Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

J P P Journal of Pharmacognosy and Phytochemistry

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2017; 6(6): 1660-1664
Received: 29-09-2017
Accepted: 30-10-2017

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GC-MS analysis and *in vitro* evaluation of methanol root extract of *Tagetes patula* against *Meloidogyne incognita*

A Kumar, KK Chahal and NK Dhillon

Abstract
The non-polar and polar fractions of methanol root extracts of *Tagetes patula* were assayed against *Meloidogyne incognita*. Methanol extract and its fractions were tested for egg hatch and mortality against second stage juveniles of *M. incognita* in the laboratory at five different concentrations. The non-polar fraction of methanol extract inhibited egg hatching and was extremely toxic to juveniles of *M. incognita* at concentration of 5 mg/mL and this lethality was highly significant compared to the other extracts. The more active non-polar fractions was analysed by GC-MS. Twenty-four compounds representing 85% of the total extract were identified of which 9,12-Octadecadienoic acid (14.87%), Ascorbic acid 2,6-dihexadecanoate (12.82%), Thianthrene (11.91%), 5-(4-acetoxy-1-butynyl)-2,2'-bithienyl (11.76%), Alpha.-terthienyl (8.77%) etc. were the major compounds. The present study suggests that hexane fraction was active against *M. incognita* due to the presence of thiophenes, fatty acid, fatty acid ester and steroids present as major compounds.

Keywords: Hatching, *Meloidogyne incognita,* mortality, root extract, *Tagetes patula*

Introduction
Plant parasitic nematodes, the hidden enemy of crops is one of the many groups of soilborne pathogens which depend on plants for their survival. It is the most destructive species which cause serious problem in various agricultural crops. *M. incognita* may affect 1700 plant species and are among the most destructive nematodes in agriculture (Oka *et al.,* 2000) [7]. Popular nematicides are volatiles, toxic and hazardous to the environment caused residue problem (Chitwood, D., 2002) [2]. Plants are an important source of naturally occurring pesticides. The plant oils received much attention due to their multifunctions as microbial (Tripathi *et al.,* 2004) [15], antifungal (Sahani *et al.,* 2009) [9], nematicidal (Ray *et al.*, 2000) [8], insecticidal (Sharma N & Chahal KK 2012) [11]. *Tagetes spp*. has been known to suppress plant-parasitic nematodes especially *Meloidogyne spp*. (Hooks *et al.,* 2010) [5]. Marigold is one of the most widely studied plant genera due to its allelopathic potential against plant parasitic nematode.
Essential oils and acetylenicthiophenes derived from roots are the most important secondary metabolites of French marigold (Szarka *et al.,* 2006) [14]. Monoterpenes, sesquiterpenes and α-terthienyl have been reported to bethe main ingredients of the stem and roots of *T. patula*, respectively. The nematicidal activities of this plant are due to the presence of high levels of sesquiterpenes and thiophene compounds in the essential oil and organic solvent extracts of roots (Bakker *et al.,* 1979) [1]. Most of the work on *T. patula* has been carried on its aqueous extracts and under greenhouse conditions in field for its nematicidal potential. So, the present study was conducted to examine the chemical composition of most active fraction of methanol extract of *T. patula* against egg hatching and juvenile's mortality of root knot nematode in laboratory conditions.

Materials and Methods
Gas chromatography/mass spectrometry (GC/MS) analysis: The hexane extract was analyzed using GC-MS (QP2010 Plus, Shimadzu, Japan), equipped with an Rtx-5 MS capillary column (30.0 m × 20 mm i.d., 0.25 µm film thickness). The injector was maintained at 250 °C and operated in split injection mode with the split valve closed for 1 min. Helium gas was used as the carrier gas at a constant pressure of 69kPa. The column oven was initially maintained at 50 °C for 2min, raised to 180 °C at 3 °C/min, then to 280 °C at 10 °C/min. The interface temperature was 260 °C and the ionization mode was electron impact (70 eV). The mass selective detector was operated in the scan mode between 40 and 600 m/z.

Data acquisition was started 3.0 min after injection. MS parameters used were; Ionization voltage (EI) 70 eV, peak width 2s, mass range 40–600 amu and detector voltage 1.5 V. Peak identification was carried out by comparison of the mass spectra with mass spectra data available on database of NIST08, WILEY8, Perfumery and Flavor and Fragrance libraries (Kataria *et al.,* 2016) [6].

Plant material: The roots of the *T. patula* were collected in the month of March from the field of the Department of Plant Pathology, Punjab Agricultural University, Ludhiana. The roots were dried under shade and powdered using electrical grinder.

Soxhlet extraction: 50 g of dried and powdered plant material was subjected to Soxhlet extraction method for eight hrs using 250 mL of methanol as solvent. A yellowish brown solution obtained was concentrated using rotary vacuum pump.5gm of yellowish brown oil was obtained. The process was carried out several batches to collect the methanol extract. The extract was stored at 4˚C in a freezer for further studies.

Partitioning of methanol extract: The concentrated methanol extract was partitioned into non-polar (hexane) and polar (chloroform) fractions using a separating funnel. 5.0 g of methanol extract of roots of marigold was transferred into 250 mL separating funnel. 100 mL of brine solution was added to the separating funnel. The oil was partitioned thrice using, 100, 50 and 50 mL of hexane. The hexane fraction formed the upper layer and separated out. The remaining water layer was partitioned thrice using 100, 50 and 50 mL of chloroform which form the lower layer and was separated. These fractions were concentrated by distillation under reduced pressure and were stored at 4˚C in a freezer for nematicidal activity.

Nematicidal Activity

Multiplication of M. incognita

Pure culture of root nematode was raisedby single egg mass technique on brinjal plant in pots. Perennial patterns of adult females were cut and the species identified were *M. incognita*. For maintenance and multiplication of pure culture the soil was autoclaved at 15 psi pressure at 121º C for at least 30 min. The autoclaved soil was filled in the pot. The pot was ethod planted with seedling of brinjal, a susceptible host for root knot nematode. Simultaneous inoculations were carried out with freshly hatched 2nd stage juveniles collected from egg masses of pure culture and the culture was multiplied on brinjal plants.

Evaluation of nematicidal activity

The non-polar and polar fractions of methanol extract of roots of *T. patula* were evaluated for percent hatching and mortality test against root knot nematode in laboratory condition.

Hatching studies

Root-knot infected brinjal plants were up-rooted from the pot house and washed gently under tap water. Five egg masses with an average of 200-250 eggs were collected from pure culture maintained in pot house were added to each petri dish containing10.00 mL of methanol, non-polar and polar fractions at different concentrations i.e. 1, 2, 3, 4 and 5 mg/mL.These concentrations were prepared by serial dilution of stock solution of 10,000 µg/mL along with 15.00 ml/L Tween 80 emulsifier. For each treatment a control was also formed in distilled water along with Tween 80 in same amount as taken for samples. Each treatment was replicated thrice. The experiment was conducted at 27±2˚C. Observations on egg hatch were recorded on 2nd, 4th, 6th, 8th and 10th day. The percentage of inhibition was calculated by the formula

$$\% \text{ Hatching} = \frac{\text{No. of egg hatch in treatment}}{\text{Total no. egg hatch in control or water}} \times 100$$

Mortality studies

For mortality test, eggs were collected and kept in water till hatching of second stage juveniles. Freshly hatched fifty juveniles were placed in each concentration of methanol extract, its non-polar and polar fractions. Tween 80 was in water used as control. Observations on mortality were recorded on 2nd, 4th, 6th, 8th and 10th day. The nematodes were considered dead if found motionless when probed with fine needle. A needle with narrow tip used to stab the nematode to check any movement. Average of three replications was taken as mortality for each concentration.

$$\% \text{ Mortality} = \frac{\text{No. of nematode died in treatment}}{\text{Total no. nematode in control or water}} \times 100$$

A revival test was performed for each treatment by decanting the test solvent and adding distilled water to the petri-plates. After 4 days of exposure, readings were counted. Again the revived juveniles were counted and dead nematodes were confirmed. The nematodes were considered dead if motionless when probed with a fine needle.

Statistical analysis

Per cent egg hatch and per cent mortality data was subjected to statistical analysis using the factorial completely randomized design statistical package. The critical differences in main effects i.e. compounds, concentrations and days as well as in their interactions were tested at P = 0.05 %.

Results and Discussion

Egg Hatching studies: Studies conducted on the effect of the methanol extract, its non-polar (hexane) and polar (chloroform) fractions on egg hatching revealed that all the fractions significantly reduced egg hatching of *M incognita* (Table 1). On comparing the different fractions, it was found that the maximum decrease in cumulative hatch count of *M. incognita* was observed in non-polar (hexane) fraction followed by methanol extract and chloroform fraction. Studies of these fractions at different concentrations revealed lowest count of hatched second stage juveniles of root knot nematode at 5mg/mL. Inhibition of egg hatching was least at 1 mg/mL concentration. However in case of control, the percent egg hatching was found to increase with increase in time of exposure. There was more inhibition of egg hatching at higher concentration but less inhibition was observed at lower concentrations.

Table 1: Percentage hatching of root knot nematode in methanol extract and its non-polar and polar fractions at different concentrations.

Compounds	Duration (days)	Percentage of egg hatchingat different concentration (mg/mL)				
		5	4	3	2	1
Methanol extract	2	80.00(09.63)	100.00(12.04)	136.66(16.46)	201.66(24.29)	250.00(30.12)
	4	178.33(17.36)	201.66(19.57)	230.00(22.40)	261.66(31.65)	383.33(37.33)
	6	180.00(15.65)	281.00(24.49)	341.66(29.70)	360.00(35.07)	441.66(40.14)
	8	200.00(20.00)	298.33(29.83)	353.33(35.33)	385.00(38.50)	490.00(49.00)
	10	215.00(19.02)	293.33(25.95)	346.66(30.67)	406.66(35.98)	485.00(42.92)
Control for methanol extract	2	830.00				
	4	1026.66				
	6	1150.00				
	8	1000.00				
	10	1130.00				
Non-polar fraction	2	41.66(05.27)	78.33(09.91)	115.00(14.55)	148.33(18.77)	171.66(21.72)
	4	70.00(08.07)	113.33(13.07)	168.33(19.42)	200.00(23.07)	255.00(29.42)
	6	78.33(08.39)	121.66(13.03)	160.00(17.14)	203.33(21.78)	270.00(28.92)
	8	73.33(07.99)	121.66(13.27)	175.00(19.09)	228.33(24.90)	296.66(32.36)
	10	76.66(08.24)	128.33(13.79)	171.66(18.45)	221.66(23.83)	291.66(31.36)
Polar fraction	2	90.00(11.39)	140.00(17.72)	210.00(26.58)	265.00(33.54)	298.33(38.60)
	4	176.66(20.38)	220.00(25.38)	261.66(29.61)	353.33(40.76)	390.00(45.00)
	6	173.33(18.57)	220.00(23.57)	291.66(31.24)	375.00(40.17)	446.66(47.85)
	8	178.33(19.45)	206.66(22.54)	273.33(29.81)	400.00(43.63)	463.33(50.54)
	10	183.33(19.71)	231.66(24.90)	310.00(33.33)	396.66(42.65)	466.66(50.17)
Control for non-polar and polar fraction	2	790.00				
	4	866.66				
	6	933.33				
	8	916.66				
	10	930.00				
C D						
A	Compound					5.85*
B	Concentration					9.85*
C	Day					6.54*
A × B	Compound x Concentration					18.51*
A × C	Compounds × Days					13.09*
B × C	Concentrations × Days					20.70*

In bracket ( ) percentage of hatching was given.
*P< 0.05

Mortality of second stage juveniles: All of the extracts tested were found to exhibit some level of toxicity toward second stage juveniles of the root-knot nematode (Table 2). Generally, the mortality rates of juveniles increased with an increase in exposure time. All of the extracts exhibited higher mortality rates when kept for longer duration of time. The non-polar fraction of methanol extract were found to be extremely toxic to juveniles of *M. incognita* at concentrations of 5 mg/mL and this lethality was highly significant as compared to the other extracts. The larval mortality increased with increase in the concentration of different extract. Methanol extract showed complete mortality on sixth day of observation at 5 mg/mL. At 1 mg/mL, 90 percent of juvenile mortality was noted upto the 10th day of treatment. All the nematodes in control remained active during the period of study.

The study conducted by Sankarimeena *et al.,* (2009) [10] on five species of *Tagetes* against *M. incognita* under microplot conditions on tomato confirmed that the root exudates from the *Tagetes* sp. had high nematicidal property against root knot nematode. The toxic compound *i.e.* α terthenyl was found associated with *Tagetes* sp. that had adverse effect on the nematode population. The study conducted by Sharma N & Chahal KK (2012) [11] also found that the non-polar hexane fraction (I) of marigold was more toxic than polar fractions of dichloromethane-hexane (1:1) (II) and dichloromethane (III) against *T. castaneum* adults. The mortality rate of adult insects was highest in non-polar fraction (I) which showed complete mortality in 4, 3 and 2 days at 2000, 3000 and 4000 µg/mL, respectively which showed insignificant mortality. Sharma *et al*., (2007) [12] also reported similar results for the insecticidal properties of *T.erecta* where comparison of the bioefficacy of the essential oil and its non-polar hexane fraction showed that the hexane fraction was more effective as insecticide than the complete flower oil.

Table 2: Corrected percentage mortality of root knot nematode in methanol extract and its non-polar and polar fractions at different concentrations.

Compounds	Duration (days)	Corrected percentage mortality at different concentration (mg/mL)				
		5	4	3	2	1
Methanol extract	2	53.32	44.66	37.32	28.00	20.00
	4	93.32	83.32	59.32	50.66	42.00
	6	100.00	100.00	85.32	73.32	64.00
	8	100.00	100.00	100.00	90.66	82.00
	10	100.00	100.00	100.00	95.32	90.00
Non-polar fraction	2	100.00	100.00	76.00	52.00	28.66

	4	100.00	100.00	90.00	70.32	53.32
	6	100.00	100.00	100.00	95.32	90.00
	8	100.00	100.00	100.00	100.00	100.00
	10	100.00	100.00	100.00	100.00	100.00
Polar fraction	2	43.32	36.00	32.00	23.32	21.32
	4	78.00	60.00	55.32	41.32	34.00
	6	90.00	80.00	75.66	69.32	50.32
	8	100.00	99.33	80.00	72.66	56.66
	10	100.00	100.00	89.32	75.66	60.00
Control	2	0	0	0	0	0
	4	0	0	0	0	0
	6	0	0	0	0	0
	8	0	0	0	0	0
	10	0	0	0	0	0
C D						
A	Compound					0.97*
B	Concentration					1.19*
C	Day					1.09*
A × B	Compound x Concentration					2.39*
A × C	Compounds × Days					2.18*
B × C	Concentrations × Days					2.67*

*P< 0.05

Chemical composition: GC-MS analysis of the most active non-polar fraction was carried out (Table 3, Fig 1). Twenty-four compounds representing 85% of the total extract were identified of which 9,12-Octadecadienoic acid (14.87%), Ascorbic acid 2,6-dihexadecanoate (12.82%), Thianthrene (11.91%), 5-(4-acetoxy-1-butynyl)-2,2'-bithienyl (11.76%), α-terthienyl (8.77%), Stigmasta-5,20(22)-dien-3-ol (7.70%), Stigmast-5-en-3-ol, (3.beta.,24S) (6.31%) etc. were the major compounds. Hexane fraction mainly consists of thiophene components (30-35%), fatty acids and fatty acid ester (40-50%) and steroids (15-20%). Sesquiterpenes (1-3%) were present in very minor quantities in roots as previously reported. Four compounds 5-(3-buten-1-ynyl)-2,2′-bithiophene (BBT), 5-(3-penten-1-ynyl)-2,2′-bithiophene (PBT), 2,2′:5′,2″- terthiophene (α-terthiophene) and 5-(4-acetoxy-1-butynyl)-2,2′-bithiophene (BBTOAc) were reported in the essential oil of roots of *Tagetes patula* L.(Szarka *et al.,* 2007) [13]. Three thiophene were obtained containing BBT, 5-(4-acetoxy-1-butynyl)-2,2'-bithienyl (BBTOAc) and 5-(4-hydroxy-1-butynyl)-2,2'-bithienyl (BBTOH) by flash chromatography from the solvent extracts of roots (Szarka *et al.,* 2006) [14]. Thianthrene was reported as the major compounds in the extract of *Tagetes minuta* (Gabda *et al.,* 2015) [4]. So the roots mainly consists of the thiophenes components. But in our study the hexane extracts also confirmed the presence of fatty acid, fatty acid ester and steroids as major components. The results are in consonance with the earlier studies on the extracts of yellow flowers of *Tagetes patula* L. against the *Heterodera zeae* (Faizi *et al.,* 2011) [3]. In studies of compounds obtained commercially, α-terthienyl and gallic and linoleic acids showed complete mortality at concentrations of 0.125% after 24 h of exposure. Assessment of structure-activity relationships revealed that an increase in the number of hydroxyl groups in phenolic acids increased the activity whereas in case of fatty acids, activity depended on chain length, the number and position of double bonds.

Table 3: Chemical composition of the non-polar extract.

S. No	Compound Name	Retention Time(min)	Area (%)
1	Caryophyllene	11.763	0.65
2	1-methyl-4-(5-methyl-1-methylene-4-hexenyl	14.194	1.05
3	Caryophyllene oxide	16.399	0.51
4	Lanceol, cis	17.059	0.44
6	Thianthrene	25.697	11.91
7	Hexadecanoic acid, methyl ester	25.752	2.46
8	L-(+)-Ascorbic acid 2,6-dihexadecanoate	27.156	12.82
9	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	29.892	4.17
10	9,12,15-octadecatrienoic acid, methyl ester	30.010	1.68
11	9,12-Octadecadienoic acid (Z,Z)-	31.269	14.87
12	Octadecanoic acid	31.765	1.51
13	2H-thiopyran-3-carboxaldehyde, 6-(4-methoxyphenyl	32.432	2.89
14	Alpha.-terthienyl	32.856	8.77
15	5-(4-acetoxy-1-butynyl)-2,2'-bithienyl	35.578	11.76
16	1-methyl-3-(4-hydroxybutyl)fluoranthene	37.165	0.56
17	1-heptacosanol	38.714	0.53
18	Docosanoic acid, methyl ester	39.482	0.81
19	Pyrido[4',3':4,5]thieno[3,2-e][1,2,4]triazolo	40.313	0.53
20	1-docosanol, acetate	41.431	1.80
21	Vitamin E	53.926	0.43
22	Stigmasta-5,20(22)-dien-3-ol	57.564	7.70
23	Stigmast-5-en-3-ol, (3.beta.,24S)	59.661	6.31

24	Stigmastanol	59.990	0.45
25	4,4,6a,6b,8a,11,12,14b-Octamethyl-1,4,4a,5,6,6a,6b,7,8,8a,9,10,11,12,12a,14,14a,14b-octadecahydro-2H-picen-3-one	61.340	0.61
26	Stigmast-4-en-3-one	64.570	0.80

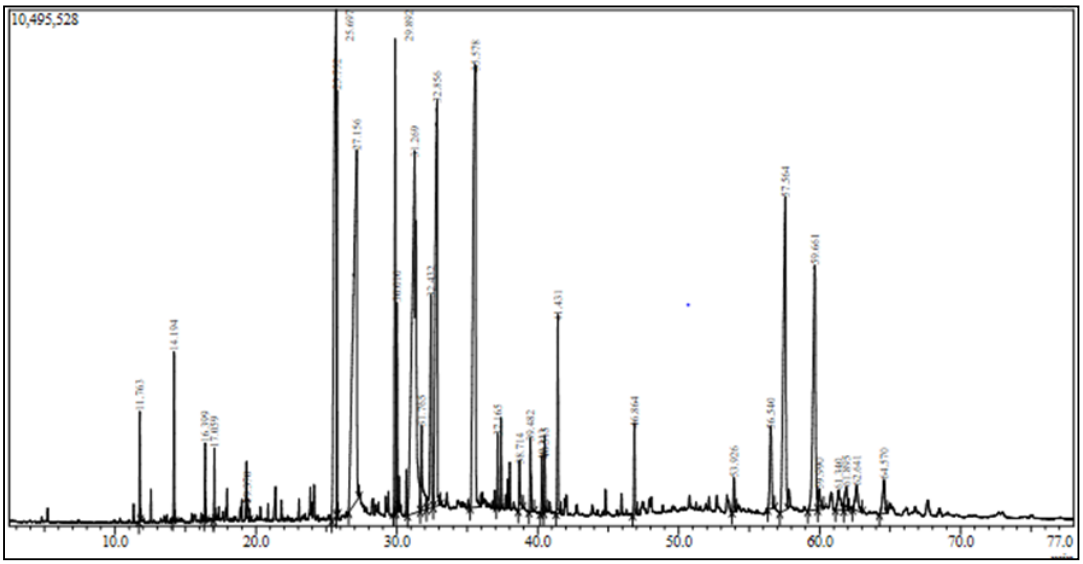

Fig 1: GC-MS chromatogram of active hexane extract.

Conclusion

The current research documented that the root extracts of *T. patula* was effective in controlling of egg hatching and mortality of root knot nematode with concentrations ranges between 1 to 5mg ml^{-1}.The nematicidal activity of hexane extract was due to the presence of high concentration of thiophene, fatty acids, fatty acid ester and steroidal compounds. The future looks bright for identifying new classes of nematicides from natural plants for replacement of the synthetic dangerous and expensive chemicals used at present. It can be concluded that non-polar fraction of the methanol extract can be used for the control of plant parasitic nematodes, especially root-knot nematode populations. Thus, the present method of control can contribute in minimizing the risks and hazards of toxic nematicides, especially on vegetables produced for fresh consumption.

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