

Lupeol induces immunity and protective efficacy in a murine model against visceral leishmaniasis

Research Article

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Abstract

The available chemotherapeutics for the cure of visceral leishmaniasis (VL) are linked with many detrimental effects. Moreover, VL is associated with the suppression of protective Th1 immune response of the host and induction of disease exaggerating Th2 immune response. Therefore, there is an urgent requirement of therapeutics which can augment the immune status of the host to cure this disease. In the current investigation, the antileishmanial potential of lupeol was monitored *in vitro* and *in vivo* in inbred BALB/c mice against *Leishmania donovani*. Lupeol showed potent antipromastigote activity *via* arresting parasites at sub G₀/G₁ phase *in vitro*. Lupeol significantly decreased the splenic parasite burden by inducing strong delayed-type hypersensitivity responses in contrary to untreated infected animals. The therapeutic efficacy of lupeol was observed to be similar to the reference drug, AmB. Treatment of infected animals with lupeol depicted enhanced levels of T cells and Th1 cytokines in contrast to only infected controls. Further lupeol treatment upregulated the levels of nuclear factor κ B and nitric oxide synthase genes and elevated the production of reactive oxygen species and nitric oxide. Unlike AmB, lupeol-treated infected animals did not show any toxicity. These findings are promising and indicate that lupeol can serve as a prototype drug for the cure of VL.

Introduction

Neglected tropical diseases (NTD) impose a huge disease burden worldwide. Visceral leishmaniasis (VL) is one of the NTD which leads to a high number of morbidity and mortality. It is marked by the existence of *Leishmania* parasites in the visceral organs such as spleen, liver and bone marrow and it accounts for clinical manifestations such as hepatomegaly, splenomegaly, pancytopenia, prolonged fever and weight loss (Tajebe *et al.*, 2017). Leishmanial parasites have the potential to subvert the immune system of the host and survive within the macrophage phagolysosome. They change the immunological and inflammatory host responses to their profit by suppressing the nuclear factor κ B (NF κ B) complex. The latter leads to the downregulation of nitric oxide synthase (iNOS) expression in infected macrophages and hinder the production of nitric oxide (NO), assisting its replication and sustained infection (Marcusso Orsini *et al.*, 2016). In recent years, many studies have been carried out to explore the possibility of development of new drugs with immunomodulatory properties.

At present, chemotherapy is the only option which can be used to control VL. For all clinical forms of leishmaniasis, pentavalent antimony is still used in Bangladesh, Nepal, West Bengal, Jharkhand and Uttar Pradesh (Sundar and Chakravarty, 2015; Singh *et al.*, 2016). The adverse outcomes such as abdominal pain, hepatic and cardiac problems and growth of resistant parasites have severely affected the efficacy of antimonial drugs (Singh *et al.*, 2016). Other available options such as amphotericin B (AmB), miltefosine, pentamidine and paromomycin are also associated with unfavourable adverse outcomes. High toxicities associated with them have restrained their use in many regions (Das *et al.*, 2001; Chattopadhyay and Jafurulla, 2011; Chawla *et al.*, 2011; Sundar *et al.*, 2012). Although lipid formulations of AmB seem efficacious, its effectiveness is still a concern due to issues of resistance and high cost (Croft *et al.*, 2006).

The landmark cytokines such as TNF- α , IFN- γ , IL-17 and IL-12 indicate Th1 immune response and IL-10 and IL-4 indicate Th2 immune response (Piscopo and Mallia Azzopardi, 2007). IL-12 along with IFN- γ plays a crucial role in the control of disease *via* parasite death (Bacellar and Carvalho, 2005). Likewise IFN- γ together with TNF- α protects the host against VL through increasing the levels of NO (Salim *et al.*, 2016). In contrary to this, high concentrations of IL-4 and IL-10 deactivate the *Leishmania* killing efficacy of macrophages. In fact, these cytokines favour the growth and multiplication of intracellular parasites (Peruhype-Magalhães *et al.*, 2005). Thus a dominant Th1 immune status over Th2 inhibits parasite growth through activated macrophages for the elimination of intracellular parasites *via* NO and other reactive oxygen species (ROS) (Piscopo and Mallia Azzopardi, 2007). Altogether the paucity of effective treatment approaches has been the driving impulse for the search of new therapeutic entities against *Leishmania*. A new approach to finding effective remedies for the cure of leishmaniasis necessitates a novel agent which not only has leishmanicidal activity but also has the potential to strengthen the immune status of the host. In this regard natural products are gaining increased attention as they are safe and many of them possess immunostimulatory efficacy (Duarte *et al.*,

2016). Many studies have researched the antileishmanial efficacy of single molecules in different murine animal models. For instance, oral administration of a clerodane diterpene to *L. donovani*-infected hamsters decreased the parasite burden in liver, bone marrow and spleen (Misra *et al.*, 2010). Similarly another compound, (-)- α -Bisabolol when administered to *L. infantum*-infected BALB/c *via* oral route at the concentration of 200 mg kg⁻¹, showed marked parasite reduction (71–89%) with no toxic effects (Corpas-Lopez *et al.*, 2015). Triterpenoids are known to have many pharmacological effects such as immunostimulatory, antiviral, antimicrobial and anticancerous. These secondary metabolites are responsible for different biological activities like immunomodulation in many plants. Several triterpenoids such as derivative of oleanolic acid are under clinical trials for the treatment of chronic diseases like tumours (Han and Bakovic, 2015). These derivatives have also been found to show a significant activity against *Mycobacterium tuberculosis* in BALB/c mice (Hong *et al.*, 2012; Jimenez-Arellanes *et al.*, 2013). In the current research work, the leishmanicidal activity of a triterpenoid, lupeol was monitored as it possesses various biological effects such as hepatoprotection, nephroprotection, antileukemic and cardioprotection. Lupeol has the ability to annihilate diseased and unhealthy human cells without affecting normal cells (Wal *et al.*, 2015). Das *et al.* (2017) also described the antileishmanial potential of lupeol against VL in BALB/c mice when administered *via* intraperitoneal route. In this study, infected mice were treated with lupeol (75 mg kg⁻¹) for 5 days alternatively after 14 days of infection. Further, the parasitological and immunological parameters were examined on 30 post treatment day (p.t.d). However in our study, the therapeutic potential of lupeol was investigated after 30 days of infection. The latter time regimen is a conventional period which allows the substantial progression of infection (Melo *et al.*, 2017). The infected mice were treated orally with lupeol (25 and 50 mg kg⁻¹ b.wt.) for 2 weeks and sacrificed on 14th p.t.d. Moreover along with parasitological and immunological studies, the safety profile of lupeol was also checked *in vitro* and in *in vivo* model. In addition to this antileishmanial activity of lupeol was also compared with the standard drug AmB. Many reports suggest that lupeol is a non-toxic agent and causes no side-effects in animals up to the concentration of 2000 mg kg⁻¹ (Siddique and Saleem, 2011).

Methods

Parasite and culture maintenance

MHOM/IN/80/Dd8 strain of promastigotes of *L. donovani* was used and it was procured from the PGIMER, Chandigarh. Promastigotes were maintained in modified Novy, McNeal and Nicolle's (NNN) medium (Rao *et al.*, 1984) and RPMI-1640 medium at 22 + 1 °C in a B.O.D. incubator.

Evaluation of antipromastigote activity

Promastigotes of *L. donovani* were seeded in a 24-well plate (2 × 10⁶ cells mL⁻¹) and incubated for 72 h with different concentrations of lupeol (10–100 µg mL⁻¹) and AmB (0.1–1 µg mL⁻¹) at 22 °C. A 0.01% of DMSO was taken as negative control. Further, the number of viable parasites was enumerated in Neubauer haemocytometer after staining with trypan blue dye. The mean percentage viability was computed as given below:

$$\text{Percentage viability} = \frac{\text{No. of viable parasites in treated well}}{\text{No. of viable parasites in blank well}} \times 100$$

$$\text{Percentage growth inhibition} = 100 - \text{percentage viability}$$

The inhibitory concentration that reduced the parasite growth by 50% (IC₅₀) was calculated by projecting percentage inhibition against concentrations of drug using software namely SPSS (Strober, 2001).

Further to study the cell cycle of promastigotes, parasites (1 × 10⁶ mL⁻¹) were treated with the IC₅₀ value of lupeol and AmB at 22 °C for 72 h. These cells were fixed with chilled 70% ethanol and kept for 1 h at 4 °C. The cells were washed with PBS and then incubated with RNase (200 µg mL⁻¹) for 1 h. Further, these cells were incubated with propidium iodide (PI, 50 µg mL⁻¹) dye for 20 min in the absence of light at 37 °C. Sample acquisition and analysis was done by using CellQuest software of BD FACSCalibur and PI was measured in the PE channel (Zakraoui *et al.*, 2017).

Cytotoxicity assay on human macrophages

PMA (phorbol-12-myristate-13-acetate) induced human THP-1 macrophages in RPMI 1640 medium in 96-well plate were incubated with various concentrations of lupeol or AmB (20–1000 µg mL⁻¹) in a CO₂ incubator. MTT (1 mg mL⁻¹) was added into each well followed by incubation of 3–4 h. Then formazan crystals were solubilized with 100 µL of DMSO and optical density was read at 550 nm. The cell viability was monitored on the basis of optical density of the untreated, treated samples and blank wells using probit analysis on SPSS software (Das *et al.*, 2008). Selectivity index was computed as the ratio between the activity of the drug against macrophages and parasites (CC₅₀/IC₅₀).

Design of experiment

Promastigotes (1 × 10⁷) of *L. donovani* were injected into the lateral tail vein of inbred BALB/c mice. After 30 days of infection, treatment was commenced. The experiment consisted of six groups and each group consisted of six animals: A – uninfected control; B – untreated infected animals; C – standard suspension vehicle; D, E – treatment of infected animals with lupeol, orally at two concentrations (25 and 50 mg kg⁻¹ b.wt.) for 14 days; F – infected mice administered intraperitoneally with AmB (2.5 mg kg⁻¹ b.wt.) for 5 days served as positive control. All the animals were euthanized on 14th p.t.d.

qPCR for determination of parasite load

DNA was extracted from the spleen (25 mg) using phenol-CHCl₃ extraction procedure as stated by Sambrook *et al.* (1989) with some alterations in the concentration of lysis buffer (10 mM Tris HCl, 0.5 mM EDTA, 20 mg mL⁻¹ proteinase K and 5% SDS). Similarly DNA was purified from the promastigotes of *L. donovani* (1 × 10¹⁰ cells mL⁻¹) to plot the calibration curve (up to 10-fold serial dilutions). The *L. donovani* microsatellite forward primer 5'-ACACGCAGAGAACTCGGTTT-3' and the reverse primer 5'-TGGAGCGAGAAAGGACAAGT-3' (accession number – FJ263505.1) of 229 bp were used. qPCR was carried out on LightCycler® 480 system (Roche) and 20 µL reaction mix was formulated by 1 µL (150 ng) of DNA, 10 µL of 2X SYBR Green (Thermo), a mixture of forward and reverse primers (1 µL, 10 µM) and PCR grade water (8 µL). The conditions used in thermal cycler were: the first step at 95 °C (5 min), then 35 cycles at 94 °C (10 s), 58 °C (20 s) and 72 °C (30 s). Each run comprised at least one run with PCR grade water as negative control. The melting curve was attained at 65–97 °C to check the amplified product without dimers (Hossain *et al.*, 2017).

Assessment of delayed-type hypersensitivity responses

Immune status was monitored in various groups of animals by delayed-type hypersensitivity (DTH) response. The right footpad was challenged subcutaneously with phenol (0.5% in phosphate-buffered saline) killed promastigotes (leishmanin 40 μL , 2×10^8 promastigotes mL^{-1}) and left footpad was injected with PBS. The thickness of both footpads was measured with Vernier caliper after 48 h. Results were interpreted as an enhancement in the thickness of right footpad as compared to left footpad of animals in millimetres (Banerjee *et al.*, 2008).

Flow cytometric immunophenotypic analysis

Spleens were taken from all the groups of animals on scheduled time point and homogenized in cell strainer. The cell suspension was then incubated with 1% NH_4Cl for lysis of erythrocytes for 20 min followed by centrifugation (3000 rpm for 5 min, 4 °C). The obtained pellet of splenocytes was washed two times with PBS. Then this suspension of splenocytes was adjusted to a concentration of 1×10^6 cells per sample and labelled with monoclonal anti-CD3-PE, anti-CD4-FITC, anti-CD8-APC antibodies for 15–20 min at 4 °C. The splenocytes were then suspended in 200 μL PBS and captured on DIVA software of FACSCalibur (Gupta *et al.*, 2006).

Estimation of cytokine profile

Various cytokines such as Th1 (TNF- α , IL-17, IL-12, IFN- γ) and Th2 (IL-4, IL-10) were checked in the sera by sandwich enzyme-linked immunosorbent assay using kits (ELISA; Diaclone, France) according to the guidelines of the manufacturer.

Measurement of ROS and NO production

In all the experimental groups, the levels of NO were assessed by Griess assay. The spleen was homogenized (10% w/v) in 0.1 M PBS (pH 7.4) followed by centrifugation (10,000 g at 4 °C) for 15–20 min and the supernatants were obtained. In 96-well plate, an equal volume (50 μL) of the supernatant and Griess reagent (0.1% of naphthyl ethylenediamine dihydrochloride in orthophosphoric acid and 1% sulfanilamide) was dispensed and kept at 37 °C for 10 min. The concentration of NO was measured at 550 nm on ELISA reader. Different concentrations of sodium nitrite (1.56–100 μM) were used for the preparation of a standard curve with the same protocol as described earlier and the amount of NO formed in various groups was calculated from this standard curve.

ROS production was monitored by cell permeant 2',7'-dichlorofluorescein diacetate (H2DCFDA) dye (Sigma, USA). Spleen from various groups of animals was homogenized and single-cell suspensions were prepared. Then these cells (1×10^6 cells mL^{-1}) were incubated with H2DCFDA dye (10 μM) in a conventional incubator (37 °C, 5% CO_2) for 25–30 min in dark. After washing the cells with PBS, these were taken up in PBS into 96-well plate and fluorometric measurements were done immediately at 485 nm of excitation and 535 nm of emission wavelength, respectively (Paik *et al.*, 2016). Fold change in ROS production was calculated by the following equation:

$$\text{Fold Change} = \frac{\text{fluorescent intensity of treated group} - \text{fluorescent intensity of uninfected group}}{\text{fluorescent intensity of uninfected group}}$$

Evaluation of in vivo toxicity

Hepatic enzymes and renal function markers were estimated in the sera of all groups by measuring serum glutamic pyruvic

transaminase (SGPT), lactate dehydrogenase (LDH), serum glutamic oxaloacetic transaminase (SGOT), uric acid, urea, blood urea nitrogen (BUN) and creatinine according to the protocol of manufacturer (Thermo Fischer Scientific. kits, India).

Further for histological analysis, liver and kidney tissues were taken out from all groups of mice and fixation of the tissues was commenced in Bouin's solution for 24 h. Then embedding of tissues was carried out in paraffin blocks followed by the cutting of sections with a microtome and the stretching of sections was carried out in hot water on albumin-coated slides. Further haematoxylin and eosin (H&E) dye was used to stain these slides and observed in the light microscope (Pearse, 1968).

Gene expression

Tri reagent (Sigma, USA) was used for the isolation of RNA from the spleen and RNA (5 μg) was reverse transcribed into cDNA by using a cDNA synthesis kit (Thermo Scientific, Lithuania). RT-qPCR was used to determine the expression of iNOS and NF κB genes. The primers used for this study were as follows: β -actin: forward primer: GCATTGCTGACAGGATGCAG, reverse primer: CCTGCTTGCTGATCCACATC; NF κB : forward primer: CTGGTGGACACATACAGGAAGAC, reverse primer: ATAGGCACTGTCTTCTTTCACCTC (annealing temperature: 57 °C); iNOS: forward primer: GATCCGATTTAGAGTCTTGGTG, reverse primer: TCCCTGGCTAGTGCTTCAG (annealing temperature: 60 °C) (accession numbers – NM_007393.5; NM_019408; NM_001313922.1 of β -actin, NF κB and iNOS, respectively). Total reaction mixture (20 μL) was comprised of cDNA (1 μL), 1 μL of a blend of forward and reverse primers (10 μM), 10 μL of 2X SYBR green master mix and molecular grade water (8 μL). The thermal conditions followed in this experiment have been already described before. The reference gene used for this study was β -actin and the relative expression of the gene was estimated by the $2^{-\Delta\Delta\text{CT}}$ method (Livak and Schmittgen, 2001).

Statistical analysis

All the experiments were assessed in triplicate and results were presented as means \pm S.E. (standard error). The differences between the groups were examined using Tukey test of one-way analysis of variance (ANOVA) and the differences were statistically significant at $P < 0.05$.

Results

In vitro leishmanicidal and cytotoxic activity

In vitro efficacy of lupeol against *L. donovani* promastigotes was assessed by trypan blue staining. Lupeol revealed potent *in vitro* efficacy with an IC_{50} value of $25.49 \pm 2.5 \mu\text{g mL}^{-1}$ at 72 h (Fig. 1). However, AmB showed highest antileishmanial activity with an inhibitory concentration of $0.055 \pm 0.003 \mu\text{g mL}^{-1}$. The solvent DMSO (0.01%) did not affect the viability of parasites. Further the cell cycle analysis of promastigotes after incubation

with lupeol or AmB at the IC_{50} concentration was performed to assess the cells in the sub- G_0/G_1 phase as cells in this phase indicate the degradation of DNA and apoptosis of the cells. The

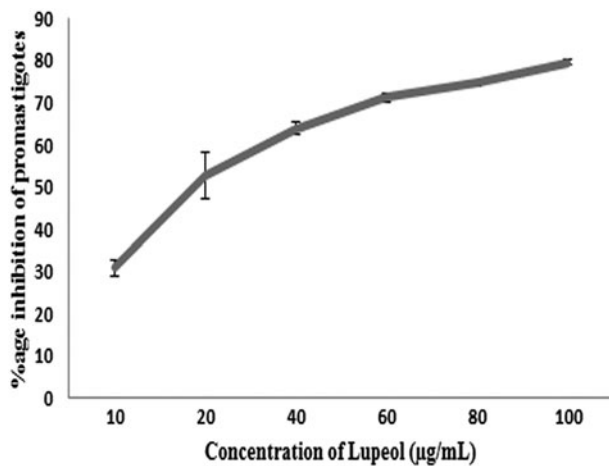


Fig. 1. Estimation of antipromastigote activity by trypan blue dye exclusion assay. The inhibitory concentration that reduced the parasite growth by 50% (IC_{50}) was calculated by projecting percentage inhibition against different concentrations of the drug.

population of promastigotes in this phase was significantly ($P < 0.05$) increased (39.23%) upon treatment with lupeol as compared to untreated controls (8.73%). However, the population of promastigotes in sub- G_0/G_1 was $53.56 \pm 2.4\%$ upon treatment with AmB. This finding clearly indicated that lupeol induced cell cycle arrest at the sub- G_0/G_1 phase (Fig. 2).

The cytotoxic potential of lupeol and AmB was determined by measuring oxidoreductase activity in human macrophage cell line THP1. Lupeol showed less cytotoxicity with a CC_{50} value of $960.04 \pm 27.64 \mu\text{g mL}^{-1}$ in comparison to AmB ($27.73 \pm 0.1 \mu\text{g mL}^{-1}$) at 72 h. Moreover, the selectivity index of lupeol and AmB was found to be more than 10.

In vivo therapeutic potential of lupeol against *L. donovani*

Parasite burden was quantified in target organ spleen, in different groups of animals at 14th p.t.d. by qPCR. It was significantly ($P < 0.05$) found to decrease in lupeol (25 and 50 mg kg^{-1} b.wt.) and AmB-treated animals as compared to infected controls. Infected mice treated with a higher dose of lupeol caused marked ($P < 0.05$) reduction of parasites than the lower dose. Mice infected with *L. donovani* receiving lupeol at 25 and 50 mg kg^{-1} b.wt. caused 87.26 ± 0.95 and $97.75 \pm 0.18\%$ parasite reduction, respectively, while $98.16 \pm 0.14\%$ parasite reduction was detected in AmB-treated animals. However, a higher dosage of lupeol led to equivalent reduction ($P > 0.05$) in parasite burden in infected mice as was observed in AmB-treated infected mice. Vehicle suspension-treated infected animals revealed the same parasite burden as observed in only infected controls (Fig. 3).

Immune response

Induction of DTH response is a symbol of cell-mediated immunity against leishmaniasis. Infected mice treated with lupeol at both doses and AmB exhibited a significant ($P < 0.05$) enhancement in footpad thickness as compared to infected control mice. DTH response was significantly ($P < 0.05$) more in infected animals treated with a higher dose of lupeol as compared to those treated with the lower dose (Fig. 4). Moreover, no significant difference ($P > 0.05$) was noticed in DTH response in infected mice after treatment of a higher dose of lupeol and AmB. To further verify the induction of cell-mediated immune response, the population of CD4+ and CD8+ T cells was monitored in the spleen of different groups of animals by flow cytometry. The percentage of CD4+ and

CD8+ T cells was significantly ($P < 0.05$) found to be enhanced in the lupeol (25 and 50 mg kg^{-1} b.wt.) and AmB-treated infected animals in comparison to untreated infected controls. The phenotypic analysis indicated that the infected mice receiving lupeol at 50 mg kg^{-1} b.wt. exhibited significantly ($P < 0.05$) higher population of these cells than lupeol at 25 mg kg^{-1} b.wt. (Fig. 5). Treatment of infected mice with lupeol at higher dose showed a similar increase ($P > 0.05$) in the percentage of CD4+ and CD8+ T cells as observed after administration of AmB.

The concentration of Th1 (IL-12, TNF- α , IFN- γ , IL-17) and Th2 (IL-4, IL-10) cytokines were measured in the serum by sandwich ELISA. The animals showed exhaustion of immune response in terms of decreased concentration of protective cytokines of Th1 type and increased levels of disease-promoting, Th2 cytokines. AmB and lupeol (at both doses) treatment significantly ($P < 0.05$) increased the Th1 cytokines (Fig. 6) and decreased the levels of Th2 cytokines as compared to infected controls. However, no significant difference ($P > 0.05$) was observed in the levels of Th2 cytokines after treatment of infected mice with a higher dose of lupeol as compared to the lower dose (Fig. 7). Treatment of lupeol (50 mg kg^{-1} b.wt.) and AmB in infected mice displayed almost equal increase ($P > 0.05$) in the levels of Th1 cytokines (IL-12, TNF- α , IFN- γ). However, AmB-treated infected mice revealed significant ($P < 0.05$) higher concentration of IL-17 in comparison to lupeol-treated infected mice. In the case of Th2 cytokines also, lupeol (50 mg kg^{-1} b.wt.) as well as AmB treatment led to a comparable decrease in the levels of IL-4 and IL-10 in the sera of infected mice.

NO and ROS are the macrophage-derived oxidative molecules which are important for the termination of growth of parasites. Treatment of infected mice with lupeol at higher dosage induced significant ($P < 0.05$) enhancement ($8.40 \pm 0.30 \mu\text{M}$) in the levels of NO in splenocytes than the only infected controls ($2.17 \pm 0.81 \mu\text{M}$). Higher levels ($17.75 \pm 1.52 \mu\text{M}$) of NO were also detected in AmB-treated infected mice. Similarly a 1.5-fold change in the levels of ROS production ($P < 0.05$) was observed in splenocytes of infected mice after lupeol treatment in contrast to only infected controls where low levels of ROS (0.74-fold) were found. Treatment of infected mice with AmB revealed maximum fold change (2.7-fold) in the levels of ROS generation (Fig. 8).

Toxicity profile

The safety potential of all the treatment groups was checked by monitoring the levels of hepatic enzymes and renal function markers in the serum. The levels of SGPT, SGOT, and LDH were observed to be increased in untreated infected control animals. In AmB- and lupeol-treated infected mice, these enzymes reverted to the normal levels depicting no side-effects of these treatments on the functioning of the liver (Table 1). However, the LDH value was still significantly lower in lupeol-treated infected mice as compared to AmB-treated. Among the kidney function markers, uric acid, creatinine, urea and BUN levels were all measured above the normal range in untreated infected control animals. Treatment of lupeol in infected animals showed normal levels of renal function markers. In addition, these metabolites were found above the normal limits in infected mice treated with AmB. The findings depicted that AmB was injurious to kidneys and lupeol had no renal toxicities (Table 2).

Further, the histological alterations in liver and kidney were estimated by H&E staining. The cross-section of a liver of infected mice exhibited Kupffer cell hyperplasia as compared to uninfected mice which revealed normal anatomical architecture. In all the treated groups, a normal histological architecture of the liver was observed as in the uninfected controls (Fig. 9). In the cross-section of the kidney of infected animals, lymphocytic infiltration

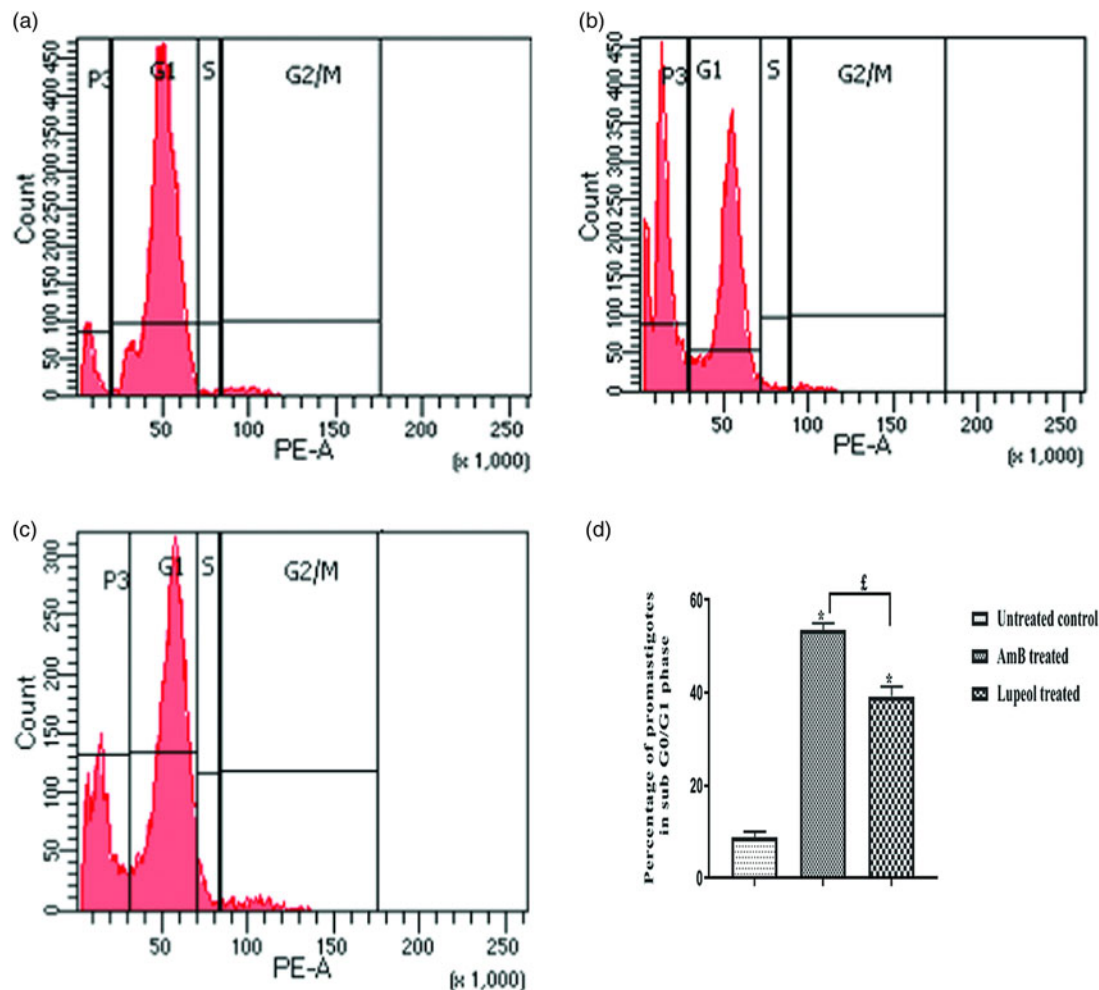


Fig. 2. Determination of sub G_0/G_1 population. Untreated or treated with IC_{50} concentration of lupeol and AmB were processed for cell cycle studies. Histograms represent the sub G_0/G_1 population in P3. (a) Untreated control, (b) AmB-treated, (c) Lupeol-treated, (d) graphical representation of the percentage of promastigotes in the sub G_0/G_1 phase of the cell cycle. Results were interpreted by mean \pm s.e. and a significant difference was tested by one-way ANOVA. * $P < 0.05$: untreated control vs AmB/Lupeol treated; $\epsilon P < 0.05$: AmB vs Lupeol treated.

was found which depicts the focal interstitial nephritis. Moreover, mild tubular disruption and casting of proteins were detected in the infected animals upon treatment with AmB. However, no such aberrations were found in the histological renal section of infected animals after treatment with lupeol (Fig. 10).

Effect of lupeol on NF κ B and iNOS mRNA expression

Further the effect of lupeol was monitored on the NF κ B and iNOS gene expression in the target tissue, spleen by reverse transcriptase qPCR. Treatment of infected mice with lupeol showed a significant ($P < 0.05$) upregulation (6.82-fold) in NF κ B when compared to only infected animals (1.6-fold). Moreover, treatment with lupeol also caused remarkable ($P < 0.05$) elevation (6.9-fold) in the expression of iNOS in contrary to infected untreated controls (1.84-fold). However, treatment with AmB in the infected mice exhibited maximum fold change in the expression of NF κ B (15.64-fold) and iNOS (12.16-fold) in contrast to lupeol-treated mice (Fig. 11).

Discussion

The available chemotherapeutic drugs are in the appalling state as the treatment options are inadequate because of their adverse effects, development of resistance and high cost. The drugs can be administered either parenterally or *via* an oral route.

However, the latter method of administration is relatively safer and more convenient to treat patients not only at the level of the community but during the times of epidemics also (Turner *et al.*, 2011). One of the lacunae related to the conventional antileishmanial drugs like sodium stibogluconate, AmB, pentamidine and paromomycin is their parenteral administration. This has complicated the treatment options as it can lead to thrombosis, severe hypersensitivity reactions and other secondary infections at the site of injection (Bruce and Wong, 2001; Perez-Victoria *et al.*, 2011).

Lupeol is pharmacologically active in treating the number of diseases in animal models irrespective of routes of administration viz. oral, topical, intravenous and intra-peritoneal (Sankaran and Jayasri, 2017). In the study of Sunitha *et al.* (2001), treatment of lupeol (orally; 150 mg kg^{-1} b.wt.) protected the liver of rats from the toxicity of cadmium. In another study by Manoharan *et al.* (2012), oral administration of lupeol at 50 mg kg^{-1} b.wt. decreased the volume and burden of tumour in hamsters treated with 7,12-dimethylbenz[a]anthracene. Thus, it is essential to find novel antileishmanial compounds which exhibit potent immunomodulatory property. In the current investigation, we evaluated the protective efficacy of lupeol against experimental murine VL and also evaluated its safety profile in comparison to the standard drug, AmB. Lupeol is an effective immunomodulatory triterpenoid found in various medicinal plants such as *Bauhinia variegata*, *Euphorbia resinifera* and *Sterculia villosa*. It is used as a

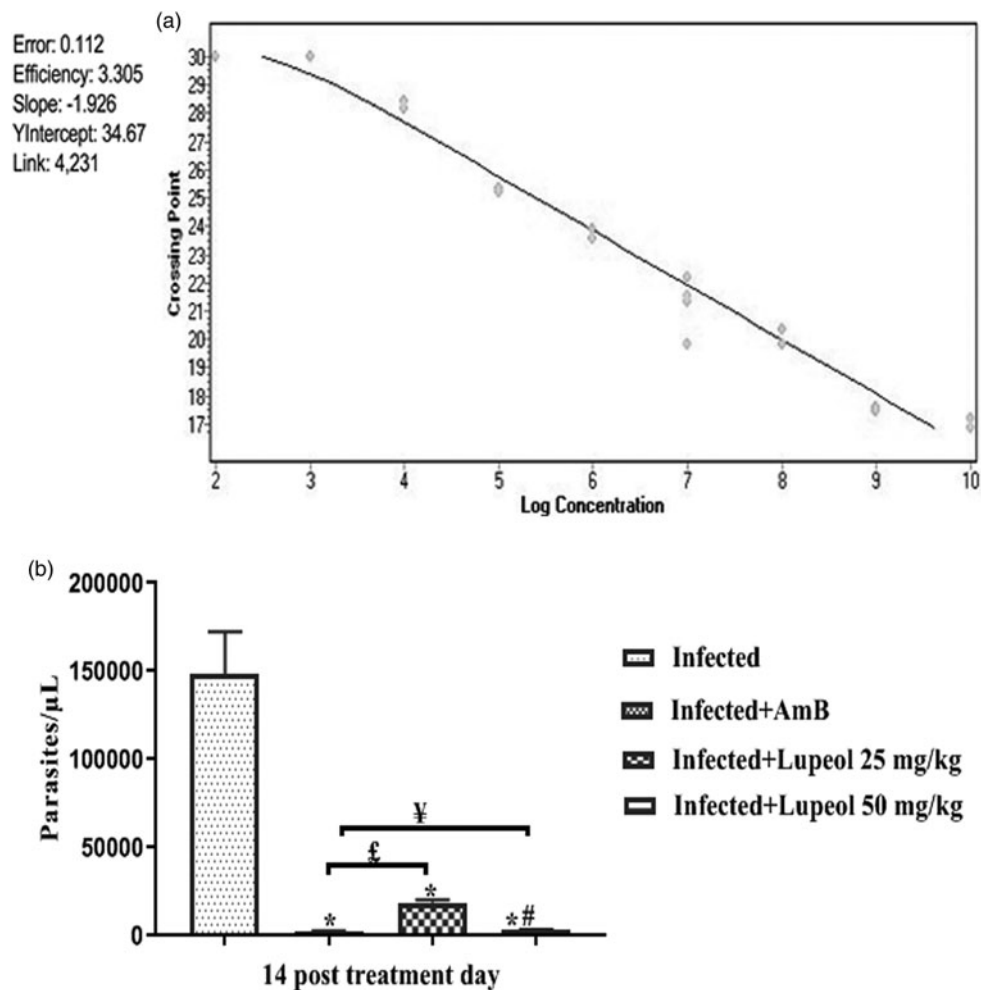


Fig. 3. Quantification of parasite load in different groups of animals. (a) Standard curve was prepared from the *in vitro* grown promastigotes of *L. donovani*. (b) Bar graph depicts the parasite burden in the spleen of various groups. Results are interpreted by mean \pm s.e. and significant difference was tested by one-way ANOVA. * $P < 0.05$: Infected control vs Infected + AmB/Infected + Lupeol 25 mg kg⁻¹/Infected + Lupeol 50 mg kg⁻¹ b.wt.; # $P < 0.05$: Infected + Lupeol 25 mg kg⁻¹ vs Infected + Lupeol 50 mg kg⁻¹; £ $P < 0.05$: Infected + AmB vs Infected + Lupeol 25 mg kg⁻¹; ¥ $P > 0.05$: Infected + AmB vs Infected + Lupeol 50 mg kg⁻¹.

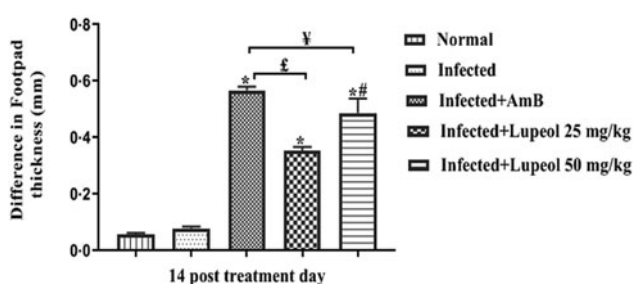


Fig. 4. Delayed type hypersensitivity response to leishmanial antigen. Footpad swelling (millimetre) was examined by measuring the difference between the right footpad (leishmanin) and left footpad (PBS) after 48 h. Results are interpreted as mean \pm s.e. for six animals per group and the difference between means for statistical significance was checked by one-way ANOVA using Tukey's test. * $P < 0.05$: Infected control vs Infected + AmB/Infected + Lupeol 25 mg kg⁻¹/Infected + Lupeol 50 mg kg⁻¹ b.wt.; # $P < 0.05$: Infected + Lupeol 25 mg kg⁻¹/Infected + Lupeol 50 mg kg⁻¹ b.wt.; £ $P < 0.05$: Infected + AmB vs Infected + Lupeol 25 mg kg⁻¹; ¥ $P > 0.05$: Infected + AmB vs Infected + Lupeol 50 mg kg⁻¹.

therapeutic and preventive agent for a wide array of diseases as it possesses many pharmacological effects such as anticancer and antimicrobial activities (Wal *et al.*, 2015).

In the *in vitro* studies, we found that lupeol was effective against promastigotes of *L. donovani* but it showed a negligible cytotoxic effect against human macrophage cell line THP1. Thus lupeol

possesses good biological potential against leishmaniasis as depicted by its SI ≥ 10 which describes that lupeol is more active against the pathogen and does not show any toxic effects on normal cells (Nagle *et al.*, 2014). In contrast to lupeol, the CC₅₀ value of AmB against mammalian macrophages was found to be very less, i.e. only 27.73 $\mu\text{g mL}^{-1}$. This depicted that lupeol is safer for human cells and can be used in *in vivo* models. Further, flow cytometric analysis revealed that lupeol arrested the promastigotes at sub G₀/G₁ phase of the cell cycle which is a hallmark of apoptosis. Many phytoconstituents such as zerumbone showed inhibition against promastigotes and induced arrest at sub G₀/G₁ phase of the cell cycle (Mukherjee *et al.*, 2016). *In vitro* activity of lupeol prompted us to further examine its antileishmanial efficacy in the *in vivo* model.

Immunotherapeutic potential of lupeol against VL was appraised in inbred BALB/c mice. Lupeol at 50 mg kg⁻¹ b.wt. was significantly ($P < 0.05$) effective in declining the splenic parasite load in infected animals as compared to the lower dose (25 mg kg⁻¹ b.wt.). Similarly, Das *et al.* (2017) also described the reduction of parasite load in the spleen of infected mice after treatment of lupeol at 75 mg kg⁻¹. In addition, our study also established that the efficacy of lupeol at 50 mg kg⁻¹ b.wt. is comparable to AmB in reducing the splenic parasite burden on 14 p.t.d. Similarly, enhancement in the DTH response in mice was found to be significant ($P < 0.05$) at a higher dose in comparison to the lower dose of lupeol. Therefore, lupeol restored the protective immune responses and showed a

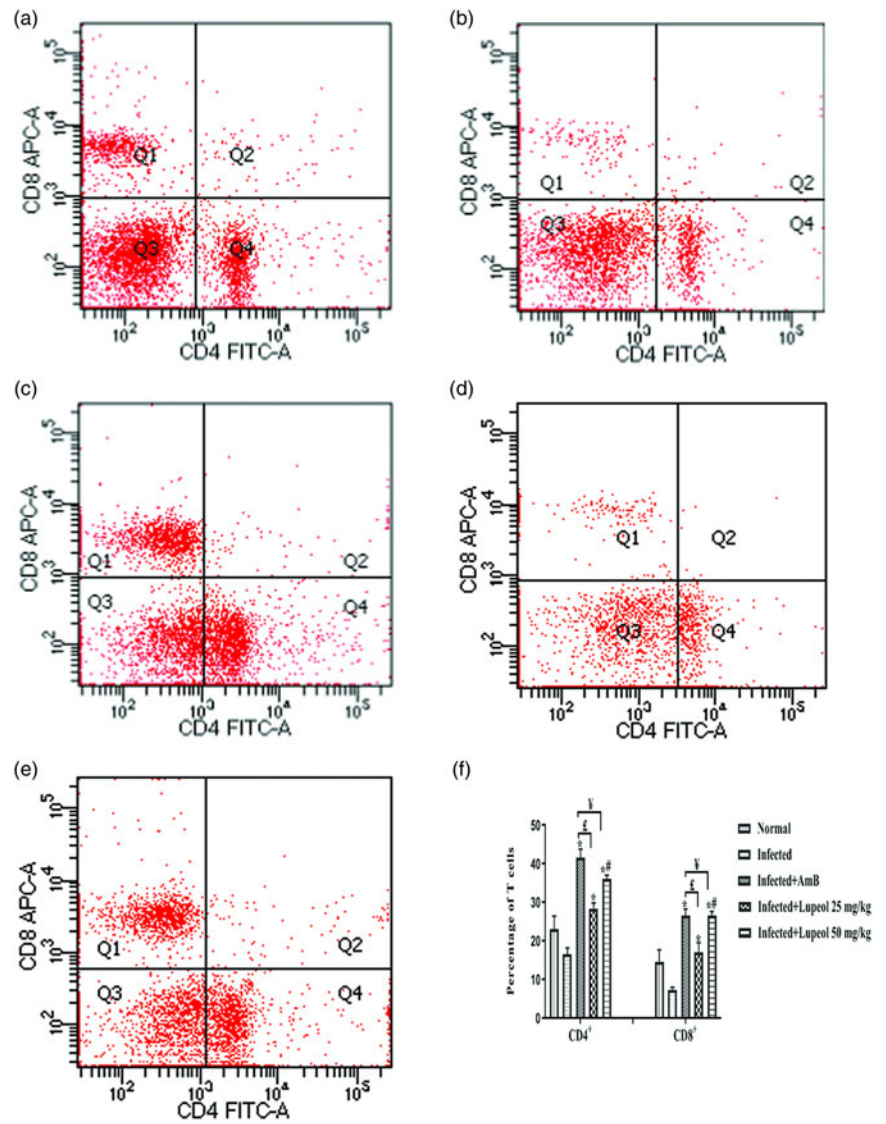


Fig. 5. Quantification of CD4+ and CD8+ T cells in different groups of animals by flow cytometry. Splenocytes were labelled with anti-CD4+ FITC and anti-CD8+ APC antibodies as explained in methodology. Dot plots represent the percentage of CD4+ and CD8+ T cells in different groups of animals. (a) Uninfected mice. (b) Infected mice. (c) Infected + AmB treated mice. (d) Infected + Lupeol 25 mg kg⁻¹ treated mice. (e) Infected + Lupeol 50 mg kg⁻¹ treated mice. (f) Bar graph is showing the mean percentage population of CD4+ and CD8+ T cells in different groups. Results are representing the mean ± s.e. and significance was analysed by ANOVA with Tukey's test. **P* < 0.05: Infected control vs Infected + AmB/Infected + Lupeol 25 mg kg⁻¹/Infected + Lupeol 50 mg kg⁻¹ b.wt.; #*P* < 0.05: Infected + Lupeol 25 mg kg⁻¹ vs Infected + Lupeol 50 mg kg⁻¹ b.wt.; †*P* < 0.05: Infected + AmB vs Infected + Lupeol 25 mg kg⁻¹; ‡*P* > 0.05: Infected + AmB vs Infected + Lupeol 50 mg kg⁻¹.

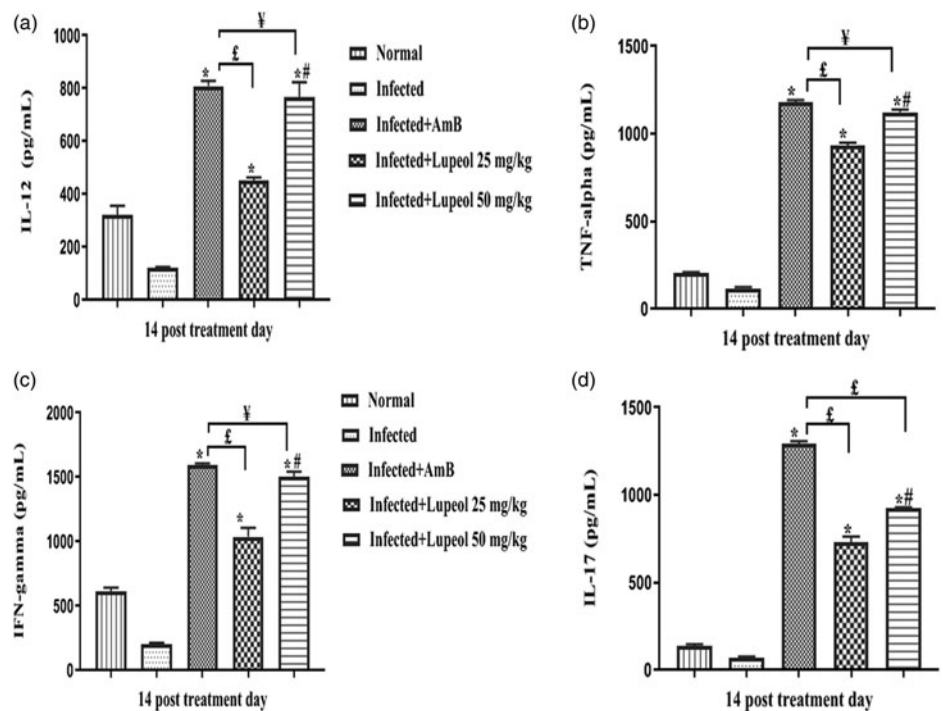


Fig. 6. Determination of Th1 cytokine levels in serum of various groups of animals by sandwich ELISA assay (Diaclone kit). (a) IL-12, (b) TNF- α , (c) IFN- γ and (d) IL-17. Data are expressed as mean ± s.e. and a significant difference was examined by one-way ANOVA using Tukey's test. **P* < 0.05: Infected control vs Infected + AmB/Infected + Lupeol 25 mg kg⁻¹/Infected + Lupeol 50 mg kg⁻¹ b.wt.; #*P* < 0.05: Infected + Lupeol 25 mg kg⁻¹ vs Infected + Lupeol 50 mg kg⁻¹ b.wt.; †*P* < 0.05: Infected + AmB vs Infected + Lupeol 25 mg kg⁻¹ and 50 mg kg⁻¹; ‡*P* > 0.05: Infected + AmB vs Infected + Lupeol 50 mg kg⁻¹.

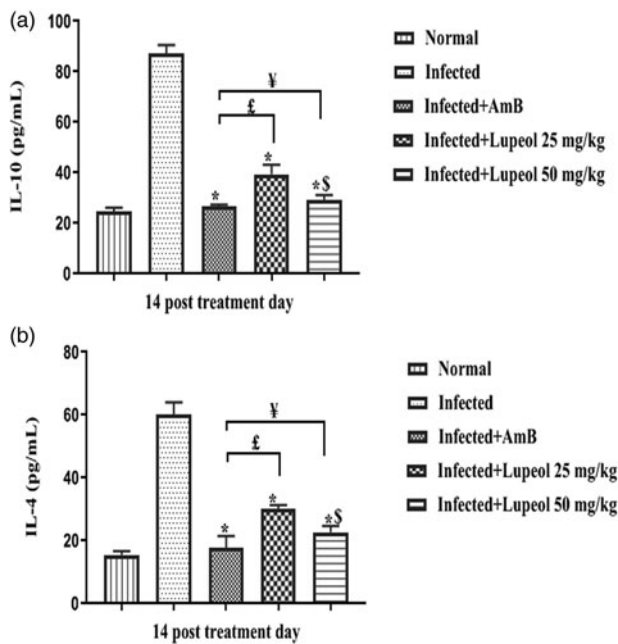


Fig. 7. Determination of Th2 cytokine levels in serum of various groups of animals. (a) IL-10 and (b) IL-4. Data are expressed as mean \pm s.e. and a significant difference was examined by one-way ANOVA using Tukey's test. * $P < 0.05$: Infected control vs Infected + AmB/Infected + Lupeol 25 mg kg^{-1} /Infected + Lupeol 50 mg kg^{-1} b.wt.; $\$P > 0.05$: Infected + Lupeol 25 mg kg^{-1} vs Infected + Lupeol 50 mg kg^{-1} b.wt.; $\text{\pounds}P < 0.05$: Infected + AmB vs Infected + Lupeol 25 mg kg^{-1} ; $\text{\textyen}P > 0.05$: Infected + AmB vs Infected + Lupeol 50 mg kg^{-1} .

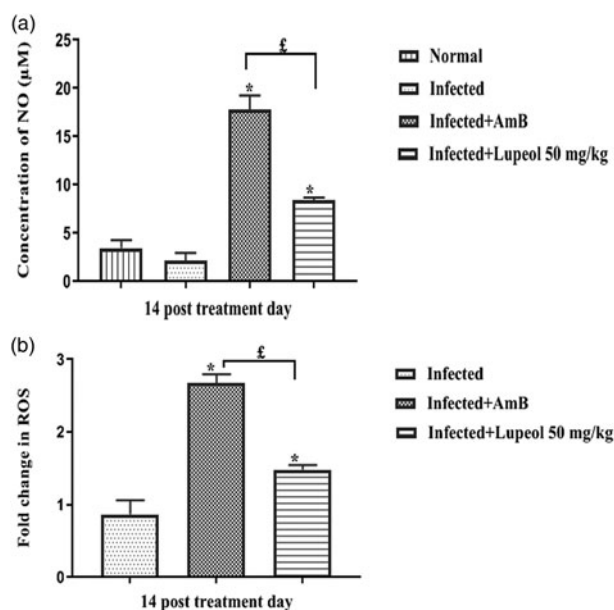


Fig. 8. Detection of NO (a) and ROS (b) production in splenocytes of various groups of animals by staining with Griess reagent and H2DCFDA dye. ROS was calculated in terms of fold change between uninfected control and treated groups. Data are presented as mean \pm s.e. and a significant difference was checked by ANOVA using Tukey's test. * $P < 0.05$: Infected control vs Infected + AmB/Infected + Lupeol 50 mg kg^{-1} b.wt.; $\text{\pounds}P < 0.05$: Infected + AmB vs Infected + Lupeol 50 mg kg^{-1} .

remarkable reduction in parasite burden. Similar observation has been noticed in oleuropein-treated infected mice (Kyriazis *et al.*, 2016) where a decrease in the parasite load was accompanied by the increase in the DTH reaction.

During VL, the functioning of CD4+ and CD8+ T cells gets impaired (Tsagozis *et al.*, 2003). Similarly in the current study too, the percentage of these cells was found to be suppressed in

Table 1. Determination of serum hepatic enzymes in various groups of animals

Groups	SGOT (U/L)	SGPT (U/L)	LDH (IU/L)
Normal	28.29 \pm 2.04	28.68 \pm 1.71	180 \pm 1.3
Infected	73.47 \pm 3.35	71.03 \pm 2.22	263 \pm 3.44
Infected + AmB	29.07 \pm 1.03	28.29 \pm 0.68	233.3 \pm 1.98
Infected + Lupeol 25 mg kg^{-1} b.wt.	25.93 \pm 1.36 ^b	23.57 \pm 0.68 ^b	156 \pm 1.99 ^a
Infected + Lupeol 50 mg kg^{-1} b.wt.	27.11 \pm 1.80 ^b	27.50 \pm 1.04 ^b	177.1 \pm 3.27 ^a

Data were represented by mean \pm s.e. of hepatic enzymes which were computed by using commercial kits and a significant difference was measured by one-way ANOVA.

Normal range: SGOT: 10–35 U/L, SGPT: 0–38 U/L and LDH: 114–240 IU/L.

^a $P < 0.05$: Infected + AmB vs Infected + Lupeol 25 and 50 mg kg^{-1} .

^b $P > 0.05$: Infected + AmB vs Infected + Lupeol 25 and 50 mg kg^{-1} .

the untreated infected controls. CD8+ and CD4+ T cells have a crucial role in the effective control of leishmanial infection as these cells produce proinflammatory cytokines such as IFN- γ and TNF- α which activate the macrophages to release the oxidative radicals like NO to kill intracellular parasites. CD8+ T cells also possess cytotoxic potential against cells expressing *Leishmania* antigens (Tsagozis *et al.*, 2003). In the present study, the percentage of CD4+ and CD8+ T cells was increased in the infected mice upon treatment with lupeol in contrast to only infected controls. The increment in the percentage of CD8+ and CD4+ T cells was more pronounced with a higher dose of lupeol than at the lower dose. Moreover, lupeol (50 mg kg^{-1} b.wt.) treatment was as effective as AmB in increasing the percentage of both T cells in infected mice. This immunopotentiating effect has also been seen in the treatment of astrakurkurone to BALB/c mice infected with *L. donovani* (Mallick *et al.*, 2016).

Switching of the immune response to Th1 or Th2 determines vulnerability or resistance towards *Leishmania* infections in mice (Oliveira *et al.*, 2014). Similarly in our study also, Th2 cytokines were found to be increased and the concentration of Th1 cytokines was observed to be suppressed in *L. donovani*-infected animals. Activation of the protective Th1 immune response leads to the secretion of many cytokines, especially IFN- γ and TNF- α . These cytokines further activate macrophages to enhance iNOS activity that in turn boosts up the generation of NO for the killing of the parasite (Salim *et al.*, 2016). In the present investigation, the lupeol's ability to differentiate CD4+ T cells to protective Th1 immune response was analysed by investigating the levels of Th1 and Th2 cytokines. Lupeol treatment developed a protective immune response through the induction of Th1 cytokines and reduction of Th2 cytokines in serum. The elevation in the levels of Th1 cytokines after lupeol treatment at 50 mg kg^{-1} b.wt. was comparatively higher than that observed at 25 mg kg^{-1} b.wt. These findings were consistent with previous studies which demonstrated that lupeol enhanced the production of Th1 cytokines and suppressed the Th2 cytokines (Wu *et al.*, 2013; Das *et al.*, 2017). Moreover, our study also showed that lupeol at higher dose and AmB had equal potential in augmenting the Th1 cytokines and repression of Th2 cytokines in the infected mice. On the other hand, the study of Sanchez-Burgos *et al.* (2015) is in contrary to our findings as this study has reported that lupeol could suppress the proinflammatory cytokines.

In view of the immunotherapeutic potential of lupeol against murine VL, toxicity studies were carried out by biochemical tests and histological studies. Lupeol-treated animals showed normal levels of liver enzymes and kidney function markers. Histological examination further verified that the lupeol treatment did not cause any injuries to the liver and kidneys. Conversely, AmB-treated infected mice resulted in the enhancement in the

Table 2. Determination of serum renal function markers in various groups of animals

Groups	Urea (mg dL ⁻¹)	Creatinine (mg dL ⁻¹)	BUN (mg dL ⁻¹)	Uric Acid (mg dL ⁻¹)
Normal	39.36 ± 2.4	1.29 ± 0.04	18.38 ± 1.09	4.6 ± 0.23
Infected	72.95 ± 1.5	1.71 ± 0.08	34.07 ± 0.71	8.6 ± 0.25
Infected + AmB	50.13 ± 2.9	1.42 ± 0.09	23.41 ± 1.40	7.5 ± 0.43
Infected + Lupeol 25 mg kg ⁻¹ b.wt.	32.54 ± 1.3 ^a	0.82 ± 0.09 ^a	15.20 ± 0.61 ^a	4.1 ± 0.13 ^a
Infected + Lupeol 50 mg kg ⁻¹ b.wt.	34.23 ± 1.8 ^a	1.09 ± 0.06 ^a	15.99 ± 0.85 ^a	3.7 ± 0.14 ^a

Renal function markers were represented by mean ± s.e. and measured by using commercial kits.

Normal range: urea: 10–45 mg dL⁻¹, creatinine: 0–1.4 mg dL⁻¹, BUN: 5–21 mg dL⁻¹ and uric acid: 2.6–7.0 mg dL⁻¹.

The significant difference was checked by one-way ANOVA.

^aP < 0.05: Infected + AmB vs Infected + Lupeol 25 and 50 mg kg⁻¹.

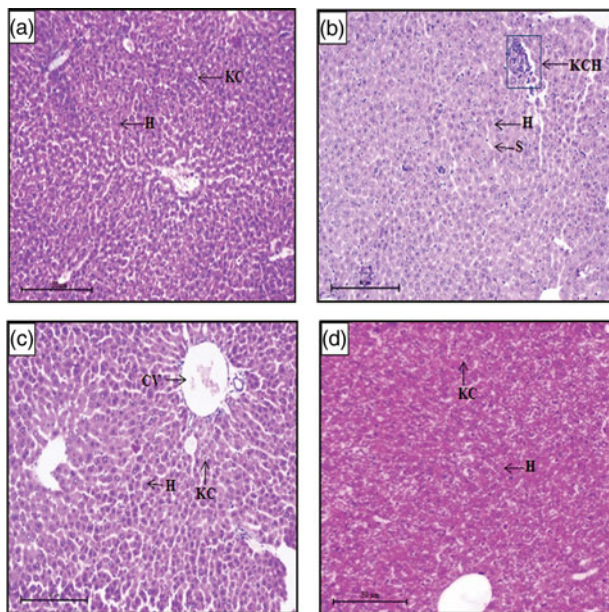


Fig. 9. Histological studies of the liver of various groups of animals. (a) Uninfected mice. (b) Infected mice. (c) Infected + AmB treated mice. (d) Infected + Lupeol treated mice. The histological pattern of the liver was normal in uninfected, AmB and lupeol treated mice (a, c and d). Hyperplasia of Kupffer cells was found in infected mice (b) (H&E staining, 100X, scale bar: 50 μm). Abbreviations: S, sinusoids; H, hepatocytes; KC, Kupffer cell; CV, central vein; KCH, Kupffer cell hyperplasia.

concentration of uric acid, creatinine, urea and BUN. In addition, AmB-treated infected mice also displayed protein casts and mild tubular disruption in kidneys. The nephrotoxic action of AmB has also been described in earlier studies (Jesus *et al.*, 2017). Thus lupeol can be considered as a safe candidate for the treatment of VL.

NFκB plays a crucial role in the host defence to leishmanial infection and is essential for the proliferation of CD4⁺ T-cells and the generation of Th1 immune responses (Artis *et al.*, 2003). The current investigation has demonstrated that the lupeol modulates the iNOS and NFκB expression at the transcriptional level in the spleen. However, downregulation of the expression of these genes was observed in the untreated infected animals. The capacity of lupeol in increasing the expression of NFκB gene might be allied to its ability to augment the levels of T cells (CD4⁺) and proinflammatory cytokines. Furthermore, the enhanced iNOS expression could be attributed to the increased expression of NFκB gene due to lupeol treatment. It demonstrated the effect of lupeol at the transcriptional level. Similar induction of iNOS expression due to increment in the expression of NFκB after the treatment of cystatin was reported

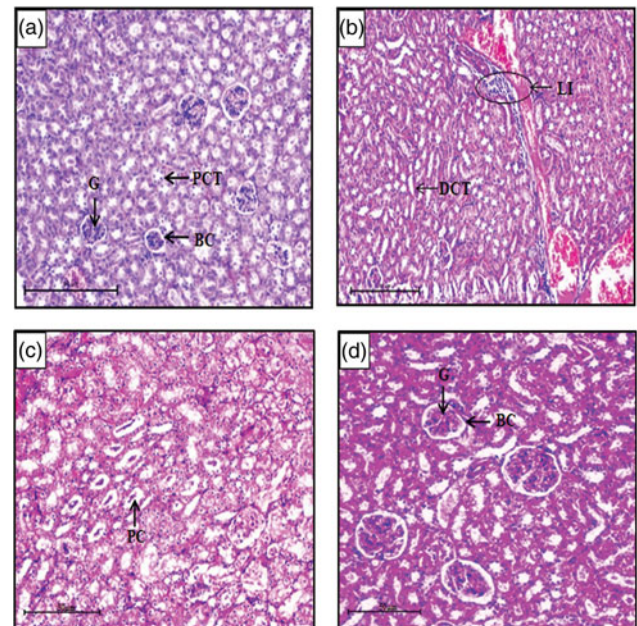


Fig. 10. Transverse sections of the kidney of different groups of animals. (a) Uninfected mice. (b) Infected mice. (c) Infected + AmB treated mice. (d) Infected + Lupeol treated mice. The renal morphology was found to be normal in lupeol-treated mice (d). Lymphocytic infiltration and some protein casts with mild damage to tubules were observed in infected mice (b) and in AmB-treated mice (c), respectively (H&E staining, 100X, scale bar: 50 μm). Abbreviations: BC, Bowman's capsule; G, glomerulus; PCT, proximal convoluted tubule; DCT, distal convoluted tubule; circle, lymphocytic infiltration; PC, protein cast.

(Kar *et al.*, 2011). In the present study, the enhancement in the proinflammatory cytokines due to lupeol treatment might have led to the activation of iNOS. It is corroborated with the investigation of Horta *et al.* (2012) which revealed that TNF-α and IFN-γ are directly involved in the induction of iNOS expression which further leads to the production of NO for the killing of the parasite. Moreover in the present study, NO was also raised in the splenocytes of infected animals treated with lupeol which is correlated with the reduction in parasite load. Likewise, Das *et al.* (2017) also demonstrated that lupeol treatment upregulated the expression of iNOS and consequently NO production in mice infected with *L. donovani*. The enhancement in the levels of NO might also be due to the activation of iNOS upon the treatment of infected mice with lupeol. In addition, lupeol treatment also resulted in ROS production which could be correlated with the reduction of parasite burden in infected mice. Similar findings were found in many studies in which the leishmanicidal activity of natural products was due to the augmentation of ROS and NO production (Rodrigues *et al.*, 2014).

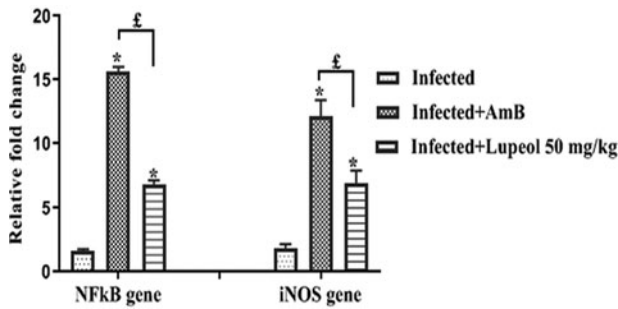


Fig. 11. Quantification of NFκB and iNOS in splenocytes of various groups of animals by reverse transcriptase qPCR. The relative fold change in mRNA expression of iNOS and NFκB was calculated relative to the β-actin (housekeeping gene). One-way ANOVA test was used to check the significant difference. * $P < 0.05$: Infected control vs Infected + AmB/Infected + Lupeol 50 mg kg⁻¹ b.wt.; £ $P < 0.05$: Infected + AmB vs Infected + Lupeol 50 mg kg⁻¹.

Currently, the usage of phytomedicines has been enhanced due to less toxic effects and therapeutic effects in comparison to the allopathic medicines. However, plant compounds reveal limited pharmacological activity in *in vivo* models because of their less solubility and lipophilicity. This leads to meager absorption and thus an insufficient distribution of the drug in tissues. In the present day, new drug delivery carriers are being developed which can enhance the bioavailability of phytoconstituents. Various drug delivery systems such as liposomes, phytosomes and nanoparticles are known to increase the distribution of herbal components (Kesarwani and Gupta, 2013). The combination of plant components in delivery systems helps in improving the stability and bioavailability. It also decreases degradation in metabolic processes which leads to more biological activity (Karimi *et al.*, 2015). Therefore in the present study, the absence of a delivery system to enhance the effect of lupeol for treating leishmaniasis is a limiting factor.

In conclusion, our data indicated that lupeol treatment efficiently impeded the growth of the parasite in infected mice which was comparable to a conventional drug, AmB. Moreover, treatment of lupeol in infected mice did not exhibit any side-effects in human cells and in infected mice. Leishmanicidal effect of lupeol was associated with the induction of iNOS-mediated NO production. The induction of iNOS might be attributed to the increased expression of NFκB due to lupeol treatment. NFκB also caused the activation of CD4⁺ T cells and subsequently proinflammatory cytokines as a result of lupeol therapy which ultimately induced the NO and ROS production. Therefore lupeol boosted the immunity of host by the production of immunostimulatory molecules (Th1 cytokines) and leishmanicidal molecules (NO, ROS) for controlling the infection. So lupeol can serve as an active and safer agent against VL. Further studies of antileishmanial potential of lupeol should be carried in higher test models.

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Conflict of interest. None.

Ethical standards. Susceptible BALB/c mice of both sexes (inbred, weight 20–25 g), 6–8 weeks age were taken in the current study. The study was approved by the Institutional Animal Ethics Committee of the Panjab University, Chandigarh (PU/IAEC/S/14/139) which has the certification from the Committee for the purpose of control and supervision of experiments on animals (CPCSEA). Mice were provided with feed and water *ad libitum* and all assays were performed as per the accepted instructions of European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific purposes.

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