Original Article

Oral Treatment with Aqueous Solution of *Coffea canephora* Induce Protective Immune Response to Reduce Parasite Burden in Experimental Visceral Leishmaniasis

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ABSTRACT

Therapeutic option for visceral leishmaniasis (VL), a potentially fatal disease prevalent in India, is limited. Successful chemotherapy with long lasting protection is ensured when the parasite clearance is associated with the induction of Th1 mediated proinflammatory immune response. Search for novel drugs utilizing natural products is considered beneficial. In the present study oral treatment with aqueous solution of soluble coffee granules of *Coffea canephora* is evaluated for its antileishmanial role against experimental VL in BALB/c mice. UV-visible spectroscopy showed that caffeine and chlorogenic acids were the two major components of the coffee solution. Continual oral intake of soluble coffee for one month showing no hepatic or cardiac toxicity caused partial but significant reduction of parasite burden from both liver and spleen in established infection. Toxicity levels and the parasite clearing efficacy of coffee treatment from liver were comparable with single shot Amphotericin B treatment. Induction of protective cell mediated immune response upon coffee treatment is evident from the formation of granuloma in liver. Decrease in anti-leishmanial IgG titre in the serum samples after treatment is also supportive for the disease cure.

Keywords: BALB/c, Visceral leishmaniasis, Therapy, *Coffea canephora*, Rubiaceae.

INTRODUCTION

Visceral leishmaniasis (VL), caused by *Leishmania donovani* involves uncontrolled parasitization of liver, spleen, bone marrow, and is potentially fatal if left untreated. The disease is characterized by hepatosplenomegaly, prolonged fever, pancytopenia etc. and prevalent in several parts of the world notably in parts of the Indian subcontinent. It is estimated to be 500,000 new annual cases, of which >90% occurs in India and Africa[1]. Therapeutic options for VL are limited. Pentavalent antimony, the long existing and the only specific drug is observed to suffer from serious unresponsiveness in India in past two decades, leaving Amphotericin B (AmB) as almost the single option for treatment in India. Other drugs like Miltefosin, Pentamidine, etc. have many toxicity related limitations. It is reported that chemotherapy without proper modulation of the immune response fails to exert long lasting protection against VL[2]. Hence, search for new potential antileishmanial candidates is an immediate necessity.

Natural products with anti-leishmanial activities are considered as important source for novel and potential therapeutic agents[3]. Coffee is a common beverage worldwide. Robusta coffee (*Coffea canephora*, syn. *Coffea robusta*), originally from central and western sub-Saharan Africa, is now produced in many countries including Vietnam, Brazil, India and Indonesia. It is a flowering plant of family Rubiaceae. Water soluble fraction of coffee is reported to enhance the differentiation of thymocytes and the activation of peripheral T-lymphocytes. Coffee extract is also reported to exert anti-mammary tumour effects[4]. The present study is designed to search any possible therapeutic and/or immunomodulatory role of coffee against experimental VL in BALB/c mice. The therapeutic efficacy of soluble coffee upon continuous oral treatment for one month is compared with single shot intravenous treatment with AmB.

MATERIALS AND METHODS

L. donovani parasites and *in vivo* maintenance in animals

L. donovani strain AG83 (MHOM/IN/1983/AG83) obtained as a gift from Dr. Nahid Ali, Indian Institute of Chemical Biology (IICB), Kolkata, was originally isolated from Indian kala-azar patients[5] and was maintained *in vivo* in BALB/c mice in the animal facilities of Visva-Bharati and used for the experimental purpose. BALB/c mice (4-6 wk) were infected via tail vein with 2×10^7 stationary phase promastigotes, suspended in 0.2 mL phosphate buffered saline (PBS). Mice were sacrificed at 2 mo established infection for *in vitro* transformation and culture. The regulations of the Animal Ethical Committee of the university (Visva-Bharati) following CPCSEA guidelines (approval number-VB/IAEC/2013/4) were maintained to protect the welfare of animals.

In vitro maintenance of *L. donovani*

Amastigotes were aseptically isolated from small pieces of spleen of infected mice after sacrifice, and were allowed to convert to promastigotes at 22°C in M199 (Himedia), pH 7.4, supplemented with 10% fetal bovine serum (FBS) (Himedia), 100 U/mL of penicillin G-sodium and 100 µg/mL streptomycin sulphate (Sigma). Parasites were subcultured at an average density of 2×10^6 cells/mL for 72 hours in M199 supplemented with 6-7% FBS at 22ºC[6].

Antigen preparation

Antigens were prepared following the methods published earlier[7] from the promastigote of *L. donovani* (AG83). In a typical preparation, ~500 g of the packed cell was suspended in 20 mL of 5 mM cold Tris-HCl buffer, pH 7.6. The suspension was vortexed 6-7 times for 2 min each, with 10 min interval on ice. The suspension was then centrifuged at 5000 rpm for 15 min at 4°C. The supernatant (i.e. cytosolic fraction) is taken out and stored. The pellet containing the crude ghost membrane was resuspended in 10 mL of the same buffer and sonicated (5-10 sec pulse) for 6-7 times at 4°C until a translucent appearance is observed. The suspension was finally centrifuged at 7000 rpm for 30 min at 4°C. The supernatant containing the solubilized membrane protein, termed 'LAg' was harvested. Cytosolic fraction of the antigen termed 'CFLAg' was lyophilized, resuspended in 1 mL of 5 mM tris-HCl. Concentration of LAg and CFLAg were determined by Lowry's method[8] and stored at -80°C until use.

Preparation of aqueous solution of coffee

Soluble coffee granules of 'NESCAFE Classic' brand (Batch no.– 10030452NB 4) were used to prepare aqueous solution of coffee. NESCAFE Classic is made up of 100% pure Robusta (*Coffea canephora*) coffee beans. To prepare soluble coffee green coffee beans are roasted, ground and dissolved in water for extraction. It is then freeze dried to produce soluble coffee powder or larger granules and packaged[9]. 2% (w/v) aqueous solution was prepared by mixing the soluble coffee granules in drinking water at ~60-65˚C for 2-3 min and cooled to room temperature.

UV-Vis-NIR absorption spectra of coffee solution

For electronic absorption measurement different concentrations of coffee samples (0.125-0.5%) in a 1 cm quartz cuvette (vol. 1 mL) and a UV-Vis-NIR scanning spectrometer (Shimadzu 31010PC) with wave length regions of 200-1000 nm were used keeping scanning speed in 'slow' mode. Data acquisition was performed by computer interfaced with the spectrometer.

In vivo toxicity assessment of oral coffee and AmB treatment

Normal healthy BALB/c mice (3 animals per group) were treated orally with 2% aqueous coffee solution, instead of drinking water, continually for 1 mo. For positive control, another group of mice were treated with single shot AmB deoxycholate (Fungizone™, 1.25 mg/kg body weight in PBS) (Sarabhai Piramal Pharmaceuticals) intravenously[2]. According to the manufacturer's pamphlet, since crystalline AmB is insoluble in water, the drug is solubilized by the adding sodium desoxycholate, (AmB: Sodium desoxycholate : Sodium phosphate buffer, in the ratio of 50:41:20.2) which following reconstitution, form a colloidal dispersion for intravenous infusion. Sera collected after one-month treatment were pooled and assessed for hepatic and cardiac toxicity through determination of Alanine aminotransferase (ALT) and Aspertate aminotransferase (AST) according to manufacturers protocol (Span Diagnostics Ltd, India).

Treatment schedule of infected BALB/c mice with aqueous coffee solution

Infected BALB/c mice (3 animals per group), at 1 mo infection, were treated orally with 2% aqueous coffee solution continually for 1 mo. Infected control group was left untreated and kept on regular drinking water up to 2 mo after infection. For positive control, infected mice at 1 mo infection were treated intravenously with single shot administration of Amphotericin B deoxycholate (1.25 mg/kg body weight).

Mice were sacrificed after 1 mo of treatment and analyzed for different histopathological and immunological assays.

Determination of splenic and hepatic parasite burden

Spleen and liver were removed after 1 mo of treatment and multiple impression smears were prepared and stained with Giemsa. Organ parasite burdens, expressed as Leishman-Donovan units (LDU), were calculated as the number of parasites per 1000 nucleated cells × organ weight (in mg).

Histological examination for granuloma formation in liver

For assessment of cell mediated immune response after treatment, microtome-sections of infected and treated mice were stained with hematoxylin and eosin and microscopically evaluated for granuloma formation.

ELISA for Leishmanial antigen-specific IgG

The LAg- and CFLAg- specific ELISA for the detection of serum IgG antibodies was carried out on polystyrene microtiter flat bottom plates (Tarsons). The plates were coated with 25 µg/mL antigen (100µL/well) 20 mM phosphate buffer (pH 7.5) at 4°C, overnight. The plates were then washed thrice with PBS supplemented with 0.05% Tween 20 (washing buffer). Excess reactive sites were blocked with 150 µL of 1% bovine serum albumin (BSA) in PBS for 1 h at room temperature. The plates were subsequently incubated for 3 h at room temperature with 100 µL of sera from mice of different experimental sets, serially diluted in PBS containing 1% BSA. After the plates were washed, peroxidase-conjugated goat anti-mouse IgG (Genei, Banglore, India) was applied at a 1:5,000 dilution with sample diluent for 1 h at room temperature. After three washes, *o*-phenylenediamine dihydrochloride (OPD) with H_2O_2 in phosphate citrate buffer (pH 5.0) was applied as the enzyme substrate and the optical density (OD) was read at A_{492} in an ELISA reader (Anthos 2020) after adding 50 µL 1N H_2SO_4 as a stop solution. For determination of serum antibody levels each serum sample was titrated at the dilution required to reach half-maximal absorbance (A_{492} = 1.0) and titre value was determined by plotting the graph on semi log graph between OD v/s antibody dilution.

Statistical Analysis

Three mice per group were used for the *in vivo* experiments. The statistical significance of differences between groups was determined by 'unpaired t test' using the online statistical program Quick Calcs. A value of $p < 0.05$ was considered statistically significant. Error bars in the graph represent the SEM.

RESULTS

Two major peaks were identified in the coffee solution by UV-visble scanning spectroscopy

UV-visble scanning spectroscopic analysis of coffee solution revealed two peaks (Figure 1) at ~280nm and ~320nm, indicating the presence of the bioactive componds. A careful literature survey on this data revealed that the first peak is due to the presence of caffeine and the second peak is of chlorogenic acids (CGA)[10]. Hence, the two major biactive components are caffeine and CGA.

Oral coffee treatment show no hepatic or cardiac toxicity

Serum ALT and AST levels are indicators of hepatic and cardiac toxicity, respectively. Continuous treatment with aqueous coffee solution for one month does not show any significant increase in the activity of these enzymes. ALT and AST levels in the treated group were 5.23 IU/mL

and 8.96 IU/mL respectively. The normal ranges of these enzymes in mice were 4-10 IU/mL and 5-15 IU/mL as found in our experimental conditions. Toxicity levels with AmB treatment, although within the normal range, were found slightly higher than the oral coffee treatment (Table 1).

Oral coffee treatment was effective to reduce parasite burden

BALB/c mice (1 mo infected) treated continually for 1 mo with aqueous coffee solution, and control infected mice (2 mo post infection) were sacrificed. Therapeutic efficacy of oral treatment with coffee was assessed for organ weight and LDU determination from spleen and liver. A significant reduction of increased weight of both spleen (57% reduction) and liver (7.4% reduction) at established infection was observed after treatment with coffee-extract ($p < 0.0001$ and $p = 0.0022$ respectively) (Figure 2A). For parasite burden determination, the amastigotes were counted in Giemsa-stained stamp smear of both the organs and LDU was calculated as mentioned in materials and methods. The parasite load was reduced significantly in spleen ($p = 0.0002$) and liver ($p = 0.0002$) in comparison to infected control in response to coffee treatment (Figure 2B). Suppression of parasite burden in response to coffee treatment was further compared with that of AmB treatment. Splenic and hepatic parasite burden with coffee treatment were reduced to 48% (i.e. 52% reduction), and 40% (i.e. 60% reduction) respectively, while the splenic and hepatic parasite burden with AmB treatment were reduced to 17.34% (i.e. 82.66% reduction) and 37.33% (i.e. 62.67% reduction) one mo after treatment (Figure 3).

Liver-histopathology of coffee-treated animals indicated protective immune reaction against *L. donovani*

Th1 cell mediated immune response is associated for protection in VL. Granuloma formation in the liver is correlated with a T cell mediated immune response to infection[11]. Hematoxylin-eosin stained sections of the liver of treated mice was analyzed for possible indication of immune responses. Intense granuloma, formed largely of infiltrating mononuclear cells, was observed in liver sections of coffee-treated mice (Figure 2A), which indicated that these mice elicited a protective cell mediated immune response against the *L. donovani* infection in response to coffee treatment.

Leishmania-specific IgG levels in mice sera is reduced after treatment

Above results prompted us to look into the anti-leishmanial humoral immune responses. LAg- specific titre of IgG in the pooled serum samples (3 mice per group) of infected and treated BALB/c mice were compared. A gradual increase in the IgG levels was observed in course of time of infection. One month after infection LAg-specific IgG titre was 6300 which further was raised at establishment of infection (2 mo) to 10500. Interestingly, the IgG levels sharply decreased after one month of treatment to a titer value of 5000. This decline in response to coffee treatment was ~52%, in comparison to ~60% decline in response to AmB, one mo after treatment (Figure 2B). CFLAg-specific IgG titre at 2 mo infection was 8100 which reduced after treatment to 2900 (Figure 2C).

DISCUSSION

It is well known that natural protection is achieved in individuals once cured of VL, though the underlying immunological mechanisms is still a subject of intense research. BALB/c mouse is a well

characterized model for VL[6]. *L. donovani* infection is associated with suppression of macrophage activating T cell function largely mediated by elevation of IL-10. Protection requires the restoration of the proin-flammatory response manifested by the production of IL-12 driven IFN-γ[12,13]. Existing effective antileishmanial drugs were reported to elevate the Th1 immune response as a key for their therapeutic success[2,7,14]. Antigen specific humoral immune response, although fails to exert protection, is known to be highly elevated at infection[15]. The present study was designed to search any possible therapeutic and/or immunomodulatory role of coffee against experimental VL in BALB/c mice. Besides being a neurostimulatory beverage filtered coffee is known to have protective role in many chronic systemic disorders. Prolonged coffee consumption is known to reverse the innate immune suppression in experimental murine models of cancer[16]. Our investigations for anti-leishmanial activity of *Coffea canephora* aqueous solutions (treated orally) showed reduction of hepatosplenomegaly as a result of suppression of parasite burden to a significant level in both liver and spleen, confirming its therapeutic potential. It is noteworthy that the parasite clearing efficacy of soluble coffee (60% reduction) from liver, in our experiments, was almost similar with that of AmB treatment (62.67% reduction), though parasite clearance from spleen with coffee treatment was lower than that of AmB treatment. Although a possible direct parasite killing mechanism is yet to be analyzed, coffee treatment probably induced proin-flammatory response as evidenced from the huge infiltration of mononuclear cells to form granuloma in liver. A recent report showed that arabinogalactan-protein (AGP) isolated from coffee is capable of inducing IFN-γ, TNF-α, and IL-2[17]. Interestingly, antileishmanial humoral response in terms of IgG titers against both membrane- and cyosolic- antigens of *L. donovani* promastigotes was markedly reduced after treatment. This is noteworthy, because *Leishmania*-speicific humoral responses although persists for long[18], tends to subside after successful therapy[2]. Since coffee is a rich source of phenolic compounds including CGA like 5-O-caffeoylquinic acid (5-CQA) and its isomers, consumption of coffee is associated with a reduced risk of chronic and degenerative diseases including cardio-vascular disorders, and diabetes[19]. Our UV-visible spectrophotometric data showed that the soluble coffee used for the treatment was rich in caffeine and CGA. Continuous oral coffee treatment for one month did not show any hepatic or cardiac toxicity in our experimental conditions. These toxicity levels with the single shot intravenous treatment with AmB deoxycholate, the standard drug used to treat the VL patients, were found slightly higher than oral coffee treatment. However, excessive intake of caffeine, a xanthine alkaloid and the main bioactive compound in coffee, may lead to some toxic effects as reported elsewhere[20]. Therefore, these novel antileishmanial therapeutic properties of *Coffea canephora* necessitates for extensive study towards identification, isolation and functional characterization of the major antileishmanial compounds from its beans. Intensive investigation for protective immunomodulatory efficacies of these components are the further mandates towards the discovery of a novel antileishmanial drug.

CONCLUSION

The present study showed the novel antileishmanial therapeutic and immune-modulatory potential of *Coffea canephora*. Since the prolonged treatment with crude soluble coffee could induce comparable therapeutic effects as single shot AmB treatment, isolation and a detailed investigation with its bioactive components

may be promising to find out a novel antileishmanial drug.

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Conflict of Interest

The authors have no financial conflict of interest.

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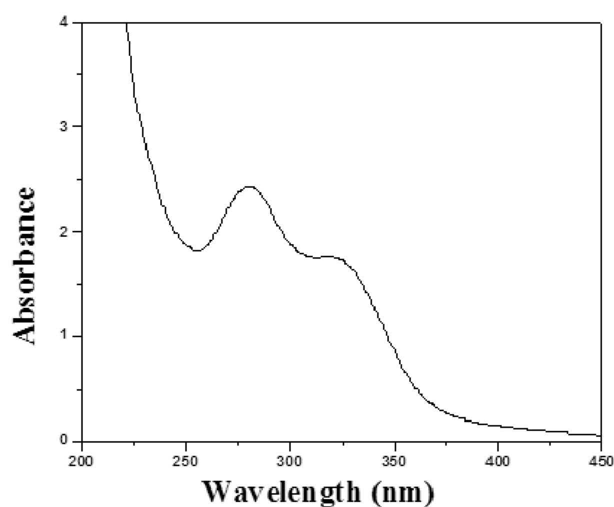

Figure 1. UV-Visible absorption spectra of coffee solution (0.25%) in 'slow' mode showed two major peaks. The absorption maxima of these peaks match with caffeine and CGA

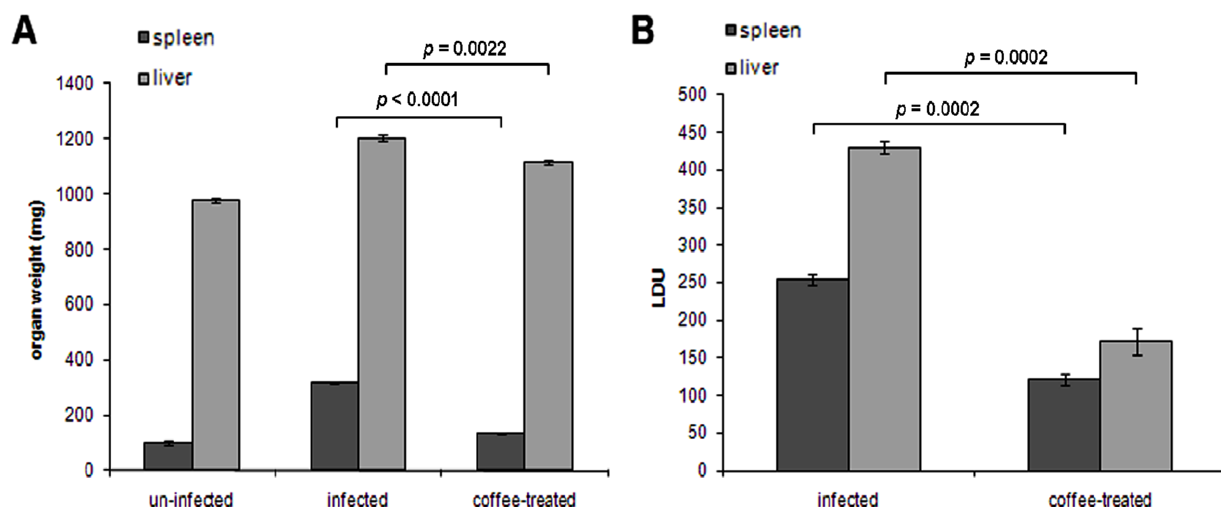

Figure 2. Oral treatment with aqueous coffee solution effectively cleared parasite burden. Untreated infected mice (2 mo established infection) were considered as controls. In treated group, coffee-extract treatment was started 1 mo post-infection and continued for another 1 mo. A, Organ weights before infection, at established infection and after treatment. B, Parasite burden (LDU) of spleen and liver in infected control and treated mice. Data represent the mean ± SE for three animals per group. Differences between the means were analyzed by Student's t test. $p < 0.05$ was considered as significant

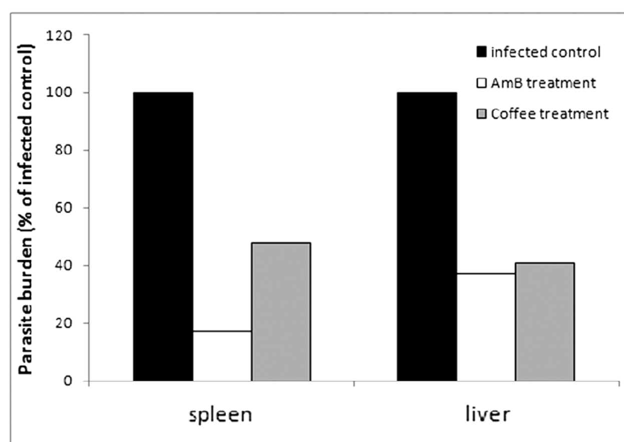

Figure 3. Comparison of efficacy of coffee treatment with respect to AmB treatment to clear the parasite burden from spleen and liver. The remaining parasite burden, measured as LDU, after two treatment modalities is presented as the percentage of parasite burden of respective experimental infected controls

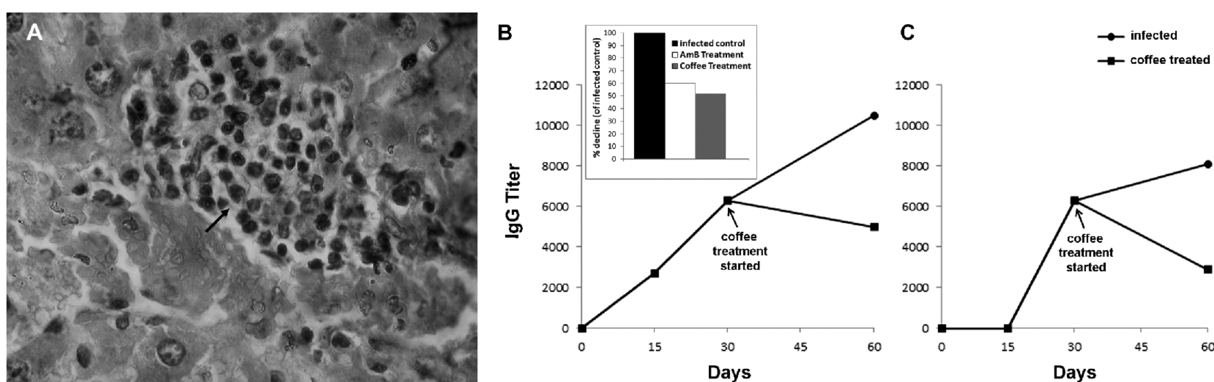

Figure 4. Protective immune response was induced after treatment. Coffee treatment was started at indicated time points. A, Formation of large granuloma by infiltrating mononuclear cells showing protective immune response in liver after treatment. B and C, Antileishmanial IgG titer against promastigote antigens in the pooled sera of coffee treated mice were markedly reduced in comparison to untreated control. B, IgG titer profile against solubilzed membrane antigens (LAg); Inset, comparison in percent decline in IgG titers against LAg after 1 mo treatment with AmB and coffee; and C, IgG titer profile against cytosolic fraction of antigens (CFLAg)

Table 1. Toxicity levels tested in normal mice upon treatment with coffee

Toxicity parameters	Normal range	AmB treatment	Coffee treatment
Serum ALT	4-10 IU/mL	8.33 IU/mL	5.23 IU/mL
Serum AST	5-15 IU/mL	10.66 IU/mL	8.96 IU/mL