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Promastigotes of *Leishmania donovani* exhibited sensitivity towards the high altitudinal plant *Cicer microphyllum*

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ABSTRACT

In this study, we explored *Cicer microphyllum* (CM), a Trans-Himalayan plant for its chemical components by GC-MS, phytochemical quantitation, and anti-leishmanial efficacy against sensitive strain (SS) and resistant strain (RS) promastigotes of *L. donovani in vitro*. The hydroethanolic extract of aerial parts of CM was screened for the presence of chemical compounds and phytochemical estimation. The antileishmanial activity of CM was assessed against the promastigotes of *L. donovani*. The cell volume and cell viability were analyzed by flow cytometry. The generation of reactive oxygen species (ROS) and lipid bodies was determined after treatment with reference and test drug. The extract of CM is complemented with major plant secondary metabolites and the quantitative assessment for phytoconstituents showed the highest concentration of phenols followed by flavonoids and terpenoids. Different biologically active chemical compounds were identified by the GC-MS analysis. The 50% inhibitory concentrations against *L. donovani* sensitive strain were 14.40 μg/ml and 23.03 μg/ml whereas for resistant promastigotes these were 49.84 μg/ml and 26.77 μg/ml after SAG (sodium stibogluconate) and CM exposure, respectively. CM treatment reduced cell viability induced by loss in plasma membrane integrity. Drug treatment resulted in higher ROS generation and production of lipid bodies. GC-MS screening of the extract revealed the richness of active chemical components in CM. The presence of diverse phytochemicals, no cytotoxicity to human macrophages, and the antileishmanial action of CM depicted its potential as an alternative future drug.

1. Introduction

Leishmaniases are multiplex diseases of tropical and subtropical zones of the world caused by *Leishmania* species and transmitted by sand flies (Goyal et al., 2021a). The parasites are endowed with immense host subversion machinery and induce a spectrum of clinical manifestations, varying from cutaneous lesions to fatal visceral leishmaniasis. According to the World Health Organization (WHO, 2018), more than 95% of the new cases occurred in ten countries: Brazil, Iraq, Ethiopia, China, Kenya, India, Nepal, Somalia, Sudan and South Sudan. Although 700,000 to 1 million cases occur yearly (WHO, 2018), leishmaniasis has been considered an unappealing drug target for industry, being predominantly the ailment of the poor (Choi et al., 2021). Drugs are the best options to control diseases and for almost 70 years, pentavalent antimonials have been the mainstays of disease control. Sodium stibogluconate (SAG) was the mainstay of antileishmanial therapy in the Indian subcontinent and its cure rate was reported to be greater than 90% (Sundar, 2001). Later, responsiveness to pentavalent antimonials decreased tremendously in Bihar, a hyperendemic state of India and nearly 50–65% of patients failed the treatment with SAG and about 24% in Nepal (Hefnawy et al., 2017). The awful side effects of SAG like anorexia, myalgia, arthralgia, abdominal pain, hepatic and cardiotoxicity, led to its cessation in the Indian subcontinent. Amphotericin B, a second-line drug treatment of leishmaniasis is toxic, costly, and had also rendered treatment failure (Srivastava et al., 2011). Drug combination therapy is an effective blueprint for the cure of visceral leishmaniasis. Reports evidenced that combination therapy is more effective against *Leishmania* than monotherapy and thus slows the drug resistance development. Co-administration of liposomal amphotericin B-miltefosine and sodium stibogluconate-paromomycin has been evaluated in India and Sudan respectively, and were found to be quite successful (Sundar and Singh, 2018). However, an *in vitro* study on *L. infantum* promastigotes has demonstrated resistance to sodium stibogluconate-paromomycin and miltefosine-paromomycin combinations (Hendrickx et al., 2016). Studies have revealed that drug-resistant parasites are becoming more tolerant to ROS damage, ATP diminution, sustained membrane integrity, have

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increased thiol levels, and resist mitochondrial membrane potential (Ponte-Sucre et al., 2017). Inability to control the vector and the rapidly emanating resistance to the currently available armamentarium of anti-leishmanial therapy is a matter of great concern. Moreover, the unavailability of an effective human vaccine is another major issue in disease control (Goyal et al., 2021b).

Natural products can be alternative to chemotherapy and may help escape from toxicity-rendered side effects. The Trans-Himalayan region is a mega diversity hotspot and a treasure trove of floristic affluence and adobe for ayurvedic drugs (Ballabh and Chaurasia, 2011). From the past, it has been evident that plants and plant-derived components are an incredible source of affordable, safe, and effective drugs (Keshav et al., 2021). Most of the high altitudinal plants have shown dynamic medicinal values over the others due to their capability to survive in environmentally harsh and stressed conditions. *Cicer microphyllum*, a member of the family Fabaceae is endemic to the high-altitude cold desert region of Leh-Ladakh, India (Singh et al., 2017). This plant is a wild relative of the cultivated chickpea (*Cicer arietinum*) and is regionally known as Kukunnory or wild gram. Traditionally, *C. microphyllum* has been used to cure various ailments such as mountain sickness, jaundice, mouth infections and stress (Angmo et al., 2012). The unripened seeds of this plant are considered as a stimulant, aphrodisiac, nootropic and neuroprotective (Sharma et al., 2017). The immunomodulatory and anthelmintic activities of *C. microphyllum* have been reported (Kour et al., 2011). Diverse plants and their extracted phytochemicals had already shown the capacity to function as resistance modifying agents (Abreu et al., 2012). In the event of finding a new treatment option, the drug must be screened for sensitive as well as the resistant strains of the parasite. The drug potency is solely pertinent if it can overcome the aforementioned constraints and restrain the springing resistant strains. To the best of our knowledge, no scientific validation of the *C. microphyllum* as an anti-leishmanial has been reported so far. Hence, this study aimed to quantitate the principal phytochemicals present, characterize the chemical composition by GC-MS, and scrutinize the potential of *C. microphyllum* to restrict the growth of SAG-resistant and SAG-sensitive promastigotes of *L. donovani*.

2. Materials and methods

2.1. Plant material, extraction and phytochemical analysis

Cicer microphyllum (aerial parts) was collected from the Trans-Himalayan region of Leh-Ladakh, India. The identification of the plant was done by Dr Om Prakash Chaurasia, (Ethnobotanist) of Defence Institute of High Altitude Research (DIHAR), Leh-Ladakh, India (voucher number 319). The plant material (aerial parts) was shade-dried, pulverized, and subjected to hydroethanolic (30:70) soxhlet extraction. The decoction was prepared by rota-evaporator and dried by lyophilization. The dried extract was stored at −20 °C until further use.

The presence or absence of different phytochemical constituents in *C. microphyllum* hydroethanolic extract (CM) was checked by standard methods (Harborne, 1973; Roy et al., 2018).

2.2. Determination of total flavonoid content (TFC)

The aluminum chloride colorimetric method was employed to determine the total flavonoid content in the crude plant extract. The CM extract (200 µl of 1 mg/ml) was dissolved in 800 µl distilled water and 60 µl of 5% sodium nitrite ($NaNO_2$) was added and incubated for 5 min. Post-incubation, 60 µl of 10% aluminum chloride ($AlCl_3$) solution was added and permitted to rest for 5 min. To this mixture, 400 µl of NaOH (1 M) solution and distilled water were added to make the volume of 5 ml. The O.D. was measured spectrophotometrically at 510 nm. The total flavonoid content was assessed by constructing a standard curve using quercetin. The result is shown as mg quercetin equivalent (QE) per g dry weight (Park et al., 2008).

2.3. Determination of total phenol content (TPC)

The total phenol present in the crude extract was determined by using Folin-Ciocalteu reagent. A total of 200 µl (1 mg/ml) of the sample was mixed with 500 µl of Folin-Ciocalteu reagent. The mixture was counterpoised by adding 2 ml of sodium carbonate (Na_2CO_3, 20% w/v) solution. The reaction mixture was kept at room temperature (25 °C) for 1 h in the dark with fitful shaking for the instigation of color. The absorbance was measured at 765 nm using a UV-VIS spectrophotometer. A calibration curve of standard reference was set up utilizing gallic acid and TPC was disclosed as gallic acid equivalents (GAE) mg per g of the extract (Zhou et al., 2009).

2.4. Determination of total terpenoid content (TTC)

The total terpenoid content in CM extract was determined by the method described by Ghorai et al. (2012). For estimation of TTC, linalool was used as the standard. For the assay, 200 µl CM extract (1 mg/ml) was added to 1.5 ml chloroform. After thorough shaking, the mixture was kept for 3 min. Concentrated H_2SO_4 (100 µl) was added to the mixture and incubated for 2 h at room temperature (25 °C) and checked for the appearance of a reddish-brown precipitate. The precipitate was dissolved in 95% methanol (1.5 ml) and read on a spectrophotometer at 538 nm. The results were expressed as mg/g of linalool equivalent (LE).

2.5. GC-MS analysis of CM

The phytocomponent inspection of hydroethanolic extract of CM (1 mg/ml) was performed *via* gas chromatography-mass spectrometry (GC-MS) using Thermo Scientific TRACE 1300 Gas Chromatograph coupled with Thermo TSQ 8000 Triple Quadrupole MS (Thermo Fisher Scientific, Austin, USA). The equipment has a TG 5MS column with dimensions of 30 mm × 0.25 mm, 0.25 µM. Helium (He) was the carrier gas with a flow rate of 1.5 ml/min and the injection volume was 1 µl. The instrument was operated at an initial temperature of 60 °C and maintained at this temperature for 2 min. The oven temperature was gradually increased to 200 °C for 4 min. The sample was run at a range of 50–700 m/z. Identification of compounds was achieved by the comparison of their mass spectra with databases stored in the National Institute of Standards and Technology (NIST) Library, Gaithersburg, USA.

2.6. Parasite

Promastigotes of *Leishmania donovani* sensitive strain (MHOM/IN/80/Dd8) were acquired from the London School of Hygiene and Tropical Medicine, London, UK, while the SAG-resistant strain (P.B.-0014) was obtained from Rajendra Memorial Research Institute of Medical Sciences (RMRIMS), Patna, Bihar, India. They were cultured and maintained in NNN medium and RPMI-1640 medium complemented with 10% fetal bovine serum (FBS) at 22 ± 2 °C in B.O.D. incubator.

2.7. Parasite growth analysis

Promastigotes of the sensitive strain (SS) and resistant strain (RS) of *L. donovani* (1×10^6 cells/ml) were incubated with different concentrations of SAG and CM (10–100 µg/ml) in 24-well culture plates at 22 ± 2 °C in B.O.D. incubator for 72 h. SAG and DMSO (0.01%) were used as a positive (reference drug) and a negative control respectively. After 72 h, 10 µl of each sample was gently mixed with 10 µl of trypan blue and loaded on Neubauer's chamber (Strober, 2001).

2.8. Cell toxicity assay using MTT

To detect cytotoxicity of CM, the human monocytic leukemia cell line THP-1 was used. The mitochondrial activity was measured by colorimetric analysis based on the depletion of tetrazolium salt or MTT (3-(4,5-

dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (yellow) to formazan crystals (purple) by virtue of metabolically active cells as explained elsewhere (Keshav et al., 2021). Complete RPMI-1640 medium was used to maintain PMA (phorbol 12-myristate 13-acetate) differentiated THP-1 cell line in an incubator at 5% CO_2 and 37 °C. Varied concentrations of CM or SAG (20–1000 μg/ml) were employed to treat the differentiated cells. A total of 10 μl of MTT (10 mg/ml) was added after 72 h and incubated for 4–5 h. DMSO was added to break down the formazan crystals and the O.D. was measured through ELISA (enzyme--linked immunosorbent assay) reader at 550 nm. The percent cytotoxicity was calculated using the following formula:

$$\text{Percentage cytotoxicity} = \frac{(\text{At} - \text{Ab})}{(\text{Ac} - \text{Ab})} \times 100$$

where At is the O.D. of treated cells, Ab is the O.D. of blank cells, and Ac is the O.D. of untreated cells.

2.9. Selectivity index (SI)

The index of selectivity (the ratio of CC_{50} and IC_{50}, see Keshav et al., 2021) was employed to identify the compound with good antiparasitic activity and lower toxicity in mammalian macrophages (as they are the host cells of the *Leishmania* parasite).

2.10. Determination of reactive oxygen species (ROS)

ROS plays a critical role in cellular physiopathology. The cell-permeant 2′,7′-dichloro-dihydrofluerescein diacetate (H_2DCFDA) dye was used to detect intracellular ROS generation according to the method already explained (Goyal et al., 2021b). Promastigotes of *L. donovani* were maintained in the presence of drugs and medium alone for 72 h. Cells were washed two times with PBS, after adding H_2DCFDA (10 μM) incubated in the dark at room temperature (25 °C) for 20 min. Relative fluorescence was measured at 504 nm (excitation wavelength) and 529 nm (emission wavelength) in a microplate reader. Measurements were made in triplicate and results are expressed as fluorescence intensity units.

2.11. Cell volume analysis by flow cytometer

Promastigotes treated with SAG or CM for 72 h were analyzed in a flow cytometer after two washings with PBS. In the area that is analogous to the promastigotes, 10,000 events were recorded (Mendes et al., 2016). The data were analyzed using the software CellQuest Pro (BD FACSDiva 8.0.1).

2.12. Propidium iodide uptake assay

The promastigotes of *L. donovani* were exposed to SAG or CM for 72 h at 22 ± 2 °C. After incubation with drugs, promastigotes were centrifuged, washed thrice, and kept in an incubator with propidium iodide (50 μg/ml, Sigma-Aldrich, St. Louis, USA) for 20–30 min in the dark at room temperature (25 °C). A minimum of 10,000 events was recorded for each sample. The cells were inspected through a flow cytometer (BD Bioscience FACSCalibur, San Jose, USA) using BD FACSDiva 8.0.1 software (Kaur and Kaur, 2018).

2.13. Lipid bodies assessment

Lipid bodies were determined using Nile Red dye (Teixeira de Macedo Silva et al., 2018). Promastigotes of *L. donovani* were incubated with a drug (either SAG or CM) for 72 h. After harvesting, cells were washed with PBS two times. Nile Red (10 μg/ml) was added to promastigotes, incubated for 30 min at room temperature (25 °C), and washed twice to remove an excess of dye. Fluorescence was read on a microplate reader at 485 nm of excitation and 535 nm of emission wavelengths.

2.14. Data analysis

Statistical analyses were carried out using one-way ANOVA with *post-hoc* tests. The results are presented as the mean ± standard deviation (SD) with a *P*-value < 0.05 considered as statistically significant.

3. Results

3.1. Phytoconstituents and quantitative phytochemical investigation

Phytochemicals are secondary metabolites of plant origin and markers for the potentiality of any herbal drug. All the major phytochemical components like alkaloids, terpenoids, flavonoids, saponins, phenols, and glycosides were present in CM.

The quantitative phytochemical investigation of CM extract was performed for total phenol, flavonoid, and terpenoid content. The highest level of total phenols was found, followed by flavonoids and terpenoids. The total phytoconstituents were computed as 52.95 ± 3.67 mg/g GAE (phenols), 31.5 ± 2.64 mg/g QE (flavonoids) and 23.06 ± 2.01 mg/g LE (terpenoids).

3.2. Chemical characterization by GC-MS

The chemical characterization of CM was carried out by gas chromatography coupled with mass spectrometry (GC-MS) and the findings are summarized in Table 1. The results of GC-MS screening of the hydroethanolic extract of CM indicated the presence of 10 different phytocomponents with a broad range of retention time (RT) values ranging from 7.25 to 33.26 min. The major components accounted together for 86.87% of total constituents and were illustrated by peaks 4, 6, 7, 8 and 9. The compounds identified with the highest and lowest percentages were didodecyl phthalate (47.84%) and 2,5-dihydroxyacetophenone, bis(trimethylsilyl) ether (0.81%) respectively.

3.3. Anti-promastigote activity

Treatment-induced parasite inhibition was checked by exposure of promastigotes to the drugs tested. The promastigotes of *L. donovani* were subjected to various concentrations of SAG or CM to measure the effects on their replication *in vitro*. The parasite percentage inhibition was observed to vary in a dose-dependent manner. At 72 h CM exposure resulted in an IC_{50} value of 23.03 ± 0.53 μg/ml while SAG exposure resulted in an IC_{50} value of 14.40 ± 0.53 μg/ml for the sensitive strain, and the difference was significant (ANOVA, $F_{(3,8)} = 1{,}021$, $P < 0.0001$). In the case of the resistant strain, the IC_{50} values were 49.84 ± 1.09 μg/

Table 1
Chemical compounds detected by gas chromatography-mass spectrometry (GC-MS) analysis of *Cicer microphyllum* extract.

No.	RT	Compound	Percentage[a]
1	7.25	2,5-dihydroxyacetophenone, bis(trimethylsilyl) ether	0.81
2	19.19	Dibutyl phthalate	2.91
3	22.88	Heptasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13-tetradecamethyl	2.75
4	24.37	9,12,15-Octadecatrienoic acid, 2,3-bis [(trimethylsilyl)oxy]-propyl ester, (Z,Z,Z)	8.43
5	24.54	Octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl	2.48
6	25.86	Squalene	10.18
7	26.41	Didodecyl phthalate	47.84
8	26.58	13-docosenamide	13.46
9	32.22	Betulin	6.96
10	33.26	3-desoxo-3,16-dihydroxy-12-desoxyphorbol 3,13,16,20-tetraacetate	4.18

Abbreviations: No., serial number; RT, retention time.
[a] Percentage of peak area.

ml for SAG exposure and 26.77 ± 0.97 μg/ml for CM exposure (Fig. 1). The IC_{50} value for CM exposure was significantly lower than the IC_{50} for SAG exposure for the resistant strain (ANOVA, $F_{(3,8)} = 1{,}021$, $P < 0.0001$). The IC_{50} for SAG exposure of the sensitive strain was significantly lower (ANOVA, $F_{(3,8)} = 1{,}021$, $P < 0.0001$) in comparison to the resistant strain whereas the difference of IC_{50} for CM exposure to both strains was less significant (ANOVA, $F_{(3,8)} = 1{,}021$, $P = 0.002$).

3.4. Toxicity profile of CM against a human cell line and selectivity index

An effective drug is acceptable when it is safe in terms of toxicity or less toxic. The tested drugs were assessed for toxicity *via* testing on human cells. THP-1 (human monocytic leukemic cell line) was employed to evaluate the toxic nature of CM and the reference drug SAG by performing an MTT assay. The test relies on mitochondrial respiration and can be used as an indicator of cellular energy measurement. The spectrophotometric analysis revealed the toxicity of SAG with a CC_{50} value of 52.34 ± 2.43 μg/ml. The CC_{50} value for CM was found to be non-toxic at 1678.07 ± 94.79 μg/ml which suggests its safety.

Any potent drug with high antiparasitic activity and low toxicity shows a selectivity index of >10 and proves to be efficacious. CM was found to be a good parasite inhibitor with a SI > 10 while SAG was toxic to the human cell line with a SI < 10.

3.5. Augmentation of ROS upon treatment

Many reactive oxygen radicals are crucial for cellular processes. Imbalance or overproduction of these radicals largely contributes to oxidative stress that subsequently leads to cell death. Reactive oxygen species (ROS) are reported to successfully combat several pathogens. The high levels of ROS are toxic to the cells or promastigotes. Therefore, ROS levels were estimated after treatment by employing the fluorogenic dye H_2DCFDA; upon diffusion into the cell, the dye is deacetylated to a non-fluorescent compound by the cellular esterases. This non-fluorogenic compound is oxidized by reactive oxygen species into highly fluorogenic 2′,7′-dichlorofluorescein (DCF). The ROS generation was found to be elevated in the CM-treated SS and RS promastigotes. The production of ROS was significantly greater in CM-treated RS than SAG-treated RS (ANOVA, $F_{(4,10)} = 191.4$, $P < 0.0001$; Fig. 2). Hence it is evident that this trans-Himalayan plant targets the mitochondria and induces endoparasitic ROS accumulation that might lead to the apoptotic-like death of promastigotes in both strains in comparison to the negative control (UT).

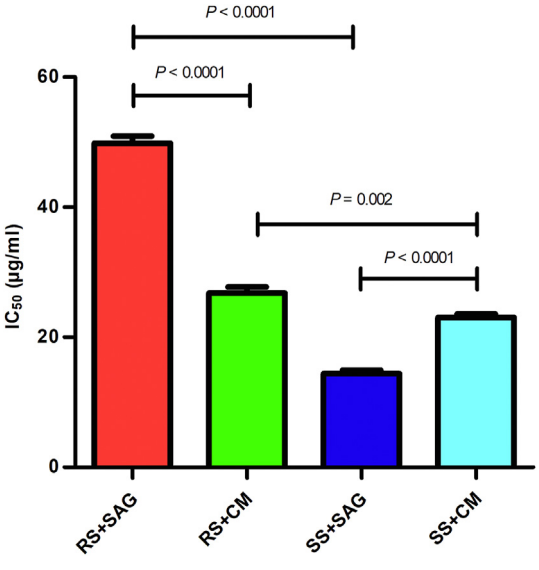

Fig. 1 Inhibitory concentration 50% (IC_{50} μg/ml) against promastigotes of *L. donovani* after 72 h of sodium stibogluconate (SAG) and *Cicer microphyllum* (CM) exposure. Data are represented as the mean ± standard deviation (SD). *Key*: UT, untreated; RS + SAG, resistant strain treated with sodium stibogluconate; RS + CM, resistant strain treated with *C. microphyllum*; SS + SAG, sensitive strain treated with sodium stibogluconate; SS + CM, sensitive strain treated with *C. microphyllum*.

3.6. Cell volume

The morphological changes in the parasites such as cell shrinkage and reduced cell volume are the hallmark of apoptosis. Hence, cell shrinkage was analyzed in both treated and untreated promastigotes of *L. donovani*. Cell shrinkage was observed in promastigotes of the sensitive strain exposed to SAG or CM in contrast to promastigotes which received no treatment. SAG was not able to induce significant shrinkage in cell volume while CM showed a reduction in the resistant strain after 72 h of treatment (Fig. 3).

3.7. Plasma membrane integrity

Further, we performed cell death analysis to confirm the treatment-induced changes. Propidium iodide (PI) was used to perform this assay. Dead parasites upon the loss in plasma membrane integrity allow the entry of PI whereas the live cells with unaffected plasma membranes do not. The results indicated that SAG was able to kill promastigotes of the sensitive strain efficiently in contrast to promastigotes of the resistant strain. SAG killed 83.7% of the promastigotes of the sensitive strain and 28.7% of the promastigotes of the resistant strain. The shift in PI-positive cells (dead parasites) in P2 on the x-axis upon CM treatment was 78.9% for the sensitive strain and 68.5% for the resistant strain (Fig. 4). CM potently killed both the strains (sensitive and resistant) showing its virtue against resistant strain as well. Untreated cells (UT) were taken as a control that showed no cell death.

3.8. CM promotes the accumulation of lipid bodies

Cellular stress is signified by the accumulation of lipid bodies. Lipid droplet production in stressed or starved cells is linked to dysfunctional mitochondria and further hampers the smooth functioning of cellular machinery. In this study, we analyzed the lipid accumulation in promastigotes before and after treatment. The metachromatic dye Nile Red was used to stain untreated and treated promastigotes for the quantification of

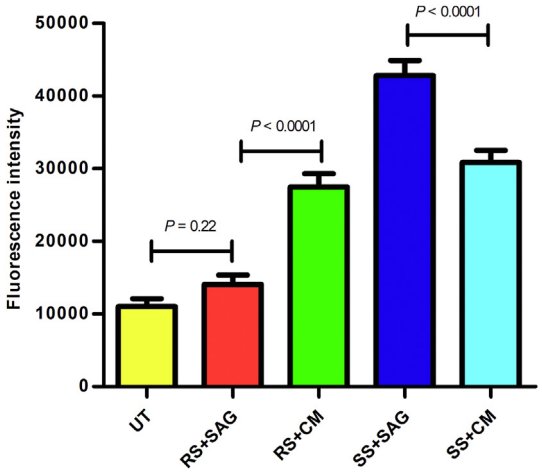

Fig. 2 Levels of ROS generation in *L. donovani* promastigotes after sodium stibogluconate (SAG) and *Cicer microphyllum* (CM) treatment. Results are expressed as the mean ± standard deviation (SD). *Key*: UT, untreated; RS + SAG, resistant strain treated with sodium stibogluconate; RS + CM, resistant strain treated with *C. microphyllum*; SS + SAG, sensitive strain treated with sodium stibogluconate; SS + CM, sensitive strain treated with *C. microphyllum*.

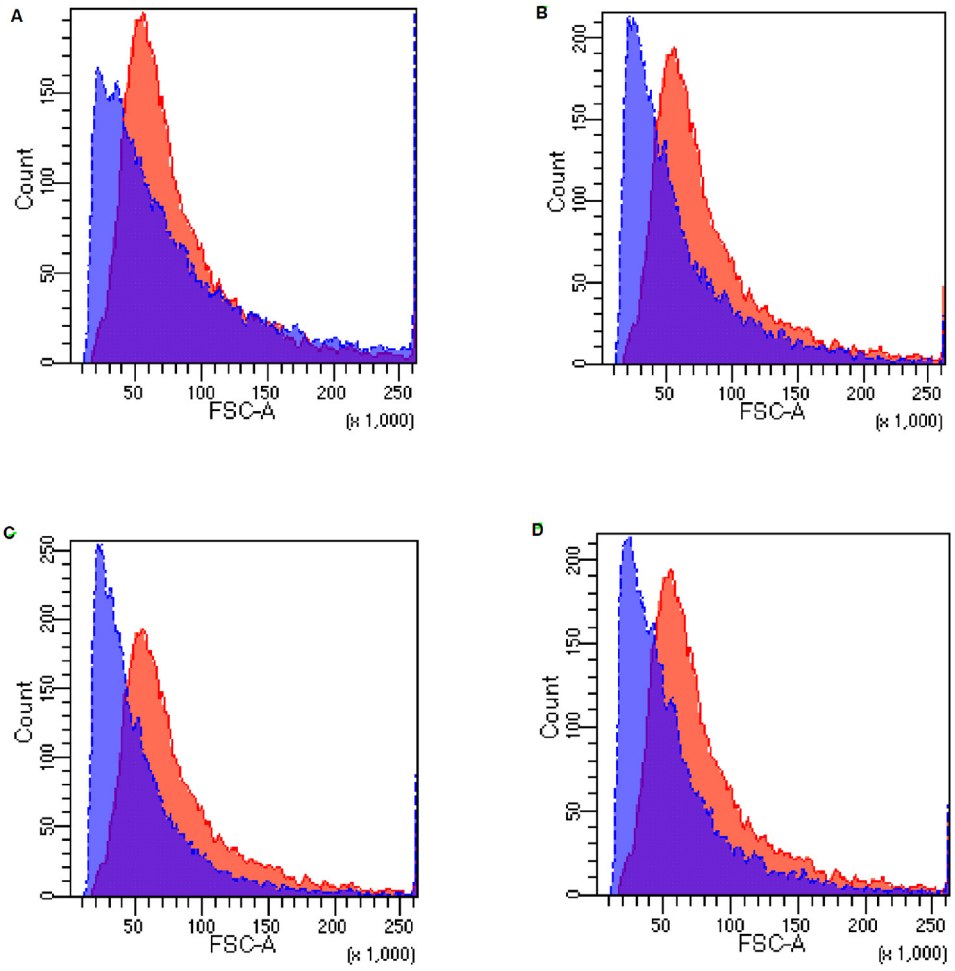

Fig. 3 Cell volume analysis of *L. donovani* promastigotes treated with *Cicer microphyllum* (CM). Forward light scatter (FSC-A) corresponds to cell size. **A** Untreated (UT) *vs* resistant strain treated with sodium stibogluconate (RS + SAG). **B** UT *vs* resistant strain treated with *C. microphyllum* (RS + CM). **C** UT *vs* sensitive strain treated with sodium stibogluconate (SS + SAG). **D** UT *vs* sensitive strain treated with *C. microphyllum* (SS + CM). Red histogram: untreated, blue histogram: treated.

neutral lipid bodies. The spectrofluorimetric analysis revealed, treatment-induced agglomeration of lipid bodies after 72 h of CM treatment (Fig. 5) in contrast to the untreated cells. A significant rise was found in the lipid bodies of RS parasites treated with CM in comparison to SAG-treated parasites (ANOVA, $F_{(4,10)} = 82.14$, $P < 0.0001$). SAG treatment was associated with significant elevation in the lipid bodies in SS than in RS promastigotes (ANOVA, $F_{(4,10)} = 82.14$, $P < 0.0001$).

4. Discussion

The major cause of drug resistance in the Indian subcontinent is the widespread misuse of the pentavalent antimonials which were freely available in India. Unrestricted availability, lack of knowledge, improper regimen, and misguided dosages by amateur health workers lead to the development of resistance (Sundar et al., 2000). The use of sodium stibogluconate, paromomycin, and the oral drug miltefosine was discontinued in some regions because of their aftermath of hepatotoxicity, nephrotoxicity, abdominal pain, teratogenicity, and the advent of resistance. At present, only a few alternative drugs have been emanated for the cure of leishmaniasis. None of the available drugs are considered good due to their lengthy treatment regimen, toxicity, high cost, and adverse effects which lead to treatment desertion (Singh et al., 2016; Goyal et al., 2021c). Different vaccine trials have been done against leishmaniasis, but still no human vaccine is accessible (Goyal et al., 2021d).

Thus, switching to alternate therapies mainly derived from plants may deliver headway in the search for better, affordable, and safer antileishmanials. Trans-Himalayas, a vast expanse of arid high altitude lands nurtures plants with a perplexing range of adaptations to sustain these extreme climatic conditions. *Cicer microphyllum* (CM), a cold desert inhabitant, is having multifarious medicinal values.

In this study, the phytoconstituents investigation of CM showed the presence of plant secondary metabolites such as saponins, phenols, flavonoids, alkaloids and terpenoids. In CM, the higher quantities of phenols, flavonoids, and terpenoids were evidenced by the quantitative phytochemical analysis. Various studies suggested the importance of the above-mentioned phytochemical components in controlling parasite growth, including *Leishmania* species (Antwi et al., 2019; Keshav et al., 2021).

Plants have explicitly revolutionized the drug discovery. Numerous new chemical entities have been developed coupled with copious potential against several pathogens such as bacteria, viruses and parasites. GC-MS analysis of plant extracts opens a new avenue in the field of drug development. Most of the compounds identified in CM by GC-MS screening have been reported to have various biological activities. The antioxidant, antitumor, and chemopreventive action of squalene have been identified (Das et al., 2008; Spanova and Daum, 2011; Casuga et al., 2016). The antileishmanial efficacy of betulin has been reported against *L. braziliensis*, *L. infantum* and *L. donovani* (Alcazar et al., 2014; Saudagar and Dubey, 2014; Sousa et al., 2014).

The above findings prompted us to investigate the antileishmanial potential of CM against SS and RS promastigotes of *L. donovani*. A less significant difference was observed in IC_{50} of CM for sensitive and

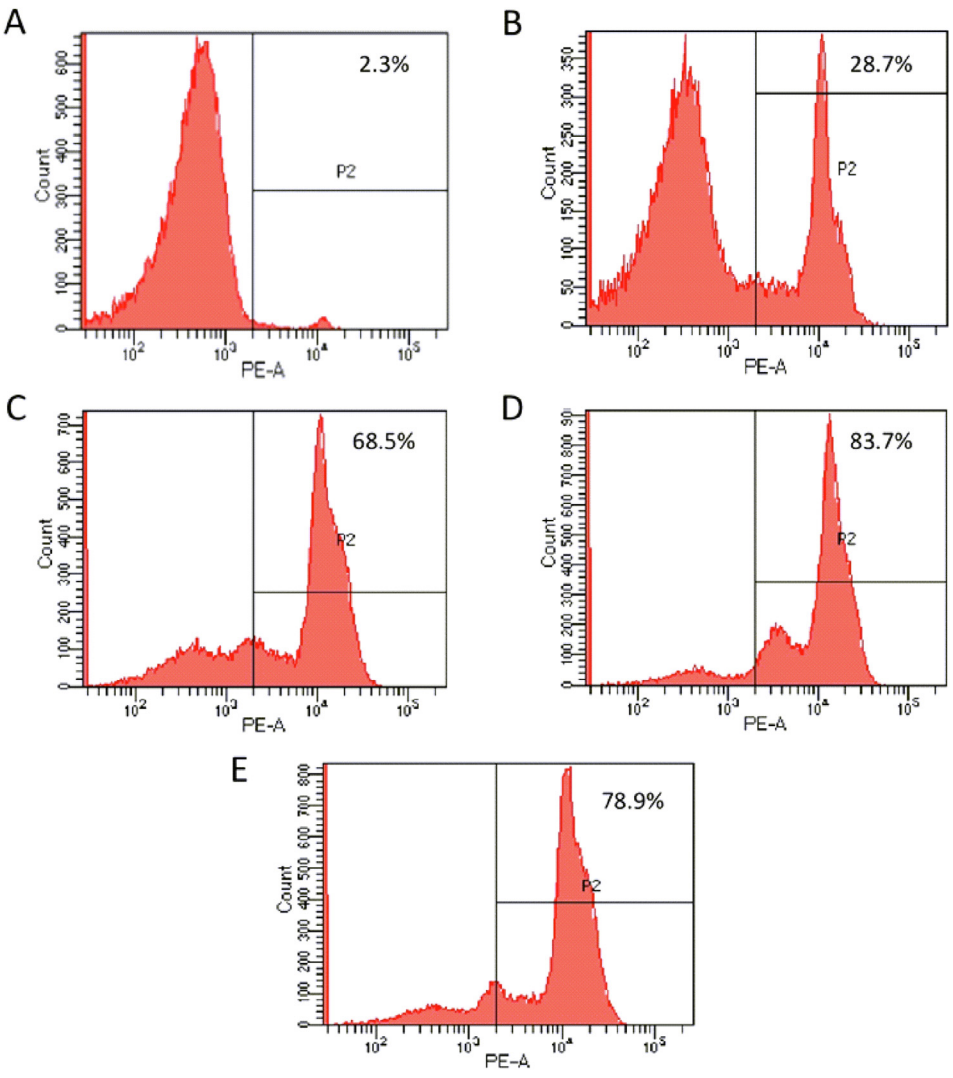

Fig. 4 Cell viability analysis was performed *via* propidium iodide uptake assay by flow cytometry after treatment with *Cicer microphyllum* (CM). Histograms depicting the dead parasites (propidium iodide-positive, P2). **A** Untreated (UT). **B** Resistant strain treated with sodium stibogluconate (RS + SAG). **C** Resistant strain treated with *C. microphyllum* (RS + CM). **D** Sensitive strain treated with sodium stibogluconate (SS + SAG). **E** Sensitive strain treated with *C. microphyllum* (SS + CM). PE-A: phycoerythrin-area for excitation at 488 nm for propidium iodide.

resistant strains while there was a highly significant difference in the IC_{50} values of SAG for SS and RS. Likewise, the Himalayan plant *Codonopsis clematidea* has been reported to exhibit antileishmanial activity against *L. donovani* promastigotes with an IC_{50} of 34.58 μg/ml (Kaur et al., 2018). CM is rich in all the major phytochemical constituents like alkaloids, flavonoids, phenols, saponins, etc. These phytochemicals have been proved to be active antileishmanials against *Leishmania* spp. The flavonoid rutin was reported with an IC_{50} value of 12.64 μg/ml and 13.07 μg/ml against the sensitive and resistant promastigotes of *L. donovani* and evidenced to be a potent antileishmanial agent (Chauhan et al., 2018). The IC_{50} of the phenolic compound gallic acid was found to be 14.48 μg/ml against *Leishmania amazonensis* promastigotes (Dutra et al., 2019). The extract was devoid of any cytotoxicity as its safety profile was found to be better than that of SAG. Lectins isolated from *C. arietinum* showed no toxicity even at higher concentrations reported in a previous study (Kumar et al., 2014).

Like all other metazoans, the protozoans also retain ROS at the basal level for different physiological functions of the cell (Kathuria et al., 2014). We assessed the intracellular ROS generation before and after treatment as the increase in the level of ROS is responsible for cell death (Mehta and Shaha, 2004). The present study demonstrated that the treatment of SAG leads to ROS generation in SS promastigotes whereas it was unable to produce ROS in RS promastigotes showing their high tolerance towards the SAG. Unlike the SAG, CM augmented ROS

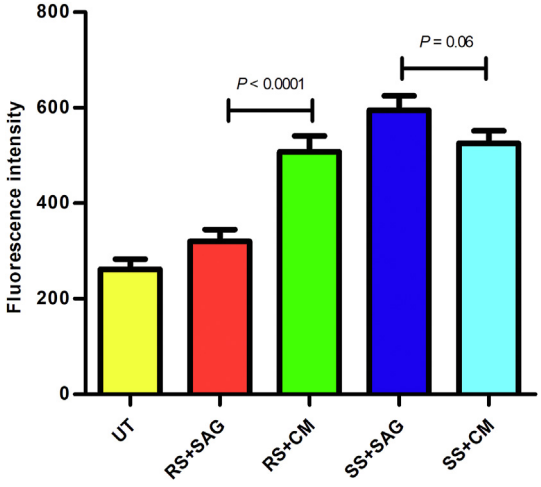

Fig. 5 Lipid bodies accumulation (Nile Red) in *L. donovani* promastigotes after treatment with *Cicer microphyllum* (CM). Results are expressed as the mean ± standard deviation (SD). *Key*: UT, untreated; RS + SAG, resistant strain treated with sodium stibogluconate; RS + CM, resistant strain treated with *C. microphyllum*; SS + SAG, sensitive strain treated with sodium stibogluconate; SS + CM, sensitive strain treated with *C. microphyllum*.

generation in both SS and RS promastigotes upon exposure. Our findings are in accordance with earlier studies where eugenol-rich oil of *Syzygium aromaticum* generated ROS, induced killing of *L. donovani in vitro* (Islamuddin et al., 2013), neem leaf extract killed *L. donovani* promastigotes after the instigation of ROS (Dayakar et al., 2015), and killing of *L. donovani* by ROS-mediated apoptotic like death by withanolides took place (Chandrasekaran et al., 2013). The ROS- and NO-generated oxidative stress leads to DNA attrition ultimately causing cell death and tissue abrasion (Tan et al., 2016).

We also examined parasite viability after treatment with SAG or CM through flow cytometry. The results confirmed reduced parasite viability upon treatment with SAG or CM in sensitive parasites whereas the growth of resistant parasites was better inhibited by CM as compared to SAG. The results are supported by a study on the antileishmanial efficacy of *Bergenia ligulata* against *L. donovani* promastigotes (Kaur and Kaur, 2018). Our results revealed that CM was rich in active phytocomponents like betulin, a terpenoid that operates against the *Leishmania* parasite, which was confirmed by GC-MS screening.

Fatty acids deposition is prevalent in all cell forms from prokaryotes to eukaryotes and is an important building block that is accumulated as lipid droplets, lipid bodies, or lipid inclusions (Lee et al., 2013; Onal et al., 2017). The formation of lipid bodies is a hallmark of cellular stress. Accumulation of lipid bodies takes place due to various disturbances to the cellular functions of the parasite. Our observations revealed that the formation of lipid bodies was significant in SS and RS parasites after CM exposure. The results are in accordance with the findings of Diniz et al. (2018) reporting on the unusual accumulation of intracellular lipids in the promastigotes of *L. amazonensis* treated with *Ocimum canum* essential oil. Similar studies of lipid body accumulation were documented in *Trypanosoma cruzi* and *L. amazonensis* after treatment with Taxol and Ravuconazole respectively (Dantas et al., 2003; Teixeira de Macedo Silva et al., 2018). Previous studies on *L. amazonensis* promastigotes treated with 4-nitrobenzaldehyde thiosemicarbazone (BZTS) and *L. donovani* treated with zerumbone reported an increased intracellular lipid accumulation, suggesting an alteration in the sterol composition of parasite membranes that might lead to loss of membrane integrity and possible death by apoptosis (Britta et al., 2014; Mukherjee et al., 2016).

In this study, we investigated activity of CM against the sensitive and resistant strains of *L. donovani*. The inhibitory concentration of CM was higher than SAG for the sensitive strain but it was lower than SAG for the resistant strain while a less difference was detected in the IC_{50} values of CM for both the strains. However, SAG showed a greater difference in IC_{50} values for the sensitive and resistant strains in contrast to CM. Therefore, the results showed the potential of CM against the resistant strain in addition to the sensitive strain of the *Leishmania* parasite.

5. Conclusions

The presence of plant secondary metabolites, their quantitation in CM and GC-MS characterization effectuated its bioactivity. CM potentially inhibited the growth of sensitive and resistant strains of *L. donovani*. It also effectively augmented ROS and lipid bodies leading to cell shrinkage and loss in plasma membrane integrity thereby demonstrating that it promotes parasite killing. Hence, CM can be a promising option for controlling visceral leishmaniasis infection. However, further *in vivo* studies are required in animal models to validate the *in vitro* studies.

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CRediT author statement

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Declaration of competing interests

The authors declare that they have no known competing financial interest or personal relationships that could have appeared to influence the work reported in this paper.

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