quantification of *L. donovani* burden in THP-1 macrophages treated with IMPA-2 and IMPA-12 (right). Each value is the mean ± SEM of three independent experiments, **p < 0.01,***p < 0.001 vs PM.

6.1.2. IMPA-2 and IMPA-12 induces apoptosis like cell death and oxidative stress in *L. donovani* promastigotes

Apoptosis or programmed cell death plays a pivotal role in maintaining cellular homeostasis by maintaining a harmony between cellular proliferation and cell death. Apoptosis protects cells from invading pathogens such as viruses and other infectious agents, such as intracellular bacteria and parasites [10]. To evade and attenuate these defensive mechanisms in host macrophages, *L. donovani* promastigotes also developed an arsenal to subvert intracellular ROS and apoptosis pathways [11]. For investigating one of the important hallmarks of apoptosis, which is DNA degradation, we have performed Acridine orange/Ethidium bromide staining of *L. donovani* promastigotes treated with IMPA-2 and IMPA-12 for 24 h. Untreated control *L. donovani* promastigotes exhibited green fluorescence indicated healthy condition. Interestingly, while IMPA-2 incubated promastigotes mostly exhibited necrotic cells (NC) as denoted by the appearance of orange fluorescence with few populations of late apoptotic (LA) cells showed orange-red fluorescence. The IMPA-12 treated promastigotes demonstrated a mixed population of early apoptotic cells (EA) and late apoptotic cells (LA) displaying both yellowish orange to orange-red fluorescence, respectively **(Fig. 6.2A)**. These results suggest that IMPA-2 and IMPA-12 treatment could induce apoptosis like cell death in *L. donovani* promastigotes at their IC_{50} concentrations. It has been shown that IMPA derivatives effectively induces apoptosis in cancer cells by promoting ROS-mediated oxidative stress [2]. We therefore interested to examine the efficacy of IMPA-2 and IMPA-12 on the induction of oxidative stress in *L. donovani* promastigotes. We measured ROS levels in IMPA-2 and IMPA-12 treated *L. donovani* promastigotes by H2DCFDA staining. Both IMPA-2 and IMPA-12 treatment markedly induced ROS production in *L. donovani* promastigotes **(Fig. 6.2B)**. These findings indicate that the efficacy of ROS generation in response to IMPA-2 was comprehensive as compared to IMPA-12. Both IMPA-2 and IMPA-12 treatment effectively induces ROS mediated oxidative stress in *L. donovani* and that may be responsible for apoptosis like cell death.

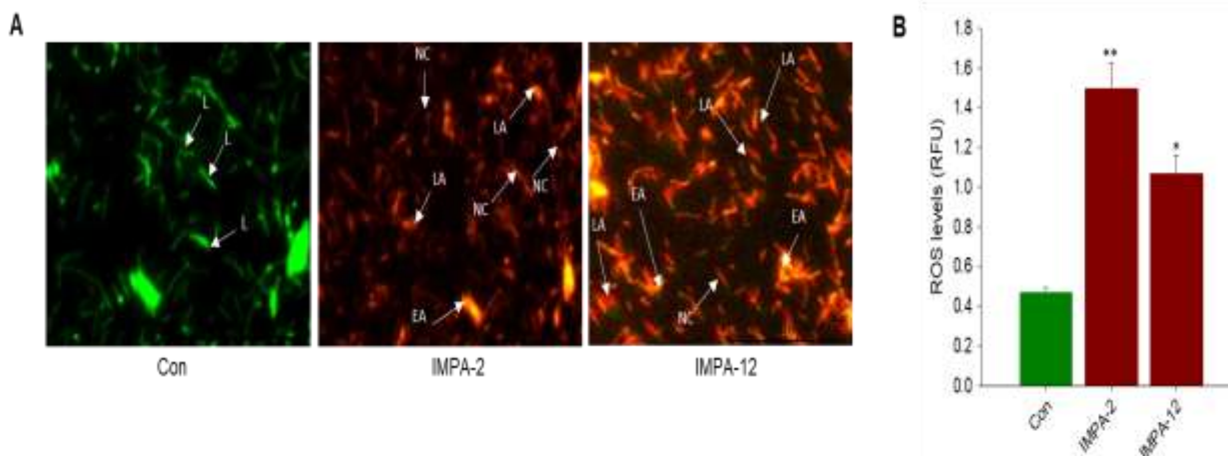


Fig 6.2: IMPA-2 and IMPA-12 incubation promote apoptosis like cell death and oxidative stress in *L. donovani* promastigotes. (A) Representative microscopic images of Acridine orange/Ethidium Bromide stained *L.donovani* promastigotes treated without (control) or with IMPA-2 or IMPA-12 .Untreated Control promastigotes exhibited green fluorescence, denoted as live parasites, whereas, IMPA-2 incubated promastigotes mostly displayed orange fluorescence denoted as necrotic cells (NC), and IMPA-12 treated promastigotes demonstrated a mixed population of cells with yellowish orange to orange-red fluorescence, indicated as early apoptotic cells (EA) and late apoptotic cells (LA), respectively(indicated by white arrows). Scale bars: 75μM. (B) *L. donovani* promastigotes treated without (control) or with IMPA-2 andIMPA-12followed by the addition of H2DCFDA. On termination of incubations, cells were lysed and ROS production was measured by Varioskan LUX multimode reader. Each value is the mean ± SEM of three independent experiments,**p < 0.01, ***p < 0.001 vs. Control.

6.1.3. Induction of apoptotic cell death in *L. donovani* by IMPA-2 and IMPA-12

Annexin V, a calcium-dependent phospholipid binding protein specifically binds to negatively charged phospholipids and labeled with Fluorescein isothiocyanate (FITC) is routinely used for detection of phosphatidylserine translocation in cells undergoing apoptosis, through flow cytometry or fluorescence microscopy [18]. To examine the efficacy of IMPA-2 and IMPA-12 on the promotion of apoptotic cell death in *L. donovani* promastigotes, we performed flow cytometric analysis of FITC-Annexin-V/Propidium iodide (PI). Flow cytometric data revealed that both IMPA-2 and IMPA-

12 treated *L. donovani* promastigotes exhibited lowered percentage of viable cells which coincided with the significant increase of dead, late apoptotic and early apoptotic cell percentage **(Fig 6.3 A,B)**. This observation further confirms the induction of apoptotic cell death in *L. donovani* promastigotes in response to IMPA-2 and IMPA-12 incubations.

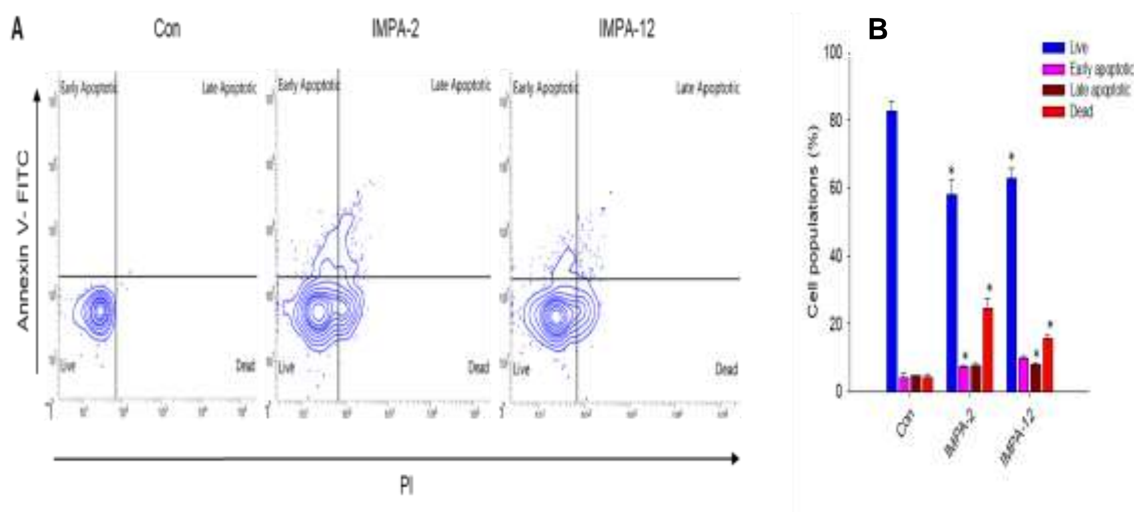


Fig 6.3: IMPA-2 and IMPA-12 treatment induces apoptosis in *L. donovani* promastigotes.

(A) Flow cytometric analyses of Annexin V-FITC/PI staining (left) (B) and their quantifications (right) indicating apoptosis of *L. donovani* promastigotes in response to IMPA-2 and IMPA-12. Graph represents the mean ± SEM of three independent experiments in triplicate, $*p < 0.05$, $**p < 0.01$ vs. Control.

6.1.4. IMPA-2 and IMPA-12 promotes loss of mitochondrial membrane potential ($\Delta\Psi_m$) in *L. donovani* promastigotes

Alteration of mitochondrial membrane potential ($\Delta\Psi_m$) is one of the key features of the onset of apoptosis [12]. To assess the loss of mitochondrial membrane potential ($\Delta\Psi_m$) in *L. donovani* promastigotes in response to IMPA-2 and IMPA-12 incubations, we performed JC-1 staining. JC-1 is a cationic dye which aggregates to form JC-1 dimer in the mitochondrial matrix, when the membrane potential is hyperpolarized, emitting red fluorescence and on the other hand, monomeric JC-1 form present in

mitochondria with low membrane potential or depolarized state emitting green fluorescence. Flow cytometric analyses of JC-1-stained *L. donovani* promastigotes exhibited fluorescence intensity shifted from red to green in response to IMPA-2 and IMPA-12 as compared to control parasites. It would be interesting to note that IMPA-12 treated promastigotes exhibited more depolarization of membranes than IMPA-2 treated promastigotes **(Fig 6.4 A,B).**

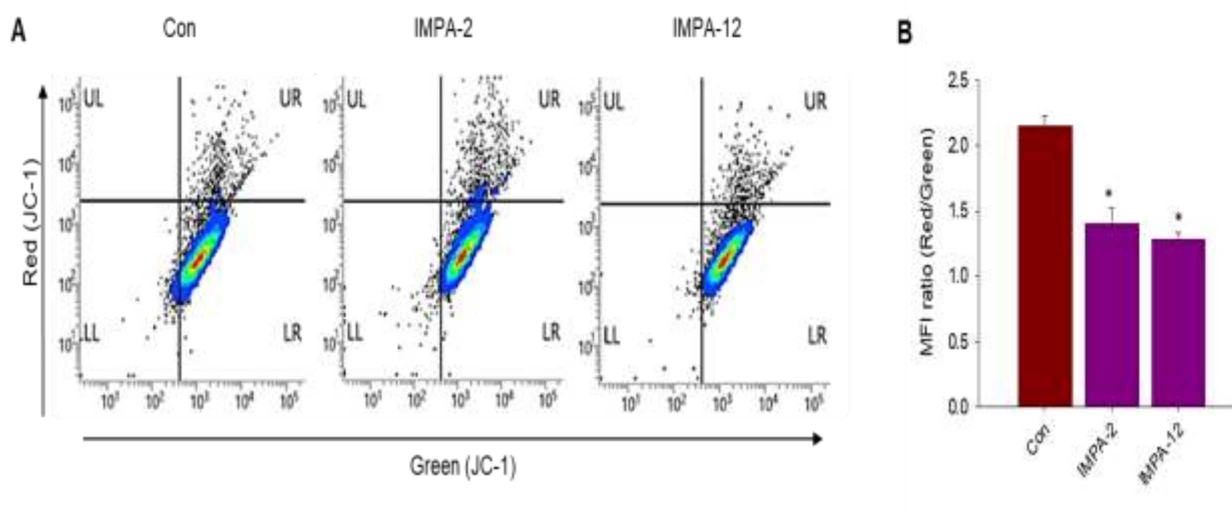


Fig 6.4: Impairment of mitochondrial membrane potential in *L. donovani* in response to IMPA-2 and IMPA-12. (A) Flow cytometric analyses (left) and (B) their quantification (right) representing JC-1 staining of *L. donovani* promastigotes exhibited loss of mitochondrial membrane potential as indicated by red to green fluorescence shifting upon treatment with IC_{50} doses of IMPA-2 and IMPA-12. Graph represents the mean± SEM of three independent experiments in triplicate, *$p < 0.05$ vs. Control.

6.1.5. IMPA-2 and IMPA-12 promotes cell cycle arrest in *L. donovani* promastigotes

Induction of cellular apoptosis mediated DNA damage known to associate with the inhibition of cell cycle progression [13]. To investigate the effect of IMPA-2 and IMPA-12 on the cell cycle impairment of *L. donovani* promastigotes, we performed Propidium iodide staining followed by flow cytometric analysis of cell cycle distribution. We found that *L. donovani* promastigote population in G0/G1 cell cycle phase was significantly increased with concomitant decrease of S and G2/M cell cycle

phase in response to IMPA-2 and IMPA-12 incubations. The cell cycle arrest at G0/G1 phase in *L. donovani* promastigotes upon IMPA-2 treatment was considerably more profound as compared to IMPA-12 incubation. These results suggest that IMPA-2 and IMPA-12 incubations suppressed the proliferation of *L. donovani* promastigote by cell cycle arrest at G0/G1 phase **(Fig 6.5)**.

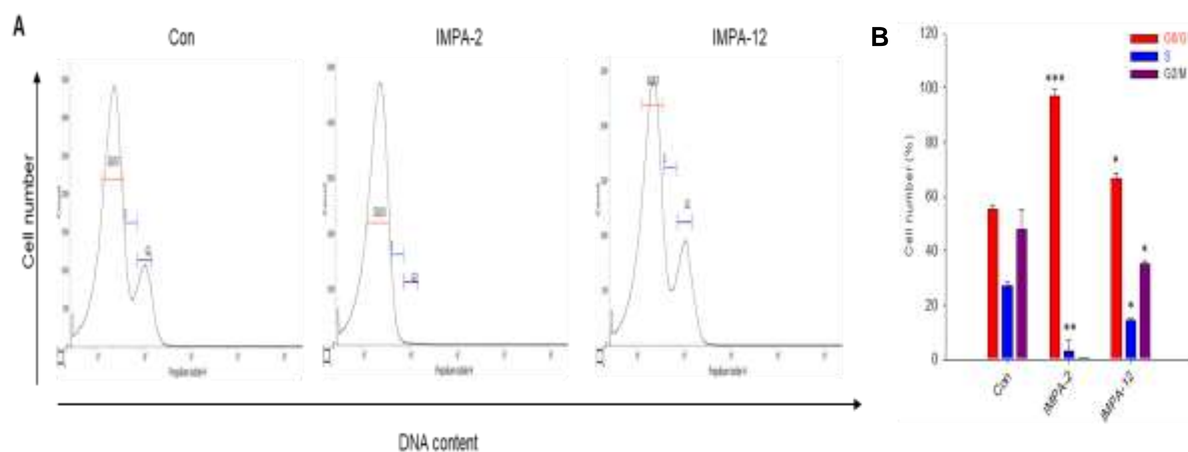


Fig 6.5: IMPA-2 and IMPA-12 persuades cell cycle arrest in *L. donovani* promastigotes. (A) Flow cytometric assessment of cell cycle distributions and (B) their quantifications exhibiting % of G0/G1, S, and G2-M cell populations in response to IMPA-2 and IMPA-12 treated *L.donovani* promastigotes. Graph represents the mean ± SEM of three independent experiments,*p < 0.05, **p < 0.01 vs. Control.

6.2 Discussion

Commercially available drugs for the management of leishmaniasis, showed drug resistance, toxicity, lack of specificity, multiorgan side effects and prolonged treatment regime [20]. First line drugs such as pentavalent antimonials, sodium stibogluconate and meglumine antimoniate although demonstrated good therapeutic effects, however often associated with acute cardiotoxicity, drug resistance and

pancreatitis [21-26]. Whereas, the second line drugs available for the treatment are pentamidine and amphotericin B, which associated with severe side effects including myalgia, breathing difficulty, nephrotoxicity, hypotension, hypokalemia, and drug resistance [20, 27-32]. To mitigate these side effects researchers are working towards the design and development of newer, safe, and effective therapeutic agents for managing leishmaniasis. In this context, many heterocyclic motifs have been explored in recent years for their therapeutic potential as anti-leishmanial agents [33-38].

Cumulative research evidence suggesting that heterocyclic organic moieties such as triazoles, chalcones, chromone, thiazoles, thiosemicarbazones, indole, imidazole and quinolines are gained popularity among the researchers for finding an effective therapeutic candidate with lesser side effects in combating visceral leishmaniasis manifestation [1]. Synthesis of IMPA scaffold and its derivatives by coupling of imidazo[1,2-α] pyridine with 2-amino-4H-pyran was previously described by Dr. Rambabu Gundla and his group [2]. They found that IMPA derivatives are potent candidate for inducing apoptosis in non-small cell lung cancer (NSCLC) [2]. Moreover, reports denoted that imidazo[1,2-α] pyridine as a potential core pharmacophore for mitigating leishmaniasis [3,4,5,6]. Particularly, amino pyran belongs to pyran family of heterocycles gained more insight for its cytotoxic and microbicidal potential and thus serve as a suitable candidate for synthesizing imidazo[1,2-α] pyridine derivatives [7,8]. For this study, we have used five IMPA derivatives, IMPA-2,-5,-6,-8, and -12, which were gifted by Prof Gundla to investigate their therapeutic potential as anti-leishmanial agents. Cell viability assay showed that, these five IMPA derivatives significantly decreased cell viability in a dose dependent manner at micromolar concentrations against *L. donovani* promastigotes. We also checked the THP1 macrophage viability in response of these IMPA derivatives, and found that IMPA-2 and IMPA-12 does not have any significant cytotoxic effect on THP-1 macrophage. These cell viability analyses demonstrated that both IMPA-2 and IMPA-12 have potent cytotoxic effect against promastigotes as indicated by their IC_{50} values of IMPA-2: 7.03 ± 0.84 μM and IMPA-12: 5.58 ± 0.74 μM. On contrary, these IMPA derivatives displayed minimal cell toxicity towards THP-1 macrophage cells. Since macrophage serve as a host cell

for *L. donovani* amastigote growth and proliferation [9], therefore, it would be important to check the efficacy of IMPA-2 and IMPA-12 for the inhibition of intra-macrophagic parasite survival. Treatment of IMPA-2 and IMPA-12 to THP-1 macrophage cells minimized the parasite load. Thus, out of five selected IMPA derivatives, IMPA-2 and IMPA-12 exhibit promising anti-leishmanial activity. From the structural point of view of this five IMPA's, IMPA-2,-5,-6,-8, and -12 contains -CH_3,-CL,-F,-Br, and -CF_3 groups, respectively. Our data suggest that IMPA-2 and IMPA-12 by having -CH_3 and -CF_3 group could be responsible for their potent anti-leishmanial activity without posing significant toxic effect on host macrophage cells. To investigate the efficacy of different synthetic drug candidates as anti-leishmanial agents, researchers primarily focused their analyses on apoptosis and oxidative stress pathways, as potential therapeutic targets for several years. Induction of apoptosis or programmed cell death plays an important role in protecting cells from invading pathogens such as viruses and other infectious agents, such as intracellular bacteria and parasites [10]. In *L. donovani*, features like nuclear condensation, DNA fragmentation and cell shrinkage shares similarity with metazoan apoptosis [11,13]. Both Acridine orange/EtBr staining and Annexin V/FITC staining of *L. donovani* promastigotes showed that IMPA-2 and IMPA-12 treatment notably promoted apoptotic cell death at their IC_{50} concentration. Externalization of phosphatidyl serine is a prominent marker of apoptosis process in cellular level. Translocation of PS from inner leaflet to the outer surface of the cell is an early mediator of cellular apoptosis. In viable cells PS is undetectable on the outer cell surface, but when a cell undergoes apoptotic cell death, PS is translocated on the outer cell surface [14-17].In our study, we also found that both IMPA-2 and IMPA-12 significantly elevated cellular oxidative stress through ROS generation and turned catastrophic for *L. donovani* promastigotes survival. As reported previously, induction of cellular ROS leads to mitochondrial dysfunction by depolarizing mitochondrial membrane potential contributing to the induction of apoptosis [14].

Mitochondria are cellular organelle which plays an important role for the cellular survival, as they function as a reservoir of ATP. Maintaining the proton gradient across the inner mitochondrialmembrane is needful for ATP production during

oxidative phosphorylation. Thus, mitochondrial membrane potential (ΔΨm) is essential for both ATP production and cell survival [12]. We therefore evaluated mitochondrial membrane potential in *L. donovani* promastigotes after treatment with IMPA-2 and IMPA-12. The JC-1 staining showed that mitochondrial membrane of the parasites was highly depolarized when treated with IMPA-2 and IMPA-12 as suggested by the increased fluorescence intensity shift from red to green ratio, at their IC_{50} concentration. The impairment of cell cycle progression is attributed for the inhibition of cell proliferation and survival [13]. In this work, we also analyzed the activity of IMPA-2 and IMPA-12 on cell cycle progression. The IMPA-2 and IMPA-12 treated promastigotes mainly arrested the cell cycle progression at G0/G1 phase although the effect is more pronounced in IMPA-2 than IMPA-12.

In summary, this present investigation highlighted the efficacy of IMPA derivatives, particularly IMPA-2 and IMPA-12 for their anti-proliferative and pro-apoptotic properties against *L. donovani* promastigotes. Both IMPA-2 and IMPA-12 are significantly induced oxidative stress and apoptosis hindering promastigote growth inside host macrophage cells. Evidence also pointed out that IMPA-2 is more proficient in inhibiting parasite growth, ROS induction, mitochondrial dysfunction, cell cycle inhibition at G0/G1 phase and promoting apoptosis as compared to IMPA-12. Thus, IMPA-2 and IMPA-12 could be used as potential therapeutics against visceral leishmaniasis.

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