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In vitro Anti-leishmanial Activity of Traditional Medicinal Plants from Cameroon and Ghana

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Abstract: The aim of the study was to screen selected traditional medicinal plants from Cameroon and Ghana for their *in vitro* anti-leishmanial activity. The ethanolic and aqueous extracts of the selected plants were assessed for their effect on the promastigote stage of *Leishmania tarentolae*. Parasites were incubated with different concentrations of the extracts and proliferation inhibitory effects were monitored after 24 h and 48 h. Preliminary phytochemical screenings were carried out on extracts of these plants. Among the plants investigated in this study, extracts from *Steganotaenia araliacea*, *Anogeissus leiocarpus*, *Phyllanthus muellerianus* and *Hoslundia opposita* affected the proliferation of *L. tarentolae* most potently. Growth inhibition was concentration-dependent with an IC_{50} after 48 h ranging between 0.41-0.68 mg mL^{-1}. Preliminary phytochemical screenings were carried out on extracts revealing the presence of flavonoids, alkaloids, saponins, carbohydrates and tannins in the selected plants. This study reveals that *S. araliacae*, *A. leiocarpus*, *P. muellerianus* and *H. opposita* extracts could lead to an alternative application in the control of *Leishmania* infections.

Key words: *Leishmania, Steganotaenia, Anogeissus, Phyllanthus, Hoslundia*

INTRODUCTION

Leishmaniasis is a zoonotic infection caused by protozoa that belong to the genus *Leishmania* and it is transmitted by sandflies (*Phlebotomus* or *Lutzomyia* species). In the human host, *Leishmania* are intracellular parasites that infect the mononuclear phagocytes. Visceral leishmaniasis is caused by *L. donovani*. About 350 million people are at risk of infection. An estimated 12 million people are now thought to be infected worldwide with an approximately 1.5 to 2.0 million new cases occurring annually (Desjeux, 2004). In addition, 1.0 to 1.5 million cases of cutaneous leishmaniasis and 500,000 cases of visceral leishmaniasis occur each year (Braunwald *et al*., 2001). The disease is endemic in many regions of the world including developing countries and least developed countries and remains a serious public health problem (Tasdemir *et al*., 2006). Currently, the disease appears to be on the rise in Africa and the incidence of leishmaniasis in Africa is extensive (Boakye *et al*., 2005). The infected populations in Africa are not familiarized with the use of herbal to treat leishmaniasis. In the absence of a vaccine, drug treatment with pentavalent antimonials is still the first line of treatment for leishmaniasis (Firdous *et al*., 2009). Pentavalent antimonials, sodium stibogluconate and meglumine antimoniate have been used for decades as first choice drugs for the treatment of the disease and the current treatments for leishmaniasis are unsatisfactory due to their route of administration, severe side effects, high cost and lastly the development of resistance of *Leishmania* to the above drugs (Berman, 1997; Ephros *et al*., 1997; Boelaert *et al*., 2002; Georgopoulou *et al*., 2007). In addition to antimonials, various formulations of amphothericin B and pentamidine are still used in the treatment of leishmaniasis, although they are more toxic than drugs mentioned earlier (Lira *et al*., 1999).

Due to damages caused by leishmaniasis, there is an urgent need to develop new classes of drugs that are effective, affordable to resource poor people in developing countries and having minimum side effects (Tasdemir *et al*., 2006; Sundar *et al*., 2007). Many plant-derived bioactive substances with considerable therapeutic benefits have attracted interest in the scientific community over the last two decades (Fabricant and Farnsworth, 2001). In Africa and other developing countries, most people depend on herbal remedies for health needs (Fabricant and Farnsworth, 2001). Traditional medicinal plants in central and South America have been used to manage some of the symptoms of visceral leishmaniasis. Most of these herbal medicines or

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preparations have been used over the years and are most at times safer than the active compounds isolated from plants (Fabricant and Farnsworth, 2001). Some medicinal plants from central and South America have been investigated and found to be active against *L. donovani* (Sauvain *et al*., 1996; Ahua *et al*., 2007; Braga *et al*., 2007; Devkota *et al*., 2007). However just few works *in vitro* or *in vivo* on leishmaniasis has been so far reported with medicinal plants from Africa (Mesia *et al*., 2008). Here, we investigated the effects of extract of some Cameroonian and Ghanaian medicinal plants known to have anthelminthic and antimalaria properties, on the nonpathogenic model trypanosomatid *Leishmania tarentolae* (Belova, 1971; Elwasila, 1988), a parasite of geckos that has been exploited for a variety of molecular, biochemical, evolutionary and pharmacological studies (Thiemann *et al*., 1998). Furthermore, we performed preliminary phytochemical screening on these extracts.

Based on informations collected from traditional healers, the following plants were selected for further investigations:

Pupalia lappacea (L.) Juss. (Amaranthaceae) is an annual herb 30-120 cm high, sometimes woody below; branches shortly and softly pubescent. Poultice of the fresh leaves is used in the treatment of boils, chronic and fresh wounds. A decoction of the black powder of the plant is drunk to cure piles and enema for fever and malaria (Dokosi, 1998; Agyare *et al*., 2009). The ethanolic extract of the whole plant has shown anticancer activity (Ayoub and Babiker, 1984).

Hoslundia opposita Vahl. (Lamiaceae) is an erect or scrambling weak shrub up to 4.5 m high. The leaves and flowers are washed and applied to herpes and other skin diseases. The juice of the crushed leaves is used as eye-drops for conjunctivitis and for treatment of vertigo and epilepsy. It is also used as febrifuge, vermifuge, diuretic and cholagogue for jaundice and yellow fever (Irvine, 1961). The *n*-hexane extract of the root bark was found to have significant activity *in vitro* against the malaria parasite *Plasmodium falciparum* (Achenbach *et al*., 1992). Crude extracts of the twigs has been shown to exhibit strong antibacterial activity (Khan *et al*., 1980).

Ficus exasperata Vahl. (Moraceae) is a tree up to 45 m tall, bole very smooth, greenish or yellowish, eventually developing massive plant buttresses. The young leaves are used as an ingredient in a vermifuge preparation in Liberia. The leaves are used as haemostatic and wound healing agents in Ghana and Cote d'Ivoire. Leaf decoction is used as an enema for intestinal pains and antidote to poison (Irvine, 1961; Burkill, 1997). Aqueous decoction of the leaves has been shown to possess anti-ulcer activity (Akah *et al*., 1998).

Phyllanthus muellerianus (Kuntze.) Exell. (Euphorbiaceae) is a glaborous or woody climber, often with recurved thorns leaves. A leaf infusion is used as an eye lotion and as a wash for fevers, malaria, skin eruptions and wounds. Ethanol leaf extract has been found to be active against chloroquine-resistant *Plasmodium falciparum* (Zirihi *et al*., 2005). Aqueous extracts of the leaves and stem bark showed antibacterial activity (Doughari and Sunday, 2008) and wound healing properties (Agyare *et al*., 2009).

Khaya senegalensis (Desr.) A. Juss. (Meliaceae) is a tree up to 30 m tall with a short bole to 3 m girth, unbuttressed or very slightly swollen at the base. Bark decoction is used for the treatment of fever, menstrual disorders, venereal diseases, worm infection, dysentery and stomach complaints (Burkill, 1997). Extracts from the leaves, stem bark and root have been reported with antimicrobial (Kubmarawa *et al*., 2008), antitumor, antioxidant activities (Karou *et al*., 2005; Zhang *et al*., 2007) and antifungal activities (Abdelgaleil *et al*., 2004).

Anogeissus leiocarpus (DC.) Guill and Perr. (Combretaceae) is a tall tree up to 20 m and girth of 2.5 m. It is used for treatment of yellow fever, jaundice and as vermifuge in folklore medicine (Irvine, 1961; Burkill, 1997). It possesses antimicrobial (Sanogo *et al*., 1998), antiplasmodial and cytotoxic activities (Vonthron-Senecheau *et al*., 2003).

Euphorbia hirta L. (Euphorbiaceae) is an annual, unarmed, hairy herb up to 70 cm tall. The stems are sparingly branched near the base. It is used for management of asthma, syphilis, dysentery, wounds and skin diseases in folklore medicine (Burkill, 1997; Dokosi, 1998). and has shown anti-asthmatic (Ekpo and Pretorius, 2007) and anti-amoebic activities (Tona *et al*., 2009).

Steganotaenia araliacea Hochst. (Apiaceae) is soft-wooded, sparsely branched, deciduous shrub and it is found widespread in tropical Africa. It is used in Eastern Uganda, Eastern of Somalia and Cameroon for the treatment of diarrhea, oedema, malaria, helminth and wound infections. The plants are commonly used by healers to treat helminth infections in animals (Musongong *et al*., 2004). Steganone isolated from the plant showed antiproliferative activity against an ovarian cancer cell line (Meragelman *et al*., 2001). Stem bark extracts exhibited diuretic (Agunu *et al*., 2005) and antibacterial properties (Lino and Deogracious, 2006).

MATERIALS AND METHODS

Plant material and chemicals: Leaves of *Phyllanthus muellerianus* (Kuntze.) Exell., *Ficus exasperata* Vahl., *Pupalia lappacea* (L.) Juss. and *Hoslundia opposita* Vahl. were collected in July, 2007 from Bosomtwi-Atwima-Kwanwoma area, Ghana and identified by Dr. A. Asase,

Table 1: IC_{50} values for plant extracts tested against cultured *L. tarentolae* promastigotes. Parasites were incubated with different concentrations of DMSO-Ethanol dissolved plant extracts for 24 and 48 h. Best fit IC_{50} were calculated from concentration-response curve using global model of non-linear regression curve fitting. Confidential intervals (CI) are shown

	Best-fit value			
	24 h		48 h	
Extract	IC_{50} (mg mL^{-1})	95% CI	IC_{50} (mg mL^{-1})	95% CI
A. leiocarpus	0.896	0.578-1.836	0.678	0.334-1.38
E. hirta (H_2O)	2.003	1.689-2.393	1.651	1.17-2.379
F. exasperata (EtOH)	>3	2.56-4.88	>3	2.35-4.66
F. exasperata (H_2O)	>6	6.27-10.21	>5	5.62-8.19
H. opposita	1.075	0.683-1.675	0.472	0.149-1.867
K. senegaleusis (leaves)	>2	1.079-4.177	>2	0.678-5.920
P. muellerianus (EtOH)	1.249	0.856-1.841	0.663	0.069-2.316
P. muellerianus (H_2O)	1.125	0.919-1.657	1.050	0.776-1.574
P. lappacea	1.758	1.404-2.226	1.589	1.005-2.183
S. araliacea	0.501	0.167-1.625	0.408	0.045-1.739
Pentamidine	0.297[a]	0.582-1.503	0.222[a]	0.331-1.494

[a]Pentamidine values are expressed in μg mL^{-1}

Department of Botany, University of Ghana and voucher specimens were deposited at the Ghana Herbarium, University of Ghana, Ghana. Leaves or stem bark of *Euphorbia hirta* L., *Anogeissus leiocarpus* (DC.) Guill and Perr., *Steganotaenia araliacea* Hochst and *Khaya senegalensis* (Desr.) A Juss were collected in February, 2009 in Ngaoundere area, Cameroon and identified by Mr. Froumsia Moksia, Department of Life Science, University of Maroua, Cameroon. Voucher specimens were deposited at the National Herbarium in Yaounde, Cameroon. If not stated otherwise all chemicals were purchased by Sigma (Deisenhofen, Germany).

Preparation of plant extracts: Plants species were shade-dried at room temperature, weighed, grounded finely and sieved on a 0.5 mm mesh screen. 10 g of the milled powder were mixed into 100 mL distilled water at 90°C for 10 min or in 100 mL of 60% ethanol at 70°C for 30 min, centrifuged for 10 min at 3.500xg and filtered with filter papers 413 (VWR International). The filtrates were evaporated and concentrated by a rotary evaporator at a temperature not exceeding 40°C under reduced pressure. Solid extracts were obtained after lyophilization and stored at 4°C. Dried extracts of plant material were dissolved in 50% ethanol, diluted in 1% DMSO to a final concentration of 100 mg mL^{-1}, centrifuged and aliquoted to determine their activity on *Leishmania tarentolae*.

***L. tarentolae* cultivation:** *L. tarentolae* (ATCC No. 30143, Bioscience, Jena, Germany) were cultured in the dark at 26°C. The protozoans were grown axenically in sterile 3.7% BHI (Brain Heart Infusion) medium (Becton, Dickinson, USA) supplemented with 0.5 mg mL^{-1} pork hemin and penicillin/streptomycin (10,000 U/ 10,000 μg mL^{-1}) (complete medium).

Anti-leishmanial *in vitro* test: Well grown *L. tarentolae* cultures (approx. 1.5-2.0×10^8) were diluted in 1:10 fresh complete medium. Subsequently, 500 μL of aliquots were transferred to 24-well plates and exposed to increasing concentrations of plant extracts (0, 0.25, 0.5, 0.75, 1, 1.25, 1.5, 1.75 and 2 mg mL^{-1}). Assays were incubated at 26°C. After 24 and 48 h the OD_{600} was determined to record the proliferation rates that were expressed relative to the growth of untreated control cells. Pentamidine (Sigma, Deisenhofen) was used as positive control (Ganguly *et al*., 2006; Singh *et al*., 2009), 0.02% DMSO and 0.05% ethanol as negative control. Each extract and each control was tested in three independent dupliclate determinations. The IC_{50} values (concentration that is required to inhibit the growth of *Leishmania* by 50%) was calculated using GraphPad analysis. Table 1 shows IC_{50} values for plant extracts tested against cultured *L. tarentolae* promastigotes. Parasites were incubated with different concentrations of DMSO-Ethanol dissolved plant extracts for 24 and 48 h. Best fit IC_{50} were calculated from concentration-response curve using global model of non-linear regression curve fitting. Confidential intervals (CI) are shown.

Phytochemical screening: Preliminary phytochemical screenings were conducted on dried leaves from *P. muellerianus*, *H. opposita*, *P. lappacea*, *F. exasperata*, *A. leiocarpus*, *E. hirta* and *S. araliacea* as well as from dried leaves and stem bark of *K. senegalensis* for flavonoids, alkaloids, carbohydrates, saponins (Wagner and Bladt, 1996; Harborne, 1998). The quantity of tannins was determined according to the method of Glasl (1983) using pyrogallol as reference compound.

RESULTS AND DISCUSSION

In the present study extracts from selected plants that are used in traditional Cameroonian and Ghanaian medicine were tested for their *in vitro* activity against *L. tarentolae* promastigote cultures. In general, the

anti-leishmanial effect of the plant extracts was found to be time- and concentration-dependent. The most effective extracts (*A. leiocarpus*, *S. araliacae*, *H. opposita* and *P. muellerianus*) killed most of the Leishmania at 2 mg mL^{-1} after 24 and 48 h, respectively. The positive controls in all the figures showed that neither the ethanol nor DMSO concentrations used in these tests had an effect on Leishmania.

According to their proliferation inhibition efficacy, extracts in Fig. 1a-d were rated as exhibiting high (IC_{50} < 1 mg mL^{-1}), moderate in Fig. 2 (IC_{50} between 1 and 2 mg mL^{-1}) and low anti-leishmanial activity in Fig. 3 (IC_{50} >2 mg mL^{-1}). Ethanolic extracts of barks of *S. araliacae* and *A. leiocarpus* as well as of leaves of *H. opposita* and *P. muellerianus* displayed the highest anti-leishmanial activity with IC_{50} values of 0.408 mg mL^{-1}, 0.678, 0.472 and 0.663 mg mL^{-1} after 48 h as shown in Table 1, respectively while aqueous extracts of *P. muellerianus* leaves and ethanolic extracts of *P. lappacea* leaves showed moderate activity (Fig. 2a-b). Plant extracts from *F. exasperata*, *E. hirta* and *K. senegalensis* hardly affected the growth of *L. tarentolae* (Fig. 3a-d). These results strongly indicate that the four extracts derived from *S. araliacae*, *A. leiocarpus*, *H. opposita* and *P. muellerianus* contain promising candidate compounds that might be useful in the control of Leishmania infections by interrupting the parasite life cycle and preventing infections. Remarkably, the anti-leishmanial activity of the *A. leiocarpus* extract is in good accordance with a study carried out by Shuaibu *et al.* (2008a), where a respective methanolic extract was tested. These authors isolated castalagin and demonstrated their potent effects as active anti-leishmanial compound from this plant.

Remarkably, none of these selected plants except *A. leiocarpus* have been tested against *Leishmania.*

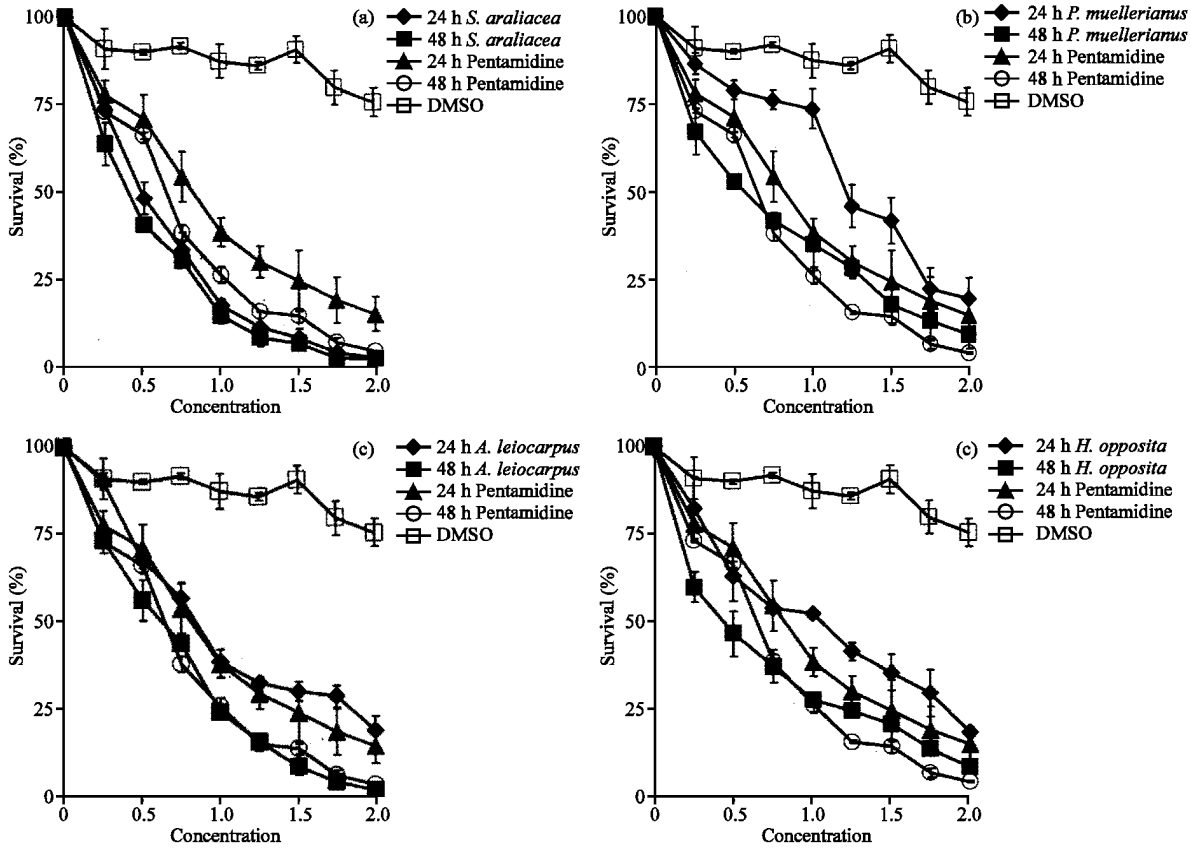

Fig. 1: (a-d) Plant extracts with high activity against *L. tarentolae*. Growth rates of *L. tarentolae* after 24 and 48 h exposure to different concentrations (0-2 mg mL^{-1}) of crude extracts from *S. araliacae*, *H. opposita*, *P. muellerianus* and *A. leiocarpus*. Positive (pentamidine) and negative (DMSO and ethanol) controls are included. The extracts affect the proliferation of *L. tarentolae* in a time- and concentration-dependent manner. Neither ethanol nor DMSO concentrations used in the tests had an effect on the growth of *L. tarentolae*. Data are Mean±SEM from three independent dupliclate experiments

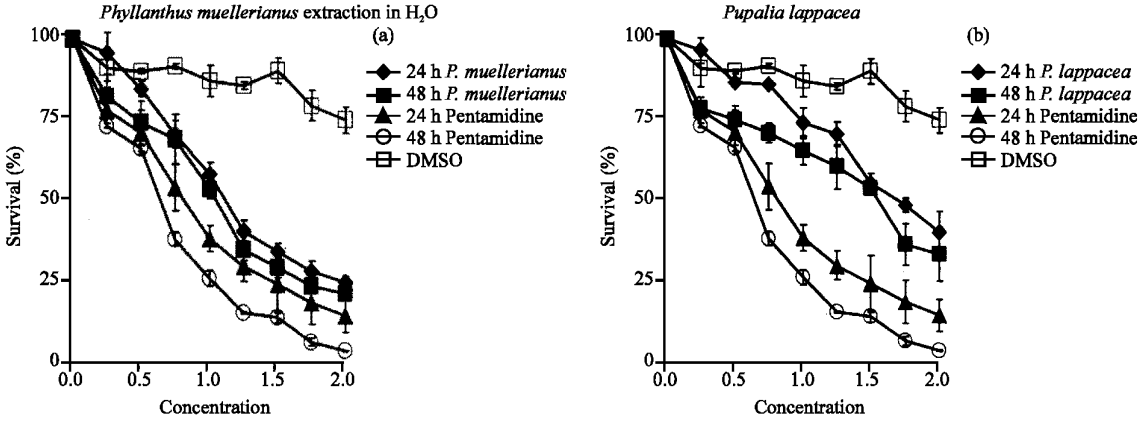

Fig. 2: (a, b) Plant extracts with moderate activity against *L. tarentolae*. *L. tarentolae* were exposed to different concentrations (0-2 mg mL^{-1}) of crude extracts from *P. muellerianus* and *P. lappacea*. The proliferation rates were determined after 24 and 48 h. Positive (pentamidine) and negative (DMSO and ethanol) controls are included. Data are Mean±SEM from three independent dupliclate experiments

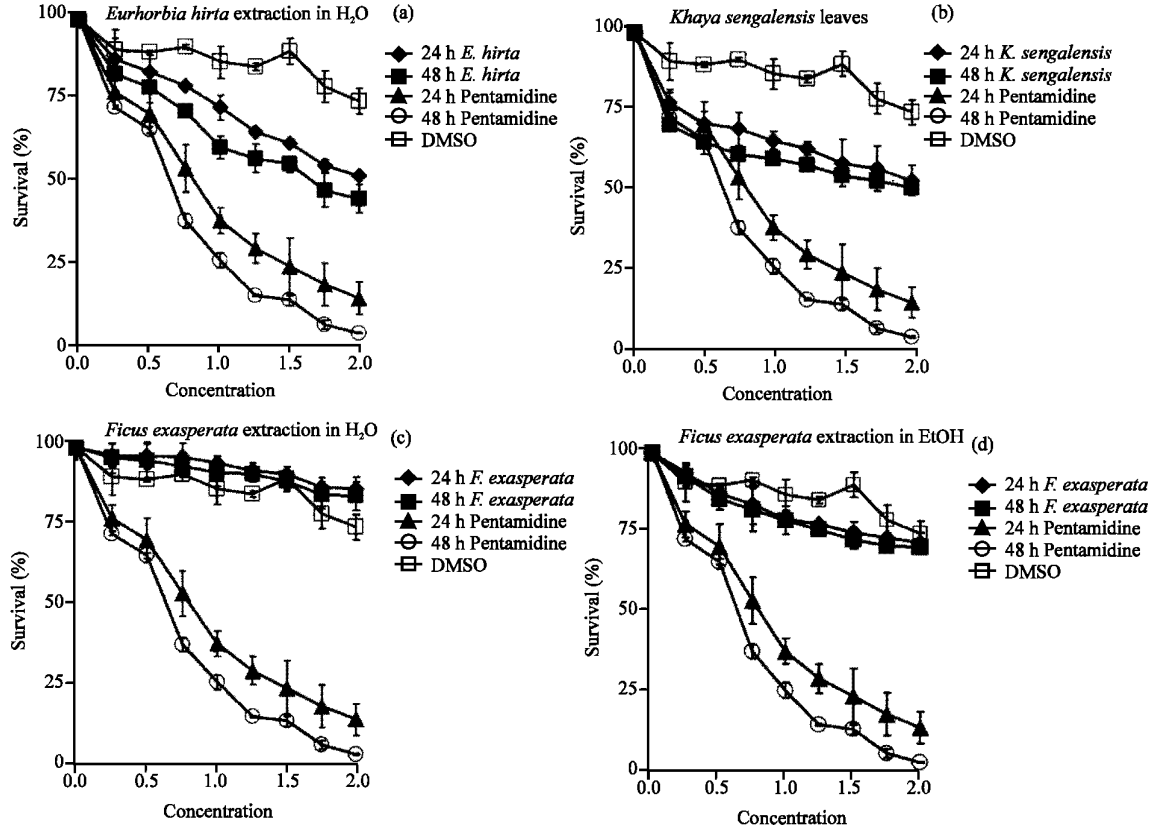

Fig. 3: (a-d) Plant extracts showing low activity against *L. tarentolae*. *L. tarentolae* were incubated in the presence of different concentrations (0-2 mg mL^{-1}) of crude extracts from *F. exasperata*, *E. hirta* and *K. senegalensis*. Cell number was determined after 24 and 48 h. Positive (pentamidine) and negative (DMSO and ethanol) controls are included. Data are Mean±SEM from three independent dupliclate experiments

However, some other plant extracts such as *Plagiochila disticha*, *Ambrosia peruviana*, *Withania somnifera*, *Allium sativum*, *Alcornea cordifolia*, *Sapium cornutum* and *Casearia sylvestris* have shown *in vitro*, inhibitory

Table 2: Phytochemical screening of selected plants. The reactions were performed as described under materials and methods

Name of plants	Plant part	Flavonoids	Saponins	Alkaloids	Carbohydrates	Tannins (% w/w)
A. leiocarpus	Bark	-	+++	-	+	14.6
P. muellerianus	Leaves	+	++	+	+	13.7
F. exasperata	Leaves	+	+++	-	+	1.1
P. lappacea	Leaves	+	+++	-	+	1.3
H. opposita	Leaves	+	++	-	+	1.8
E. hirta	Leaves	+	-	-	+	3.2
S. araliacea	Bark	-	+	-	+	1.3
K. senegaleusis	Leaves	+	++	-	+	4.1

properties against *Leishmania*, *Trypanosoma cruzi*, *Plasmodium falciparum* and *Mycobacterium tuberculosis* (Mesquita *et al*., 2005; Mesia *et al*., 2008; Sharma *et al*., 2009; Aponte *et al*., 2010). Similar results were reported on the inhibition of Protozoa activity with plant extracts of *Albizia zygia*, *Harungana madagascarensis* and *Triclisia patens* on *Plasmodium falciparum* K1 chloroquine-resistant strain, *Leishmania donovani*, *Trypanosoma cruzi* and *Trypanosoma brucei rhodesiense* (Camacho *et al*., 2003). All those studies reported are not directly comparable to our results, due to differences in plant materials. However, recent studies using some of plants mentioned above have also demonstrated efficacity against nematodes (Adenola *et al*., 2004; Musongong *et al*., 2004; Monglo *et al*., 2006).

Some plants used in this study, such as *A. leiocarpus* have been used as antimalarial, antitrypanocidal, antileishmanial or anthelmintic agents (Monglo *et al*., 2006; Shuaibu *et al*., 2008a, b). Crude extracts of *A. leiocarpus* have been shown to display good potentiality against eggs and infective larvae of *Haemonchus contortus* (Monglo *et al*., 2006). Musongong *et al*., 2004 have also reported the toxicity of *S. araliacea* to infective larvae of *Strongyloides papillosus*.

All these results confirm our finding with the selected plant extracts, reinforce the existing knowledge and the regular use of the plants by traditional healers for the treatment of leishmania infections.

Treatment of Leishmania infection in humans is hampered by Pentamidine. Pentamidine is very toxic and still in use because other drugs have developed resistance, variability and inefficacity against the parasites (Lira *et al*., 1999). Since pentamidine has been reported to be very toxic, this opens a new opportunity to further investigate these plant extracts for the isolation of their active compounds and the study of their toxicity.

As shown in Table 2, the results of the phytochemical screening revealed the presence or the absence of flavonoids, alkaloids, saponins, carbohydrates and the quantities of tannins in the selected plants.

However, further fractionation is required in order to analyze whether such secondary metabolites are responsible for the anti-leishmanial activity found in this study. Mishra *et al*. (2009a, b) have speculated that in the future, alkaloids may serve as one of the main anti-leishmanial drugs. These speculations support our finding with the plant extract *P. Muellerianus* which contains alkaloids and has revealed activity against *Leishmania*. But are in contradiction with our results with the plant extracts *A. leiocarpus*, *S. Araliacea* and *H. opposita* that have shown good activities against *Leishmania* and do not contain alkaloids, their activity on *Leishmania* may be due to the presence of tannins, flavonoids, saponins or carbohydrates. However, these results are supported with the observations of Marin *et al*. (2009) and Firdous *et al*. (2009), who have shown, the antileishmanial activity of flavonoids isolate from *Consolida oliveriana* and the efficacy of carbohydrates in the treatment of leishmaniasis. Also, tannins like castalagin, flavogallonic and ellagitannins isolated from *A. leiocarpus* have been shown to display good activity against *Leishmania*, *Plasmodium* and *Trypanosoma* (Shuaibu *et al*., 2008b). Many constituents like terpenoids (sesquiterpene lactones) have been reported also to be active against these infective protozoa (Schmidt *et al*., 2002; Van Miert *et al*., 2005; Schmidt, 2006; Nour *et al*., 2009).

CONCLUSION

In conclusion, this study revealed four plants used in traditional medicine against *Plasmodium* and nematodes that contain promising candidate compounds with *in vitro* anti-leishmanial activity. In future studies, the active compounds will be isolated by bioactivity guided fractionation and characterization. Although results from the growth inhibition assays are encouraging, the *in vivo* effects of these plant extracts on *Leishmania* merits further investigation.

In addition, since promastigotes transform into amastigotes, we are also planning to test extracts inside macrophages to determine if these extracts kill intracellular amastigotes while leaving the mammalian or the lizard cell intact.

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