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Isolation of Nematicidal Compounds from *Tagetes patula* L. Yellow Flowers: Structure−Activity Relationship Studies against Cyst Nematode *Heterodera zeae* Infective Stage Larvae[†]

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ABSTRACT: Bioassay-guided isolation studies on the extracts of yellow flowers of *Tagetes patula* L. against the *Heterodera zeae* were carried out to identify phytochemicals lethal to this economically important cyst nematode. In vitro investigation of a polar extract and fractions showing activity led to the isolation of phenolic compounds (flavonoids and phenolic acids). In the nonpolar extract, a few fatty acids, their methyl esters, and thiophenes (including α-terthienyl) were detected. In studies of compounds obtained commercially, α-terthienyl and gallic and linoleic acids showed 100% mortality at concentrations of 0.125% after 24 h. Assessment of structure−activity relationships revealed that an increase in the number of hydroxyl groups in phenolic acids increased the activity; with fatty acids, activity depended on chain length and the number and position of double bonds. Crude extracts of the flowers of different colors also have promising activity.

KEYWORDS: *Tagetes patula* L., marigold, gallic acid, linoleic acid, α-terthienyl, patuletin, nematicidal activity, cyst nematode, *Heterodera zeae*

INTRODUCTION

Cyst-forming nematodes are one of the most economically important pests and as pathogenic as root-knot nematodes[1] produce losses to agricultural crops mainly in the temperate regions of the world.[2] *Heterodera zeae*, a cyst nematode, is very widespread from North Africa to the Indo-Pakistan subcontinent and affects maize, sugar cane, and sorghum.[1,2] The production of cysts, which enclose the eggs, poses special problems in their control.[2,3] Traditionally, management of nematode-induced crop destruction has been achieved with the utilization of plant resistance,[3,4] crop rotation, biological control, and cultural practices including solarization and various organic amendments and use of pesticides.[3−6] Mainly, there are two groups of chemical pesticides in use: low molecular weight soil fumigants (e.g., methyl bromide) and carbamates (e.g., carbofuran, aldicarb) or organophosphates (e.g., diamidafos).[1,3−6] Since the 1950s, farmers have relied mainly on synthetic pesticides rather than on other approaches.[6] Resistance development and the potential for adverse ecological impact from pesticide use create a continuing need for the development of new products and alternative nematode control strategies.

Several benefits may result from the identification of the specific phytochemicals involved in plant−nematode interactions (including repellents, attractants, hatching stimulants or inhibitors, and nematotoxicants, either constitutive or formed in response to nematode presence), whether they occur in a field or in a laboratory. These compounds can be developed for use as nematicides themselves, or they can serve as model compounds for the development of chemically synthesized derivatives with enhanced activity and reduced environmental impact.[5,7] Moreover, plants usually produce a complex mixture of defense chemicals attacking different targets in pests; it is more difficult for a herbivore or pathogen to develop resistance to them, compared to the situation with synthetic regulators that consist of a single compound only.

The genus *Tagetes* is recognized as a source of essential oils[8,9] and very interesting biologically active chemical constituents, that is, acetylenes,[8] alkaloid,[10] carotenoids,[8] citric and malic acids,[11] flavonoids,[8,12−15] terpenoids,[8] and thiophenes.[5,8,16−19] The marigold species *Tagetes patula*, *Tagetes erecta*, and *Tagetes minuta* are most often used for nematode control,[9,20] whether utilized as a poor host, cover crop, rotation crop, green manure, or source of nematode-antagonistic extracts.[5,9,20,21] In general, they are used in crop rotation but, intercropping is very effective in many situations.[9] They are capable of suppressing a wide range (up to 14 genera) of nematode pests.[20,21] Of the 14 genera, *Pratylenchus* and *Meloidogyne* are most consistently affected by marigolds.[21] The nematicidal potential varies with the *Tagetes* species, the nematode species targeted, and soil temperature.[20] The bioactive compounds of different *Tagetes* species may differ in composition, quality, and quantity. *T. patula* usually gives better nematode control than the other species.[20] It is known to be effective against *Meloidogyne* spp., *Pratylenchus* spp., and *Radopholus* spp.[9,22] It has been reported that thiophene derivatives, widely distributed in *T. patula*, and *T. erecta*, are accountable for the nematicidal activity of the plant.[16,17] Tetrachlorothiophene, a simple analogue, was once a registered nematicide in

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Table 1. Compounds Identified by GC-MS in Nonpolar Fractions of *T. patula* Yellow Flowers and Their Relative Percentages of Total Chromatogram Area

sample	compound[a]	mol formula	RI[b]	RI[c]	basis of identification or mass fragments of its mass spectrum[d]	fractions[e]	%
1	1-octanol	$C_8H_{18}O$	1065	1068	MS, RI	JFYP	2.30
2	(*Z,Z*)-α-farnesene	$C_{15}H_{24}$	1461	1460	MS, RI	JFYP	1.20
3	dodecanoic acid, ethyl ester	$C_{14}H_{28}O_2$	1648	1597	MS	JFYP	0.70
4	tetradecanoic acid, methyl ester	$C_{15}H_{30}O_2$	1785	1728	MS	JFYP	0.80
5	tetradecanoic acid, ethyl ester	$C_{16}H_{32}O_2$	1854	1805	MS	JFYP	2.20
6	hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	2002	1933	MS	JFYP	5.70
7	hexadecanoic acid	$C_{16}H_{32}O_2$	2063	2021	MS, Std	JFYP	0.99
8	hexadecanoic acid, 2-methyl-, methyl ester	$C_{18}H_{36}O_2$	2075	1972	MS	JFYP	9.00
9	5′-methyl, 5-(3-buten-1-ynyl) 2,2′-bithiophene[f]	$C_{13}H_{10}S_2$	2150		MS	JFYP	1.46
10	8,11-octadecadienoic acid, methyl ester	$C_{19}H_{34}O_2$	2180	2196	MS, RI	JFYP	3.10
11	7,10,13-hexadecatrienoic acid, methyl ester[f]	$C_{17}H_{28}O_2$	2169		MS	JFYP	1.92
12	(*Z,Z,Z*)- 9,12,15-octadecatrienoic acid, methyl ester[f]	$C_{20}H_{32}O_2$	2169		MS	JFYP	
13	(*Z*)-2-docosene[f]	$C_{22}H_{44}$	2228	2223	MS, RI	JFYP	7.00
14	(*Z,Z,Z*)- 9,12,15-octadecatrienoic acid, ethyl ester	$C_{20}H_{34}O_2$	2238	2214	MS, RI	JFYP	5.00
15	octadecanoic acid, ethyl ester	$C_{20}H_{40}O_2$	2264	2199	MS	JFYP	2.90
16	α-terthienyl (**1**)[g]	$C_{12}H_8S_3$	2244	2243	MS, RI, Std	JFYP	1.99
						JYM-PV1	3.49
17	2-hydroxy-3-tridecenoic acid[f]	$C_{13}H_{24}O_3$	2332		MS	JFYP	1.64
18	eicosanoic acid, ethyl ester	$C_{22}H_{44}O_2$	2374	2400	MS, RI	JFYP	0.65
19	2-hydroxy-3-pentadecenoic acid[f]	$C_{15}H_{28}O_3$	2526		MS	JFYP	7.80
						JYM-PV29	9.83
20	α- or β-amyrin[h] (**2a**, **2b**)	$C_{30}H_{50}O$	2590		MS	JFYP	1.40
21	15,23-pentacosadienoic acid, methyl ester[f]	$C_{26}H_{48}O_2$	2664		MS	JFYP	5.00
22	7,10,13-pentacosatrienoic acid, methyl ester[f]	$C_{26}H_{46}O_2$	2672		MS	JFYP	4.70
23	3,3-diethyltricosane[f]	$C_{27}H_{56}$	2690	2673	MS, RI	JFYP	1.20
24	tetracosanoic acid, methyl ester	$C_{25}H_{50}O_2$	2713	2731	MS, RI	JFYP	1.70
						JYM-PV29	8.74
25	tetracosanoic acid, ethyl ester	$C_{26}H_{52}O_2$	2755	2779	MS	JFYP	2.20
						JYM-PV29	4.19
26	3,3-diethylpentacosane[f]	$C_{29}H_{60}$	2844	2877	MS, RI	JFYP	7.10
27	hexacosanoic acid, methyl ester	$C_{27}H_{54}O_2$	2927	2941	MS, RI	JFYP	0.43
28	2-methylpentacosanoic acid, ethyl ester[f]	$C_{28}H_{56}O_2$	3002		MS	JFYP	1.10
29	*n*-hentriacontane	$C_{31}H_{64}$	3100	3100	MS, RI	JFYP	5.60
30	α-tocopherol	$C_{29}H_{50}O_2$	3111	3111	MS, RI	JFYP	0.43
						JYM-PV29	52.82
31	hexacosanoic acid, *i*-propyl ester[f]	$C_{29}H_{58}O_2$	3115		MS	JFYP	0.30
32	5-hydroxy-2-methyl-3-hexadocosenoic acid[f]	$C_{29}H_{56}O_3$	3146		MS	JFYP	0.60
33	14-methylpentadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	1876	1884	MS, RI	JYM-P1	14.72
34	hexacosanoic acid, ethyl ester	$C_{18}H_{36}O_2$	1948	1954	MS, RI	JYM-P1	7.01
35	9,12-octadecadienoic acid, methyl ester	$C_{19}H_{34}O_2$	2062	2075	MS, RI	JYM-P1	46.0
						JYM-DC1	6.53
36	octadecanoic acid, methyl ester	$C_{19}H_{38}O_2$	2084	2104	MS, RI	JYM-P1	6.09
37	9,12-eicosadienoic acid[f]	$C_{20}H_{36}O_2$	2114		MS	JYM-P1	15.88
38	methyl, 7-methoxytetradecanone[f]	$C_{16}H_{32}O_2$	2413		MS	JYM-P1	2.41
39	docosanoic acid, methyl ester	$C_{23}H_{46}O_2$	2456	2459	MS, RI	JYM-P1	0.92
40	5,9-heptadecadienoic acid[f]	$C_{18}H_{32}O_2$	2538		MS	JYM-P1	3.68
41	2,3-dimethyl-1-butanol	$C_6H_{14}O$	923	835	MS	JYM-DC1	10.20
42	unknown		1198		120 (36), 55 (100), 45 (10), 64 (9), 77 (8), 86 (8), 105 (8)	JYM-DC1	9.29
43	benzoic acid	$C_7H_6O_2$	1220	1193	MS, RI	JYM-DC1	4.79
44	1-(3-methoxyphenyl)ethanone	$C_9H_{10}O_2$	1284	1295	MS, RI	JYM-DC1	11.19

Table 1. Continued

sample	compound[a]	mol formula	RI[b]	RI[c]	basis of identification or mass fragments of its mass spectrum[d]	fractions[e]	%
45	unknown		1399		261 (5), 84 (100), 77 (17), 78 (22), 91 (61), 92 (82), 103 (30), 121 (19),122 (15)	JYM-DC1	11.59
46	ethylparaben[h]	$C_9H_{10}O_3$	1467		MS	JYM-DC1	0.47
47	dihydrojasmone[f]	$C_{11}H_{18}O$	1570		MS	JYM-DC1	4.65
48	4-(3-hydroxy-1-propenyl)-2-methoxyphenol	$C_{10}H_{12}O_3$	1722	1726	MS, RI	JYM-DC1	48.60
49	3,4,5-trimethoxybenzyl methyl ether[h]	$C_{11}H_{16}O_4$	1739		MS	JYM-DC1	7.69
50	benzoic acid, octyl ester	$C_{15}H_{22}O_2$	1780	1792	MS, RI	JYM-DC1	4.13
51	3,4,5-trimethoxybenzenemethanol[i]	$C_{10}H_{14}O_4$	1850		MS	JYM-DC1	5.46
53	1,2-dipalmitin[i,j]	$C_{35}H_{68}O_5$	1938		MS	JYM-DC1	0.58
54	(*Z,Z*)-9,12-octadecadienoic acid	$C_{18}H_{32}O_2$	2112	2104	MS, RI	JYM-DC1	6.70
55	unknown		2255		576 (38), 522 (19), 516 (23), 331 (15), 313 (32), 295 (13), 261 (38), 253 (53), 219 (30), 194 (17)	JYM-DC1	4.52
56	(*Z*)-3-decen-1-ol[f]	$C_{10}H_{20}O$	1792		MS	JYM-PV1	17.56
57	5,5-diethylheptadecane[f]	$C_{21}H_{44}$	2016	2006	MS, RI	JYM-PV1	8.15
58	7,7-diethylnonadecane[f]	$C_{23}H_{48}$	2189	2187	MS, RI	JYM-PV1	25.98
59	6,6-diethylicosane[f]	$C_{24}H_{50}$	2286	2296	MS, RI	JYM-PV1	1.52
60	5-butyl-5-ethylhenicosane	$C_{27}H_{56}$	2533	2543	MS, RI	JYM-PV1	6.63
61	3,7,11-trimethyl-2,6,10-dodecatrien-1-ol[f]	$C_{15}H_{26}O$	2660		MS	JYM-PV1	8.96
62	3,7-dimethylnonacosane	$C_{31}H_{64}$	3021	3010	MS, RI	JYM-PV1	7.17
63	β-tocopherol[i]	$C_{28}H_{48}O_2$	2966		MS	JYM-PV29	11.29

[a] Order of elution on column ZB-5 is given. [b] Calculated retention indices of compounds. [c] Compounds were identified by comparison with retention indices from the literature available in the NIST database. [d] MS, identification by comparing EI mass spectrum with NIST mass spectral database; RI, identification by retention indices with literature data; Std, mass spectrum agreed with standard injected under the same conditions. For mass fragments, the proportion of the mass fragment is given in parentheses. [e] JFYP, petroleum ether (PE) extract; JYM-P1, first PE phase of methanol extract; JYM-DC1, dichloromethane phase of methanol extract; JYM-PV1, JYM-PV29, VLC fractions of petroleum ether phase. [f] Compound tentatively identified according to mass spectrum only. [g] Szarka et al.[19] [h] RI available but on different/nonequivalent columns. [i] RI not available. [j] NIST mass spectral search program, 2000.

the United States.[5] The essential oil of *T. patula* has also been found to possess nematicidal activity.[9] It has been reported that the aqueous leaf extract of *T. patula* had a nematostatic property;[22] however, until now no formulation based on *Tagetes* extract has been marketed as a nematicide.[1]

In this paper, the bioassay-guided isolation work on the extracts of yellow flowers of *T. patula* is described for the first time, which led to the identification of active principles of the flowers in relation to their nematicidal activity against cyst nematode, *H. zeae* infective stage larvae. Moreover, structure−activity relationship (SAR) studies have also been carried out. This investigation may provide interesting molecular probes for the biochemist and lead structures to be developed into alternative nematicidal agents for the organic chemist.

MATERIALS AND METHODS

Chemicals. α-Terthienyl (**1**), myristic acid (**5**), palmitic acid (**6**), cetyl alcohol (**7**), stearic acid (**8**), oleic acid (**9**), methyl oleate (**10**), linoleic acid (**11**), quercetin (**12**), rutin (**13**), benzoic acid (**14**), 4-hydroxybenzoic acid (**15**), 3,4-dihydroxybenzoic acid (**16**), methyl 3,4-dihydroxybenzoate (**17**), 3,4,5-trihydroxybenzoic acid (gallic acid) (**18**), 4-methoxybenzoic acid (**19**), 3,4-dimethoxybenzoic acid (**20**), and 3,4,5-trimethoxybenzoic acid (**21**) were used in SAR studies. Compounds **1**, **5**−**9**, and **11**−**13** were purchased from Sigma-Aldrich (Germany), and compounds **14**−**16** and **18**−**21** were bought from Merck (Germany). Methyl derivatives **10** and **17** were prepared by employing the usual method.[14] A mixture of α-amyrin (**2a**) and β-amyrin (**2b**) was isolated in this work, whereas pure patuletin (**3**) and patulitrin (**4**) used were previously isolated from *T. patula*.[12,14] The conventional nematicide carbofuran[23] was purchased from Sigma-Aldrich (Germany).

Analytical Studies. GC-MS was taken with an Agilent 6890N (USA) JMS 600H (JEOL, Tokyo, Japan) [EI mode; ionizing potential, 70 ev; capillary column ZB-5 (30 m × 0.32 mm × 0.22 μm film) (Zebron, Phenomenex); oven temperature, 50−250 °C (rate of temperature increase = 5 °C/min); carrier gas, He; flow rate, 1.8 mL/min; split ratio, 30]. Most of the compounds were identified using two different analytical methods: (a) retention indices (RI) in reference to *n*-alkanes (C_9−C_{32}) (Sigma-Aldrich, Germany) using the equation given in the literature;[24] (b) mass spectra [(authentic chemicals and National Institute of Standards and Technology (NIST) database (http://webbook.nist.gov/chemistry)]. Identification was considered to be tentative when based on mass spectral data only (Table 1). The EIMS and HREIMS spectra were recorded on Finnigan MAT 112 (Finnigan, Germany) and JMS HX-110 (JEOL, Japan) spectrometers. The ^{1}H NMR spectrum was run on a Bruker AC-500 instrument operating at 500 MHz. TLC was performed on silica gel 60 F_{254} (Merck). Silica gel HF 60_{254} was used for vacuum liquid chromatography (VLC). All of the chemicals and solvents used were of analytical grade.

Determination of Nematicidal Activity. A soil sample (500 g) was collected from pure culture of *H. zeae*, maintained on maize plants (CWM-19), the commercially available cultivar, in microplots of a screen house, National Nematological Research Centre, University of Karachi. The emerged larvae were collected from the soil by Baermann funnel technique.[25] After larvae had been counted in a counting

Table 2. Nematicidal Activity[a] of Extracts and Fractions of Yellow Flowers of *Tagetes patula* against Cyst Nematode *Heterodera zeae*

				mortality[e] (%)						mortality at 5% concn[j] (mean ± SEM, $N = 9$)	
				light[f]			dark[f]			light	dark
sample	sample code[b]	solvent[c]	concn[d] (%)	24 h	48 h	72 h	24 h	48 h	72 h	24 h	24 h
1	JFYP	PE	10	0	50	50	20	50	70		
			5	0	20	20	0	10	20	12.40 ± 3.5c[k]	7.6 ± 2.4d
			2	0	0	0	0	0	10		
			1	0	0	0	0	0	0		
2	JFYM	MeOH	10	100	–[g]	–	80	100	–		
			5	100	–	–	80	100	–	100 ± 0.00a	90.5 ± 5.2b
			2	0	100	–	50	100	–		
			1	0	0	0	0	0	0		
3	JYM-P1	PE	10	100	–	–	80	100	–		
			5	100	–	–	70	100	–	100 ± 0.00a	83.9 ± 4.9b
			2	50	50	50	10	50	100		
			1	10	50	50	0	50	100		
4	JYM-DC1	PE	10	0	50	70	20	70	100		
			5	0	20	50	0	20	50	9.5 ± 5.0c	12.8 ± 3.3d
			2	0	0	20	0	0	10		
			1	0	0	0	0	0	0		
5	JYM-EA1	MeOH	10	100	–	–	100	–	–		
			5	50	100	–	100	–	–	85.0 ± 7.5b	100 ± 0.00a
			2	0	70	100	80	100	–		
			1	0	40	100	50	70	100		
6	JYM-EA2M[h]	DMSO	10	100	–	–	0	100	–		
			5	100	–	–	0	100	–	100 ± 0.00a	100 ± 0.00a
			2	100	–	–	0	100	–		
			1	70	70	70	0	100	–		
7	JYM-EA2D	DMSO	10	100	–	–	100	–	–		
			5	100	–	–	100	–	–	100 ± 0.00a	100 ± 0.00a
			2	100	–	–	100	–	–		
			1	100	–	–	100	–	–		
8	JYM-Bu1	MeOH	10	100	–	–	100	–	–		
			5	100	–	–	60	100	–	100 ± 0.00a	82.5 ± 3.5b
			2	80	100	–	–	100	–		
			1	0	0	100	0	100	–		
9	JYM-aq	H_2O	10	0	30	80	0	40	100		
			5	0	0	50	0	10	60	0.00 ± 0.00	10 ± 0.00d
			2	0	0	10	0	0	10		
			1	0	0	0	0	0	10		
10	JYM-PV1	DMSO	10	100	–	–	90	100	–		
			5	100	–	–	90	100	–	100 ± 0.00a	95.4 ± 2.0b
			2	40	100	–	–	100	–		
			1	0	50	50	0	50	50		

Table 2. Continued

sample	sample code[b]	solvent[c]	concn[d] (%)	mortality[e] (%)						mortality at 5% concn[j] (mean ± SEM, N = 9)	
				light[f]			dark[f]			light	dark
				24 h	48 h	72 h	24 h	48 h	72 h	24 h	24 h
11	JYM-PV29	DMSO	10	20	30	50	50	50	70		
			5	0	10	50	10	50	50	00 ± 0.00	45 ± 11.6c
			2	0	0	10	10	30	50		
			1	0	0	0	0	0	20		
12	JYM-PV65[i]	DMSO	1	0	0	0	0	0	0		
			0.5	0	0	0	0	0	0	0 ± 0.00	0 ± 0.00
			0.25	0	0	0	0	0	0		
			0.125	0	0	0	0	0	0		
13	JYM-PV101	DMSO	10	50	100	–	40	50	100		
			5	50	100	–	10	50	70	75.5 ± 10.5b	35.6 ± 5.6c
			2	10	50	100	0	50	50		
			1	0	50	100	0	10	50		
14	carbofuran	DMSO	10	100	–	–	100	–	–		
			5	100	–	–	100	–	–	100 ± 0.00	100 ± 0.00
			2	100	–	–	100	–	–		
			1	100	–	–	100	–	–		
			0.5	100	–	–	100	–	–		
			0.25	80	100	–	80	100	–		
			0.125	40	100	–	60	100	–		
15	MeOH	H_2O	5	0	0	0	0	0	0	0 ± 0.00	0 ± 0.00
16	DMSO	H_2O	5	0	0	0	0	0	0	0 ± 0.00	0 ± 0.00

[a] The bioassay was conducted in agar medium for the petroleum ether soluble samples and in a glass cavity block for the remaining samples. [b] JFYP, petroleum ether (PE) extract; JFYM, methanol extract; JYM-P1, first PE phase; JYM-DC1, dichloromethane phase of methanol extract; JYM-EA1, first ethyl acetate phase; JYM-EA2M, insoluble fraction of second ethyl acetate phase; JYM-EA2D, soluble fraction of second ethyl acetate phase; JYM-Bu1, first butanol phase; JYM-aq, aqueous phase; JYM-PV1, 29, 65, and 101, VLC fractions of PE phase. [c] PE, petroleum ether; MeOH, methanol; DMSO, dimethyl sulfoxide. [d] Concentration of stock solution was 30 mg/mL. [e] Values represent the mean of three experiments. [f] No mortality during 5–60 min. [g] No further observation. [h] Ten percent activity after 60 min (concn = 10%). [i] A mixture of α- (**2a**) and β-amyrins (**2b**), and concentration of stock solution was taken as 1 mg/mL. [j] Means of mortality rate in light and dark conditions for each treatment were compared. [k] Means within a column (belonging to the same bioassay) and row followed by the same letters are not significantly different (P = 0.05).

chamber, 100 larvae were placed in a cavity block with a minimum amount of water for bioassay. Stock solutions for plant extract and fractions (30 mg/mL) were prepared in their suitable solvents (Tables 2 and 5) and then diluted in their respective solvent to make the amount of 5 mL of different concentrations (10, 5, 2, and 1%); likewise for the pure compounds, 1 mg/mL was the stock solution (Tables 3 and 4), and 1.0, 0.5, 0.25, and 0.125% dilutions were prepared from the stock. The standard nematicide carbofuran was used for the comparison, and distilled water was used as the control. Stock solution of positive control, that is, carbofuran was also prepared in the same manner as for extracts and pure compounds. The nematicidal activities of 5% dimethyl sulfoxide (DMSO) and 5% methanol used as the solvent and as negative controls were also determined (Tables 2–5).

To determine the nematicidal effect of methanol and DMSO soluble fractions and pure compounds, 100 freshly hatched second-stage juveniles were introduced separately in each 3 × 3 glass Petri plates with the three replicates. Petri plates were kept at room temperature (28 ± 2 °C) in the laboratory and dark box conditions. Each treatment was observed in light and dark conditions separately and replicated three times. The mortality of the nematodes was examined under a stereoscopic microscope at 4× after 24 h. In the case of immobile larvae, their irreversible immobility was confirmed by transferring them to distilled water. The percent mortality was calculated from an average of three replicates (N = 3). This procedure was repeated after 48 and 72 h.

The methodology for the nonpolar extracts/compounds (petroleum ether soluble constituents) was followed as reported by Kyo et al.[17] *H. zeae* larvae were cultured on agar. Aliquots of 5 mL of different concentrations of extracts were placed on a glass cavity block and evaporated to dryness in air. The agar medium on which the nematodes had been cultured was cut into pieces of about 1 cm^2 and placed with the upper surface of the agar in contact with the area where the sample had been dried. Then a piece of agar was removed from the glass cavity block. In this way nematodes were placed on the area. Glass cavity blocks were kept at saturated humidity for 72 h. The number of wiggling nematodes

Table 3. Nematicidal Activity[a] of Fatty Acids, an Ester, and a Thiophene against the Cyst Nematode *Heterodera zeae*

				mortality[d] (%)						mortality at 0.5% concn[g] (mean ± SEM $N = 9$)	
				light[e]			dark[e]			light	dark
sample	sample code	solvent[b]	concn %[c]	24 h	48 h	72 h	24 h	48 h	72 h	24 h	24 h
1	α-terthienyl (**1**)	DMSO	1	100	–[f]	–	100	–	–		
			0.5	100	–	–	100	–	–	100.00 ± 0.00a[h]	100.00 ± 0.0.00a
			0.25	100	–	–	100	–	–		
			0.125	100	–	–	100	–	–		
2	myristic acid (**5**)	DMSO	1	40	50	80	50	60	90		
			0.5	30	30	50	50	50	80	30.00 ± 1.9c	42.50 ± 2.2c
			0.25	20	20	50	30	30	80		
			0.125	20	20	40	20	20	50		
3	palmitic acid (**6**)	PE	1	0	70	80	80	80	80		
			0.5	0	50	70	0	50	70	28.00 ± 2.0c	68.00 ± 0.7bc
			0.25	0	10	50	0	20	70		
			0.125	0	0	0	0	0	50		
4	cetyl alcohol (**7**)	PE	1	70	80	100	70	80	100		
			0.5	60	80	100	60	80	100	75.60 ± 0.3b	76.4 ± 4.4b
			0.25	0	50	80	0	50	80		
			0.125	0	0	40	0	0	40		
5	stearic acid (**8**)	PE	1	100	–	–	100	100	–		
			0.5	50	70	100	50	70	100	90.80 ± 0.00ab	63.00 ± 1.9bc
			0.25	40	70	100	40	70	100		
			0.125	50	50	70	40	50	70		
6	oleic acid (**9**)	PE	1	50	100	–	50	100	–		
			0.5	50	100	–	40	100	–	100.00 ± 0.00a	85.00 ± 4.0b
			0.25	10	50	100	10	10	10		
			0.125	10	50	100	10	10	10		
7	methyl oleate (**10**)	PE	1	50	100	–	50	100	–		
			0.5	50	100	–	40	100	–	100.00 ± 0.00a	88.00 ± 3.9b
			0.25	10	50	100	10	10	10		
			0.125	10	50	100	10	10	10		
8	linoleic acid (**11**)	DMSO	1	100	–	–	100	–	–		
			0.5	100	–	–	100	–	–	100.00 ± 0.00a	100.00 ± 0.00a
			0.25	100	–	–	100	–	–		
			0.125	100	–	–	100	–	–		
9	carbofuran	DMSO	1	100	–	–	100	–	–		
			0.5	100	–	–	100	–	–	100.00 ± 0.00a	100.00 ± 0.00a
			0.25	80	100	–	80	100	–		
			0.125	40	100	–	60	100	–		
10	DMSO	H_2O	5	0	0	0	0	0	0	0.00 ± 0.00	0.00 ± 0.00

[a] The bioassay was conducted in agar medium for the petroleum ether soluble samples and in a glass cavity block for the remaining samples. [b] DMSO, dimethyl sulfoxide; PE, petroleum ether. [c] Concentration of stock solution was 1 mg/mL. [d] Values represent the mean of three experiments. [e] No mortality during 5–60 min time exposures. [f] No further observation. [g] Means of mortality rate in light and dark conditions for each treatment were compared. [h] Means within a column (belonging to the same bioassay) and row followed by the same letters are not significantly different ($P = 0.05$).

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Table 4. Nematicidal Activity of Phenolic Compounds against the Cyst Nematode *Heterodera zeae*

				mortality[b] (%)						mortality at 0.5% concn[f] (mean ± SEM $N = 9$)	
				light[c]			dark[c]			light	dark
sample	sample code	solvent	concn %[a]	24 h	48 h	72 h	24 h	48 h	72 h	24 h	24 h
1	patuletin (**3**)	DMSO[d]	1	40	40	100	20	60	60		
			0.5	20	70	100	0	20	20	44 ± 6.65c[g]	20 ± 2.0d
			0.25	20	70	100	0	10	10		
			0.125	20	50	100	0	0	0		
2	patulitrin (**4**)	DMSO	1	0	20	50	0	40	50		
			0.5	0	0	50	0	20	40	0 ± 0.00	20 ± 2.25d
			0.25	0	20	20	0	10	20		
			0.125	0	0	10	0	0	10		
3	quercetin (**12**)	DMSO	1	50	50	70	50	70	70		
			0.5	50	50	80	40	50	70	50 ± 0.00c	55 ± 9.9c
			0.25	40	50	70	40	50	50		
			0.125	40	50	80	40	40	40		
4	rutin (**13**)	H_2O	1	100	100	–[e]	100	–	–		
			0.5	100	100	–	100	–	–	100 ± 0.00a	100 ± 0.80a
			0.25	0	100	–	0	70	70		
			0.125	0	100	–	0	70	70		
5	benzoic acid (**14**)	DMSO	1	0	50	70	0	70	80		
			0.5	0	10	40	0	50	80	10 ± 2.00d	50 ± 1.1c
			0.25	0	0	0	0	10	50		
			0.125	0	0	0	0	0	0		
6	4-hydroxybenzoic acid (**15**)	DMSO	1	0	50	100	0	40	70		
			0.5	0	40	50	0	40	50	40 ± 0.00c	40 ± 0.5c
			0.25	0	40	50	0	40	50		
			0.125	0	40	50	0	10	40		
7	3,4-dihydroxybenzoic acid (**16**)	DMSO	1	100	–	–	40	100	–		
			0.5	50	100	–	40	100	–	72.7 ± 3.0b	89.7 ± 19.45b
			0.25	50	100	–	10	100	–		
			0.125	50	100	–	10	100	–		
8	methyl 3,4-dihydroxy-benzoate (**17**)	DMSO	1	100	–	–	60	100	–		
			0.5	70	100	–	20	100	–	89.5 ± 4.5b	65.8 ± 20.00bc
			0.25	50	100	–	10	100	–		
			0.125	50	100	–	10	100	–		
9	3,4,5-trihydroxy-benzoic acid (**18**)	DMSO	1	100	–	–	100	–	–		
			0.5	100	–	–	100	–	–	100 ± 0.00a	100 ± 0.00a
			0.25	100	–	–	100	–	–		
			0.125	100	–	–	100	–	–		
10	4-methoxybenzoic acid (**19**)	DMSO	1	40	50	80	50	60	90		
			0.5	30	40	50	50	50	80	33.2 ± 6.4c	50 ± 2.5c
			0.25	10	20	50	30	40	80		
			0.125	10	10	40	10	20	50		

Table 4. Continued

				mortality[b] (%)						mortality at 0.5% concn[f] (mean ± SEM $N = 9$)	
				light[c]			dark[c]			light	dark
sample	sample code	solvent	concn %[a]	24 h	48 h	72 h	24 h	48 h	72 h	24 h	24 h
11	3,4-dimethoxy-benzoic acid (**20**)	DMSO	1	80	100		50	60	90		
			0.5	60	80	100	50	50	80	82.5 ± 2.2b	50 ± 0.00c
			0.25	10	50	100	30	40	80		
			0.125	10	50	100	10	20	50		
12	3,4,5-trimethoxybenzoic acid (**21**)	DMSO	1	100	–	–	100	–	–		
			0.5	100	–	–	90	100	–	100 ± 0.00a	92 ± 1.2ab
			0.25	90	100	–	90	100	–		
			0.125	90	100	–	80	100	–		
13	carbofuran	DMSO	1	100	–	–	100	–	–		
			0.5	100	–	–	100	–	–	100 ± 0.00a	100 ± 0.00a
			0.25	80	100	–	80	100	–		
			0.125	40	100	–	60	100	–		
14	DMSO	H_2O	5	0	0	0	0	0	0	0.00 ± 0.00	0.00 ± 0.00

[a] Concentration of stock solution was 1 mg/mL. [b] Values represent the mean of three experiments. [c] No mortality during 5–60 min time exposures. [d] DMSO, dimethyl sulfoxide. [e] No further observation. [f] Means of mortality rate in light and dark conditions for each treatment were compared. [g] Means within a column and row followed by the same letters are not significantly different ($P = 0.01$).

and the total number were counted to calculate the percent mortality of the nematodes. The assay was conducted three times ($N = 3$) with each sample in light and dark conditions for time lengths of 24–72 h.

Plant Material. Plants of *T. patula* in full bloom were collected from the campus of University of Karachi in the year 2004 and identified by plant taxonomist Dr. Rubina Dawar of the Department of Botany, University of Karachi. A voucher specimen (KUH GH No. 67280) was deposited in the herbarium of the same department.

Extraction and Isolation of Compounds. Shade-dried yellow flowers (i.e., capitulum and involucres, 621 g) of *T. patula* were extracted three times with petroleum ether followed by methanol at room temperature. Petroleum ether extracts were combined and evaporated under vacuum, giving a gummy residue, JFYP (Scheme 1). The methanol extract was concentrated in vacuo into a thick liquidish form (JFYM), dissolved in water, and subjected to liquid–liquid extraction with organic solvents furnishing phases of different polarities, that is, petroleum ether (five times, JYM-P1–JYM-P5), dichloromethane (three times, JYM-DC1–JYM-DC3), ethyl acetate (four times, JYM-EA1–JYM-EA4), butanol (three times, JYM-Bu1–JYM-Bu3), and aqueous (JYM-aq) phases (Scheme 1).

The petroleum ether phases (JYM-P1–JYM-P5) showing similar spots on TLC were evaporated at room temperature, combined together (5.9039 g), and subjected to vacuum liquid chromatography (VLC) eluting with petroleum ether/dichloromethane (100:0 → 81:19, 143 fractions), and ethyl acetate/dichloromethane (81:19 → 0:100, 88 fractions) with increasing order of 0.05 mL, and ethyl acetate/methanol (100:0 → 0:100, 23 fractions) with increasing order of 1 mL, which yielded a total of 254 fractions. Fraction 1 (JYM-PV1) showed a major spot of α-terthienyl (**1**) on TLC, which was compared with the authentic sample. There were white crystals deposited in fractions JYM-PV65–JYM-PV67 (petroleum ether/dichloromethane, 9.8:0.2) on evaporation at room temperature, which were washed with a small amount of petroleum ether as they dissolved instantly in excess solvent. The crystals showed a single iodine active spot on TLC ($R_f = 0.48$, choloroform). The spectral studies (EIMS, HREIMS, ^{1}H NMR) revealed them to be a mixture of α- (**2a**) and β-amyrins (**2b**).[26] The ethyl acetate and butanol phases were completely evaporated at room temperature except the second ethyl acetate phase, which was concentrated at room temperature and decanted into decantate (JYM-EA2D) and deposit (JYM-EA2M); apparently, these two fractions were the same on TLC, showing patuletin (**3**) and patulitrin (**4**) as the main constituents.[12,14]

Crude Extracts of Mixed Color Flowers. Mixed flowers (fresh flowers of different colors) of *T. patula* were divided into three piles, each extracted separately at room temperature with methanol, acetone, and ethanol.

Statistical Analysis. The most promising results have been obtained after 24 h at 5% (Tables 2 and 5) and 0.5% concentrations (Table 3 and 4) for extracts/fractions and pure compounds, respectively. Nematicidal activities at these concentrations were subjected to one-way ANOVA (Tables 2–5), and means of mortality rate in light and dark conditions for each treatment were compared by performing LSD at $P = 0.01$.

RESULTS AND DISCUSSION

Nematicidal activity of the extracts, fractions, and pure compounds of yellow flowers of *T. patula* was conducted against the cyst nematode *H. zeae* following two different methods of bioassay, that is, agar medium[17] for petroleum ether soluble compounds and glass cavity block for compounds soluble in methanol, dimethyl sulfoxide, and water. The data obtained from these two different methods could not be compared. The activity of all the samples was also examined in relation to their phototoxic effect, which revealed significant results in most cases (Tables 2–5).

The first nonpolar petroleum ether extract, JFYP, which mainly consisted of ethyl and methyl esters of long-chain fatty acids and hydrocarbons (Table 1), exhibited low nematicidal activity, with only 50% mortality after 48 h of incubation at the

Table 5. Nematicidal Activity of Crude Extracts of Mixed Color Flowers of *Tagetes patula* against the Cyst Nematode *Heterodera zeae*

				mortality (%)[d]						mortality at 5% concn[f] (mean ± SEM, *N* = 9)	
				light			dark			light	dark
sample	sample code[a]	solvent[b]	concn %[c]	24 h	48 h	72 h	24 h	48 h	72 h	24 h	24 h
1	JFM	DMSO	10	100	–[e]	–	100	–	–		
			5	100	–	–	100	–	–	100 ± 0.00a[g]	100 ± 0.00a
			2	100	–	–	100	–	–		
			1	100	–	–	100	–	–		
			0.5	0	60	–	–	–	–		
			0.25	0	60	–	–	–	–		
			0.125	0	40	–	–	–	–		
2	JF-Ace	DMSO	10	100	–	–	100	–	–		
			5	100	–	–	100	–	–	100 ± 0.00a	100 ± 0.00a
			2	100	–	–	100	–	–		
			1	60	–	–	100	–	–		
			0.5	0	50	–	–	–	–		
			0.25	0	40	–	–	–	–		
			0.125	0	40	–	–	–	–		
3	JF-Eth	DMSO	10	70	100	–	50	100	–		
			5	50	100	100	50	70	100	50 ± 0.00b	50 ± 0.00b
			2	50	100	100	50	70	100		
			1	50	100	70	30	70	100		
			0.5	0	0	0	0	0	0		
			0.25	0	0	0	0	0	0		
			0.125	0	0	0	0	0	0		
4	carbofuran	DMSO	10	100	–	–	100	–	–		
			5	100	–	–	100	–	–	100 ± 0.00a	100 ± 0.00a
			2	100	–	–	100	–	–		
			1	100	–	–	100	–	–		
			0.5	100	–	–	100	–	–		
			0.25	80	100	–	80	100	–		
			0.125	40	100	–	60	100	–		
5	DMSO	H_2O	5	0	0	0	0	0	0	0 ± 0.00	0 ± 0.00

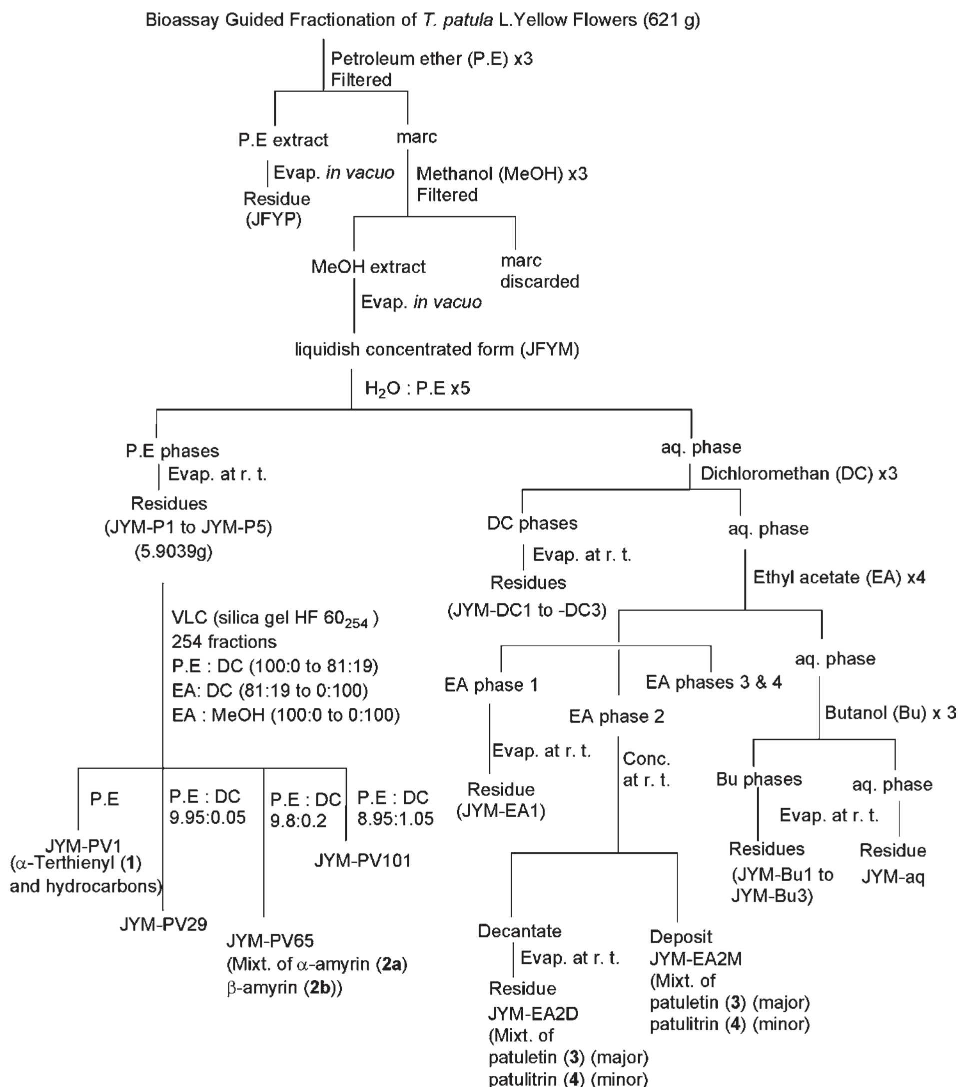
[a] JFM, methanol extract; JF-Ace, acetone extract; JF-Eth, ethanol extract. [b] DMSO, dimethyl sulfoxide. [c] Concentration of stock solution was 30 mg/mL. [d] Values represent the mean of three experiments. [e] No further observation. [f] Means of mortality rate in light and dark conditions for each treatment were compared. [g] Means within a column and row followed by the same letters are not significantly different (*P* = 0.01).

highest concentration of 10% under both light and dark conditions (Table 2).

On the other hand, the methanol extract (JFYM) showed 100% mortality after 24 h at 5% concentration. It was subjected to liquid–liquid extraction, furnishing different organic phases. The first nonpolar petroleum ether phase (JYM-P1) mainly consisted of methyl esters of fatty acids (Table 1) and exhibited 100% activity at 5% concentration in contrast to JFYP, whereas the subsequent dichloromethane phase (JYM-DC1) showed activity comparable with that of JFYP (Table 2), although they have quite different GC-MS profiles (Table 1). All of the petroleum ether phases (JYM-P1–JYM-P5) were combined together on the basis of TLC profile and subjected to VLC, affording 254 fractions. The first fraction, JYM-PV1, eluted with petroleum ether, exhibited activity as good as that of JYM-P1 (Table 2). Its active constituent identified by GC-MS was α-terthienyl (**1**, Figure 1; Table 1), a commercial sample of which was also evaluated; the details are given in the following text.

Moderately polar ethyl acetate phases, JYM-EA1, JYM-EA2D, JYM-EA2M, and JYM-Bu1, caused 100% larval mortality of *H. zeae* after 24 h at concentrations of 10, 1, 2, and 5%, respectively (Table 2). Among these phases, only JYM-EA2M (concentration = 10%) was able to impart 10% mortality within 1 h of incubation (result was not shown in Table 2). They all contained patuletin (**3**) and its glucoside, patulitrin (**4**), which have been previously isolated and purified from the flowers.[12,14] They individually showed nonsignificant effects on *H. zeae* within 48 h of incubation (Table 4); therefore, remarkable activities of these phases containing **3** and **4**

Scheme 1. Bioassay-Guided Fractionation of *T. patula* L. Yellow Flowers (621 g)



were probably due to their synergistic effect (combined effect of these flavonoids).

All of the samples except JYM-EA2D and JYM-EA2M showed significant differences in activities under light and dark conditions (Table 2).

Evaluation of Structure−Activity Relationship. The nematicidal activity of all of these compounds isolated or identified in the present studies and some of the commercially available analogues have been discussed with respect to the SAR under the following headings.

Thiophene. Thiophenes are abundantly present in marigold tissues, and roots of the plants had the highest diversity and contents of this class of compounds.[8,16−18] α-Terthienyl is one of the most studied thiophenes in *Tagetes*.[5,16,20,21] It has nematicidal, insecticidal, fungicidal, antiviral, and cytotoxic activities.[20] In this connection, a commercial sample of α-terthienyl (**1**) was tested that caused 100% mortality after 24 h even at the concentration of 0.125%, at which the activity of a conventional nematicide, carbofuran, was only 40% (Table 3). α-Terthienyl, being a component of JYM-PV1 (Table 1), appears to be responsible for the activity of this fraction (Scheme 1). It has been reported that **1** was most active against *Heterodera rostochiensis*, *Ditylenchus dipsaci*, and *Anguina tritici* in an in vitro nematicidal activity test.[16] However, this thiophene and its analogues have limited nematicidal activity when incorporated into the soil.[16,20] With regard to its mode of action, experiments have indicated that the overall activities of peroxidases in the roots increase after nematode invasion and may excite terthienyl, which is capable of producing singlet oxygen in or near the nematode's body. The generation of singlet oxygen is probably responsible in these plants for the death of nematodes.[16] However, Topp et al. disqualified this mechanism against soil microorganisms.[27] Recently, *T. minuta* and *T. lucida* were proved to be the most promising *Tagetes* species considering both thiophene concentrations and biomass yields for their use as biocidal crops in integrated pest management systems.[18]

Triterpenoid. A 1:1 mixture of triterpenes, α- (**2a**) and *β*-amyrins (**2b**) (JYM-PV65), was isolated in white crystalline form. The ^{1}H NMR (500 MHz, $CDCl_3$) spectrum of the sample indicated the characteristic signals of vinylic proton at δ 5.16 (t, J = 3.5 Hz, H-12) and methine proton at δ 3.19 (m, H-3) for α-amyrin and at δ 5.12 (t, J = 3.5 Hz, H-12) and δ 3.21 (m, H-3) for

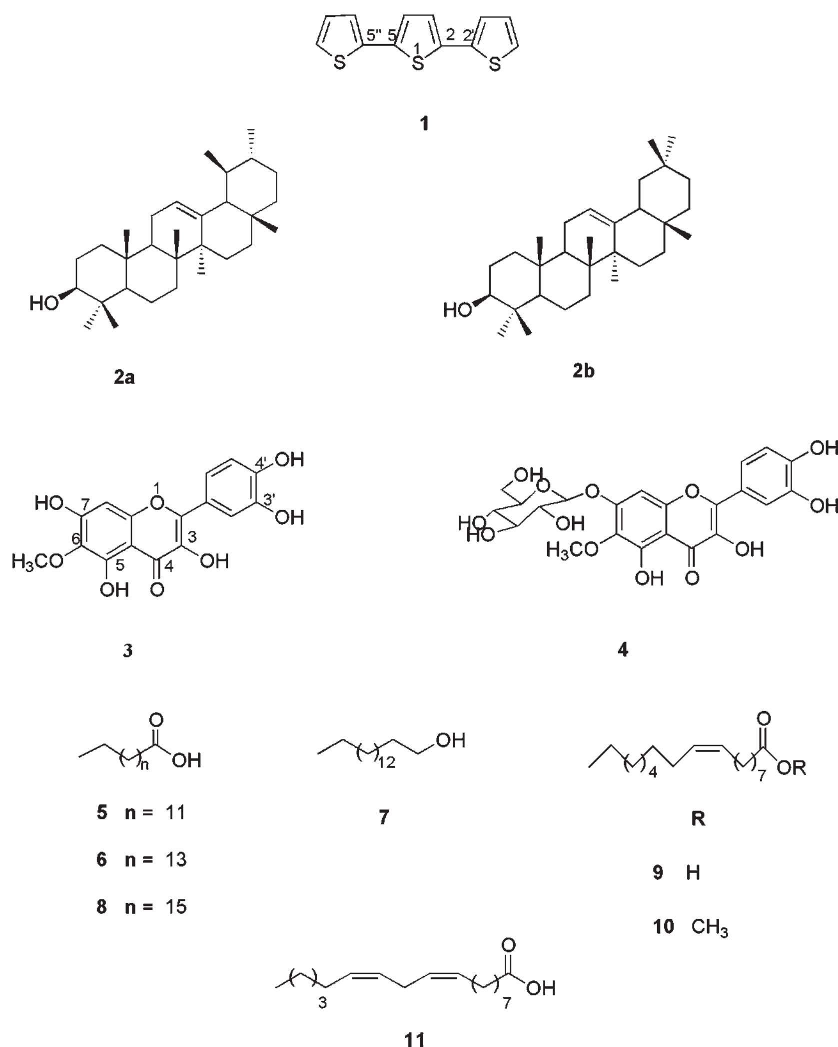


Figure 1. Structures of natural, synthetic, and commercial compounds (**1**−**11**) used in this study.

β-amyrin.[26] Signals of 16 methyls appeared in the range of *δ* 1.05−0.77. The EI-MS and HR-EIMS spectra showed the molecular ion at *m/z* 426, consistent with the formula $C_{30}H_{50}O$. The mixture (**2a** + **2b**) was found to be inactive against the nematode (Table 2). However, there are papers that mention the activity of other triterpenes against a number of nematodes.[5] This is the first report of the isolation of α- and *β*-amyrins from *T. patula* and their evaluation against a nematode.

Fatty Acids. Fatty acids and their derivatives are considered to be nematicidal phytochemicals.[5] They were identified in some of the VLC fractions such as JYM-PV29 (Table 1) and JYM-PV101 (mixture of fatty acids, C-16, -18, -20, and -24 by EI-MS). In this regard, a few commercial fatty acids were investigated on *H. zeae* larvae. Fatty acids with carbon chains of 14, 16, and 18 were evaluated, and the results were compared in terms of 100% mortality after 24 h of exposure. The results revealed that stearic acid (C_{18}) (**8**) and oleic acid ($C_{18:1}$) (**9**) are more active than palmitic acid (C_{16}) (**6**). In comparison to **8**, compound **9** with one double bond at C-9 exhibited a slight reduction in nematicidal activity (Table 3). On the other hand, myristic acid (C_{14}) (**5**) could not produce 100% mortality, whereas it was achieved by linoleic acid ($C_{18:2}$) (**11**) with four more carbons and two double bonds. Moreover, cetyl alcohol (C_{16}) (**7**), which is a fatty alcohol, was found to be more active than palmitic acid (**6**) (Table 3). The activity of fatty acids may depend on several factors such as chain length and number and position of double bonds. Dodecanoic and myristic acids, being the isolates of *T. erecta*, were reported to be active toward *Meloidogyne incognita*.[9] The methyl derivative of oleic acid (**10**) was also prepared and evaluated and showed the same activity as oleic acid (Table 3). Compounds **8**, **9**, and **10** exhibited significant phototoxic effect (Table 3).

Phenolic Compounds. (a) Flavonoids. Patuletin (**3**) and patulitrin (**4**) are the signature compounds of *T. patula* and belong to the flavonoid class, which has diverse pharmacological effects.[12−14] Fractions JYM-EA 2D and JYM-EA2M, which have higher concentrations of **3** than **4**, were found to be more active than JYM-EA1 and JYM-Bu1, which have lesser amounts of **3** (Table 2). The pure patuletin at various dilutions, that is, 1, 0.5, 0.25, and 0.125%, exhibited the highest nematicidal activity, that is, 100% in 72 h, whereas patulitrin induced mortality in the range of 10−50% only. It has been reported that **3** is usually more potent than **4** in other biological assays such as antimicrobial[12] and antioxidant.[14] Quercetin (**12**, Figure 2), which has the same skeleton as patuletin except that it lacks a methoxy group at the C-6 position, could not reach 100% activity. It caused a maximum

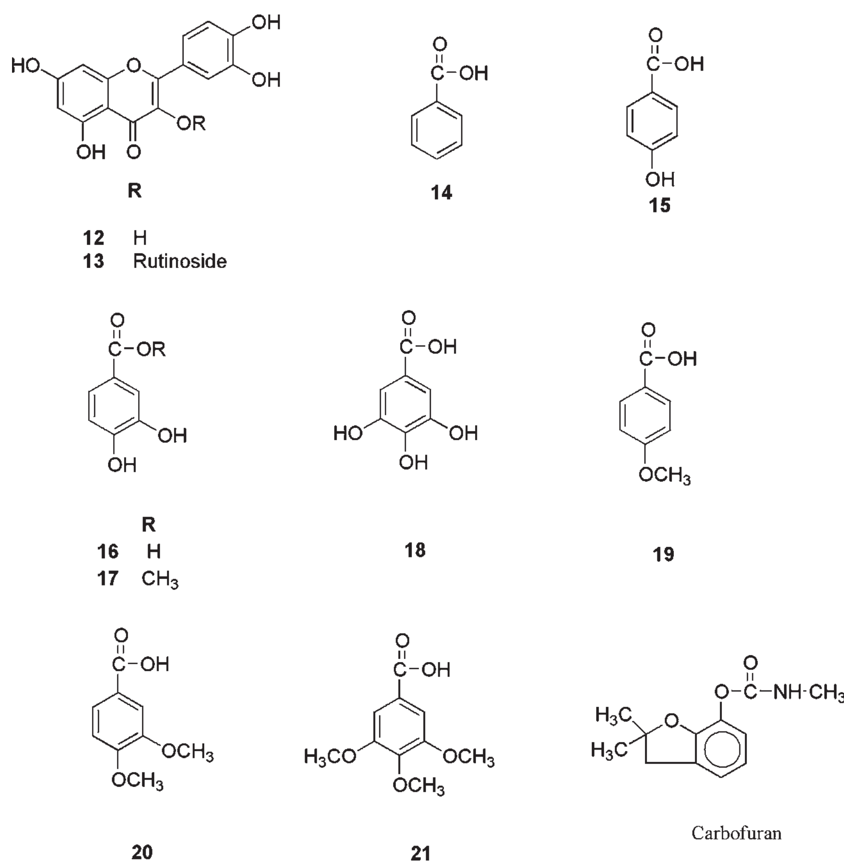


Figure 2. Structures of natural, synthetic, and commercial compounds (**12**–**21** and carbofuran) used in this study.

activity of 80% in 72 h (at a concentration of 0.125%). Rutin (3-rutinoside of quercetin) (**13**), on the other hand, was able to show 100% activity (up to 0.5% concentration) after 24 h of exposure (Table 4). The effective use of the flavonoid-rich extract of *T. patula* against *Meloidogyne* spp. has previously been reported.[15] In plant–nematode interaction, inducible flavones have also been mentioned as defense compounds against *Heterodera avenae* and *Pratylenchus neglectus*.[28]

It is known that phenolic compounds with a catechol moiety readily oxidize to orthoquinones, which have an inhibitory effect on nematodes.[29] This phenomenon seemed to be one of the reasons in the case of the above tested active flavonoids, having a catechol moiety in ring B. This moiety is also one of the important structural features necessary for the antioxidant activity of flavonoids.[14] However, the nematicidal activity of these compounds certainly depends on various other factors that cannot be discussed here with only a few tested compounds. Any firm conclusion needs a number of phenolic compounds to be evaluated and compared.

(b) Phenolic Acid. It has previously been reported that the total level of free phenolcarboxylic acids may serve as one of the integral criteria of evaluation of the allelopathic potential of *Tagetes* species.[30] Therefore, a simple aromatic acid such as benzoic acid (**14**) and its hydroxyl analogues, that is, 4-hydroxybenzoic acid (**15**), 3,4-dihydroxybenzoic acid (**16**) and its methyl ester (**17**), and 3,4,5-trihydroxybenzoic acid (**18**) along with their methoxyl analogues 4-methoxybenzoic acid (**19**), 3,4-dimethoxybenzoic acid (**20**), and 3,4,5-trimethoxybenzoic acid (**21**), were chosen for the evaluation of their activity. The results showed that benzoic acid containing no −OH group was unable to show appreciable toxicity in either light or dark over the period of 72 h. The activity increased with increasing −OH groups in the benzene ring (Table 4). Therefore, gallic acid (**18**) with three −OH exhibited 100% mortality after 24 h at a concentration as low as 0.125%. The activities of methoxyl (−OMe) analogues **20** and **21** were not appreciably different from those of their hydroxyl counterparts (**16**, **18**) at the concentrations used. Whereas compound **19** (mono −OMe) was slightly more active than **15** (mono −OH), it did not reach 100% activity at the given time exposures (Table 4). It is important to note that methyl-3,4-dihydroxybenzoate (**17**), which is a genuine chemical constituent of *T. patula* and possesses strong antioxidant activity,[14] has almost the same nematicidal activity as its corresponding acid (Table 4), indicating that the presence of the methyl group as methyl ester did not affect the sensitivity of the compound. The results showed that the hydroxyl as well as methoxyl groups in the molecules are essential for killing the nematode. Compounds **17**, **20**, and **21** were significantly active under light conditions as compared to dark conditions (Table 4).

The plant extracts/fractions and pure compounds were studied under light and dark conditions, keeping two points in focus. First, *H. zeae* is a semiendoparasite and feeds on specialized cells within plant roots.[2,3] Second, the chemistry of plant extracts is extremely complex, constituting a vast number of chemicals belonging to several classes of compounds, some of which are unstable when separated or sensitive to the environment such as light and heat.[10] To determine the influence of biocompounds, conditional reservoirs were adapted for the pest. It was also

correlated with the efficiency of compounds in light and dark conditions. From the data mentioned in the Tables 3 and 4, it could be suggested that the activities of some compounds are influenced by their surroundings.

Crude Extracts of Mixed Color Flowers. In subsistence agriculture, the toxicity of crude plant extracts is more interesting because farmers can use all of the potential active compounds from the whole plant rather than single toxic elements from the purified material. Furthermore, crude extracts are cheaper than commercial pesticides, as they do not require a lot of investment. They can also reduce the potential of resistance development, as mixtures of bioactive compounds act synergistically. It has been reported that all of the aerial parts (flower, leaf, and stem) of *T. lucida*, *T. minuta*, and *T. tenuifolia*, when incorporated into the soil, reduced root galls caused by *M. incognita*.[21] In the case of *T. lucida*, among the activities of different parts, flower extracts were more deleterious to the reniform, lance, and spiral nematodes.[21] On the basis of these facts and the results mentioned in Table 2, *T. patula* flowers of different colors were mixed together and extracted with different polar organic solvents (i.e., acetone, ethanol, and methanol) and evaluated in vitro at concentrations of 10, 5, 2, and 1% against *H. zeae* larvae under light and dark conditions. After 24 h, the methanol extract (JFM) showed 100% lethal activity at 5% concentration, whereas the acetone extract (JF-Ace) produced the same activity at concentration up to 2% which reduced to 60% (at 1%) in light and increased to 100% in the dark (Table 5). Both extracts revealed their 50% activities up to the concentration of 0.125% (48 h). The ethanol extract (JF-Eth), on the other hand, exhibited 50−70% lethal activity after 24 h, which reached a maximum in 48 h at the concentration range of 10 to 1%; however, it showed no activity below this concentration. They all showed nonsignificant phototoxic effects (Table 5). It could be suggested that the acetone and methanol extracts may serve as nematicidal potentials in the pot or field experiments. Least significant results were found for the mortality rate of *H. zeae* second-stage larvae at $P = 0.01$ after 24 h of treatment between JFM and JF-Ace, whereas JF-Eth showed a significant difference of mortality rate in comparison with JFM and JF-Ace at $P = 0.01$ (Table 5).

In conclusion, a polar extract (JFYM) and phases (JYM-EA1 and JYM-Bu1) of yellow flowers of *T. patula* L. have comparable nematicidal activities, whereas the nonpolar phase (JYM-P1) and fraction (JYM-PV1) showed almost similar activities against the cyst nematode *H. zeae* infective stage larvae. This in vitro bioassay-guided isolation led to the identification of several active principles that belong to different classes of compounds. In polar extract and phases, active constituents were phenolic compounds (phenolic acids and flavonoids), whereas nonpolar ones consisted of fatty acids, their methyl esters, and thiophene. Structure−activity relationship studies in relation to their nematicidal activity led to the conclusion that an increase in the number of hydroxyl groups in phenolic acids was accountable for a positive change in the activity, and their replacement with methoxyl did not impart any drastic changes. The activity of fatty acids depended on several factors such as chain length and the number and position of double bonds. The esterification of the aromatic acids and fatty acids did not affect the activity. Light and dark experiments were also carried out, and significant differences in activities were observed in some of the treatments. This study would be extended to the pot followed by field experiments to formulate *Tagetes*-based pesticides.

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DISCLOSURE

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DEDICATION

This paper is dedicated to the fond memory of Prof. Salimuzzman Siddiqui *FRS* (1897−1994), the founding director of the HEJ Research Institute of Chemistry, University of Karachi.

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