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Nematicidal Effect of Paeonia Suffruticosa Constituents

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ABSTRACT

Due to their harmful effects on the environmental phases, synthetic nematicides are now reappraised and the public concerns are tending to the natural nematicides as an alternative safe strategy for nematode control. The methanol extract of *Paeonia suffruticosa* leaves was tested for its activity at a concentration of 1000 ppm on the L_1 larva and egg-bearing adult stages of *Caenorhabditis elegans* nematode and it proved to be active on both stages. The extract was fractionated to ten fractions by liquid partitioning followed by chromatographic separation on Sephadex LH-20 open column chromatography. The most active fraction (0.61 %) was hydrolyzed to its components with concentrated sulfuric acid and the resultant constituents were purified by thin layer chromatography (TLC) and paper chromatography (PC). These components were identified with ^{1}H and ^{13}C NMR spectroscopy to gallic acid and D-glucose, so the active constituent is a gallotannin derivative.

INTRODUCTION

Invasion of nematodes on plants is a very serious problem as they affect as one of the most dangerous causal agents decreasing crop yields. Rovira *et al.* (1981) reported that, the cereal cyst nematode, *Heterodera avenae* severely decreased the wheat yield with 64%. *Tylenchulus semipenetrans* at population densities above the economic threshold caused altered patterns of root and shoot growth in citrus trees. (Hamid *et al*., 1985). Orui (1998) showed that, 13 species of root knot nematodes, *Meloidogyne species* are very agriculturally important plant parasitic nematodes infest many plant species in Japan. Generally, many crops have been attacking with nematodes in many countries losing the agriculture productivity with 77 billion dollars annually in the world (Sasser and Freckman, 1987). Therefore, many efforts have been doing for controlling nematodes including agricultural methods like crop rotation and resistant cultivars; physiological active agents as antibiotic and anthelminic drugs; plant growth regulators, inhibitors and synthetic nematicides (Osman and Viglierchо, 1988). Synthetic nematicides are low specificity neurotoxic compounds, negatively affect the environmental phases are using to control nematodes (Gonzalez and Estevez, 1998) Aldicarb suppressed the plant-growing-promoting bacteria that enhance potatoes growth due to its long-term use (Sturz and Kimpiski, 1999). Fatigue and light-headedness, acute progressive respiratory, gastrointestinal and neurologic symptoms caused by methyl bromide among the field workers (Herzstein and Cullen, 1990). So, public concerns over biological control to replace using chemicals have been growing as several plant species reduced nematode populations when either planted together or added to soil (Matsuki *et al.,* 1997). Nishizawa *et al.* (1983) revealed the effect of *Paeoniae radix*, family paeoniaceae on the urea-nitrogen concentration and owed this effect to its content of galloyl glucoses. So this research was carried out to evaluate the activity of its original extract and the ether-free fraction against both survival and reproduction of C. *elegans* L_1 larva and egg-bearing adult stages as a recommended experimental animal at different exposure times as well as the active extract was re-fractionated to its constituents to reach the chemical structure of the nematicidally active constituent.

MATERIALS AND METHODS

A) Extraction and separation of *Paeonia suffruticosa* leaves constituents:

As shown in scheme (1), 0.6 kg of the air-dried leaves were blinded and extracted with 1.2 liter of methanol at room temperature and kept in the dark for a week. After filtration, the remained plant tissues were re-soaked in methanol (1.0 Liter) for another week. The combined filtrates was concentrated under vacuum at 40^{o} C to 140 ml and centrifuged at 3000 rpm for 10 minutes. The supernatant was concentrated to 36 gm of dried oven extracted materials. The resulted extract (26 gm in 240 ml water) was partitioned twice with diethyl ether followed by petroleum ether (100 ml of each). The organic layers were combined and dried over anhydrous Na_2SO_4 to 3.3 gm. The aqueous layer was concentrated

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to 22.7 gm. The two fractions were 12.7 % and 87.3 % of the oven-dried extracted materials.

The aqueous extract (5.0 gm) was chromatographed on Sephadex LH-20 (2.5 × 70 cm) with ethanol (1500 ml, 20 ml/hr); then water (500 ml, 35 ml/hr); followed by 10%, 50% and 60% aqueous methanol (600 ml of each) at 35 ml/hr. The adsorbed materials were recovered with 70 % aqueous acetone. Based on the UV spectrum measured at 280 nm except for the acetonic fraction (fraction X) the eluate was fractionated into ten successive fractions as follows:

Fraction	I	II	III	IV	V
(mg)	3.5	394	650	345	422
Fraction	VI	VII	VIII	IX	X
(mg)	363	1311	58	70	585

B) Chemical hydrolysis of the acetonic fraction:

The acetonic fraction (fraction 10) was refluxed in 1N H_2SO_4 at 110^o C for 2 hours. After cooling, the reaction mixture was extracted with diethyl ether and ethyl acetate successively. The combined organic layers were concentrated under vacuum and the resulted concentrate was purified on thin layer chromatography (TLC) using the silica gel (Merck Kiesel gel 60 PF 254). The sample was diffused with the solvent system benzene: propionic acid: water at a ratio of (4: 4: 0.1) in volume. The purified compound was collected at 3.0 – 4.2 cm against 10.5 cm of the used solvent system, separated from the silica gel and dried to give 68 mg of amorphous powder identified as gallic acid. The aqueous layer was neutralized by $Ba(OH)_2$ to pH 6.8. After removal of barium hydroxide at 3000 rpm for 10 minutes, two drops of the product were added to phenol (two drops) in a test tube, then five milliliters of sulfuric acid were added to the test tube. Orange color appeared indicated the presence of glucose, which was purified as follows:

The filtrate was concentrated and applied to (20 × 15 cm) sheet of Advantec Toyo No. 526 paper chromatography. The suitable elution system was prepared by mixing ethyl acetate (EtOAc), pyridine and water at a ratio of (2: 1: 2) in volume and separating the mixture in a separatory funnel. The upper layer was used as the elution system. The glucose concentrate was charged by syringe needle to the paper sheet and diffused with the composed solvent system. The paper sheet was cool-air dried until disappearance of the pyridine smell and two narrow strips were cut on each side and sprayed with the aniline spray. The aniline spray was composed as (Islam *et al*., 1997) by mixing 1.6 gm of phthallic acid, 0.92 ml of aniline, 49 ml of n-butanol, 49 ml of diethyl ether and 2 ml of water. After keeping these strips at 110^o C for 10 minutes in an oven, the sugar band turned to brown. Comparing the strips with the chromatographic paper sheet, the glucose band was collected at 4.5–9.0 cm against solvent system. The sugar band was cut into small squares and dissolved in re-distilled water and then filtered. The filtrate was concentrated and dried under vacuum to give 27.9 mg of glucose.

C) Composition of the active fraction:

The components were identified as gallic acid and D-glucose as follows:

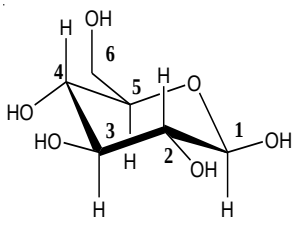
1- Gallic acid

In the ^{1}H NMR spectra (acetone-d_6), decoupled OH protons show a broad band centered at 8.25 ppm. The two aromatic protons (2-H and 6-H) give a singlet band at 7.19 ppm. From the ^{13}C NMR spectrum, signals at 110.1, 138.7 and 146.0 ppm are arisen from C-2 or C-6, C-4 and C-3 or C-5 carbon atoms, respectively. As the quaternary carbon atom (C-1) gives the signal at 121.99 ppm, the carbon atom of COOH group gives its signal at 168.01 ppm.

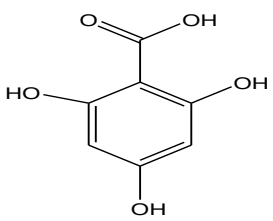
2- D-Glucose

From its ^{1}H NMR spectrum (methanol-d_4), bands at 5.1 ppm (d, J = 3.9 Hz) and at 4.47 ppm (d, J = 7.6 Hz) are assigned to 1-Hα and 1-Hβ anomers respectively. The multiplet bands at 3.23–3.9 ppm are resulted from both anomers of 2-H, 3-H 4-H, 5-H, 6-H and 6-H' protons. While ^{13}C spectrum (D_2O) shows that, signals of both α and β anomers of C-1, C-4 and C-6 rotationally occur at 92.9 and 96.7, 70.4 and 61.5 ppm. The β-anomer signals of C-3 and C-5 atoms overlapped at 76.6 ppm. Signal of C-3α occurs at 73.5 ppm. Overlapped signals at 72.2 ppm are due to the overlapping of C-2α and C-5α. The β-anomer of C-2 gives signal occurs at 74.9 ppm.

Based on the ^{1}H NMR and ^{13}C NMR spectroscopic measurements of the resulted components, the active fraction is supposed to be a galloyl glucose derivative (gallotannins).



D-Glucose



Gallic acid

D) Culture of the tested nematode stage:

The tested nematode cultured on S medium (Sulston and Brenner, 1974) spread with *E. coli* as food from dauer larvae. The consistent dauer larvae were produced by treatment with 0.2% aqueous sodium dodecyl sulfate (SDS) for 10 minutes (Golden and Riddle, 1984), washed twice with M9 buffer and once with S medium and collected at 2000 rpm for 2 minutes as a moist pellet. After removing the over moisture the

consistent dauer pellet was incubated into the 3.5 cm NGM agar plate spread with *E. coli* at 20°C. The dauer larvae resumed its developmental growth within 72 hrs to the egg-bearing adults, which were transferred under the binocular microscope to the bioassay test.

E) Bioassay technique:

A suspension of *E. coli* OP50 in S medium was used as a bioassay medium. Egg-bearing adults of *C. elegans* were picked up under the microscopic observation in 20 µl of the used medium to a hole slide glass. The slide was placed in 9 cm petri dish supported on two glass rods, 15 ml of distilled water was added to the dish. A strip of filter paper (26 × 1.5 cm) was surrounded on the inner wall of the dish. The food (*E. coli*) was introduced to the slide in 160 µl of the used medium (2 gm wet /80 ml). The tested sample was transferred to the slide in 20 µl. The total volume was 200 µl. The tested concentration was 1000 ppm. Twenty-seven adults were individually treated in each treatment, while larvae were treated in three replicates for each treatment. Control was concurrently conducted by using 20 µl of S medium instead of the toxic material. Both mortality percents and reduction in the treated adults reproduction were determined after 8, 16, 24, 36, 48, 65 and 72 hours. Recording data was continued to 132 hours in case of larvae. Mortality percentages were calculated according to the following formulas:

Natural death % = 100 (X / Y).

Survival % = 100 (C / [D – (D × natural death)].

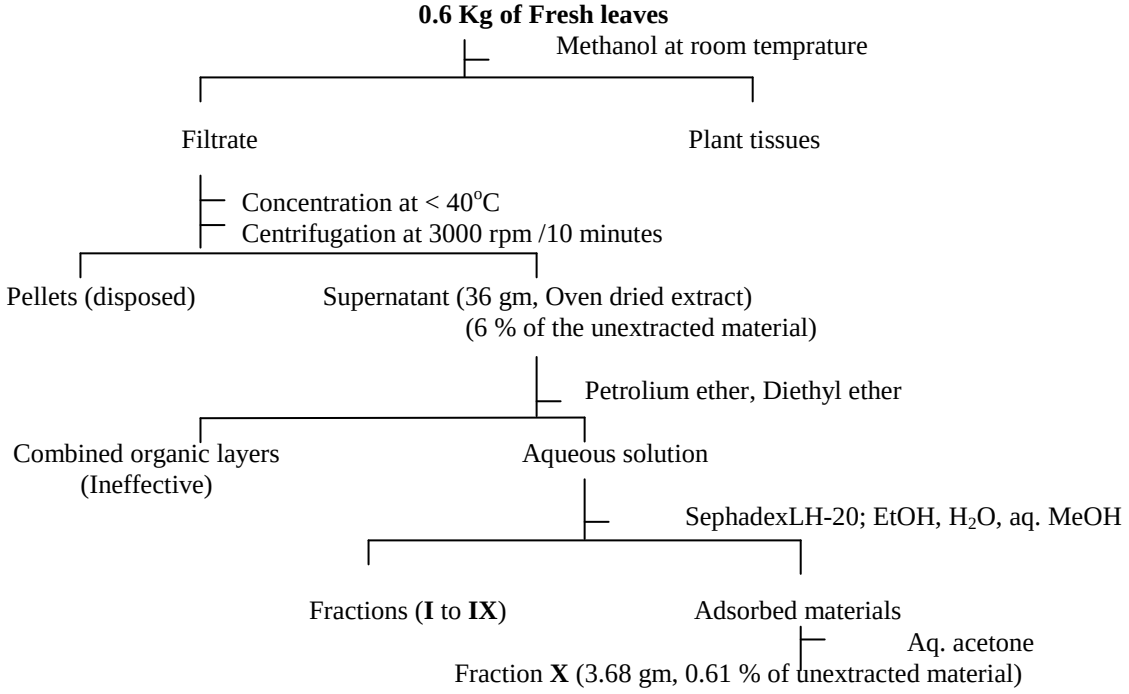
Where:

X, the dead nematodes in control

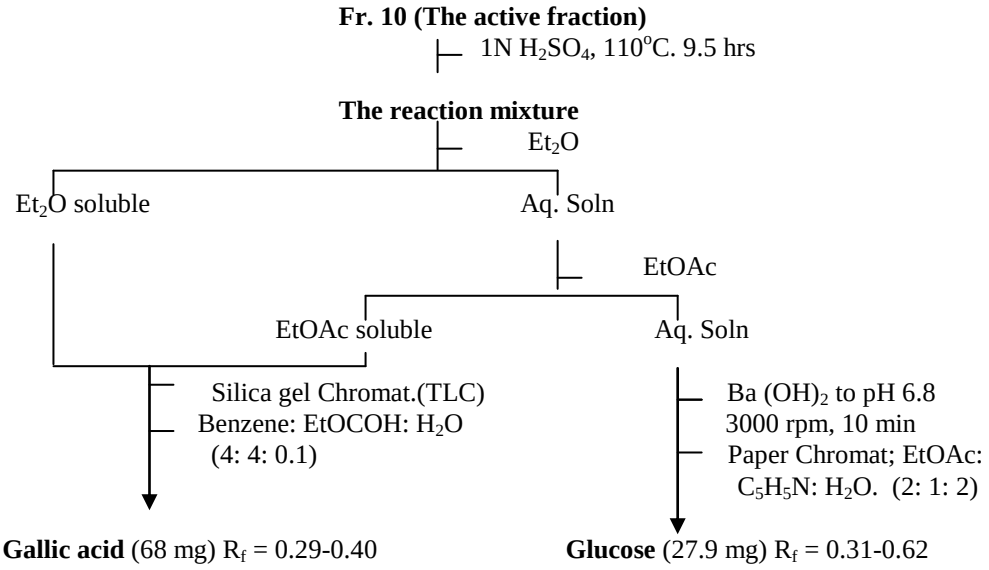
Y, the applied nematodes in control;

C, the survivors in treatment

D, the applied nematodes in treatment



Scheme (1): Extraction and chromatographic separation of *Paeonia suffruticosa*



Scheme (2): Chemical hydrolysis of the active fraction.

Reduction in the offspring number was calculated for each treatment comparing with the control using the following formula:

Reduction % = 100 × (C-T / C)

C: An adult offspring number in control

T: An adult offspring number in treatment

RESULTS AND DISCUSSION

A) Nematicidal activities of un-fractionated extracts:

The effects of both original and ether free extracts of *P. sufruticosa* leaves on both L_1 larva and egg-bearing adult stages of *C. elegans* are recorded in Tables (**1&2**). Comparing with the untreated population, the original extract caused 90% mortality after 5 days on L_1 larva with lethal effective period of 50% population (LEP_{50}) equals 1.51 days lowering its reproduction to 64% of control, while the ether-free extract completely killed the population with complete suppression of their reproduction within the same treatment period with LEP_{50} equals 1.92 days. The effect was slightly differed against the egg-bearing adult stage as the original extract exceeded the ether-free extract killing all the treated population in two days with LEP_{50} equals 1.1 days comparing with 89% mortality with LEP_{50} equals 1.8 days after three days treatment. They gave nearly the same effect against the reproduction on the treated populations. These results encouraged the separation of the ether-free extract to its constituents to reach the active structure in this plant.

B) Nematicidal activitiy of the separated fractions:

Comparing the untreated animals that were completely active with normal movement and behavior, the separated fractions differently affected the tested nematode. Fraction 8 appeared to be inactive on both mortality and reproduction of the treated population as it caused no lethal effect or any inhibiting effect on its reproduction through 72 hours exposure. Although fractions 2 and 9 caused a very weak and no mortal effect after 72 hours exposure at the tested concentration, they respectively inhibited the treated population reproduction to 56% and 75% of control within the experimental period. Both fractions 3 and 4 killed 22% of nematodes until 72 hours reducing their reproduction to 79% and 60% of control in systematic arrangement **(Table 3).**

On the other hand, although fraction 5 gave the same lethal effect (22% mortality), its effect appeared after three days exposure and its effect on the reproduction was deeper (47% of control). Vice versa, fraction 7 gave the same mortality % in an earlier time with no reduction in reproduction. Fraction 6 reduced the reproduction to 44% of the untreated population with 56% mortality within the same experimental period.

Among the separated fractions, fraction 10 was the most active affecting locomotion and activity of the treated population preventing them from the normal growth. Some irregular shapes of nematodes appeared staying nematodes in their places with only shaking their heads. It caused 33% mortality after 16 hours exposure only. It completely killed the treated animals after 24 hours exposure to the tested concentration exceeding the un-fractionated original extract, which gave 89%mortality after 72 hours exposure. Fraction 10 also

Table 1. Effect of *P. suffruticosa* extracts on *C. elegans* L_1 larva at 1000 ppm; shown as control activity.

Extract	Effect as	Time (hours)													LEP_{50} days
		0	8	16	24	36	48	65	72	84	96	108	120	132	
Control	A	0	0	0	0	0	0	0	0	0	0	0	0	0	
	B									100	100	100	100	100	
Original Extract	A	0	9	9	19	33	62	90	90	90	90	90	90	90	1.51
	B									0	0	8	35	64	-
Ether-Free Extract	A	0	10	10	10	16	53	58	68	89	89	95	95	100	1.92
	B									0	0	0	0	0	-

A: mortality %; B: reproduction%

Table 2. Effect of *P. suffruticosa* extracts on *C. elegans* egg-bearing adult at 1000 ppm; shown as control activity.

Plant species	Effect as	Time (hours)								LEP_{50} (EP_{50})
		0	8	16	24	36	48	65	72	
Control	A	0	0	0	0	0	0	0	0	
	B			100	100	100	100	100	100	
Original Extract	A	0	0	44	44	70	100	100	100	1.1
	B			42	37	33	29	23	19	< 7.0
Ether-Free Extract	A	0	0	0	11	33	56	78	89	1.8
	B			48	42	39	29	23	17	>3.0

A: mortality %; B: reproduction %

completely suppressed the treated population reproduction comparing to 17 % in case of the un-fractionated mother extract. The observed data translated the different biologically active contents of the separated fractions constituents and the active nematicidal compounds appeared be contained as large as in fraction 10. Effects of some separated fractions on the reproduction may be due to their interaction with oogenesis or spearmatogenesis pathways by binding some of proteins as three or more families called the major sperm proteins (MSPs) and other polypeptides that appear to be sperm specific because they were not detected in other worm tissues (Kimple and Ward, 1988). Such proteins increase the area of sites of action and effect deepness. At the same time, spermatogenesis is an energy-dependent process, which is blocked by metabolic energy inhibitors explaining another mechanism of action at this stage.

These results go with that revealed by Mohamed *et al*. (2000) as they isolated galloyl glucoses (mono-to hexa-galloyl glucoses) that showed nematicidal activity against *caenorhabditis elegans*). Inhibition of β-glucosidase, peroxidase, catalase, alcohol dehydrogenase and lactic dehydrogenase enzymes by tannin compounds was referred to the formation of substrate-tannin complex which is not attacked with enzyme. Compounds with a greater number of hydroxyl groups will be more efficient as their action as uncouplers of oxidative phosphorylation through the acidity of their hydroxyl groups.

Table 3. Effect of the separated *P. suffruticosa* fractions on C. elegans adult at 1000 ppm; shown as control activity.

Fraction	Effect as	Time (hours)							
		0	8	16	24	36	48	65	72
Control	A	0	0	0	0	0	0	0	0
	B			100	100	100	100	100	100
Fraction 2	A	0	0	0	0	0	0	11	11
	B			31	60	49	56	65	56
Fraction 3	A	0	0	11	11	11	11	22	22
	B			77	111	53	73	85	79
Fraction 4	A	0	0	0	0	11	22	22	22
	B			100	93	62	61	69	60
Fraction 5	A	0	0	0	0	0	0	0	22
	B			62	44	45	45	54	47
Fraction 6	A	0	0	33	33	33	44	44	56
	B			100	80	60	47	45	44
Fraction 7	A	0	0	0	11	22	22	22	22
	B			100	100	100	100	100	100
Fraction 8	A	0	0	0	0	0	0	0	0
	B			100	100	100	100	100	100
Fraction 9	A	0	0	0	0	0	0	0	0
	B			87	59	58	60	72	75
Fraction 10	A	0	0	33	100	100	100	100	100
	B			0	0	0	0	0	0
Unfract. Aq. Soln	A	0	0	0	11	33	56	78	89
	B			48	42	39	29	23	17

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الملخص العربي

النشاط الابادي لمكونات مستخلص نبات البيونيا علي نيماتودا الـ Caenorhabditis elegans

أحمد صبرى عبد العاطى

قسم كيمياء المبيدات – كلية الزراعة – جامعة الإسكندرية – الشاطبى – الاسكندرية- مصر

نظرا للتاثير الضار لمبيدات النيماتودا المخلقة علي كل أوجه البيئة تتجه الأنظار الي المركبات الطبيعية كبدائل لهذه المبيدات آمنة علي أوجه البيئة 0 أظهر نبات البيونيا Paeonia تاثيرا ابادیا علي النيماتودا محل الدراسة0 تم استخلاص أوراق هذا النبات بنقعها فى الميثانول على درجة حرارة الغرفة لمدة أسبوع مرتين متتاليين و بعد تبخير المذيب كاملا تم دراسة تاثيره علي الطور اليافع الحامل للبيضلنيماتودا الـ *Caenorhabditis elegans* 0 تم عمل تجزئة Fractionation لهذا المستخلص بالتوزيع بين الماء و الاثير البترولى (Petrolium ether) ثم ايثير ثنائي الايثيل(diethylether) و لوحظ تركز السمية في الجزء المائي الذي تم عمل فصل كروماتوجرافي لمكوناته باستخدام جيل الـ Sephadex LH-20 الي 10 قطفات ten fractions مختلفة باستخدام الايثانول والماء و الميثانول المائى كمذيبات ازاحة0

أظهرت 10 fraction أعلي سمية حيث تعدت المستخلص الام مسببة 100% موت لكل أفراد العشيرة المعاملة بعد فقط 24 ساعة تعرض مع اخماد كامل لقدرتها على التكاثر على كل أزمنة التعرض المختلفة مقارنة ب 11% موت فقط مع خفض القدرة على التكاثر الى 42% فقط من العشيرة الغير معاملة عند نفس زمن التعرض فى حالة المستخلص الأم0

أجرى تحلل حامضى لهذه الـ fraction بعمل refluxing لها مع حامض الكبريتيك 1 ع لمدة ساعة على 110 ^{5}م ثم التنقية بالتحليل الكروماتوجرافى بالطبقة الرقيقة (TLC) و الورق الكروماتوجرافى (PC) لمكونات هذه الـ fraction الفعالة0 بالتعرف عليهذه المكونات بواسطة جهاز الرنين النووى المغناطيسى ^{1}H NMR and ^{13}C NMR على أنها حمض الجاليك Gallic acid و الجلوكوزD-glucose مما يؤكد على أن المكون الفعال فى هذا النبات هو أحد مشتقات الـ Galloyl glucose (Gallotannins)**0**