Journal of Traditional and Complementary Medicine 13 (2023) 611–622

Contents lists available at ScienceDirect

Journal of Traditional and Complementary Medicine

journal homepage: http://www.elsevier.com/locate/jtcme

Prosopis juliflora (Sw.) DC.induces apoptotic-like programmed cell death in *Leishmania donovani* via over production of oxidative stress, mitochondrial dysfunction and ATP depletion

Bhimashankar Utage [a, **], Milind Patole [a], Punam Nagvenkar [a], Rajesh Gacche [b, *]

[a] National Centre for Cell Science, NCCS Complex, Pune, 411007, MS, India
[b] Department of Biotechnology, Savitribai Phule Pune University, Pune, 411007, MS, India

ARTICLE INFO

Article history:
Received 29 January 2023
Received in revised form
3 May 2023
Accepted 29 June 2023
Available online 29 June 2023

Keywords:
Visceral leishmaniasis
Natural products
Prosopis juliflora
Apoptosis
Cell cycle
GC-MS

ABSTRACT

Background: Leishmaniasis is endemic in more than 60 countries with a large number of mortality cases. The current chemotherapy approaches employed for managing the leishmaniasis is associated with severe side effects. Therefore there is a need to develop effective, safe, and cost affordable anti-leishmanial drug candidates.

Purpose of the study: This study was designed to evaluate the *in vitro* antileishmanial activity of a *Prosopis juliflora* leaves extract (PJLME) towards the *Leishmania donovani* parasites.

Material and methods: PJLME was evaluated for its cytotoxicity against the *L. donovani* parasites and the mouse macrophage cells. Further, various *in vitro* experiments like ROS assay, mitochondrial membrane potential assay, annexin v assay, cell cycle assay, and caspase 3/7 assay were performed to understand the mechanism of cell death. Phytochemical profiling of *P. juliflora*was performed by utilizing HPTLC and GC-MS analysis.

Results: PJLME demonstrated antileishmanial activity at a remarkably lower concentration of IC_{50} 6.5 μg/mL. Of note, interestingly PJLME IC_{50} concentration has not demonstrated cytotoxicity against the mouse macrophage cell line. Performed experiments confirmed ROS inducing potential of PJLME which adversely affected the mitochondrial membrane potential and caused loss of mitochondrial membrane potential and thereby ATP levels. PJLME also arrested the cell cycle and induced apoptotic-like cell death in PJLME treated *L. donovani* promastigotes.

Conclusion: The results clearly established the significance of *Prosopis juliflora* as an effective and safe natural resource for managing visceral leishmaniasis. The findings can be used as a baseline reference for developing novel leads/formulations for effective management of visceral leishmaniasis.

1. Introduction

Leishmaniasis is caused by several species of trypanosomatid protozoan parasites that are transmitted by the bite of a female phlebotomine sandfly. More than 20*Leishmania* species are reported to cause human infections.[1] The most prominent symptoms of *Leishmania* infection include skin ulcers, mucosal lesions, and fatal visceral infections. Leishmaniasis is currently endemic in 88 countries and threatens 350 million people, with a worldwide prevalence of 12 million cases. WHO (World Health Organization) included this disease in neglected tropical diseases and made a combating plan for 2030. In India, visceral leishmaniasis (VL) is also called a kala-azar, and it is caused by an intracellular protozoan parasite belonging to the genus *Leishmania*. Human VL has no prophylactic vaccine; thus, treatment relies only on chemotherapeutic drugs. *Leishmania* parasite has two morphological forms:

Abbreviations: GC-MS, Gas chromatography–mass spectrometry; PJLME, *Prosopis juliflora* leaves methanol extract; TM, Traditional Medicine; VL, visceral leishmaniasis; XTT, Sodium-2,3-bis-[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide.

* Corresponding author.
** Corresponding author.
E-mail addresses: bhima@nccs.res.in (B. Utage), rngacche@unipune.ac.in (R. Gacche).

Peer review under responsibility of The Center for Food and Biomolecules, National Taiwan University.

https://doi.org/10.1016/j.jtcme.2023.06.003

promastigotes and amastigotes, and only the amastigote form is responsible for human pathogenesis, which affects various organs, including the spleen, liver, lymph nodes, etc., and sometimes it becomes fatal if it is not treated correctly.

In the current mainstream of leishmaniasis treatment, there are no vaccines available for the prevention of leishmaniasis. The development of effective vaccines could help to reduce the burden of the disease. Unfortunately, there is availability of only a handful of antileishmanial drugs for the treatment of *Leishmania*, wherein the majority of them are associated with severe side effects, off-target toxicities, unaffordable cost, and prolonged hospitalization owing to invasive drug administration routes. Patients with leishmaniasis may also be co-infected with other diseases, such as HIV, malaria, and tuberculosis, which can complicate the line of treatment and disease management. Moreover, there are other challenges related to controlling disease-spreading vectors, need for adequate and prompt diagnosis methods, and limited treatment modalities.

Nevertheless, the efficacy of the available antileishmanial drugs is limited due to evolving drug resistance and treatment failure. In fact, treatment failure is the more frequently observed in almost all types of leishmaniasis worldwide. Of note, drug resistance is more particularly described in the case of visceral leishmaniasis in South East Asia.[2] The current chemotherapeutic approach still relies on prescribing pentavalent antimonials, miltefosine, paromomycin, and liposomal amphotericin B. However, the efficacy of these drugs is limited owing to life-threatening severe side effects such as arthralgias, m-+9yalgias, leukopenia, pancreatitis, hypoglycemia, diabetes mellitus, hypotension, abscess formation at the site of injection, cardiotoxicity, cardiac arrhythmia.[3] Moreover, the induction of parasite resistance, duration of treatment, and unaffordable high-cost constraints are a few more adversities associated with the effective management of leishmaniasis. Looking towards the efforts invested in developing vaccines against leishmaniasis, it seems that antileishmanial vaccines may not be made available shortly; therefore, excursion for novel and effective leads from natural sources is a significant hope for the treatment of leishmaniasis.

Natural products and drug discovery have remained a proven association since ancient times, and plant-derived drugs have contributed significantly to treating various infectious and noninfectious human ailments. Novel antileishmanial agents should be non-toxic or less toxic, safe, efficient, and economically affordable to low-income populations of developing countries. From ancient times to till date, many countries have relied on their traditional system of medicine, wherein medicinal herbs have occupied an important place in the mainstream of traditional therapeutics. Nevertheless, WHO has formulated a '*Traditional Medicine Strategy* 2014–2023′ with a clear mandate of supporting member countries towards harnessing the potential contribution of *Traditional Medicine* (TM) for community health; this mandate is also for promoting the safe and effective use of TM by making certain norms, doing the required research, and integrating TM products, practitioners, and practice into National health care systems, where appropriate.[4] Medicinal herbs possess diverse secondary metabolites, which have been explored for several pharmacological properties such as anti-microbial, antiparasitic, anticancer, antidiabetic, and antioxidant activities.[5]

Developing combinatorial therapy of anti-leishmanial drugs with natural products is currently appreciated most promising and rational approaches for the amelioration of drug toxicity, drug resistance, and treatment failure rates, and also for increasing the drug efficacy by synergic effects of natural products.[6] Variety of botanicals such as *Artemisia annua, Allium sativum*, *Curcuma longa*, *Baccharis uncinella*, *Olea europaea*, *Handroanthus* species, *Artemisia aucheri*, *Urtica dioica*, *Piper marginatum*, *Tabernaemontana coronaria*, *Picramnia gracilis*, etc. have demonstrated significant antileishmanial effect under *in vitro* and *in vivo* settings.[7] These herbs can be explored as complementary and alternative combination therapeutic approaches for effectively managing leishmaniasis. The biologically active secondary metabolites such as polyphenols, flavonoids, terpenoids, coumarins, and a variety of alkaloids have been shown to act against a variety of human ailments, including inhibition of the growth of several *Leishmania* species.[8–10] *Prosopis juliflora (Sw.),* DC is a xerophytic plant species that extensively grow in Asia, Africa, Australia, and Brazil. Besides the uses of *Prosopis* species as furniture wood, firewood, and household animal feed, the plant's pods are also used in the diet in many parts of the world owing to a large amount of sugar.[11] From ancient times, *P. juliflora* has been helpful in various folk remedies, such as wound healing, fever, inflammation, measles, excrescences, diarrhea, and dysentery.[12] Previously, we have demonstrated the anticancer efficacy of *Prosopis juliflora* against triple-negative breast cancer cells under *in vitro* and *in vivo* settings.[13] Recently, we have also investigated its mechanistic activity against melanoma cell lines and showed that its phytoconstituents induce apoptosis, inhibit cell proliferation, signaling pathways, epithelial-mesenchymal transition, migration, invasion, angiogenesis and stem cell markers of B16F10 mouse melanoma cells.[14] Owing to its tolerance to extreme drought conditions, we hypothesized the presence of diversified bioactive metabolites. We were inspired to undertake the present investigation of assessing the efficacy of *Prosopis juliflora* against visceral leishmaniasis caused by *Leishmania donovani*parasites.

2. Material and methods

2.1. Chemicals, cell culture media, and reagents

Fetal bovine serum (FBS), Dimethyl sulfoxide (DMSO), Sodium pyruvate, Sodium-2,3-bis-[2-methoxy- 4-nitro-5-sulfophenyl]-2H-tetrazolium- 5-carboxanilide (XTT), 2′,7′-Dichlorodihydrofluorescein diacetate (DCFH-DA), RPMI-1640 cell culture medium (R6504 and R8755), Annexin FITC/PI kit, RNase A, and Hoechst 33258 from Sigma Chemical Co. (St. Louis, MO, USA), Caspase 3/7 assay kit procured from Promega, USA. All other chemicals used were of AR grade and were obtained from commercial sources.

2.2. Collection and preparation of the plant material

Leaves of the *Prosopis juliflora* (Sw.), DC. (family-Mimosaceae) were collected from the campus of Swami Ramanand Teerth Marathwada University, Nanded (MS), following standard practice, and permission was obtained. Plant study complies with relevant institutional, national, and international guidelines and legislation. The plant sample was taxonomically identified by RNG and **voucher specimen (SLS/BOT/PJ02)** deposited at the Department of Botany, SRTM University, Nanded (MS). The extraction method of the plant sample was reported previously.[13] In brief, in the summer season, leaves were collected, washed with distilled water, and shed dried leaves (10g) of *P. juliflora* plant were extracted in methanol using Soxhlets apparatus. PJLME was stored at −20 °C until the experiments.

2.3. Total phenolic content (TPC)

The total phenolic content (TPC) in PJLME plant extract was determined using the Folin-Ciocalteu method.[15] Standard solutions of Gallic acid of concentration 1.56–100 μg/mL were prepared in water. 50 μl of extract (1 mg/mL) or standard solution was added to 50 μl of distilled water. 50 μl of 10% Follin–Cicocalteu's (F–C)

phenol reagent and 50 μl of 1 M sodium carbonate solution were added to the mixture in a 96-well plate with distilled water as a blank. Reactions were incubated for 60 min at room temperature and protected from light. The absorbance was measured at 750 nm with a Microplate Reader (Biotek, USA.). Total phenolic contents were expressed as Gallic Acid Equivalents (GAE) μg/mg of dry plant material.

2.4. Quantitative analysis of the total flavonoid contents

Total flavonoid content was determined by the aluminum chloride colorimetric assay.[15] Quercetin (Standard) concentration 1.56–100 μg/mL was prepared in 80% ethanol. PJLME 50 μl (1 mg/mL) or quercetin standard solution was added to 10 μl of 10% aluminum chloride solution and followed by 150 μl of 95% ethanol. In the above mixture, 10 μl of 1 M sodium acetate was added in a 96-well plate. Ethanol (80%) was used as a reagent blank. All reagents were mixed and incubated for 40 min at room temperature, protected from light. The absorbance of the plate containing samples was measured at 415 nm with a Microplate Reader (Thermo, USA). Total flavonoid contents were expressed as μg Quercetin Equivalents (QE) per mg dry of plant material.

2.5. HPTLC (high performance thin layer chromatography) fingerprint profiling and GC-MS analysis of the PJLME

The HPTLC analysis was performed at Agharkar Research Institute's facility in Pune, India. The flavonoid fingerprint analysis was carried out by using PJLME and Rutin **(**as a standard flavonoid**)**. PJLME and Rutin were loaded (20 μl) on silica gel HPTLC plates (20 × 10 cm, 60 F254, Merck, Germany). After drying the spots, these plates were developed in a Camag twin-trough chamber previously saturated with the solvent for 30 min with mobile phase chloroform: toluene [ratio 9.5: 0.5 (v/v)]. After development, these plates were dried at 105 °C for 15 min, and then plates were derivatized for flavonoids with freshly prepared 95% methanol and 5% H_2SO_4 in the chamber for 20 s. Oven-dried plates were then scanned at 366 nm by a Camag TLC Scanner equipped with WinCATS software.

GC-MS analysis of the PJLME was carried out using the GCMS-TQ8030 Triple Quadrupole Gas Chromatograph Mass Spectrometer analysis system (Shimadzu, Japan) detailed procedure is previously explained.[13]

2.6. Maintenance of parasites and macrophage cell line

The*Leishmania donovani* parasite strain (MHOM/IN/1983/AG83) and mouse macrophage cell line RAW-264.7 were procured from National Centre for Cell Science, Pune (MS), India. Promastigotes were maintained at 26 °C in the complete RPMI-1640 medium (Sigma-R6504) containing 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 4500 mg/L glucose, 1500 mg/L sodium bicarbonate, 100 μg/mL streptomycin, 100 IU/mL penicillin and 10% fetal bovine serum (FBS). Mouse macrophage RAW-264.7 cell line was maintained in the RPMI-1640 medium supplemented with 2 mM L-glutamine, 1500 mg/L sodium bicarbonate, 100 μg/mL streptomycin, 100 IU/mL penicillin, and 10% FBS 37 °C incubator with 5% CO_2.

2.7. Anti-promastigote activity of the PJLME

Cytotoxicity of the PJLME towards the*L.donovani* promastigotes was analyzed by performing the colorimetric XTT assay.[16] Log phase promastigotes ($1x10^6$ parasites/mL) were added into 96 well plate containing 200 μl of complete RPMI-1640 (Sigma-R8755) cell culture medium (phenol red free). *L. donovani* promastigotes were treated with different concentrations of the PJLME (prepared in 0.1% DMSO). Plates were incubated for 72 h at 26 °C; Giemsa-stained promastigotes were examined under the light microscope (100X objectives, Nikon) and digitized. After the treatment in each well, 50 μl of XTT solution (5 mg/mL in PBS) was added, and then plates were incubated for up to 4 h. Absorbance was recorded at 450 nm and 650 nm as a reference, and the IC_{50} value was obtained from the concentration-response linear curve. All experiments were carried out in triplicates by performing independent assays. Experimental findings were expressed as mean ± standard deviation.

2.8. Anti-amastigote activity of PJLME

The anti-amastigote activity of the PJLME was determined on*L.donovani* parasite-infected macrophage cell line (RAW264.7). Macrophages were cultured on a glass coverslip in the wells of 24 well plates (1×10^6 cells/mL) and kept for 24 h for incubation. Afterwards, the macrophages were infected with stationary phase promastigotes using a cell-to-parasite ratio of 1:10 and kept in the complete RPMI-1640 medium (Sigma-R6504) at 37 °C for 4 h. After the incubation, free motile promastigotes (non-phagocytosed) were removed by washing with a cell culture medium, and for the complete transformation of the promastigotes into the amastigotes, cells were incubated further for 24 h at 37 °C. After 24 h of incubation, intracellular amastigotes were treated with 6.5 μg/mL of PJLME for 72 h. After the treatment, cells were fixed in 4% paraformaldehyde and stained with Hoechst 33258 stain (5 μg/mL in PBS; pH 7.4) for 10 min in a dark chamber. Following the staining procedure, cells were washed once with PBS, coverslips were mounted with a fluorescence mounting medium (Dako, Glostrup, Denmark), and cells were visualized under a confocal microscope (LSM 510 Meta, Carl Zeiss). Captured images were analyzed with LSM 5 Image Examiner software (Zeiss). To assess the activity of PJLME against the amastigotes, total numbers of intracellular amastigotes were counted in hundred infected macrophages. The percentage number of amastigotes in vehicle control (0.1% DMSO) and PJLME were calculated using the following formula.

$$\text{Amastigotes } (\%) = \frac{\text{Number of amastigotes in PJLME treated macrophages}}{\text{Number of amastigotes in 0.1\% DMSO}} \times 100$$

2.9. Cytotoxicity assay against mouse macrophage cells

Cytotoxicity of the PJLME on mouse macrophage cells was determined by using XTT assay. Macrophage cells (RAW-264.7) were maintained in the complete RPMI-1640 medium (Sigma-R6504). Macrophages were seeded into the 96-well plates at a concentration of $1x10^5$ cells/mL in a RPMI-1640 medium

containing 10% FBS and treated with the different concentrations of PJLME and vehicle control (0.1% DMSO). After that, treated macrophages were incubated for 72 h at 37 °C in an atmosphere of 5% CO_2. Cytotoxicity was determined by using the XTT assay as described for the anti-promastigote activity screening assay.

2.10. Effect of PJLME on mouse macrophages cell and nuclear morphology

Extracellular and intracellular events of the cytotoxicity of PJLME towards the mouse macrophages were observed for cell and nuclear morphology as described in our previous report.[13] Briefly, macrophages ($5x10^4$) were cultured onto the sterile glass coverslips in the complete RPMI-1640 medium (Sigma-R6504) for 24 h. After the incubation period, cells were treated with different concentrations of PJLME for 72 h. Treated cells were fixed in 4% paraformaldehyde and stained with Hoechst 33258 nuclear stain. The stained cells were observed under a confocal microscope (LSM 510 Meta, Carl Zeiss), and images were analyzed with LSM 5 Image Examiner software (Zeiss).

2.11. Detection of externalized phosphatidylserine 'apoptosis marker'

Externalized phosphatidylserine in PJLME treated *L. donovani* promastigotes was observed by using Annexin V Apoptosis Detection Kit (Sigma) according to the manufacturer's instructions and described previously.[17] Briefly, *L. donovani* promastigotes ($1x10^7$/mL) were treated with two different concentrations of the PJLME, i.e., 6.5 and 13.0 μg/mL for72 h. After completion of the treatment period, parasites were harvested and washed twice with PBS. Then parasites were resuspended in a binding buffer (100 μl) and transferred to FACS tubes. Then FITC Annexin V (5 μl) was added, and the cells were incubated for 15 min at room temperature (25 °C) in the dark. After incubation, 400 μl of binding buffer was added to each tube along with 5 μl Propidium iodide. Stained cells were then analyzed on a flow cytometer (BD FACS Calibur, USA), and results were analyzed using the BD Cell Quest Pro software.

2.12. Detection of DNA fragmentation in PJLME treated L.donovani promastigotes

A qualitative DNA fragmentation assay was performed to detect DNA fragmentation in PJLME treated *L.donovani* promastigotes. Assay was performed by using FragEL DNA Fragmentation Detection Kit, Millipore (Calbiochem). In brief, promastigotes (1×10^7 cells) were incubated with the vehicle control (0.1% DMSO), PJLME (6.5 μg/mL IC_{50}), and amphotericin B (4 μg/mL) for 72 h. After the treatment, promastigotes were fixed in 4% paraformaldehyde and stained with reagents supplied in FragEL DNA Fragmentation Detection Kit. Stained promastigotes were observed under a confocal microscope (Leica TCS SP5 II, Germany).

2.13. Cell cycle analysis

The PJLME's effect on*L.donovani* promastigotes cell cycle phases were determined as described previously with some modifications.[18] Briefly, exponential phase *L.donovani* promastigotes (2×10^6) were treated with the PJLME (6.5 and 13.0 μg/mL) for 24 h and 72 h. After the treatment, parasites were harvested and fixed in 70% methanol/PBS; after fixation, cells were stained with Propidium Iodide (10 μg/mL) and RNaseA (10 μg/mL). Different cell cycle phases were determined by flow cytometer (BD Canto TM II System), and data was analyzed using CellQuestPro software.

2.14. Detection of reactive oxygen species (ROS) level

The fluorogenic marker 2′, 7′-Dichlorodihydrofluorescein diacetate (H_2DCFDA) is live-cell-permeable acetate ester, and upon entry, it is cleaved by cellular esterases, reacts with cellular ROS (reactive oxygen species) and emits green fluorescence. ROS levels in the PJLME treated *L.donovani* promastigotes were determined by performing fluorimetric analysis as previously described[19] with some modifications. Briefly, *L.donovani* promastigotes (1×10^7) were cultured in RPMI complete medium supplemented with 10% FBS and incubated at 26 °C with different concentrations of PJLME, i.e., 6.5 and 13.0 μg/mL for 24 h. After the treatment, parasites were washed twice with PBS, and the parasite number was equilibrated in PBS (200 μl). Then, 10 μM H2DCFDA (Sigma-Aldrich) was added, and the samples were incubated for 30 min under dark conditions. The fluorescence was measured using a fluorescence microplate reader (Molecular Devices, CA, USA) at excitation and emission wavelengths of 507 and 530 nm, respectively.

2.15. Analysis of mitochondrial membrane potential($\Delta\Psi m$)

To elucidate the mitochondrial membrane potential ($\Delta\Psi m$) of PJLME treated *L.donovani* promastigotes, a cell-permeable JC-1 dye was used as described previously with some modifications.[20] Briefly, log-phase promastigotes (2×10^6/mL) were incubated with PJLME (6.5 and 13.0 μg/mL) for 24 h at 26 °C. After completion of the treatment, cells were suspended in phosphate-buffered saline (PBS) and stained with the JC1 dye (5 μg/mL) for 10 min at 37 °C and analyzed by using FACS Canto TM II System (BD Biosciences).

2.16. Measurement of intracellular ATP levels in PJLME treated promastigotes

To determine the mitochondrial ATP levels (adenosine triphosphate) in PJLME treated and untreated promastigotes, ATP assay was performed using the firefly luciferase bioluminescence-based ATP detection assay kit (ATP determination kit; Invitrogen) and as described previously[21] with some modifications. Briefly, active promastigotes (10^6/mL) were treated with the vehicle control and PJLME (6.5 and 13.0 μg/mL) for 6 h at 26 °C. After completion of the treatment, promastigotes were lysed using 0.5% Triton X-100 and mixed with a standard reaction buffer (ATP Determination Kit, Molecular Probes) containing DTT (1 mM), luciferin (0.5 mM) and firefly luciferase (1.25 μg/mL). Luminescence intensity was measured by using a fluorescence microplate reader (Molecular Devices, CA, USA), and the amount of ATP was calculated from an ATP standard curve.

2.17. Determination of caspase-3/7 protease-like activity

Caspase-3/7 protease activity was measured using the Apo-1 homogenous caspase-3/7 activity assay kit (Promega, Madison, WI). The assay was performed according to the manufacturer's instructions and with some minor modifications. In brief, 10^7 *L. donovani* promastigotes were treated with PJLME (6.5 and 13.0 μg/mL) and vehicle control (DMSO 0.1%) for 72 h; after treatment, cells were washed once with cold PBS, and after centrifugation, 'cells' were suspended in a 100 μl of reaction buffer containing caspase substrate Z-DEVD-R110 and incubated for 4 h in the dark at room temperature (25 °C). After completion of the incubation period, the increase in fluorescence caused by cleavage of the Z-DEVD-R110 substrate was measured fluorometrically at excitation and emission wavelengths of 485 and 530 nm, respectively.

2.18. Statistical analysis

The 50% inhibitory concentration (IC_{50}) values were calculated using the dose-response curve in SigmaPlot software. Results are represented as Mean ± SD. The results were considered statistically significant at P value < 0.05.

3. Results

3.1. Quantitative analysis of phytochemicals, HPTLC profiling, and GC-MS analysis of P. julifloraextract

3.1.1. Observed quantity of total phenolic and flavonoids contents in the PJLME

Phenolics are secondary products of plant metabolism, and their antioxidant activity is mainly due to their redox properties and chemical structure, which can play an essential role in chelating transitional metals and scavenging free radicals, so; quantification of phenolic compounds in a plant extract is always essential. The total phenolic acid content (TPC) and total flavonoid content (TFC) in the *P.juliflora* leaves extract was calculated using Gallic acid and Quercetin. Observed values in the quantitative test are Phenolic contents 15.5 ± 1.67 Mg GAE/g, Flavonoid contents 35.51 ± 0.94 Mg QE/g ($n = 3$).

3.1.2. HPTLC fingerprint profile and GC-MS analysis of PJLME

HPTLC methods are commonly applied for the identification or content uniformity of herbal raw materials and their formulations.[22] Similarly, the flavonoid fingerprint of the PJLME was developed by the HPTLC method using Rutin as a marker phytochemical (flavonoid). The Rutin flavonoid band peak in the test sample was seen single in the chromatogram for standard and in the test sample very near to the standard. The chromatogram and derivatized HPTLC plate photographs are shown (Fig. 1 B). To know possible phytochemicals present in PJLME a GC-MS (Gas chromatography-mass spectrometry analysis) was performed and results are shown in (Fig. 1C). GC-MS analysis explored some phytochemicals like phthalic acid, myo-Inositol (4-C-methyl), l-(+)-ascorbic acid 2,6-dihexadecanoate, phytol (2-hexadecen-1-ol), isophytol acetate and cyclohexanol (5-methyl-2-(1-methylethyl) and other phytochemicals are published previously.[13]

3.2. Anti-promastigote activity of PJLME

Antileishmanial activity of the PJLME was analyzed by performing XTT cell viability assay with *L.donovani* promastigotes. The assay is based on the conversion of tetrazolium salts to the hydrosoluble form of formazan crystals by metabolically active cells. PJLME was dissolved in DMSO and applied on stationary phase promastigotes for 72 h. The result of the cell viability assay (Fig. 2C) indicates the concentration-dependent anti*leishmanial* activity of the PJLME against *L. donovani* promastigotes with an IC_{50} concentration of 6.5 μg/mL. This antileishmanial effect of the PJLME against *L. donovani* promastigotes was also confirmed by observing Giemsa-stained promastigotes under a phase-contrast microscope. Microscopic images of PJLME treated promastigotes (Fig. 2B) showed abnormal cell morphology, loss of flagellum, granulation, and rounding of the cells. Other abnormal changes like decreased promastigotes motility, size shrinkage with dense cytoplasm, etc., were also observed in PJLME treated parasites. In contrast, vehicle-treated (0.1% DMSO) promastigotes were seen with normal morphology possessing elongated shapes and longer motile flagella (Fig. 2A).

3.3. Anti-amastigote activity of PJLME

The anti-amastigotes activity of the PJLME was screened against *L. donovani* amastigotes. The stationary phase *L. donovani* promastigotes were infected with the mouse macrophage cells (RAW 264.7); after the formation of the intracellular amastigotes, cells were treated with the PJLME (6.5 μg/mL) for 72 h. Captured microphotographs of the anti-amastigote activity assay (Fig. 2D–I)

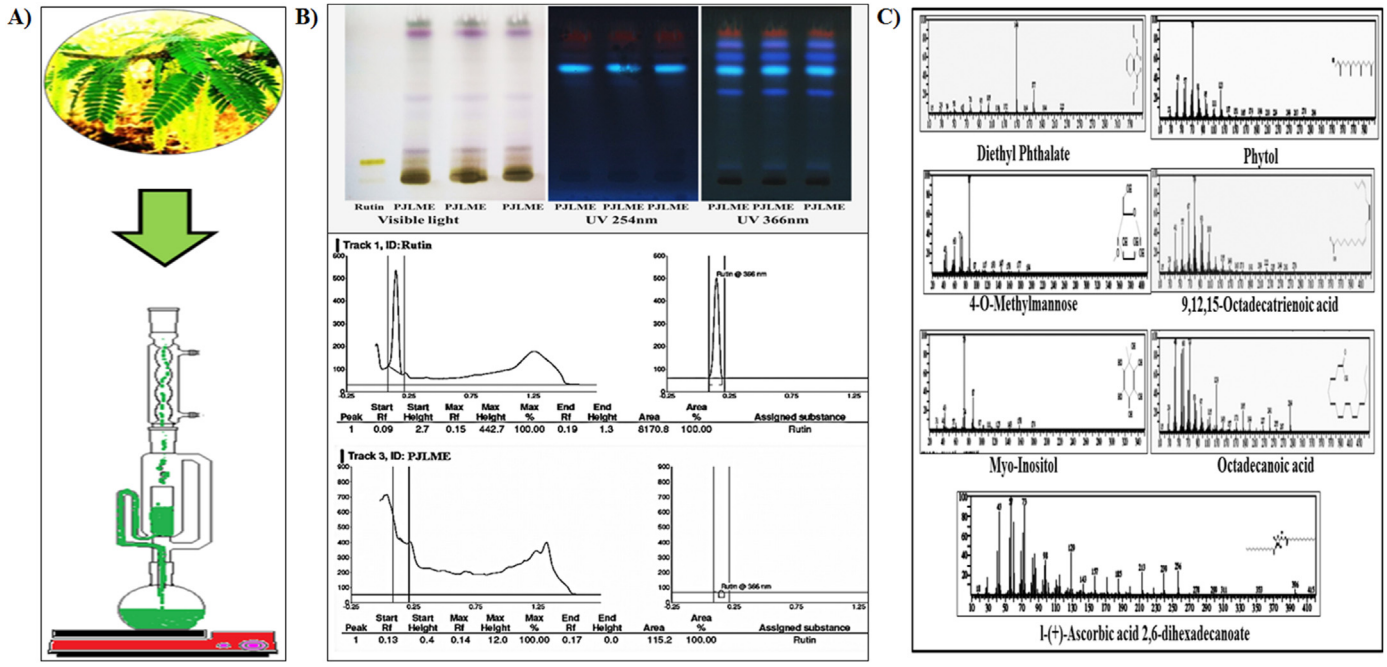

Fig. 1. PJLME preparation, HPTLC fingerprint profile, and GC-MS analysis. (A) *P.juliflora* leaf methanol extract preparation. **(B)** HPTLC fingerprint profile of PJLME and **(C)** GC-MS analysis of PJLME.

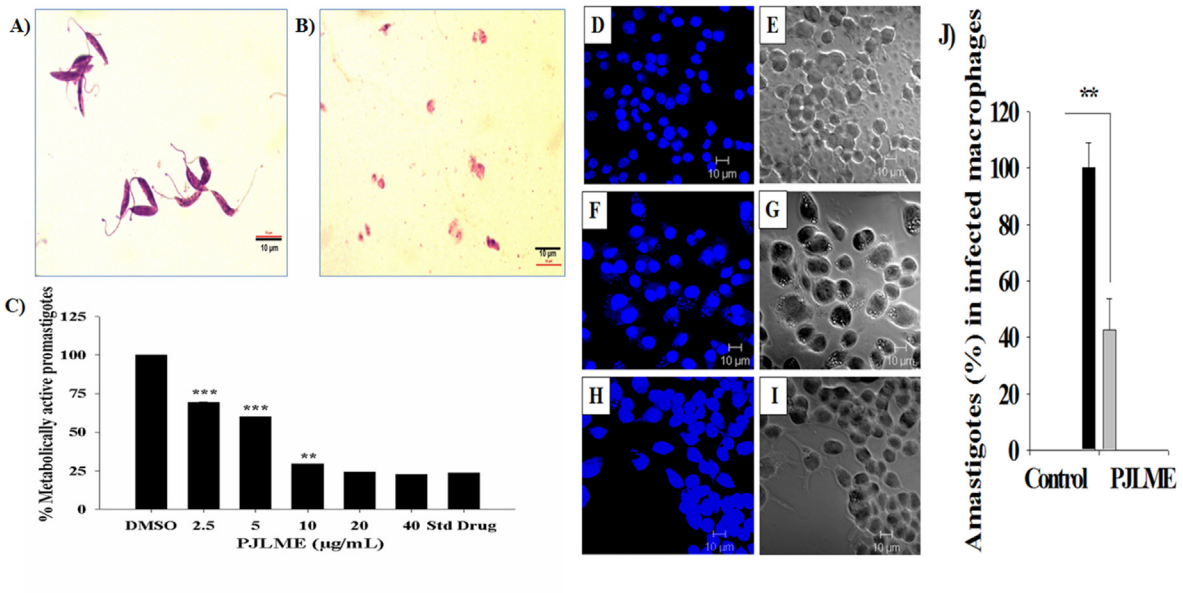

Fig. 2. Antileishmanial effect of PJLME. A) Morphology of vehicle control (0.1% DMSO) promastigotes. **(B)** PJLME treated promastigotes, and **(C)** Graph of cell viability (%) of PJLME treated *L.donovani* promastigotes after 72 h. (Scale bars: 10 µm). **Antiamastigote activity of PJLME (D–J)**. For evaluation of the antiamastigote activity of PJLME, mouse macrophage cells were infected with stationary phase *L. donovani* promastigotes, and after forming amastigotes, cells were treated with PJLME (6.5 µg/mL) for 72hr. After PJLME treatment, cells were stained with nucleic acid stain Hoechst 33258, and images were captured under a confocal microscope at 63x magnifications (LSM 510 Meta, Carl Zeiss). Macrophages without L. *donovani* infection and without PJLME treatment **(D&E)**, Cells with L. *donovani* amastigotes & without PJLME treatment **(F&G)**.Cells with L. *donovani* amastigotes & with PJLME treatment 6.5 µg/mL **(H&I)**. (Scale bars: 10 µm). Bar graph showing amastigotes (%) after PJLME treatment **(J)**. p-Values ≤0.005 (**) were considered significantly different from that of the control.

clearly indicated that PJLME reduced the intracellular amastigotes replication, which is also validated by statistical analysis (Fig. 2J). Digitized microphotographs of cellular and nuclear morphology of PJLME treated *L. donovani*-infected mouse macrophage cells also confirmed the non-cytotoxic nature of PJLME against the mouse macrophage cells.

3.4. Effect of PJLME on mouse macrophage cells

For the evaluation of the cytotoxic effects of the PJLME on mouse macrophage cells (RAW 264.7), an XTT assay was performed. Macrophages were treated with different concentrations of the PJLME for 72 h. The results of the XTT assay (Fig. 3A) exhibited the PJLME-induced dose-dependent cytotoxic effects against the mouse macrophage cells with an IC_{50} concentration of 22.5 µg/mL. The cytotoxic potential of PJLME towards the mouse macrophage cell line at 6.25 and 12.5 µg/mL concentrations was confirmed by staining cells with nucleic acid stain (Hoechst 33258) and observing cellular and nuclear morphology under a confocal microscope (LSM 510 Meta, Carl Zeiss). Stained cells (Fig. 3B) did not show any morphological as well as nuclear abnormalities (chromatin condensation and fragmentation) and looked similar to the vehicle-treated cells (0.1% DMSO).

3.5. PJLME induces apoptotic cell death and DNA fragmentation in L.donovani promastigotes

The externalized phosphatidylserine, which is considered a hallmark of apoptosis, was determined in PJLME treated *L.donovani* promastigotes by staining with annexin V assay kit. FACS analyzed data (Fig. 4A–E) showed a large number of late apoptotic cells population [annexin V (+), PI (+)] in PJLME treated parasites 72% (6.5 µg/mL) than the untreated cell population. Interestingly, significant necrotic cell number was not detected in both PJLME treated (6.5 µg/mL and 13 µg/mL) apoptotic cells population. These results strongly point out apoptotic-like cell death mechanism by externalizing phosphatidylserine in PJLME treated *L.donovani* promastigotes. Further, for qualitative analysis of apoptosis, a DNA fragmentation assay was performed. Fragmentation in cellular DNA is an indicator of apoptosis; it was observed in *L.donovani* promastigotes by using FragEL DNA Fragmentation Detection Kit, Millipore (Calbiochem), followed by observing cells under confocal microscopy. After the attachment of fluorescein-labeled deoxynucleotides at the site of exposed 3′-OH ends of fragmented DNA, the fluorescence of these deoxynucleotides can be visualized under a fluorescence microscope. PJLME (IC_{50}) treated *L.donovani* promastigotes showed DNA fragmentation, and it was observed with the fluorescence of fluorescein-labeled deoxynucleotides after the treatment of 72 h. Similar kinds of DNA fragments were also observed in the amphotericin B-treated promastigotes. It is also important to note that fluorescence of the DNA fragmentation in the vehicle control (0.1% DMSO) treated cells had not been observed (Fig. 4F–H). This specifies that promastigotes death is a cause of the PJLME-induced apoptosis, which strongly endorses the efficacy of PJLME-encompassed phytochemicals property.

3.6. PJLME inhibits the cell cycle of L.donovani promastigotes

For evaluation of the effect of the PJLME on different cell cycle phases of*L.donovani* promastigotes, cell cycle analysis was performed by flow cytometry. PJLME treated promastigotes exhibited increased accumulation of cells at sub G0 phase being 35.2 ± 1.47% at 24 h and 69.4 ± 1.0% at 72 h for 6.5 µg/mL and 68.6 ± 3.96% at 24 h and 82.0 ± 3.70% at 72 h for 13 µg/mL respectively compared to untreated promastigotes (2.4 ± 0.17% at 24 h and 3.1 ± 1.0% at 72 h). In contrast, the number of cells at G1, S and M phase were decreased at both concentrations and both time points than the untreated control cells (Fig. 5 A, B). Fragmented phenotype of DNA

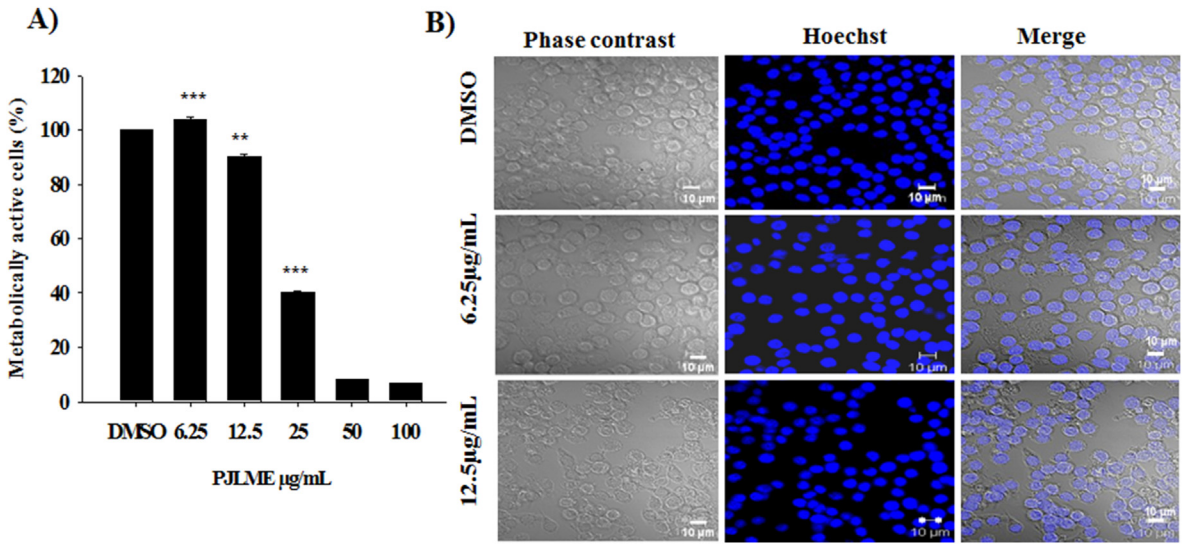

Fig. 3. Cytotoxicity of PJLME against mouse macrophage cells (RAW 264.7). (A) Cytotoxicity of the PJLME against mouse macrophage cells was evaluated by performing XTT cell viability assay. **(B)** Macrophages were treated with control (DMSO 0.1%), and different concentrations of PJLME for 72 h, and cells were stained with nucleic acid stain Hoechst 33258, and images were captured under Zeiss LSM510 META confocal microscope. (Scale bars: 10 µm). p-Values ≤0.005 (**) and ≤0.0001 (***) were considered significantly different from that of the control.

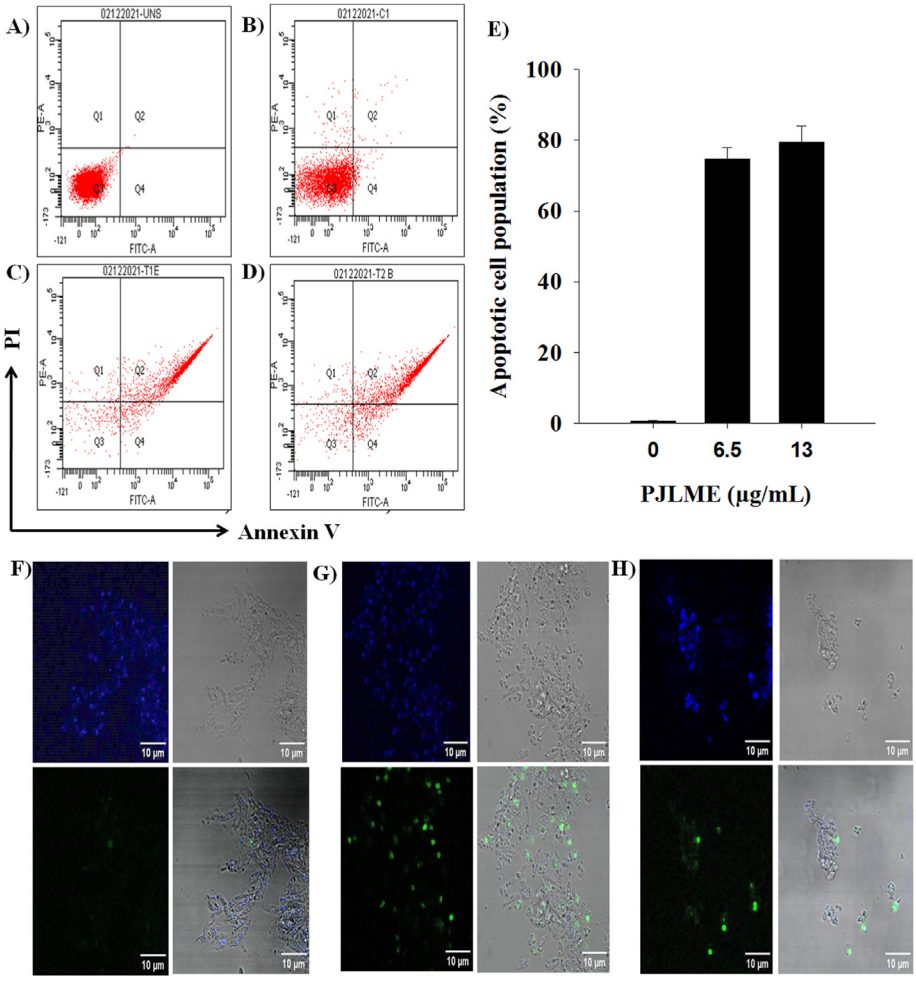

Fig. 4. PJLME induces apoptosis and DNA fragmentation in *L. donovani* promastigotes. (A–E) FACS profile of Annexin V-FITC assay. **(A)** Untreated unstained cells. **(B)** Untreated vehicle control. **(C)** PJLME (6.5 µg/mL). **(D)** PJLME (13 µg/mL). **(E)** Bar graph of apoptotic cells. **DNA fragmentation assay.** Images **(F–H)** are of the assay performed by using FragEL DNA Fragmentation Detection Kit. Upper quadrants images are of DAPI and phase contrast, lower quadrants images are of Fluorescein and merge **(F)** Vehicle control, **(G)** PJLME treated, **(H)** Amphotericin B treated. (Scale bars: 10 µm).

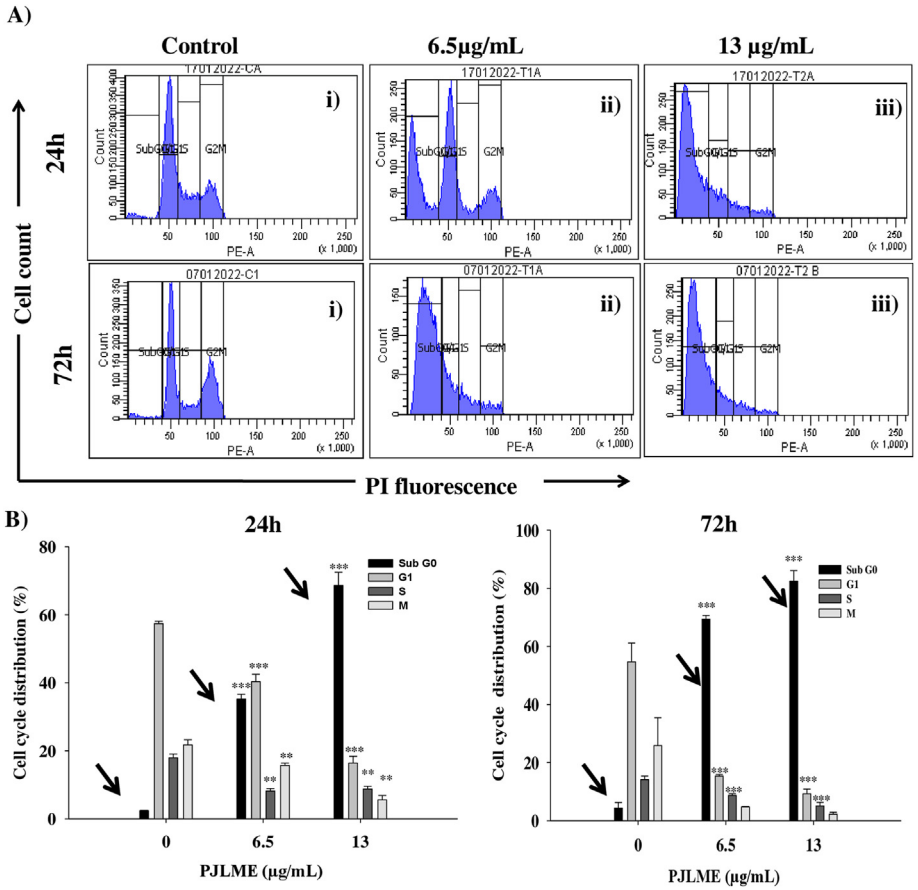

Fig. 5. Arrest of cell cycle progression at G0 phase in *L. donovani* promastigotes after the PJLME treatment. (A) Histograms of cell cycle distribution of PJLME treated and untreated *L.donovani* promastigotes at 24 h and 72 h. (i) Untreated cells. (ii) 6.5 µg/mL (iii) 13 µg/mL **(B)** The bar diagram depicts the mean percentage of cells in different cell cycle phases. The histograms are representative of three independent experiments (*P < 0.05, **P < 0.01, ***P < 0.001).

is an indicator of cells undergoing apoptosis, and it translates into the phases of the cell cycle leading to dysregulation. The increased population of PJLME treated *Leishmania* promastigotes in the G0 phase indicates cell cycle arrest at G0 phase.

3.7. PJLME induces reactive oxygen species (ROS) levels

Reactive Oxygen Species (ROS) are essential for cell cycle progression and proliferation under normal physiological conditions; however, an excessive level of ROS activates the death signaling pathway. For detection of the ROS level in PJLME treated *L.donovani* promastigotes, parasites were stained with non-fluorescent 2′, 7′ dichlorofluorescin (DCFH-DA) which, after reacting with reactive oxygen species, it transforms into the fluorescent product 2′, 7′ dichlorofluorescein. Results of the assay exhibited two-fold percentages (272%) of enhanced ROS—DCFH-DA fluorescence in PJLME treated (6.5 µg/mL) promastigotes (Fig. 6 F) as compared to untreated promastigotes (100%). These findings correlate with previously reported various phytochemicals like clerodane diterpene compound isolated from the *Polyalthia longifolia plant* leaves, a bioactive fraction of *Azadirachta indica* and carbazole alkaloid purified from *Murraya koenigii* plant have shown antileishmanial properties by inducing surplus ROS production in *Leishmania* parasite.[23–25]

3.8. Mitochondrial membrane potential($\Delta\Psi m$) and inhibition of ATP production

Leishmania mitochondrion is a primary target for therapeutic candidates since it is intimately related to the parasite's metabolism and ATP production. We measured **mitochondrial membrane potential and** ATP levels of the PJLME treated *L.donovani* promastigotes. For detection of the mitochondrial membrane potential, parasites were treated with the PJLME and after completion of the treatment, cells were stained with JC-1 dye. JC-1 dye is a cationic carbocyanine dye; when it accumulates in the mitochondria of low membrane potential in monomer form, it produces green fluorescence. At high concentrations in high mitochondrial membrane potential, it forms 'J-aggregates', which produces red fluorescence. PJLME treatment (6.5 µg/mL) in *L.donovani* parasites resulted in (Fig. 6A–E) reduction in the red fluorescence (20%) and an increase in the green fluorescence (80%). Comparatively, red fluorescence was increased in untreated parasites (97%) and decreased in green fluorescence (2.1%); red fluorescence is an indicator of high mitochondrial membrane potential. Experimental results undoubtedly revealed the loss of the mitochondrial membrane potential (green fluorescence) in PJLME treated promastigotes.

ATP level is a direct marker of the energy state, and the health of the cell's mitochondrion, and detection of the ATP level in PJLME treated parasites were observed by a bioluminescence ATP

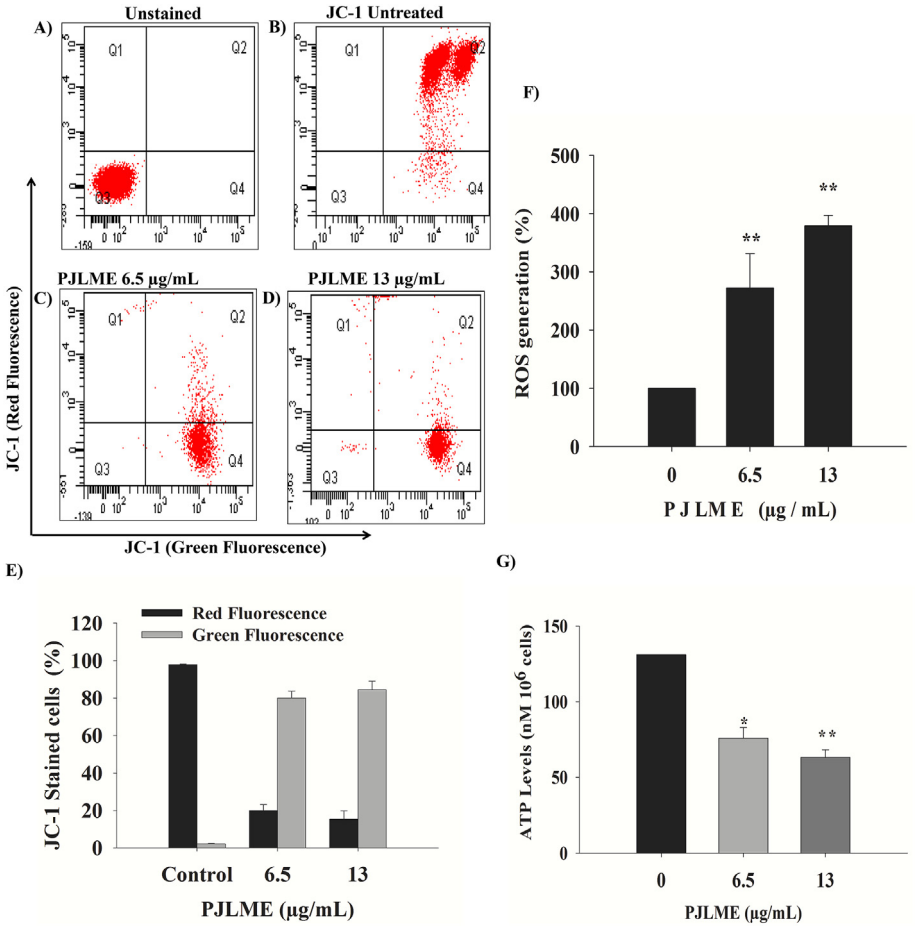

Fig. 6. PJLME-induced ROS causes loss of mitochondrial membrane potential and ATP depletion in *L. donovani* promastigotes. (A–E) FACS profile of JC-1stained PJLME treated promastigotes (72 h). **(A)** Unstained. **(B)** Untreated and stained promastigotes. **(C & D)** PJLME treated and stained promastigotes. **(E)** Bar graph of statistical representation of JC-1 Assay. **(F)** Bar graph showing enhanced ROS levels in **(F)** and ATP levels (6 h) **(G)** in PJLME treated *L.donovani* promastigotes. (*P < 0.05, **P < 0.01, ***P < 0.001).

detection assay kit. In this ATP-luminescent reaction, generated luminescence is proportional to the amount of ATP present, based on the utilization of ATP by luciferase. PJLME treated parasites showed a marked reduction in the ATP levels of *L.donovani* promastigotes. The total ATP content of the untreated promastigotes was 131 nm/10^6 cells, and PJLME treated (6.5 and 13 µg/mL) were 75.8 nm/$10^6$and 63.2 nm/10^6, which is significantly less than the untreated promastigotes (Fig. 6G).

3.9. PJLME induces caspase independent cell death in L.donovani promastigotes

There are various reports of miltefosine and other phytochemicals that induces apoptosis in *L.donovani* promastigotes with activation of Caspase 3/7-like protease activity.[26–28] To know the mechanism of apoptosis-like cell death in PJLME treated *L.donovani* promastigotes, parasites were treated with different concentrations of the PJLME (6.5 and 13 µg/mL), and after the treatment, parasites were lysed with Z-DEVD-R110 substrate and fluorescence was measured. PJLME treatment not induced caspase 3/7 protease-like activity in *L.donovani* promastigotes treated with both concentrations as compared with vehicle control (0.1% DMSO) (Fig. 7). This was also confirmed after 6 h of PJLME treatment and results are similar to the 72 h treated promastigotes (Data not showed).

4. Discussion and conclusion

On the eve of finding novel resources for the management of leishmaniasis, our experimental settings clearly demonstrated the antileishmanial potential of PJLME in general and anti-amastigote (virulent form in humans) in specific. Usually, plants belonging to the family Mimosaceae have previously been reported for their antibacterial, antifungal, antihelmintic, antimalarial, and molluscicidal activities.[29] The previous report describing the antileishmanial activities of *P. juliflora* fruit extract against *L. infantum* has reported activity at IC_{50} 35.3± 2.6[30] and the activity of hydroalcoholic extract of leaves or twigs at IC_{50} of 312 µg/mL.[31]

In this study, we explored antileishmanial activity and the mechanism of action of the PJLME *(P. juliflora* leaves methanol extract) against visceral leishmaniasis-causing *L.donovani* parasites. For confirmation of the antileishmanial activity of the PJLME, initially, we performed cell viability XTT assay (72 h) against *L. donovani* promastigotes, and the experimental results clearly demonstrated the concentration-dependent anti-*L. donovani* promastigotes activity of PJLME at a remarkably lower IC_{50} value of 6.5 µg/mL. The results of the XTT assay were also confirmed by observing the effect of PJLME IC_{50} concentration (6.5 µg/mL) on the cellular morphology of the parasite, wherein the adverse effects were observed with the cellular morphology of *L. donovani* (Fig. 2 A, B). The results of the XTT cell viability assay performed for 72 h with

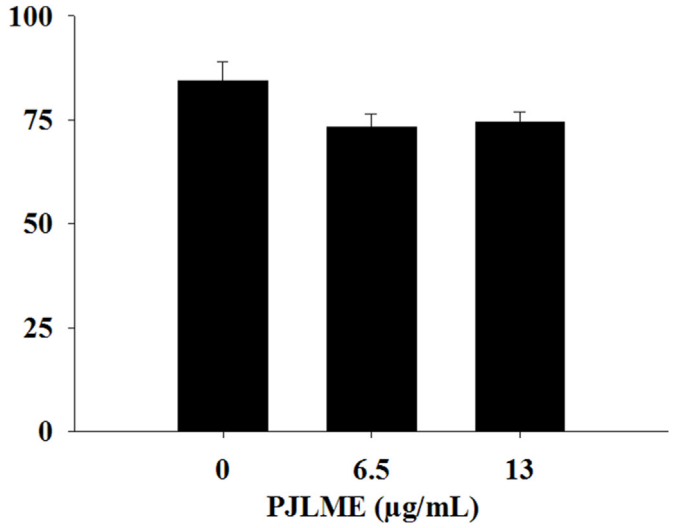

Fig. 7. PJLME induces caspase-independent apoptosis-like cell death in *L.donovani* promastigotes. For detection of Caspase, 3/7 activity in PJLME treated *L.donovani* promastigotes (72 h); after the treatment, parasites were lysed with Z-DEVD-R110 substrate, and fluorescence was measured at 521 nm. Data represent the mean ± SD of three independent experiments.

mouse macrophage cells (RAW 264.7) exhibited dose-dependent cytotoxicity of PJLME with an IC_{50} value of 22.5 µg/mL.

It is a well-established fact that, during the life cycle and pathogenesis of *Leishmania*, the promastigote form of the parasite can be infected with several host cell types such as neutrophils, dendritic cells, fibroblasts, etc.; however, the disease is mainly propagated primarily within macrophages. Internalized promastigotes are delivered to the mature phagolysosome compartment of host macrophages, where they transform into non-motile amastigote form. Disease progression occurs when the propagation of intracellular amastigotes of macrophages gets entry into other macrophages following cell division or lysis of the host cell. It has been reported that *Leishmania* is a stealthy pathogen that either avoids or actively suppresses macrophage microbicidal activity.[32] Therefore, the antileishmanial agents having significant activity against the anti-amastigote form of the parasite can be a valuable addition to increasing the therapeutic index of antileishmanial drugs.

In the present investigation, we have confirmed the anti-amastigote activity of PJLME against the *L.donovani* parasite. In *in-vitro* conditions, mouse macrophages were infected with the *L.donovani* promastigotes, and after the formation of the intracellular amastigotes, cells were treated with the PJLME (6.5 µg/mL) for 72 h. The obtained results clearly showed a significant reduction of the amastigotes in the infected macrophages (Fig. 2H–J). These results correlate with previously reported anti-amastigote activity exhibiting plants.[24,33]

The DNA fragmentation and externalized phosphatidylserine has been considered essential signatures of apoptosis, and the majority of the phytochemicals present in PJLME, especially phenolics, flavonoids, alkaloids, terpenes, steroids, and tannins, are reported to induce apoptosis in a variety of animal cells.[13,34] Apoptosis and cell cycle arrest properties of the PJLME were also observed in PJLME treated *L. donovani* promastigotes. Results of the flow cytometric analysis of the annexin v assay exhibited a significant difference in the number of externalized phosphatidylserine positive cells after PJLME treatment (72%) than the untreated cells (Fig. 4A–E). For more confirmation of the apoptosis, we also performed qualitative DNA fragmentation analysis with PJLME treated cells. Images of the assay clearly exhibited a higher number of green fluorescence in PJLME treated *L.donovani* promastigotes than in the untreated cells (Fig. 4F–H). To explore the cell cycle arrest properties of the PJLME, we performed a cell cycle assay with PJLME treated *L.donovani* parasites. Results of the cell cycle analysis confirmed cell cycle arrest in the G0 phase (Fig. 5 A, B). These findings correlate with previously reported crude plant extract and secondary metabolites such as *Cinnamomum cassia*, *Azadirachta indica*, clerodane diterpene, sesamol, berberine chloride, Spinigerin, β-sitosterol and *Embilica officinalis* L fruit extract which induces DNA fragmentation, cell cycle arrest and apoptosis-like cell death in *Leishmania donovani* parasite.[17,20,23,24,33,35]

ROS are known to cause oxidative damage to the parasite's DNA, proteins, and lipids. The ROS generated in the phagolysosome by the host cell can directly kill the parasite or initiate a cascade of events leading to its death. ROS are also implicated in inducing the parasite's programmed cell death-like mechanism by generating lipid peroxides which subsequently leads to mitochondrial dysfunction and, thereby, depletion in ATP production.[36] Of note, *Leishmania* parasites have a unique thiol-based antioxidant system that helps them to neutralize ROS and get rescued from oxidative damage. However, the excessive production of ROS supersedes the parasite's antioxidant defense mechanism and leads to its death.[37] The present-day therapeutic antileishmanial drugs and previously reported phytochemicals are known to induce apoptotic cell death in leishmanial parasites by generating reactive oxygen species, a drop of mitochondrial membrane potential, and ATP depletion. PJLME treated *L. donovani* parasites also exhibited a concentration-dependent increase in ROS production (Fig. 6F), decreased mitochondrial membrane potential (Fig. 6A–E), and depletion in ATP levels (Fig. 6G).

Leishmania evades the host immune system for entry and survival within host cells, like macrophages. Apoptosis, a kind of programmed cell death, is essentially a physiological process needed for the normal function and development of multicellular organisms. However, many intracellular pathogens and parasites, like *Leishmania*, have developed evolved mechanisms to delay or inhibit host cell apoptosis, which otherwise will adversely affect the survival and replication within their host cell. *Leishmania* does not possess a classical cascade of programmed cell death.[38] One possible reason as to why *Leishmania* does not follow the classical apoptosis may be because it possesses a unique repertoire of cell death regulating mechanisms. More precisely, *Leishmania* has a complex network of pathways and proteins implicated in regulating cell death mechanisms such as apoptosis-like cell death, necrosis-like, and autophagic cell death. However, the exact molecular underpinnings by which *Leishmania* regulates cell death mechanisms are poorly understood and is one of the active areas of *Leishmania* research. Furthermore, it has been suggested that the survival of *Leishmania* within the host cells may be associated with the inhibition of host cell apoptosis and its ability to interfere with host cell signaling pathways and regulate the expression of pro-inflammatory cytokines. Possibly these mechanisms may rescue the parasite from the host immune system and allow them to establish infection and replication.[38,39]

We have performed the Caspase 3/7 assay and revealed the caspase-independent and ATP depletion-related mitochondrial mechanism of apoptosis-like cell death in PJLME treated promastigotes. The results of the caspase assay strongly correlate with the previously reported phytochemicals showing caspase-independent and ATP depletion-mediated apoptosis in *Leishmania*.[40–42] All these experimental results correlate with previously reported phytochemicals and various plant extracts that induce apoptosis-like cell death, cell cycle arrest via increasing ROS levels, reduction in mitochondrial membrane potential, and thereby reduction in ATP levels.[41,43,44]

Structurally diverse groups of bioactive compounds belonging to different classes of phytochemicals, such as phenolics, flavonoids, alkaloids, terpenes, steroids, and tannins, have been reported from different *Prosopis* species.[45] *Prosopis* plant-derived compounds had already been reported for their bioactivities and specifically for anti-leishmanial activities.[46,47] Moreover, in our previous report, the GC-MS analysis of PJLME revealed the presence of different phytochemicals such as phthalic acid and its variety of esters, myo-Inositol (4-C-methyl), l-(+)-ascorbic acid 2, 6-dihexadecanoate, phytol (2-hexadecane-1-ol), isophytol acetate, and cyclohexanol.[13] Perhaps the bioactivity of components mentioned above or the synergistic effects of the phytoconstituents of PJLME might be associated with the antileishmanial effects observed in the present investigation. The set of *in vitro* experiments performed and the outcome of the present investigation clearly established the significance of *Prosopis juliflora* as an effective and safe natural resource for the effective management of visceral leishmaniasis caused by *L. donovani* parasites.

Author contribution statement

GRN and PMS designed the study, UBG performed the experiments, NPV maintained cell cultures for the study, UBG and GRN wrote the MS, PMS edited the MS, and all authors approved the final version.

Declaration of conflict of interest

The authors declare that there is no conflict of interest to disclose.

Acknowledgments

RNG thanks the financial assistance from RUSA Phase-II grant of Savitribai Phule Pune University, Pune. UBG thanks Director, NCCS, Pune, for allowing access to laboratory facilities available at NCCS, Pune.

References

1. Akhoundi M, Kuhls K, Cannet A, et al. A historical overview of the classification, evolution, and dispersion of Leishmania parasites and sandflies. *PLoS Neglected Trop Dis*. 2016;10(3). https://doi.org/10.1371/journal.pntd.0004349.
2. Wijnant G-J, Dumetz F, Dirkx L, et al. Tackling drug resistance and other causes of treatment failure in leishmaniasis. *Frontiers in Tropical Diseases*. 2022;3. https://doi.org/10.3389/fitd.2022.837460.
3. Chakravarty J, Sundar S. Drug resistance in leishmaniasis. *J Global Infect Dis*. 2010;2(2):167. https://doi.org/10.4103/0974-777x.62887.
4. World Health Organization. *WHO Traditional Medicine Strategy: 2014–2023*. 2013.
5. Wink M. Modes of action of herbal medicines and plant secondary metabolites. *Via Medici*. 2015;2(3):251–286. https://doi.org/10.3390/medicines2030251.
6. Oryan A. Plant-derived compounds in the treatment of leishmaniasis. *Iran J Vet Res*. 2015 Winter;16(1):1–19. PMID: 27175144; PMCID: PMC4789233.
7. Sharma L, Dhiman M, Singh A, Sharma MM. Green approach: "a forwarding step for curing leishmaniasis—a neglected tropical disease. *Front Mol Biosci*. 2021;8. https://doi.org/10.3389/fmolb.2021.655584.
8. Soosaraei M, Fakhar M, Hosseini Teshnizi S, Ziaei Hezarjaribi H, Banimostafavi ES. Medicinal plants with promising antileishmanial activity in Iran: a systematic review and meta-analysis. *Annals of Medicine and Surgery*. 2017;21:63–80. https://doi.org/10.1016/j.amsu.2017.07.057.
9. Delgado-Altamirano R, Monzote L, Piñón-Tápanes A, et al. In vitro antileishmanial activity of Mexican medicinal plants. *Heliyon*. 2017;3(9). https://doi.org/10.1016/j.heliyon.2017.e00394.
10. De Queiroz AC, Dias Tde, Da Matta CB, et al. Antileishmanial activity of medicinal plants used in endemic areas in northeastern Brazil. *Evid base Compl Alternative Med*. 2014;2014:1–9. https://doi.org/10.1155/2014/478290.
11. Sathiya M, Muthuchelian K. Investigation of phytochemical profile and antibacterial potential of ethanolic leaf extract of Prosopis juliflora DC. *Eth Leafle*. 2008 Dec;12:1240–1245. https://opensiuc.lib.siu.edu/ebl/vol2008/iss1/167/.
12. Tene V, Malagón O, Finzi PV, Vidari G, Armijos C, Zaragoza T. An ethnobotanical survey of medicinal plants used in Loja and Zamora-Chinchipe, Ecuador. *J Ethnopharmacol*. 2007;111(1):63–81. https://doi.org/10.1016/j.jep.2006.10.032.
13. Utage BG, Patole MS, Nagvenkar PV, Kamble SS, Gacche RN. Correction: prosopis juliflora (sw.), DC induces apoptosis and cell cycle arrest in triple negative breast cancer cells: in vitro and in vivo investigations. *Oncotarget*. 2018;9(68). https://doi.org/10.18632/oncotarget.26095, 33050-33050.
14. Choudhari J, Nimma R, Nimal SK, Totakura Venkata SK, Kundu GC, Gacche RN. Prosopis juliflora (sw.) DC phytochemicals induce apoptosis and inhibit cell proliferation signaling pathways, EMT, migration, invasion, angiogenesis and stem cell markers in melanoma cell lines. *J Ethnopharmacol*. 2023;312, 116472. https://doi.org/10.1016/j.jep.2023.116472.
15. Pawar SS, Dasgupta D. Quantification of phenolic content from stem-bark and root of hugonia mystax linn. using RP-HPLC. *J King Saud Univ Sci*. 2018;30(3):293–300. https://doi.org/10.1016/j.jksus.2016.09.002.
16. Williams C. Hydrosoluble Formazan XTT: its application to natural products drug discovery for Leishmania. *J Microbiol Methods*. 2003;55(3):813–816. https://doi.org/10.1016/j.mimet.2003.08.013.
17. Shadab M, Jha B, Asad M, Deepthi M, Kamran M, Ali N. Apoptosis-like cell death in Leishmania Donovani treated with kalsometm10, a new liposomal amphotericin B. *PLoS One*. 2017;12(2). https://doi.org/10.1371/journal.pone.0171306.
18. Ambit A, Fasel N, Coombs GH, Mottram JC. An essential role for the Leishmania major metacaspase in cell cycle progression. *Cell Death Differ*. 2007;15(1):113–122. https://doi.org/10.1038/sj.cdd.4402232.
19. Cunha-Júnior EF, Andrade-Neto VV, Lima ML, et al. Cyclobenzaprine raises ROS levels in leishmania infantum and reduces parasite burden in infected mice. *PLoS Neglected Trop Dis*. 2017;11(1). https://doi.org/10.1371/journal.pntd.0005281.
20. Afrin F, Chouhan G, Islamuddin M, Want MY, Ozbak HA, Hemeg HA. Cinnamomum cassia exhibits antileishmanial activity against Leishmania donovani infection in vitro and in vivo. *PLoS Neglected Trop Dis*. 2019;13(5). https://doi.org/10.1371/journal.pntd.0007227.
21. Amaral M, de Sousa FS, Silva TA, et al. A semi-synthetic Neolignan derivative from dihydrodieugenol B selectively affects the bioenergetic system of Leishmania infantum and inhibits cell division. *Sci Rep*. 2019;9(1). https://doi.org/10.1038/s41598-019-42273-z.
22. Senguttuvan J, Subramaniam P. HPTLC fingerprints of various secondary metabolites in the traditional medicinal herb hypochaeris radicata L. *Journal of Botany*. 2016;2016:1–11. https://doi.org/10.1155/2016/.
23. Kathuria M, Bhattacharjee A, Sashidhara KV, Singh SP, Mitra K. Induction of mitochondrial dysfunction and oxidative stress in Leishmania donovani by orally active Clerodane diterpene. *Antimicrob Agents Chemother*. 2014;58(10):5916–5928. https://doi.org/10.1128/aac.02459-14.
24. Chouhan G, Islamuddin M, Want MY, et al. Erratum to: apoptosis mediated leishmanicidal activity of azadirachta indica bioactive fractions is accompanied by th1 immunostimulatory potential and therapeutic cure in vivo. *Parasites Vectors*. 2016;9(1). https://doi.org/10.1186/s13071-016-1593-3.
25. Roy S, Dutta D, Satyavarapu EM, et al. Mahanine exerts in vitro and in vivo antileishmanial activity by modulation of redox homeostasis. *Sci Rep*. 2017;7(1). https://doi.org/10.1038/s41598-017-03943-y.
26. Kumar R, Tiwari K, Dubey VK. Methionine aminopeptidase 2 is a key regulator of apoptotic like cell death in Leishmania Donovani. *Sci Rep*. 2017;7(1). https://doi.org/10.1038/s41598-017-00186-9.
27. Paris C, Loiseau PM, Bories C, Jaqueline Bréard. Miltefosine induces apoptosis-like death in Leishmania donovani promastigotes. *Antimicrob Agents Chemother*. 2004;48(3):852–859. https://doi.org/10.1128/aac.48.3.852-859.2004.
28. Verma NK, Dey CS. Possible mechanism of Miltefosine-mediated death of leishmania donovani. *Antimicrob Agents Chemother*. 2004;48(8):3010–3015. https://doi.org/10.1128/aac.48.8.3010-3015.2004.
29. Gurib-Fakim A. Medicinal plants: traditions of Yesterday and drugs of tomorrow. *Mol Aspect Med*. 2006;27(1):1–93. https://doi.org/10.1016/j.mam.2005.07.008.
30. Al-Musayeib NM, Mothana RA, Al-Massarani S, Matheeussen A, Cos P, Maes L. Study of the in vitro antiplasmodial, antileishmanial and antitrypanosomal activities of medicinal plants from Saudi Arabia. *Molecules*. 2012;17(10):11379–11390. https://doi.org/10.3390/molecules171011379.
31. Kheiri Manjili H, Jafari H, Ramazani A, Davoudi N. Anti-leishmanial and toxicity activities of some selected Iranian medicinal plants. *Parasitol Res*. 2012;111(5):2115–2121. https://doi.org/10.1007/s00436-012-3059-7.
32. Naderer T, McConville MJ. The leishmania-macrophage interaction: a metabolic perspective. *Cell Microbiol*. 2007;10(2):301–308. https://doi.org/10.1111/j.1462-5822.2007.01096.x.
33. Ali R, Tabrez S, Akand SK, et al. Sesamol induces apoptosis-like cell death in Leishmania Donovani. *Front Cell Infect Microbiol*. 2021;11. https://doi.org/10.3389/fcimb.2021.749420.
34. Kumar M, Kaur V, Kumar S, Kaur S. Phytoconstituents as apoptosis inducing agents: Strategy to combat cancer. *Cytotechnology*. 2015;68(4):531–563. https://doi.org/10.1007/s10616-015-9897-2.
35. Sardar AH, Das S, Agnihorti S, et al. Spinigerin induces apoptotic like cell death in a caspase independent manner in Leishmania Donovani. *Exp Parasitol*. 2013;135(4):715–725. https://doi.org/10.1016/j.exppara.2013.10.011.
36. Menna-Barreto RF. Cell death pathways in pathogenic trypanosomatids: lessons of (over)kill. *Cell Death Dis*. 2019;10(2). https://doi.org/10.1038/s41419-019-1370-2.
37. Dumas C. Disruption of the trypanothione reductase gene of leishmania decreases its ability to survive oxidative stress in macrophages. *EMBO J*.

1997;16(10):2590–2598. https://doi.org/10.1093/emboj/16.10.2590.
38. Basmaciyan L, Azas N, Casanova M. Different apoptosis pathways in Leishmania parasites. *Cell Death Discovery*. 2018;4(1). https://doi.org/10.1038/s41420-018-0092-z.
39. Solano-Gálvez S-G, Álvarez-Hernández D-A, Gutiérrez-Kobeh L, leishmania Vázquez-López R. Manipulation of signaling pathways to inhibit host cell apoptosis. *Therapeutic Advances in Infectious Disease*. 2021;8, 204993612110149. https://doi.org/10.1177/20499361211014977.
40. Gervazoni LF, Barcellos GB, Ferreira-Paes T, Almeida-Amaral EE. Use of natural products in leishmaniasis chemotherapy: an overview. *Front Chem*. 2020;8. https://doi.org/10.3389/fchem.2020.579891.
41. Saha P, Sen R, Hariharan C, Kumar D, Das P, Chatterjee M. Berberine chloride causes a caspase-independent, apoptotic-like death inleishmania donovani promastigotes. *Free Radic Res*. 2009;43(11):1101–1110. https://doi.org/10.1080/10715760903186124.
42. Dutta A, Bandyopadhyay S, Mandal C, Chatterjee M. Aloe vera leaf exudate induces a caspase-independent cell death in Leishmania Donovani promastigotes. *J Med Microbiol*. 2007;56(5):629–636. https://doi.org/10.1099/jmm.0.47039-0.
43. Moreira W, Leprohon P, Ouellette M. Tolerance to drug-induced cell death favours the acquisition of multidrug resistance in Leishmania. *Cell Death Dis*. 2011;2(9). https://doi.org/10.1038/cddis.2011.83.
44. Ali R, Islamuddin M, Tabrez S, et al. Embilica officinalis L. inhibits the growth and proliferation of Leishmania donovani through the induction of ultrastructural changes, mitochondrial dysfunction, oxidative stress and apoptosis-like cell death. *Biomed Pharmacother*. 2021;143, 112156. https://doi.org/10.1016/j.biopha.2021.112156.
45. Singh S. Phytochemical analysis of different parts of Prosopis juliflora. *IJCPR*. 2012;4:59–61. https://innovareacademics.in/journal/ijcpr/Issues/Vol4Issue3/532.pdf.
46. Antwi CA, Amisigo CM, Adjimani JP, Gwira TM. In vitro activity and mode of action of phenolic compounds on Leishmania donovani. *PLoS Neglected Trop Dis*. 2019;13(2). https://doi.org/10.1371/journal.pntd.0007206.
47. Sharifi-Rad J, Kobarfard F, Ata A, et al. Prosopis plant chemical composition and pharmacological attributes: targeting clinical studies from preclinical evidence. *Biomolecules*. 2019;9(12):777. https://doi.org/10.3390/biom9120777.