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Nematicidal Compounds from the Leaves of *Schinus terebinthifolius* Against Root-knot Nematode, *Meloidogyne incognita* Infecting Tomato

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Abstract – The root-knot nematode, *Meloidogyne incognita* caused a serious damage to many plants. The phenolic components of the leaves of *Schinus terebinthifolius* were investigated as potential nematicidal agents for *M. incognita*. Nine compounds were isolated and characterized as viz., 1,2,3,4,6-pentagalloyl glucose (**1**), kaempferol-3-*O*-α-L-rhamnoside (Afzelin) (**2**), quercetin-3-*O*-α-L-rhamnoside (Quercetrin) (**3**), myricetin (**4**), myricetin-3-*O*-α-L-rhamnoside (Myricetrin) (**5**), methylgallate (**6**), protocatechuic acid (**7**), quercetin (**8**), and gallic acid (**9**) using nuclear magnetic resonance (NMR) spectroscopy. Compound **1** showed pronounced nematicidal activity compared to Oxamyl as a positive control. It showed the lowest eggs-hatchability (34%) and the highest mortality in nematode population (21% after 72 hours of treatment) at a concentration of 200 μg/mL. It exhibited the best suppressed total nematode population, root galling and number of eggmasses in infected tomato plants. The total carbohydrates and proteins were also significantly induced by **1** with reduction in total phenolics and increase in defense-related proteins. Thus, compound **1** could be a promising, more safe and effective natural nematicidal agent for the control of root-knot nematodes.

Keywords – *Meloidogyne incognita*; *Schinus terebinthifolius*; pentagalloyl glucose; nematicidal activity; root-knot nematode

Introduction

Schinus terebinthifolius Raddi (Anacardiaceae), known as "Brazilian pepper" is an evergreen tree, native to Brazil and introduced in different parts of the world. It has been used in folk medicine as anti-inflammatory, immunomodulatory, antipyretic, analgesic, detoxifying agent, chemopreventive and in wound healing. In addition, it has been used in treating skin, mucous membranes, genitourinary, respiratory and GIT infections due to its antifungal and antibacterial properties.[1-8] Moreover, the ethanolic extract of leaves and stems of *S. terebinthifolius* inhibits quorum-sensing activity in methicillin-resistant *Staphylococcus aureus* infections and thus inhibits the production of virulence factors.[9] The leaves extract exhibits anticancer properties[10] and the fruits extract inhibits nitric oxide production and shows antioxidant activity.[4] Also, the ethyl acetate fraction of leaves of *S. terebinthifolius* exhibits anti-allergic properties and inhibits histamine release and edema development in mice.[11]

Diverse groups of phytochemicals including phenolics and terpenoids have been reported from leaves, barks and fruits of *S. terebinthifolius*. Several phenolic compounds including mono- and biflavonoids, coumaric acid and gallic acid derivatives have been reported.[6,13-16] A biphenyl compound namely, 4'-ethyl-4-methyl-2,2',6,6'-tetrahydroxy [1,1'-biphenyl]-4,4'-dicarboxylate, has been also isolated from leaves and stem of *S. terebinthifolius*.[6] However, fewer studies addressed the triterpenoid contents of this plants. For instance, tirucallane-type triterpenes such as masticadienoic acid and schinol have been isolated from the leaves *S. terebinthifolius* in large amounts.[17,18] Other triterpenoid groups including ursane-type (α-amyrene, α-amyrenone and ursolic acid), bauerane-type (bauerenone) and adianane-type (simiarenol) triterpenes have been also isolated from this plant.[18,19]

Plant parasites especially root-knot nematode is one of

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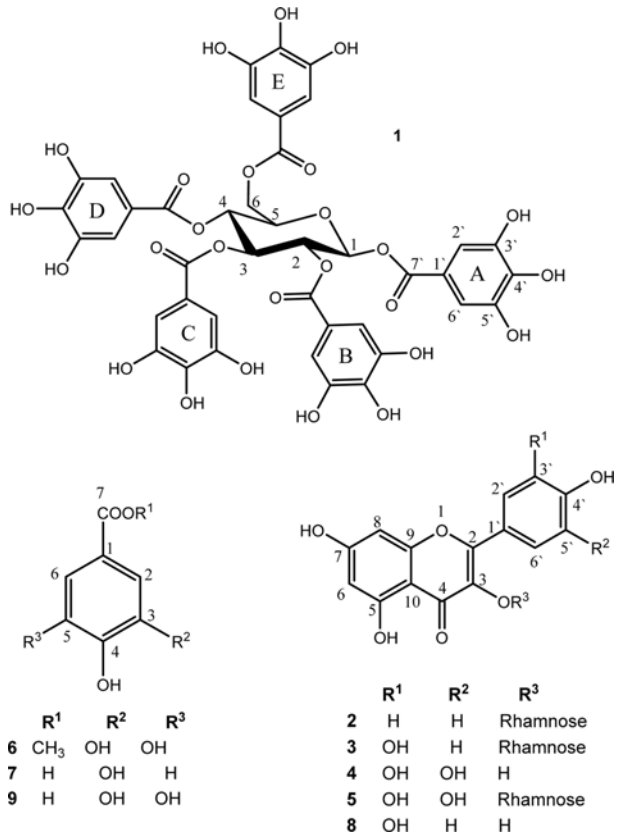

Fig. 1. Structures of the isolated compounds.

the leading problems in production of vegetables and other essential crops causing serious plant damage. *Meloidogyne* sp. produced knots that prevent water and nutrients from reaching the leaves and fruits. *Meloidogyne incognita*, one of the widely distributed species, has been considered the most damaging of ten main genera of plant parasitic nematodes. It causes about 12.3% loss of the annual yield of world's major crops.[20] The damage caused by *M. incognita* to tomato plant is massive and require safe and efficient control.

In the current research, the phenolic content of the methanol extract of the leaves of *S. terebinthifolius* was investigated. Nine phenolic compounds were isolated and characterized using spectroscopic methods. The isolated phenolic compounds (Fig. 1) **1 - 9** were evaluated for their nematicidal activity against root-knot nematode (*M. incognita*) infecting tomato plant using *in vitro* and greenhouse experiments. The *in vitro* experiments were used to evaluate the number of eggmasses and larvae, while the greenhouse experiments were used to evaluate plant growth parameters including shoot length, shoot and root weight. Additionally, chemical analysis was used to assess carbohydrates, proteins, defense-related proteins such as peroxidase (PO) and polyphenol oxidase (PPO) in both infected and control plants.

Experimental

General experimental procedures – The NMR spectroscopic experiments were performed on a Bruker Avance III 500 MHz Spectrometer (Bruker BioSpin Corp., Rheinstetten, Germany). For column chromatography, stationary phases were silica gel G60, 70 - 230 mesh, (Catalogue # 1077345000, Merck, Germany) and Sephadex LH 20 (Catalogue # 17-0090-01, Sigma-Aldrich, USA). For TLC, silica gel plates F_{254} (Catalogue # 1055540001, Merck, Germany) were used. Vanillin-sulfuric acid was used as a spray reagent. All chromatographic solvents were of reagent grade (El-Nasr Company, Egypt). The HR-MS experiments were conducted with a Bruker MicrOTof-Q (Bruker Daltonics, Bremen, Germany), where analytes were ionized using electrospray ionization (ESI) interface operated in the positive mode.

General methods

Phytochemical isolation of the phenolic compounds from the leaves of *Schinus terebinthifolius* – The leaves of *S. terebinthifolius* Raddi were collected from a farm at Damietta city, Egypt in July 2012, shade-dried, powdered and kept for further investigation. The plant was authenticated by Prof. Ibrahim Mashaly, at Ecology and Botany Department, Faculty of Science, Mansoura University. A voucher specimen was deposited at Pharmacognosy Department, Faculty of Pharmacy, Mansoura University (012-Mansoura-3). Powdered leaves of *S. terebinthifolius* Raddi (4.0 kg) were macerated in a glass jar with methanol (4 × 6 L). The obtained methanol extract was concentrated using rotary evaporator at 45 C^{o} and further dried to obtain a crude extract (817.4 g). The crude methanol extract was then dissolved in a suitable amount of methanol, and an equal amount of dist. H_2O and partitioned with *n*-hexane, CH_2Cl_2 and EtOAc. The obtained crops, were evaporated and dried to afford *n*-hexane (n-Hex, 124.7 g), CH_2Cl_2 (34.7 g) and EtOAc (372 g) extracts. Finally, the left aqueous extract was passed over Diaion®, an ion exchange adsorbent, washed with water, eluted with methanol and evaporated under vacuum at 45 C^{o} to afford a dry aqueous extract (86.0 g). For isolation of the phenolic compounds, only the aqueous and the EtOAc extracts were nominated to proceed an intensive phytochemical isolation using different chromatographic techniques. Fractionation and purification of the components of the aqueous extract afforded six compounds (**1 - 6**) as demonstrated in Fig. 2. Similarly,

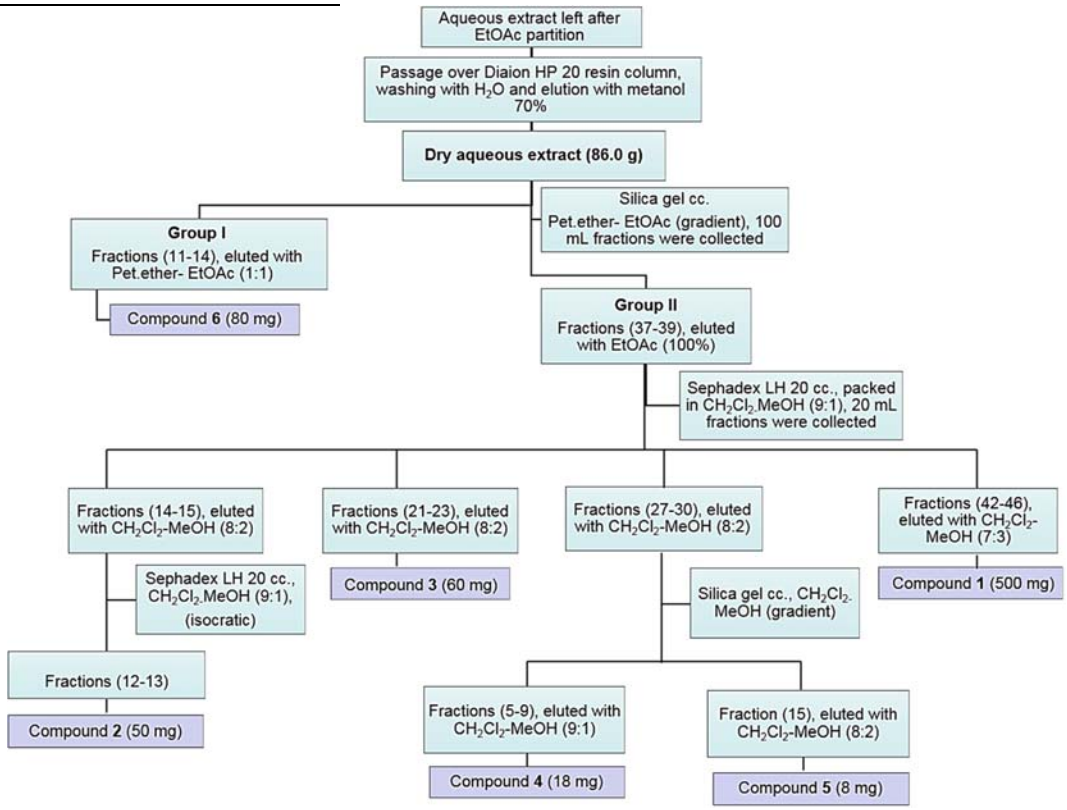

Fig. 2. Isolation scheme of compounds **1 - 6** from the aqueous extract left after partitioning of the total MeOH extract using pet.ether, CH_2Cl_2 and EtOAc, respectively.

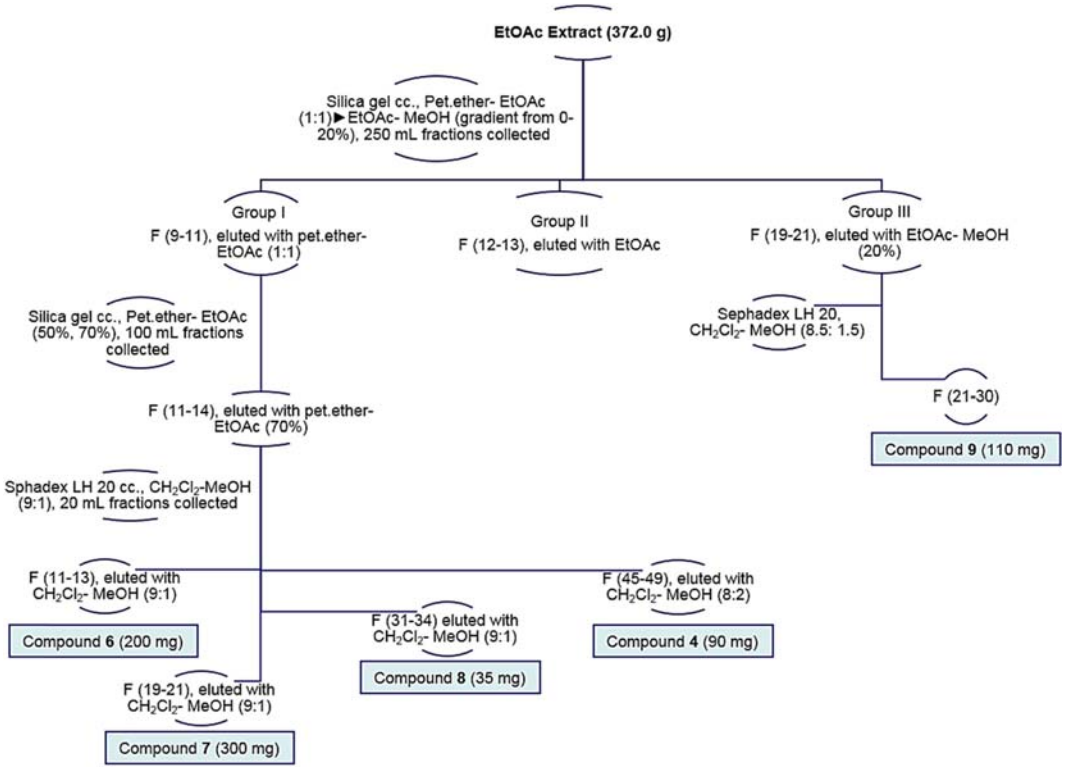

Fig. 3. Isolation scheme of compounds **4**, **6** and **7 - 9** from the ethyl acetate extract.

the EtOAc extract was also purified to afford three other compounds (**7 - 9**), in addition to compounds **4** and **6** that were isolated before from the aqueous extract (Fig. 3).

Evaluation of the nematicidal activity of the extracts and the isolated compounds against root-knot nematode (*M. incognita*)

Preparation of *Meloidogyne incognita* inocula[21]

***M. incognita* eggs inoculum** – Eggmasses were collected from the roots of coleus plants, *Coleus blumei* L., heavily infected with *M. incognita* grown in horticultural nursery under greenhouse condition and propagated on coleus plants, a susceptible host, for 3 months at Nematological Research Unit (NERU), Faculty of Agriculture, Mansoura University, Mansoura, Egypt. Sodium hypochlorite (NaOCl) extraction method was used in order to collect the eggs of *M. incognita*. Infected roots were washed and cut into 2 - 3 cm parts and shacked in 200 mL of 1.0% NaOCl solution for 1 - 2 minutes.[21] NaOCl solution was quickly filtered through a 60-mesh sieve nested over a 400-mesh sieve to collect freed eggs and quickly placed under a stream of tap water to remove residual NaOCl.

***M. incognita* juveniles' inoculum[22]** – Fresh second stage juveniles of the root-knot nematode, *M. incognita* were obtained from pure culture maintained on coleus roots. Roots were incubated in a modified Baermann technique for hatching at room temperature for 5 - 7 days.[22]

Experimental design

Laboratory Experiments

Impact against eggs of root-knot nematode (*M. incognita*)[23] – In order to evaluate the nematicidal properties of the extracts and isolated compounds against the eggs of root-knot nematode, *M. incognita*, the treatments were tested in Petri dishes, as triplicates at concentrations of 200, 100 and 50 µg/mL. Whereas, solvent and nematodes alone were used as negative control. To each dish, inoculum of 1 mL containing 100 eggs (*M. incognita*) was added to each treatment. The dishes were left for 10 days then the effect on egg hatching was examined under a microscope and compared to control.

Impact against larva of root-knot nematode (*M. incognita*)[23] – In separate experiment, the extracts and the isolated compounds were added in Petri dishes, as triplicates at concentrations of 200, 100 and 50 µg/mL. Solvent and nematodes alone were used as negative control. To each dish, inoculum of 1 mL containing 100 larvae (*M. incognita*) was added. The dishes were examined after 24, 48 and 72 h using microscope to study the effect of these treatments on the activity of larval second stage of root-knot nematode.

Greenhouse Experiment[24]

Impact of the extracts and the isolated compounds on tomato plants infected with root-knot nematode (*M. incognita*) – A greenhouse experiment was conducted using sandy clayey soil in order to evaluate the nematicidal activity of the isolated compounds against the root-knot nematode, *M. incognita*. The resulting effects on plant growth parameters and on induced resistance (IR) were assayed through chemical composition and enzyme activities. In fifty-seven pots (15 cm in diameter) containing 800 g of sterilized soil, one seedling per pot of tomato plant was placed. Seedlings were then infected with 1000 viable larvae of *M. incognita*. After one week, infected plants were treated with the isolated compounds and extracts (200, 100 and 50 µg/mL) as soil drench. Three pots were treated with Oxamyl® 10G as a standard nematicide at a concentration of 0.3 g per pot and three other pots were treated with DMSO (10 mL/pot) as a standard solvent. Additionally, three pots were left without nematode infection or any treatment and serve as control (N free). To three other pots nematodes were added alone without any treatment and served as control (N alone). After then, in a greenhouse at 27 ± 3 ºC, pots were settled in three replicates as a random complete block design where they received water as required. After 45 days of nematode inoculation, plants were harvested and washed from adhering soil. Plant growth parameters including weight and length of both fresh or dried shoot and root were recorded. Nematodes were recovered from soil using sieving and modification of the Baermann method.[22] Root galling (eggmasses) were ranked according to the number of galls per root system using a scale of 0-5; 0 means "no galls", 1 means "1 - 2 galls", 2 means "3 - 10" galls, 3 means "11 - 30" galls, 4 means "31 - 100" galls and finally, 5 means "> 100 galls".[23]

Chemical analysis – For each treatment, dried shoot (1 g) was subjected to chemical analysis in order to evaluate crude protein, total carbohydrate and total phenols. For the enzyme assays, enzyme extracts were prepared grinding dried root (0.5 g) of each treatment in 3 mL sod.phosphate buffer (pH 6.8) in a mortar and centrifuge at 1,500 × g 20 min at 6 ºC. The obtained supernatant was used for the evaluation of peroxidase (PO) and polyphenol oxidase (PPO) activities.[25]

Crude protein – The protein content in supernatant of fresh tomato plant from each treatment was estimated according to the method of Bradford (1976) [26] by using bovine serum albumin as a standard protein. Protein content was adjusted to 2 mg/mL per sample.

Total carbohydrate – Total soluble sugars (reducing and non-reducing) were determined using the spectrophotometeric method described by Thomas and Dutcher, (1924)[27] using picric acid at λ_{max} 540 nm.

Total phenols – Total phenols were determined after harvesting of fresh whole plant using Folin-Ciocalteau reagent.[28] Total content of phenolic compounds in plant ethanolic extracts was calculated as catechol equivalents by the following equation:

$$T = \frac{CXV}{m} \times 100$$

Where, T; Total content of phenolic compounds (mg of catechol/100 g of fresh weight material). C; concentration of catechol established from the calibration curve (mg/mL). V; volume of extract (mL). m; weight of pure plant ethanolic extract (g).

Peroxidase activity (PO) – Peroxidase was evaluated using the spectrophotometric method described by Amako et al. (1994).[29] The reaction was started by adding 20 μL of the enzyme solution to a mixture of 1,500 μL phosphate buffer, 480 μL H_2O_2 and 1,000 μL pyrogallol. Blank was prepared using phosphate buffer instead of the enzyme extract. Absorbance of the solution was measured at λ_{max} 430 nm and compared to blank. One unit of enzyme activity was defined as the amount of the enzyme, which changing the optical density at 430 nm per min. at 25 ºC under standard assay conditions. Specific activity was expressed in units by dividing it to mg protein.

Polyphenol oxidase (PPO) – Polyphenol oxidase was evaluated using the spectrophotometric method described by Coseteng and Lee (1987).[30] The reaction was started by adding 0.05 mL of the enzyme solution to a mixture of 2.7 mL K.phosphate buffer (90.05 M, pH 6.2) and 0.25 mL catechol (0.25 M). Blank was prepared using phosphate buffer instead of the enzyme extract. Absorbance of the solution was measured at λ_{max} 420 nm and compared to blank. One unit of enzyme activity is defined as the amount of the enzyme that causes an increase of 0.001 absorbance unit per minute at 25 ºC.

Data Analysis – Statistical analysis of the obtained data was carried out using analysis of variance (ANOVA),[31] followed by Duncan's multiple range tests (MRT) to test for significant difference between means (Duncan, 1955).[32]

Result and Discussion

Identification of the isolated compounds – Compound **1** was identified based on its spectral data including ^{1}H, ^{13}C NMR, IR and HR-ESIMS and by comparison with previously reported data[33] as 1,2,3,4,6-pentagalloyl glucose which has been identified before from *S. terebinthifolius*.[16,34] Similarly, characterization of compounds **2 - 9** was established using their ^{1}H and ^{13}C NMR data and by comparison with previously reported data for similar structures. Based on these data, compound **2** was characterized as kaempferol-3-*O*-α-L-rhamnoside (Afzelin), compound **3** as quercetin-3-*O*-α-L-rhamnoside (Quercetrin), compound **4** as myricetin and compound **5** as myricetin-3-*O*-α-L-rhamnoside (Myricetrin),[15,35,36] compound **6** methylgallate,[12,34,35] compound **7** as protocatechuic acid which is isolated for the first time from *S. terebinthifolius*,[37] compound **8** as quercetin,[34,36] and finally compound **9** as gallic acid.[12]

***1,2,3,4,6-Penta-O-galloyl-β-D-glucose* 1** – ^{1}H NMR (CD_3OD, 500 MHz); δ_H 6.22 (1H, d, J = 8.3 Hz, H-1), 5.56 (1H, dd, J = 9.7, 8.5 Hz, H-2), 5.88 (1H, t, J = 9.7 Hz, H-3), 5.60 (1H, t, J = 9.6 Hz, H-4), 4.50 (1H, d, J = 10.8 Hz, H-5), 4.36 (2H, m, H-6).^{13}C NMR (CD_3OD, 125 MHz); δ_C 92.4 (C-1), 70.8 (C-2), 72.7 (C-3), 68.4 (C-4), 73.0 (C-5), 62.9 (C-6), Gal-A; 118.3 (C-1'), 109.2 (C-2'/6'), 139.4 (C-3'/5'), 145.2 (C-4'), 164.8 (C-7'), Gal-B; 118.8 (C-1'), 109.0 (C-2'/6'), 139.0 (C-3'/5'), 145.0 (C-4'), 165.6 (C-7'), Gal-C; 118.9 (C-1'), 109.0 (C-2'/6'), 138.8 (C-3'/5'), 144.9 (C-4'), 165.9 (C-7'), Gal-D; 118.8 (C-1'), 109.1 (C-2'/6'), 139.0(C-3'/5'), 145.1 (C-4'), 165.5 (C-7'), Gal-E; 119.6 (C-1'), 108.9 (C-2'/6'), 138.6 (C-3'/5'), 145.0 (C-4'), 166.5 (C-7'); HRMS at m/z 963.1048 $[M+Na]^+$, Calcd. for $C_{41}H_{32}NaO_{26}$ 963.0180.

***Kaempferol-3-O-α-L-rhamnoside (Afzelin)* 2** – ^{1}H NMR (CD_3OD, 500 MHz); δ_H 6.16 (1H, brs, H-6), 6.31 (1H, brs, H-8), 6.92 (2H, d, J = 7.6, H-3'/5'), 7.72 (2H, d, J = 7.7 Hz, H-2'/6'), 5.39 (1H, brs, H-1"), 4.28 (1H, brs, H-2"), 3.77 (1H, brd, J = 3.0 Hz, H-3"), 3.37 (1H, brs, H-4"), 3.33 (1H, brs, H-5"), 0.95 (3H, brs, H_3-6"). ^{13}C NMR (CD_3OD, 125 MHz); δ_C 158.2 (C-2), 136.2 (C-3), 179.5 (C-4), 163.0 (C-5), 99.9 (C-6), 94.9 (C-8), 159.2 (C-9), 105.9 (C-10), 122.6 (C-1'), 132.0 (C-2'/6'), 116.5 (C-3'/5'), 161.4 (C-4'), 103.5 (C-1"), 72.0 (C-2"), 72.1 (C-3"), 73.3 (C-4"), 72.0 (C-5"), 17.7 (C-6").

***Quercetin-3-O-α-L-rhamnoside (Quercetrin)* 3** – ^{1}H NMR (CD_3OD, 500 MHz); δ_H 6.19 (1H, brs, H-6), 6.34 (1H, brs, H-8), 7.4 (1H, brs, H-2'), 6.92 (1H, d, J = 8.0 Hz, H5'), 7.30 (1H, d, J = 8.0 Hz, H-6'), 5.37 (1H, brs, H-1"), 4.31 (1H, brs, H-2"), 3.80 (1H, d, J = 8.8, H-3"), 3.33 (1H, m, H-4"), 3.43 (1H, m, H-5"), 0.97 (3H, d, J = 5.5 Hz, H_3-6"). ^{13}C NMR (CD_3OD, 125 MHz); δ_C 159.3 (C-2), 136.3 (C-3), 179.6 (C-4), 163.1 (C-5), 99.9 (C-6), 165.9 (C-7), 94.8 (C-8), 158.4 (C-9), 105.9 (C-10), 123.0 (C-1'), 116.4 (C-2'), 149.7 (C-3'), 146.3 (C-4'), 117.0 (C-

5'), 123.0 (C-6'), 103.5 (C-1"), 72.1 (C-2"), 72.1 (C-3"), 73.3 (C-4"), 71.9 (C-5"), 17.7 (C-6").

Myricetin 4 – ^{1}H NMR (CD_3OD, 500 MHz); δ_H 6.20 (1H, brs, H-6), 6.40 (1H, brs, H-8), 7.37 (2H, s, H-2'/6'). ^{13}C NMR (CD_3OD, 125 MHz); δ_C 148.0 (C-2), 136.9 (C-3), 177.2 (C-4), 162.4 (C-5), 99.3 (C-6), 165.5 (C-7), 94.4 (C-8), 158.1 (C-9), 104.5 (C-10), 123.1 (C-1'), 108.6 (C-2'/6'), 146.7 (C-3'/5'), 137.4 (C-4').

Myricetin-3-O-α-L-rhamnoside (Myricetrin) 5 – ^{1}H NMR (CD_3OD, 500 MHz); δ_H 6.19 (1H, brs, H-6), 6.34 (1H, brs, H-8), 6.99 (2H, s, H-2'/6'), 5.35 (1H, brs, H-1"), 4.30 (1H, brs, H-2"), 3.86 (1H, d, J = 7.1 Hz, H-3"), 3.18 (1H, m, H-4"), 3.54 (1H, m, H-5"), 0.99 (3H, d, J = 5.0 Hz, H_3-6"). ^{13}C NMR (CD_3OD, 125 MHz); δ_C 146.3 (C-

Table 1. Impact of treatments with *n*-hexane, EtOAc extracts and compounds (**1 - 9**), isolated from *Shinus terbenthifolius* leaves, against eggs and larvae of root-knot nematode *Meloidogyne incognita* under laboratory conditions.

Compds.	Conc. (μg/mL)	Unhatched eggs	Dead larvae		
			24 hr	48 hr	72 hr
	200	66[a]	14[d]	16[c]	21[a]
1	100	59[a]	9[f]	10[e]	16[c]
	50	59[a]	7[g]	5[h]	10[c]
	200	27[e]	3[i]	5[h]	6[g]
2	100	20[d]	5[h]	7[g]	14[d]
	50	10[i]	2[i]	3[i]	8[f]
	200	21[f]	3[i]	4[h]	4[h]
3	100	35[c]	5[h]	11[e]	5[h]
	50	44[b]	2[i]	8[g]	10[e]
	200	19[g]	3[i]	8[f]	9[f]
4	100	23[d]	11[e]	9[f]	12[d]
	50	10[i]	9[f]	7[g]	4[h]
	200	29[e]	2[i]	5[h]	4[h]
5	100	34[c]	7[g]	8[f]	8[f]
	50	30[d]	6[g]	2[i]	5[h]
	200	58[c]	5[h]	8[f]	13[d]
6	100	48[b]	8[f]	14[d]	11[e]
	50	32[d]	6[g]	3[i]	8[f]
	200	59[c]	4[h]	9[f]	13[d]
7	100	43[b]	8[f]	7[g]	19[b]
	50	22[f]	4[h]	4[h]	7[g]
	200	26[e]	5[h]	7[g]	9[f]
8	100	33[c]	8[f]	13[d]	15[d]
	50	26[e]	7[g]	3[i]	5[h]
	200	23[f]	4[h]	9[f]	12[d]
9	100	36[c]	8[f]	9[f]	17[c]
	50	12[g]	3[i]	7[g]	5[h]
	200	60[b]	5[h]	7[g]	9[f]
EtOAc	100	39[c]	9[f]	10[e]	9[f]
	50	31[d]	8[f]	5[h]	4[h]
	200	47[d]	6[g]	6[g]	6[g]
n-Hex	100	53[a]	7[g]	9[f]	10[e]
	50	31[d]	5[h]	4[h]	5[h]
DMSO solvent	---	17[h]	2[i]	2[i]	2[i]
Water Control	---	10[i]	2[i]	2[i]	2[i]

*Each value presented the mean of three replicates.
M. incognita; eggs (100 eggs / plant) or larvae (100 second stage larva / plant)
Means in each column followed by the same letter(s) did not differ at $P \leq 0.05$ according to Duncan's multiple range test.

2), 136.3 (C-3), 179.6 (C-4), 158.4 (C-5), 100.0 (C-6), 165.7 (C-7), 94.9 (C-8), 158.2 (C-9), 105.8 (C-10), 122.0 (C-1'), 109.9 (C-2'/6'), 146.7 (C-3'/5'), 137.9 (C-4'), 103.5 (C-1"), 72.1 "(C-2"), 72.1 (C-3"), 73.4 (C-4"), 71.9 (C-5"), 17.8 (C-6").

Methylgallate 6 – ^{1}H NMR (DMSO-d_6, 500 MHz); δ_H 7.00 (2H, s, H-2/6), 3.72 (3H, s, OCH_3). ^{13}C NMR (DMSO-d_6, 125 MHz); δ_C 119.4 (C-1), 108.6 (C-2/6), 145.4 (C-3/5), 138.3 (C-4), 166.4 (C-7), 51.3 (OCH_3).

Protocatechuic acid 7 – ^{1}H NMR (DMSO-d_6, 500 MHz); δ_H 6.81 (1H, d, J = 6.9 Hz, H-5), 7.33 (1H, d, J = 6.7 Hz, H-6), 7.38 (1H, brs, H-2), 9.62 (1H, OH). ^{13}C NMR (DMSO-d_6, 125 MHz); δ_C 121.6 (C-1), 116.4 (C-2), 144.7 (C-3), 149.9 (C-4), 115.2 (C-5), 122.1 (C-6), 167.4 (C-7).

Quercetin 8 – ^{1}H NMR (CD_3OD, 500 MHz); δ_H 6.20 (1H, brs, H-6), 6.40 (1H, brs, H-8), 7.75 (1H, brs, H-2'), 6.89 (1H, d, J = 8.0 Hz, H-5'), 7.64 (1H, d, J = 8.0 Hz, H-6'). ^{13}C NMR (CD_3OD, 125 MHz); δ_C 148.8 (C-2), 137.3 (C-3), 177.3 (C-4), 162.5 (C-5), 99.2 (C-6), 165.6 (C-7), 94.4 (C-8), 158.2 (C-9), 104.5 (C-10), 124.1 (C-1'), 116.0 (C-2'), 146.2 (C-3'), 148.0 (C-4'), 116.2 (C-5'), 121.7 (C-6').

Gallic acid 9 – ^{1}H NMR (CD_3OD, 500 MHz); δ_H 7.09 (2H, s, H-2/6). ^{13}C NMR (CD_3OD, 125 MHz); δ_C 122.0 (C-1), 110.4 (C-2/6), 146.4 (C-3/5), 139.6 (C-4), 170.5 (C-7).

Impact against eggs and larva of root-knot nematode (*M. incognita*) – Data in Table 1 document the impact of screened materials (compounds **1 - 9**, EtOA and *n*-hexane extracts) on *in vitro* controlling *M. incognita*. Results revealed that nematode population were significantly suppressed by all tested treatments. Out of the tested materials, compound **1** at the concentration of 200 μg/mL achieved the lowest hatchability in eggs of root-knot nematode (34.0%) followed by EtOAc (40.0%) then compound **7** and **6** (41.0 and 42%, respectively) at the same concentration (Table 1, Fig. 4). Also, compound **1** at the concentration of 200 μg/mL achieved the highest mortality in root-knot nematode population after 72 hr (21.0%) followed by compound **7** and **6** (13.0%, each) at the same concentration after 72 hr of inoculation (Table 1, Fig. 5). Similar trend was noticed at the concentration of 100 and 50 μg/mL for the same treatments. Thus, compound **1**, **6**, **7**, *n*-hexane and EtOAc extracts were selected for *in vivo* investigation on plant growth response of tomato plant var. 162 infected with *M. incognita*.

Impact of the extracts and the isolated compounds on tomato plants infected with root-knot nematode (*M. incognita*) – *M. incognita* infection caused a significant reduction in plant growth parameters (shoot and root length, shoot weight). Patel et al.[23] reported the effect of several plant extracts on the suppression of the population of *M. incognita* and *M. javanica* infecting tomato plants. In this study, compounds **1**, **6** and **7**, in addition to *n*-hexane and EtOAc extracts from *Schinus terebenthifolius* showed remarkable increase in plant growth parameters in terms of shoot length, shoot and root weight with various degrees with all treatments (Table 2). It was evident that compound **1** enhanced plant growth parameters and caused

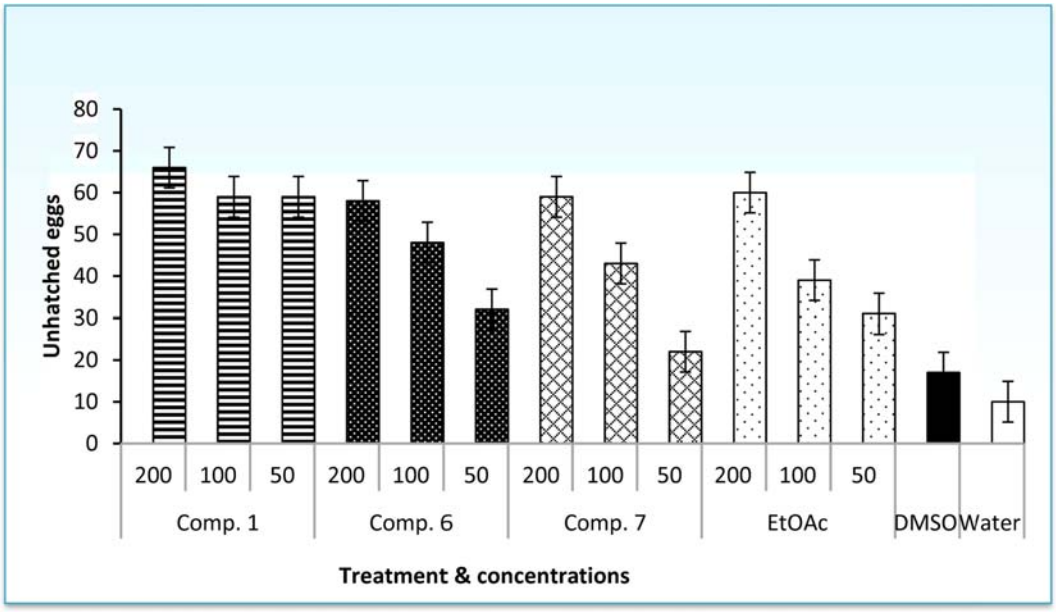

Fig. 4. Impact of treatments with EtOAc extracts and compounds (**1**, **6**, **7**), isolated from *Shinus terbenthifolius* leaves, on egg hatchability of root-knot nematode *Meloidogyne incognita* under laboratory conditions.

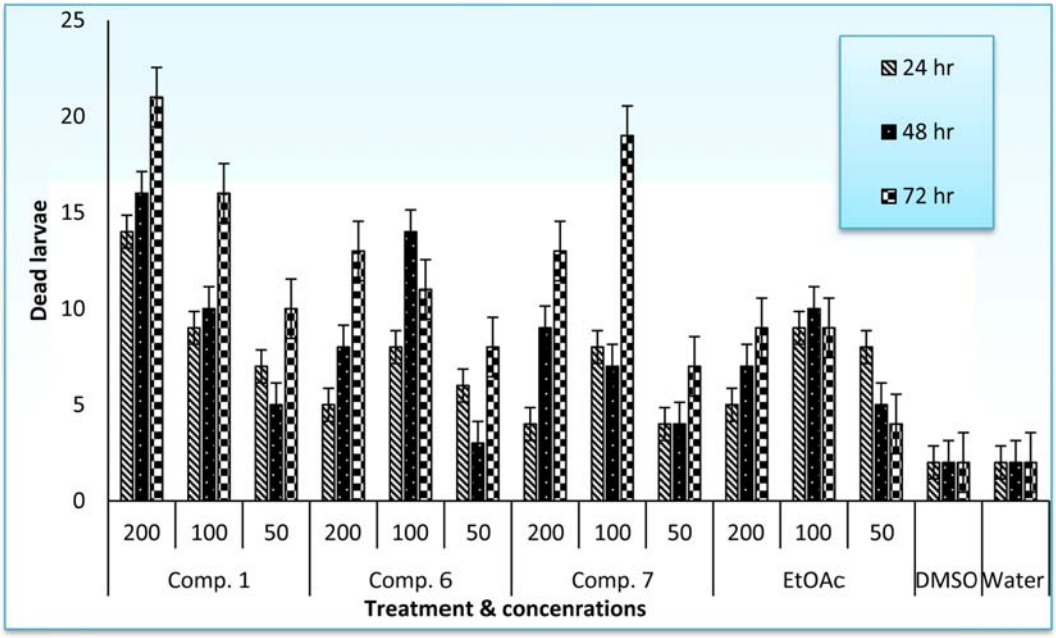

Fig. 5. Impact of treatments with EtOAc extracts and compounds (**1**, **6**, **7**), isolated from *Shinus terbenthifolius* leaves, against larvae of root-knot nematode *Meloidogyne incognita* under laboratory conditions.

Table 2. Impact of treatment with *n*-hexane, EtOAc extracts and compounds **1**, **6** and **7**, isolated from *Shinus terbenthifolius* leaves, on plant growth response of tomato var. 162 infected with *Meloidogyne incognita* under greenhouse conditions (27 ± 3 °C).

Compds.	Treatment (µg/mL)	*Plant growth response									
		Length (cm)				Plant fresh wt. (g)				Shoot Dry wt. (g)	Inc.%
		Shoot	Inc.%	Root	Inc.%	Shoot	Root	Total	Inc.%		
	50	16.0^{d}	45.5	9.0^{c}	50.0	2.3^{c}	1.6^{b}	3.6	143.75	1.3^{b}	116.7
1	100	19.0^{b}	72.7	10.0^{b}	66.7	2.4^{c}	1.4^{c}	3.8	137.5	1.0^{d}	66.7
	200	23.0^{a}	109.1	11.5^{a}	91.7	3.0^{a}	1.9^{a}	4.9	206.25	1.5^{a}	150.0
	50	11.7^{g}	6.4	9.0^{c}	50.0	2.3^{c}	0.8^{f}	3.1	93.75	0.8^{e}	33.3
6	100	13.0^{f}	18.2	10.0^{b}	66.7	2.9^{b}	0.8^{f}	3.7	131.25	1.1^{c}	83.3
	200	16.0^{d}	45.5	10.5^{b}	75.0	3.0^{a}	1.2^{d}	4.2	162.5	1.4^{b}	133.3
	50	15.0^{e}	36.4	9.5^{c}	58.3	2.7^{b}	1.0^{e}	3.7	131.25	0.9^{e}	50.0
7	100	16.0^{d}	45.5	10.0^{b}	66.7	2.6^{b}	1.6^{b}	4.2	162.5	1.3^{b}	116.7
	200	17.5^{c}	59.1	10.5^{b}	75.0	3.1^{a}	1.3^{c}	4.4	175.0	1.5^{a}	150.0
	50	13.0^{f}	18.2	6.0^{e}	0.0	1.8^{d}	1.2^{d}	3.0	87.5	0.7^{f}	16.7
n-Hex	100	13.0^{f}	18.2	6.0^{e}	0.0	2.6^{b}	1.2^{d}	3.8	137.5	0.9^{e}	50.0
	200	17.0^{d}	54.5	8.0^{d}	33.3	2.7^{b}	1.5^{c}	4.2	162.5	1.0^{d}	66.7
	50	13.0^{f}	18.2	9.0^{b}	50.0	1.0^{f}	1.0^{e}	2.0	25.0	0.7^{f}	16.7
EtOAc	100	16.0^{d}	45.5	11.0^{a}	83.3	1.5^{e}	1.1^{d}	2.6	62.5	0.9^{e}	50.0
	200	18.0^{b}	63.6	11.0^{a}	83.3	1.8^{d}	1.3^{c}	3.1	93.75	1.1^{c}	83.3
DMSO		17.0^{c}	54.5	10.0^{b}	66.7	1.9^{d}	1.2^{d}	3.1	93.75	1.1^{c}	83.3
Oxamyl		18.5^{b}	68.2	10.0^{b}	66.7	2.6^{b}	1.1^{d}	3.7	131.25	0.7^{f}	16.7
N-free		12.5^{f}	13.6	9.0^{b}	50.0	1.8^{d}	1.2^{d}	3.0	87.5	1.1^{c}	83.3
N alone		11.0^{g}	0.0	6.0^{e}	0.0	1.0^{f}	0.6^{g}	1.6	0.0	0.6^{g}	0.0

*Each value presented the mean of three replicates.
N: *M. incognita* (1000 second stage (larva) / plant).
N-free: Plants free of *M. incognita*
Means in each column followed by the same letter (s) did not differ at $P \leq 0.05$ according to Duncan's multiple range test.

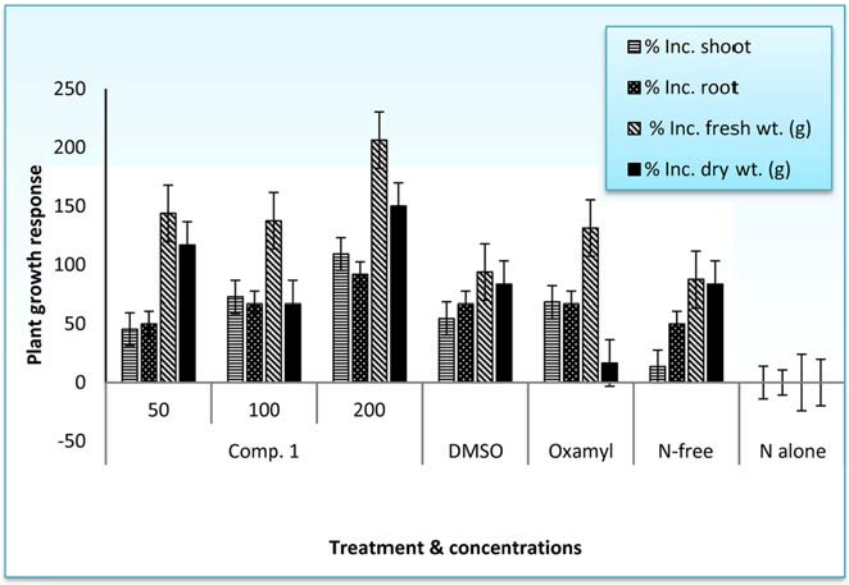

Fig. 6. Impact of compounds **1**, isolated from *Shinus terbenthifolius* leaves, on plant growth response of tomato var. 162 infected with *Meloidogyne incognita* under greenhouse conditions (27 ± 3°C).

Table 3. Impact of treatments with *n*-hexane, EtOAc extracts and compounds **1**, **6** and **7**, isolated from *Schinus terbenthifolius* leaves, on the development and reproduction of *Meloidogyne incognita* infecting tomato var. 162 grown under greenhouse conditions at 27 ± 3 °C.

Compds.	Treatment (µg/mL)	Nematode population			Final population (Pf)	RF**	Red. %	No. of galls	***RGI	No. of Eggmass	***EI
		Soil	Root								
			Developmental stages	Females							
	50	1200.0^{d}	0.0^{b}	33.0^{cd}	1233.0	1.2	66.3	30.0^{b}	3.0	1.5^{d}	1.0
1	100	600.0^{g}	0.0^{b}	25.0^{e}	625.0	0.63	82.9	22.0^{d}	3.0	1.0^{e}	1.0
	200	400.0^{h}	0.0^{b}	19.0^{i}	419.0	0.42	88.6	16.0^{f}	3.0	0.5^{f}	0.5
	50	1600.0^{c}	0.0^{b}	28.0^{d}	1628.0	1.6	55.5	25.0^{c}	3.0	2.0^{c}	1.0
6	100	800.0^{f}	0.0^{b}	25.0^{e}	825.0	0.83	77.5	20.0^{e}	3.0	1.5^{d}	1.0
	200	750.0^{fg}	0.0^{b}	20.7^{h}	770.7	0.77	78.9	16.0^{f}	3.0	1.5^{d}	0.5
	50	1350.0^{cd}	0.5^{ab}	22.1g	1372.6	1.4	62.5	20.0^{e}	3.0	1.0^{e}	1.0
7	100	1000.0^{de}	0.5^{ab}	24.4^{f}	1024.9	1.0	72.0	20.0^{e}	3.0	1.0^{e}	1.0
	200	950.0^{e}	0.0^{b}	28.2^{d}	978.2	0.98	73.3	25.0^{c}	3.0	0.5^{f}	0.5
	50	1400.8^{ab}	0.8^{ab}	34.0^{c}	1434.8	1.4	60.8	30.0^{b}	3.0	2.0^{c}	1.0
n- Hex	100	1000.5^{de}	0.5^{ab}	28.0^{d}	1029.0	1.0	71.9	25.0^{c}	3.0	1.5^{d}	1.0
	200	810.0^{f}	0.5^{ab}	24.0^{f}	834.5	0.84	77.2	16.0^{f}	3.0	1.0^{e}	1.0
	50	1880.0^{bc}	0.0^{b}	30.0^{cd}	1910.0	1.9	47.8	25.0^{c}	3.0	1.5^{d}	1.0
EtOAc	100	1200.0^{d}	0.0^{b}	28.0^{d}	1228.0	1.2	66.4	25.0^{c}	3.0	1.5^{d}	1.0
	200	900.0^{e}	0.0^{b}	28.0^{d}	928.0	0.93	74.6	25.0^{c}	3.0	1.0^{e}	1.0
DMSO		2000.0^{ab}	0.0^{b}	39.0^{b}	2039.0	2.0	44.3	30.0^{b}	3.0	3.0^{b}	2.0
Oxamyl		300.0^{i}	0.0^{b}	28.0^{d}	328.0	0.33	91.0	25.0^{c}	3.0	0.5^{f}	0.5
N alone		3500.0^{a}	1.4^{a}	56.0^{a}	3660.0	3.67	0.0	54.0^{a}	4.0	10.0^{a}	2.0

*Each value presented the mean of three replicates.
N = *M. incognita* (1000 larva/ plant).
Means in each column followed by the same letter (s) did not differ at $P \leq 0.05$ according to Duncan's multiple range test.
** Reproduction factor (RF) = Nematode population in soil + No. of developmental stages + No. of females + No. of eggmasses.
No. of eggs inocula, * ** Root gall index (RGI) or egg-masses index (EI) was determined according to the scale given by Taylor and Sasser (1978) as follows : 0 = no galls or eggmasses, 1 = 1-2 galls or eggmasses , 2 = 3-10 galls eggmasses, 3 = 11-30 galls or eggmasses, 4 = 31-100 galls or eggmasses and 5 = more than 100 galls or eggmas

a significant improvement in shoot length (109.1%), plant fresh weight (206.25%) and shoot dry weight (150.0%) at 200 µg/mL (Fig. 6). Also, compound **6** and **7** at 200 µg/mL resulted in a pronounced improvement in plant growth (Table 2). Oxamyl as a standard nematicide showed moderate improvement in pervious criteria of tomato grown with shoot length (68.2%), plant fresh weight (131.25%) and dry shoot weight (16.7%).

Data in Table 3 displays the impact of screened materials on controlling *M. incognita* infecting tomato var. 162 grown in potted sandy-clayey soil. Results revealed that nematode population within soil and root were significantly suppressed by all tested treatments with reproduction factor ranged from 0.42 to 1.60. Compound **1** exhibited the best suppressed total nematode population (RF = 0.42), root galling (RGI = 3.0) and number of eggmasses (EI = 0.5), respectively at 200 µg/mL (Fig. 7). Also, compound **6** at 200 µg/mL resulted in significant reduction in galling formation (78.9%). On the other hand, total nematode population was suppressed with oxamyl introduced to (RF = 0.33) root galling (RGI = 3.0) and number of eggmasses (EI = 0.5), respectively.

Biochemical activities

Total carbohydrates – Root knot nematode infection cause the formation of typical root galls that affect nutrients uptake and translocation of food materials in plants such as protein and sugar.[38] The adverse effect of *M. incognita* infection on total carbohydrates in leaves of tomato was investigated with percentage of reduction reached (Table 4). However, a detectable induction in total carbohydrates was recorded with all treatments. The greatest % induction in total carbohydrates was recorded for compound **1** (61.4%) at a concentration of 200 µg/mL compared to untreated plants (N alone).

$$The\ \%\ induction = \frac{(Treated - Control)}{\text{Control}} \times 100$$

Crude proteins – Untreated tomato infected with *M. incognita* exhibited significant reduction in total proteins as compared with untreated plants with percentage of reduction reached (Table 4). However, a detectable induction in total proteins was recorded with all treatments. The greatest induction in total proteins was recorded with compound **1** (33.66%) at the concentration of 200 µg/mL compared to untreated plants (N alone).

Defense mechanism against root knot nematode

Plant nematodes, spend most of their life cycles in the roots of the host plant and thus subjected to diverse types of defense responses. Consequently, the root-knot nematode infection increased phenol content, peroxidase activity and polyphenol oxidase activities.[39] Thus, total phenols and defense-related proteins including peroxidase and polyphenol oxidase are biochemical markers in infested plants.

Total phenols – The total phenolics increased in infected plants compared to non-infected plants (Table 4). However, all treatments showed a reduction in total phenolics to various extents relative to infected plants. It was observed

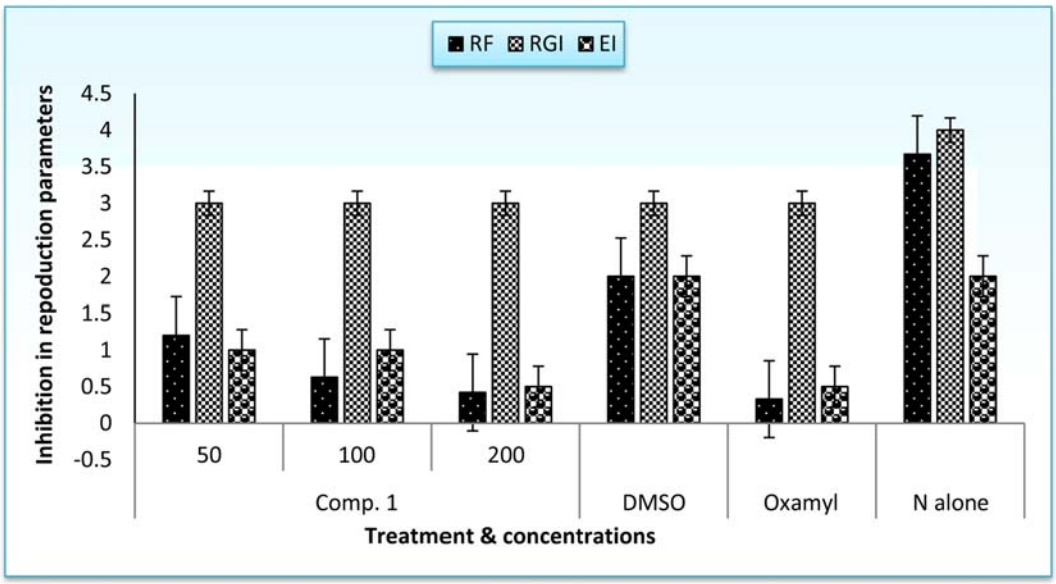

Fig. 7. Impact of compound **1** isolated from *Schinus terbenthifolius* leaves, on the reproduction of *Meloidogyne incognita* infecting tomato var. 162 grown under greenhouse conditions at 27 ± 3 ºC. Reproduction factor (RF), Root gall index (RGI) or egg-masses index (EI) were determined according to the scale given by Taylor and Sasser (1978).

Table 4. Impact of treatment with *n*-hexane, EtOAc extracts and compounds **1**, **6** and **7**, isolated from *Schinus terbinthifolius* leaves, on the biochemical activities of the leaves of tomato var. 162 infected with *Meloidogyne incognita* and grown under greenhouse conditions at 27 ± 3 °C.

Compds.	Treat. (μg/mL)	T. carb (mg/g)*	C. proteins (mg/g)*	T. phenols (mg/g)*	PO (Units/ mg protein)*	PPO (Units/ mg protein)*
	50	15.5^{f}	20.53 ^{cd}	0.368^{h}	0.429	0.030
1	100	18.5^{d}	20.87 ^{c}	0.290^{i}	0.783	0.066
	200	20.5^{b}	22.99 ^{b}	0.132^{j}	1.554	0.028
	50	16.8^{e}	17.88 ^{f}	0.369^{h}	0.662	0.083
6	100	18.6^{d}	18.17 ^{e}	0.305^{i}	1.312	0.097
	200	19.5^{c}	18.63 ^{e}	0.256^{ij}	1.467	0.049
	50	15.8^{f}	18.75 ^{e}	0.540^{d}	0.710	0.030
7	100	18.9^{d}	20.31 ^{cd}	0.495^{e}	1.311	0.036
	200	20.2^{b}	20.54 ^{cd}	0.440^{g}	1.416	0.034
	50	13.6^{g}	18.09 ^{ef}	0.579^{c}	0.470	0.078
n-Hex	100	16.4^{e}	18.23 ^{e}	0.495^{e}	1.669	0.085
	200	18.9^{d}	18.28 ^{e}	0.436^{g}	1.034	0.083
	50	16.8^{e}	19.41 ^{d}	0.576^{c}	0.636	0.016
EtOAc	100	18.4^{d}	19.53 ^{d}	0.569^{cd}	1.320	0.036
	200	18.6^{d}	19.65 ^{d}	0.481^{ef}	1.523	0.025
DMSO		13.5^{g}	17.5^{g}	0.635^{ab}	0.812	0.086
Oxamyl		19.5^{c}	19.91^{d}	0.367^{h}	0.118	0.047
N-free		22.5^{a}	23.62^{a}	0.240^{ij}	2.001	0.027
N alone		12.7^{h}	17.2^{h}	0.654^{a}	0.619	0.020

*Each value presented the mean of three replicates. T. carb; total carbohydrates, C. proteins; crude proteins, T. phenols; total phenols, PO; peroxidase, PPO; polyphenol oxidase. N = *M. incognita* (1000 larvae / plant). N-free: Plants free of *M. incognita*. Means are followed by the same letter (s) did not differ at $P \leq 0.05$ according to Duncan's multiple range test.

that pots receiving compound **1** showed reduction in total phenolics comparable to the healthy plant.

Defense-related proteins – Data presented in Table 4 revealed that the least induction of PO and PPO was recorded in tomato plants untreated and inoculated with *M. incognita*. Oxamyl differed in its ability to stimulate peroxidase (PO) and polyphenol oxidase (PPO) activities in plant inoculated with nematodes. However, increased PO activity was observed for compound **1** followed by the EtOAc, compound **6** then **7** compared to untreated inoculated plants. On the other hand, the increased activity of PPO remained higher in plants treated with compound **6**.

Conclusions

Biological control of plant pests including nematodes continues to inspire researchers in this field. Natural products are promising resources for biocontrol agents that are safer, environmentally friendly and cost-effective. In this study, phenolic compounds isolated from the leaves of *Schinus terebenthifolius* were investigated for their nematicidal activity against the root-knot nematode *M. incognita* and its infested tomato plant. The isolated compounds from *S. terebenthifolius* extract showed promising nematicidal activity compared to Oxamyl as a positive control. Compound **1** namely, 1,2,3,4,6-pentagalloyl glucose showed significant nematicidal activity in both *in vitro* and *in vivo* studies. It exhibited the best suppressed total nematode population, root galling and number of eggmasses. It also restored total carbohydrates, proteins and phenolics to normal and increased defense-related proteins. Thus, 1,2,3,4,6-pentagalloyl glucose could be a promising nematicidal agent against the root-knot nematode *M. incognita*.

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