Screening and Characterization of Bioactive Molecules Derived From Medicinal Plants for Antileishmanial Activities for *Leishmania donovani*

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Abstract- Leishmaniasis is a vector-borne disease caused by genus *Leishmania*. It causes significant morbidity and mortality in the endemic areas of several developing countries. Due to multidrug resistance in *Leishmania sp a*nd unavailability of an effective vaccine, discovery of new drugs is urgently needed. The aim of the present study was screening of medicinal plants used as Indian traditional medicine for leishmanicidal activity. Promastigote forms of Leishmania parasite were cultured in-vitro in NNN medium and further sub-cultured and maintained in RPMI-1640 medium containing 10% Fetal Bovine Serum for the screening of medicinal plants. A total of 26 medicinal plants were collected and screened for leishmanicidal activity. The methanolic extracts showing antileishmanial activity were subjected to LC-MS analysis to identify the major phyto-constituents in the crude methanolic extracts. The compounds were detected in the LC-MS of active extracts. The plant extract showing maximum antileishmanial activity was further fractionated to isolate the major compound(s).The compound isolated was characterised by IR, Mass spectrometry and subjected to *in vitro* antileishmanial activity against *L. donovani* promastigotes. *In vitro* antileishmanial assay revealed that crude methanolic extracts of 2/10 plants were active against *L. donovani* promastigotes. Methanolic root extracts of *Inula racemosa were* found active (54.83%) against the parasite while T. *Terrestris* were found least active with such as toxicity, percent inhibition 43.10% at concentration of 500 µg/ml. *Inula racemosa* methanolic extract further fractionated subjected for LC-MS analysis. The compound isolated from the methanolic root extract of *I. racemosa was* isoalantolactone, which did not show any antileishmanial activity against *L. donovani*. The study suggested that crude extract of *Inula racemosa* and *T. Terrestris* have shown potent antileishmanial activity while extracted bioactive molecules does not show efficacy against *Leishmania* parasite. Hence the antileishmanial activity could be due to any other compound which could not be detected, so further study is undertaken.

Key words: *Inularacemosa, T. terrestris, L. donovani,* MDR, LC-MS and IR.

Introduction

Leishmaniasis is a major public health problem caused by an intracellular obligate protozoan parasite of the genus *Leishmania*. Leishmanias is, by its various clinical forms, causes significant morbidity and mortality in the developing countries of the world every year. It is estimated that 0.2 to 0.4 million new cases of visceral leishmaniasis, 0.7 to 1.3 million new cases of cutaneous leishmaniasis with 20,000 to 30,000 deaths occur every year worldwide (Rama et al 2015). Despite the severity of this parasitic disease till date, there is no vaccine available to efficiently prevent or cure leishmaniasis. The currently used chemotherapy has unpleasant side effects variable efficiency between different species and development of drug resistance in the parasite [Camacho et al 2003, Croft et al 2006]. People living in rural areas of the developing countries are still dependent on traditional medicines to health eirailments (Chan-Bacab et al 2006). There is a need to explore the potential of natural products obtained from the plants used by traditional healers which may eliminate the parasite from the host without causing any side effects. Several plant species have been screened for leishmanicidal activity after extraction with different solvents like petroleum ether, chloroform, hexane, ethyl acetate, methylene chloride, methanol and tested *in vitro* and *in vivo* against *Leishmania* parasite (Fournet et al 1996, Pereira et al 2010, Ghosh et al 2011).The discovery and development of leishmanicidal biomolecules is also aided by the biochemical studies of the metabolic pathways of the parasite. In this approach, the enzymes essential for the survival of *Leishmania* are identified and targeted by lead molecules (Ogungbe et al 2013, Sidana et al 2015, 2018). The Indian plants have not been screened extensively for leishmanicidal activity and there is a need to search for new therapeutic agent(s) from the diverse range of medicinal plants found in North India against *Leishmania spp*. The extracts of different parts of medicinal plants may be screened *in vitro* against *Leishmania spp*. to evaluate their potency and efficacy to inhibit the parasite. The biological activity of plant extracts will be attributed to compounds belonging to diverse chemical groups including alkaloids, flavonoids, phenyl-propanoids, steroids and terpenoids (Iwu*et al.*,1994; Rocha *et al.*, 2005; Wang *et al.*, 2009).The present study was aimed to assess the antileishmanial potential of methanolic extract of different medicinal plants *in vitro* against *L. donovani* and further extraction and characterization of bioactive molecules from active plant extract against *L. donovani* to recognize the compounds responsible for the antileishmanial activity.

Material and Methods

Collection of plant material

The medicinal plants used for antileishmanial activity were obtained from Y. S. Parmar University of Horticulture and Forestry, Nauni, HP and Arya Vastu Bhandar, Dehradun, UK, India. A total of 10 authenticated medicinal plants were procured (Table-1).

Table-1 Medicinal plants screened for *in vitro* antileishmanial activity

S. No	Botanical name	Family	Local name	Part used
1	*Acoruscalamus*	Acoraceae	Boiye	Leaves
2	*Alstoniascholaris*	Apocynaceae	Chitvan	Leaves

3	*Inularacemosa*	Asteraceae	Pushker	Root
4	*Tribulusterestris*	Zygophyllaceae	Gokhru	Leaves
5	*Aegle marmelos*	Rutaceae	Bael	Dried fruit
6	*Albiziaprocera*	Fabaceae	Safed Siris	Leaves
7	*Andrographispaniculata*	Acanthaceae	Kalmegh	Stem
8	*Asparagus abscendens*	Liliaceae	Safed musli	Roots
9	*Cassia fistula*	Fabaceae	Amaltash	Fruit
10	*Embeliaribes*	Primulaceae	Vidanga	Fruits

Preparation of plants extracts

The parts of plants were washed with tap water and then with distilled water, followed by drying on absorbing paper at room temperature in open air under shade for 10-15 days. The dry plant parts were ground to yield coarse powder and stored at ambient temperature in amber glass bottles until use. The powder of each plant part was extracted with methanol using hot Soxhlet extraction for 24 hours. After extraction, the extracts were concentrated under reduced pressure using rotary evaporator. The concentrated extracts were further dried in a desiccat or using calcium chloride as a desiccant. The dried extracts were weighed to obtain the percentage yield and stored in air tight bottles at 4°C until use.

Antileishmanial assay

In Vitro Assessment of Antileishmanial Activity Parasite stock culture

The Axenic culture of L. donovani (LdMIPL-1) was maintained at 25°C in RPMI 1640 (Himedia) medium supplemented with 10% heat inactivated Fetal Bovine Serum (FBS, Himedia), streptomycin (150 µg/ml), penicillin G (100 µg/ml) and gentamycin (150 µg/ml) at pH 7.2.

For antileishmanial activity, promastigotes of L. donovani were sub-cultured in Schneider's Insect Medium (Himedia) supplemented with 10% heat inactivated FBS, streptomycin (150 µg/ml), penicillin G (100 µg/ml) and Gentamycin (150 µg/ml). The anti leishmanial screening was performed in 96-well flat bottom tissue culture plates (Corning Life Sciences, USA). One hundred microliters of cell suspension containing 2 ×106 to 3 ×106 cells/ml was poured in each well of the plate. Four different concentrations of the methanolic root extract i.e. 100, 250, 350 and 500µg/ml dissolved in dimethyl sulfoxide (<0.025% v/v) were added to the culture. The plates were then incubated at 25°C for 24-48 hours. Amphotericin B and sodium stibugluconate were used as positive controls and cell suspension with 0.025% DMSO was used as a negative control. The inhibition of the promastigotes was assessed by measuring the cleavage of 10mg/ml of MTT [3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyl tetrazolium bromide]. The absorbance was measured by using ELISA plate reader (BioTek, USA) at 595nm. The percent

growth inhibition was calculated by the following formula:

$$\% \textbf{ of inhibition} = \frac{\textbf{OD control} - \textbf{OD treated}}{\textbf{OD control}} \times \textbf{100}$$

IR, Mass spectrometry studies IR spectrum was taken on an FT-IR spectrophotometer (Model RZX, Perkin Elmer) using KBr pellet. Mass spectrum was recorded on Model Q-ToF Micro (Waters) spectrometer. Isolation and characterization of pure compound from methanolic root extract of *I. racemosa.* The methanolic extract of the dried and powdered roots of *I. racemosa* was fractionated by using ethyl acetate and petroleum ether. The fraction in petroleum ether was dried and fine crystals of the compound were obtained. The crystalline white compound was characterized on the basis of its spectral data. Statistical analysis: The antileishmanial assay was performed in triplicate with three replicates of each concentration tested. The results were expressed as mean ± standard error of mean. The overall variation in a set of data was analysed by one way analysis of variance (ANOVA). A value of $P < 0.05$ was considered significant.

Result

In vitro antileishmanial activity of medicinal plants. A total of 10 methanolic extracts of different parts of medicinal plants used as traditional medicine in India were evaluated for their antileishmanial activity by using MTT reduction assay. Out of the 10 extracts, only two methanolic extracts of *Inula racemosa* roots, and *Tribulus terrestris* leaves showed significant *in vitro* antileishmanial activity against *L. Donovani* promastigotes (Figure-1 and 2). The remaining 8 plant extracts have not shown any activity against *L. donovani*, since the highest concentration (500 µg/ml) of all 8 extracts could not inhibit the growth of the parasite.

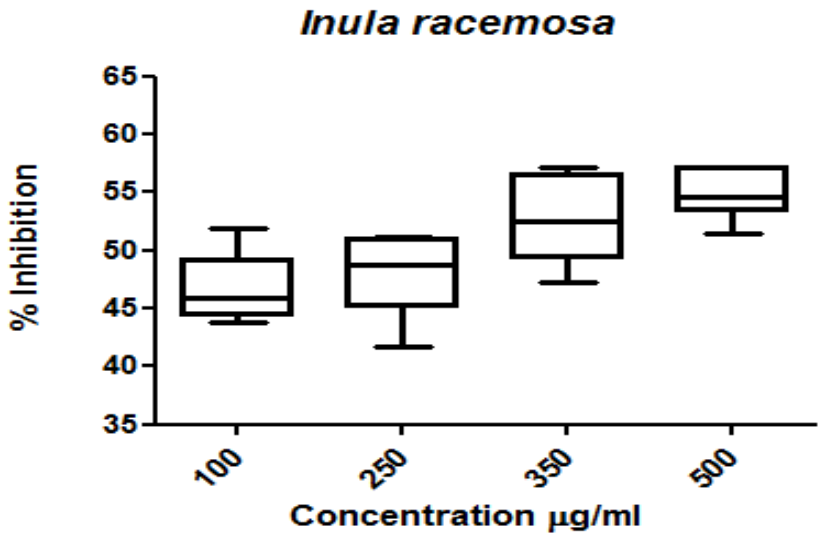


Figure-1 Antileishmanial activity of crude methanolic roots extract of *I. racemosa*

Table-2 Percent inhibition of *L. donovani* promastigotes after 24 hours of incubation with four different concentrations of methanolic root extract of *I. racemosa* and standard error of the mean.

Conc (µg/ml)	100	250	350	500	P value
Mean % Inhibition	46.72	47.91	52.63	54.83	0.0007
SEM	1.224	1.454	1.536	0.892	***

The most active extract was that of the *Inula racemosa* roots which showed 54.83% inhibition of *L. Donovani* promastigotes within 24 hours of incubation at 25°C.The inhibition of the parasite was concentration dependent, as it increases with the increase in extract concentration. The means of the percent inhibition were considered significant with a P value of 0.0007 (P<0.05) (Table-2).

Methanolic leaves extract of *Tribulus terrestris* also showed significant activity against *L. donovani promastigotes* with a percent inhibition of 42.77% at a concentration of 500 µg/ml followed by 40.29, 38.14 and 30.50% inhibition at 350, 250 and 100 µg/ml, respectively. The means of percent inhibition were considered significant with a P value of 0.0003 (P<0.05) (Table-3).

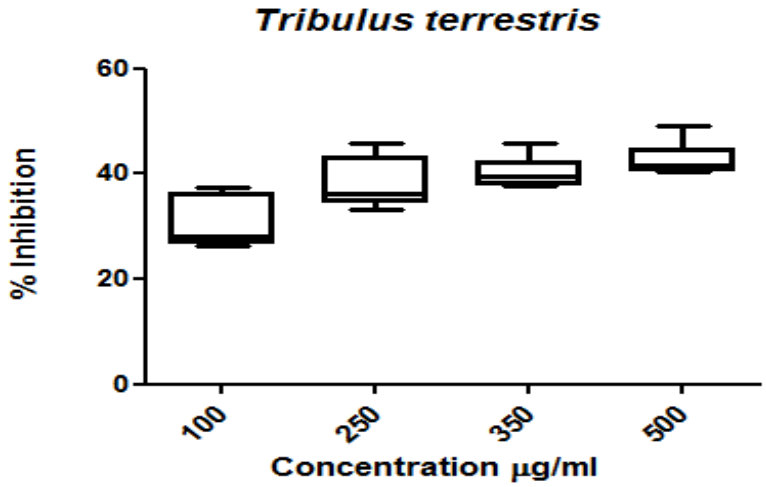


Figure-2 Antileishmanial activity of crude methanolic leaves extract of *T. Terrestris*

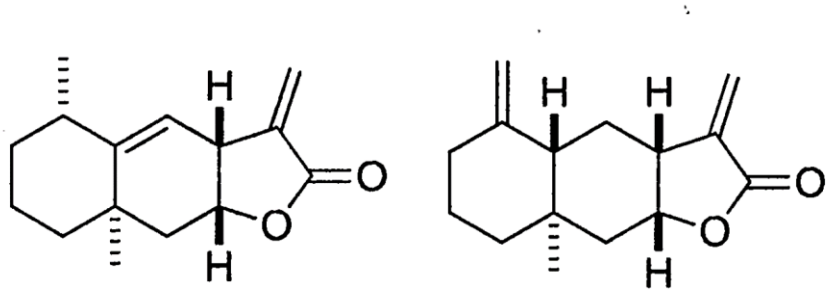
Table-3 Percent inhibition of *L. donovani*promastigotes after 24 hours of incubation with four different concentrations of methanolic leaves extract of *T.Terrestris*and standard error of the mean.

Conc (µg/ml)	100	250	350	500	P value
Mean % Inhibition	30.50	38.14	40.29	42.77	0.0003
SEM	1.978	1.986	1.219	1.324	***

Out of the two active methanolic extracts, minimum antileishmanial activity was observed with the leaves extracts of *Tribulus terestris*. While the methanolic root extracts of *I. racemosa* have shown maximum antileishmanial activity against *L. donovani*. Therefore, the methanolic root extract of *I. racemosa* was further subjected for fractionation for the isolation of compound(s) which may be responsible for antileishmanial activity.

LC-MS analysis of active methanolic extracts

The LC-MS analysis of active methanolic extracts *Inula racemosa* was carried out to identify the major compounds present in the extracts which may be responsible for antileishmanial activity.



A B

Figure-3-Structure of compounds indicated by LC-MS analysis.

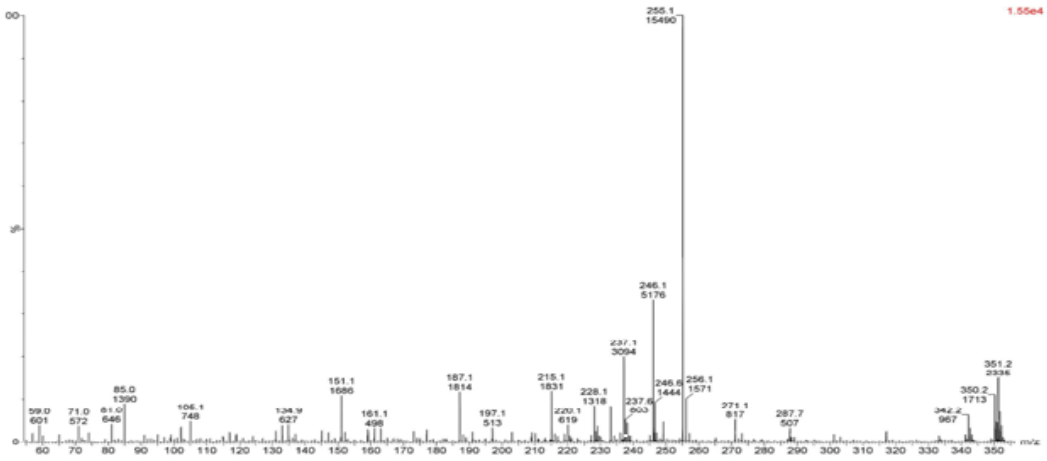


Figure-4 Mass Spectrum of methanolic root extract of *I. recimosa*

Isolation and characterisation of pure compound from methanolic root extract of *I. racemosa*

The methanolic extract of the dried andpowdered roots of*I. racemosa*was fractionated by using ethyl acetate and petroleum ether. The fraction in petroleum fractionated by using ethyl acetate and petroleum ether. The fraction in petroleumether was dried and fine crystals of the compound were obtained. The crystalline white compound was characterized on the basis of its spectral data(Figure-5).

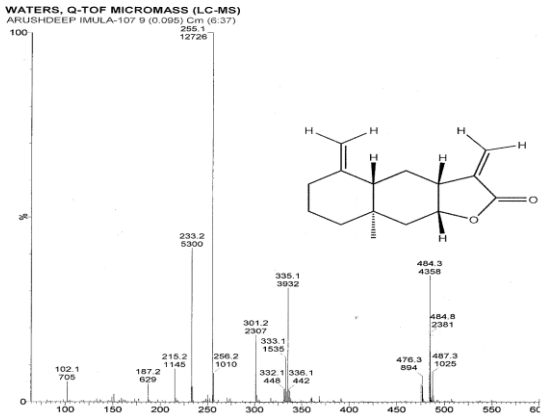


Figure-5 Mass spectrum of Isolantolactone

The IR spectrum indicated the presence of a lactone in thestructure (1752 cm-1). Other functional groups such as hydroxyl, amino andcarboxylic were not present in the IR spectrum. The mass spectrum presented the base peak at 255 [M^+Na^+] and the molecular ion peak at 233[M^+H^+] depicting. The molecular mass to be 232. This molecular mass corresponded to isolantolactone and lantolactone, the two isomeric lactones reported from *Inula* species (Figure-6).

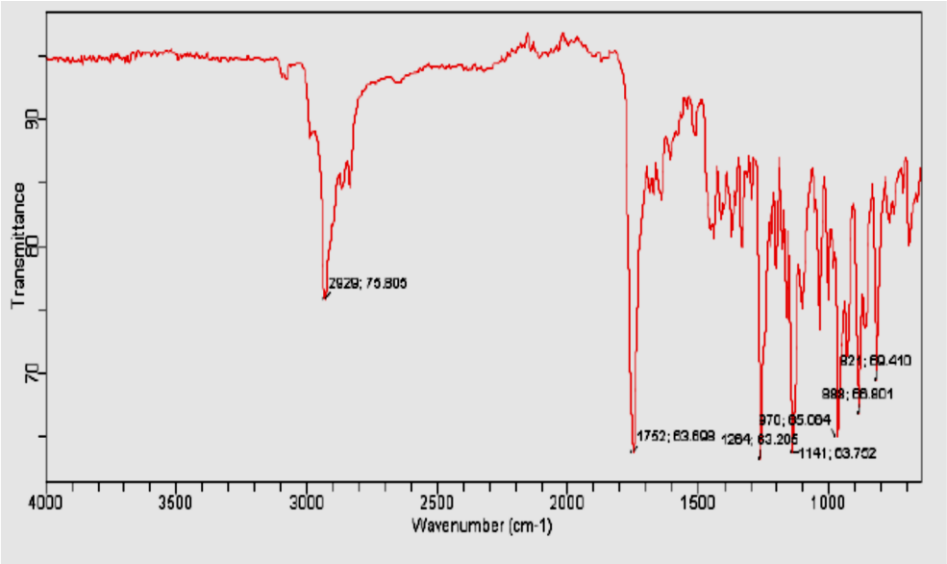


Figure-6 Infrared spectrum of compound isolated from methanolic root extract of *I. racemosa*

Further, the compound was identified by comparison of physical properties and the obtained spectral data with the literature values of isoalantolactone (Table-4).

Table-4 Physical properties of the compound isolated from methanolic roots extract of *I. racemosa*.

S. No	Physical Properties of Compound	Observation
1	Color	White
2	Melting Point	112°C
3	Solubility	Methanol

Discussion

Leishmaniasis, being prevalent in developing countries, creates a worldwide health problem. Control of the disease becomes more difficult due to lack of an effective vaccine and safe and economical antileishmanial drugs. The current available antileishmanial drugs including pentavalent antimonials, liposomal formulations of amphotericin B, penta-midine and miltifosine, all of these are associated with one or more drawbacks which limit their uses (Santos et al 2008). In the search of new antileishmanial drugs, research is going on worldwide and is more concentrated towards the use of natural products rather than synthetic chemicals. Biological molecules extracted from plant and marine natural products represent an important source of novel therapeutics against various forms of leishmaniasis (Rocha et al 2005, Tempone et al 2011).The survey of the plant kingdom for finding a potent antileishmanial agents is going on from decades and several active biomolecules have been exposed with strong antileishmanial effect (Sen and Chatterjee, 2011). The present study was aimed to screen medicinal plants used in traditional medicine in India for antileishmanial

activity against *L. donovani*. A total of 26 methanolic extracts were prepared from different parts of the plants. Out of the 26 crude methanolic extracts, two have shown significant leishmanicidal activity by inhibiting 35-55% promastigotes within 24 hours of application.

Tribulus terrestris (Zygophyllaceae) has shown 42.77% inhibition of the *L. donovani* promastigotes at a concentration of 500 µg/ml. In earlier studies, extract of *Peganumharmala* (Zygophyllaceae) was reported to show potent *in vitro* antileishmanial activity against *L. major* (Mirzaie *et al.,* 2007). Similarly, potent *in vitro* and *in vivo* antileishmanial activities of crude hydro-alcoholic extract of *Peganum harmala* seeds were observed against *L. major* (Rahimi-Moghaddam*et al.,* 2011). The ethanolic fruit extract of *T. Terrestris* was reported to have very potent antibacterial activity against *Bacillus subtilis*, *Bacillus cereus*, *Proteus vulgaris* and *Coryne bacterium diphtheria* (Al-Bayati and Al-Mola, 2008). Similarly, we have also reported potential antileishmanial activity of crude methanolic extract of *Acoruscalamus*, *Alstonia scholaris* and *Berberi saristate* (Sidana et al 2015). The methanolic root extract of *Inula racemosa* (Asteraceae) was found most active extract with 54.83% inhibition of the *L. donovani* promastigotes. Similarly, the ethanolic root extract of *Echinacea purpurea*of family Asteraceae has been reported to possess potent antileishmanial activity against promastigotes of *L. major* (Soudi*et al.,* 2007).The dichloromethane extracts of aerial parts of *Acanthospermumhispidum* (Asteraceae) has also been reported to show potent *in vitro* antileishmanial activity against *L. Mexicana* (Bero*et al.,* 2011). The LC-MS analysis of methanolic root extract of *I. racemosa*revealed the presence of alantolactone and iso alantolactone. The antileishmanial activity of these compounds has not been reported so far. In the present study, owing to the potent antileishmanial activity of *I. racemosa*, we have fractionated the methanolic root extract of this plant to isolate the major compound(s) present in it which may be responsible for the antileishmanial activity. The isolated compound was then characterized by its physical properties and spectral studies like IR, Mass and NMR.

The compound obtained was white in colour with molecular mass of 232 and melting point of 112°C. The structural formula of the compound was C15H20O2. The isolated compound was identified as isoalantolactone. Further, this compound was subjected for *in vitro* antileishmanial activity against promastigotes of *L. donovani*. However, it did not show any antileishmanial activity. Contrary to this, the isomer of this compound i.e. alantolactone has been reported to exhibit potent antibacterial activity against *Pseudomonas aeruginosa* and *Bacillus cereus* (Lokhande*et al.,* 2007). Both alantolactone and isoalantolactone have also been reported to show antifungal and anthelmintic activities (Satyawati*et al.,* 1987). This suggests that the strong antileishmanial activity of the crude methanolic root extract of *I. racemosa* may not be due to these two compounds; however, it may be due to some other compound which could not be detected in the LC-MS analysis and could not be isolated by fractionation. The survey of the plant kingdom for finding a potent antileishmanial agents is going on from decades and several active biomolecules have been exposed with strong antileishmanial effect (Sen and Chatterjee, 2011). The present study was aimed to screen medicinal plants used in traditional medicine in India for antileishmanial activity against *L. donovani*. A total of 26 methanolic extracts were prepared from different parts of the plants. Out of the 26 crude methanolic extracts, two have shown significant leishmanicidal activity by inhibiting 35-55% promastigotes within 24 hours of application.

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Conclusion

We discovered that the crude extracts of *Inula racemosa* and *T. terrestris* showed substantial antileishmanial activity in this investigation. Furthermore, isoalantolactone, a bioactive component derived from the methanolic root extract of *I. racemosa* has no antileishmanial action against *L. donovani*. As a result, the antileishmanial activity could be attributed to any other chemical that was not found, hence more research is needed.

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