Scientific Research

Antileishmanial Potential of *Piper nigrum* Seed Extracts against *Leishmania donovani*

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Abstract

Visceral leishmaniasis (VL) is a pestilent form of leishmaniasis that chiefly impinges the poorest sections of the society. The prototypical therapeutic interventions in vogue are handicapped due to toxicity and alarming increase in drug resistance. In the absence of vaccines, progressive emergence of HIV-VL co-infection and relapse in the form of post kala-azar dermal leishmaniasis, have fuelled the quest for alternative therapies. Herein, we report antileishmanial activity of *Piper nigrum*, which is endowed with multifarous medicinal properties. Hexane (PNH) and ethanolic (PNE) extracts of *P. nigrum* substantially inhibited the growth of *Leishmania donovani* promastigotes with 50% inhibitory concentration (IC_{50}) of 31.6 and 37.8 μg·ml^{-1}, respectively. Growth reversibility analysis revealed the leishmanicidal effect of PNH which caused cell shrinkage and flagellar disruption. In contrast, PNE treated promastigotes showed partial effect. PNH and PNE also abrogated the growth of intra-macrophagic *Leishmania* amastigotes with IC_{50} of 14.6 and 18.3 μg·ml^{-1}, respectively. Anti-amastigote efficacy of PNH was accompanied by higher selectivity over host macrophages than PNE. Gas-Chromatography-Mass Spectrometry showed the presence of several secondary metabolites such as trans-*β*-caryophyllene, piperine, *β*-bisabolene and other sesquiterpenes in PNH and piperine, *δ*-(sup 9)-cis oleic acid and piperyline in PNE. Conclusively, our work revealed discernible antileishmanial activity of *P. nigrum* extracts.

Keywords

Visceral Leishmaniasis, *Leishmania donovani*, *Piper nigrum*, Antileishmanial, Plant Extracts

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1. Introduction

Leishmaniasis is a neglected, usually poverty associated complex vector-borne disease that causes huge morbidity and economic losses all over the world. Visceral leishmaniasis (VL), which is the fatal form of leishmaniasis, is responsible for an annual death toll of 60,000 and incidence of 500,000 cases per annum. It is estimated that around 200 million people living in 109 districts in India, Bangladesh, and Nepal are at risk of developing visceral infection. The antiquated chemotherapeutics in use for antileishmanial therapy either cause noxious effects or are rendered ineffective due to growing parasite resistance. Escalation in leishmaniasis burden owing to multiple factors such as co-infection with HIV, occurrence of Post Kala-Azar Dermal Leishmaniasis (PKDL) as relapse infection, lack of vaccine based approaches and difficulties in controlling vectors, have left chemotherapeutic intervention as the sole alternative [1].

Plants offer ample sources to develop new antileishmanials and replace synthetic drugs. Herbal remedies have been utilized by human population residing in leishmaniasis endemic regions since ancient times. Plants of Piperaceae family have long served the world civilization as wealthy source of ingredients for food spices and traditional medicines. *Piper nigrum* is a well-known medicinal plant that possesses secondary metabolites with antibacterial, immunomodulatory, cytotoxic, and antimalarial activities [2]. Plant extracts from various species of the genus *Piper* have demonstrated potent antileishmanial efficacy and leishmanicidal molecules apart from piperine have also been identified [3]-[6]. This prompted us to evaluate the antileishmanial efficacy of *P. nigrum* seed extracts against *L. donovani* and to check the presence of plant secondary metabolites other than piperine, which could be responsible for the leishmanicidal effect.

2. Materials

2.1. Parasite Culture and Maintenance

L. donovani strain AG83 (MHOM/IN/83/AG83) was maintained *in vivo* in BALB/c mice. The promastigote form of the parasites was cultured in Medium 199 (M199), pH 7.4 supplemented with penicillin G sodium (100 U·ml^{-1}), streptomycin sulfate (100 µg·ml^{-1}), HEPES (25 mM) and 10% heat-inactivated FBS (complete M199). Log phase promastigotes were subcultured every 72 - 96 h, in the same medium at 22˚C, inoculum being 2×10^6 cells·ml^{-1} [7].

2.2. Plant Material and Preparation of Extracts

P. nigrum dried fruits were purchased locally, and authenticated by Dr. H.B. Singh (voucher no. NISCAIR/RHMD/Consult/-2010-11/1440/38), Taxonomist, NISCAIR, CSIR, New Delhi. The dried fruits were thoroughly washed with water, shade dried and ground to powder form. The powdered plant material (100 g) was sequentially extracted with solvents of increasing polarity, including hexane, ethanol and water by percolation. The powder was initially soaked in hexane (500 ml) and four washes (250 ml each, at 24 h interval) were performed, followed by extraction in ethanol and water in a similar way. Filtrate at each step was passed through Whatman filter paper (No. 1), pooled and then concentrated to dryness under vacuum by rotary evaporation at 35˚C for hexane (PNH) and ethanolic (PNE) extracts and lyophilized to obtain the aqueous (PNA) extract. The dried extracts were stored at −20˚C until used for bioassay.

2.3. Evaluation of Anti-Promastigote Potential of *P. nigrum* Extracts and Assessment of Growth Reversibility

Growth kinetics assay was performed to appraise the anti-leishmanial efficacy of *P. nigrum* extracts. Freshly transformed *L. donovani* promastigotes (2×10^6 cells·ml^{-1}) were incubated at 22˚C in the presence or absence of test extracts (PNH, PNE and PNA) at 500 µg·ml^{-1}. Piperine, one of the known constituents of *Piper* species and pentamidine, a standard antileishmanial drug, (positive control used for *in vitro* studies) were also tested at 500 µg·ml^{-1} for all the experiments. DMSO (0.5%), which was used to solubilize the extracts, was taken as solvent control to determine any unspecific parasite death. The bioactivity of the extracts was assessed by enumerating the viable parasites under phase contrast microscope (40×) after every 24 h for 7 days. Erythrosin B stain (0.2%, 1:1) was used to distinguish live and dead cells. In a parallel set of experiment, untreated and treated parasites were harvested, washed twice with M199 and finally resuspended in fresh complete M199 at 22˚C. After 96 h,

the cell viability was determined by counting the motile, viable, flagellated cells under the microscope [8].

2.4. Determination of 50% Promastigote Growth Inhibitory Concentration (IC$_{50}$) of the Bioactive Extracts and Study of Cellular Morphology

Exponential phase promastigotes (2×10^6 ml^{-1}) were incubated in triplicates with the bioactive extracts, piperine, pentamidine or medium alone at serial three fold dilutions (500 to 2.05 µg·ml^{-1}). After 96 h, parasite survival was assessed at different concentrations of the drugs by enumerating the live promastigotes and percent viability calculated according to the formula:

$$\% \text{ Viability} = \frac{\text{Average viable cell count per ml (treated samples)}}{\text{Average viable cell count per ml (parasite control)}} \times 100$$

IC$_{50}$, the concentration that decreased parasite growth by 50% was determined by graphical extrapolation. In parallel, at 96 h, the parasites were harvested, washed twice (3000 ×g, 10 min, 4˚C) with phosphate buffered saline (PBS, 0.02 M, pH 7.4), fixed in 80% chilled ethanol, stained with erythrosin B and observed under Nikon Eclipse 80i microscope (100× objective) for morphological alterations. Since the cells were fixed prior to staining, erythrosin B was not used to distinguish between live and dead cells but for staining the cells for optimal imaging.

2.5. Evaluation of Anti-Amastigote Activity *ex Vivo* and Determination of Nitric Oxide (NO) Production

Leishmania infected macrophage cells (RAW 264.7) were used to test intracellular efficacy of the bioactive extracts. Macrophages (5×10^6 ml^{-1}, 100 µl per well) in RPMI-1640 medium were plated on round cover slips in 24-well plates. The cells were allowed to adhere for 24 h in carbon dioxide incubator with 5% CO$_2$ at 37˚C. Non adherent cells were removed followed by infection with stationary phase promastigotes, maintaining a *Leishmania*: macrophage ratio of 10:1. After 24 h, the non-phagocytosed promastigotes were washed with serum-free RPMI 1640 and the infected macrophages were incubated with RPMI 1640 (infected control) or with serial four-fold dilutions of PNH, PNE, piperine (200 to 3.12 µg·ml^{-1}) or pentamidine (100 to 0.39 µg·ml^{-1}) in triplicates at 37˚C. After 48 h, the cover slips were washed with PBS, dried, fixed with chilled methanol (analytical grade) and giemsa-stained. At least 200 macrophages per cover slip from triplicate cultures were counted to calculate the percentage of infected macrophages. Percent amastigote infectivity was determined using the formula:

$$\% \text{ Reduction} = \frac{\text{Number of amastigotes per 200 macrophages}_{\text{(treated samples)}}}{\text{Number of amastigotes per 200 macrophages}_{\text{(infected control)}}} \times 100$$

IC$_{50}$, the concentration of drug that is cytotoxic to 50% of the amastigotes, was obtained by plotting the graph of percent infectivity versus drug concentrations tested [9]. NO was estimated from supernatants of treated and untreated macrophages by Griess reaction [10].

2.6. Cytotoxic Potential of the Bioactive Extracts and Determination of Selectivity Index

To assess the cytotoxicity, the macrophages (2×10^6 ml^{-1}) in RPMI 1640 were seeded (200 µl·well^{-1}) and left for adherence. After 24 h, the non-adherent macrophages were removed by washing with serum-free RPMI and the adherent macrophages were incubated with the bioactive extracts at serial two fold dilutions (500 to 7.81 µg·ml^{-1}) in triplicates for 48 h. MTT reagent (5 mg·ml^{-1}, 50 µl per well) was added for a further 4 - 6 h, following which the formazan crystals were dissolved in isopropanol:dimethylsulfoxide (1:1) and the absorbance taken in ELISA plate reader at 570 nm. The absorbance by macrophages in the absence of any treatment was considered to be 100%. CC$_{50}$, the cytotoxic concentration at which 50% of macrophages are viable was calculated according to the formula:

$$CC_{50} = \frac{\text{Mean specific absorbance}_{\text{(treated samples)}}}{\text{Mean specific absorbance}_{\text{(control samples)}}} \times 100$$

Selectivity index (SI) as a measure of specificity of the leishmanicidal effect of the extracts against internalized parasites over the host macrophages was determined as the ratio of CC_{50} to IC_{50} against intramacrophagic amastigotes.

2.7. Gas Chromatography-Mass Spectrometry (GC-MS) of Secondary Metabolites in Bioactive Extracts

The secondary metabolites from PNH and PNE were characterized by GC-MS using a Shimadzu QP2010 with a DB-5 column (30 m, film 0.25 mm, ID 0.25 mm). The temperature of the column was increased gradually from 45˚C to 270˚C at 5˚C·min^{-1}, and the injector and detector temperature for the analysis was about 250˚C. Helium was used as the carrier gas at a flow rate of 1.5 ml·min^{-1}. Identification of the chemical constituents was based on correlation of the recorded mass spectra with those obtained from WILEY8.LIB and NIST08.LIB library spectra provided with the software of the GC-MS system [8].

2.8. Statistical Analysis

All experiments were performed at least thrice in triplicate. The results shown are from one of three independent experiments and expressed as mean ± standard error of mean (SEM) of the samples in triplicate. Statistical analysis was performed using Graph-Pad software and statistical significance was calculated by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. Antiproliferative Effect of PNH and PNE on *L. donovani* Promastigotes

PNH was the most potent in inducing parasite death. PNE also exhibited killing of *L. donovani* promastigotes but was less effectual than PNH. Pentamidine, completely arrested the parasite growth after 72 h of incubation (**Figure 1(a)**). No cell death was apparent in PNA, parasite control, and 0.5% DMSO (solvent control) treated parasites. Piperine initially induced marginal reduction in the promastigote proliferation rate after which the parasites expanded normally.

3.2. Growth Inhibitory Effect of PNH on *L. donovani* Promastigotes Is Leishmanicidal

The cytocidal (lethal) or cytostatic (growth inhibitory) effect of *P. nigrum* extracts was confirmed in a growth reversibility assay. Analysis of parasite count after 96 h revealed negligible reversion of growth in PNH treated parasites, indicating that the mode of PNH induced killing was essentially leishmanicidal (**Figure 1(b)**). However, low parasite count observed in case of PNE treatment reflected its cytostatic effect. High parasite counts were observed in parasites receiving no treatment (parasite control) or upon treatment with 0.5% DMSO (solvent
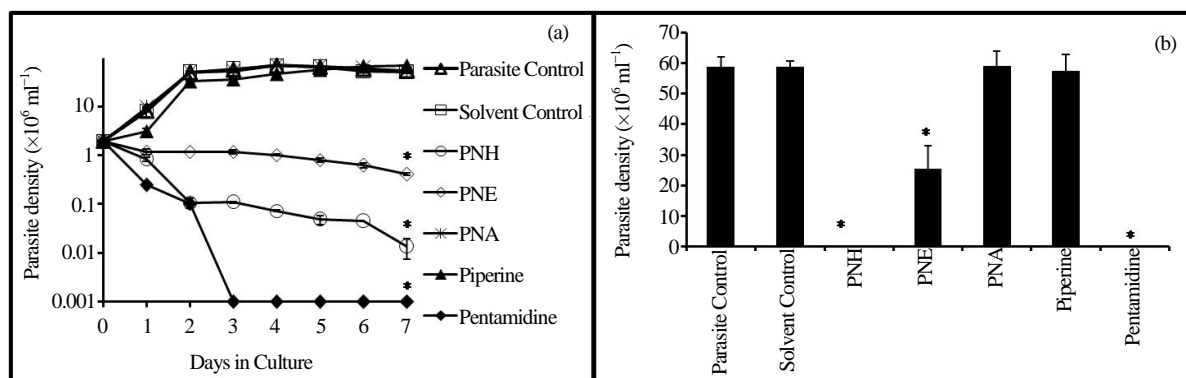

Figure 1. (a) Antiproliferative effects of *P. nigrum* extracts against *L. donovani* promastigotes. Promastigotes (2×10^6 ml^{-1}) were incubated with 500 µg·ml^{-1} of extracts or 0.5% DMSO (solvent control) and viable cells enumerated at different time points; (b) Growth reversibility analysis of *P. nigrum* extracts after 96 h. PNH and pentamidine treated promastigotes exhibit no reversion in parasite growth upon drug withdrawal. *$P < 0.001$ with respect to parasite control.

control) or PNA whereas in pentamidine treated parasites, there was absolutely no reversion in growth of *L. donovani* promastigotes.

3.3. PNH Induced Morphological Alterations in *L. donovani* Promastigotes

Morphological changes in *L. donovani* promastigotes in the presence or absence of *Piper* extracts were examined microscopically (100×) after 96 h. The photomicrographs depicted that the control untreated parasites retained their long flagella, slender and elongated shape, and were highly motile (**Figure 2(a)**). PNH treated promastigotes however, had an atypical appearance owing to shortening of flagella, cell shrinkage and consequent circularization. Similar observations were found after pentamidine treatment whereas such changes were less pronounced in PNE treated samples. No such morphological alterations occurred in piperine treated promastigotes.

3.4. IC$_{50}$ of Bioactive Extracts against *L. donovani* Promastigotes

To assess IC$_{50}$ concentration of the bioactive extracts of *P. nigrum*, parasites were incubated for 96 h with different concentrations of the extracts (2.05 to 500 µg·ml^{-1}) and control was set without any treatment. PNH and PNE induced dose dependent inhibition of parasite growth with IC$_{50}$ value corresponding to 31.6 and 37.83 µg·ml^{-1}, respectively (**Figure 2(b)**, **Table 1**).

3.5. *P. nigrum* Extracts Abrogated Intracellular Amastigote Form of *L. donovani* in *ex Vivo* Macrophage—Amastigote Model

The anti-leishmanial activity of PNH and PNE was tested against intracellular amastigotes in *L. donovani*
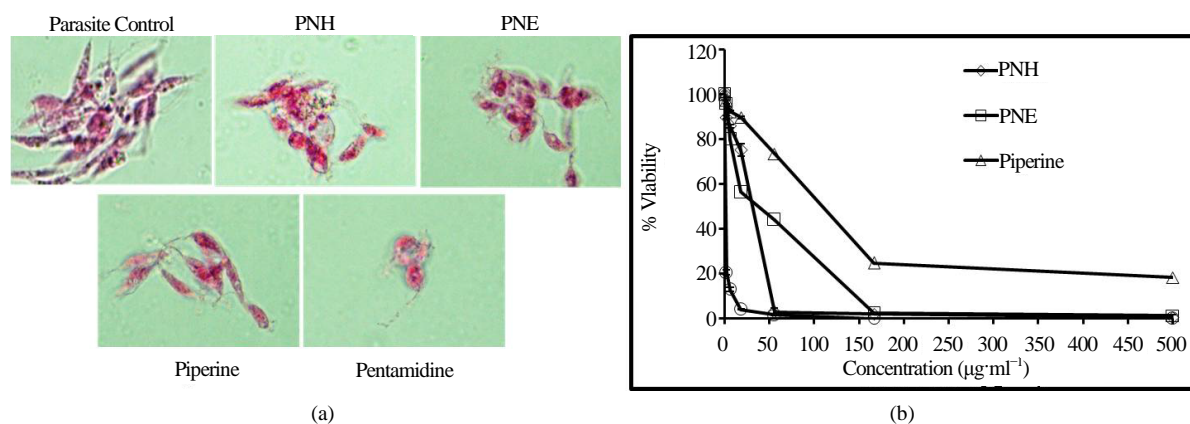
Figure 2. (a) Morphological alterations in *L. donovani* promastigotes after treatment with *P. nigrum* extracts. Changes in cellular morphology 96 h post treatment with the bioactive extracts, piperine and pentamidine as observed under microscope (100×); (b) Dose-dependent antipromastigote activity of bioactive extracts. Promastigotes (2×10^6 ml^{-1}) were incubated with increasing concentrations of test extracts (2.05 to 500 µg·ml^{-1}) and the IC$_{50}$ was determined as described in methods. Inset shows mean IC$_{50}$ values of treated promastigotes.

Table 1. Anti-promastigote and amastigote activity of *P. nigrum* extracts and determination of Selectivity Index (SI).

P. nigrum extracts/compounds	IC$_{50}$ (µg·ml^{-1}) promastigotes (Mean ± SE)	IC$_{50}$ (µg·ml^{-1}) amastigotes (Mean ± SE)	CC$_{50}$ (µg·ml^{-1}) macrophages (Mean ± SE)	SI
PNH	31.6 ± 0.13	14.63 ± 1.58	379.7 ± 8.19	25.95
PNE	37.83 ± 2.58	18.33 ± 0.82	200 ± 1.69	10.91
PNA	>500	>500	-	-
Piperine	108.66 ± 1.96	41.33 ± 1.44	>500	-
Pentamidine	1.09 ± 0.055	0.84 ± 0.16	45.2 ± 2.86	53.80

SI of bioactive extracts was determined as a measure of their toxicity against RAW 264.7 macrophages. SI = CC$_{50}$ against macrophages/IC$_{50}$ against amastigotes, SE = Standard error.

infected RAW 264.7 cells in comparison to pentamidine (100 to 0.39 µg·ml^{-1}), owing to its cytotoxicity. Percent inhibition for each group (PNH, PNE, piperine, and pentamidine) was plotted in a concentration-response curve to calculate the IC$_{50}$ as elaborated in methods. PNH and PNE exhibited a dose dependent reduction in parasite load with IC$_{50}$ of 14.63 and 18.83 µg·ml^{-1}, respectively. The IC$_{50}$ of pentamidine was found to be 0.84 µg·ml^{-1} and that of piperine was achieved at 41.33 µg·ml^{-1} (**Figure 3**, **Table 1**), which is indicative of its moderate anti-leishmanial activity.

3.6. Antileishmanial Activity of *P. nigrum* Extracts Is Independent of NO Generation

Since NO is one of the major effector microbicidal molecules responsible for clearance of *Leishmania* amastigotes, we probed the role of NO in PNH and PNE induced death. We found that our bioactive extracts along with piperine and pentamidine failed to generate NO in *L. donovani* infected RAW macrophages ($P > 0.05$, data not shown).

3.7. Cytotoxicity of *P. nigrum* Extracts towards Mammalian Macrophages

To assess the safety of bioactive extracts of *P. nigrum* against macrophages, the RAW 264.7 cells were incubated with increasing concentrations of PNH and PNE (0 to 500 µg·ml^{-1}). In order to examine the specificity of these extracts against *Leishmania* amastigotes over host macrophages, the SI was calculated as a ratio of CC$_{50}$ against macrophages to IC$_{50}$ against amastigotes. PNH was found to be most selective with SI of 25.95 (**Table 1**). PNE exhibited SI = 10.91, whereas piperine was non-toxic against RAW macrophages.

3.8. GC-MS Analysis of PNH and PNE

GC-MS profiling of PNH and PNE was performed to identify the leishmanicidal plant secondary metabolites in these bioactive extracts. PNH constituted 52 compounds, the most abundant being trans-β-caryophyllene (22.28%). The other constituents included β-bisabolene (6.08%), tetrahydropiperine (5.66%), 9Z-9-tetra-decenal (5.32%), α-copaene (4.76%), 3-amino-4-piperonyl-5-pyrazolone (4.14%), 1,1-dimethyldecahydronap-thalene (3.42%), 14-methyl-8-hexadcyn-1-ol (3.04%), δ-cadinol (2.21%), 1-methyldecahydronapthalene (2.75%), α-caryophyllene (2.06%), 1-(12-octadecanoyl) pyrrolidine (2.03%), δ-cadinene (1.81%), α-selinene (1.80%), β-elemene (1.62%) and elemol (1.37%). Piperine content of PNH was observed to be 8.65% (Supplementary
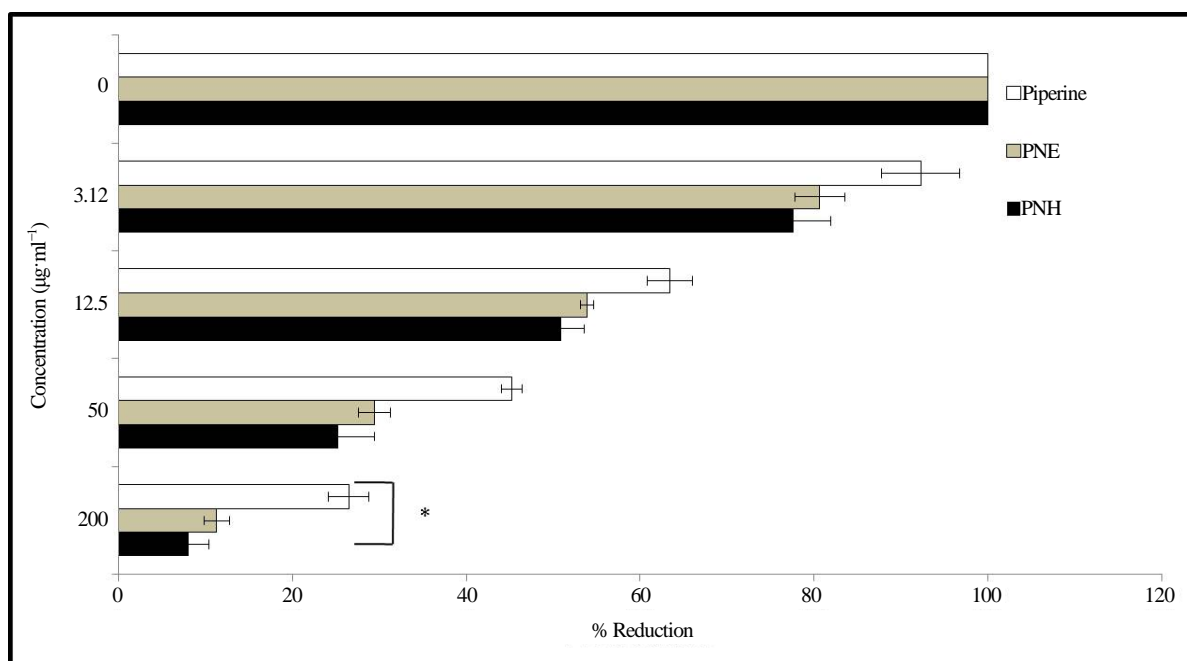

Figure 3. Antiamastigote efficacy of PNH and PNE. Antiamastigote efficacy was evaluated in RAW macrophages parasitized with *L. donovani* promastigotes as described in methods. $^*P < 0.001$ with respect to infection control.

File 1). However, PNE was dominated by piperine (70.36%) though other components such as δ-(sup 9)-cis oleic acid (3.71%), piperoylpiperidine (3.70%), piperyline (3.02%), 3-amino-4-iperonyl-5-pyrazolone (2.13%), tetrahydropiperine (1.64%), trans-β-caryophyllene (1.33%) were also detected (Supplementary File 2).

4. Discussion

In the present work, we found that hexane (PNH) and ethanolic (PNE) extracts of *P. nigrum* were found to be more effectual in reducing *in vitro* proliferation of *L. donovani* promastigotes and amastigotes in comparison to piperine. Our data elaborated the cytocidal effect of PNH that is preferred over cytostatic mode of cell death as evident with PNE, for advantages such as rapid elimination of parasites, lesser risk of resistance development or resurgence of infection. Alterations in parasite morphology were also observed and the irreversible changes in morphology accompanying minimal growth reversion further corroborated the leishmanicidal action of PNH in comparison to PNE. Pentamidine was used as a known antileishmanial, that has been earlier reported by us [8] [9] and others [11] for *in vitro* studies.

Further, the antiamastigote efficacy of the extracts was independent of NO generation. Trans-β-caryophyllene, a major plant secondary metabolite found in PNH has also been reported to be active against *L. amazonensis* in NO independent manner [12]. Piperine also failed to induce NO production and similar observation has also been demonstrated by others [13]. SI values were calculated to assess the adverse cytotoxicity of bioactive extracts and SI values above 10 were considered specific in accordance with those reported for antiprotozoal compounds including *Leishmania* [14]. In accordance, PNH was found to be highly specific and least toxic, whereas PNE was found to be moderately specific.

GC-MS analysis of PNH revealed that the major portion of the extract was constituted by cyclic sesquiterpenes. Leishmanicidal activity of this class of compounds has been well documented [15]. Tetrahydropiperine, a derivative of piperine present in PNH (5.66%) and to a lesser extent in PNE (1.64%) has exhibited antileishmanial activity against *L. amazonensis* [13]. In addition to being antileishmanial, trans-β-caryophyllene has also been reported to be antitrypanosomal [16] and antitumoricidal [17]. Leishmanicidal potential of piperine was reported as early as 1993 against an attenuated *L. donovani* UR6 strain where it completely inhibited its growth after 5 days of incubation at 1.2 mg·ml^{-1} [18]. IC$_{50}$ of piperine against *L. donovani* promastigotes has been reported to be 2.558 mM [19]. We also found that piperine was moderately active against *L. donovani* promastigotes even at 500 µg·ml^{-1}. Thus, the GC-MS data further demonstrates that *P. nigrum* extracts are a source of potent leishmanicidal plant secondary metabolites besides piperine.

5. Conclusion

Conclusively, our studies have demonstrated promising antileishmanial potential of *P. nigrum* extracts. Hexane (PNH) and ethanolic (PNE) extracts exhibited antileishmanial activity with PNH being highly selective towards intracellular stage of the parasite. Thus, the active principles present in *P. nigrum* seeds may be a source of promising leishmanicidal compounds and may serve as structural templates for synthesis of various synthetic and semi-synthetic derivatives with improved antileishmanial activity.

Acknowledgements

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