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COMBINED NEMATOCIDAL EFFECT OF NONACOSAN-10-OL AND 23A-HOMOSTIGMAST-5-EN-3*B*-OL ON *MELOIDOGYNE INCOGNITA* (KOFOID AND WHITE) CHITWOOD

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ABSTRACT

The synergistic effect of three phytochemicals, nonacosan-10-ol, 23a-homostigmast-5-en-3*β*-ol and *cis*- and *trans*-protopinium were assessed in four combinations and three concentrations (5, 50 and 100 µg ml^{-1}) against *Meloidogyne incognita*, *in vitro* and *in planta* on the tomato cultivar Riogrande. Additionally, an *in vitro* phytotoxicity test on tomato seedlings was performed with the same phytochemical combinations. A combination of nonacosane-10-ol and 23a-homostigmast-5-en-3*β*-ol inhibited egg hatching at the highest rate and induced 100.0% mortality of second-stage juvenile (J2) at the concentration of 100 µg ml^{-1}. The egg hatch inhibition and J2 mortality were positively correlated with an increase in the concentrations of phytochemical as well as with the incubation time. All phytochemical mixtures displayed a nematicidal effect against *M. incognita* in *in planta* studies. The combine application of nonacosan-10-ol and 23a-homostigmast-5-en-3*β*-ol resulted in the highest reduction in nematode parameters (viz., galls, gall index, females g^{-1} of root and eggs g^{-1} of root) and promoted the greatest increase in the plant parameters (such as shoot and root lengths, fresh and dry shoot weights, and number of flowers $plant^{-1}$) at the highest concentration of 100 µg ml^{-1}. The phytochemical mixtures did not induce phytotoxic effects on tomato seed germination and seedling growth in *in vitro* tests. It was concluded from the present study that such phytochemical mixtures offer nematocidal potential in organic vegetable production systems or could be integrated with other management tools as a part of sustainable control strategies for plant-parasitic nematodes.

Keywords: Phytochemical combinations, nonacosane-10-ol, sterol, *cis*- and *trans*-protopinium, *Meloidogyne incognita*, tomato.

INTRODUCTION

The southern root-knot nematode (SRKN), *Meloidogyne incognita* infects a wide range of cultivated crops including tomato and is responsible for crop losses worth millions of dollars annually (Sasser and Freckman, 1987). Globally, the impact of this species is greater by its broad host range, which comprises more than 5,500 host plant species (Trudgill and Blok, 2001). Consequently, crop rotation is not an effective management tool (Moens and Perry, 2009), and hence other methods must be utilized. Although soil solarization may reduce the nematode populations in the top 30cm of soil and thereby minimize early plant infection (Katan, 1981), the presence of eggs at deeper soil levels makes this management tool ineffective as well (Katan, 1981). Much current research has focused on the need for development of more sustainable and less toxic alternatives to chemical nematicides for plant-parasitic nematode management. Amongst such tools, phytochemicals have great potential as sources for new compounds that could be safely developed into novel commercial nematicides (Naz *et al*. 2013a) and used in organic agriculture. Such phytochemicals are generally safe for the environment as well as humans (Chitwood, 2002).

Fumaria parviflora Lam. (Fumariaceae) is an annual herbaceous weed that grows in wheat fields (Shah *et al*., 2006) and is a non-host for *M. incognita* (Naz *et al*. 2015a). Application of this plant as a green manure or as a dry amendment reduces the inoculum density of *M. incognita* on tomato, both under controlled screen house and under field conditions (Naz *et al*. 2013b and 2015b). In addition, root and stem extracts of the plant showed stronger nematicidal activity against *M. incognita* than leaf extracts in *in planta* greenhouse experiments (Naz *et al*. 2013a). Seven classes of nematicidal constituents (Naz *et al*. 2013a) nclude three nematicidal phytochemicals from roots: nonacosan-10-ol, 23a-

homostigmast-5-en-3*β*-ol (Naz *et al*. 2013b) and *cis*- and *trans*-protopinium (Naz *et al*. 2016).

Although the nematode-antagonistic effects of the three individual phytochemicals from *F. parviflora* have been reported, the effects of their mixtures have not. The purpose of this investigation was to determine the optimal concentrations of alcohol (nonacosan-10-ol), sterol (23a-homostigmast-5-en-3*β*-ol) and alkaloid (*cis*- and *trans*-protopinium) applied as mixtures in the *in vitro* and *in planta*, and to evaluate their phytotoxic effects on tomato seed germination and seedling growth.

MATERIALS AND METHODS

Maintenance of nematodes: The inoculum of *M. incognita* was obtained from tomato (*Solanum lycopersicum* cutivar, Riogrande) production fields at Dargai, Khyber Pakhtunkhwa, Pakistan (Naz *et al*. 2013a). Inocula were maintained on *S. lycopersicum* Riogrande via single egg mass inoculation in a greenhouse at 25 ºC and 16 h of daylight. Eggs and second-stage juveniles (J2) were extracted from tomato roots by the Sodium Hypochlorite (NaOCl) (Hussey and Barker, 1973). Eggs and freshly hatched juveniles were separately used in laboratory bioassays whereas eggs and J2s were used in the screenhouse experiments.

***In vitro* nematicidal bioassay:** The nonacosan-10-ol (an alcohol), 23a-homostigmast-5-en-3*β*-ol (a sterol homolog) and *cis*- and *trans*-protopinium (an alkaloid) previously isolated from *F. parviflora* (Naz *et al*., 2013b and 2016) roots were tested in mixtures *in vitro* and *in planta* greenhouse experiments against *M. incognita* on tomato (*cv*. Riogrande). Stock solutions of each phytochemical were prepared by reconstituting the respective dried chemical (5.0 mg) in DMSO (1%; v/v) and then diluting with sterile water for the preparation of final concentrations of 5, 50 and 100 µg mL^{-1} (each chemical has a final concentration of 2.5, 25 and 50 µg mL^{-1} in a mixture). Four combinations of these phytochemicals (nonacosan-10-ol + 23a-homostigmast-5-en-3*β*-ol + *cis*- and *trans*-protopinium, nonacosan-10-ol + 23a-homostigmast-5-en-3*β*-ol, nonacosan-10-ol+ *cis*- and *trans*-protopinium and 23a-homostigmast-5-en-3*β*-ol + *cis*- and *trans*-protopinium) were used. Control treatments included simple distilled water (SDW) dissolved in DMSO (1.0 %). Nematode stock suspension was prepared by diluting the egg and J2s with sterile water to approximately 1000 ± 50 eggs mL^{-1} and 50 J2s mL^{-1}. Eggs and J2s were exposed to the phytochemical combinations at the above mentioned concentrations and incubated (~ 27˚C) for 6, 12, 24 and 48 h in 24-microwell plates (Multiwell™ 24 well, Becton Dickinson, USA) (Naz *et al*. 2013a) with each dilution replicated five times in a completely randomized (CR) design. Both experiments (egg hatching and J2 mortality assays) were performed as separate simultaneous experiments and performed twice. Finally, eggs and J2s were transferred to microwell plates (each well containing 1 mL distilled water) with micropipette to assess further hatching or recovery of mobility after 48 h. J2 were regarded as dead only if movement was not restored upon transfer to water.

***In planta* studies:** Three-week old seedlings of *S. lycopersicum* cv. Riogrande, grown in steam sterilized soil (100 ºC for 6 h) were transferred (one seedling/pot) to 15cm-diameter earthen clay pots (1000 cm^3). Three days later, 5,000 eggs were pipetted into three equidistant holes approximately 3-cm-deep surrounding the base of each plant stem. Then 50 mL of each phytochemical mixture (either two or three chemicals combined @ 5, 50 or 100 µg mL^{-1}, as discussed before) was applied to the rhizosphere of each transplant with a sterilized plastic pipette. Plants inoculated with only sterile distilled water served as controls. Seedlings were fertilized with optimum level of N:P:K and micronutrients and were watered thrice (weekly) with fresh water (300 mL of water per pot). The experiment was performed twice using a CR design with a factorial arrangement. After 60 days in the greenhouse at 25 ºC and 16 h of daylight, the nematode parameters (such as the number of galls $plant^{-1}$, galling index (GI), females g^{-1} of root, eggs g^{-1} of root) and plant parameters (for example shoot and root lengths (cm), fresh and dry shoot weights (g), fresh root weight (g) and number of flowers $plant^{-1}$) were assessed. The GI was scored using a 0–5 galling scale and eggs were extracted from the roots as described by Hussey and Barker (1973).

Phytotoxicity test on tomato seedlings: Uniformly sized seeds of tomato (cv. Riogrande) were surface disinfested with 5% sodium hypochlorite for one minute and washed for about 5 min in sterile water. Five sterile Petri dishes were lined with circular Whatman No. 1 filter paper. The filter papers in four Petri dishes were moistened with 20 ml of phytochemical mixes at a final concentration of 5, 50 and 100 µg ml^{-1} for each, with one sterile distilled water control. Ten surface-disinfested tomato seeds were placed in each Petri dish and incubated at 25 ˚C for two weeks. Phytotoxicity tests (Ghani, 1998; Ayeni and Yahaya, 2010) were performed by measuring the mean lengths of plumule and radicle by selecting five plants at random. The radicles and plumules were then wrapped up in separate envelopes and incubated at 80 °C in Gallenkamp oven (Model IH-150) to a constant weight to obtain the dry weight. The experiment was performed twice with five replicates per experiment arranged in a CR design with factorial arrangement

Data analysis: All the experimental data were analyzed by ANOVA using Statistix (Version 8.1, Analytical Software, Roseville, MN) (Campbell and Madden, 1990).

Treatment means were compared using Fisher's protected LSD test at p = 0.05 (Gomez and Gomez, 1984).

RESULTS

Egg Hatch inhibition of *Meloidogyne incognita:* Increase in concentration and exposure time of secondary metabolites extracted from *Fumaria parviflora* were found toxic to *Meloidogyne incognita* eggs (Table 1). Combined treatments of (alcohol + sterol) and 23a-homostigmast-5-en-3*β*-ol + *cis*- and *trans*-protopinium were recorded as highly toxic at all concentrations and eliminated hatching by 100 and 97.3 %, respectively at the highest concentration (100 µg ml^{-1} after 48h incubation). The combination of alcohol + sterol inhibited egg hatching more quickly than the other concentrations, as shown in the Table 1. Eggs, exposed to sterile distilled water as control, showed the least egg hatch inhibition. Importantly, there was no egg hatching after the eggs were submerged in the phytochemical mixtures and later incubated in sterile distilled water for three days.

Nematocidal activity against J2s of *Meloidogyne incognita:* In experiments investigating the combined effects of the three compounds from *F. parviflora* against the *M. incognita* J2s, the alcohol + sterol mixture at the highest concentration killed all nematodes after 48 h of incubation (Table 2). This combination was statistically superior to the other treatments. Increasing the concentrations of the tested compounds and incubation times significantly ($P < 0.05$) increased death of J2s of *M. incognita*; the majority of J2s killed, were observed in all the mixtures at all concentrations, except in the distilled water controls (Table 2). Maximum J2s mortality (100%) was observed at 100 µg ml^{-1} after 48h incubation in alcohol + sterol combinations and minimum in controls (16.0 %) (Table 2).

Table 1. Effect of combinations of phytochemicals from *Fumaria parviflora* on percent egg hatch inhibition of *Meloidogyne incognita*[a].

Compounds	Concentration (µg ml^{-1})	Percent egg hatch inhibition			
		6 h	12 h	24 h	48 h
(Nonacosan-10-ol + 23a-homostigmast-5-en-3*β*-ol) + (*cis*- and *trans*-protopinium)	**0 (control)[b]**	11.0 ^{j}	13.0 ^{i}	14.00 ^{f}	16.0 ^{g}
	5.0	59.80 ^{iA}	65.8 ^{hB}	72.3 ^{eBC}	78.3 ^{fC}
	50	72.30 ^{fgB}	74.5 ^{efB}	79.3 ^{dB}	85.8 ^{dB}
	100	81.50 ^{deC}	84.3 ^{cdC}	88.5 ^{cC}	91.5 ^{cB}
(Nonacosan-10-ol) + (23a-homostigmast-5-en-3*β*-ol)	**0 (control)**	11.0 ^{j}	13.0 ^{i}	14.00 ^{f}	16.0 ^{g}
	5.0	78.0 ^{efB}	79.8 ^{deA}	88.3 ^{cA}	91.3 ^{cA}
	50	88.5 ^{bcA}	92.5 ^{bA}	95.0 ^{abA}	99.0 ^{aA}
	100	99.9 ^{aA}	100.0 ^{aA}	100.0 ^{aA}	100.0 ^{aA}
(Nonacosan-10-ol) + (*cis*- and *trans*-protopinium)	**0 (control)**	11.0 ^{j}	13.0 ^{i}	14.00 ^{f}	16.0 ^{g}
	5.0	62.0 ^{hiB}	68.3 ^{ghB}	71.5 ^{eC}	80.3 ^{efBC}
	50	72.3 ^{fgB}	75.8 ^{efB}	78.5 ^{dB}	84.5 ^{deB}
	100	85.0 ^{cdC}	87.5 ^{bcBC}	90.7 ^{bcC}	92.8 ^{bcB}
(23a-homostigmast-5-en-3*β*-ol) + (*cis*- and *trans*-protopinium)	**0 (control)**	11.0 ^{j}	13.0 ^{i}	14.00 ^{f}	16.0 ^{g}
	5.0	67.0 ^{ghB}	73.8 ^{fgAB}	80.0 ^{dAB}	85.0 ^{dAB}
	50	84.8 ^{cdA}	88.3 ^{bcA}	92.3 ^{bcA}	93.8 ^{bcA}
	100	91.5 ^{bB}	91.5 ^{bB}	95.3 ^{abB}	97.3 ^{abA}
LSD (P < 0.05)		5.8	5.7	5.1	4.7

[a]Data are means of ten replicates combined from two experiments (spring and autumn, 2011). [b]Control treatments (0) utilized simple distilled water (SDW) dissolved in DMSO (1%). Means followed by the same lowercase or same uppercase letter do not differ significantly ($P \geq 0.05$) according to Fisher's protected LSD test. Lower case letters represent comparisons between different concentrations of the same phytochemical combination; upper case letters represent comparisons between the same concentrations of different phytochemical combinations.

Effects of phytochemicals on plant growth and *M. incognita* infectivity: At all concentrations tested, the phytochemical combinations significantly ($P < 0.05$) reduced nematode parasitism (Table 3). The Galling Index (1.3), and numbers of nematode galls (42.5), females g^{-1} of root (44.5) and eggs g^{-1} of root (1432.5) were the lowest in plants treated with the 100 µg ml^{-1}, of the alcohol + sterol mixture, followed by the 23a-homostigmast-5-en-3*β*-ol + *cis*- and *trans*-protopinium mixture. All concentrations of the phytochemical combinations increased ($P < 0.05$) tomato growth parameters (*viz*., shoot and the root lengths, fresh and dry shoot weights and the number of flowers $plant^{-1}$) except for the fresh root weight, which was the greatest in the control treatments.

Phytotoxicity tests on tomato seedlings: There was no apparent phytotoxic effect of any of the tested

combinations of phytochemicals on germination of tomato seeds or seedling (Table 4). All phytochemical combinations increased plumule dry weight, radical fresh weight and radical dry weight. Other parameters were also increased by some specific combinations; e.g., alcohol + sterol combination increased all growth measurements and 23a-homostigmast-5-en-3β-ol + *cis*- and *trans*-protopinium increased plumule length. However, differences amongst the means of the other treatments such as fresh root weight were not significant (Table 4).

Table 2. Effect of combinations of phytochemicals from *Fumaria parviflora* on mortality of *Meloidogyne incognita* J2s [a].

Compounds	Concentration (µg ml^{-1})	Percent J2s mortality			
		6 h	12 h	24 h	48 h
(Nonacosan-10-ol) + (23a-homostigmast-5-en-3*β*-ol) + (*cis*- and *trans*-protopinium)	**0 (control)[b]**	12.0 ^{g}	14.0 ^{g}	15.0 ^{g}	16.0 ^{h}
	5.0	48.0 ^{fB}	53.0 ^{fB}	59.7 ^{fC}	71.3 ^{gB}
	50	65.8 ^{deB}	70.8 ^{deB}	77.3 ^{deB}	81.5 ^{defB}
	100	81.5 ^{bB}	85.5 ^{bB}	89.8 ^{bB}	92.5 ^{bcB}
(Nonacosane-10-ol) + (23a-homostigmast-5-en-3*β*-ol)	**0 (control)**	12.0 ^{g}	14.0 ^{g}	15.0 ^{g}	16.0 ^{h}
	5.0	67.3 ^{deA}	72.5 ^{deA}	82.3 ^{cdAC}	91.5 ^{bcA}
	50	83.0 ^{bA}	84.3^{beA}	93.7 ^{abA}	97.5 ^{abA}
	100	96.3^{A}	97.5 ^{aA}	99.5 ^{aA}	100.0 ^{aA}
(Nonacosane-10-ol) + (*cis*- and *trans*-protopinium)	**0 (control)**	12.0 ^{g}	14.0 ^{g}	15.0 ^{g}	16.0 ^{h}
	5.0	46.0 ^{fB}	55.0 ^{fB}	62.0 ^{fC}	75.0 ^{fgB}
	50	61.3 ^{eB}	66.7 ^{eB}	72.0 ^{eB}	79.5 ^{efB}
	100	72.0 ^{cdC}	76.5 ^{cdC}	81.7 ^{cdC}	88.0 ^{cdB}
(23a-homostigmast-5-en-3*β*-ol) + (*cis*- and *trans*-protopinium)	**0 (control)**	12.0 ^{g}	14.0 ^{g}	15.0 ^{g}	16.0 ^{h}
	5.0	61.0 ^{eA}	65.7 ^{eA}	71.7 ^{eB}	79.0 ^{efB}
	50	67.8 ^{deB}	73.5 ^{deAB}	78.0 ^{deB}	83.7 ^{deB}
	100	79.8 ^{bcBC}	84.0 ^{bcBC}	88.7 ^{bcBC}	93.0 ^{abcAB}
LSD (P < 0.05)		8.1	8.3	7.4	7.1

[a]Data are means of ten replicates combined from two experiments (spring and autumn, 2011). [b]Control treatments (0) utilized simple distilled water (SDW) dissolved in DMSO (1%). Means followed by the same lowercase or same uppercase letter do not differ significantly ($P \geq 0.05$) according to Fisher's protected LSD test. Lower case letters represent comparisons between different concentrations of the same phytochemical combination; upper case letters, comparisons between the same concentrations of different phytochemical combinations.

Table 3. Effect of combination application of phytochemicals from *Fumaria parviflora* on *Meloidogyne incognita* population and growth measurements of *Solanum lycopersicum*[a].

Compounds	Concentration (µg ml^{-1})	Galls	GI[c]	Females g^{-1} of root	Eggs g^{-1} of root	Shoot length (cm)	Root length (cm)	Fresh shoot weight (g)	Dry shoot weight (g)	Fresh root weight (g)	Number of flowers plant^{-1}
(Nonacosan-10-ol)+(23a-homostigmast-5-en-3*β*-ol)+(*cis*- and *trans*-protopinium)	**0 (control)[b]**	125.0 a	5.0 ^{a}	117.0 ^{a}	5120.0 ^{a}	49.0 ^{e}	22.0 ^{i}	48.0 ^{g}	18.0 ^{i}	54.0 ^{a}	41.0 ^{i}
	5.0	93.5 ^{bcA}	3.8 ^{bA}	99.7 ^{bA}	3002.5 ^{bA}	54.8 ^{bcdA}	26.0 ^{hC}	50.5 ^{efB}	26.8 ^{fgBC}	23.3 ^{cNS}	47.5 ^{hC}
	50	82.5 ^{deAB}	3.3 ^{cdeAB}	93.8 ^{bcA}	2825.0 ^{cA}	56.0 ^{abcA}	27.5 ^{gC}	51.5 ^{deB}	27.8 ^{efBC}	24.5 ^{bcNS}	48.8 ^{ghC}
	100	72.0 ^{gA}	3.0 ^{efA}	91.8 ^{cd}	2625.0 ^{deA}	56.8 ^{abA}	28.8 ^{efgC}	53.0 ^{dB}	29.0 ^{deB}	26.3b^{NS}	51.3 ^{fgC}
(Nonacosan-10-ol)+(23a-homostigmast-5-en-3*β*-ol)	**0 (control)**	125.0 ^{a}	5.0 a	117.0 ^{a}	5120.0 ^{a}	49.0 ^{e}	22.0 ^{i}	48.0 ^{g}	18.0 ^{i}	54.0 ^{a}	41.0 ^{i}
	5.0	73.0 ^{fgB}	2.5 ^{gB}	76.3 ^{fgC}	1925.0 ^{hD}	54.2 ^{bcdB}	30.0 ^{defA}	56.5 ^{cA}	33.3 ^{cA}	24.8 ^{bcNS}	53.8 ^{defB}
	50	57.5 ^{hC}	1.9 ^{hC}	64.3 ^{iC}	1745.0 ^{iD}	56.0 ^{abcA}	32.5 ^{bcA}	59.3 ^{bA}	35.0 ^{bA}	26.5 ^{bNS}	58.8 ^{bA}
	100	42.5 ^{iB}	1.3 ^{iB}	44.5 ^{jC}	1432.5 ^{jC}	60.0 ^{aB}	37.3 ^{aA}	62.3 ^{aA}	37.5 ^{aA}	26.3 ^{bNS}	66.0 ^{aA}
(Nonacosan-10-ol)+ (*cis*- and *trans*-protopinium)	**0 (control)**	125.0 ^{a}	5.0 a	117.0 ^{a}	5120.0 ^{a}	49.0 ^{e}	22.0 ^{i}	48.0 ^{g}	18.0 ^{i}	54.0^{a}	41.0 ^{i}
	5.0	94.8 ^{bA}	3.8 ^{bcA}	91.8 ^{cdAB}	2732.5 ^{cdB}	50.5 ^{deB}	28.5 ^{fgB}	49.3 ^{fgB}	25.0 ^{hC}	26.0^{bNS}	52.5 ^{efB}
	50	86.5 ^{cdA}	3.5 ^{bcdA}	77.5 ^{fgB}	2639.5 ^{cdeB}	51.5 ^{deB}	29.5 ^{defB}	51.5 ^{defB}	26.0 ^{ghC}	26.5^{bNS}	54.3 ^{cdefB}
	100	80.8 ^{defA}	3.3 ^{deA}	70.5	2540.0	52.0	30.5 ^{dC}	52.5 ^{deB}	27.0 ^{fgC}	27.0^{bNS}	55.5

				ghiC	efA	cdeB					bcdeBC
(23a-homostigmast-5-en-3*β*-ol)+(*cis*- and *trans*-protopinium)	**0 (control)**	125.0 [a]	5.0 a	117.0 [a]	5120.0 [a]	49.0 [e]	22.0 [i]	48.0 [g]	18.0 [i]	54.0 [a]	41.0 i
	5.0	85.0 [dA]	3.5 bcdA	83.5 efBC	2525.0 efC	53.3 bcdeAB	30.8 [dA]	50.5 [efB]	28.0 [efB]	24.5 [bcNS]	56.5 [bcdA]
	50	76.3 [efgB]	3.0 [efB]	73.5 [ghB]	2415.0 [fC]	53.5 bcdAB	32.3 [cA]	52.0 [deB]	29.5 [dB]	25.8 [bcNS]	57.5 [bcAB]
	100	71.3 [gA]	2.9 [fA]	67.0 [hiB]	2270.0 [gB]	54.8 bcdAB	33.7 [bB]	53.3 [dB]	30.3 [dB]	26.5 [bNS]	58.8 [Bb]
LSD (P < 0.05)		8.16	0.38	7.73	139.87	4.32	1.3	2.2	1.4	2.7	3.3

[a]Data are means of ten replicates per treatment recorded in the spring 2011 greenhouse trial. Plants were inoculated with 5000 eggs + J2s of *Meloidogyne incognita* for each concentration. Means followed by the same lowercase or same uppercase letter do not differ significantly ($P \geq 0.05$) according to Fisher's protected LSD test. Lower case letters represent comparisons between different concentrations of the same phytochemical combination; upper case letters, the same concentrations of different phytochemical combinations. NS represents non-significant. [b]Control (nematode-inoculated without compound application). [c]Galling index: 0 = no galls on roots; 1 = 1-2; 2 = 3-10; 3 = 11-30; 4 = 31-100; 5 = more than 100 galls per root.

Table 4. Effect of phytochemical combinations from *Fumaria parviflora* on the growth of seedlings of *Solanum lycopersicum* L. (*cv*. Riogrande).

Compounds	Concentration (µg ml^{-1})	Phytotoxicity test						
		Germination (%)	Plumule length (cm)	Radicle length (cm)	Plumule fresh weight (g)	Plumule dry weight (g)	Radicle fresh weight (g)	Radicle dry weight (g)
(Nonacosan-10-ol) +	**0 (control)[b]**	100.0	4.7 [c]	5.7 [c]	0.45 [c]	0.1 [e]	0.49 [e]	0.1 [d]
(23a-homostigmast-5-	5.0	100.0	4.7 [c]	5.7 [c]	0.49 [bc]	0.1 [e]	0.51 [de]	0.16 [cd]
en-3*β*-ol) + (*cis*- and	50	100.0	4.8 [bc]	5.8 [bc]	0.49 [bc]	0.1 [e]	0.56 [cd]	0.20 [bcd]
***trans*-protopinium)**	100	100.0	4.9 [bc]	5.9 [abc]	0.49 [bc]	0.13 [de]	0.59 [bc]	0.23 [abc]
(Nonacosan-10-ol) +	**0 (control)**	100.0	4.7 [c]	5.7 [c]	0.45 [c]	0.1 [e]	0.49 [e]	0.1 [d]
(23a-homostigmast-5-	5.0	100.0	5.3 [b]	6.2 [ab]	0.5 [b]	0.2 [cd]	0.59 [bc]	0.20 [bcd]
en-3*β*-ol)	50	100.0	5.3 [b]	6.3 [a]	0.5 [b]	0.30 [b]	0.61 [ab]	0.26 [abc]
	100	100.0	5.8 [a]	6.3 [a]	0.59 [a]	0.4 [a]	0.65 [a]	0.33 [a]
(Nonacosan-10-ol) +	**0 (control)**	100.0	4.7 [c]	5.7 [c]	0.45 [c]	0.1 [e]	0.49 [e]	0.1 [d]
(*cis*- and *trans*-	5.0	100.0	4.9 [bc]	5.7 [c]	0.49 [bc]	0.1 [e]	0.59 [bc]	0.20 [bcd]
protopinium)	50	100.0	4.9 [bc]	5.8 [bc]	0.49 [bc]	0.16 [de]	0.59 [bc]	0.23 [abc]
	100	100.0	4.9 [bc]	5.9 [abc]	0.5 [bc]	0.26 [bc]	0.60 [bc]	0.23 [abc]
(23a-homostigmast-5-	**0 (control)**	100.0	4.7 [c]	5.7 [c]	0.45 [c]	0.1 [e]	0.49 [e]	0.1 [d]
en-3*β*-ol) + (*cis*- and	5.0	100.0	5.0 [b]	5.8 [bc]	0.5 [bc]	0.1 [e]	0.59 [bc]	0.23 [abc]
***trans*-protopinium)**	50	100.0	5.0 [b]	5.9 [abc]	0.5 [bc]	0.20 [cd]	0.59 [bc]	0.23 [abc]
	100	100.0	5.0 [b]	5.9 [abc]	0.5 [bc]	0.3 [b]	0.59 [bc]	0.23 [abc]
LSD (P < 0.05)		100.0	0.37	0.45	0.05	0.09	0.04	0.11

[a]Data are the means of ten replicates from two experiments (spring and autumn, 2011). [b]Control treatments (0) utilized sterile distilled water (SDW) dissolved in DMSO (1%). Means followed by the same lowercase letter do not differ significantly ($P \geq 0.05$) according to Fisher's protected LSD test.

DISCUSSION

Phytochemicals have been regularly investigated as potential sources of herbicides, pesticides and pharmaceuticals; these secondary metabolites possess diverse structures and many such compounds have displayed biological activity (Chitwood, 2002). Most secondary metabolites have their origin in the shikimate or acetate pathways or they are derived from components of both (Einhellig, 2002). Plants produce more than twenty different classes of secondary metabolites, many of which are stored and released into the rhizosphere where they affect other organisms (Einhellig, 2002).

In the current research findings, combinations of three phytochemicals exhibited nematicidal activity against *Meloidogyne incognita*, both in the laboratory and greenhouse trials. Nonacosane-10-ol ($C_{29}H_{60}O$) has been reported as a major nemoticidal phytochemical component of a *n*-hexane extract of *F. parviflora* roots (Naz *et al*. 2013b). Other *F. parviflora* components such as 23a-homostigmast-5-en-3*β*-ol ($C_{30}H_{52}O$) and *cis*- and *trans*-protopinium or protopine ($C_{20}H_{19}N^{+}O_5$) were previously isolated through bioactivity-guided isolation (Naz *et al*. 2013b and 2016). When tested individually, nonacosan-10-ol and 23a-homostigmast-5-en-3*β*-ol inhibited hatching (15-95%) and induced J2 mortality

(20-100%); *cis*- and *trans*-protopinium caused 100% hatch inhibition and J2 mortality at 200 µg mL^{-1} (Naz *et al*. 201b and 2016). In the present study, mixtures of these phytochemicals showed nematocidal effects at concentrations as low as 5 µg mL^{-1}. Each phytochemical combination appeared to exhibit nematocidal effect towards hatch inhibition and juvenile mortality.

The alcohol + sterol (nonacosane-10-ol + 23a-homostigmast-5-en-3*β*-ol) combination was the most active nematocidal combination at the three tested concentrations; hatch inhibition and J2s mortality both approached 100.0% at 50 and 100 µg mL^{-1}. Results of the present study on hatch inhibition and J2s mortality are consistent with the previous findings, where individual applications of 50 µg mL^{-1} nonacosane-10-ol and 23a-homostigmast-5-en-3*β*-ol inhibited egg hatch (61.3 and 59.7%, respectively) and induced juvenile mortality (73.3 and 60.0%, respectively) after 120 h (Naz *et al*. 2013b). Hatch inhibition and J2 mortality reached 100 and 97.5%, respectively, after 12 h. These results are similar to the previous findings in which both highest hatch inhibition and mortality occurred when the concentration and incubation time were the highest (Naz *et al*. 2013b).

The toxic effects of phytochemical mixtures have been reported frequently. For example, a 9:1 mixture of the plant sterols *β*-sitosterol and stigmasterol exhibited strong toxicity toward *M. incognita* J2s, when applied at a concentration of 5.0 µg mL^{-1} (Barbosa *et al*. 1999). The hatching inhibition obtained with the nonacosan-10-ol + 23a-homostigmast-5-en-3*β*-ol mixture was very similar to that observed for the 23a-homostigmast-5-en-3*β*-ol + *cis*- and *trans*-protopinium mixture. However, the former combination had a greater effect on J2 mortality than the latter, except for the higher concentration (100 µg mL^{-1}) for 48 h, where the effects were similar. The nematicidal activity of alcohols, sterols and alkaloids against *M. incognita* has been described by Chitwood (2002). Another aliphatic alcohol, 1-octanol from *Allium grayi* (Liliaceae) is active against *M. incognita*; *Evodia rutaecarpa* contains two alkaloids toxic to *M. incognita*: evodiamine (LC_{50} = 73.55 µg ml^{-1}) and rutaecarpine (LC_{50} = 120.85 µg ml^{-1}) (Liu *et al*. 2012).

Greenhouse trials with the application of the mixtures of nonacosan-10-ol + 23a-homostigmast-5-en-3*β*-ol resulted in dose-dependent reduction in nematode eggs, galls, GI and females in tomato roots and a dose-dependent increase in plant growth parameters. Nonacosan-10-ol + 23a-homostigmast-5-en-3*β*-ol were the strongest nematocidal combination *in planta*. The suppression of nematode damage by the phytochemical combinations possibly resulted in the observed increases in shoot and root lengths, fresh and the dry shoot weights and number of flowers per plant. The results of the present study support the previous findings, in which individual applications of the three compounds reduced GI and numbers of galls, females and eggs in tomato roots (Naz *et al*. 2013b and 2016).

The three compounds used in the present study were found to be structurally different. We previously suggested that the highest nematocidal activity of nonacosan-10-ol and the 23a-homostigmast-5-en-3*β*-ol could be ascribed to the presence of an OH group at C-10 or C-3 (Naz *et al*. 2013b). Some suggestions as to the mode of action can be based on the structures of *cis*- and *trans*-protopinium and 23a-homostigmast-5-en-3*β*-ol. The ring systems of these compounds are similar to those of the neurotransmitters dopamine and serotonin found in the nematodes. The structural similarities may suggest that these compounds act at neurotransmitter receptor sites as agonists or antagonists (Groger and Floss, 1998). The alkaloid nicotine, once widely used as an insecticide in agriculture, acts as an acetylcholine inhibitor and interfering with neurotransmission (Ntalli, 2011). The isoquinoline alkaloids protopine, allocrytopine and stylopine inhibit human acetylcholinesterase and butyrylcholinesterase (Cahlíková *et al*. 2010). The neurotransmitters acetylcholine, dopamine and serotonin have been detected in the microbivorous nematode *Caenorhabditis elegans* and play a significant role in nematode behavior. Dopamine has also been detected in *M. incognita* and cyst nematodes (Sharpe and Atkinson, 1980; Stewart *et al*., 2001). Exposure of *C. elegans* to exogenous dopamine causes adverse effects such as paralysis, changes in egg laying and defecation (Schafer and Kenyon, 1995; Weinshenker *et al*. 1995; Chase *et al*. 2004). Although the alkaloid protopine inhibits serotonin and noradrenaline transporters in mice (Xu *et al*. 2006), its mode of action in nematodes may be connected to receptor sites, which expectedly vary depending on nematode species.

Although phytotoxicity is a major problem impeding the development of utilization of phytochemical compounds (Chitwood, 2002), the three *F. parviflora* phytochemicals exhibited no phytotoxic effects on tomato seed germination and seedling growth and actually increased some measures of growth.

Discovery of novel antinematodal phytochemicals such as nonacosane-10-ol and 23a-homostigmast-5-en-3*β*-ol and their effects will provide new insights increasing the use of natural products in plant protection as nematode management tools. In this case, we have demonstrated that the combination of nonacosan-10-ol and 23a-homostigmast-5-en-3*β*-ol is the best of the three tested combinations of *F. parviflora* phytochemicals, even at a low concentration. The optimization of suitable dose of phytochemicals mixtures in laboratory and greenhouse trials will provide practical and useful information to growers.

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