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 IC₅₀ 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. araliacea, 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 amphotericin 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
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 (Savain et al., 1996; Ahua et al., 2007; Draga 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 Cameroon and Ghanaian medicinal plants known to have anthelmintic and antimalarial 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 information 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).

Hosundia 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-drop 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; Burkhill, 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 glabrous 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 (Zirili et al., 2005). Aqueous extracts of the leaves and stem barks 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, unbudded 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 (Abdulgaleel 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; Burkhill, 1997). It possesses antimicrobial (Sanogo et al., 1998), antiproliferative and cytotoxic activities (Vounthi-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 treatment of asthma, syphilis, dysentery, wounds and skin diseases in folklore medicine (Burkill, 1997; Dokosi, 1998). and has shown anti-asthmatic (Exko and Pretorius, 2007) and anti-amoebic activities (Tona et al., 2009).

Steganotaenia aralacea 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 (Agujo et al., 2005) and antibacterial properties (Lino and Deogracias, 2006).

MATERIALS AND METHODS

Plant material and chemicals: Leaves of Phyllanthus muellerianus (Kuntze.) Exell., Ficus exasperata Vahl., Puplia lappacea (L.) Juss. and Hosundia opposita Vahl. were collected in July, 2007 from Bosomtwi-Atwima-Kwanwoma area, Ghana and identified by Dr. A. Asase,
Table 1: IC₅₀ 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 IC₅₀ were calculated from concentration-response curve using global model of non-linear regression curve fitting. Confidential intervals (CI) are shown.

<table>
<thead>
<tr>
<th>Extract</th>
<th>IC₅₀ (mg mL⁻¹)</th>
<th>95% CI</th>
<th>IC₅₀ (mg mL⁻¹)</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. leucocarpus</td>
<td>0.896</td>
<td>0.578-1.836</td>
<td>0.678</td>
<td>0.334-1.38</td>
</tr>
<tr>
<td><em>E. hirta</em> (EtOH)</td>
<td>2.003</td>
<td>1.680-2.393</td>
<td>1.651</td>
<td>1.147-2.137</td>
</tr>
<tr>
<td><em>P. exasperata</em> (EtOH)</td>
<td>&gt;3</td>
<td>2.56-4.88</td>
<td>&gt;3</td>
<td>2.35-4.66</td>
</tr>
<tr>
<td><em>H. opposita</em></td>
<td>1.075</td>
<td>0.683-1.675</td>
<td>0.472</td>
<td>0.149-1.867</td>
</tr>
<tr>
<td><em>K. senegalensis</em> (leaves)</td>
<td>&gt;2</td>
<td>1.079-1.177</td>
<td>&gt;2</td>
<td>0.678-5.920</td>
</tr>
<tr>
<td><em>P. muellerianus</em> (EtOH)</td>
<td>1.249</td>
<td>0.856-1.841</td>
<td>0.663</td>
<td>0.089-2.316</td>
</tr>
<tr>
<td><em>P. muellerianus</em> (H₂O)</td>
<td>1.125</td>
<td>0.919-1.657</td>
<td>1.050</td>
<td>0.776-1.574</td>
</tr>
<tr>
<td><em>P. lapagea</em></td>
<td>1.758</td>
<td>1.404-2.226</td>
<td>1.589</td>
<td>1.005-2.183</td>
</tr>
<tr>
<td><em>S. aralacea</em></td>
<td>0.501</td>
<td>0.382-1.503</td>
<td>0.408</td>
<td>0.045-1.739</td>
</tr>
<tr>
<td>Pentamidine</td>
<td>0.297*</td>
<td>0.382-1.503</td>
<td>0.222*</td>
<td>0.331-4.194</td>
</tr>
</tbody>
</table>

*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 leucocarpus* (DC.) Guill and Perr., *Steganotaenia aralacea* Hochst. and *Khaya senegalensis* (Desr.) A Juss were collected in February, 2009 in Ngaoundere area, Cameroon and identified by Mr. Frounsia Moksa, 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, ground finely and sieved on a 0.5 mm mesh screen. 10 g of the milled powder were mixed into 100 mL distilled water at 50°C for 10 min or in 100 mL of 60% ethanol at 70°C for 30 min, centrifuged for 10 min at 3,500g 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). 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 OD₅₇₃ 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 duplicate determinations. The IC₅₀ values (concentration that is required to inhibit the growth of *Leishmania* by 50%) was calculated using GraphPad analysis. Table 1 shows IC₅₀ 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 IC₅₀ 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*, *F. lapagea*, *F. exasperata*, *A. leucocarpus*, *E. hirta* and *S. aralacea* as well as from dried leaves and stem bark of *K. senegalensis* for flavonoids, alkaloids, carbohydrates, saporins (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. araliaceae, 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 (IC₅₀ < 1 mg mL⁻¹), moderate in Fig. 2 (IC₅₀ between 1 and 2 mg mL⁻¹) and low anti-leishmanial activity in Fig. 3 (IC₅₀ > 2 mg mL⁻¹). Ethanolic extracts of barks of S. araliaceae and A. leiocarpus as well as of leaves of H. opposita and P. muellerianus displayed the highest anti-leishmanial activity with IC₅₀ 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. araliaceae, 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. araliaceae, 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 duplicate 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 duplicate 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 duplicate experiments.

However, some other plant extracts such as *Plagiochila disticha*, *Ambrosia peruviana*, *Withania somnifera*, *Allium sativum*, *Alcornoque cordifolia*, *Saputum cornutum* and *Casearia sylvestris* have shown *in vitro*, inhibitory
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 madagascariensis* and *Triclisia patens* on *Plasmodium falciparum* K1 chloroquine-resistant strain, *Leishmania donovani, Trypanosoma cruzi* and *Trypanosoma brucei rhodesiense* (Camarcho 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 efficacy against nematodes (Adenola et al., 2004; Musongong et al., 2004; Monglo et al., 2006).

Some plants used in this study, such as *A. leioicarpus* have been used as antimalarial, antitrypanosomal, antileishmanial or anthelmintic agents (Monglo et al., 2006; Shuaibu et al., 2008a, b). Crude extracts of *A. leioicarpus* 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 leishmaniasis infections.

Treatment of Leishmaniasis infection in humans is hampered by Pentamidine. Pentamidine is very toxic and still in use because other drugs have developed resistance, variability and inefficacy 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. leioicarpus, 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 Marín 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, flavagallonic and ellagittamin isolated from *A. leioicarpus* 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.

**ACKNOWLEDGMENTS**

This research was supported by the fellowship of the Alexander von Humboldt Foundation (AVH) to
D. Ndjonka and of the German Academic Exchange Service (DAAD), to C. Aygare. Further support was provided by the Deutsche Forschungsgemeinschaft (DFG) grant L1 793/5-1 to the Cameroonian-German Cooperation Project (CGCP) http://www.cameroon.uni-muenster.de.

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