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PROTOCATECHUIC ACID AND SAPONIN MIXTURE FROM *STEGANOTAENIA ARALIACEA* STEM BARK

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Abstract
Steganotaenia araliacea Hochst (Apiaceae / Umbelliferae) is used in East and West African ethnomedicine for treating gastro-intestinal disorders, peptic ulcer, rheumatism and various diseases of microbial origin. The plant was therefore investigated for its chemical constituents while testing for possible antimicrobial, antioxidant, spasmolytic and anti-inflammatory activities. Through bioactivity-driven fractionation, protocatechuic acid was isolated from the ethyl acetate fraction as the main antimicrobial (agar diffusion) and antioxidant (radical scavenging-DPPH) principle. The crude extract exhibited spasmolytic activity, which was found to reside exclusively in the aqueous fraction. Further fractionation of the aqueous fraction yielded a saponin mixture. The observed spasmolytic effect was found to be antihistaminic rather than anticholinergic. The saponin mixture also demonstrated significant anti-inflammatory activity. At a dose of 1 mg/kg *i.p.* it gave a 77.7% inhibition of carrageenan-induced rat-paw oedema.

Keywords: *Steganotaenia araliacea*; Saponins; Antimicrobial; Antioxidant; Spasmolytic; Antiinflammatory

INTRODUCTION

Steganotaenia araliacea Hochst (Synonyms: *Peucedanum araliaceum* Benth. & Hook f.; *P. fraxinifolium* Hiern), commonly called carrot tree, belongs to the family Apiaceae / Umbelliferae. It is a small, soft-wooded tree up to 12m high. The leaves are simply pinnate having 3-5 pairs of serrate leaflets with a terminal one. It produces tiny white flowers in compound umbels during the dry season. The fruits are small, flat and 2-winged (Hutchinson and Dalziel, 1958; Irvine, 1961; van Jaarsveld, 2004; Botanical garden, 2004). The root is used to treat: snakebites in India (Selvanayahgam *et al.,* 1994); menstrual problems, abdominal pains, malaria and snakebite in Tanzania (Chhabra *et al.,* 1993); bilharzias, sore throat and swellings caused by allergies in East Africa. It is used in multicomponent prescriptions to treat heart palpitations, severe abdominal pains and gonorrhoea (Hedberg *et al*., 1983). The bark is used to treat: asthma by the Zigula and Sukuma people of South Africa (Watt and Breyer-Brandwijk, 1962); leukaemia and malaria in Tanzania (Chhabra *et al.,* 1993; Gessler *et al.,* 1995); rheumatism by rubbing the ash into scarifications, dysentery and flatulence by taking a decoction mixed with milk in East Africa (Hedberg *et al*., 1983). The water extract of the leaf is used to treat: gonorrhoea, sore eyes and sore throat in

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Zimbabwe (Wild and Gelfand, 1959; Gelfand *et al.,* 1985); convulsions in Gambia (Irvine, 1961). The whole plant is used to treat lung and liver diseases of cows in East Africa. It is also reputed to cause abortion in goats (Watt and Breyer-Brandwijk, 1962). The central core of the root, when wrapped around the penis is claimed to increase the size of the latter (Buchanan, 1975). Pharmacognostic/ phytochemical screening of the plant revealed the presence of tannins, resins, flavonoids and saponins while alkaloids and anthraquinones were not found (Mohammed *et al*., 1999). The ethanol extract and chromatography fractions have been shown to possess antiviral (Beuscher *et al.,* 1994) and cytotoxic properties (Taafrout *et al.,* 1983a). The leaves and bark were also shown to have molluscicidal activity (Kupchan *et al.,* 1973; Kloos *et al.,* 1987). The leaf extracts demonstrated insect repellent/ antifeedant properties (Abubakar *et al.,* 2001). The 70% ethanol extract of the fresh root bark was however inactive in antibacterial tests against *B. subtilis* and *E. coli* at 100μg/mL. It was also inactive in antiviral (against rhinovirus Type 2), antifungal (*Penicillium crustosum*), and anti-yeast (*Saccharomyces cerevisiae*) test systems (Taniguchi *et al.,* 1978). Several lignans have been isolated from the stem bark and entire plant. These include: araliangine (Taafrout *et al.*, 1983a), neoisostegane (Hicks and Sneden, 1983; Taafrout *et al.*, 1983b; 1984b), prestegane A (Taafrout *et al.*, 1983c), prestegane B (Taafrout *et al.*, 1984a), steganacin, steganangin (Kupchan *et al.,* 1973), steganol (Wickramaratne *et al.,* 1993), steganolide A (Taafrout *et al.*, 1986), steganolides B, steganolide C (Robin *et al.,* 1986), steganone (Hughes and Raphael, 1976) and 10-demethoxystegane (Meragelman *et al.,* 2001). Also, triterpenoid glycosides (saponins) have been isolated from the leaves. These include: glycosides of barrigenol R_1 (now known as barringtogenol C) and steganogenin with glucose, galactose and rhamnose in their sugar portions (Lavaud *et al.,* 1992). The specific saponins are: 21-*O*-angeolyl-3-*O*-[β-D-glucopyranosyl-(1→2)-β-D-xylopyranosyl-(1→3)-β-D-glucuronopyranosyl] barringtogenol; 21-*O*-tigloyl-3-*O*-[β-D-galactopyranosyl-(1→2)-β-D-galactopyranosyl (1→3)-β-D-glucuronopyranosyl] barringtogenol; 21-*O*-tigloyl-3-*O*-[β-D-glucopyranosyl-(1→2)-β-D-xylopyranosyl-(1→3)-β-D-glucuronopyranosyl] barringtogenol; 3-*O*-[β-D-galactopyranosyl-(1→2)-β-D-glucuronopyranosyl]-28-*O*-β-D-glucopyranosyl- olean-12-en-28-oic acid and 3-*O*-[β-D-glucopyranosyl-(1→2)-β-D-glucopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranosyl] steganogenin. The present study aimed at isolating bioactive compounds, through activity-guided fractionation, from the stem bark extract of *S. araliacea* with a view to justifying some of its claimed uses.

MATERIALS AND METHODS

General procedures: Melting point (uncorrected) was determined on a Baird Tatlock melting point apparatus. Thin-layer Chromatography (TLC) was carried out on Si gel 60 F_{254} Merck®. Accelerated Gradient Chromatography - AGC (Bæckström, 1993), a form of Medium Pressure Liquid Chromatography (MPLC) was carried out on columns packed with Si gel 60, 0.040-0.063mm Merck®. The MPLC workstation was from Bæckström Separo Ab, Sweden. Sephadex LH-20 for gel filtration was a product of Pharmacia®. NMR was carried out on a Brucker 400 MHz spectrometer.

Plant material: The stem bark of *S. araliacea* was collected from mature trees in Jos, Nigeria between December and March. The plant was authenticated by Mr. T. K. Odewo of the Forestry Research Institute of Nigeria (FRIN), Ibadan where voucher specimens had been deposited at the herbarium.

Extraction, fractionation and isolation: The dried, pulverized plant material

(3.3 kg) was extracted with 50% ethanol by percolation at room temperature for 48h. The extract was concentrated to dryness *in vacuo* and dried over silica in a desiccator to yield a crude extract (242 g or 7.3%). This was fractionated by separately partitioning between water and each of the following organic solvents - CH_2Cl_2, EtOAc, *n*-BuOH. The EtOAc fraction, being the most active, was further examined. It was subjected to gel filtration chromatography on a Sephadex LH 20 column eluted with PhMe/MeOH (3:1). This yielded needle shaped crystals, characterized as protocatechuic acid (83mg). The aqueous fraction from EtOAc partition (i.e. EtOAc mother liquor) was found to be rich in saponins (froth test). It was therefore further purified by ether precipitation (achieved by dissolving in a small volume of MeOH and adding excess Et_2O). The precipitate collected was subjected to AGC employing a CH_2Cl_2-EtOAc-MeOH gradient. This afforded a saponin mixture (0.49 g).

Test for antimicrobial activity: Screening of extracts, fractions and pure compounds against typed organisms was carried out by agar diffusion cup-plate method (B.P. 1988).

Test for antioxidant (radical scavenging) activity: Extracts, fractions and isolated compounds were tested. The tests were carried out by running the TLC of the samples (in duplicate). Ascorbic acid was spotted along to serve as positive control. One chromatogram was sprayed with β-carotene (0.1% w/v in MeOH or EtOH) while the other was sprayed with 1,1-diphenyl-2-picrylhyrazyl i.e. DPPH (0.1% w/v in MeOH or EtOH). When the plate sprayed with β-carotene is irradiated with UV light at 366nm for 15 minutes, antioxidant spots appear yellow on a bleached background. For the DPPH-sprayed plate, antioxidant spots appear yellow against a purple background (Burits and Bucar, 2000).

Test for spasmolytic activity: Rabbit jejunum and guinea pig ileum were used (Amos *et al.*, 1998). The tissue was mounted in a 20mL organ bath containing Tyrode's solution of the following composition (mM): NaCl (136.8), KCl (2.7), $CaCl_2$ (1.3), $NaHCO_3$ (12.0), $MgCl_2$ (0.5), Na_2PO_4 (0.14) and glucose (5.5). The solution was continuously aerated and maintained at 37 ^{0}C. A tension of 0.5g was applied. A 1h equilibration period was allowed during which the physiological solution was changed every 15 min. At the end of the equilibration period, the effect of acetylcholine (ACh), histamine, and/or test samples were evaluated.

Test for antiinflammatory activity: The method described for carrageenan-induced rat paw oedema (Kosuge *et al.,* 1985; Williamson *et al.,* 1996) was employed. Male albino Wistar rats weighing 130-150g were used for the test. The samples to be tested were administered by intraperitoneal injection as a 1% Tween 80 suspension at a dose of 1mg/kg body weight. Rats in the control group received only Tween 80. Thirty minutes later, subplantar injection of 0.1mL of 1% carrageenan in normal saline was given to the right hind-paw. The volume of the foot, up to the tibio-tarsal articulation, was measured with a plethysmometer every hour for 5h and the percent swelling (foot oedema) was calculated. Since the swelling of the paw peaks at 3h after carrageenan treatment, results were expressed as percentage inhibition of swelling at 3h, relative to the control group, which received only the vehicle. Student t-test for significance was carried out at P=0.05 level.

RESULTS AND DISCUSSION:

Fractionation, isolation and bioactivity. The entire fractionation process was bioactivity-driven. The crude extract was separately partitioned between CH_2Cl_2 and H_2O, EtOAc and H_2O, and *n*-BuOH and H_2O. Antimicrobial test on the extract and fractions

(Table 1) revealed that the activity of the extract partitioned preferentially into the organic phase with the EtOAc fraction being the most active. Antioxidant activity also followed the same pattern. The EtOAc fraction was therefore subjected to further fractionation by gel filtration chromatography. This led to the isolation of protocatechuic acid as the main antimicrobial and antioxidant principle. The compound had activity against both Gram-positive and Gram-negative bacteria (Table 1). It also demonstrated strong activity in the DPPH assay.

The extract and fractions were also tested for spasmolytic activity. Results showed that the crude extract and aqueous fraction (i.e. mother liquor from from EtOAc partition) caused a concentration-dependent decrease in amplitude of contractions (i.e. relaxation) of the rabbit jejunum (Fig. 1). The aqueous fraction had no effect on ACh-induced contraction of the rabbit jejunum (Table 2) but significantly attenuated histamine-induced contraction of the guinea pig ileum. From the aqueous fraction, the saponin mixture obtained, greatly diminished histamine-induced contraction (Table 3). The saponin mixture was also tested for anti-inflammatory activity, employing rat-paw oedema measurements. At a dose of 1mg/kg *i.p.*, there was a 77.7% inhibition of carrageenan-induced oedema (Table 4).

Characterization of protocatechuic acid. Appearance/ solubility:- White to pale brown solid; m.p. 208^{o}C with decomposition (Literature: m.p. ~200^{o}C with decomposition - Merck, 1996); soluble in EtOAc, Me_2CO and MeOH; insoluble in CH_2Cl_2.

^{1}H NMR (250 MHz, CD_3OD): δ 6.80 (1H, *d*, *J* = 8.9 Hz, H-5); δ7.43 (1H, *dd*, *J* = 8.9, 2.3 Hz, H-6); δ7.46 (1H, *d*, *J* = 2.3 Hz, H-2).

The ^{1}H NMR spectrum has protons in three different chemical environments. The coupling pattern is a typical AMX type, indicative of a 1,3,4- trisubstituted benzene ring. This data is consistent with the spectrum of protocatechuic acid in the Aldrich library of NMR spectra.

TABLE 1: Zones of inhibition* of *S. araliacea* extract, fractions and isolated compound against selected typed organisms.

Samples	1	2	3	4	5	6	7	8	9	10	11
E. coli NCTC 10418	0.0	0.0	2.5	0.0	1.0	0.5	0.5	9.5	0.0	10.0	-
Staph. aureus NCTC 6571	3.3	0.0	5.0	0.0	5.0	1.0	2.0	11.3	0.0	16.3	-
Ps. aeruginosa ATCC 10145	0.0	0.0	0.8	0.0	0.0	0.0	0.0	8.0	0.0	0.0	-
B. subtilis NCTC 8236	5.0	0.0	5.3	0.5	4.5	3.0	5.0	10.3	0.0	10.0	-
C. pseudotropicalis NCYC 6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	16.5
Concn. (mg/mL)	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0		1.0	6.0

* Mean of duplicate readings; diameter, mm, less cup size. 1 = n-BuOH fraction; 2 = n-BuOH mother liquor; 3 = EtOAc fraction; 4 = EtOAc mother liquor; 5 = CH_2Cl_2 fraction; 6 = CH_2Cl_2 mother liquor;7 = *S. araliacea* crude extract; 8 = protocatechuic acid; 9= solvent [MeOH/H_2O (2:1)]; 10 = streptomycin; 11 = acriflavine

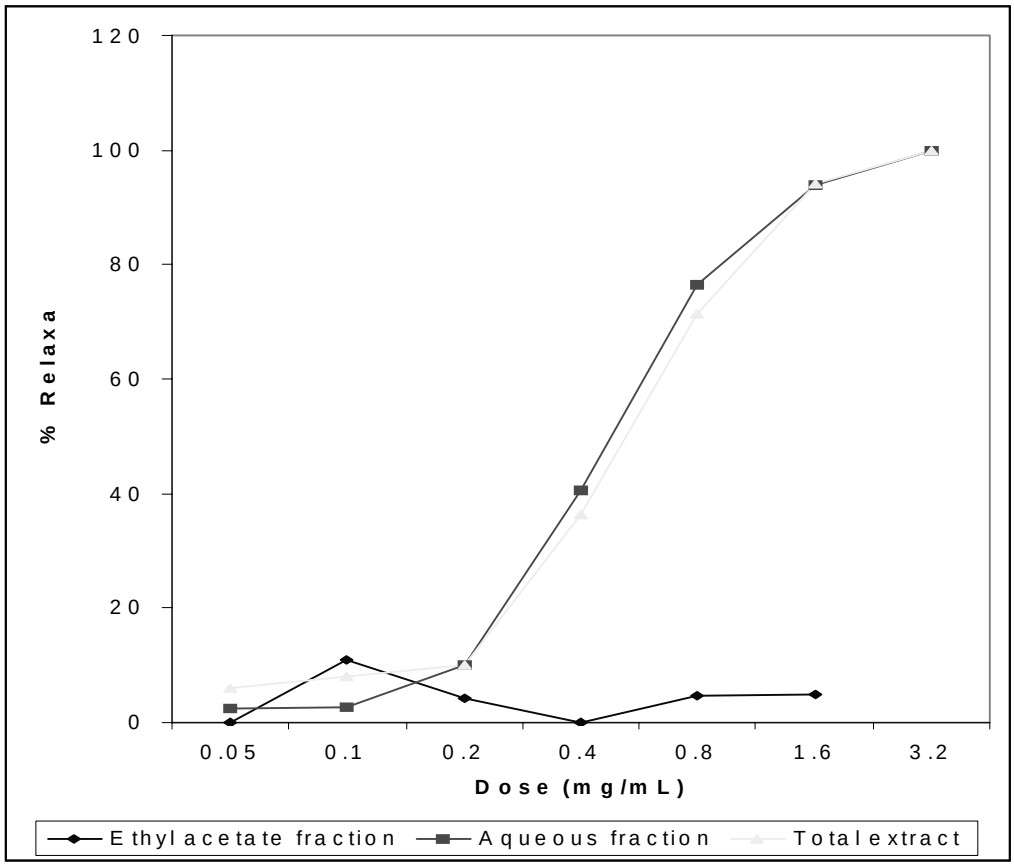

Table 2: Spasmolytic effect of *S. araliacea* aqueous fraction on ACh-induced contraction of the rabbit jejunum.

	Concentration	% Maximum contraction
ACh alone	2.75 x 10^{-6} M	64.7
	5.50 x 10^{-6} M	95.6
	5.50 x 10^{-6} M	100.0
	5.50 x 10^{-6} M	100.0
Aqueous fraction (+ 5.50 x 10^{-6}M Ach)	0.80mg/mL	104.4
	1.60mg/mL	97.1

Table 3: Spasmolytic effect of *S. araliacea* saponin mixture on histamine-induced contraction of the guinea pig ileum

	Concentration	% Maximum contraction
Histamine alone	2.17 x 10^{-6} M	100
Saponin mixture (+ 2.17 x 10^{-6}M histamine)	0.40mg/mL	35.7
	0.80mg/mL	7.1

Table 4: Antiinflammatory effect of *S. araliacea* saponin mixture - inhibition of rat paw oedema

Treatment	*Mean* paw volume ± SEM (mL) at 0 h*	Mean* paw volume ± SEM (mL) at 3 h	Inhibition of oedema at 3 h (%)
Tween 80 (1% v/v *i.p.*)	2.00 ± 0.2	3.97 ± 0.6**	-
Indomethacin (5 mg/kg *i.p.*)	2.01 ± 0.2	2..20 ± 0.2**	90.4
Saponin mixture (1mg/kg *i.p.*)	1.90 ± 0.2	2.34 ± 0.4**	77.7

* n = 5; ** Statistically significant compared to Control

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