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Effect of different concentrations of *Eriobotrya japonica* extract on control of infection by *Meloidogyne incognita* and *Cephalobus litoralis*

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This study discusses and developed methods for obtaining plant extracts/pure compounds and its usages as a nematicidal agent. Freshly hatched second-stage juveniles of two nematode species, *Meloidogyne incognita* and *Cephalobus litoralis* were used. A bioassay guided isolation of the extract, fractions and pure compounds were done for their nematicidal activity at different concentrations in comparison with *Azadirachta indica,* while distilled water was taken as control. The crude extract showed 90% and ethyl acetate fraction 97% mortality rate after 48 h at 1% concentration against *M. incognita* species and 81 and 50% against *C. litoralis* specie at the same concentration. Among the pure compounds, 4 and 9 showed maximum mortality of 90 and 91% and compounds 8, 3, 6, 2, 5, 7 and 1 showed 89, 88, 88, 82, 80, 80 and 69% mortality, respectively after 48 h in *M. incognita* sp. In *C. litoralis*, compounds 8 and 9 showed 72 and 75%, significant mortality, while 7, 4, 3, 5, 6, 2 and 1 showed 70, 70, 70, 68, 62, 60 and 58% mortality, respectively after 48 h. The plant is of economic importance with nematicidal value.

Key words: *Meloidogyne incognita*, *Cephalobus litoralis*, compound, mortality, crude extract.

INTRODUCTION

Eriobotrya japonica is been used to treat several diseases in East Asia. The leaves of *E. japonica* is widely used in traditional medicine for the treatment of many diseases including cough and asthma. It protects against oxidative stress and cognitive deficits induced by the Aβ peptide. *E. japonica* improves hyperlipidemia and reverses insulin resistance in high-fat-fed mice (Shih, 2010). Agricultural countries study the agricultural productivity which is appropriately protected from pests and diseases caused by insects, nematodes, fungi, viruses and bacteria; (Nasira and Shanina, 2007). Among these, nematodes have been considered universally, as one of the important microscopic organism which play significant role in the agriculture production in different diseases (Alam et al., 1979; Sultana et al., 2010a, b). In the form of plant parasitic nematodes, sometimes, it play very destructive role and causes loss of billions globally (Shurtleff and Averre, 2000).

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Some of the important nematodes species cause severe damage to the economically important crops e.g. *Heterodera avenae, Rotylenchulus reniformis, Pratylenchus* spp., *Hoplolamus* spp., *Xiphinema* spp., *Trichodorus* spp. and root-knot nematodes (Pathan et al., 2008). These nematodes attack almost all parts of plant, including roots, stem, leaves and seeds or fruits and as such damage all variety of crops; some of them are responsible for transmission of soil born viruses which produce deadly diseases in many plants (Shahid et al., 2007).

The realization has prompted increased studies all over the world on nematodes parasites to plants and their control (Amponsah, 2008). Small-scale farmers have limited access to the commercially available nematicidal and pesticidal services owing either to their unavailability or to their high cost. According to different researchers, the plant possesses not only beneficial characteristics but also pesticidal and insecticidal properties (Chitwood, 2003; Javed and Zaki, 2003; Javed et al., 2007). Many modern drugs are derived from plant but there are also an increasing number of herbal products commercially available (Javed et al., 2007, 2008).

MATERIALS AND METHODS

Preparation of plant extracts

The whole plant of *E. japonica* (25 kg) was collected from Swat valley in February. A voucher specimen (KUH # 139(678) was deposited in the Herbarium of Department of Botany, University of Karachi.

Extraction

The whole plant of *E. japonica* (25 kg) was dried in a dryer for three days at 50°C, ground, sieved and socked in 50 L ethanol for one week. The ethanolic extract was concentrated to a gummy material weighed to about 520 g.

Fractions

Crude ethanolic extract was further fractionated into hexane, chloroform, ethyl acetate and methanol.

Preparation of nematode *Cephalobus litoralis* culture

Culture of *C. litoralis* which reproduces parthogenetically was prepared using a single egg. Green peas (*Pisum sativum*) were mashed in small Petri dishes. A single egg was carefully picked under stereoscopic binocular and placed beside pea meal paste (PMP) in a Petri dish.

Nematode eggs hatched within 72 h and after 10 days, large number of nematodes in various stages of life cycle were obtained.

Preparation of nematode root-knot culture

Experiments were performed under laboratory conditions at 28± 2°C. Fresh egg masses collected from stock culture maintained on tomato root tissues were kept in water for egg hatching. The larvae emerged after 48 h from the egg masses incubated at 30°C and were used at test species for larval mortality studies. To determine the nematicidal effect of the various fractions and the pure compounds, freshly hatched second-stage juveniles were taken in tap water. The movements of the nematodes were checked by touching them with the needle.

Preparation of substrate for bioassay

Glass tubes, 15 cm long having a diameter of 8 cm were taken for bioassay. 2, 1 and 0.5 % solution of plant extracts and compounds were prepared in ethanol from stock solution. This solution was passed through Whatman filter paper No. 1 and 3 ml of it was taken in each tube. Four tubes were taken for each treatment whereas another four served as control set.

Inoculation

Nematodes larvae were isolated through modified Baermann funnel technique using Whatman filter paper No. 41 and larvae were counted in a dish with 0.5 cm square at the outer surface to determine their concentration. The required amount of nematode suspension was poured into the tubes each of which contain equal amount of plant extract, fractions and pure compounds 2, 1 and 0.5% had already been added. In other four tubes, distilled water with nematode larvae was taken as control. The experiment was run on benches under room temperature.

Experimental work

Column chromatography was carried out using silica gel of 70-230 mesh and flash chromatography on silica gel 230-400 mesh. Aluminium sheets precoated with silica gel 60 F_{254} (20 x 20 cm, 0.2 mm thick; E-Merck) were used for TLC to check the purity of the compounds and were visualized under UV light (254 and 366 nm) followed by ceric sulfate as spraying reagent. Optical rotations were measured on a Jasco DIP-360 digital polarimeter. The UV spectra were recorded on a Hitachi UV-3200 spectrometer (λ_{max} in nm). IR spectra were recorded on Shimadzu IR-460 spectrophotometer (v in cm^{-1}). EIMS, HREIMS, FABMS and HRFABMS spectra were recorded on Jeol JMS-HX 110 spectrometer with data system. The ^{1}H NMR spectra were recorded on Bruker AMX-400 MHz instruments using TMS as an internal reference. The chemical shift values are reported in ppm (δ) units and the scalar coupling constants *(J)* are in Hz.

RESULTS AND DISCUSSION

A bioassay guided isolation of the alcoholic extract, hexane, ethyl acetate, chloroform, methanol fractions and pure compounds were done for their nematicidal activity at 0.25, 0.5 and 1% concentrations, respectively, in comparison with *Azadirachta indica.* Structures of pure compounds (1-9, Figure-1) were earlier reported by chemical and spectroscopic methods including one dimensional (^{1}H-NMR, ^{13}C-NMR broad band and DEPT) and two dimensional (COSY-45. NOESY, *J-resolved*, hetero COSY) NMR techniques (Sohail et al., 2008; Kang et al., 2008) Figure 1. The nematicidal activity of the crude ethanolic extract, its fractions (hexane, ethyl acetate, chloroform, methanol) as well as pure compounds (1-9) were tested against *M. incognita* and *C.*

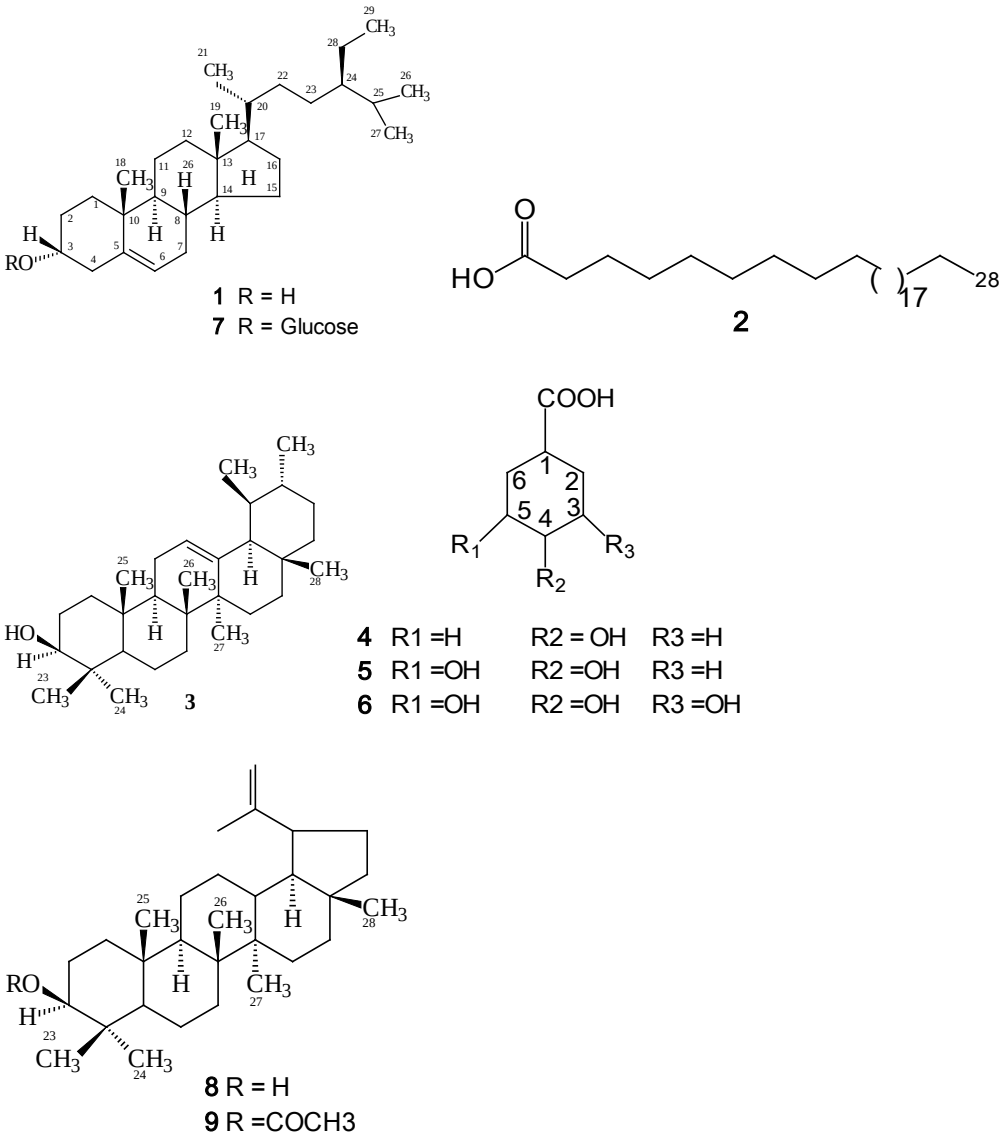

Figure 1. Structures of pure compounds (1 - 9) isolated from *E. japonica.*

litoralis (Noweer and Hasabo, 2005).

The nematicidal action of *E. japonica* extract, fractions and compounds in *in vitro* investigation against second stage juveniles of both species is shown in Tables 1 to 4. The 1% of crude extract showed 78% mortality and fractions of hexane 18%, ethyl acetate 69%, chloroform 35% and methanol 15% after 24 h, while after 48 h, crude extract showed 90%, fraction of hexane 19%, ethyl acetate 97%, chloroform 45%, methanol 25% at same concentration against *M. incognita* species. Nematicidal activity showed 1, 0.5, 0.25% concentration and control as shown in Table 1.

The pure compounds 1, 2, 3, 4, 5, 6, 7, 8 and 9 showed 57, 73, 71, 81, 70, 75, 72, 74, 76% mortality, respectively after 24 h, while after 48 h, compounds showed 69, 82, 88, 90, 80, 88, 80, 89 and 91% mortality, respectively. Nematicidal activity on 1, 0.5, 0.25% concentration and control is given in Table 2.

The 1% of crude extract, hexane fraction, chloroform fraction, ethyl acetate and methanol soluble fraction showed 77, 30, 29, 50 and 40% mortality against *C. litoralis* respectively after 24 h and 81, 35, 33, 50 and 48% mortality after 48 h, respectively. Nematicidal activity of other concentrations is given in Table 3.

The pure compounds (1-9) were isolated from *E. japonica* and tested for their nematicidal activity on *C. litoralis* larvae. The results of *in vitro* evaluation are shown in Table 4. Compound 9 showed 61%, 8- 54%, 7- 62%, 6- 55%, 5- 58%, 4- 66%, 3- 65%, 2- 50% and 1- 45% mortality after 24 h in 1%concentration while after 48 h, compounds showed 75, 72, 70, 62, 68, 70, 70, 60, 68 and 58% mortality in the same concentration.

Table 1. The larval mortality of root-knot *M. incognita*.

Fraction	Concentration after 24 h				Concentration after 48 h			
	Percent Mortality				Percent Mortality			
	1%	0.5%	0.25%	Control	1%	0.5%	0.25%	Control
Hexane	18	15	10	1	19	16	10	3
Chloroform	35	29	22	1	45	30	27	4
Ethyl acetate	69	48	30	2	97	72	45	5
Methanol	15	12	11	2	25	20	15	3
Crude	78	50	32	3	90	68	50	5

Table 2. The larval mortality of *M. incognita* (root-knot) nematodes.

Compound	Concentration after 24 h				Concentration after 48 h			
	Percent mortality				Percent mortality			
	1%	0.5%	0.25%	Control	1%	0.5%	0.25%	Control
β-sitosterol	57	44	42	2	69	64	45	3
Octacosanoic acid	73	52	30	1	82	57	62	3
Ursolic acid	71	64	50	4	88	70	68	5
4-hydroxybenzoic acid	81	68	57	2	90	73	62	5
3,4-dihydroxybenzoic acid	70	62	48	1	80	77	59	2
Gallic acid	75	66	50	2	88	71	58	4
β-sitosterol' *3-0-,β-D* glucopyranoside	72	55	47	4	80	73	50	5
Lupeol	89	41	36	2	74	70	57	3
Lupeol acetate	91	61	55	4	76	71	53	2

Table 3. The larval mortality of *Cephalobus litoralis* nematodes.

Fraction	Concentration after 24 h				Concentration after 48 h			
	Percent mortality				Percent mortality			
	1%	0.5%	0.25%	Control	1%	0.5%	0.25%	Control
Hexane	30	17	11	1	33	20	18	2
Chloroform	35	10	7	2	29	18	15	3
Ethyl acetate	55	22	14	2	50	30	22	3
Methanol	49	30	22	3	48	33	20	5
Crude	81	50	28	4	77	56	37	5

Nematicidal activity of other concentrations is given in Table 4.

The plant is of economic importance with nematicidal value. Phytochemicals are used in many drugs, insecticides, pesticides especially for plant diseases. The plants which have these proportions can be used in the manufacture of nematicide (Javed et al., 2006).

It is evident from the above discussion that there is a great likelihood of use of bio-control agents for disease control by nematodes (Akhtar et al., 1991; Javed et al., 2007). Although several potential bio-control agents have been isolated and tested for their efficacy against soil born root pathogens, there is need to discover new potential antagonists or improve strains of already isolated antagonists for better crop production. Possible environmental hazards due to the use of microorganisms as bio-control agents should also be looked into (Jiskani et al., 2005). Development of a simple, cheap and effective method for mass production of bio-control agents is a pre-requisite for the replacement of chemical fungicides by a bio-control agent which also needs investigation (Akhtar et al., 1991; Shakeel et al., 2010).

Table 4. The larval mortality of *Cephalobus litoralis* nematodes.

Compound	Concentration after 24 h				Concentration after 48 h			
	Percent mortality				Percent mortality			
	1%	0.5%	0.25%	Control	1%	0.5%	0.25%	Control
β-sitosterol	45	38	25	1	58	43	30	2
Octacosanoic acid	50	30	16	2	60	38	20	4
Ursolic acid	65	45	34	1	70	50	38	2
4-hydroxybenzoic acid	66	53	20	1	70	58	30	2
3,4-dihydroxybenzoic acid	58	34	24	0	68	40	32	1
Gallic acid	55	40	27	1	62	50	34	3
β -sitosterol' *3-0-,β-D* glucopyranoside	62	53	28	0	70	58	37	2
Lupeol	72	19	16	0	54	20	16	1
Lupeol acetate	75	49	37	1	61	56	49	2

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