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Antitrichomonal activity of *Acanthospermum hispidum* D. C. (Asteraceae)

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***Acanthospermum hispidum* (Asteraceae), used ethnomedically in the treatment of inflammatory conditions and fever, was evaluated for antiprotozoal activities such as trypanocidal and antiplasmodial effects. This study was carried out to investigate the anti-trichomonal potential of the plant. The air-dried leaf was extracted successively with petroleum ether, chloroform, ethylacetate and methanol using the soxhlet extraction method. Bioactivity-guided fractionation of the most active extract was carried out using the vacuum liquid chromatographic technique for antitrichomonal activity using *Trichomonas gallinae in vitro.* The ethyl acetate extract (A3) was the most active extract with LC50-LC90 values of 0.58-1.06 and 0.58-1.05 mg/ml at 24 and 48 h, respectively. Subfraction C7 had the highest antitrichomonal activity with 0.25-0.66 and 0.25-0.54 mg/ml at 24 and 48 h, respectively comparable to the activity of metronidazole at 0.20-0.39 and 0.16-0.36 mg/ml at 24 and 48 h, respectively. *A. hispidum* possessed antitrichomonal activity which resided in the chloroform portion of the ethyl acetate extract of the plant.**

Key words: *Trichomonas gallinae*, vacuum liquid chromatography, antiprotozoal.

INTRODUCTION

Diseases such as trypanosomiasis and trichomoniasis are taking their toll in terms of mortality and morbidity on human and animal populations in the developing countries. Available data showed that the annual incidence of trichomoniasis is more than 170 million cases worldwide (WHO, 1995). Trichomoniasis encompasses a broad range of symptoms ranging from a state of severe inflammation and irritation with a frothy malodorous discharge to a relatively asymptomatic carrier state (Swygard et al., 2004). The emergence of drug-resistant strains and dose-limiting toxic effects of existing drugs have complicated the treatment of parasitic protozoan diseases. Medicinal plants are a reservoir of bioactive compounds and therefore, effort is focused on them for potentially useful anti-infective agents. *Acanthospermum hispidum* DC (Asteraceae) is commonly called Bristly starburr, bristly tee or hispid starburr has its synonym as *A. humile.*

Acanthospermum is from the Greek words 'acantha' (thorn) and 'sperma' (seed) and refers to the prickly fruit while *hispidum* is Latin which means rough, bristly or prickly (David et al., 1989). Ethnomedicinally, *A. hispidum* is used in the treatment of yellow fever, malaria and stomach disorder (Denis, 2002; Mann et al., 2003). It is also used in some parts of South America as sudorific and diuretic. The plant has been scientifically investigated for its antibacterial and antiviral (Summerfield et al., 1997; Anani et al., 2000; Kamanzi et al., 2002; Fleischer et al., 2003; Hoffman et al., 2004), abortive and teratogenic (Lemonica and Alvarenga, 1994), antifeedant (Kraus et al., 1994; Rai and Achanya, 1999), antimalarial (Sanon et al., 2003; Gafon et al., 2012), immunostimulatory

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(Summerfield and Sallmuller, 1998), antitrypanosomal, antileishmania (Kamanzi et al., 2004; Ganfon et al., 2012) activities.

The plant has been reported to possess sesquiterpene lactones such as acanthospermal B, acanthospermal B epoxide, hispidunolide A and B (Herz and Kalyanaraman, 1975; Jakupovic et al., 1986; Kraus et al., 1994; Cartagena et al., 2000; Arena et al., 2011). In addition, glycosides and flavonoids have been reported to be present in the aerial part of the plant (Nair et al., 1976; Edewor and Olajire, 2011). *A. hispidum* has been shown to be a potentially useful plant in the treatment of protozoan infections; though, the antitrichomonal activity had not been reported. This study was carried out to investigate the activity of various extracts and fractions of *A. hispidum* on a protozoan parasite, *Trichomonas gallinae*.

MATERIALS AND METHODS

Drugs, reagents and solvents

Metronidazole tablet (May and Baker, Nigeria; Batch No. IU 268), methanol, ethyl acetate, chloroform, petroleum ether (BDH, UK), dimethylsulphoxide, sodium chloride, potassium chloride, calcium chloride, glucose, sodium hydrogen phosphate (BDH), sodium hydrogen bicarbonate (East Anglia Chemicals, UK) and sulphuric acid (Scharlau, Spain).

Plant collection and preparation

A. hispidum D. C. (Asteraceae) was collected at Ile-Ife, Nigeria in July. Plant authentication was done by Dr. H. C. Illoh of the Botany Department, Obafemi Awolowo University and compared with herbarium specimen IFE 5986. The leaf was oven-dried at 40°C, powdered using the grinding machine (Christy Norris) and stored appropriately in an amber-coloured bottle until required.

Plant extraction

The powdered leaf of *A. hispidum* (2.0 kg) was successively extracted with petroleum ether, chloroform, ethyl acetate and methanol for 48 h each using the soxhlet extraction method. The extracts were concentrated *in vacuo* at 35°C to give petroleum ether (A_1), chloroform (A_2), ethyl acetate (A_3) and methanol (A_4) extracts. The yields obtained were 0.95, 2.63, 1.00 and 1.27%, respectively.

Vacuum liquid chromatography (VLC)

The ethyl acetate extract (A_3, 18 g) was subjected to vacuum liquid chromatography (VLC) using silica (Burgoyne, India) and eluted with gradient solvents of petroleum ether/chloroform, 100% chloroform, chloroform:methanol (1:1) to give sub fractions B_{1-7}. Further fractionation of B_2 (2.7 g) by VLC using gradient solvent systems of petroleum ether and chloroform, yielded purified fractions C_{1-8}.

Thin layer chromatography analysis of A_3 and its fractions

Extracts and fractions were monitored by thin layer chromatography (TLC) on precoated silica gel 60 F_{254} plates (Merck®) eluted with the following solvent systems: I) petroleum ether: chloroform (1:1), II) petroleum spirit:chloroform (1:9), III) chloroform:ethyl acetate (4:1), IV) chloroform:methanol (3:2) and V) ethyl acetate:methanol (3:2). The chromatograms were examined under the UV light at 254 and 366 nm then sprayed with 10% H_2SO_4.

Preparation of metronidazole (positive control), extracts/fraction

Metronidazole, extract/fraction (4 mg) was dissolved in 0.25 ml dimethyl sulfoxide (DMSO) and made up to 1 ml solution using the Locke-egg (LE) medium to give 4000 µg/ml. Serial dilution was done to obtain 2000, 1000, 500, 250, 125, 62.5, 31.25, 15.625 and 7.8125 µg/ml. The LE medium was prepared by thoroughly mixing 50 ml Ringer's solution, 1 ml bovine serum and 1 ml 10% glucose solution.

Parasite

T. gallinae parasites were isolated from the mouth and upper crop of *Columba livia* (local pigeon) using sterile cotton-tipped swab sticks immersed in physiological saline solution (Narcisi and Secor, 1996). The parasites were cultured in egg slant tubes suspended in LE medium and incubated vertically at 37°C until ready for use (Omisore et al., 2005).

Antitrichomonal bioassay

For each extract/fraction, 50 µl of 10^4 organisms/ml of *T. gallinae* parasites was added to 150 µl of test extract/fraction in a sterile 96-microwell flat bottom plate (Nunc) with metronidazole and DMSO-LE medium as positive and negative controls, respectively. The plates were incubated at 37°C. At 24 and 48 h, surviving (motile) parasites were counted per ml with the aid of a microscope. At least each concentration was done in triplicate analyses.

Data and statistical analysis

The percentage mortality of the parasites was calculated as $100 \times [100-(A/B)]$, where A is the number of motile organisms in the test groups and B is the number of motile organisms in the negative control group. The LC_{50} and LC_{90} values were derived from the respective percentage mortality values using Microsoft Excel (2007) and subjected to statistical analysis using the one-way analysis of variance (ANOVA) followed by the post-hoc Dunnett test (Graphpad Instat, 2003).

RESULTS

The results are presented in Tables 1 to 3. The LC_{50} and LC_{90} values of A_1, A_2 and A_3 reduced non-significantly ($P>0.05$) over time from 24 to 48 h. The activities of A_1, A_2 and A_3 were comparable to the positive control, metronidazole; however, A_4 was significantly different [F

Table 1. Antitrichomonal activity of the extracts of *Acanthospermum hispidum* DC. (Asteraceae) using *Trichomonas gallinae*.

Extract	24 h		48 h	
	LC_{50} (mg/ml)	LC_{90} (mg/ml)	LC_{50} (mg/ml)	LC_{90} (mg/ml)
A_1	0.81±0.23	1.44±0.30*	0.61±0.02	1.16±0.08*
A_2	0.63±0.05	1.23±0.13*	0.60±0.02	1.15±0.08*
A_3	0.58±0.02	1.06±0.05*	0.58±0.00	1.05±0.00*
A_4	1.07±0.27*	1.66±0.54*	0.89±0.04*	1.06±0.17*
Metronidazole	0.20±0.02	0.39±0.01	0.16±0.01	0.36±0.02

LC_{50}, $LC_{90:\ values}$ are mean ± standard error of the mean (SEM). *Significantly different from metronidazole. A_1, Petroleum spirit extract; A_2, chloroform extract; A_3, ethyl acetate extract; A_4- methanol extract.

Table 2. Antitrichomonal activity of the fractions of the ethyl acetate extract (A_3) of *A. hispidum* at 24 and 48 h.

Extract	24 h		48 h	
	LC_{50} (mg/ml)	LC_{90} (mg/ml)	LC_{50} (mg/ml)	LC_{90} (mg/ml)
A_3	0.58±0.02	1.06±0.05	0.58±0.00	1.05±0.00
B_1	1.13±0.15	2.00±0.27	0.70±0.10	1.44±0.17
B_2	1.15±0.15	1.97±0.21	0.99±0.14*	1.77±0.11*
B_3	1.09±0.25	2.03±0.39	0.79±0.15	1.54±0.18*
B_4	1.54±0.46*	2.70±0.86	1.36±0.37*†	2.43±0.58*†
B_5	1.04±0.13	1.95±0.27	0.77±0.04	1.42±0.09*
B_6	1.06±0.13	2.05±0.25	0.85±0.15	1.60±0.10*
B_7	1.87±0.57*†	3.11±1.11*	0.99±0.23*	1.86±0.29*
Metronidazole	0.20±0.02	0.39±0.01	0.16±0.01	0.36±0.02

LC_{50}, $LC_{90:}$ values are mean ± standard error of the mean (SEM). *Significantly different from metronidazole. †Significantly different from A_3.

(4, 10) 3.96, P = 0.035] from metronidazole at 24 h (Table 1). A_3 gave the highest activity as it had the lowest LC_{50} and LC_{90} values at 24 and 48 h. Thus, A_3 (22 g) was fractionated using VLC and sixteen eluates were obtained which were bulked into 7 fractions (B_{1-7}) according to their TLC profiles. At 24 h, all the fractions except B_4 and B_7 had similar activity compared with metronidazole while only the LC_{50} of B_7 was significantly different [F(8, 18) 3.099, P = 0.022] at 24 h from the mother extract, A_3 (Table 2). At 48 h, the LC_{50} values of B_2, B_4 and B_7 were significantly different from metronidazole while only B_4 was different from A_3 [F (8, 18) = 3.624, P = 0.011). All the fractions except B_1 were significantly different from metronidazole when their LC_{90} values were compared. Although, B_{1-3} and B_{5-6} had comparable activities, B_2 (2.9 g), which had the highest weight, was further purified. Eight bulked fractions were obtained (C_{1-8}) according to their TLC characteristics.

At 24 h, all the sub-fractions except C_5, C_6 and C_7 were significantly different [F (9, 20) = 5.52, P = 0.0007] from metronidazole while at 48 h, only C_6 and C_7 were comparable [F (9, 20) 41.46, P<0.0001] to metronidazole. However, they were significantly different [F (9, 20) 34.29, P<0.0001) from A_3 (Table 3). Thus, the activities of the two sub-fractions were significantly comparable to metronidazole at both time points and showed better activity than A3.

DISCUSSION

The extracts and fractions of *A. hispidum* gave moderate to remarkable levels of mortality when tested on the protozoa, *T. gallinae*. The activity of A1, A2 and A3 implies the bioactive component may be non-polar while the polar methanolic extract exhibited minimal activity when compared with metronidazole. It thus, appeared that the bioactive component (s) is/are relatively apolar. In addition, it seemed the extracts exhibited biostatic action on the protozoa which was not sustained after 24

Table 3. Antitrichomonal activity of subfractions of B_2 using *Trichomonas gallinae* at 24 and 48 h.

Extract/sub-fraction	24 h		48 h	
	LC_{50} (mg/ml)	LC_{90} (mg/ml)	LC_{50} (mg/ml)	LC_{90} (mg/ml)
A_3	0.58±0.02	1.06±0.05	0.58±0.00*	1.05±0.00*
C_1	1.00±0.08*	1.70±0.09*†	0.73±0.06*†	1.40±0.09*†
C_2	1.10±0.12*	1.80±0.10*†	0.67±0.08*	1.25±0.09*
C_3	0.74±0.38*	1.44±0.03*†	0.68±0.05*	1.29±0.06*
C_4	0.94±0.16*	1.73±0.20*†	0.85±0.04*†	1.49±0.13*†
C_5	0.62±0.00	1.21±0.01*	0.59±0.01*	1.14±0.04*
C_6	0.26± 0.03	0.60±0.05†	0.28 ± 0.01†	0.52± 0.01†
C_7	0.25 ± 0.01	0.66± 0.01†	0.25± 0.01†	0.54± 0.01†
C_8	0.81±0.04*	1.56±0.01*†	0.62±0.03*	1.18±0.10*
Metronidazole	0.20±0.02	0.39±0.01	0.16±0.01	0.36±0.02

LC_{50}, $LC_{90:}$ values are mean ± standard error of the mean (SEM). *Significantly different from metronidazole. †Significantly different from A_3.

h. The increasing resistance to metronidazole in the treatment of trichomoniasis, with the various adverse effects observed in the use of the drug, has led to the search for bioactive agents in medicinal plants with potential antitrichomonal activity. Trichomonad species readily obtained for laboratory study are *T. muris* in mice and rats and *T. gallinae* from crop of pigeons (Smyth, 1996). *T. gallinae* was used because of its availability and morphological similarity to T. vaginalis *T. vaginalis*, the causative parasite for human trichomoniasis. Since the duration of survival and growth rate is inversely proportional to inoculum density, trichomonads can sometimes overgrow the media and die off within 36 to 48 h, thus the choice of the time points.

The most active subfractions are from the chloroform portion of the ethyl acetate extract. Deepa et al. (2004) reported that the ethyl acetate extract of *A. hispidum* possessed antibacterial and antifungal activities comparable to ciprofloxacin and clotrimazole, respectively. Non-polar compounds have been found effective as potential agents in the treatment of trichomoniasis. The pentacyclic triterpenoid, hederagenin, was reported as the antitrichomonal component of *Cussonia holtsi* (Araliaceae) with an IC_{50} of 2.8 µM (He et al., 2003). In addition, bartericins A and B as well as isobavachalcone (isolated from *Dorstenia barteri*) were reportedly active at 0.121 to 31.25 µg/ml, against *T. gallinarum* (Omisore et al., 2005). The antibacterial and antimalarial activities of *A. hispidum* have been ascribed to Acanthospermal B and other sesquiterpene lactones (Arena et al., 2011; Ganfon et al., 2012). It is therefore possible that the putative antitrichomonal constituent of *A. hispidum* belongs to the class of sesquiterpene lactones which abound in the plant.

Sesquiterpene lactones (SQLs) have been reported from 10 families of flowering plants; with the greatest numbers derived from the Asteraceae. The α-methylene γ-lactone moiety of this group of compounds is very reactive with the thiol groups of important biological components such as enzymes which make SQLs have diverse biological activity. Further studies on the most active subfractions may reveal a more active compound than the standard drug, metronidazole.

Conclusion

Bioactivity-directed purification of the leaf of *A. hispidum* using anti-trichomonal assay yielded subfractions C_6 and C_7 which had activity comparable to metronidazole, the positive control and had better activity than the mother ethyl acetate extract. The study further showed the potential usefulness of *A. hispidum* in treating protozoal infections.

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