

## Anti-leishmanial activity of *Agave americana* L.– A traditional Indian medicinal plant

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Due to lack of safe treatment and developing resistance to the available drugs for visceral leishmaniasis or *Kala-azar* - a fatal parasitic disease caused by *Leishmania donovani* - the search for drugs from natural resources is imperative. In the present study, the comparative *in vitro* anti-leishmanial activity of various fractions of *Agave americana* extracts has been evaluated. Extracts were prepared through successive solvent extraction through Soxhlet apparatus using benzene, chloroform, ethyl acetate and methanol respectively. Among those extracts ethyl acetate fraction was capable of selectively inhibiting both stages of *Leishmania donovani* by generation of reactive oxygen and nitrogen species. Ethyl acetate fraction *Agave americana* showed significant antileishmanial activity ( $IC_{50}$ ~25  $\mu$ g/ml complete inhibition ( $IC_{90}$ ) at 50  $\mu$ g/ml. This observation emphasizes the need to extend studies related to traditional medicines from botanicals for better and safe alternatives to the available anti-leishmanials.

**Keywords:** Visceral leishmaniasis, *Agave Americana* L., Promastigotes, MTT assay

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Visceral leishmaniasis (VL), commonly called as *kala-azar*, caused by an intracellular protozoan parasite *Leishmania donovani*. This parasite is mostly found in two morphological forms: promastigotes and amastigotes, the former being the developmental and infective stage, occur only in the insect vector i.e., female sandflies. VL is symptomised by fever, hepatosplenomegaly, cachexia, and blood cytopenia. VL has also been emerging as an opportunistic infection in several immunosuppressive diseases especially, HIV infection<sup>1</sup>. It is a systemic infection and serious public health problem in developing countries which affects most of the vital organs such as spleen, liver, lymph nodes, etc. About 2-4 lakh new cases occur every year around the world and causing more than 10% deaths, out of which 90% occur in the Indian subcontinent, Sudan, South Sudan, Ethiopia and Brazil<sup>2</sup>.

The chemotherapy of VL has limitations as resistance, variable efficacy, toxicity, parenteral administration, and requirement for long courses of

administration and relapses. In India, sodium antimony gluconate (SAG) is no longer useful as a drug because > 64% of VL patients fail to respond or promptly relapse due to development of resistance to the parasite<sup>3</sup>. Alternative chemotherapeutic treatments with amphotericin B and its lipid formulation have severe limitations due to their toxic effect and prohibitive high cost of treatment. There is an urgent need for new chemotherapeutic drugs for the treatment of these diseases with the recent emphasis of the World Health Organization on the development of anti-leishmanial agents from natural products. Therefore, an urgent search for a safe active drug derived from terrestrial plants as well as marine flora and fauna has been initiated.

*Agave americana* L. (Family-Agavaceae) is traditional medicinal plant which has been used in Ayurvedic medicine for fish poison, it is given internally as a febrifuge in malaria and various other fevers; externally it is applied to wounds as an antiseptic and tonic<sup>4</sup>. This plant is an important source of saponins and polyphenols which have several potential pharmacological activities. *Agave americana*

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has a wide range of pharmacological activities such as anticancer<sup>5</sup>, antifungal, anti-microbial, antioxidant, antidiabetic, anti-inflammatory, hepatoprotective activity and anti-anxiety activity<sup>6,7</sup>. In the present study, the antileishmanial activity of *Agave americana* a xerophitic plant of genus *Agave* of Indian brought up was evaluated.

## Methodology

### Procurement and authentication of plant material

Plant material was collected from hills of Bihar and Jharkhand by Balaji Utthan Sansthan, Uma complex, Fraser Road, Patna. Collected plant material was identified and authenticated as *Agave americana* L. by Botanical Survey of India (BSI), Howrah. Voucher specimen (no. SNPS/JU/1416) has been preserved at School of Natural Product Studies, Jadavpur University, Kolkata, India for further reference.

### Extraction and fractionation of plant material

The dried plant material (200 gm) was broken into small pieces and powdered. Successive extraction of plant materials was done by Soxhlet apparatus in different solvents like benzene, chloroform, ethyl acetate and methanol. Soxhlet process was run for 10-15 hrs for each solvent for effective and proper extraction. Solvents were removed under reduced pressure and temperature by using rotary evaporator (EYELA, Japan) to get the concentrated extract. Further plant extracts were lyophilized to get dried extract and obtained extracts was kept in desiccators for further use. The yield in different fractions of plant material, benzene fraction (S1-AAB), Chloroform fraction (S2-AAC), ethyl acetate fraction (S3-AAE) and methanol fraction (S4-AAM) were found to be 1.60, 0.81, 0.44 and 6.14% w/w, respectively.

### *In vitro* maintenance of promastigotes

Late log phase promastigotes of clinical isolates of *Leishmania donovani* and reference strain Ag 83 were maintained at Balaji Utthan Sansthan, kala azar research center. Parasites were cultured at a concentration of  $2 \times 10^6$  cells/ml in RPMI 1640 supplemented with 10 % heat-inactivated fetal bovine serum, 100 U penicillin and 100 mg streptomycin at 26 °C<sup>8</sup>.

### *In vitro* activity of extracts of *Agave americana* against Promastigotes

Extracts of *Agave americana* with different solvents were prepared under sterile conditions. Late log phase of promastigotes ( $1 \times 10^6$  cells/ml) were

seeded into 24-well tissue culture plates (Nunc), and different concentrations of standard drugs as well as test samples were administered. Untreated cells were served as control. After treatment, microscopic analysis of cells was done using a 0.1 Nauber chamber (Fein Optic, JENA, Germany) at different time intervals (24-72 hrs). Each assay was performed in triplicate<sup>9</sup>.

### *In vitro* activity of extracts of *Agave americana* against intracellular amastigotes

The *in vitro* bioassay testing of different *Agave* extracts at different concentrations was performed in the mouse (Balb/C) peritoneal macrophage cells which were procured after 2% induction of starch solution in peritoneum for 24 hrs (Fig. 1). For procurement of cells, the starched mice were sacrificed by suffocation (CO<sub>2</sub>) and the peritoneum of mice was filled with cold RPMI medium. After gently dislodging the cells in peritoneum, the medium containing cells was aspirated gently and collected in sterile centrifuge tubes. The cells were washed thrice with incomplete medium, suspended in complete medium and cultured to a cell density of  $2.5 \times 10^6$  cells/well. These were further infected with late log phase promastigotes at a ratio of 20:1 (parasite/macrophage) and incubated at 37°C in 5% CO<sub>2</sub> for 8-12 hrs. Wells were later on washed thrice with incomplete medium to remove non-phagocytosed parasites, and finally supplemented with complete medium. Different concentrations of test materials were added to the wells in triplicate and incubated for 24, 48 and 72 hrs. Chamber slides were fixed in absolute methanol, stained with Giemsa, and examined for parasite load in each macrophage<sup>9</sup>.

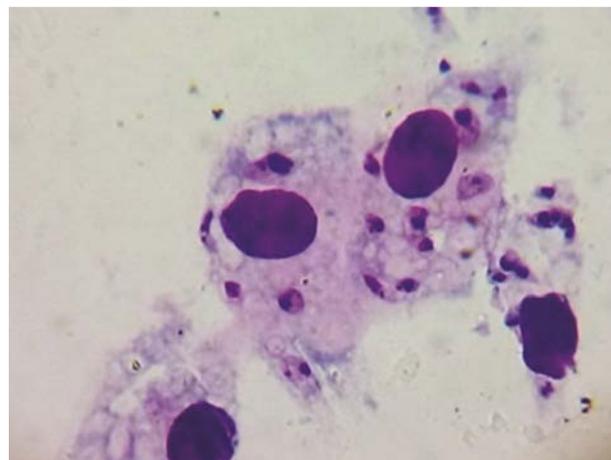


Fig. 1— Amastigotes of peritoneal macrophage cells

## Cytotoxicity against mammalian mononuclear cells

### Mitochondrial dehydrogenase-based assay

MTT assay was performed at IC<sub>50</sub> concentration and many folds higher concentration than IC<sub>50</sub> dose using healthy mammalian mononuclear cells so as to assess the cytotoxicity of these fractions against host macrophages<sup>10</sup> by using MTT-based *in vitro* toxicology assay kit (Sigma Aldrich, USA). The treated cells with various fractions of agave extracts were subsequently supplemented with 100 µl of MTT solution and incubated for 2 hrs in a CO<sub>2</sub> incubator at 35±1<sup>0</sup> C and 95% humidity. The culture was removed after incubation and supplemented with 1 ml MTT solubilising solution. The cells were analyzed with a plate reader at a wavelength of 570 nm using 690 nm as references. In a viable cells mitochondrial dehydrogenase cleaved the tetrazolium ring of MTT yielding insoluble purple formazan was indicative of high level of mitochondrial dehydrogenase.

### Nitric oxide (NO) production and reactive oxygen species (ROS) level in macrophages against the effective concentration of *Agave americana* extracts

NO production by treatment with above said fractions was assessed using Griess reagents in the culture supernants of human macrophages after the incubation with effective concentration of all the fractions and LPS (10 µg/ml Sigma, USA) was used as mitogen<sup>11</sup>. Isolated peripheral macrophages were suspended in culture medium and plated at 10<sup>6</sup> cells/well and exposed to different fraction for 48 hrs. The supernatants (100 µl) collected from macrophages culture 48 hrs after incubation was mixed with an equal volume of Griess reagents (Sigma USA) and left for 10 min at room temperature. The absorbance of the reaction was measured at 540 nm in an ELISA reader.

To evaluate the generation of ROS in macrophages followed by treatment with different fraction, the cell-permeant probe 2', 7'-dichlorodihydrofluorescein di-acetate (H2DCFDA) was used<sup>12</sup>. H2DCFDA is a nonpolar compound that reading diffuses into cells, where it is hydrolyzed to the non-fluorescent derivatives dichlorodihydrofluorescein and is thereby trapped within the cells. In the presence of proper oxidant dichlorodihydrofluorescein is oxidized to the highly fluorescents 2', 7' dichlorofluorescein. Cells treated with fraction (at the IC<sub>50</sub> and twice IC<sub>50</sub> dose) for 48 hrs were re-suspended in 100 ml RPMI and labeled with 10mM H2DCFDA for 15 min. in the

dark. Cells were analyzed for intracellular ROS by using a FACS caliber flow cytometer with Flowjo software.

## Results

### Determination of IC<sub>50</sub> dose of potential extracts against *Leishmania* promastigotes

The adapted and maintained laboratory clinical isolates of *Leishmania donovani*, isolated from *Kala-azar* patients were used for *in vitro* screening and calculating IC<sub>50</sub> and IC<sub>90</sub> activities of different extracts of *Agave americana*. Among the all the fractions ethyl acetate extract showed potential anti-leishmanial activity. The drug Amphotericin-B was used as a positive control. Various doses of the *Agave americana* extracts were tested against promastigote stage in range of 200, 100, 50, 25, 12.5 µg/ml. Ethyl acetate extract of *Agave americana* (S3-AAE) showed potential antileishmanial activity with IC<sub>50</sub> dose of ~25 µg/ml and complete inhibition (IC<sub>90</sub>) at 50 µg/ml obtained results have been given in Table 1. Standard drug Amphotericin-B showing antileishmanial activity with IC<sub>50</sub> dose of 0.1 and 1.2µg/ml served as a positive control (Fig. 2).

### Determination of IC<sub>50</sub> dose of potential extracts against *Leishmania* promastigotes

Similar to promastigotes, various concentrations of all the above said extracts were evaluated for their

Table 1— *In vitro* effect of *Agave americana* extracts on *Leishmania donovani* promastigotes

AA	S1-AAB	S2-AAC	S3-AAE	S4-AAM
No Extract	2x10 <sup>6</sup>	2x10 <sup>6</sup>	2x10 <sup>6</sup>	2x10 <sup>6</sup>
12.5µg/ml	2x10 <sup>6</sup>	1.5x10 <sup>6</sup>	1.8x10 <sup>6</sup>	2.6x10 <sup>6</sup>
25 µg/ml	1.9x10 <sup>6</sup>	1.4x10 <sup>6</sup>	0.9x10 <sup>6</sup>	2.4x10 <sup>6</sup>
50 µg/ml	1.7x10 <sup>6</sup>	1.3x10 <sup>6</sup>	0	1 x10 <sup>6</sup>
100 µg/ml	1 x10 <sup>6</sup>	0.8x10 <sup>6</sup>	0	0.9x10 <sup>6</sup>
200 µg/ml	0.6x10 <sup>6</sup>	0.5x10 <sup>6</sup>	0	0.4x10 <sup>6</sup>
1 µg/ml AMB	0	0	0	0

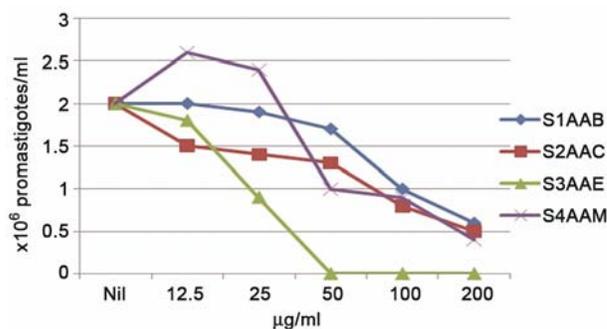


Fig. 2— Evaluation of IC<sub>50</sub> of various extracts against the promastigotes of late log phase (2x10<sup>6</sup>/ml) after incubation with different concentration of extracts

anti-leishmanial potential against intracellular pathogenic form of parasite, i.e., amastigotes. The extract concentration of  $\sim 12.5$   $\mu\text{g/ml}$  was observed  $\text{IC}_{50}$  and  $\sim 25$   $\mu\text{g/ml}$  as  $\text{IC}_{90}$ , results are shown in the Fig. 3 and Table 2.

### MTT assay against human mononuclear cells

After treating the cells with effective concentration of *Agave americana* about three fold decreased dehydrogenase activities of mitochondria was observed than untreated cells of mononuclear cells (Fig. 4).

### Evaluation of nitric oxide response

NO production in peritoneal macrophages after 48 hrs of incubation in response to S3-AAE was quantified using griess reagent. For comparison, NO production in LPS (mitogen) stimulated and unstimulated cells served as positive and negative controls respectively. Above said extract showed 1.5 folds increase in NO production which was at par

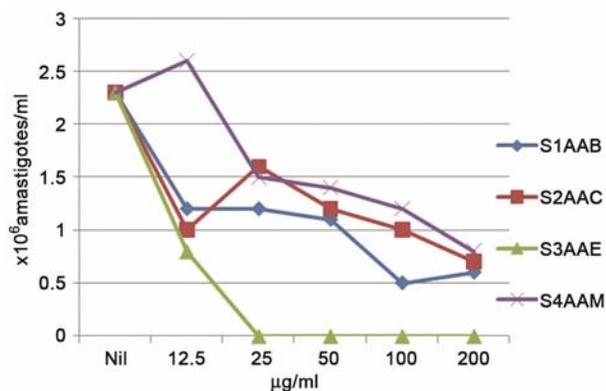


Fig. 3—  $\text{IC}_{50}$  of various extracts against intracellular amastigotes ( $2.3 \times 10^6/\text{ml}$ ) after incubation with different concentration of extracts

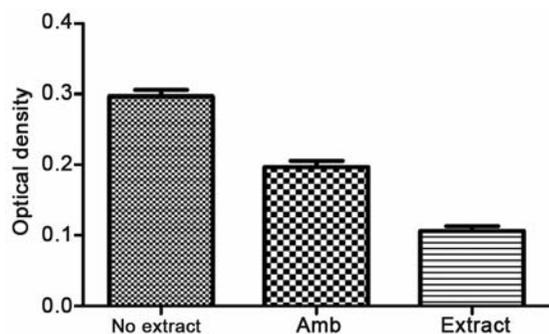


Fig. 4— MTT assay (Mean  $\pm$  SD) [No extract = untreated cell; Amb = positive control; Extract = treated with *A. americana* extract]

with that produced by LPS stimulation which served as positive control (Fig. 5).

### ROS generation in human macrophages through *Agave americana* extract

$\text{H}_2\text{DCFDA}$ , a non-polar compound, is converted on oxidation to the highly fluorescent 2', 7'-dichlorofluorescein and this property has been utilized to monitor ROS generation. Treatment with ethyl acetate fraction of *Agave americana* extract lead to more than 2 folds increase in ROS up to 48 hrs when compared with unstimulated control (Fig. 6).

Table 2— *In vitro* effect of *Agave americana* extracts on *Leishmania donovani* intracellular amastigotes

AA	S1-AAB	S2-AAC	S3-AAE	S4-AAM
No Extract	$2.3 \times 10^6$	$2.3 \times 10^6$	$2.3 \times 10^6$	$2.3 \times 10^6$
12.5 $\mu\text{g/ml}$	$1.2 \times 10^6$	$1 \times 10^6$	$0.8 \times 10^6$	$2.6 \times 10^6$
25 $\mu\text{g/ml}$	$1.2 \times 10^6$	$1.6 \times 10^6$	0	$1.5 \times 10^6$
50 $\mu\text{g/ml}$	$1.1 \times 10^6$	$1.2 \times 10^6$	0	$1.4 \times 10^6$
100 $\mu\text{g/ml}$	$0.5 \times 10^6$	$1 \times 10^6$	0	$1.2 \times 10^6$
200 $\mu\text{g/ml}$	$0.6 \times 10^6$	$0.7 \times 10^6$	0	$0.8 \times 10^6$
1 $\mu\text{g/ml}$ AMB	0	0	0	0

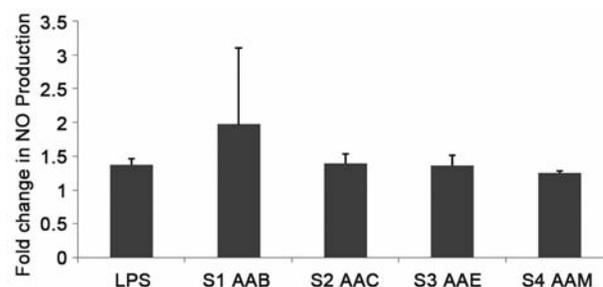


Fig. 5— NO generation in human macrophages after treatment with *Agave americana* extract for 48 hrs. (Each bar represents  $\text{MFI} \pm \text{SD}$ ) [S1AAB = benzene fraction; S2AAC= Chloroform fraction; S3AAE= ethyl acetate fraction; S4AAM= methanol fraction]

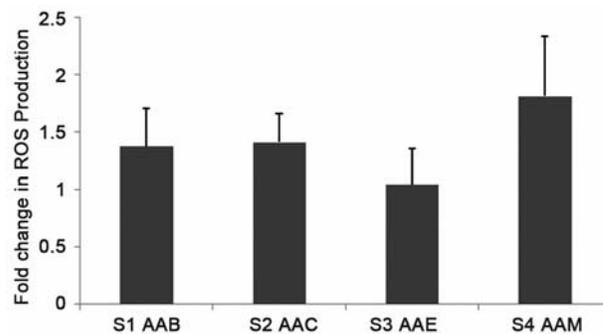


Fig. 6— ROS generation in human macrophages after 48 h. (Each bar represents  $\text{MFI} \pm \text{SD}$ ) [S1AAB = benzene fraction; S2AAC= Chloroform fraction; S3AAE= ethyl acetate fraction; S4AAM= methanol fraction]

## Discussion

Many plants have been explored to possess interesting antileishmanial activities, validating their use in folk medicine. It was observed that ethyl acetate fraction of *Agave americana* extract (S3-AAE) showed potent anti-leishmanial activity against pathogenic form of the parasite, i.e., amastigotes along with promastigote form whereas no activity was observed with other extracts. This indicates the medicinal applicative value of ethyl acetate fraction of *Agave americana* extract. However, some level of cytotoxicity and hemolytic activity was observed in this fraction. Further studies are underway to identify the component responsible for cytotoxicity and its removal so as to develop this fraction as a safe alternative for antileishmanial treatment.

Further clarifying the S3-AAE mode of action against *L. donovani*, lead us to evaluate whether PB-BMM could induce oxidative stress in promastigotes as well as in infected macrophages. It has been observed that metabolic activation of some plant extracts can lead to the production of toxic pro-oxidants and cause cell injury under different conditions<sup>13</sup>. The increase in ROS after exposure of human macrophages to S3-AAE suggested that S3-AAE-mediated generation of ROS might be a major pathway responsible for the death of promastigotes and amastigote-infected macrophages. Generation of NO after drug treatment in macrophages further indicated the involvement of reactive nitrogen species in amastigote death. It is the well known fact that activation of NOS and further production of nitric oxide is major antimicrobial mechanism initiated by activated macrophages for parasite killing. Increased production of NO post S3-AAE treatment indicates that S3-AAE activates the macrophages and induces NO production for parasite killing. The plant is grown in areas with a high incidence of leishmaniasis, and has given good lead as an anti-leishmanial showing that it possesses bioactive components. Further identification, characterization, purification and biological evaluation of these are in progress by our group. Steps are more focused on identifying the components responsible for some level of cytotoxicity and removing those components so as to develop this as a safe herbal drug against Visceral Leishmaniasis.

## Conclusion

Present finding showed that extract of the *Agave americana* have potential anti-leishmanial activities in both the stages of parasite, viz. promastigote stage and pathogenic intracellular amastigote stage. The IC<sub>50</sub> indicates that the ethyl acetate fraction have potential efficacy even at very low concentration. The observed results indicate that further fractionation and exploration of the potential extracts can lead to identification of novel and safe drug against VL.

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