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Cedrus deodara: *In vitro* antileishmanial efficacy & immumomodulatory activity

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***Background & objectives*: The existing antileishmanial drugs for complete cure of visceral leishmaniasis (kala-azar) are limited. The available drugs are either toxic or less effective leading to disease relapse or conversion to post-kala-azar dermal leishmaniasis. Several herbal extracts have been shown to have antileishmanial activity, but a herbal drug may not always be safe. In the present study, the extract of *Cedrus deodara* leaves has been standardized and tested for immunomodulatory antileishmanial activities.**

***Methods*: The extracts of *C. deodara* leaves with different solvents such as benzene, chloroform, ethyl acetate and methanol were made by soxhlation process. Solvents were removed under reduced pressure and temperature using rotary evaporator. The antileishmanial bioassay test was performed with *in vitro* maintained parasites. Immunomodulatory activity of different extracts was tested by flow cytometry. Standardization of the effective fraction was performed with Linalool as a marker compound through reverse-phase high-performance liquid chromatography.**

***Results*: The extract with the use of benzene solvent showed strong antileishmanial activities within a dose 25-200 µg/ml culture with non-significant haemolytic activities and significant immunomodulant activities against the host cells. Linalool was found to be 1.29 per cent in the effective extract of *C. deodara*.**

***Interpretation & conclusions*: The antileishmanial activity of *C. deodara*, as assessed by bioassay testing on *Leishmania donovani* parasites and immunomodulatory effect of benzene extract of leaves on host cells indicated that it might be a potential new safe therapeutic target to cure the visceral leishmaniasis.**

Key words Arginase - *Cedrus deodara* - linalool - reverse-phase high-performance liquid chromatography - visceral leishmaniasis

The vector-borne disease - leishmaniasis are of cutaneous, mucocutaneous and visceral forms and caused by kinetoplastid protozoan parasite *Leishmania donovani*. Mainly, it is a disease of tropical regions such as Asia, Africa and some part of South America. The countries such as India, Bangladesh, Nepal and some

part of Bhutan are affected by the visceral leishmaniasis (VL) which is fatal if not treated. In 88 countries, about 350 million people are at risk of this disease and 12 million are infected cases, of which 90 per cent are from India, Bangladesh, Southern Sudan, Nepal and North East Brazil[1]. The cases of visceral leishmaniasis (kala azar) are decreasing in India[2], but the cases of post-kala-azar dermal leishmaniasis (PKDL) are increasing[3]. The effective drugs for the treatment of this disease are very limited and development of resistance to the existing drugs is a common feature. The parasite developed resistance towards the first line of drug sodium antimony gluconate even after increasing the doses and drug regimen between 1970s and 1990s[4]. Gradually, the drug became toxic to the patient. The next successful drug was amphotericin B but it was highly toxic to the patient and needed to be administered under a closed monitoring of a skilled practitioner. This drug was the last choice of treatment if the patient became non-responder or relapsed or converted into PKDL. At present, drugs such as miltefosine as an oral drug and single-dose therapy of AmBisome are in practice but sensitivity is low[5]. Due to the drug resistance and host toxicity of existing drugs, the attention has been focused towards the herbal drugs. Several investigations on the antiprotozoal activity of plants from West and Central Africa have been conducted. The anti-leishmanial and antimicrobial activities of Nigerian medicinal plants have been evaluated[6]; however, safe biologically active compounds for the treatment of protozoan disease have not been certainly identified. The triterpenoid saponin extract from Vietnamese plant *Maesa balansae* was the most promising lead for fulfilment of herbal antileishmanial drug[7], but it was also found highly toxic. In our previous study, we found an active antileishmanial activity of Indian *Agave americana* against *in vitro* culture of *Leishmania donovani*[8], but it also showed toxicity against the host cells. Herbal treatment did not mean always safe[9].

Cedrus deodara, also known as Himalayan cedar belongs to the family of Pinaceae and has been widely used in Indian system of medicine due to its nutritional and pharmaceutical effects. Pine needle of *C. deodara* is recognized as a healthy food material with abundant protein, vitamins and minerals[10]. It has been traditionally used for the treatment of tic, fever, cough, bronchitis, ulcer and tuberculosis. Major chemical constituents reported from the *C. deodara* include α-terpineol, linalool, limonene, anethole, caryophyllene, and eugenol, taxifolin, cedeodarin, wikstromal, deodarone, cedrinoside and flavonoids[11,12]. *C. deodara* has a different pharmacological activity such as anti-inflammatory, analgesic, anti-hyperglycaemic, antiulcer, antispasmodic, antibacterial, insecticidal, molluscicidal and anticancer activity[13].

We report here the *in vitro* antileishmanial efficacy and immunomodulatory activities of Indian *C. deodara* plant leaf extract.

Material & Methods

Plant collection and identification: Leaves of *C. deodara* were collected from Shimla, Himachal Pradesh, India, and authenticated by Dr. S. Rajan, field Botanist, Ooty, Tamil Nadu, Government of India. Voucher specimen (SNPS-JU/1068) was deposited at the School of Natural Product Studies, Jadavpur University, Kolkata, for the future reference[14]. Plant material was washed under current water, then Milli-Q water and dried under room temperature.

Extraction and fractionation of plant material: Dried plant leaves (400 g) were broken into small pieces and powdered. Successive extraction of plant materials was done by Soxhlet apparatus in different solvents such as benzene, chloroform, ethyl acetate and methanol[8]. Soxhlation process was run for 10-15 h for each solvent for effective and proper extraction. Solvents were removed under reduced pressure and temperature using rotary evaporator (EYELA, Japan) to get the concentrated extract. Further, plant extracts were lyophilized to get dried extract and obtained extracts were kept in desiccators for further use.

Bioassay testing of extracts against Leishmania donovani parasites

Procurement of *Leishmania* parasites: The cryopreserved reference strain (MHOM/N/1983/AG83) and freshly obtained isolates of *L. donovani* from VL patients who came to the Balaji Utthan Sansthan, Kala-azar Research Centre, Patna, India, in 2014 for treatment, were cultured and adjusted on a concentration of 1.9×10^6 cells/ml in Schneider's insect tissue culture medium (Sigma, USA) supplemented with 10 per cent heat-inactivated foetal bovine serum (FBS) and 100 U penicillin, 50 mg gentamycin and 100 mg streptomycin/l at 24°C±1°C. The maintained promastigotes were used for bioassay testing. The intracellular amastigotes were procured from established promastigotes infection in peritoneal macrophage cells of BALB/c mice, purchased

from Central Drug Research Institute, Lucknow and maintained at animal house facilities of Rajendra Memorial Research Institute of Medical Sciences (ICMR-RMRIMS), Patna. The inoculum (2×10^6 intracellular amastigotes/ml) was maintained for bioassay testing of *C. deodara* extracts.

The study was conducted after taking prior approval from human ethics committee of Balaji Utthan Sansthan and animal ethics committee of RMRIMS, Patna.

In vitro activity of *Cedrus deodara* extracts against promastigotes and intracellular amastigotes: The different fractions of the plant extracts such as benzenes, chloroform, ethyl acetate and methanol were diluted in dimethyl sulphoxide (DMSO) (1% w/v). The early stationary phase of *L. donovani* promastigotes (1.9×10^6/ml) was seeded into 24-well tissue culture plate (Nunc, Denmark) and different concentration of extracts (12.5, 25, 50, 100 and 200 µg/ml) as test, and amphotericin B as standard drug (1 µg/ml) for positive control were administered properly in each well of parasites. Parasites (1.9×10^6/ml) without drug and addition of equal amount of normal saline were used for negative and DMSO vehicle controls. The plates were incubated at 24°C±1°C for 24, 48 and 72 h. For each time scale, three plates were used and, on each time interval, one was observed. The number of experimental repeats was in triplicate of each group (n=3). The microscopic analysis using Neubauer chamber (Fein, Optic, JENA, Germany) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) assay were done in triplicate to get the standard deviation (SD)[15]. The *in vitro* screening as IC_{50} (50% inhibitory concentration) and IC_{90} value of different extracts was calculated[16,17]. Activities of *C. deodara* extracts against intracellular amastigotes were performed in the Lab-tek® Chamber Slide™ (Nunc, USA). The peritoneal exudates of inbred BALB/c mice-derived macrophages were used for establishment of intracellular amastigotes infection in the ratio of (1:10) macrophage and infective stage of promastigotes. About 90 per cent macrophages became infected on completion of 4 h incubation at 36°C ±1°C with 5 per cent CO_2 and 95 per cent humidity. The free promastigotes were removed by three gentle washes with incomplete medium and finally suspended the macrophages with complete RPMI 1640 tissue culture medium supplemented with 10 per cent FBS. The intracellular amastigotes were adjusted to 2×10^6 parasites/ml[18] and seeded in test, positive, negative and DMSO vehicle control wells. The different concentrations (12.5, 25, 50, 100 and 200 µg/ml) of test extracts of *C. deodara* were added in each test well. The re-incubation was done at 36°C±1°C in 5 per cent CO_2 incubator with 95 per cent humidity for 24, 48 and 72 h. LabTek Chamber Slides were fixed in absolute methanol stained with Giemsa and examined the parasites load and macrophage morphology. The cultures were examined for IC_{50} and IC_{90} for each extract concentration.

MTT assay for extract treated promastigotes: The parasites viability was checked by MTT assay. For this, 100 µl of MTT solution was added to the extract-treated parasites at the concentration for IC_{50} and incubated at 24°C±1°C temperature for 2 h and absorbance was measured at 570 nm wavelengths in spectrophotometer[19].

Effect on mammalian mononuclear cells after extract treatment

Cytotoxic effects: The mononuclear cells were collected from a healthy person after getting the informed written consent from individual and ethical clearance by the Institutional Ethical Committee. The cells were treated with IC_{50} concentration of effective benzene fraction of *C. deodara* and the level of cleaved tetrazolium ring which forms formazan crystal was observed at the wavelength of 570 nm using spectrophotometer. The treated mammalian cells with various fractions of *C. deodara* extracts were subsequently supplemented with 100 µl of MTT solution and incubated for 2 h in a CO_2 incubator at 36°C±1°C and 95 per cent humidity[15].

Immunomodulatory effects: Nitric oxide (NO) produced by mammalian mononuclear cells was assessed using Griess reagent in the culture supernatant of macrophages after incubation with effective concentration of *C. deodara* extract and lipopolysaccharide (LPS) (10 mg/ml, Sigma, USA) as mitogen. For this, the peripheral blood mononuclear cells (PBMCs) were suspended in RPMI 1640 medium supplemented with 10 per cent FBS and plated at 1×10^6 cells/well. PBMCs were challenged with different concentration of extracts and incubated for 48 h at 36°C±1°C in CO_2 incubator with 95 per cent humidity. Then, equal volume of Griess reagents (Sigma, USA) was mixed to each well. The supernatant (100 µl) was collected from each well and left for 10 min at room temperature. The absorbance was measured at 540 nm in spectrophotometer.

Regarding the cytokines production, the human mononuclear cells were categorized as extract

unstimulated, stimulated with LPS and extract stimulated. The 1×10^6 mononuclear cells were taken for experimentation and stimulated with extract in the concentration of IC_{50} dose/ml of effective extract. The interferon-gamma (IFN-γ) monoclonal antibodies (BD, USA) were used for the detection of $CD4^+$ cells of IFN-γ production by flow cytometry (FACSCalibur, BD, USA). The study of interleukin-10 (IL-10) production was also done by the same process in the presence of IL-10 monoclonal antibodies (BD). Experiments were performed in triplicate[20].

Haemolytic effect: For assessment of haemolytic activity of *C. deodara* extract, blood sample (2 ml) of a healthy volunteer was collected. One ml of 2 per cent blood suspension was added with the tested concentration of antileishmanial *C. deodara* extract as experiment or saline as control[21]. The per cent haemolysis noted in saline control was subtracted from all groups. The 50 per cent of the maximum haemolysis was considered as HD_{50}. Experiments were performed in triplicate at each concentration[22]. The intact red blood cells (RBCs) were estimated by counting chamber.

Standardization of plant extracts: Effective fraction (benzene) of the *C. deodara* extract was standardized through reverse-phase high-performance liquid chromatography (RP-HPLC) at School of Natural Product Studies, Department of Pharmaceutical Technology, Jadavpur University, Kolkata. Lyophilized extract was accurately weighted, and volume of the solution was adjusted in a volumetric flask to obtain a final concentration of 10 mg/ml. Linalool (Sigma-Aldrich, UK) was used as a standard marker for the standardization of plant extract. Stock solution was prepared by dissolving 10 mg linalool in 10 ml solvent to get a concentration of 1 mg/ml. The optimized mobile phase was composed of methanol: Milli-Q water with 1 per cent acetic acid in the ratio of 80:20 (v/v) with a flow rate of 1 ml/min. The HPLC system comprised rheodyne-7725 injection valve with a sample loop (20 ml), Vacuum Degasser, quaternary pump and photo-diode array detector was used, with data acquisition by Empower™ 2 software (Waters 600, Milford, MA, USA). Chromatography was performed on a Spherisorb C18 column (250 mm×4.6 mm, 5 mm; Waters, Ireland) fitted with a C18 guard column (10×3.0 mm). The sample elution was performed at 25°C and detected at the UV wavelength of 254 nm. Sample was analyzed by HPLC using 20 µl injection volume of a syringe (Hamilton Microliters; Switzerland). The *p*H meter (Orion 3 Star, Thermo-Scientific, USA) was used to adjust the *p*H of the mobile phase. Amount of the biomarker present in the sample was determined through the calibration curve. Calibration curve constructed by corresponding peak area of the standard was plotted against the concentration of standards by means of linear regression.

Statistical analysis: The data were analyzed by one-way analysis of variance (ANOVA) and repeated-measure ANOVA and appropriate post-tests (Tukey, Newman-Keuls) were carried out using GraphPad Prism 5.0 software (GraphPad, USA).

Results

Bioassay evaluation

Determination of effective extracts of *C. deodara* and their IC_{50} and IC_{90} values against promastigotes and intracellular amastigotes: All the four fractions of the *C. deodara* extract were screened for *in vitro* antileishmanial activities along with controls. Benzene fraction of *C. deodara* extract showed calculated 50 per cent antileishmanial activities (IC_{50}) with the concentration of 25 µg/ml (Fig. 1A) and almost 100 per cent antileishmanial activity (IC_{90}) with the concentration of 200 µg/ml for promastigotes (Fig. 1A). The calculated 50 per cent elimination of intracellular amastigotes was found with 25 µg/ml and almost complete elimination (IC_{90}) with 100 µg/ml only (Fig. 1B). The complete elimination of parasites was not achieved by chloroform, ethyl acetate and methanol fraction of *C. deodara* (Fig. 2).

Confirmation of antileishmanial activity of *C. deodara* extract by MTT assay on promastigotes: On IC_{50} concentration of benzene extract of *C. deodara*, only the 50 per cent parasites showed insoluble purple formazan which indicated the process of mitochondrial dehydrogenase in live promastigotes compared ($P<0.001$) to untreated parasites (Fig. 3).

Immunomodulatary evaluation

Nitric oxide (NO) production: In human PBMCs after treating with IC_{50} concentration of benzene extract of *C. deodara,* the NO production was quantified using Griess reagent. The NO production in human PBMCs by stimulation with LPS (mitogen) served as positive and unstimulated cells as negative control. In comparison to unstimulated cells, the production of NO was 1.3-fold ($P<0.001$) more with effective extract; however, it was less than LPS-stimulated cells (Fig. 4).

Cytokines production: Regarding the protective cytokine (IFN-γ) production on stimulation of $CD4^+$ cells with effective extract, the IFN-γ-producing

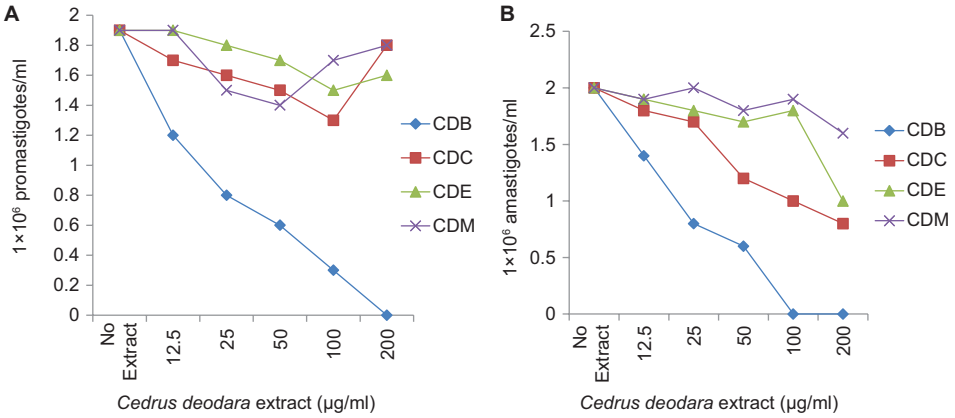

Fig. 1. Benzene fraction of *Cedrus deodara* extract (CDB) showing antileishmanial activities. (**A**) *Leishmania donovani* promastigotes of late log phase challenged with different concentration of extract showed IC_{50} and IC_{90} concentrations of 25 and 200 µg/ml, respectively. (**B**) IC_{50} and IC_{90} concentrations of *Leishmania donovani* intracellular amastigotes were found at 25 and 100 µg/ml, respectively. CDB, *Cedrus deodara* benzene; CDC, *Cedrus deodara* chloroform; CDE, *Cedrus deodara* ethanol; CDM, *Cedrus deodara* methanol.

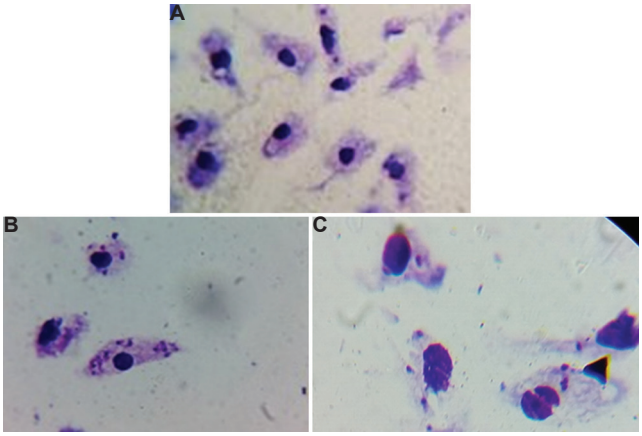

Fig. 2. Intracellular parasite burden in the absence or presence of benzene extract of IC_{50} concentration. (**A**) Macrophages (×100) without the infection treated as control, (**B**) macrophages (×100) infected with $2\times10^3 \pm 85/100$ cells, (**C**) infected macrophages (×100) treated with benzene extract to inhibit parasitic load $2\times10^2 \pm 25/100$ cells.

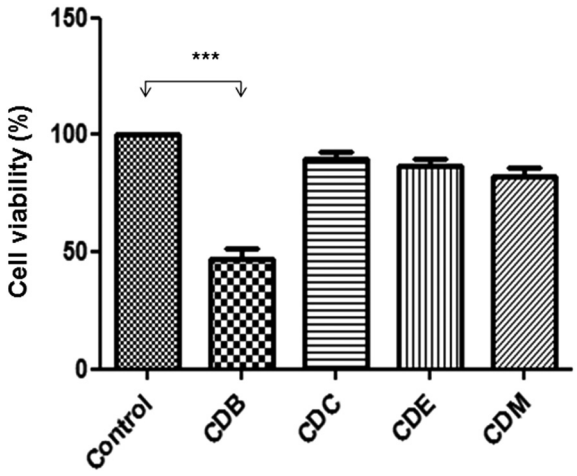

Fig. 3. MTT assay to confirm antileishmanial activity of benzene extract of *Cedrus deodara*. Parasites treated with IC_{50} concentration of extract. Only the 50 per cent parasites showed insoluble purple ($P<0.001$), indicating decreased dehydrogenase activities. CDB, *Cedrus deodara* benzene; CDC, *Cedrus deodara* chloroform; CDE, *Cedrus deodara* ethanol; CDM, *Cedrus deodara* methanol.

$CD4^+$ cells were 2-fold higher ($P<0.05$) than unstimulated and 1.6-fold higher ($P<0.05$) than LPS. The disease promoting cytokine (IL-10) within the $CD4^+$ cells after treating with effective concentration was non-significantly less than unstimulated cells and significantly less ($P<0.05$) than LPS (Fig. 5A and B).

Cytotoxicity evaluation: Cytotoxicity of effective *C. deodara* extract was assessed on human PBMCs by MTT assay. The absorbance values of test samples were almost similar to the negative control after exposure of effective extract of *C. deodara* to the PBMCs for 24 h. It indicated that the effective concentration of extract did not inhibit mitochondrial dehydrogenase (Fig. 6).

Haemolytic activity: The haemolytic activities of effective concentration of *C. deodara* extract (benzene extract, CDB) were observed on 2 per cent healthy human blood after incubation at 36°C±1°C overnight. The final concentration of RBCs number was found 5-10 per cent less than the healthy control cells (4.3×10^6/µl) (Fig. 7).

Extraction and fractionation of plant material: Plant extract was prepared in different solvents in the order of their polarity index. The percentage yields the *C. deodara* in different fractions such as benzene fraction (CDB), chloroform fraction (CDC), ethyl acetate fraction (CDE) and methanol fraction (CDM) were found to be 11.79, 2.64, 2.31 and 14.00 per cent w/w, respectively. Extract regimen was fixed 12.5-200 µg/ml culture for all experiments.

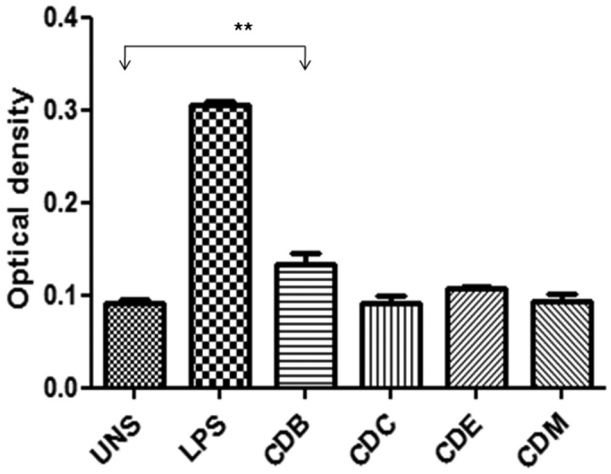

Fig. 4. Nitric oxide (NO) production in human cells after treating with *Cedrus deodara* extracts. In comparison to unstimulated cells (UNS) the nitric oxide production was 1.3-fold more with effective (benzene) extract (P<0.01); however, it was less than lipopolysaccharide (LPS) stimulated cells (P<0.001).

Standardization of *Cedrus deodara* extract: Effective fraction of *C. deodara* extract was standardized by RP-HPLC under the isocratic conditions using the external standard calibration technique. Marker compound was identified by comparing with the respective retention time (Rt) of the linalool as standard. Calibration curve was plotted by plotting peak areas against concentrations and five standard marker ranges from 100 to 500 µg/ml. Standard compound showed a good linearity between concentrations and the peak area, with the correlation coefficient (r^2) of 0.997. Obtained chromatograms from RP-HPLC analyses of the plant extracts and marker are shown in Fig. 8. The optimum separation was achieved using the mobile system at the volume ratio of methanol and Milli-Q water in the ratio of 80:20 (v/v) with a flow rate of 1 ml/min. The percentage amount of standard constituent (linalool) present in benzene fractions of plant extracts was found to be 1.29 per cent. Rt of linalool was found to be 5.32 min.

Discussion

Herbal medicines are extensively used in the developing nation due to wide availability and affordability[6]. Several extracts and their active components have been screened for antileishmanial activity[7,23,24]. We have earlier observed significant antileishmanial activities of Indian grown *A. americana* but found significant cytotoxic and haemolytic activities against the human cells[8]. Thus, several plants though show antileishmanial activities but are not safe for human. Benzene fraction of *C. deodara* leaf extract was found effective at the concentration of 25 µg/ml for IC_{50} and 200 ug/ml for IC_{90} against the promastigotes and 25 µg/ml for IC_{50} and 100 µg/ml for IC_{90} against the intracellular amastigotes with high production of NO in the macrophage cells whereas chloroform, ethyl acetate and methanol extracts were inefficient to completely inhibit the parasites. High inhibition of parasites was observed by MTT assay similar to other study[25]. The cytotoxic effect was not significant as also reported by others[26]. The protective cytokine (IFN-γ) production by $CD4^+$ cells was about 2-fold higher in stimulated than the unstimulated cells and the disease-promoting cytokine (IL-10) was insignificantly downregulated than the unstimulated cells. The upregulated IFN-γ production is the sole cause of more NO production in the presence of TNF-α. Apart of this, in *C. deodara* leaf, significant amount of terpenoids and flavonoids (stigmasterol, oleanolic acid, linalool, syringaresinol, daucosterol, benzoic acid, gallicin and gallic acid, beta-sitosterol, shikimic acid, methylconiferin and ferulic acid) have

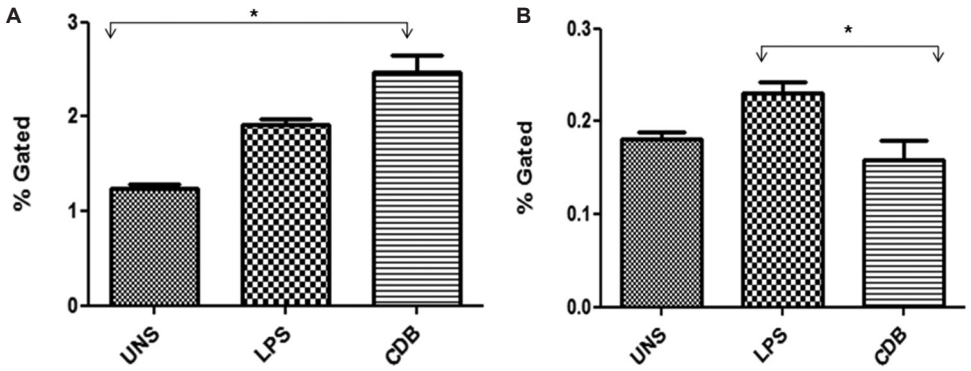

Fig. 5. Percentage of T helper cells producing (**A**) interferon-gamma (IFN-γ) and (**B**) interleukin-10 (IL-10) after PBMCs were stimulated with effective concentration of *Cedrus deodara* extracts and compared with unstimulated control and LPS stimulated positive control. The IFN-γ producing $CD4^+$ cells were 2-fold higher (P<0.05) than unstimulated and 1.6-fold higher (P<0.05) than LPS. IL-10 production within the $CD4^+$ cells was less (not significant) than unstimulated cells and significantly less (P<0.05) than lipopolysaccharide. CDB, *Cedrus deodara* benzene; LPS, lipopolysachride; UNS, unstimulated.

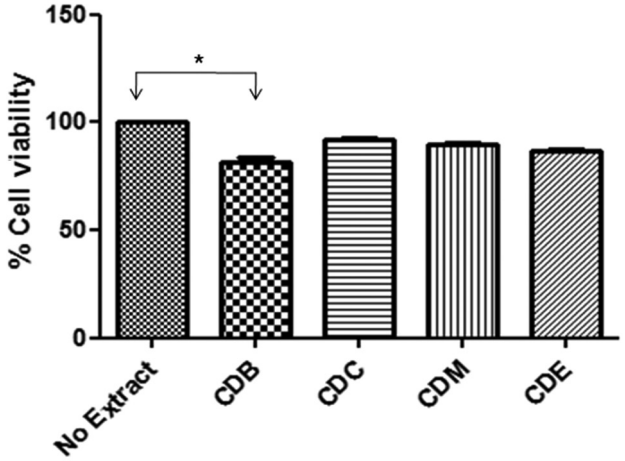

Fig. 6. MTT assay showing no effect on dehydrogenase activity in different extracts treated host cells compared to control. The absorbance values of test samples after exposure of effective extract of *Cedrus deodara* to the peripheral blood mononuclear cells for 24 h were almost similar to the negative control which indicated the effective concentration of extract did not inhibit mitochondrial dehydrogenase (P<0.01).

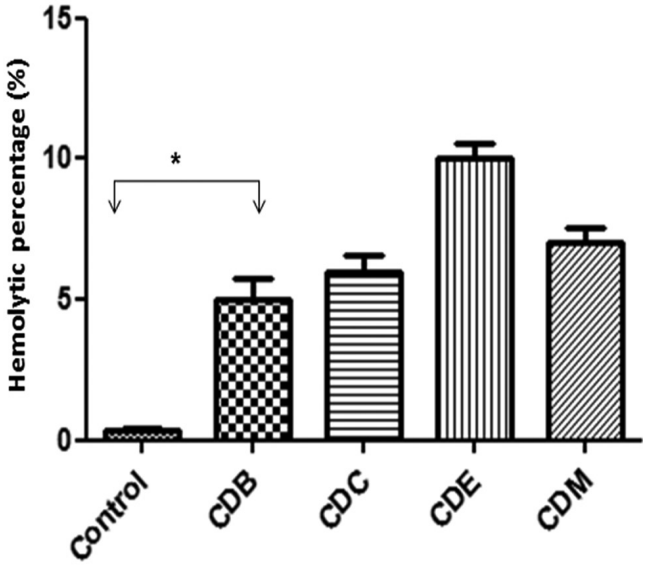

Fig. 7. Hemolytic activities of *Cedrus deodara* against human RBC. The final concentration of red blood cells number was calculated 5-10 per cent less compared to the healthy control cells (P<0.05).

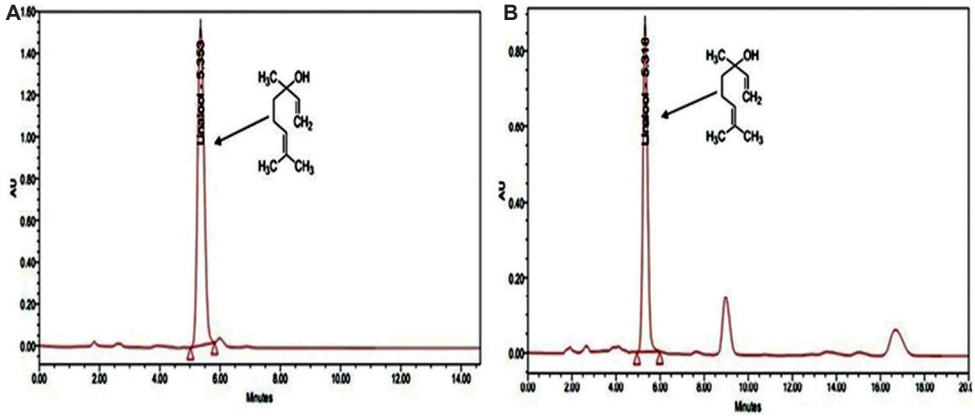

Fig. 8. Reverse phase-HPLC chromatograms. The percentage amount of standard constituent (linalool) present in benzene fractions of plant extracts was found to be 1.29 per cent. Retention time of linalool had been generated by the author which was found to be 5.32 min. (**A**) Linalool, (**B**) *Cedrus deodara*.

been reported[27] and the flavonoids are an active arginase inhibitor of *Leishmania* parasites[28] but could not affect the arginase of mammalian cells which could be the reason of non-cytotoxic effect of *C. deodara* leaf extracts. Arginine plays a key role in survival of *Leishmania* parasites[29]. Therefore, flavonoids showed specific inhibition against the arginase enzyme to affect the metabolic pathway of *Leishmania* parasites[30]. Possibly, the flavonoid as reported previously and terpenoids (linalool) present in the extract of Indian *C. deodara* leaves as estimated in the present study might be the cause of synergistic potency and strong inhibition of *L. donovani* parasites. In conclusion, the benzene extract of *C. deodara* leaves showed potential antileishmanial activities and immunomodulatory effect on host cells *in vitro.* This could be studied further as a potential therapeutic target for the cure the visceral leishmaniasis.

Acknowledgment

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***Conflicts of Interest*:** None.

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