

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
19 April 2007 (19.04.2007)

PCT

(10) International Publication Number
WO 2007/042902 A2

(51) International Patent Classification:

A61K 36/63 (2006.01) A61K 31/7048 (2006.01)
A61K 31/7028 (2006.01) A61K 31/4453 (2006.01)

(21) International Application Number:

PCT/IB2006/002805

(22) International Filing Date: 9 October 2006 (09.10.2006)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

2725/DEL/2005 10 October 2005 (10.10.2005) IN

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(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LV, LY, MA, MD, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declaration under Rule 4.17:

— as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))

Published:

— without international search report and to be republished upon receipt of that report

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: A PHARMACEUTICAL COMPOSITION USEFUL AS A LEISHMANICIDAL AGENT

(57) Abstract: This invention relates to the leishmanicidal activity of night jasmine leaf extract, wherein the said method comprises the steps of, preparing the methanolic extract of night jasmine leaf, chromatographic purification of said extract to obtain bioactive fraction containing calceolarioside A, isolation and characterization of calceolarioside A, leishmanicidal activity of calceolarioside A against axenic promastigote culture of Leishmania donovani, treatment of *L. donovani* infected chronic golden hamster with bioactive fraction for a period of 21 days for detection of leishmanicidal activity in vivo, administering of PE in normal hamster to obtain non-toxic nature, combination therapy of bioactive fraction + SAG against said model for synergistic potentiation of antileishmanial activity and complete clearance of parasitemia from liver and spleen as reflected by LDU wherein comprising a potential medicament against kala-azar.

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A PHARMACEUTICAL COMPOSITION USEFUL AS A LEISHMANICIDAL AGENT

Field of the invention:

5 The present invention relates to a bioactive fraction obtained from the leaves of the plant *night jasmine*.

More particularly, it relates to the use of said bioactive fraction in the treatment of visceral leishmaniasis.

The present invention also relates to the use of the compound calceolarioside A as a
10 leishmanicidal agent.

It also relates to a pharmaceutical composition useful as a leishmanicidal agent, wherein the said composition comprising the therapeutically effective amount of said bioactive fraction obtained from the leaves of the plant *night jasmine* or selected from the combination comprising the bioactive fraction and sodium antimony gluconate or
15 bioactive fraction and piperine or bioactive fraction and sodium antimony gluconate and piperine optionally along with one or more pharmaceutically acceptable carriers, additives, lubricant and diluents.

Further, it relates to a method of treating visceral leishmaniasis in a subject using the said pharmaceutical composition.

20 The present invention also relates to a method for the isolation of compound calceolarioside A from the bioactive fraction obtained from the leaves of the plant *Nyctanthes arbortristis*.

Background and prior art of the invention:

25 Infection with the protozoan parasite *Leishmania donovani* is a major health problem with significant morbidity and mortality. Leishmaniasis is estimated to afflict 12 million people worldwide, causing disease ranging from skin lesion in cutaneous leishmaniasis to a progressive and fatal hepatosplenomegaly in visceral leishmaniasis (C.R Davies, P. Kaye, S.L.Croft and S. Sundar, Leishmaniasis: new approaches to
30 disease control, British Medicinal Journal. (2003) Vol. 15 pp. 377-382). Chemotherapy is still a major challenge to combat leishmaniasis despite the rapid and tremendous development of medicinal and pharmaceutical industry in recent decades. Even today, the pentavalent antimonials sodium antimony gluconate (SAG) has remained the first-

line treatment of visceral leishmaniasis (VL) or kala-azar with its extended treatment regimens, peritoneal administration and toxic side effects (Santu Bandyopadhyay, Bikash Pal, Samir Bhattacharya, Mitali Ray, Keshab Chandra Roy (2003) U.S.Patent no. 6,610,332). Non-availability of antileishmanial vaccine makes the situation more difficult. Recently, co-infection of visceral leishmaniasis patients with HIV makes the search for new agents for the treatment of leishmaniasis more urgent. Compounding this problem is the fact that many countries and regions where the disease is endemic are economically poor.

This lack of an effective antileishmanial drug and urgency of the present drug situation has generated a renewed interest in the study of traditional remedies to develop new antileishmanial chemotherapeutic agents with better activity and less toxicity. Few medicinal plants have been studied for the treatment of this parasitic disease but their value as drug candidates cannot be fully assessed since systemic in vivo studies on animal models were not generated. In addition, extensive use of herbal therapy in leishmania endemic regions has created interest in evaluation of plant remedies used in traditional medicine as sources of potential antileishmanial agent (M.M. Iwu, J.E. Jackson and B.G. Schuster, Medicinal Plants in the Fight against Leishmaniasis, Parasitology Today, (1994) Vol.10.no.2, pp.65-68). *Nyctanthes arbortristis*, is a traditionally used medicinal plant of India commonly used as an anthelmintic, antibilious, diaphoretic etc. and for the treatment of various diseases such as fever, rheumatism, intestinal worm disinfections etc. (The Wealth of India, Raw Materials, (1966) Vol. 7, pp 69-70). Tribal people of central India used various parts of this plant for relieving cough, dysentery, snakebite, sores etc. (S.K.Jain, Dictionary of Indian folk medicine and Ethnobotany, 1991). Most of the recent bioactivity studies on *N. arbortristis* has been carried out from seed extract and iridoid glycosides (arbortristoside A, B, C, D, E) were isolated which showed antiviral (Anita Rathore, Vandita Srivastava, K.C.Srivastava and J.S. Tandon, Phytochemistry (1990) Vol.29 pp.1917-1920) or antileishmanial activity (J.S.Tandon, Vandita Srivastava and P.Y.Guru, Journal of Natural Products, (1991) Vol.54 pp.1102-1104). Wherein, in the present study calceolarioside A, a new class of compound to be reported from this plant, was isolated and purified from the methanolic extract of night jasmine leaf and showed significant antileishmanial activity both in vitro and in vivo test systems. This

is the first isolation report of calceolarioside A from this plant as well as the first antileishmanial activity report of calceolarioside A.

Objects of the invention:

5 The main object of the present invention is to provide a bioactive fraction obtained from the leaves of the plant *night jasmine*.

Another object of the present invention is to provide the use of said bioactive fraction in the treatment of visceral leishmaniasis.

10 Further object of the present invention is to provide the use of the compound calceolarioside A as a leishmanicidal agent.

Yet another object of the present invention is to provide a pharmaceutical composition useful as a leishmanicidal agent, wherein the said composition comprising the therapeutically effective amount of said bioactive fraction obtained from the leaves of the plant *night jasmine* or selected from the combination comprising the bioactive
15 fraction and sodium antimony gluconate or bioactive fraction and piperine or bioactive fraction and sodium antimony gluconate and piperine optionally along with one or more pharmaceutically acceptable carriers, additives, lubricant and diluents.

Still another object of the present invention is to provide a method for the isolation of compound calceolarioside A from the bioactive fraction obtained from the leaves of the
20 plant *Nyctanthes arbortristis*.

Summary of the invention:

The present invention deals with a bioactive fraction obtained from the leaves of the plant *night jasmine* comprising a) calceolarioside A b) unidentified compounds c)
25 kaempferol glycoside. It also provides the use of the said fraction and the compound calceolarioside A in the treatment of visceral leishmaniasis. Further, it also relates to a pharmaceutical composition and method of treating visceral leishmaniasis using said composition. The invention also relates to a method of isolation of said compound from the bioactive fraction obtained from the leaves of the plant *Nyctanthes arbortristis*.

30

Brief description of the figures:

Figure 1 represents the effect of crude methanolic extract of *Nyctanthes arbortristis* leaf and PE on the growth of *Leishmania donovani* promastigotes in vitro in time and dose

dependent manner. Viable cells, i.e., promastigotes ($\times 10^6$)/ml of culture. Data are shown for one representative experiment out of three with similar results. Solvent was DMSO+PBS.

Figure 2 represents the effect of *Nyctanthes arbortristis* crude methanolic leaf extract against established infection of visceral leishmaniasis in golden hamster model. Two weeks infected golden hamsters were treated with crude extract at the dose of 10mg/kg, 20mg/kg, 40 mg/kg and 60mg/kg body weight for 21 days. Controls were infected untreated animals. Crude extract was administered through i.p route. Treated animals received total six shots of crude extract during three weeks of treatment schedule. Animals were sacrificed after six weeks of infection. Levels of parasite burden in spleen and liver are expressed in Leishman-Donovan units (LDU).

Figure 3 represents the effect of methanolic extract of *Nyctanthes arbortritis* leaf and SAG in established infection of *Leishmania donovani*. Four weeks infected golden hamsters were treated intra-peritoneally with crude methanolic extract at the doses of 10mg/kg body weight, 20 mg/kg body weight and SAG 10mg/kg body weight for 7 days respectively. Controls were infected untreated animals. Treated animals received one shot in one week to complete the treatment. Level of parasite burden in spleen is expressed in Leishman-Donovan units (LDU). DMSO: PBS=DMSO+PBS as solvent of crude extract

Figure 4 represents the effect of bioactive fraction and combined therapy of bioactive fraction + SAG in established infection of visceral leishmaniasis. Two weeks infected golden hamsters were treated intra-peritoneally with bioactive fraction at the dose of 100mg/kg body weight, SAG at dose of 5mg/kg body weight and bioactive fraction +SAG at the dose of 100+5mg/kg body weight for 21 days. Controls were infected untreated animals. Treated animals received total three shots during three weeks of treatment schedule. Animals were sacrificed after six weeks of infection. Level of parasite burden in spleen and liver are expressed in Leishman-Donovan units (LDU).

Figure 5 represents the therapeutic efficacy of bioactive fraction and combined therapy of bioactive fraction + SAG in chronic visceral leishmaniasis. Eight weeks infected golden hamsters were treated intra-peritoneally with bioactive fraction at the doses of 100,150,200mg/kg body weight and bioactive fraction +SAG at the doses of 150+5,200+5,200+10mg/kg body weight respectively for 21 days. Controls were

infected untreated animals. Treated animals received total six shots during three weeks of treatment schedule. Animals were sacrificed after 12 weeks of infection. Level of parasite burden in spleen and liver are expressed in Leishman-Donovan units (LDU).

Figure 6 represents the effect of calceolarioside A on the growth of *Leishmania donovani* promastigotes in vitro in time and dose dependent manner. Viable cells, i.e., promastigotes ($\times 10^6$)/ml of culture. Data are shown for one representative experiment out of three with similar results. NA1=CalceolariosideA; Solvent=DMSO+PBS

Figure 7 represents the effect of bioactive fraction and combined therapy of bioactive fraction, SAG and piperine against established infection of visceral leishmaniasis in golden hamster model. Effect of bioactive fraction, combined therapy of bioactive fraction + piperine, bioactive fraction + SAG and bioactive fraction + SAG + piperine against established infection of visceral leishmaniasis in golden hamster model. Two weeks infected golden hamsters were treated with bioactive fraction at the dose of 100mg/kg body weight, bioactive fraction + piperine at the dose of 100 + 5 mg/kg body weight and bioactive fraction + SAG at the dose of 100 + 5mg/kg body weight and bioactive fraction + SAG + piperine at the dose of 100mg+2.5mg+5mg/kg body weight were given for 21 days. Controls were infected untreated animals. Bioactive fraction and SAG were administered i.p. and free piperine was administered orally as dry powder. Treated animals received total six shots of bioactive fraction and SAG (i.p.) and free piperine was administered orally as dry powder before two hours of each bioactive fraction dose during three weeks of treatment schedule. Animals were sacrificed after six weeks of infection. Levels of parasite burden in spleen and liver are expressed in Leishman-Donovan units (LDU).

Fig 8: HPLC chromatogram of bioactive fraction obtained from methanolic extract of night jasmine leaf.

First Peak: Calceolarioside A

Second Peak: Kaempferol glycoside

Two unidentified very small peak

Fig 9: HPLC chromatogram of calceolarioside A obtained from methanolic extract of night jasmine leaf.

Detailed description of the invention:

Accordingly, the present invention provides a bioactive fraction obtained from the leaves of the plant *night jasmine*, wherein the said fraction comprising a) calceolarioside A 39.62% b) kaempferol glycoside 34.8%, c) two unidentified compound 5.2% & 5.8% and d) unidentified unresolved fraction 14.6%, wherein said weight percentages are percentages in the total weight of the fraction.

In an embodiment of the present invention, the said fraction is useful as a leishmanicidal agent.

In another embodiment of the present invention, the said fraction is non toxic to liver and kidney at the dose of 200mg/Kg body weight / day at least for three weeks.

The present invention also provides the use of the bioactive fraction in the treatment of visceral leishmaniasis.

Further, it provides the use of the compound calceolarioside A as a leishmanicidal agent.

In an embodiment of the present invention, the said compound reduces the viability of *L. donovani* promastigotes in vitro upto 100 % at the dose of 80 µg/ml of culture media for 24 hrs.

The present invention also provides a pharmaceutical composition useful as a leishmanicidal agent, wherein the said composition comprising the therapeutically effective amount of said bioactive fraction obtained from the leaves of the plant *night jasmine* or selected from the combination comprising the bioactive fraction and sodium antimony gluconate or bioactive fraction and piperine or bioactive fraction and sodium antimony gluconate and piperine optionally along with one or more pharmaceutically acceptable carriers, additives, lubricant and diluents.

In an embodiment of the present invention, the additives used are selected from the group comprising of proteins, carbohydrates, sugar, talc, magnesium stearate, cellulose, calcium, carbohydrate, starch-gelatin paste.

In another embodiment of the present invention, the said composition further comprising the therapeutically effective amount of bioactive fraction obtained from the leaves of the plant *night jasmine* optionally along with one or more pharmaceutically acceptable carriers, additives and diluents.

Further, in another embodiment of the present invention, the dosage of the said composition is administered at a unit dose of at least 100 to 200 mg/Kg body weight.

In yet another embodiment of the present invention, the said composition is preferably administered at a unit dose of 100 mg/Kg body weight at least for three weeks wherein
5 the said composition reduces the parasitic load of spleen up to 81.6%.

In still another embodiment of the present invention, the said composition is preferably administered at a unit dose of 100 mg/Kg body weight at least for three weeks wherein the said composition reduces the parasitic load of liver up to 85.42%.

In still another embodiment of the present invention, the said composition further
10 comprising the therapeutically effective amount of bioactive fraction and sodium antimony gluconate optionally along with one or more pharmaceutically acceptable carriers, additives and diluents wherein the ratio of bioactive fraction and sodium antimony gluconate is in the range of 200:1 to 20:1.

In still another embodiment of the present invention, the dosage of the said composition
15 is administered at a unit dose of at least 150mg/Kg body weight of bioactive fraction and 5 mg/Kg body weight of sodium antimony gluconate to 200mg/Kg body weight of bioactive fraction and 10 mg/Kg body weight of sodium antimony gluconate.

In still another embodiment of the present invention, wherein the dosage of the said composition is preferably administered at a unit dose of 150mg/Kg body weight of
20 bioactive fraction and 5 mg/Kg body weight of sodium antimony gluconate at least for three weeks wherein the said composition reduces the parasitic load of spleen up to 95.1%.

In still another embodiment of the present invention, wherein the dosage of the said composition is preferably administered at a unit dose of 150mg/Kg body weight of
25 bioactive fraction and 5 mg/Kg body weight of sodium antimony gluconate at least for three weeks wherein the said composition reduces the parasitic load of liver up to 99.85%.

In still another embodiment of the present invention, wherein the dosage of the said composition is preferably administered at a unit dose of 200mg/Kg body weight of
30 bioactive fraction and 10 mg/Kg body weight of sodium antimony gluconate at least for three weeks wherein the said composition reduces the parasitic load of liver and spleen up to 100%.

In still another embodiment of the present invention, wherein the said composition further comprising the therapeutically effective amount of bioactive fraction and piperine optionally along with one or more pharmaceutically acceptable carriers, additives and diluents wherein the ratio of bioactive fraction and piperine is in the range of 200:1 to 20:1.

In still another embodiment of the present invention, wherein the dosage of the said composition is administered at a unit dose of 100mg/Kg body weight of bioactive fraction and 0.5 mg/Kg body weight of piperine to 100 mg/Kg body weight of bioactive fraction and 5.0 mg/Kg body weight of piperine.

In still another embodiment of the present invention, wherein the dosage of the said composition is preferably administered at a unit dose of 100mg/Kg body weight of bioactive fraction and 0.5 mg/Kg body weight of piperine at least for three weeks wherein the said composition reduces the parasitic load of spleen up to 98.8%.

In still another embodiment of the present invention, wherein the dosage of the said composition is preferably administered at a unit dose of 100mg/Kg body weight of bioactive fraction and 0.5 mg/Kg body weight of piperine at least for three weeks wherein the said composition reduces the parasitic load of liver up to 79.8%.

In still another embodiment of the present invention, the dosage of the said composition is preferably administered at a unit dose of 100mg/Kg body weight of bioactive fraction and 5.0 mg/Kg body weight of piperine at least for three weeks wherein the said composition reduces the parasitic load of liver and spleen up to 100%.

In still another embodiment of the present invention, the said composition further comprising the therapeutically effective amount of bioactive fraction and sodium antimony gluconate and piperine optionally along with one or more pharmaceutically acceptable carriers, additives and diluents wherein the ratio of bioactive fraction and sodium antimony gluconate and piperine is in the range of 40: 1: 0.2 to 40: 1: 2

In still another embodiment of the present invention, the dosage of the said composition is administered at a unit dose of 100mg/Kg body weight of bioactive fraction and 2.5 mg/Kg body weight of sodium antimony gluconate and 0.5 mg/Kg body weight of piperine to 100mg/Kg body weight of bioactive fraction and 2.5 mg/Kg body weight of sodium antimony gluconate and 5.0 mg/Kg body weight of piperine.

In still another embodiment of the present invention, the dosage of the said composition is preferably administered at a unit dose of 100mg/Kg body weight of bioactive fraction and 2.5 mg/Kg body weight of sodium antimony gluconate and 5.0mg/Kg body weight of piperine at least for three weeks wherein the said composition reduces the parasitic
5 load of liver and spleen up to 100%.

Further, the invention provides a method of treating visceral leishmaniasis in a subject, wherein the said method comprising the step of administering to the subject a pharmaceutical composition comprising the therapeutically effective amount of said bioactive fraction obtained from the leaves of the plant *night jasmine* or selected from
10 the combination comprising the bioactive fraction and sodium antimony gluconate or bioactive fraction and piperine or bioactive fraction and sodium antimony gluconate, and piperine optionally along with one or more pharmaceutically acceptable carriers, additives, lubricant and diluents.

In an embodiment of the present invention, the said subject is mammal including
15 human.

In another embodiment of the present invention, the additives used are selected from the group comprising of proteins, carbohydrates, sugar, talc, magnesium state, cellulose, calcium, carbohydrate, starch-gelatin paste.

In another embodiment of the present invention, the said method further comprising the step of administering to the subject a pharmaceutical composition comprising
20 therapeutically effective amount of bioactive fraction obtained from the leaves of the plant *night jasmine* optionally along with one or more pharmaceutically acceptable carriers, additives and diluents.

Further, in an embodiment of the present embodiment of the present invention, the said
25 composition is administered at a unit dose of at least 100-to 200-mg/Kg-body weight.

In yet another embodiment of the present invention, the said composition is preferably administered at a unit dose of 100-mg/Kg body weight at least for three weeks wherein the said composition reduces the parasitic load of spleen up to 81.6%.

In still another embodiment of the present invention, the said composition is preferably
30 administered at a unit dose of 100-mg/Kg body weight at least for three weeks wherein the said composition reduces the parasitic load of liver up to 85.42%.

In still another embodiment of the present invention, the said method further comprising the step of administering to the subject a pharmaceutical composition comprising the therapeutically effective amount of bioactive fraction and sodium antimony gluconate optionally along with one or more pharmaceutically acceptable carriers, additives and diluents wherein the ratio of bioactive fraction and sodium antimony gluconate is in the range of 200:1 to 20:1.

In still another embodiment of the present invention, the said composition is administered at a unit dose of at least 150mg/Kg body weight of bioactive fraction and 5 mg/Kg body weight of sodium antimony gluconate to 200mg/Kg body weight of bioactive fraction and 10 mg/Kg body weight of sodium antimony gluconate.

In still another embodiment of the present invention, the said composition is preferably administered at a unit dose of 150mg/Kg body weight of bioactive fraction and 5 mg/Kg body weight of sodium antimony gluconate at least for three weeks wherein the said composition reduces the parasitic load of spleen up to 95.1%.

In still another embodiment of the present invention, the said composition is preferably administered at a unit dose of 150mg/Kg body weight of bioactive fraction and 5 mg/Kg body weight of sodium antimony gluconate at least for three weeks wherein the said composition reduces the parasitic load of liver up to 99.85%.

In still another embodiment of the present invention, the said composition is preferably administered at a unit dose of 200mg/Kg body weight of bioactive fraction and 10 mg/Kg body weight of sodium antimony gluconate at least for three weeks wherein the said composition reduces the parasitic load of liver and spleen up to 100%.

In still another embodiment of the present invention, the said wherein the said method further comprising the step of administering to the subject a pharmaceutical composition comprising the therapeutically effective amount of bioactive fraction and piperine optionally along with one or more pharmaceutically acceptable carriers, additives and diluents wherein the ratio of bioactive fraction and piperine is in the range of 200:1 to 20:1.

In still another embodiment of the present invention, the said composition is administered at a unit dose of 100mg/Kg body weight of bioactive fraction and 0.5 mg/Kg body weight of piperine to 100 mg/Kg body weight of bioactive fraction and 5.0 mg/Kg body weight of piperine.

In still another embodiment of the present invention, the said composition is preferably administered at a unit dose of 100mg/Kg body weight of bioactive fraction and 0.5 mg/Kg body weight of piperine at least for three weeks wherein the said composition reduces the parasitic load of spleen up to 98.8%.

- 5 In still another embodiment of the present invention, the said composition is preferably administered at a unit dose of 100mg/Kg body weight of bioactive fraction and 0.5 mg/Kg body weight of piperine at least for three weeks wherein the said composition reduces the parasitic load of liver up to 79.8%.

- 10 In still another embodiment of the present invention, the said composition is preferably administered at a unit dose of 100mg/Kg body weight of bioactive fraction and 5.0 mg/Kg body weight of piperine at least for three weeks wherein the said composition reduces the parasitic load of liver and spleen up to 100%.

- 15 In still another embodiment of the present invention, the said wherein the said method further comprising the step of administering to the subject a pharmaceutical composition comprising the therapeutically effective amount of bioactive fraction and sodium antimony gluconate and piperine optionally along with one or more pharmaceutically acceptable carriers, additives and diluents wherein the ratio of bioactive fraction and sodium antimony gluconate and piperine is in the range of 40: 1: 0.2 to 40: 1: 2.

- 20 In still another embodiment of the present invention, the said composition is administered at a unit dose of 100mg/Kg body weight of bioactive fraction and 2.5 mg/Kg body weight of sodium antimony gluconate and 0.5 mg/Kg body weight of piperine to 100mg/Kg body weight of bioactive fraction and 2.5 mg/Kg body weight of sodium antimony gluconate and 5.0 mg/Kg body weight of piperine.

- 25 In still another embodiment of the present invention, the said composition is preferably administered at a unit dose of 100mg/Kg body weight of bioactive fraction and 2.5 mg/Kg body weight of sodium antimony gluconate and 5.0mg/Kg body weight of piperine at least for three weeks wherein the said composition reduces the parasitic load of liver and spleen up to 100%.

- 30 In still another embodiment of the present invention, the administration route used is selected from the group consisting of oral, intraperitoneal, intravenous, intra muscular.

In still another embodiment of the present invention, the said form for oral route is selected from the group consisting of capsule, syrup, concentrate, powder and granules.

In still another embodiment of the present invention, the effective dose of the said composition is administered in such a manner that it does not interfere the other physiological activities.

In still another embodiment of the present invention, the effective dose of the said composition is administered in such a manner that it does not create any clinical and pathological toxicity to the subject.

Still the present invention also provides a method for the isolation of compound calceolarioside A from the bioactive fraction, wherein the said method comprising the steps of:

- a) extracting the powdered dry mass of fresh leaves of *Nyctanthes arbortristis* with alcohol,
- b) concentrating the extract obtained from step (a) under reduced pressure at at least 40°C to obtain semi solid mass;
- c) performing repeatedly chromatography using organic solvent of the said mass as obtained from step (b) to get bioactive fraction;
- d) performing thin layer chromatography to get desired compound calceolarioside A.

In an embodiment of the present invention, the leaves of *Nyctanthes arbortristis* are collected from Jhargram, West Bengal and voucher specimen submitted to Botanical Survey of India, Shibpur, Howrah (vide voucher specimen no. NA101, dated 28.06.2006).

In another embodiment of the present invention, the alcohol used is selected from the group comprising of methanol, ethanol, n-butanol.

Further, in an embodiment of the present invention, the semi solid mass of step (b) is chromatographed over a silica gel column having mesh size 60nm-120nm.

In yet another embodiment of the present invention, the organic solvent used is chloroform.

The present invention is related to a method for novel drug development from natural sources without any clinical and pathological toxic effects, wherein the said method comprising preparing methanolic extract of night jasmine leaf, chromatographic

purification of said extract, isolation bioactive fraction of containing a compound calceolarioside A , treatment of *L. donovani* infected chronic golden hamster model by administering (i.p) bioactive fraction at 100mg, 150mg and 200mg/kg body weight/day for three weeks, determination of LDU for the detection of parasite load in liver, spleen and bone marrow, combination therapy treatment of bioactive fraction + sodium antimony gluconate at a dose of 150mg + 5mg and 200mg + 10mg/kg body weight/day respectively for three weeks to obtain complete clearance of parasitemia, testing the leishmanicidal activity of calceolarioside A detected against axenic culture of *L. donovani* promastigotes, and administering bioactive fraction in normal healthy animal to obtain clinical and pathological non-toxic nature.

A method for using night jasmine leaf extract as leishmanicidal agent wherein the said method comprising

- a) methanolic extraction of night jasmine leaf and chromatographic purification to isolate calceolarioside A containing bioactive fraction (PE).
- b) administering bioactive fraction at least 100-200mg/kg body weight/day through i.p for a period of at least three weeks to the *L. donovani* infected chronic golden hamster model.
- c) administering bioactive fraction at least 100-200mg/kg body weight/day and SAG at least 5 and 10mg/kg body weight/day through i .p for a period of at least three weeks to the *L. donovani* infected chronic golden hamster model.
- d) antileishmanial activity of calceolarioside A against axenic *L. donovani* promastigote culture.

A pharmaceutical composition of the present invention is useful as leishmanicidal agent against chronic visceral leishmaniasis.

Abbreviations used:

SAG=Sodium Antimony Gluconate

LDU=Leishman-Donovan Unit

PE =Calceolariside A containing bioactive fraction

The following examples are given by way of illustration of the present invention and should not be construed to limit the scope of present invention

EXAMPLE 1

8 KG of fresh leaves of *Nyctanthes arbortristis* was thoroughly washed in sterile water, shed dried and powdered, then extracted overnight with 8 L of methanol at room temperature. The process was repeated thrice. The total pool of crude extract was
5 filtered through Whatman filter paper No 1 and the filtrate was collected. The combined filtrate was evaporated to dryness under reduced pressure below 40° C and kept at room temperature in vacuum desiccator under high vacuum yielding a semi-solid mass weighing 1360gm. Successive bio-activity guided fractionation and purification were performed with this dried extract.

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EXAMPLE 2

The crude extract was triturated with Ethyl Acetate (GR Merck) and the rest was again triturated with n-Butanol (GR Merck). The n-Butanol fraction was evaporated to dryness under reduced pressure at 40°C. This extract was chromatographed over a silica
15 gel column (mesh size 60-120) beginning the elution with Chloroform (GR Merck). Successive fractions were eluted from the column with increasing percentages of methanol in chloroform. When the fractions with 8-10% methanol were evaporated, a slightly brown colored amorphous powder was collected as a semi-pure bioactive fraction. All the in-vivo experiments were carried out with this bioactive fraction.

20

EXAMPLE 3

The pure compound calceolarioside A was collected by preparative thin layer chromatography from this semi-pure bioactive fraction of example 2. Identification of the pure compound was done with ¹H, and ¹³C Nuclear Magnetic Resonance (NMR).
25 Spectra recorded with a Bruker DPX-300 machine. Both positive and negative ion-mode mass spectrometry were done with Micromass Q-Tof microTM to determine the molecular weight.

¹H NMR in CD₃OD(300MHz)

δ 7.61(1H,d,J=15.9Hz), 7.07(1H,d,J=1.3Hz), 6.98(1H,br d, J=8.1Hz), 6.79(1H,d,J=8.1Hz),
30 6.69(2H, overlapping signal), 6.59(1H, br d, J=8.1 Hz), 6.31(1H, d, J=15.9Hz),
4.38(1H, d, J=7.8Hz), 4.07(1H, dt, J=8.7, 7.8Hz), 3.4-3.8 (several protons),
2.82(2H, T, J=7Hz), solvent signals at 4.9, 3.32 and 2.17 Hz.

¹³CNMR in CD₃OD(75MHz)

8 167.6(C), 148.7(C), 146.6(C), 145.8(C), 145.1(C), 143.7(C), 130.5(C), 126.7(C),
122.1(CH), 120.3(CH), 116.1(CH), 115.5(CH), 115.3(CH), 114.3(CH),
113.8(CH), 103.4(CH), 75.1(CH), 74.8(CH), 74.3(CH), 71.5(CH), 71.2(CH₂),
5 61.5 (CH₂), 35.6 (CH₂).

ESI MS(positive mode)

Here, $m/z=501(M+Na)^+$

ESI MS(negative mode)

Here, $m/z=477(M-H)^-$

10

Yield of Methanolic crude extract from Dry leaf =17.48%

Yield of n-Butanol fraction from Dry leaf =14.96%

Yield of calceolarioside A containing Bioactive fraction (PE) from Dry leaf =3.2%

Yield of calceolarioside A from Dry leaf =1.237%

15 Yield of kaempferol glycoside from Dry leaf =1.088%

Yield of calceolarioside A from PE =39.62%

Yield of kaempferol glycoside from PE =34.8%

Yield of Other unidentified compounds from PE =5.2% & 5.8%

20 **EXAMPLE 4**

Leishmania donovani Ag83 was isolated from an Indian kala-azar patient and maintained in Golden hamster (*Mesocricetus auratus*) model (A.K Ghosh, F. K. Bhattacharya and D.K Ghosh, *Leishmania donovani*: amastigote inhibition and mode of action of berberine (1985) Experimental Parasitology, Vol. 60 pp 404-413). Liquid
25 parasite culture was done in M199 media (Sigma, USA) supplemented with 10% Fetal Bovine Serum (Sigma, USA) at 22°C culture room. Stationary phase promastigotes of *L. donovani* were used for in-vitro assay. The in-vitro activity of crude extract, bioactive fraction and pure compound calceolarioside A was determined on stationary phase promastigotes of *L. donovani* Ag83 strain. The stationary phase promastigotes were
30 collected from parasite culture by centrifugation and re-suspended with 10% FBS supplemented M199 medium. For drug sensitivity assay 50mg/ml stock solution for each test material were prepared with minimum amount of DMSO and PBS and serially diluted with sterile PBS to achieve the desired dose. Assay was performed in sterile

tubes with 450µl of parasite suspension. Then 50µl of test solutions were added to each tube and one control only with solvent (DMSO and PBS) was maintained. Tubes were incubated at 22°C for 72hrs and data was collected after each 24hrs. Mortality rate of the parasite was determined microscopically on Neubauer Counter Chamber (HBG, Germany).

EXAMPLE 5

In-vivo antileishmanial activity of bioactive fraction in the L. donovani infected golden hamster model:

Male 4-6 weeks old Golden hamster (*M. auratus*) were infected with 2.5×10^7 amastigotes of *L. donovani* intracardially on day 0 and randomly allocated into groups of 6 animals each. The amastigotes were obtained from the spleen of a two months infected donor hamster for infection. The bioactive fraction was dissolved in minimum amount of DMSO and then diluted accordingly with sterile PBS. SAG was diluted in sterile PBS. The formulations were administered, once or twice per week for one or three successive weeks, intraperitoneally at different doses after two weeks or four weeks or eight weeks of infection according to the experiment. Fresh solutions were made before each experiment. One untreated group and one solvent (DMSO and PBS) treated group were always maintained simultaneously.

EXAMPLE 6

In-vivo antileishmanial activity of bioactive fraction and piperine as bioenhancer in the L. donovani infected golden hamster model:

Male 4-6 weeks old Golden hamster (*M. auratus*) were infected with 2.5×10^7 amastigotes of *L. donovani* intracardially on day 0 from the spleen of a two months infected donor hamster for infection. The bioactive fraction was dissolved in minimum amount of DMSO and then diluted accordingly with sterile PBS. The bioactive fraction was administered twice per week for three successive weeks intraperitoneally at 100mg/Kg body weight dose after two weeks of infection. Piperine was administered orally as dry powder two hour before the intraperitoneal injection of bioactive fraction. Fresh solutions were made before each experiment. One untreated group and one solvent (DMSO and PBS) treated group were always maintained simultaneously.

EXAMPLE 7***In-vivo toxicity testing of bioactive fraction, SAG and bioactive fraction+ SAG in the *L. donovani* infected golden hamster model:***

Male 4-6 weeks old Golden hamster (*M. auratus*) were infected with 2.5×10^7 amastigotes of *L. donovani* intracardially on day 0 from the spleen of a two months infected donor hamster for infection. The bioactive fraction was dissolved in minimum amount of DMSO and then diluted accordingly with sterile PBS. The bioactive fraction was administered twice per week for three successive weeks intraperitoneally at 200mg/Kg body weight dose, SAG at 5mg/Kg body weight dose and bioactive fraction SAG at 200mg+5mg/Kg body weight dose after eight weeks of infection. Fresh solutions were made before each experiment. One untreated group was always maintained simultaneously. Animals were sacrificed after twelve weeks of infection. Blood was collected and sera isolated for assessment of specific enzyme level as Serum Glutamate Pyruvate Transaminase, Alkaline Phosphatase, Serum Creatinine and Blood Urea. All assay were done using Reddy's Laboratory kit according to manufacturer's protocol.

TABLE 1: Specific enzyme levels in sera of golden hamster undergoing experimental visceral leishmaniasis indicating non-toxic nature of bioactive fraction

Group and final Concentration	SGPT (Unit/ml)	Alkaline Phosphatase (King-Armstrong unit)	Serum creatinine (mg %)	Blood Urea (mg %)
Normal	49.6±11.63	8.8 ±1.5	0.37±0.06	33.9±2.31
Infected	156.74±10.93	19.98±4.73	0.58±0.11	68.43±13.29
PE treated (200mg/kgBW)	131.37±27.38	16.3±5.77	0.39±0.04	36.64±5.63
SAG treated (5mg/kgBW)	123.78±27.34	16.9±5.62	0.66±0.07	66.35±13.95
PE+SAG treated (200mg+5mg/kgBW)	115.33±35.78	19±4.8	0.38±0.06	51.77±9.7

Combined therapy of bioactive fraction and SAG is significantly reducing the SGPT level where $p=0.015$.

EXAMPLE 8

HPLC (Shimadzu, Japan) chromatogram of bioactive fraction containing calceolarioside A is obtained from methanolic crude extract of night jasmine leaf. The analysis was performed with C₁₈ column (250x4 mm I. D; SGE International PTY, Australia) attached with a Guard column (Supelco) a Rheodyne injection valve with a 100- μ l loop and PDA detector (SPD-M 10 Avp). A linear gradient elution was carried out with methanol: water mixtures from 0:100,(v/v) to 100:0,(v/v) within 0 to 60 min at a flow rate of 0.5 ml/min. The column was maintained at room temperature and the detection was performed at UV 210 nm and 254 nm.

EXAMPLE 9

HPLC chromatogram of was performed calceolarioside A from methanolic extract of night jasmine leaf with the same method as mentioned in Example 6.

Advantages:

The main advantages of the present invention are:

1. A potential medicament against kala-azar, visceral leishmaniasis.
2. Herbal antileishmanial chemotherapeutic agent with better activity and less toxicity.
3. Calceolarioside A, novel strikingly potential antileishmanial drug candidate obtained from natural source.

We claim:

1. A bioactive fraction obtained from the leaves of the plant *night jasmine*, wherein the said fraction comprising a) calceolarioside A 39.62% b) kaempferol glycoside 34.8% c) two unidentified compound 5.2% & 5.8% and d)
5 unidentified unresolved fraction 14.6%, wherein said weight percentages are percentages in the total weight of the fraction.
2. A bioactive fraction as claimed in claim 1, wherein the said fraction is useful as a leishmanicidal agent.
3. A bioactive fraction as claimed in claim 1, wherein the said fraction is non toxic
10 to liver and kidney at the dose of 200mg/Kg body weight / day at least for three weeks.
4. Use of the bioactive fraction as claimed in claim 1 in the treatment of visceral leishmaniasis.
5. Use of the compound calceolarioside A as a leishmanicidal agent.
- 15 6. Use of the compound calceolarioside A as claimed in claim 5, wherein the said compound reduces the viability of *L. donovani* promastigotes in vitro upto 100 % at the dose of 80 µg/ml of culture media for 24 hrs.
7. A pharmaceutical composition useful as a leishmanicidal agent, wherein the said composition comprises a therapeutically effective amount of bioactive
20 fraction as claimed in claim 1 or selected from the combination comprising bioactive fraction and sodium antimony gluconate or bioactive fraction and piperine or bioactive fraction and sodium antimony gluconate and piperine optionally along with one or more pharmaceutically acceptable carriers, additives, lubricant and diluents.
- 25 8. A pharmaceutical composition as claimed in claim 7, wherein the additive used are selected from the group comprising of proteins, carbohydrates, sugar, talc, magnesium stearate, cellulose, calcium, carbohydrate, starch-gelatin paste.
9. A pharmaceutical composition as claimed in claim 7, wherein the said composition further comprising the therapeutically effective amount of
30 bioactive fraction obtained from the leaves of the plant *night jasmine* optionally along with one or more pharmaceutically acceptable carriers, additives and diluents.

10. A pharmaceutical composition as claimed in claim 9, wherein the dosage of the said composition is administered at a unit dose of at least 100-to 200-mg/Kg-body weight.
- 5 11. A pharmaceutical composition as claimed in claim 10, wherein the said composition is preferably administered at a unit dose of 100-mg/Kg body weight at least for three weeks wherein the said composition reduces the parasitic load of spleen up to 81.6%.
- 10 12. A pharmaceutical composition as claimed in claim 10, wherein the said composition is preferably administered at a unit dose of 100-mg/Kg body weight at least for three weeks wherein the said composition reduces the parasitic load of liver up to 85.42%.
- 15 13. A pharmaceutical composition as claimed in claim 7, wherein the said composition further comprising the therapeutically effective amount of bioactive fraction and sodium antimony gluconate optionally along with one or more pharmaceutically acceptable carriers, additives and diluents wherein the ratio of bioactive fraction and sodium antimony gluconate is in the range of 200:1 to 20:1.
- 20 14. A pharmaceutical composition as claimed in claim 13, wherein the dosage of the said composition is administered at a unit dose of at least 150mg/Kg body weight of bioactive fraction and 5 mg/Kg body weight of sodium antimony gluconate to 200mg/Kg body weight of bioactive fraction and 10 mg/Kg body weight of sodium antimony gluconate.
- 25 15. A pharmaceutical composition as claimed in claim 14, wherein the dosage of the said composition is preferably administered at a unit dose of 150mg/Kg body weight of bioactive fraction and 5 mg/Kg body weight of sodium antimony gluconate at least for three weeks wherein the said composition reduces the parasitic load of spleen up to 95.1%.
- 30 16. A pharmaceutical composition as claimed in claim 14, wherein the dosage of the said composition is preferably administered at a unit dose of 150mg/Kg body weight of bioactive fraction and 5 mg/Kg body weight of sodium antimony gluconate at least for three weeks wherein the said composition reduces the parasitic load of liver up to 99.85%.

17. A pharmaceutical composition as claimed in claim 14, wherein the dosage of the said composition is preferably administered at a unit dose of 200mg/Kg body weight of bioactive fraction and 10 mg/Kg body weight of sodium antimony gluconate at least for three weeks wherein the said composition reduces the parasitic load of liver and spleen up to 100%.
18. A pharmaceutical composition as claimed in claim 7, wherein the said composition further comprising the therapeutically effective amount of bioactive fraction and piperine optionally along with one or more pharmaceutically acceptable carriers, additives and diluents wherein the ratio of bioactive fraction and piperine is in the range of 200:1 to 20:1.
19. A pharmaceutical composition as claimed in claim 18, wherein the dosage of the said composition is administered at a unit dose of 100mg/Kg body weight of bioactive fraction and 0.5 mg/Kg body weight of piperine to 100 mg/Kg body weight of bioactive fraction and 5.0 mg/Kg body weight of piperine.
20. A pharmaceutical composition as claimed in claim 19, wherein the dosage of the said composition is preferably administered at a unit dose of 100mg/Kg body weight of bioactive fraction and 0.5 mg/Kg body weight of piperine at least for three weeks wherein the said composition reduces the parasitic load of spleen up to 98.8%.
21. A pharmaceutical composition as claimed in claim 19, wherein the dosage of the said composition is preferably administered at a unit dose of 100mg/Kg body weight of bioactive fraction and 0.5 mg/Kg body weight of piperine at least for three weeks wherein the said composition reduces the parasitic load of liver up to 79.8%.
22. A pharmaceutical composition as claimed in claim 19, wherein the dosage of the said composition is preferably administered at a unit dose of 100mg/Kg body weight of bioactive fraction and 5.0 mg/Kg body weight of piperine at least for three weeks wherein the said composition reduces the parasitic load of liver and spleen up to 100%.
23. A pharmaceutical composition as claimed in claim 7, wherein the said composition further comprising the therapeutically effective amount of bioactive fraction and sodium antimony gluconate and piperine optionally along

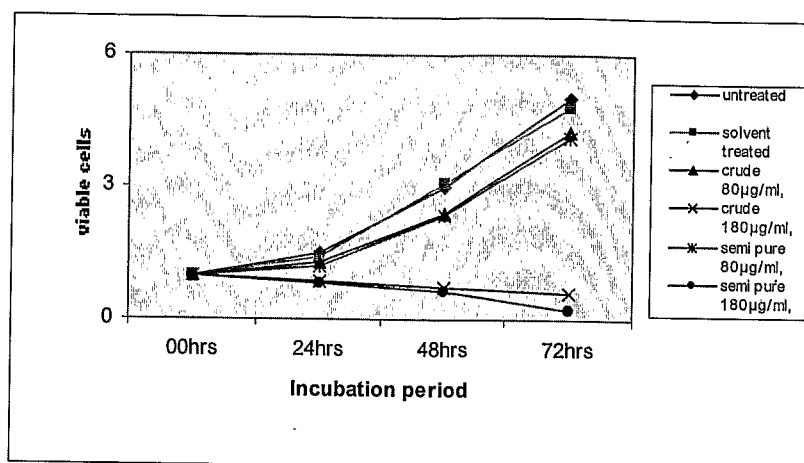
with one or more pharmaceutically acceptable carriers, additives and diluents wherein the ratio of bioactive fraction and sodium antimony gluconate and piperine is in the range of 40: 1: 0.2 to 40: 1: 2 .

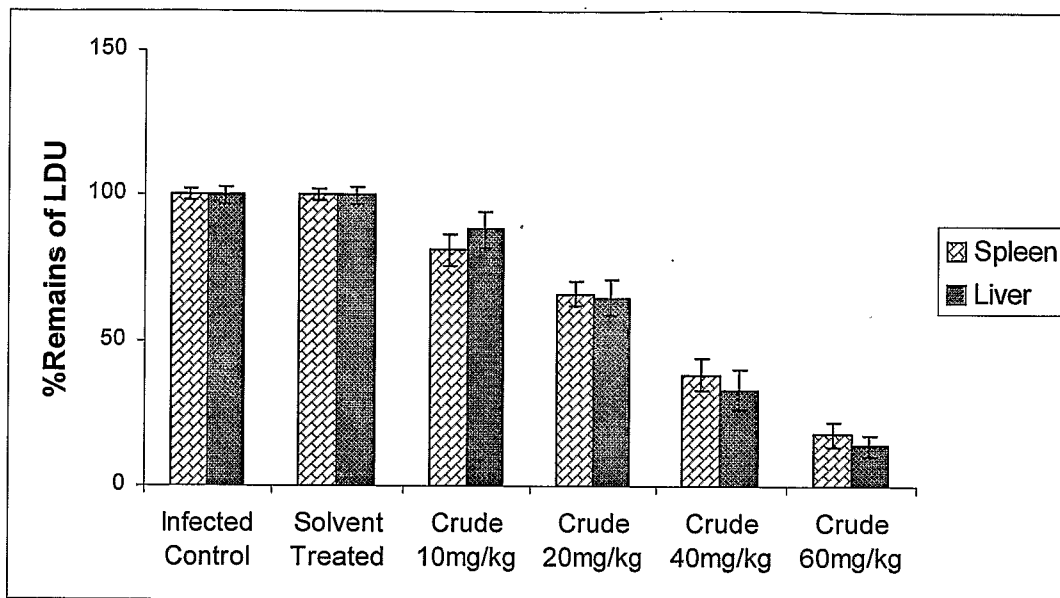
- 5 24. A pharmaceutical composition as claimed in claim 23, wherein the dosage of the said composition is administered at a unit dose of 100mg/Kg body weight of bioactive fraction and 2.5 mg/Kg body weight of sodium antimony gluconate and 0.5mg/Kg body weight of piperine to 100mg/Kg body weight of bioactive fraction and 2.5 mg/Kg body weight of sodium antimony gluconate and 5.0 mg/Kg body weight of piperine.
- 10 25. A pharmaceutical composition as claimed in claim 24, wherein the dosage of the said composition is preferably administered at a unit dose of 100mg/Kg body weight of bioactive fraction and 2.5 mg/Kg body weight of sodium antimony gluconate and 5.0mg/Kg body weight of piperine at least for three weeks wherein the said composition reduces the parasitic load of liver and spleen up to 100%.
- 15 26. A method of treating visceral leishmaniasis in a subject, wherein the said method comprising the step of administering to the subject a pharmaceutical composition comprising a therapeutically effective amount of bioactive fraction as claimed in claim 1 or selected from the combination comprising bioactive fraction and sodium antimony gluconate or bioactive fraction and piperine or bioactive fraction and sodium antimony gluconate and piperine optionally along with one or more pharmaceutically acceptable carriers, additives, lubricant and diluents.
- 20 27. A method as claimed in claim 26, wherein the said subject is mammal including human.
- 25 28. A method as claimed in claim 26, wherein the additive used are selected from the group comprising of proteins, carbohydrates, sugar, talc, magnesium state, cellulose, calcium, carbohydrate, starch-gelatin paste.
- 30 29. A method as claimed in claim 26, wherein the said method further comprising the step of administering to the subject a pharmaceutical composition as claimed in claim 9.

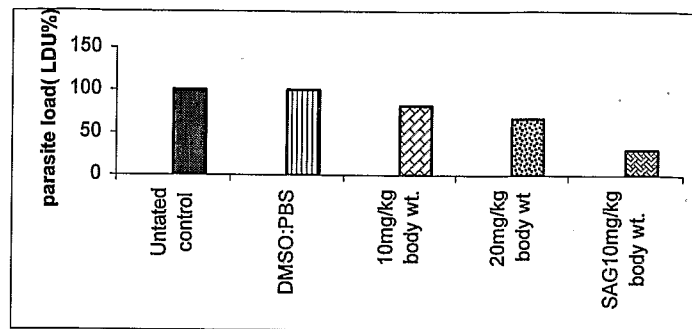
30. A method as claimed in claim 29, wherein the said composition is administered at a unit dose of at least 100-to 200-mg/Kg-body weight.
31. A method as claimed in claim 30, wherein the said composition is preferably administered at a unit dose of 100 mg/Kg body weight at least for three weeks wherein the said composition reduces the parasitic load of spleen up to 81.6%.
32. A method as claimed in claim 30, wherein the said composition is preferably administered at a unit dose of 100-mg/Kg body weight at least for three weeks wherein the said composition reduces the parasitic load of liver up to 85.42%.
33. A method as claimed in claim 26, wherein the said method further comprising the step of administering to the subject a pharmaceutical composition as claimed in claim 11.
34. A method as claimed in claim 33, wherein the said composition is administered at a unit dose of at least 150mg/Kg body weight of bioactive fraction and 5 mg/Kg body weight of sodium antimony gluconate to 200mg/Kg body weight of bioactive fraction and 10 mg/Kg body weight of sodium antimony gluconate.
35. A method as claimed in claim 34, wherein the said composition is preferably administered at a unit dose of 150mg/Kg body weight of bioactive fraction and 5 mg/Kg body weight of sodium antimony gluconate at least for three weeks wherein the said composition reduces the parasitic load of spleen up to 95.1%.
36. A method as claimed in claim 34, wherein the said composition is preferably administered at a unit dose of 150mg/Kg body weight of bioactive fraction and 5 mg/Kg body weight of sodium antimony gluconate at least for three weeks wherein the said composition reduces the parasitic load of liver up to 99.85%.
37. A method as claimed in claim 34, wherein the said composition is preferably administered at a unit dose of 200mg/Kg body weight of bioactive fraction and 10 mg/Kg body weight of sodium antimony gluconate at least for three weeks wherein the said composition reduces the parasitic load of liver and spleen up to 100%.
38. A method as claimed in claim 26, wherein the said wherein the said method further comprising the step of administering to the subject a pharmaceutical composition as claimed in claim 13.

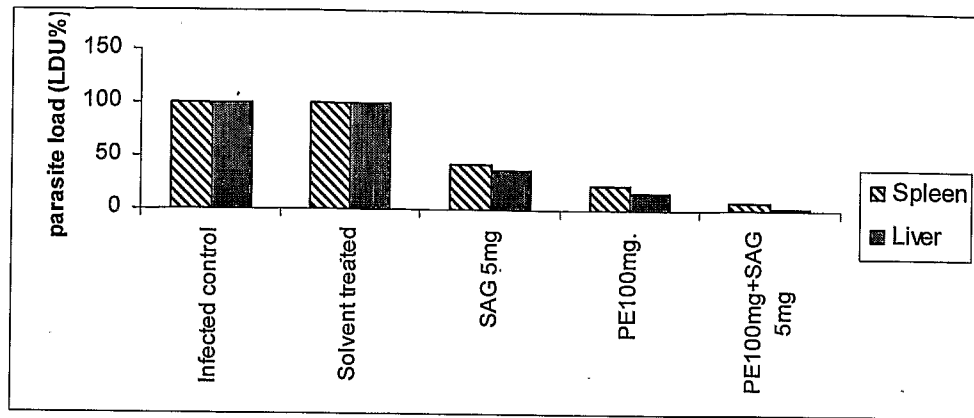
39. A method as claimed in claim 38, wherein the said composition is administered at a unit dose of 100mg/Kg body weight of bioactive fraction and 0.5 mg/Kg body weight of piperine to 100 mg/Kg body weight of bioactive fraction and 5.0 mg/Kg body weight of piperine.
- 5 40. A method as claimed in claim 39, wherein the said composition is preferably administered at a unit dose of 100mg/Kg body weight of bioactive fraction and 0.5 mg/Kg body weight of piperine at least for three weeks wherein the said composition reduces the parasitic load of spleen up to 98.8%.
- 10 41. A method as claimed in claim 39, wherein the said composition is preferably administered at a unit dose of 100mg/Kg body weight of bioactive fraction and 0.5 mg/Kg body weight of piperine at least for three weeks wherein the said composition reduces the parasitic load of liver up to 79.8%.
- 15 42. A method as claimed in claim 39, wherein the said composition is preferably administered at a unit dose of 100mg/Kg body weight of bioactive fraction and 5.0 mg/Kg body weight of piperine at least for three weeks wherein the said composition reduces the parasitic load of liver and spleen up to 100%.
43. A method as claimed in claim 26, wherein the said wherein the said method further comprising the step of administering to the subject a pharmaceutical composition as claimed in claim 21.
- 20 44. A method as claimed in claim 43, wherein the said composition is administered at a unit dose of 100mg/Kg body weight of bioactive fraction and 2.5 mg/Kg body weight of sodium antimony gluconate and 0.5mg /Kg body weight of piperine to 100mg/Kg body weight of bioactive fraction and 2.5 mg/Kg body weight of sodium antimony gluconate and 5.0 mg /Kg body weight of piperine.
- 25 45. A method as claimed in claim 44, wherein the said composition is preferably administered at a unit dose of 100mg/Kg body weight of bioactive fraction and 2.5 mg/Kg body weight of sodium antimony gluconate and 5.0mg/Kg body weight of piperine at least for three weeks wherein the said composition reduces the parasitic load of liver and spleen up to 100%.
- 30 46. A method as claimed in claim 26, wherein the administration route used is selected from the group consisting of oral, intraperitoneal, intramuscular, intravenous.

47. A composition as claimed in claim 7, wherein the composition is in form of capsule, syrup, concentrate, powder and granules.
48. A method for the isolation of compound calceolarioside A from the bioactive fraction of claim 1, wherein the said method comprising the steps of:
- 5 a. extracting the powdered dry mass of fresh leaves of *Nyctanthes arbortristis* with alcohol;
- b. concentrating the extract obtained from step (a) under reduced pressure at atleast 40°C to obtain semi solid mass;
- 10 c. performing repeatedly chromatography using organic solvent of the said mass as obtained from step (b) to get bioactive fraction;
- d. performing thin layer chromatography to get desired compound caleorlaroside A.
49. A method as claimed in claim 48, wherein the alcohol used is selected from the group comprising of methanol, ethanol, n-butanol.
- 15 50. A method as claimed in claim 48, wherein the semi solid mass of step (b) is chromatographed over a silica gel column having mesh size 60nm-120nm.
51. A method as claimed in claim 48, wherein the organic solvent used is chloroform.

**Figure 1**

**Figure 2**

**Figure 3**

**Figure 4**

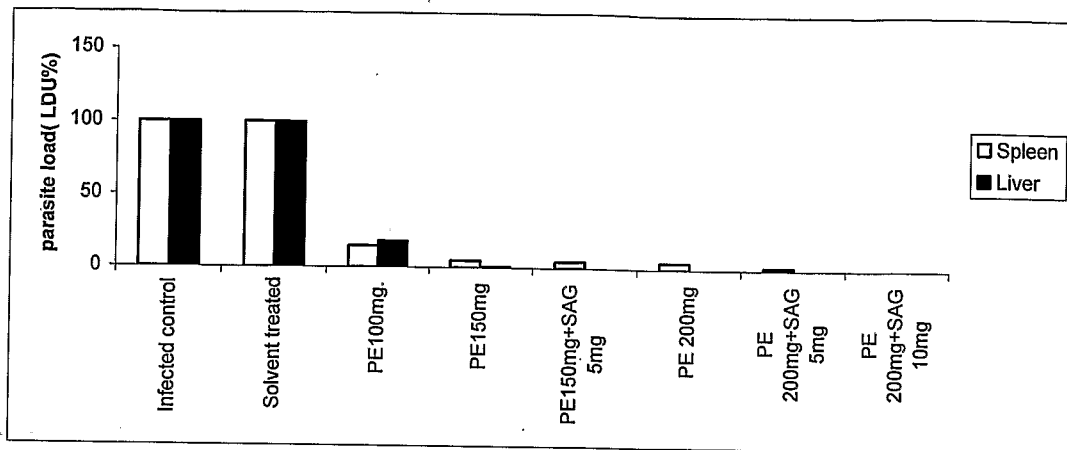


Figure 5

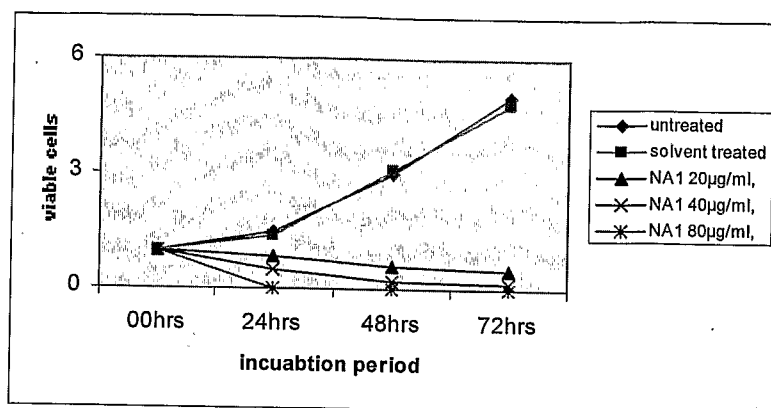


Figure 6

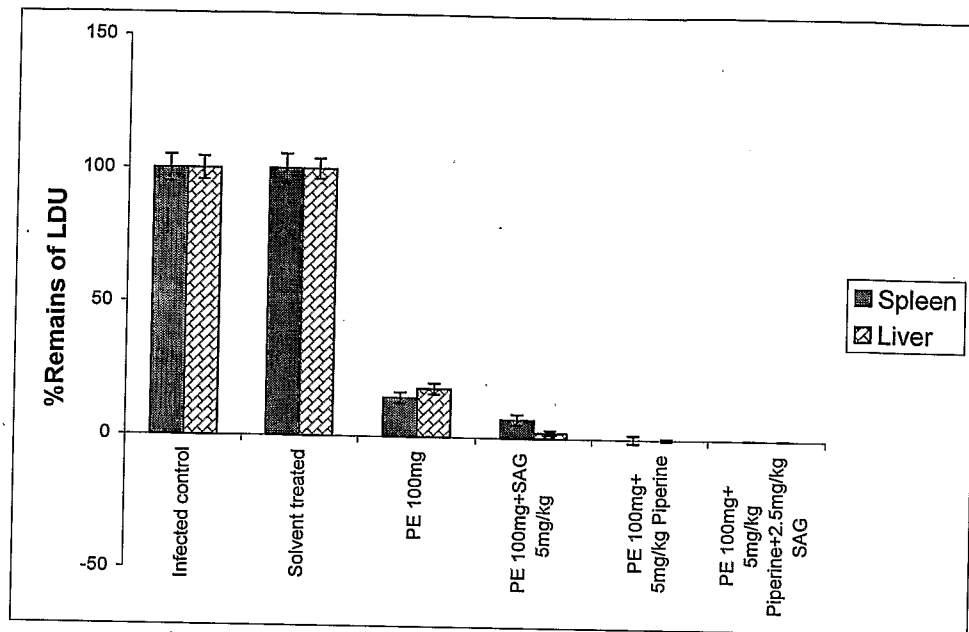


Figure 7

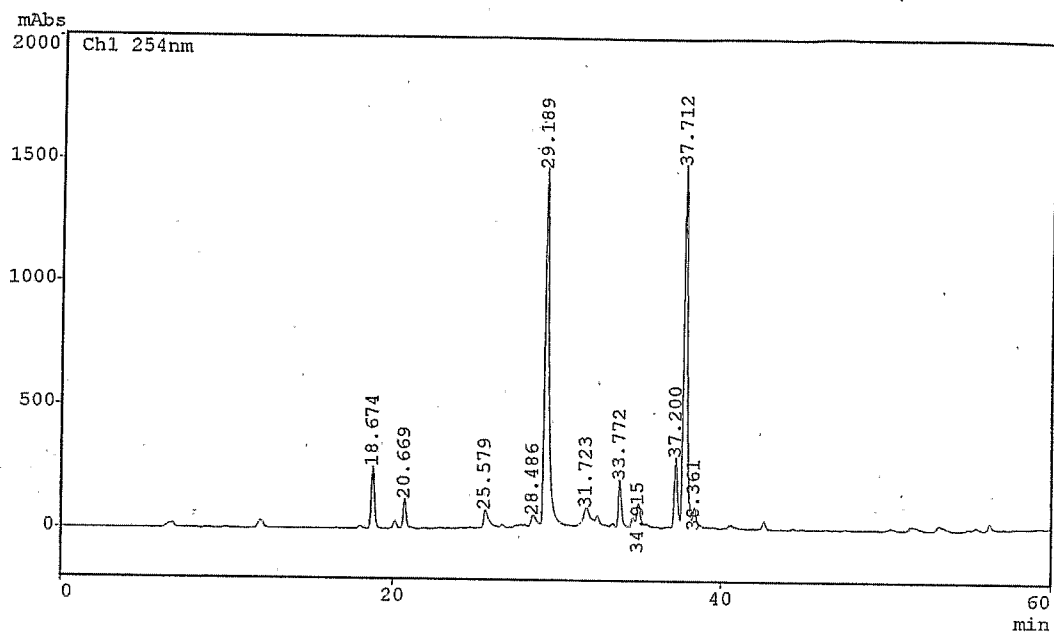


Figure 8

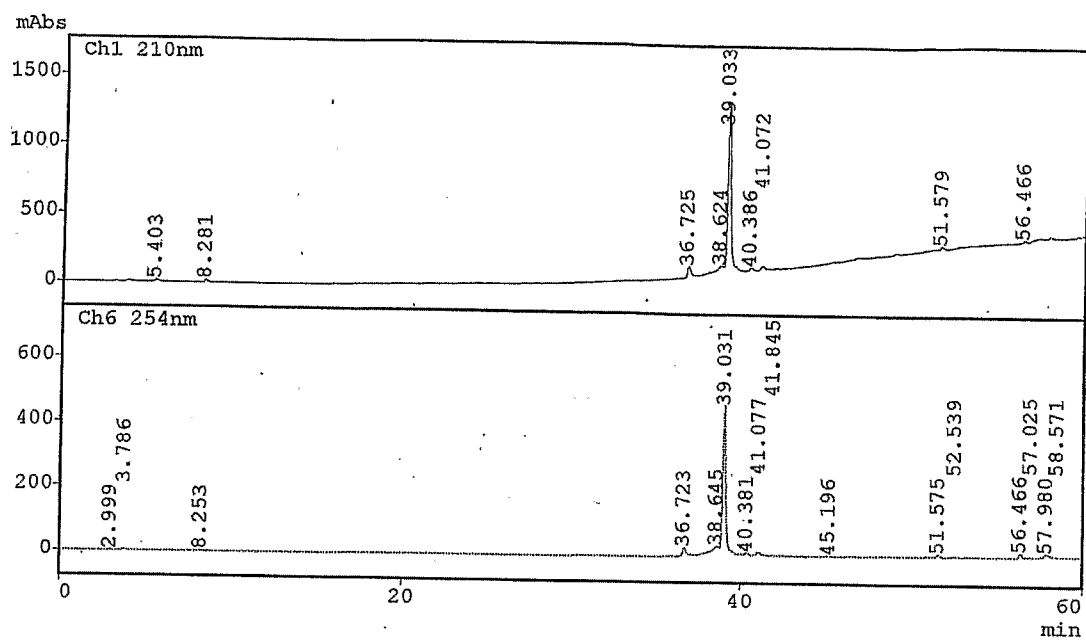


Figure 9