

New nematicidal compounds from *Mentha spicata* L. against *Meloidogyne incognita*

Partha Chandra Mondal

Vijay Kumar

Parshant Kaushik

Najam Akthar Shakil

Pankaj ,

Virendra Singh Rana

ranavs2000@yahoo.com

<https://orcid.org/0000-0002-0414-113X>

Research Article

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Abstract

Meloidogyne incognita (root-knot nematode) is one of the most destructive pests of crops worldwide. Injudicious use of synthetic nematicides has led to environmental toxicity, development of resistance and disruption of ecological balance, necessitating the need for safer and effective alternatives. Essential oil from *Mentha spicata* has been reported to have nematicidal activity but the activity of its polar constituents is unexplored against the nematodes, infesting agricultural crops. Six compounds, Stigmasterol (**1**), β -Sitosterol-3-O- β -D-glucoside (**2**), 3 β ,13 β -Dihydroxy-urs-11-en-28-oic acid (**3**), 5,6-Dihydroxy-7,8,4'-trimethoxyflavone (**4**), 5,6,4'-Trihydroxy-7,8,3'-trimethoxyflavone (**5**), and 5,6,4'-Trihydroxy-7,8-dimethoxyflavone (**6**) were isolated from methanolic extract of *Mentha spicata* (spearmint) and identified by NMR (1D & 2D) spectral analysis. Compound **3** was identified for the first time from genus *Mentha*. *In vitro* nematicidal activity of pure compounds (**2**, **4–6**) was carried out against *Meloidogyne incognita* and results showed significant mortality (LC₅₀ values ranging between 62.64 and 74.19 ppm after 96 hours). Molecular docking study revealed the favorable binding of the test compounds with acetylcholinesterase enzyme, facilitated by H-bond, electrostatic interactions, and hydrophobic interactions. The binding energy of the tested compounds with the target protein was found highest in **5** (-10.0 kcal/mol), **6** (-9.9 kcal/mol) followed by **2** (-9.2 kcal/mol) and **4** (-8.9) kcal/mol. The minimum binding energy was recorded in carbofuran (-7.5 kcal/mol), a positive control and was also corroborated with *in vitro* nematicidal activity. This study showed the potent nematicidal activity of compounds (**2,4–6**), which can be further used for the development of nematicides against *M. incognita* in agriculture.

Introduction

Mentha (Family: Lamiaceae), a genus of shrubs and trees, is distributed mainly in the tropical regions of the world. Several species of *Mentha* have been introduced into various countries and cultivated for their aromatic leaves and seeds. About six species of *Mentha* are recorded in India and are mainly cultivated for essential oil and medicinal purposes (Anon 2009). *Mentha spicata* L., commonly known as spearmint, is a glabrous aromatic perennial shrub, up to 30–90 cm in height and is widely cultivated throughout the plains of India for spearmint oil, flavour, culinary and medicinal purposes (Krishnamurthi et al. 1969; Chopra et al. 2006). It is also considered as stimulant, carminative, and antispasmodic. Its leaves are used for fevers, bronchitis, hysteria, vomiting and as an antidote for poison. Its oil is rich in carvone and used in flavoring of chewing gum, toothpaste, confectionary, and pharmaceutical products (Krishnamurthi et al. 1969; Chopra et al. 2006). It is also used in nausea, liver complaints, and colitis and found to be stimulant, antiemetic, diaphoretic, anti-inflammatory, analgesic, emmenagogue, and anti-catarrhal (İşcan et al. 2002; Moreno et al. 2002; Agarwal 2008). The essential oil has been reported to possess excellent insecticidal activity against store grain insects, mosquitoes, and houseflies and have acaricidal, nematicidal, fungicidal, and antibacterial activities (Kedia et al. 2014; Lee et al. 2002; Soltani and Aliabadi 2013; Choi et al. 2004). Earlier chemical analysis showed the presence of about 0.25 to 0.5% volatile oil. Its volatile oil has been analyzed and was found to be rich in monoterpenoids and contain carvone as the most abundant compound (Krishnamurthi et al. 1969). Earlier work also showed

the presence of flavonoids such as quercetin, kaempferol, luteolin, apigenin, naringin, and myricetin from its leaves and aerial parts (Krishnamurthi et al. 1969; Mahendran and Rahman 2020).

The plant parasitic nematodes are one of the most important pests of agricultural and horticultural crops worldwide, resulting in huge crop losses under field conditions (Elling 2013; Kumar et al. 2020). Of these, *Meloidogyne incognita* (root-knot nematodes) is one of the most economically important species infecting various field and horticultural crops accounting for the loss of over 100 billion USD worldwide (Singh et al. 2019). Currently, the management of nematodes under field conditions is being carried out by use of synthetic nematicides, which are reported to be toxic to non-targeted organisms, persistent and hazardous to the environment and human health (Feng et al. 2023) due to lack of effective and environmentally benign products of biological origin. Many of these nematicides are either banned, restricted, or listed to be banned in many countries in the future (Lilley et al. 2011; Desaegeer et al. 2020). Since the last few decades, researchers have been working to identify and develop effective and environment friendly nematode control agents from plants and microbes as an alternative to synthetic nematicide, which can be used for the management of nematodes. The secondary metabolites present in different extracts and oils of the plants are effective alternatives against the nematodes (Jardim et al. 2020; Kumar et al. 2023). Earlier work showed the promising activity of volatile oils against root-knot nematodes (Andrés et al. 2012; Ntalli and Caboni 2012) but limited studies were reported on nematicidal potential of non-volatile compounds from the plants. Two prenylated flavanones, 8-(3-Methyl-but-2-enyl)-2-phenyl chroman-4-one and 2-(4-hydroxyphenyl)-8-(3-methyl-but-2-enyl)-chroman-4-one isolated from the hexane extract of *Phyllanthus niruri* showed potent nematicidal activity against *Meloidogyne incognita* (LC_{50} = 79.9 and 14.5 ppm), and *Rotylenchulus reniformis* (LC_{50} = 102.9 and 3.3 ppm) under *in-vitro* conditions (Shakil et al. 2008). Two flavone-C-glycosides, schaftoside and isoschaftoside were found to have nematicidal activity against root-knot nematode (Du et al. 2011). Another study also showed up to 90% mortality of nematodes by triterpenoids isolated from *Lantana camara* after 72 hours (Begum et al. 2015). Recent studies showed that essential oils and some of their major compounds have potent nematode control activity (de Freitas Silva et al. 2020; Ajith et al. 2020). Nematicidal activity of flavonoids and their structure activity relationship were also reported (Bano et al. 2020).

However, fewer studies have been reported on the nematicidal activity of non-volatile or polar compounds and need to be explored for their utilization in the management of nematodes. Therefore, the present study aimed to isolate and identify bioactive compounds from the leaves of *M. spicata*, evaluate the major compounds against *M. incognita* under *in-vitro* conditions, and determine the possible mode of action of each compound using molecular docking studies.

Materials and methods

General

All the chemicals and solvents used for extraction of plant materials and column chromatography were of analytical grade, purchased from Rankem and Merck. Silica gel (100–200 mesh) of column

chromatography grade were purchased from CDH-India while precoated aluminium TLC plate (Silica-gel 60 F₂₅₄ (Merck)) were used for thin layer chromatography. The evaporation of the organic solvents from different fractions was done in BUCHI Labortechnik AG 9230 rotary evaporator. The TLC plates were visualized in SunStar UV-vis chamber and by spraying 5% sulphuric acid followed by heating at 105°C till appearance of spots. NMR data were recorded in suitable deuterated solvents (CDCl₃, CD₃OD, C₅D₅N, and C₂D₆OS) using JEOL 400 MHz NMR spectrometer (400 MHz for ¹H-NMR and 100 MHz for ¹³C-NMR) equipped with Delta 5.3.1 software.

Plant material

The leaves of *M. spicata* (4.0 kg) were obtained from M/s Creation Biotech, Bareilly, Uttar Pradesh, India. The leaves were cleaned, washed with tap water, and shed dried. The air-dried leaves (1.0 Kg) powdered and used for extraction with different solvents.

Preparation of plant extract

The dried and powdered leaves (1.0 kg) of *M. spicata* was first defatted with petroleum ether and then subsequently extracted with methanol using the Soxhlet apparatus. The solvents were removed using vacuum in a rotary evaporator. The yield of the petroleum ether and methanolic extracts was found to be 5.26 and 12.97% respectively. The extracts were stored at 4°C till further analysis.

Isolation pure compounds from methanolic extract

The pure compounds from the crude methanolic extract were isolated by column chromatography over silica gel. The methanolic extract (25.0 g) of *M. spicata* was dissolved in minimum quantity of methanol and adsorbed on silica-gel (400 g, 100–200 mesh) and dried and poured on the top of silica-gel packed glass column using chloroform. The elution of the column was started with chloroform and the polarity of the eluting solvent was gradually increased with addition of methanol. Different fractions were collected and pooled together based on their similar TLC pattern. The fractions were further chromatographed over silica-gel and six compounds **1** (85 mg), **2** (210 mg), **3** (250 mg), **4** (75 mg), **5** (4.2 g), and **6** (2.3 g) were crystalised. The purity of the isolated compounds was determined by TLC and their structure were elucidated using NMR (1D & 2D) spectral data and were also compared with those reported earlier.

In-vitro nematocidal activity of the extract and isolated compounds

Collection and isolation of *M. incognita*

The infected roots of *Solanum lycopersicum*, var. Pusa Ruby (tomato) were collected from the sick pot maintained under Division of Nematology, ICAR-IARI, New Delhi, India and the subsequently washed in tap water to remove the dirt. The egg masses were then separated with a pair of sterile forceps, washed with distilled water, and kept over mesh sieve wrapped in double layer of tissue paper in Petri dishes filled with water and incubated at 25 ± 2°C for hatching second stage juveniles (J2s).

Preparation of test solutions

The stock solutions (1.0%) of methanolic extract and four pure compounds (**2,4–6**) were prepared by mixing them with 0.5% Triton X-100 and distilled water for 30 minutes at 1000 rpm using a lab stirrer. Triton X-100 (0.5%) in water was used as a negative control and carbofuran (31.25 to 500 ppm) was used as positive control. Different test concentrations of the methanolic extract (62.5 to 1000 ppm) and compounds (31.25 to 500 ppm) were prepared by serial dilution of the stock solution for *in-vitro* study.

In-vitro activity

The nematode suspension (120 J2s/0.1 ml) was added to the different concentrations of the test solutions (1.0 ml) in a 24-well plate in triplicates. The mortality of J2s of *M. incognita* was recorded after 24, 48, 72, and 96 hours of the treatment.

Molecular docking studies

Molecular docking analysis provides an insight into the possible mechanism of interactions of the compound or ligand binding with the target protein. In the present study, acetylcholine esterase was chosen as target protein as it plays an important role in hydrolysing and inactivation of acetylcholine, a major neurotransmitter controlling motor activities in nematodes (Selkirk et al., 2005). For this study, a target sequence of the protein was obtained from the protein database of the NCBI website (<https://www.ncbi.nlm.nih.gov/>). The in-built blastP server of NCBI website was used to find suitable templates for the target protein keeping the protein data bank (<https://www.rcsb.org>) as the source. Based on the degree of similarity one or more proteins were chosen for further modelling purposes using the Modeller 10.4 software. The generated models were ranked based on the DOPE score and the best fitted model was chosen to perform the molecular docking studies. Selected proteins were prepared using the AutoDock Vina software where the additional water molecules were deleted followed by the addition of polar hydrogen and necessary charges. The 3D .sdf files of the isolated compounds were downloaded from the PubChem website and then converted to .pdb format using PyMol software and the further ligand preparation was done with the help of AutoDock Vina. After the preparation step was over, the ligand molecules were allowed to dock onto the target proteins and their affinity towards each other was ranked based on the binding energy. The protein-ligand interactions were visualized using the BIOVIA Discovery Studio software to spot the interacting amino acid residues as well as the type of interaction between the residues and the ligands.

Statistical analysis

The data of nematicidal activity methanolic extract and compounds obtained were analysed and their LC₅₀ values were calculated using probit analysis using the IBM SPSS Statistics 20 software ($\alpha = 0.05$). The dose response curves were prepared using Polo Plus LeOra software version 2.0.

Results

Extraction and isolation of compounds

The yield of the methanolic extract in the powdered leaves of *M. spicata* was found to be 12.97% (on dry weight basis). The methanolic extract was column chromatographed over silica-gel using chloroform and chloroform-methanol (100:0 to 80:20) mixture and six pure compounds **1** (85 mg, m.p. 131–133°C), **2** (210 mg, m.p. 270–272°C), **3** (250 mg, m.p. 236–238°C) **4** (75 mg, m.p. 141–143°C), **5** (4.2 g, m.p. 224–227°C) and **6** (2.3g, m.p. 240–242°C) were isolated and were identified as Stigmasterol (**1**) (Khatun et al. 2012), β -Sitosterol-3-O- β D-glucoside (**2**) (Gohar et al. 2000), 3 β ,13 β -Dihydroxy-urs-11-en-28-oic acid (**3**) (Hao et al. 1996), 5,6-Dihydroxy-7,8,4'-trimethoxyflavone (**4**) (Sato and Tamura 2015), 5,6,4'-Trihydroxy-7,8,3'-trimethoxyflavone (**5**) (Sato and Tamura 2015), and 5,6,4'-Trihydroxy-7,8-dimethoxyflavone (**6**) (Sato and Tamura 2015). The compound **3** was identified as rare and an unusual $\Delta^{11(12)}$ -dihydroxy ursane type triterpenoid, in which a double bond was present between C-11 and C-12 instead of between C-12 and C-13, while a second hydroxyl group at C-13 was present in addition to the one present at C-3 atom. Compound **3** was identified for the first time from the genus *Mentha*. The compound **4** and **6** have been previously isolated from *Mentha piperita* (Sato and Tamura 2015) and reported first time from *Mentha spicata*. The isolated (**1–6**) compounds were identified using 1D (^1H , ^{13}C and DEPT) and 2D (HSQC and HMBC) NMR spectra (Table 1a and 1b, Fig. 1) and by comparing their spectral data reported earlier. The compound **1–3** belonged to triterpenoid group while the compounds **4–6** were flavonoid in nature.

Table 1

a ^1H -NMR and ^{13}C -NMR spectral data of pure compounds 1, 2 and 3 from the leaves of *Mentha spicata*

C/H	Compound 1		Compound 2		Compound 3	
	$\delta^{13}\text{C}$ [ppm]	$\delta^1\text{H}$ [ppm]	$\delta^{13}\text{C}$ [ppm]	$\delta^1\text{H}$ [ppm]	$\delta^{13}\text{C}$ [ppm]	$\delta^1\text{H}$ [ppm]
1	37.33	1.25 (m)	34.74	0.96 (m) 1.30(m)	38.56	0.98 (m) 1.78 (dd, J = 7.6 Hz, 4.4 Hz)
2	29.80	1.67(m)	30.80	1.36 (m) 1.68 (t, J = 10.5Hz)	23.14	1.34 (m), 2.1 (m)
3	71.89	3.53 (m)	78.60	3.95 (m)	77.91	3.42 (dd, J = 8.8 Hz)
4	42.36	2.26(m)	39.88	2.43 (t, J = 10.9Hz) 2.70 (d, J = 10.9Hz)	39.49	-
5	140.82	-	141.44		55.04	0.83 (m)
6	121.81	5.35 (d, J = 5.4 Hz)	122.49	5.30 (br d, J = 5.4Hz)	17.74	0.81 (m), 1.56 (m)
7	31.72	1.84 (m)	32.72	1.36 (m) 1.50(m)	30.94	0.98 (m), 1.36 (m)
8	31.98	1.48 (m)	32.59	1.47(m)	41.98	-
9	51.32	1.48 (m)	50.88	0.85(m)	53.35	2.01 (m)
10	36.58	-	37.47	-	36.58	-
11	21.16	1.48 (m)	21.82	0.85(m)	133.71	5.64 (dd, J = 13.6 Hz, 4.8 Hz)
12	39.85	2.00 (m)	40.48	2.70 (d, J = 10.9Hz) 1.93 (m)	129.33	6.00 (d, J = 13.6 Hz)
13	42.40	-	43.02		89.38	-
14	56.84	1.10 (m)	57.36	1.08 (m)	42.19	-
s = singlet, d = doublet, t = triplet, m = multiplet dd = double doublet, br = broad, J = coupling constant, Hz = Hertz						

C/H	Compound 1		Compound 2		Compound 3	
	$\delta^{13}\text{C}$ [ppm]	$\delta^1\text{H}$ [ppm]	$\delta^{13}\text{C}$ [ppm]	$\delta^1\text{H}$ [ppm]	$\delta^{13}\text{C}$ [ppm]	$\delta^1\text{H}$ [ppm]
15	24.39	1.56 (m)	25.06	1.23 (m)	27.87	1.21 (m), 1.84 (m)
16	28.34	1.84 (m)	26.89	1.36 (m)	25.83	1.16 (m), 1.78 (m)
17	56.12	1.10 (m)	56.77	1.08 (m)	45.17	-
18	11.95	0.68 (s)	12.52	0.61(s)	60.45	1.64 (m)
19	19.49	1.01 (s)	19.74	0.82 (s)	38.13	1.78 (m)
20	40.60	2.00 (m)	36.95	1.30 (m)	40.30	0.83 (m)
21	19.91	0.92 (d, J = 6.6Hz)	19.56	0.99 (d, J = 7.0 Hz)	31.48	1.18 (m), 1.36 (m)
22	138.41	5.15 (dd, J = 15.2, 8.6)	38.02	1.36 (m)	31.92	1.57 (m), 1.84 (m)
23	129.34	5.01 (dd, J = 15.3, 8.8)	29.10	1.61 (m)	28.36	0.84 (s)
24	50.20	0.97 (m)	46.57	0.94 (d, J = 6.5 Hz)	15.67	1.01 (s)
25	29.21	1.84 (m)	29.98	2.08 (d, J = 12.6 Hz)	15.97	1.18 (s)
26	23.14	0.85 (d, J = 7.6 Hz, 3H)	19.97	0.94 (d, J = 6.5 Hz)	18.97	1.19 (s)
27	19.11	0.82 (d, J = 2.1 Hz, 4H)	20.54	0.94 (d, J = 6.5 Hz)	18.01	1.21 (s)
28	26.12	1.48 (m)	23.92	0.88(m)	179.39	
29	12.07	0.80 (t, J = 7.0Hz)	12.70	1.04 (t, J = 7.5Hz)	17.93	0.96 (d, J = 5.6 Hz)
30					19.12	0.83 (d, J = 5.6 Hz)
1'			103.13	5.03 (d, J = 7.7 Hz)		
2'			75.91	4.03 (t, J = 8.5 Hz)		
s = singlet, d = doublet, t = triplet, m = multiplet dd = double doublet, br = broad, J = coupling constant, Hz = Hertz						

C/H	Compound 1		Compound 2		Compound 3	
	$\delta^{13}\text{C}$ [ppm]	$\delta^1\text{H}$ [ppm]	$\delta^{13}\text{C}$ [ppm]	$\delta^1\text{H}$ [ppm]	$\delta^{13}\text{C}$ [ppm]	$\delta^1\text{H}$ [ppm]
3`			79.09	4.27(m)		
4`			72.22	4.27(m)		
5`			79.19	3.95 (m)		
6`			63.37	4.54 (dd, $J=$ 11.8, 2.5 Hz) 4.39 (dd, $J=$ 11.8, 5.2 Hz)		
s = singlet, d = doublet, t = triplet, m = multiplet dd = double doublet, br = broad, J = coupling constant, Hz = Hertz						

Table 1

b ^1H -NMR and ^{13}C -NMR spectral data of pure compounds 4, 5, and 6 from the leaves of *Mentha spicata*

C/H	Compound 4		Compound 5		Compound 6	
	$\delta^{13}\text{C}$ [ppm]	$\delta^1\text{H}$ [ppm]	$\delta^{13}\text{C}$ [ppm]	$\delta^1\text{H}$ [ppm]	$\delta^{13}\text{C}$ [ppm]	$\delta^1\text{H}$ [ppm]
2	164.21		163.79		163.98	
3	103.53	6.6 (s)	102.69	6.82 (s)	102.40	6.7 (s)
4	182.86		182.59		182.58	
5	142.18		143.08		141.80	
6	133.19		134.14		134.21	
7	146.62		148.02		147.96	
8	132.98		132.86		132.95	
9	142.96		141.79		143.12	
10	106.62		106.07		106.09	
1'	123.59		121.63		121.34	
2'	128.03	7.88 (d, $J = 8.8$ Hz)	109.96	7.44 (d, $J = 2$ Hz)	128.42	7.80 (d, $J = 8.8$ Hz)
3'	114.56	7.29 (d, $J = 8.8$ Hz)	147.94		116.11	6.82 (d, $J = 8.8$ Hz)
4'	162.69		150.83		161.33	
5'	114.56	7.29 (d, $J = 8.8$ Hz)	115.89	6.85 (d, $J = 8.8$ Hz)	113.11	6.82 (d, $J = 8.8$ Hz)
6'	128.03	7.88 (d, $J = 8.8$ Hz)	120.22	7.45 (dd, $J = 8.8$ Hz, 2 Hz)	128.42	7.80 (d, $J = 8.8$ Hz)
7-OCH ₃	61.44	4.14 (s)	61.78	3.83 (s)	60.95	3.82 (s)
8-OCH ₃	62.16	3.99 (s)	60.93	3.80 (s)	61.88	3.78 (s)
3'-OCH ₃			55.78	3.77 (s)		
4'-OCH ₃	56.12	3.89 (s)				
s = singlet, d = doublet, dd = double doublet, J = coupling constant, Hz = Hertz						

In-vitro nematocidal activity of extracts and isolated compounds

The methanolic extract of the leaves of *M. spicata* and the compound **2,4–6** were evaluated for their nematocidal activity against second stage juveniles (J2s) of *M. incognita* under *in-vitro* conditions and results showed the concentration and time of exposure dependent mortality of J2s of *M. incognita* (Figure S1). In methanolic extract, the mortality of J2s varied between 12.78 and 66.11% at 62.5–1000 ppm concentration after 24–96 hours of treatment while pure compounds **2, 4–6** exhibited mortality of J2s, which ranged from 82.22–85.28% at 1000 ppm after 96 hours of treatment. The LC₅₀ values of the methanolic extract and compounds **2,4–6** were calculated and are given in Table 2. Compound **5** (LC₅₀ = 62.64ppm) and **6** (LC₅₀ = 65.00ppm) showed highest mortality of J2s of *M. incognita* followed by **2** (LC₅₀ = 69.42ppm) and **4** (LC₅₀ = 74.19 ppm) while the methanolic extract (MSM) showed minimum activity (LC₅₀ = 327.55 ppm) after 96 hours of treatment compared to carbofuran (LC₅₀ = 85.27 ppm). All the tested compound (**2, 4–6**) showed better nematocidal activity than carbofuran and thus showed their potential in nematode management.

Table 2
Nematicidal activity of different treatments after variable time of exposure

Treatment	Exposure duration (hours)	LC ₅₀ (ppm)	Upper bound	Lower bound	Chi-square
MSM	24	482.31	697.73	366.23	5.56
	48	428.94	609.49	326.71	5.02
	72	366.93	497.24	284.47	4.79
	96	327.55	434.61	255.16	4.21
Compound 2	24	183.03	144.19	242.55	0.282
	48	125.09	93.96	166.51	0.942
	72	89.70	66.74	115.52	0.792
	96	69.42	50.09	89.53	1.68
Compound 4	24	194.09	151.55	262.24	0.96
	48	133.47	172.33	104.10	1.27
	72	95.10	119.86	73.31	0.315
	96	74.19	94.45	55.03	0.753
Compound 5	24	175.09	230.59	138.08	0.119
	48	113.98	150.01	85.38	0.399
	72	79.22	103.11	57.09	0.424
	96	62.64	80.86	44.76	1.85
Compound 6	24	175.08	230.96	137.82	2.06
	48	118.71	88.78	157.39	0.742
	72	84.68	61.90	109.83	0.945
	96	65.20	46.81	84.08	1.49
Carbofuran	24	202.16	271.58	158.81	1.738
	48	153.69	203.28	119.26	1.07
	72	107.60	138.07	82.31	0.094
	96	85.27	107.98	64.57	1.49
MSM = Methanolic extract of <i>Mentha spicata</i>					

Molecular docking studies

Molecular docking studies revealed the favourable binding of the all the tested compounds (**2,4–6**) with the target protein. The protein 6QAA was chosen as a template for the modelling from PDB website based on the similarity percentage for determination of anti-acetylcholine esterase activity. The isolated compounds (**2, 4–6**) were found strongly bonded to the target site, even stronger than carbofuran used as positive control, as evident from their difference in binding energy (Table S2). For all the tested compounds, H bond (conventional and C-H type) played major role in successful binding of the ligand, while GLN 332, SER 124, THR 127, PRO345, GLU 251, SER 174, and HIS 514 amino acid residues were found to form H-bond with the ligand molecules under study. This binding was further stabilized with hydrophobic interactions such as π - σ and π -alkyl interactions. For the flavonoid compounds (**4–6**) additional hydrophobic interactions such as π - π T shaped, π - π stacked and π -amide interactions were observed. However, the strongest binding of the compound **5** and **6** can be attributed towards the additional π -anion electrostatic forces working between the ligand and ASP 126 amino acid residue of the target protein (Fig. 2). Distance of H-bond was also found crucial for stronger binding. The compound **2** with shorter bond distance (~ 2 Å) had stronger binding compared to the compound **4** with relatively longer bond distance (~ 3.5 Å) (Table S2). The binding energy of the tested ligands with the target protein exhibited the trend as **5** (-10.0 kcal/mol), $\sim$ **6** (-9.9 kcal/mol) > **2** (-9.2 kcal/mol), > **4** (-8.9 kcal/mol) > carbofuran (-7.5 kcal/mol), which was also corroborated *in-vitro* nematocidal activity as well.

Discussion

The leaves of *Mentha spicata* is a main source spearmint oil, used in flavoring and therapeutic purposes in India. The phytochemical investigation of the methanolic extract of the leaves of *M. spicata* led to the isolation and identification six pure compounds, which belonged to the terpenoid and flavonoid groups. The identified compounds were Stigmasterol (**1**), β -Sitosterol-3-O- β D-glucoside (**2**), 3 β ,13 β -Dihydroxyurs-11-en-28-oic acid (**3**), 5,6-Dihydroxy-7,8,4'-trimethoxyflavone (**4**), 5,6,4'-Trihydroxy-7,8,3'-trimethoxyflavone (**5**), and 5,6,4'-Trihydroxy-7,8-dimethoxyflavone (**6**). Compound **3** was identified for the first time from genus *Mentha* while other compounds are known to be present in its different species (Sato and Tamura 2015; Ezzat et al. 2014).

Nematicidal of the methanolic extract of leaf and its isolated pure compounds revealed their variable but potent nematicidal activity against *M. incognita* under in-vitro conditions. Comparing the nematicidal activity of the compounds purified from *M. spicata*, two polar compounds, 5,6,4'-Trihydroxy-7,8,3'-trimethoxyflavone (**5**) and 5,6,4'-Trihydroxy-7,8-dimethoxyflavone (**6**) were found to have promising nematicidal activity against J2s of *M. incognita*, compared to β -Sitosterol-3-O- β D-glucoside (**2**), 5,6-Dihydroxy-7,8,4'-trimethoxyflavone (**4**). Its methanolic extract (MSM) showed minimum activity after 96 hours of treatment compared to carbofuran. Most of the tested compound (**2, 4–6**) showed better nematicidal activity than carbofuran, a commercial nematicide. Earlier studies showed that 8-(3-Methyl-but-2-enyl)-2-phenyl chroman-4-one and 2-(4-hydroxyphenyl)-8-(3-methyl-but-2-enyl)-chroman-4-one, isolated from *Phyllanthus niruri* possessed potent nematicidal activity against *M. incognita* and *Rotylenchulus reniformis* under *in-vitro* conditions (Shakil et al. 2008) while triterpenoids from *Lantana camara* were found effective against nematodes (Begum et al. 2015). Schaftoside and isoschaftoside,

the C-flavonoide glycosides were also found to have nematicidal activity against root-knot nematode (Du et al. 2011).

Further, acetylcholinesterase was identified as one of the major target sites in root-knot nematode in molecular docking study (Pacule et al. 2022). This study also showed strong bonding of tested compounds to acetylcholinesterase in docking analysis as reported earlier, where sesquiterpenoid compounds bond strongly to the target site with binding energy of -3.23 to -5.26 kcal/mol, resulting in juvenile mortality (Keerthiraj et al. 2021). In a recent study, three aliphatic acids obtained from cotton seed were found to bind favourably with acetylcholinesterase during *in-silico* studies, with binding energy ranging between - 4.9 and - 5.3 kcal/mol (Almutairi et al. (2022). In case of the isolated pure flavonoids, stronger binding was observed with increasing number of hydroxy groups in the compound. These results are in accordance with previous studies, wherein, the inhibition of acetylcholinesterase enzyme by flavonoids increased with increasing number of hydroxy (-OH) substituents whereas methoxy (-OCH₃) substituents reduced the inhibitory action of the flavonoids (Xie et al. 2014; Karmakar et al. 2019). This study showed that the leaves of *M. spicata* contain important and rare compounds (**3–6**) including nematicidal compounds (**2,4–6**). These potent nematicidal compounds (**2,4–6**) can be evaluated for the development of nematicides of plant origin against *M. incognita*.

Conclusion

The leaves of *Mentha spicata* is a main source spearmint oil used in flavoring and therapeutic purposes in India and abroad. This study showed that its leaves can also be used as source of some natural nematicidal compounds against *Meloidogyne incognita* (root-knot nematode), infecting several crops in field conditions together with rare compounds. The pure compounds isolated from its leaves were found to show better activity than carbofuran under *in-vitro* conditions. The molecular docking studies also confirmed that pure compounds have higher binding affinity than carbofuran with possible similar mode of action. These potent nematicidal compounds (**2,4–6**) can be explored for the development of nematicides against *M. incognita*.

Declarations

Conflict of interest statement

All the authors confirmed that there is no conflict of interest.

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Data availability statement

Data will be made available on request to the corresponding author.

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Figures

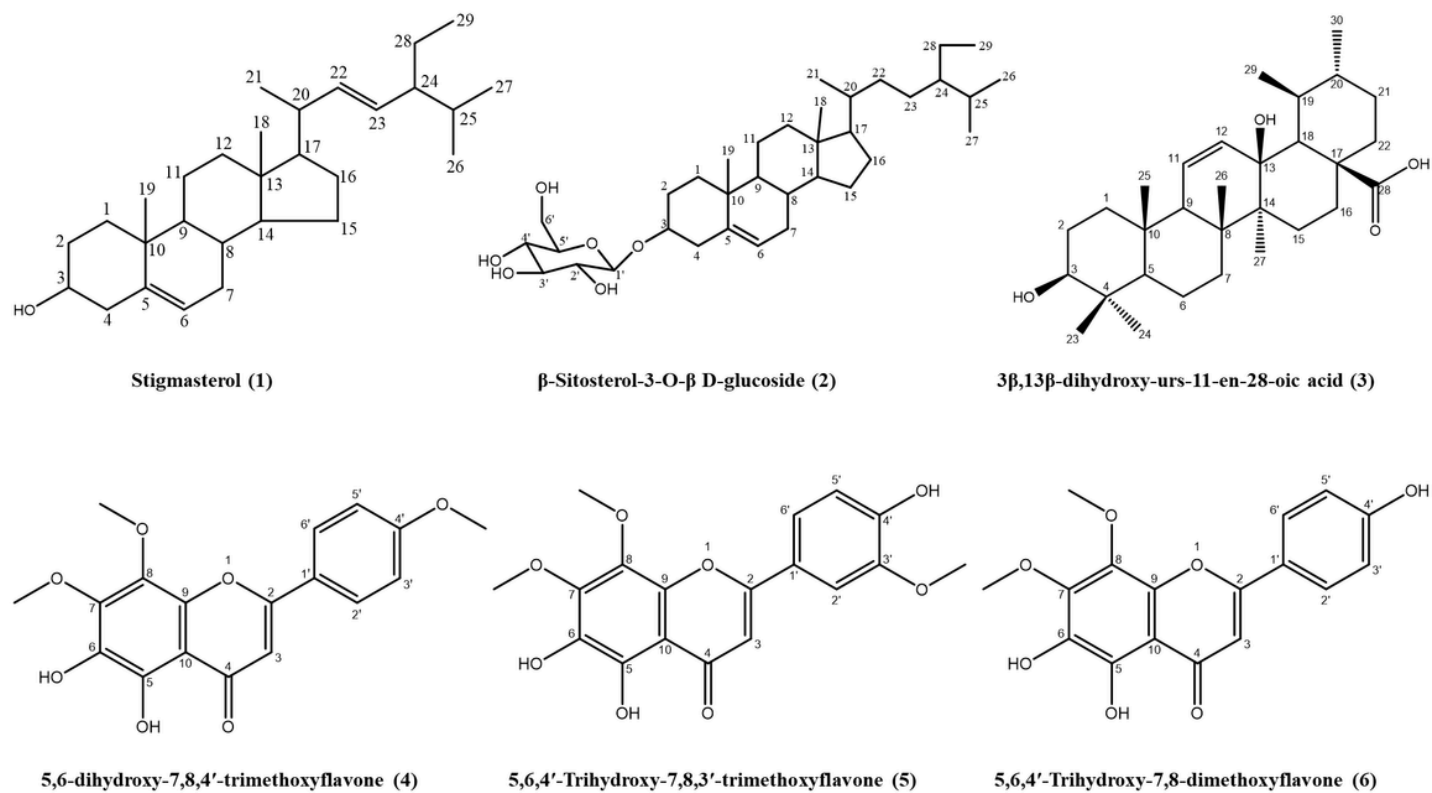


Figure 1

Structure of the pure compounds isolated from *Mentha spicata* leaves

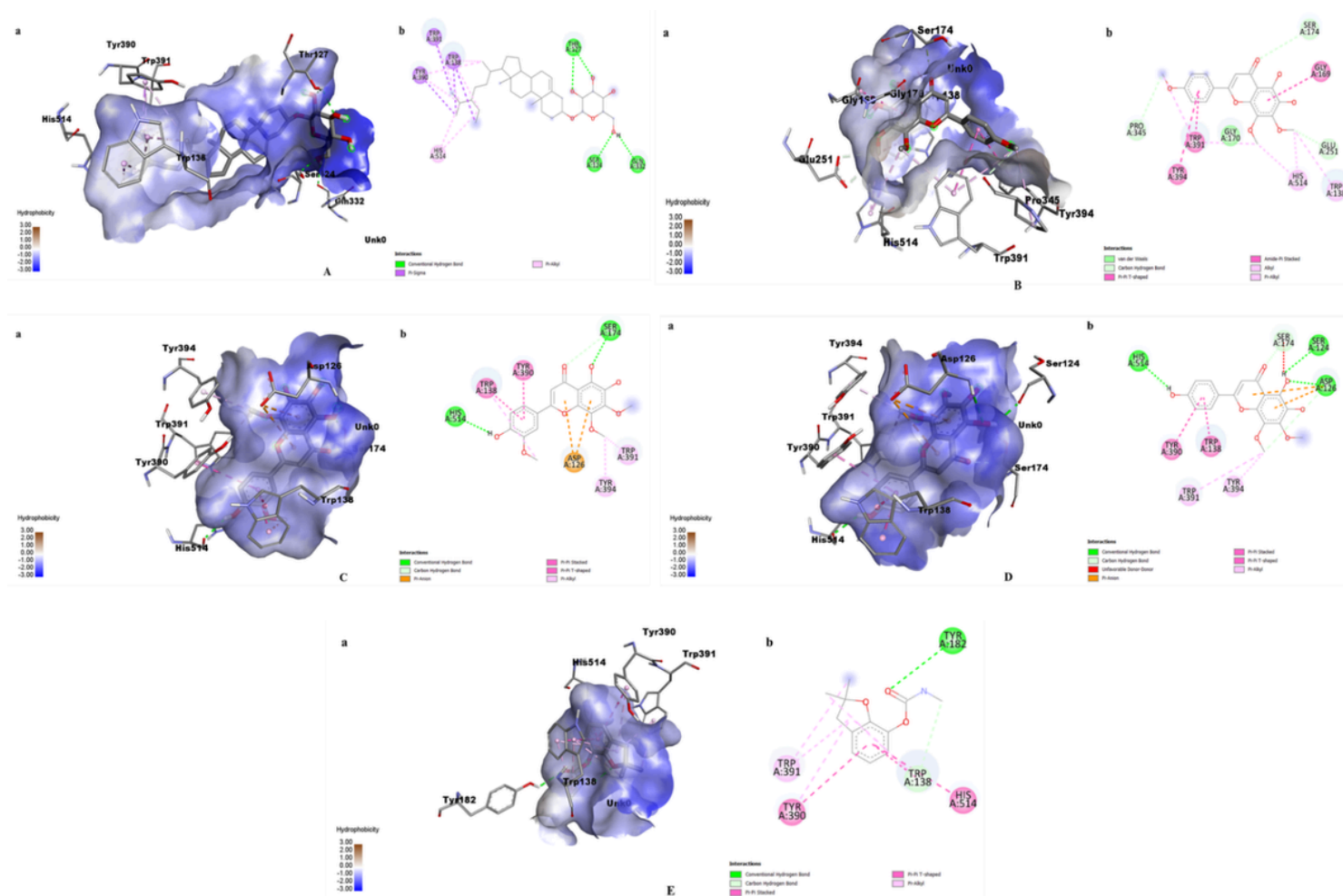


Figure 2

Protein-ligand binding (a) and 2D interaction (b) of the isolated pure compounds: 2 (A), 4 (B), 5 (C), 6 (D), and carbofuran (E)

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