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Anthelmintic properties of *Polygonum glabrum*

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A pure anthelmintic substance (PGA) has been isolated from the methanol–aqueous extract of the leaf of *Polygonum glabrum* Willd., a semi-aquatic Sudanese species of the family Polygonaceae. The antiparasitic in-vitro activity of several fractions isolated from the plant, has been examined comparatively with that of PGA. PGA also showed molluscicidal activity against *Biomphalaria glabrata* and *Limnea truncatula* Müll. Structural determination of PGA was attempted following data analysis of UV, IR, ^{13}C NMR, ^{1}H NMR and MS spectra and suggests that PGA is a terpenoid.

The genus *Polygonum* includes 150 species (Graham 1958). According to geographical origin, the literature reports different synonyms for the plant. *Polygonum glabrum* Willd. of Asia is similar to that of tropical Africa and Egypt, but the Asian species has absolutely glabrous leaves with eciliate margins. *Polygonum senegalense* Meisn. (syn *Polygonum glabrum* Willd.) is found in Senegal; the leaves are glabrous with the limb surface exuding a viscous yellow liquid, occasionally, young leaves having deciduous tomentous pubescence are found (Hutchinson & Dalziel 1963; Berhaut 1967). In Kenya, there are two forms of the plant: *Polygonum senegalense* forma *senegalense*, distinguished by dark green leaves with a yellow sticky oily residue covering the surface, and *Polygonum senegalense* forma *albotomentosum*, distinguished by leaves covered with white hairs (Graham 1958). The species of *P. glabrum* found in Sudan and India seem morphologically comparable. The plant is a perennial, persistent herb with stems 90–150 cm, procumbent then erect and leaves glabrous with no apparent sticky residue on the surface (Andrews 1950; Kirtikar & Basu 1975). In Sudan, the leaves of this plant are used to treat roundworm and tapeworm infections (O. Abdel-Moneim, Sudan National Council for Research 1979, personal communication), while in India they are used for colic pain (Tiwari et al 1979). The Sudanese species has received little phytochemical and pharmacological examination but molluscicidal properties and principles of the Kenyan species have been reported (Dossaji et al 1976: Maradufu & Ouma 1978; Dossaji & Kubo 1980). The genus *Polygonum* is a rich source of flavonoids (Tiwari et al 1979) and phytochemical investigations of such compounds from *P. glabrum* of divers origins have been reported (Tiwari et al 1979; Dossaji & Kubo 1980; Abdel-Gawad & El-Zait 1981).

We have attempted a phytochemical examination of the leaf of *P. glabrum* and isolated a pure active substance (PGA) which showed anthelmintic properties especially against *Hymenolepis nana* var. *fraterna*. All extraction methods used were monitored by TLC and biological screening for antiparasitic activity of the isolated fractions.

MATERIALS AND METHODS

Plant material

Samples collected in November 1979 from the banks of the Nile in Khartoum Province, where the plant grows partly covered with water in alluvial soil, were authenticated at the Department of Botany (University of Khartoum).

Extraction and fractionation

The leaves and stems of the shade-dried plant were finely powdered and extracted separately as 3 kg batches that were exhaustively extracted by percolation with 70% methanol in water. The percolate, concentrated under reduced pressure, when left for 24–36 h at 4 °C gave two phases which were separated by decantation. The leaf extract gave a brown viscous residue (fraction B, 127 g) and the stem extract gave a precipitate (fraction B', 19·5 g) when dried under vacuum. The mother liquor was freeze- or spray-dried to yield fractions A (306 g) from leaves and A' (76·5 g) from stems. The marc was dried at room temperature (20 °C) and percolated with 95% ethanol. The percolate on evaporating to

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dryness, under vacuum, yielded fraction C (10 g), a dark green residue from leaves, and C′ (1 g) from stems.

Fractionation of B (5–20 g) was attempted by refluxing exhaustively with solvents of decreasing polarity (acetone, ethyl acetate, and dichloromethane). After cooling and filtration, the fractions were dried under vacuum and tested for antiparasitic activity. B, 62 g, refluxed with dichloromethane (3 × 150 mL) gave an insoluble fraction (4·2 g) and a soluble fraction D (3 g) which proved to contain the active principle(s). Fraction D (10 g), dissolved in diethyl ether (200 mL), was washed successively with M KOH (100 then 2 × 50 mL) then water. After the fraction had been dried over anhydrous Na_2SO_4, the remaining solvent was evaporated on a water bath to yield a sticky dark brown residue, fraction E. The alkaline layer, acidified with M HCl, gave an abundant yellow suspension which was separated by centrifugation; the supernatant was extracted with diethyl ether and washed with water to yield fraction F, on removal of solvent. The precipitate, when dissolved in diethyl ether, afforded a dry fraction, G.

Thin layer chromatography (TLC)

TLC aluminium sheets (Merck) precoated with F-254, silica gel, cellulose or aluminium oxide (neutral-type E), were used with developing systems (A) benzene–methanol (50:6), (B) benzene–dioxane–acetic acid (90:25:4) and (C) benzene–ethyl acetate (75:25) for silica gel, and aluminium oxide adsorbents and (D) isopropanol–boric acid 0·03 M (85:15) for cellulose. Detection: UV light at 254 & 366 nm was used for all adsorbents; spray reagents: 1% vanillin-H_2SO_4 and conc. H_2SO_4 for silica gel and aluminium oxide, 1% $AlCl_3$ in ethanol and 5% $FeCl_3$ in methanol for cellulose.

Column chromatography

Fraction E (5 g) was chromatographed over 75 g of silica gel 60 (70–230 mesh ASTM, Merck), using the graded elution system: light petroleum (b.p. 30–40 °C)–benzene–methanol (50:50:0), (25:75:0), (0:100:0), (0:99:1), (0:97:3), (0:0:100). Fractions collected were biologically assayed after removal of solvent under vacuum.

Preparative liquid chromatography (PLC)

Fractionation of E was attempted with PLC on Chromatospac-Prep 10 Jobin-Yvon over silica gel (Lichroprep-Si 60) using the elution system: benzene–methanol (99:1), (98:2), (97:3), (96:4), (95:5) and (0:100), column compression, 10 bars.

Isolation of PGA

The active substance PGA (*Polygonum glabrum* anthelmintic) was crystallized from E_1 (a viscous transparent greenish fraction collected from chromatography of E) as follows: E_1 (2·67 g) in methanol (30 mL) was treated with neutral charcoal (0·3 g), then passed over 30 g of aluminium oxide F-254 neutral type 'E' using 80% methanol at 45 °C. After TLC monitoring, similar fractions were grouped into fraction E_2, a yellow transparent liquid at 45 °C, which turned whitish and turbid when concentrated under vacuum and gave PGA as white crystals when this liquid cooled slowly (24–48 h) in the dark.

Bioassays

In-vitro methods

(a) *Hymenolepis nana* var. *fraterna*: The parasite, isolated from mice, was maintained in a survival medium at 37 °C and subjected to the fractions at decreasing doses, according to the methods described by Steward (1955), Sen & Hawking (1960), Crowley (1961), Cavier & Leger (1966) and Un (1966). Mice, 20–25 g, were experimentally infected with mature eggs of the parasite and 2–3 weeks later adult worms were isolated from the intestines, washed and maintained in the medium of Sen & Hawking (1960) for 30 min. They were then distributed to petri dishes (3 worms each) with 10 mL of medium in which the required amount of test substance had been dissolved. After 24 h incubation at 37 °C, activity was assessed by comparison of binocular readings of the lethal dose (LD) for the reference and control tests. (A dose was considered lethal when all worms incubated lost their vitality; however, for all in-vitro assays, when the LD was found ≥1 mg, the result was considered non-significant.)

(b) *Fasciola hepatica*. The methods described by Benex (1966), Notteghem et al (1974) and Julien (1979) were used. The parasite was isolated from the livers of recently slaughtered cattle by dissection of the bile canals. Adult parasites were collected, placed in a sterile beaker containing the survival medium described by Cavier & Leger (1966), Benex (1966), Cavier & Notteghem (1968) and Julien (1979), washed and incubated for 30 min at 37 °C, then distributed to sterile petri dishes each with two worms in 10 mL of medium with the required amount of test substance. Activity was assessed as before.

(c) *Entamoeba histolytica*. Amoeba culture was maintained in a monophasic medium (Jones 1952) preinoculated with 10^4 amoebae mL^{-1}, in a buffer solution at pH 7·2. Decreasing doses of a fraction to be tested were prepared in test tubes each containing 10 mL of the medium described by Taylor & Baker (1968). Biological activity was considered significant when an inoculated culture was inhibited (Cavier & Cenac 1972). To determine the minimal inhibitory dose (MID), we sought the smallest amount of a fraction which completely stopped culture development within 48 h at 37 °C. As a confirmation, 1 mL from a tube containing the MID was added to 10 mL of fresh medium and if no culture developed after 48 h at 37 °C, the dose was considered effective (Jones 1952; Cavier & Cenac 1972).

In-vivo methods

We followed the methods described by Cavier & Notteghem (1968), to test the anthelmintic activity of PGA in white mice infected with *Hymenolepis nana* var. *fraterna*. Eggs in faeces were counted after 15–20 days of infection, then the animal was fasted for 24 h and oral treatment begun with PGA in suspension in water with 1% arabic gum. After three days, animals were killed and the dead worms counted. Ten mice were used in each test; results were compared with anthelmintic reference substances studied under the same conditions (Cavier & Notteghem 1968).

Evaluation of molluscicidal activity

PGA and fraction A were tested for molluscicidal activity against *Biomphalaria glabrata* and *Limnea truncatula* Müll. (bred in our laboratories), according to the WHO method (WHO Expert Committee on Bilharzia 1965).

RESULTS AND DISCUSSION

We began to trace the antiparasitic activity of *P. glabrum* with a fractionation of leaves and stems, then the most potent fractions were selected, purified further and bioassayed thus leading to the isolation of a pure anthelmintic substance (PGA) from the leaf. PGA had highly significant taenicidal activity, in-vitro, whereas it had weaker trematodicidal and amoebicidal activities. The anthelmintic activity was mainly studied against *Hymenolepis nana* var. *fraterna* of the white mouse.

Preliminary evaluation of the fractions showed no trematodicidal activity when tested against *Fasciola hepatica*, while fraction B was active against *Hymenolepis nana* var. *fraterna*. Biological assays of extracts obtained from fraction B confirmed its good solubility in solvents of varying polarity but dichloromethane was the most suitable (Table 1). TLC (silica gel, system A) of fraction E revealed twelve spots and column fractionation gave fraction E_2 which corresponded to the largest spot on the chromatogram R_F (0·62) in yields (w/w) of 60% from E and 0·38% from the leaf. The ultimate purification of E_2 gave the substance PGA. Anthelmintic assays demonstrated the highest potency in E_2 when compared with other impure fractions so far obtained. E_2 is a viscous greenish transparent residue.

Fractionation of E was attempted with PLC but collected products proved to be different from E_2, according to TLC. Moreover, bioassays confirmed that E_2 was four times more potent than each of the new fractions which might possibly have been artifacts. Hence fraction E was exclusively purified by column chromatography, PGA being finally isolated as white crystals, m.p. 110–115 °C, using the system CH_3OH–H_2O. TLC of PGA in various developing systems confirmed the isolation of a pure substance which was unstable, new spots with lower R_F values appearing in the chromatogram (systems A and C), when PGA was kept for three weeks under vacuum in a desiccator at 22 °C in the dark.

The in-vitro screening for antiparasitic activity of divers fractions isolated from the leaf of *P. glabrum*, confirmed the properties of PGA, the anthelmintic activity against *Hymenolepis nana* var. *fraterna* being observed at LD $\geqslant 2 \times 10^{-3}$ mg mL^{-1} which is ten times lower than that of the crude extract B. Pure helenin (Carl Roth Labs.) and pure santonin (Fluka Labs.), used as control anthelmintics, were active at LD $\geqslant 10^{-2}$, 10^{-1} mg mL^{-1}, respectively.

The trematodicidal activity of PGA against *Fasciola hepatica* was observed at LD $\geqslant 6 \times 10^{-1}$ mg mL^{-1} while the crude extract B was inactive. The activities of reference products were: santonin LD $\geqslant 1 \times 10^{-1}$ and helenin LD $\geqslant 5 \times 10^{-3}$ mg mL^{-1}; readings were after 24 h contact with the parasite.

The amoebicidal activity of PGA against *Entamoeba histolytica* was observed at MID $\geqslant 5 \times 10^{-1}$ mg mL^{-1} while metronidazole (Flagyl) was active at MID $\geqslant 5 \times 10^{-3}$ mg mL^{-1}.

Santonin, which we used as a reference anthelmintic, is a sesquiterpene lactone (Herz 1971), and as the spectral analysis of PGA suggested a lactone functional group, we observed the effect of alkaline hydrolysis on the anthelmintic activity of PGA. The assay of products resulting from this hydrolysis was against *Hymenolepis nana* var. *fraterna*, in-vitro. Alkaline hydrolysis should open a lactone ring,

Table 1. Anthelmintic activity of preliminary fractions and extracts obtained from fraction B, as studied in-vitro against *Hymenolepis nana* var. *fraterna*.

	Leaf extracts					Stem extracts		
Fractions	A		B		C	A′	B′	C′
LD ⩾ x in mg mL^{-1}	1		2×10^{-2}		6×10^{-1}	(—)	6×10^{-2}	(—)
Fractions	Acetone extract		Ethyl acetate extract		Dichloro-methane extract			
LD ⩾ x in mg mL^{-1}	2×10^{-2}		2×10^{-2}		1×10^{-2}			
Fractions	E	F	G	E_2	PGA			
LD ⩾ x in mg mL^{-1}	1×10^{-2}	(—)	5×10^{-2}	3×10^{-3}	2×10^{-3}			

(—) = Absence of activity.

besides other possible structural modification, and it was thought that this might increase the biological activity, but the products tested had no activity.

The in-vivo anthelmintic activity of PGA was assayed against *Hymenolepis nana* var. *fraterna* at 200–600 mg kg^{-1} and gave no positively significant results, however, we observed a good tolerance of PGA in white mice at doses ⩽400 mg kg^{-1}. Reference products, mepacrine (Quinacrine—SPECIA) and helenin, were active, in-vivo, against *Hymenolepis nana* var. *fraterna* at 220 mg kg^{-1} (100% effectiveness) and 300 mg kg^{-1} (60% effectiveness), respectively. One consideration is that the active principle(s) should reach the small intestine in effective concentrations.

Extracts from the leaf (PGA and fraction A crude extract: water-soluble) were molluscicidal when tested against *Biomphalaria glabrata* and *Limnea truncatula* Müll, while El-Tohami (1979) found aqueous extracts from the leaf of the same plant not to be molluscicidal against *Biomphalaria pfeifferi* and *Bulinus truncatus* (from Sudanese breeding), when assayed at 250 ppm. Fraction A gave 100% mortality of *B. glabrata* and 40% mortality of *L. truncatula*, after 24 h exposure at 100 ppm. PGA gave 100% mortality of *B. glabrata* at 1–0·5 ppm and 100% mortality of *L. truncatula* at 2–1 ppm, after 24 h exposure. At similar concentrations, $CuSO_4$, the first widely used chemical molluscicide, was reported by Ayad (1961) to be lethal to some aquatic snails, after 48 h exposure. Hopf et al (1963) found copper salts to be lethal to *B. glabrata* adults at 0·1 ppm after 24 h exposure (pH < 7) in hard water. However, the molluscicidal activity of our extracts compared well with the activity of other natural products. The fruits of an Ethiopian species *Phytolacca dodecandra* L'Herit (Phytolacaceae), are active against some snail species with a lethal dose of 120 ppm (calculated as dry fruit extract) and some other plant materials such as saponin and rotenone are lethal to snails only at doses ⩾150 ppm (Lemma 1970).

Analysis of the results obtained by testing the activity of fractions isolated from the leaf of *P. glabrum* of Sudan confirm the medicinal value of this plant. The structure elucidation analysis made so far, suggests that PGA could possibly be a tetracyclic sesquiterpene bearing the functions indicated by the ^{13}C NMR and other spectra. Fukuyama et al (1985) have reported the isolation of novel terpenoids from a plant species belonging to the same genus. Due to the complexity of structure of PGA, isolation of large quantities of the material and spectral studies to determine the exact structure and stereochemistry of PGA, are in progress.

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Appendix

Spectroscopic data

The infrared spectra were determined on a Perkin-Elmer 125 spectrophotometer using KBr pellets. Ultraviolet spectra were determined in methanol. Magnetic resonance spectra were recorded in $CDCl_3$ using either a Cameca spectrometer operating at 250 MHz (^1H) or a Varian FT 80 A (^{13}C). Chemical

shifts δ (ppm) were relative to TMS as internal standard. The coupling constants *J* were reported in Hz. Mass spectra were run on an AEI MS 50 under 70 eV using a direct insertion probe.

Melting points were taken on a Buchi-Tottoli apparatus and were uncorrected.

IR: (KBr pellets), ν_{max} 3350, 2960–2860, 1730, 1710, 1695, 1645, 1450, 1260, 1230, 1170–1140, 1080, 1040, 1010 cm^{-1}.

UV: $\lambda_{max}^{CH_3OH}$; 217 nm, (ε_{max}; 125).

^1H NMR: ($CDCl_3$) δ 0·9–1·10 (*m*, 10 H), 1·25–1·36 (*m*, 2 H), 1·72–1·88 (*m*, 5 H), 1·93–2·17 (*m*, 8 H), 3·62 (*s*, 1 H), 4·13 (*d*, *J* = 11 Hz, 1 H), 4·45 (*d*, *J* = 11 Hz, 1 H), 4·90 (*dd*, *J* = 12·5 and 10 Hz, 1 H), 5·14 (*qd*, *J* = 10 and 4 Hz, 1 H), 5·26 (*t*, *J* = 2·5 HZ, 1 H), 5·52 (*s*, 1 H) and 6·03 (*qi*, *J* = 7·5 Hz, 1H).

Table 2. ^{13}C NMR ($CDCl_3$) data of PGA.

Carbon	δ	Multiplicity	Carbon	δ	Multiplicity
1	14·2	q	14	68·7	d
2	15·7	q	15	79·7	d
3	17·1	q	16	99·1	d
4	17·2	q	17	116·5	d
5	20·5	q	18	127·7	s
6	23·4	t	19	128·0	s
7	28·1	q	20	136·5	s
8	34·4	s	21	138·1	d
9	39·4	s	22	138·8	d
10	43·1	t	23	139·2	d
11	49·1	d	24	167·3	s
12	61·1	d	25	172·5	s
13	68·6	t			

MS (70 eV): *m/z* (rel. int. %), M^+: 414 (2), 186 (23), 171 (12), 91 (10), 85 (11), 83 (100), 82 (16), 59 (11), 57 (16), 55 (58), 43 (28), 41 (21), 39 (10), 29 (15), 28 (21).

m.p.: 110–115 °C. Found: C; 69·08%, H; 8·16%, $C_{25}H_{34}O_5$ required: C; 72·4%, H; 8·21%.

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