Journal of Traditional and Complementary Medicine xxx (2017) 1–10

Contents lists available at ScienceDirect

Journal of Traditional and Complementary Medicine

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

Evaluation of *in vitro* and *in vivo* antileishmanial potential of bergenin rich *Bergenia ligulata* (Wall.) Engl. root extract against visceral leishmaniasis in inbred BALB/c mice through immunomodulation

Rupinder Kaur, Sukhbir Kaur*

Parasitology Laboratory, Department of Zoology, Panjab University, Chandigarh-160014, India

ARTICLE INFO

Article history:
Received 24 March 2017
Received in revised form
19 June 2017
Accepted 20 June 2017
Available online xxx

Keywords:
Extract
HPLC
Parasite load
Immunological
Biochemical
Histopathological studies

ABSTRACT

Background: Medicinal plants with immunomodulatory properties can provide good alternative therapeutics for curing visceral leishmaniasis. *Bergenia ligulata* (Wall.) Engl. is an interesting plant with strong antioxidant, antimicrobial, immunomodulatory and hepatoprotective properties.
Aim: The present study was planned to determine the antileishmanial activity of plant extract by modulating the immune responses of inbred BALB/c mice.
Methodology: Bergenin, the principle active component of *B. ligulata*, was quantitated in crude extract by performing RP-HPLC. The therapeutic potential was assessed through *in vitro* antileishmanial activity and in mice model through parasite load, cytokine assays, IgG antibody levels, DTH responses, histopathology and biochemical enzyme assays.
Results: B. ligulata showed the presence of glycosides, saponins, carbohydrates, tannins, flavonoids and bergenin which contributed to the antileishmanial activity of extract with IC50 of 22.70 μg/mL. Furthermore, the higher dose significantly reduced the parasite load by 95.56 %. The reduction was further associated with significant enhancement of IL-12 and IFN-γ levels in comparison to IL-10 and IL-4 cytokines. The switching towards Th1 type of immune response was also confirmed by elevated antibody levels of IgG2a isotype as compared to IgG1 as well as increased DTH responses. The histology of liver and kidney further complimented the non toxic nature of plant extract in addition to its negligible toxicity on HeLa cells.
Conclusions: The current study revealed the significant antileishmanial and immunomodulatory properties of this plant extract against murine visceral leishmaniasis. Further, the bioactive components will be explored to assess their efficacy for the development of safe and cost effective drug.

1. Introduction

Medicinal plants have been used in developing countries for the treatment of infectious diseases, cancer and parasitic diseases since ancient times. Numerous studies have been conducted on the therapeutic potential of plant extracts to evaluate their antifungal, antiprotozoal and antihelminthic properties.[1] These medicinal plants contain numerous bioactive components which play an active role in the treatment of various diseases. Most of the antimalarial drugs available today are derived from plants. The ether extract of *Artemisia annua* was used for isolating an active component named artemisinin.[2] These studies introduced a new era of using plant materials as medication for treatment of various diseases.

According to WHO, visceral leishmaniasis is one of the most neglected diseases and approximately 1.3 million new cases of leishmaniasis occur every year, of which 300,000 cases are of VL. Moreover, 90 % of VL cases occur only in Bangladesh, Brazil, Ethiopia, India, Sudan, South Sudan and Somalia. It is estimated

Abbreviations: DMSO, Dimethyl sulphoxide; SSG, Sodium stibogluconate; BLEE, *Bergenia ligulata* ethanolic extract; CMI, Cell mediated immune responses; p.t.d., Post treatment days; p.i.d., Post infection days; PCT, Proximal convoluted tubules; DCT, Distal convoluted tubules; T.S, Transverse section; SRBC, Sheep red blood cells; VL, Visceral leishmaniasis.

* Corresponding author.
E-mail address: puzoology@yahoo.com (S. Kaur).

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

http://dx.doi.org/10.1016/j.jtcme.2017.06.006

Please cite this article in press as: Kaur R, Kaur S, Evaluation of *in vitro* and *in vivo* antileishmanial potential of bergenin rich *Bergenia ligulata* (Wall.) Engl. root extract against visceral leishmaniasis in inbred BALB/c mice through immunomodulation, Journal of Traditional and Complementary Medicine (2017), http://dx.doi.org/10.1016/j.jtcme.2017.06.006

that 20,000 to 40,000 deaths occur every year due to this disease.[3] In addition, one of the major consequences of VL is suppression of protective T-helper (Th)-1 cells and induction of disease-promoting Th-2 cells.[4] The treatment of visceral leishmaniasis relies primarily upon chemotherapeutic drugs and most of these drugs cause severe side effects. Resistance to first line drug i.e. antimonials (sodium stibogluconate and sodium antimony gluconate) is a serious concern.[5] Alternative drugs like amphotericin B and pentamidine also have unpleasant side effects.[6] Therefore, during the past decade the treatment of this disease has become a bewildering step. Unfortunately, there is no antileishmanial drug which is derived from any plant resource. Recently, a number of studies have been conducted to explore the potential of natural plant extracts and their lead compounds against different forms of leishmaniasis. However, most of the studies are restricted to *in vitro* effects of plant part extracts on promastigote stage of *Leishmania*.[7] Thus, treatment of VL with natural plant products through immunomodulation is a good option. Therefore, the present study has been planned to evaluate *in vitro* and *in vivo* efficacy of traditional medicinal plant *B. ligulata* (Wall.) Engl. which is known to possess antiplasmodial, immunomodulatory, hepatoprotective and diuretic properties.[8-11]

2. Methodology

2.1. Maintenance of promastigote culture

Indian strain of *Leishmania donovani* i.e. MHOM/IN/80/Dd8 was obtained from the London School of Hygiene and Tropical Medicine, U.K. and was used for present study. Log phase promastigotes were used for the maintenance of strain in modified NNN medium at 22 ± 1 °C. The culture was checked for any contamination and sub cultured after every 48–72 h by transferring 0.5–1.0 mL of culture suspension in Mc Cartney vials containing NNN medium and then supplemented with 3–4 mL of MEM. The pH of 7.2 of medium was maintained by adding 7.5 % $NaHCO_3$. The promastigote culture was maintained in B.O.D. incubator at 22 ± 1 °C.[12]

2.2. Plant material

Rhizomes of *B. ligulata* (Wall.) Engl. were collected from Shimla district of Himachal Pradesh. Permission for collection of plant was taken from forest department of Himachal Pradesh, India. Specimens were authenticated by herbarium incharge of Department of Botany, Panjab University, Chandigarh. Voucher specimen was submitted in the herbarium of Department of Botany, Panjab University, Chandigarh and voucher no. 5328 was obtained.

2.3. Preparation of the extract

Rhizomes were washed thoroughly with water and dried at room temperature and then powdered. Ethanolic extract was prepared by Soxhlet extraction method. Approximately 100 g dried and powdered rhizomes were extracted with 250 mL of ethanol. Crude extract was filtered through Whatman filter paper no. 41. Filtrate was concentrated under vacuum in a rotary evaporator (Buchi, USA). Residue obtained was then lyophilized. It was equivalent to 10.5 % of the dry mass of original rhizome powder. It was stored at −20 °C till further use.

2.4. Phytochemical screening of plant extract

Phytochemical screening of the plant extract was carried out to detect alkaloids, saponins, phenols, terpenes, flavonoids, glycosides, tannins and polysterols by employing standard procedures.[13,14]

2.5. Determination of bergenin in crude extract

Bergenin is the principle active component of *B. ligulata*, therefore reverse phase-high performance liquid chromatography was performed for this compound. RP-HPLC fingerprint profile was established for ethanolic extract using RP-HPLC instrument (Shimadzu, Kyoto, Japan). The validated analytical method was used for the quantification of bergenin in plant extract. Components were separated on a Waters C18 3.9 × 150 mm I.D. 5 μm Symmetry column. Elution was performed at a flow rate of 1 mL/min with a gradient prepared from water–phosphoric acid 99.7:0.3 (v/v) (component A) and acetonitrile–water–phosphoric acid 79.7:20:0.3 (v/v) (component B). Gradient used was: 0–5 min, 88–85 % A; 5–10 min, 85–75 % A; 10–20 min, 75–70 % A. The results were acquired and processed using Shimadzu LC-solution version 6.42 software.

Bergenin standard (total purity 98%) was purchased from TCI chemicals (India) Pvt. Ltd. Amount of active compound in plant extract was determined by plotting calibration plots established by chromatography of bergenin standards at five different concentrations. Each solution was chromatographed in triplicate. Peaks were identified by comparison of retention times and UV absorption spectra with those of standards. Linearity of detector response for the standards was assessed by linear regression analysis of each amount of standard and area of the corresponding peak on chromatogram. Linearity was also confirmed for extract. After chromatographic separation, peak areas obtained were plotted against extract concentration by linear regression analysis. Purity of peaks was checked by acquisition of spectra ($\lambda = 200–400$ nm) by use of PDA detector and multivariate analysis. Spectra were acquired at the upslope, apex, and downslope of each peak, computer normalized, and superimposed. Peaks were considered pure when there was coincidence between three spectra (match factor ≥98 %).

RP-HPLC method was validated by determination of linearity, peak purity, and limits of quantification and detection. For qualitative purposes, the method was evaluated by taking into account retention time precision, peak purity and selectivity for standards. Peak purity was studied for major peaks. Impurities or co-elution were not observed (match factors ≥95 %). Linearity and limits of detection (LOD) and quantification (LOQ) were evaluated for quantitative purposes. LOD for bergenin was 2.10 and LOQ was 6.37 respectively, implying the method was suitable for quantification of this compound. R^2 values for the compound was >0.99, confirming the linearity of the method.

2.6. Estimation of parasite viability by flowcytometry

L. donovani promastigotes at a concentration of 2×10^6/mL were dispensed into 24-well culture plates. Each well was then supplemented with specific concentration (10–100 μg/mL) of plant extract. Test was performed in duplicate series. Negative control cultures were supplemented with an equal volume of 1 % DMSO. In addition, positive control cultures were incubated with 10–100 μg/mL sodium stibogluconate (SSG). Plates were incubated at 22 ± 1 °C for 48 h. After 48 h, promastigotes of *L. donovani* were stained with propidium iodide.[15] The percentage viability was calculated by probit analysis using SPSS 18.0 software.

2.7. Cytotoxic effect on HeLa cells

Growth of HeLa cells was quantitated by studying the ability of living cells to reduce yellow dye 3-(4, 5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) to a blue formazan

Please cite this article in press as: Kaur R, Kaur S, Evaluation of *in vitro* and *in vivo* antileishmanial potential of bergenin rich *Bergenia ligulata* (Wall.) Engl. root extract against visceral leishmaniasis in inbred BALB/c mice through immunomodulation, Journal of Traditional and Complementary Medicine (2017), http://dx.doi.org/10.1016/j.jtcme.2017.06.006