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Nematicidal Characterization of *Solanum nigrum* and *Mentha arvensis* Leaf Extracts Using *Caenorhabditis elegans* as a Model Organism

Naqeeb Ullah Khan, Muhammad Sajid,* Saira Bibi, Wajid Rehman,* Mohammed M. Alanazi, and Magda H. Abdellatif

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ABSTRACT: Considering foremost global issues instigated by parasitic nematodes, *Solanum nigrum* (*S. nigrum*) and *Mentha arvensis* (*M. arvensis*) nematicidal potential at the gene level has been explored herein. Methanol, ethyl acetate, chloroform, *n*-hexane, and distilled water were used for extract preparation. *Caenorhabditis elegans* (*C. elegans*) was used as the model organism. Nematicidal and anti-egg hatching assays, fluorescence microscopy, and quantitative real-time PCR were done. *S. nigrum* chloroform (LD_{50} = 1.21 mg/mL) and *M. arvensis* methanol (LD_{50} = 2.47 mg/mL) extracts exhibited excellent nematicidal potential. Both plants showed potent anti-egg hatching activity (1 mg/mL). *S. nigrum* methanol and *M. arvensis* ethyl acetate extracts showed high apoptotic effect in muscles, gonads, and uterus (eggs). Stress genes, that is, *gst*-4, *hsp*-16.2, and *gpdh*-1 were highly expressed in affected *C. elegans* (treated with *S. nigrum* and *M. arvensis* leaf extracts) when compared with normal *C. elegans*. Phytochemicals and bioactive compounds present in plants may be the major cause of their excellent nematicidal potential, which further confirmed that both plants could be an alternative candidate(s) for novel broad-scale anthelmintic drug(s).
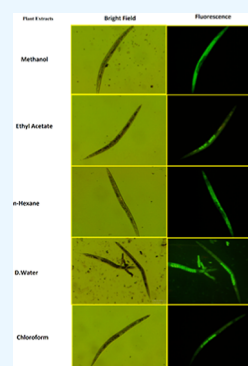
1. INTRODUCTION

Parasitic nematodes have severe negative effects on human health and productivity of animals and plants, which affect the socio-economic progress worldwide.[1] In the human population all over the world, parasitic nematodes cause high morbidity and low mortality.[2] The annual agricultural loss due to parasitic nematode infections is about US$157 billion worldwide. There are about 4000 nematode species that affect plants worldwide.[3] In developed countries, parasitic nematode infections are controlled by improved sanitation and use of synthetic nematicidal compounds.[4] Imidazothiazoles, benzimidazoles, and macrocyclic lactones have been used as anthelminthic drugs in humans and animals for more than 50 years. Nowadays, amino-acetonitrile derivatives are used against gastrointestinal nematodes. Many nematode species have developed partial to high resistance to these synthetic anthelmintic drugs. To control nematode infections, very higher doses are required which are toxic to humans, livestock, and animals.[5] High toxicity, environmental health risk, high cost, and limited availability of synthetic nematicidal drugs have created a new interest in scientists to prepare plant-based nematicides which are environmental-friendly, nontoxic, having low cost, and easily available in the market.

Recently, numerous plant-based natural products have been commercialized for the treatment of plant-parasitic nematodes.[6] In Pakistan, more than 600 plants are used for medicinal purposes.[7] In comparison with chemical compounds, the medicines prepared from medicinal plants showed "miracle" results.[8] In the context of the abovementioned issues, the current research work aims to identify the nematicidal potential of locally available plants. *Solanum nigrum* from the Solanaceae family and *M. arvensis* from the Lamiaceae family were selected. Both plant leaf extracts were tested while using *Caenorhabditis elegans* as the model organism. *C.elegans* is a free-living and nonpathogenic nematode. Its selection is due to its fast life cycle, easy and rapid culturing, simple anatomy, low testing cost, and easy maintenance.[9−11]

2. MATERIALS AND METHODS

2.1. Chemicals and Instruments. All chemicals (reagents and solvents) were purchased from Merck (Germany) and Sigma-Aldrich (USA) except RT-PCR products, which were purchased from Biotium (USA) and Thermo Fisher Scientific (USA). The plant grinding machine (HR40B-China), rotary evaporator (SD-RE 52A China), Micro Centrifuge (5425-R Hamburg, Germany), Mini Centrifuge (5453 Hamburg,

Germany), inverted microscope (Eclipse TS-100, Nikon, Japan), fluorescence microscopes (B-510FL, Italy, and BX-60, USA), and qRT-PCR system (7500 Applied Biosystems, Foster City, CA) were used in this research.

2.1.1. C. elegans and Bacterial Strains. N2, VP596, SJ4005, SJ4100, QV65, VP604, and CL2122 strains were used, which have been purchased from the *Caenorhabditis* Genetic Center, University of Minnesota, USA. All strains have been used as received. Bacterial strains *Escherichia coli* (OP50) and NA22 were also purchased from the Genetic Center, University of Minnesota, USA.

2.2. Plant Collection and Extract Preparation. *S. nigrum* and *M. arvensis* plants were collected from the Hazara Division (Khyber Pakhtunkhwa, Pakistan). Both plants were identified by the Department of Botany, Hazara University, Mansehra, Pakistan. *S. nigrum* and *M. arvensis* were prepared as herbarium samples with the accession numbers 2169 and 05134, respectively, and submitted to the herbarium of Hazara University.

Leaves of both plants were shade-dried and ground into powder using a Thomas Willey milling machine. The extracts were prepared in five different solvents [methanol, distilled water (D-water), chloroform, ethyl acetate, and *n*-hexane] by soaking 500 g of dried leaf powder in 2 L of each solvent separately for up to 15 days (at room temperature). The selection of different solvents to extract all kinds of bioactive compounds is based on polarity. Essential oils present in plants are usually soluble in non-polar solvents such as *n*-hexane etc., and for the extraction of polar substances water is used. After 15 days, extracts were filtered through filter paper (Whatman no 42, 125 mm), then this filtrate was concentrated by using a rotary evaporator, and stored for further use.[12,13] Dimethyl sulfoxide (DMSO) (≤1%) was used as a negative control; for further uses, all extracts were dissolved in DMSO. Phytochemical screening of *S. nigrum* and *M. arvensis* leaf extracts was done with the standard procedure used by Siddiqui *et al.* (2009).[14] Gas chromatography–mass spectrometry (GC–MS) analysis was used for the detection of volatile and semi-volatile compounds. For GC–MS, the standard protocol used by Makkar *et al.* (2018) was followed.[15]

2.3. Characterizations. *2.3.1. Nematicidal Assays.* To investigate the antinematodal activity of *S. nigrum* and *M. arvensis* leaf extracts, the test nematode dilution [100 µL of nematode growth media (NGM) buffer with 20 to 30 *C. elegans*] was added to each well of the sterile 96-well plate and then various concentrations of leaf extracts (0.312, 0.625, 1.25, 2.5, 5 to 10 mg/mL) were added. The plates were incubated at 20 °C for 24 h. After 24 h, *C. elegans'* survival was measured. The nematode viability was calculated by counting mobile worms using an inverted microscope and those considered dead which showed no movement on physical stimuli with a fine needle.[16,17]

2.3.2. Anti-egg Hatching Assay. 2.3.2.1. Isolation of Eggs. The N2 strain *C. elegans* was grown on an NGM plate seeded by *E. coli* (OP50). NGM buffer was used for rinsing off the worms from agar plates and placed in a 50 mL tube. Worms were washed two times *via* centrifugation (1000 rpm, 1 min) and again suspended in the NGM buffer. Up to 0.5 mL of gravid worms was processed in a single 15 mL tube with 5 mL of hypochlorite solution (3.75 mL sterile water, 1 mL household bleach, and 250 µL 10 M NaOH) for 5 min. All *C. elegans* were dissolved, and only eggs remained. Eggs were washed three times with NGM buffer *via* centrifugation (1000

rpm, 1 min) and re-suspended in 10 mL of NGM buffer. *S. nigrum* and *M. arvensis* leaf extracts were tested against eggs to determine their effect on egg hatching.[18]

2.3.2.2. Effect of Leaf Extracts on Egg Hatching. To observe the effect of *S. nigrum* and *M. arvensis* leaf extracts, the experiment was carried out in sterile 96-well plates. Almost 50–70 *C. elegans* eggs were added to each well having 100 µL NGM buffer with the concentration of 1 mg/mL *S. nigrum* and *M. arvensis* leaf extracts. In the control sample, there were only eggs and NGM buffer. At 20 °C, plates were incubated for 24 h. After the incubation period, plates were examined under an inverted microscope by counting the number of eggs and larvae.[19]

2.3.3. Fluorescence Microscopy. Fluorescence microscopy was carried out to study the apoptosis in *C. elegans* caused by *S. nigrum* and *M. arvensis* leaf extracts. For fluorescence microscopy, acridine orange (AO) dye was used for *C. elegans* staining.

2.3.3.1. AO Staining of Affected C. elegans. *C. elegans* was treated with *S. nigrum* and *M. arvensis* leaf extracts (at LD_{50} concentrations). After 24 h of incubation, the dead *C. elegans* was exposed to AO dye. From the AO stock solution (10 mg/mL), 2 µL was added in 01 mL S-medium, then 500 µL of AO was added to *C. elegans* and incubated for 1 h at 37 °C in dark. After 1 h, *C. elegans* was washed with NGM buffer and fixed on the slide for fluorescence microscopic (OPTIKA B-510FL Italy) examination.[20,21]

2.3.4. Screening Analysis. GFP and RFP reporter gene strains were used to screen different stress gene expressions and pathways induced by *S. nigrum* and *M. arvensis* leaf extracts (at 0.625 mg/mL after 24 h). The following six transgenic stress gene reporter strains **VP596** (vsIs33[dop-3p::DsRed2];dvIs19[gst-4p::GFP]), **SJ4005** (zcIS4 [hsp-4p::GFP]), **SJ4100** (zcIs13[hsp-6p::GFP]), **QV65** (gpIs1-[hsp-16.2p::GFP];vsls33[dop-3p::DsRed2]), **VP604** (kbIs24 [gpdh-1p::DsRed2;myo-2p::GFP;unc-119 rescue]), and **CL2122** (dvIs15 [mtl-2::GFP, pPD30.38 (unc-54 expression vector)]) were used to screen the six stress response gene expression (*gst*-4, *hsp*-4, *hsp*-6, *hsp*-16.2, *gpdh*-1 and *mtl*-2).[18,22–24]

2.3.4.1. Screening Analysis Assay. About 0.5 mL of adult worms of each transgenic *C. elegans* strain was processed with 5 mL of hypochlorite solution for 5 min in a 15 mL tube. All *C. elegans* was dissolved and only eggs remained. Eggs were removed and washed three times *via* centrifugation. Then, eggs of each transgenic *C. elegans* strain were added to the NA22 bacteria-seeded NGM plate and labeled. After 48–55 h, L4 and young adult *C. elegans* of each strain were removed and washed three times with NGM buffer, then NGM buffer (50 µL) containing 50–60 transgenic *C. elegans* was added to each well of 384-well plates, and then 0.625 mg/mL *S. nigrum* and *M. arvensis* leaf extracts were added and used as standards. The plate was sealed and placed in an incubator at 20 °C for assay for 24 h.[18] After 24 h, fluorescence was scored manually by a microscope (BX60) for estimation of the percent penetrance.

2.3.5. C. elegans Gene Expression Analysis. *C. elegans* gene expression analysis was done to determine biochemical pathways and molecular functions which were affected by the leaf extract. The selection of these genes was based on their use as stress response genes and available microarray expression data, that is, *gst*-4, *hsp*-4, *hsp*-6, *hsp*-16.2, *gpdh*-1, *mtl*-2 and *rpl*-2.[22,25–27]
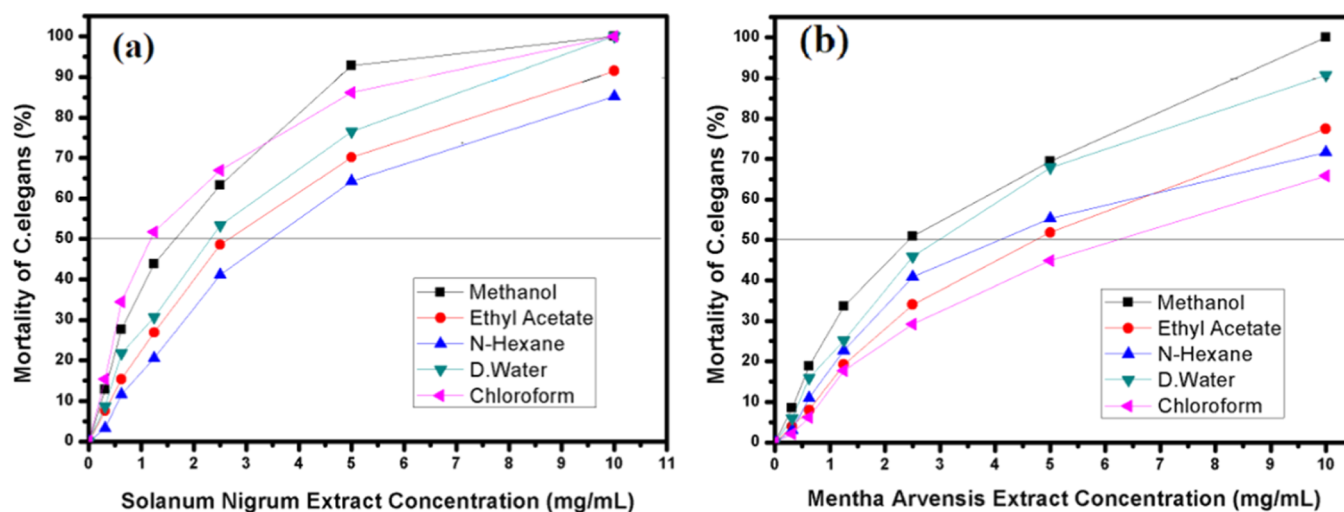
Figure 1. Nematicidal potential of *S. nigrum* (a) and *M. arvensis* (b) leaf extracts.

Table 1. Nematicidal Potential of *S. nigrum* Extracts in % at Different Concentrations

concentration	methanol	ethyl acetate	*n*-hexane	D-water	chloroform
10 mg/mL	100.00 ± 00.00	91.52 ± 02.08	85.20 ± 03.15	100.00 ± 00.00	100.00 ± 00.00
05 mg/mL	92.78 ± 02.31	70.15 ± 02.33	64.27 ± 01.83	76.53 ± 03.05	86.17 ± 01.93
2.5 mg/mL	63.26 ± 03.04	48.62 ± 02.56	41.10 ± 02.89	53.31 ± 01.76	66.96 ± 01.33
01.25 mg/mL	43.79 ± 02.09	26.91 ± 02.41	20.53 ± 02.74	30.67 ± 01.53	51.78 ± 02.28
0.625 mg/mL	27.58 ± 01.72	15.33 ± 02.19	11.65 ± 02.90	21.78 ± 01.89	34.54 ± 01.47
0.312 mg/mL	12.84 ± 02.35	07.55 ± 01.16	03.29 ± 01.40	08.95 ± 01 16	15.36 ± 01.25
negative control (≤1% DMSO)	02.67 ± 01.36	02.00 ± 01.00	01.36 ± 01.33	01.00 ± 01.00	02.10 ± 01.07
positive control (levamisole 20.35 μg/mL)			50.38 ± 2.32		

Table 2. Nematicidal Potential of *M. arvensis* Extracts in % at Different Concentrations

concentration	methanol	ethyl acetate	*n*-hexane	D-water	chloroform
10 mg/mL	100.00 ± 00.00	77.46 ± 03.05	71.65 ± 02.00	90.74 ± 03.36	65.87 ± 02.95
5 mg/mL	69.37 ± 03.20	51.85 ± 03.22	55.38 ± 02.27	67.89 ± 02.56	44.92 ± 04.34
2.5 mg/mL	50.95 ± 02.33	34.05 ± 03.22	40.89 ± 02.40	45.86 ± 02.95	29.18 ± 03.11
1.25 mg/mL	33.67 ± 03.20	19.24 ± 03.13	22.67 ± 03.63	25.17 ± 04.23	17.75 ± 03.86
0.625 mg/mL	18.82 ± 03.38	07.98 ± 04.01	10.93 ± 03.72	15.92 ± 03.86	06.21 ± 03.39
0.312 mg/mL	08.57 ± 02.25	03.95 ± 01.02	03.04 ± 01.60	05.97 ± 02.13	02.15 ± 01.40
negative control (≤1% DMSO)	03.30 ± 01.50	02.25 ± 01.25	02.50 ± 01.90	01.50 ± 02.13	01.50 ± 01.00
positive control (levamisole 20.35 μg/mL)			50.38 ± 2.32		

2.3.5.1. Isolation of RNA and Synthesis of cDNA. Newly adult or L4 stage *C. elegans* was incubated for 3 h with 0.312 mg/mL concentration of *S. nigrum* and *M. arvensis* leaf extracts. After 3 h, *C. elegans* was washed three times with NGM buffer to remove bacteria. Moving *C. elegans* was collected in centrifuge tubes, and total RNA was extracted by the GeneJET RNA Purification Kit (Thermo Scientific Inc. USA). The concentration of RNA was determined using the Qubit RNA HS Assay Kit (Thermo Fisher Scientific Inc. USA). Isolated total RNAs were treated with DNase (Merck & Co., Germany) to digest the unwanted gDNA. Total RNA (1 μg) was used for the synthesis of cDNA in 20 μL reaction by RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific Inc. USA).[28]

2.3.5.2. Quantitative Real-Time Polymerase Chain Reaction. The quantitative expression of six gene transcripts was examined by qRT-PCR. qRT-PCR assays were carried out in a 10 μL reaction mixture containing 2 μL template cDNA, 1 μL (10 μM/μL) of both forward and reverse primers, 5 μL 2× real-time SYBR Green PCR master mix (Promega Inc., USA),

and 2 μL RNase-free water. qRT-PCR was carried out in a quantitative RT-PCR instrument. The reaction was started with the initial activation of the polymerase at 95 °C for 10 min, followed by denaturation of the template at 95 °C for 15 s; annealing and elongation were done at 60 °C for 60 s. After the completion of 40 cycles, the melting curve was achieved at 60 to 95 °C to assess the presence of a unique final product.[28]

2.4. Statistical Analysis. Origin (version 8) was used for graphing. Statistix (version 8.1) was used for mean and standard deviation calculations. Prism GraphPad (version 9) was used to calculate the gene expression calculations.

3. RESULTS AND DISCUSSION

3.1. Antinematodal Potential of Leaf Extracts. *C. elegans* was incubated at 20 °C for 24 h with *S. nigrum* and *M. arvensis* leaf extracts ranging from 0.312, 0.625, 1.25, 2.5, 5 to 10 mg/mL. The results are shown in Figure 1. It can be easily observed from the figure that all five leaf extracts of both plants showed high nematicidal activities. In the case of *S. nigrum*, the

Table 3. Phytochemical Analyses of *S. nigrum* and *M. arvensis* Leaf Extracts

s. no	phytochemicals	*S. nigrum*					*M. arvensis*				
		methanol	ethyl acetate	*n*-hexane	D-water	chloroform	methanol	ethyl acetate	*n*-hexane	D-water	chloroform
1	alkaloids	+++	++	+	+	++	+++	+++	+++	++	++
2	flavonoids	+++	+	−	++	++	++	−	−	++	−
3	tannin	++	+	−	++	+	+	++	−	−	−
4	terpenoids	++	+	−	+	+++	+++	++	−	+	++
5	glycosidase	++	++	+++	+	+++	++	+	+	+	−
6	volatile oil	++	+	−	+	++	+	+	++	−	+
7	phenol	+	+	−	++	+	+	−	−	−	−
8	steroids	++	++	+	++	+	+	−	+	−	++
9	carotenoids	+	−	+		−	+	+	+	−	+
10	saponins	++	−	−	++	+	+	−	−	++	+

chloroform extract of leaves exhibited high nematicidal activity showing an LD_{50} value of 1.21 mg/mL (Figure 1a, Table 1). In the case of *M. arvensis*, the methanol extract of leaves exhibited high nematicidal activity. The LD_{50} of methanol extract was 2.47 mg/mL (Figure 1b, Table 2).

Both plants' leaf extracts showed excellent nematicidal potential. These high nematicidal activities were due to the presence of phytochemicals and nematicidal bioactive compounds (limonene, carvone, α-terpinene, eugenol, and thymol in *M. srvensis* extracts, while tetradecanoic acid, *n*-hexadecanoic acid, 9,12,15-octadecatrienoic acid, and *tert*-hexadecanethiol in *S. nigrum* extracts, as shown in Tables 3 and 4). Liu *et al.* (2011) reported the GC−MS and phytochemical screening of *Ficus carica* (*Moraceae*) leaf methanol extract, which confirmed that the nematicidal potential of *F. carica* was due to the presence of phytochemicals and bioactive compounds, that is, alkaloids, sesquiterpenoids, diterpenoids, triterpenoids, fatty acids, cyanogenic glycosides, polyphenols, polyacetylenes, quassinoids, steroids, phenolics, isothiocyanates, and glucosinolates.[29]

In most of the plants, the nematicidal activities were due to the presence of most potent phenolic compounds reported earlier.[30] The essential oil present in the plants may also be responsible for enhanced antinematodal activity.[31,32] The phytochemical and GC−MS analyses of *S. nigrum* and *M. arvensis* confirmed that they contained all the potent phytochemicals and bioactive compounds responsible for nematicidal activities.[15,33−35] The detailed information is listed in Table 3 (phytochemicals) and Table 4 (chemical compounds). The leaf extracts of *S. nigrum* and *M. arvensis* showed different nematicidal activities because the solubility of the abovementioned phytochemicals and compounds was different in different solvents.

3.2. Effect on Egg Hatching. *S. nigrum* and *M. arvensis* leaf extracts with a concentration of 1 mg/mL were tested against *C. elegans* eggs along with a control [negative control (≤1% DMSO), positive control (ivermectin 100 μg/mL)] sample, results are shown in Figure 2 and Table 5. The unhatched eggs found in *S. nigrum* methanol, ethyl acetate, *n*-hexane, D-water, and chloroform extracts and the control sample were 67.43 ± 04.23, 45.72 ± 03.96, 39.20 ± 02.09, 52.41 ± 03.58, 58.45 ± 03.69, 04.15 ± 01.69, and 100.00 ± 0.00%, respectively. Methanol, D-water, and chloroform extracts showed more than 50% unhatched eggs (EC_{50}). It can be observed from Figure 2b that the unhatched eggs found in *M. arvensis* methanol, ethyl acetate, *n*-hexane, D-water, and chloroform extracts, and the control sample were 64.65 ± 03.99, 79.45 ± 04.05, 51.87 ± 04.78, 27.47 ± 02.08, 34.93 ±

Table 4. GC−MS Analysis of *S. nigrum* and *M. arvensis* Leaf Extracts

s. no	compounds in *S. nigrum*	s. no	compounds in *M. arvensis*
1	cyclopentasiloxane, decamethyl-	1	DL-limonene
2	L-proline, ethyl ester	2	eucalyptol
3	2-ethyl-1-butanol, methyl ether	3	3-hexen-1-ol
4	dodecanal	4	isoeugenol
5	tetradecanoic acid	5	limonene
6	9-octadecenamide	6	L-menthone
7	octadecane, 3-ethyl-5-(ethyl butyl)	7	neo-menthol acetate
8	5-keto-2,2-dimethylheptanoic acid, ethyl ester	8	4-terpineol
9	phthalic acid, di(2-propylpentyl)ester	9	linalool
10	E-9-tetradecenoic acid	10	mentha-2,8-dien-1-ol
11	Z-8-methyl-9-tetradecenoic acid	11	carvone
12	butane dioic acid, hydroxyl, diethyl, ester	12	isopulegol
13	dodecanoic acid, 3-hydroxy-, ethyl ester	13	isomenthone
14	myristoleic acid	14	menthone
15	*n*-hexadecenoic acid	15	menthol
16	phytol	16	α-terpinene
17	9,12,15-octadecatrienoic acid	17	cyclopentanone
18	α-D-glucopyranoside, O-α-D-glucopyranosyl-β-D-fructofuranosyl	18	pulegone
19	1,2-propanediol, 3-(tetradecyloxy)	19	piperitone
20	*tert*-hexadecane thiol	20	menthyl acetate
21	1-dodecanamine, N,N-dimethyl	21	neomenthyl acetate
22	cyclooctasiloxane, hexadecamethyl	22	isopulegol acetate
23	ethanol, 2-(tetradecyloxy)	23	eugenol
24	heptadecane, 9-hexyl	24	thymol
25	myoinositol, hexaacetate	25	caryophyllene
26	valeric acid, 4-pentadecyl ester	26	germacrene
27	benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-, octadecyl ester	27	cadinene
28	17-(1,5-dimethylhexyl)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H		

02.62, 03.84 ± 01.49, and 100.00 ± 0.00%, respectively. Methanol, ethyl acetate, and *n*-hexane extracts showed more than 50% unhatched eggs (EC_{50}).

Adamu *et al.*, studied 13 plants' acetone extract against *C. elegans* egg hatching. Among these, five plant species, that is, *Heteromorpha trifoliata*, *Maesa lanceolata*, *Leucosidea sericea*, *Clausena anisate*, and *Clerodendrum glabrum*, were most potent and had anti-hatching EC_{50} values against *C. elegans* eggs
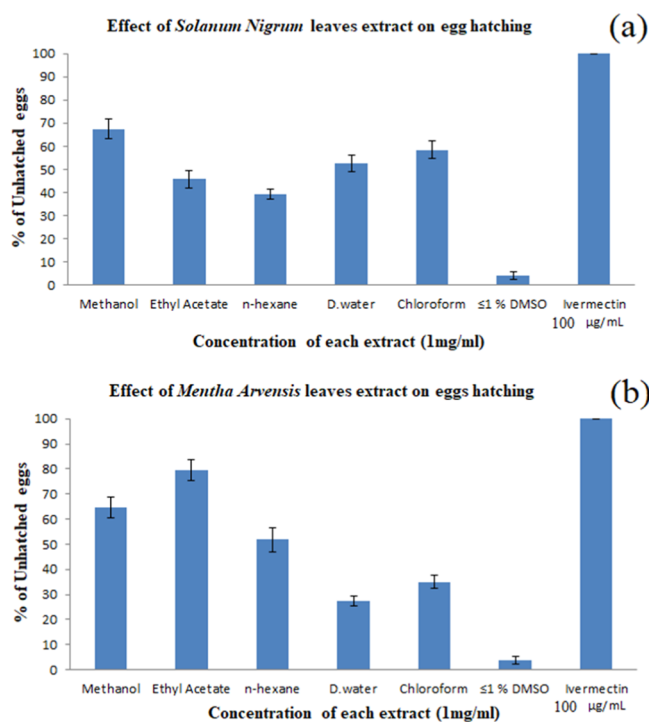
Figure 2. Effect of *S. nigrum* (a) and *M. arvensis* (b) leaf extracts on egg hatching.

Figure 3. Fluorescence microscopy of *S. nigrum* leaf extracts.

between 0.62 and 1.48 mg/mL, respectively.[19] In contrast to the previously reported literature, *S. nigrum* and *M. arvensis* showed excellent results. The enhanced egg anti-hatching activity in these plants might be due to the presence of phytochemicals, bioactive compounds, and many secondary metabolites.

3.3. Fluorescence Microscopy. Fluorescence microscopy is the best technique to study apoptosis in *C. elegans* affected by *S. nigrum* and *M. arvensis* leaf extracts. Fluorescence microscopic images of *S. nigrum* extracts in various solvents against *C. elegans* are shown in Figure 3. The methanol extract displays the fluorescence of green color in gonadal cells, indicating apoptosis in gonad arms only. Ethyl acetate and chloroform extracts display the fluorescence of green color in the uterus (eggs) and yellowish green color in gonadal cells only. *n*-Hexane extract displays the fluorescence of green color in muscle cells only. D-water extract displays the fluorescence of green color in the uterus (eggs). It is worth noting here that apoptosis occurred in those parts which display the fluorescence of green color and yellowish green color and leading to cell death. All extracts showed excellent apoptosis. Fluorescence microscopic images of *M. arvensis* extracts in various solvents against *C. elegans* are shown in Figure 4. The methanol and ethyl acetate extracts display the fluorescence of green color in the uterus (eggs), muscle cells, and gonadal cells and yellowish green color in gonadal cells, indicating apoptosis in the uterus (eggs), muscles, and gonad arms only. The *n*-hexane extract displays the fluorescence of green color in the
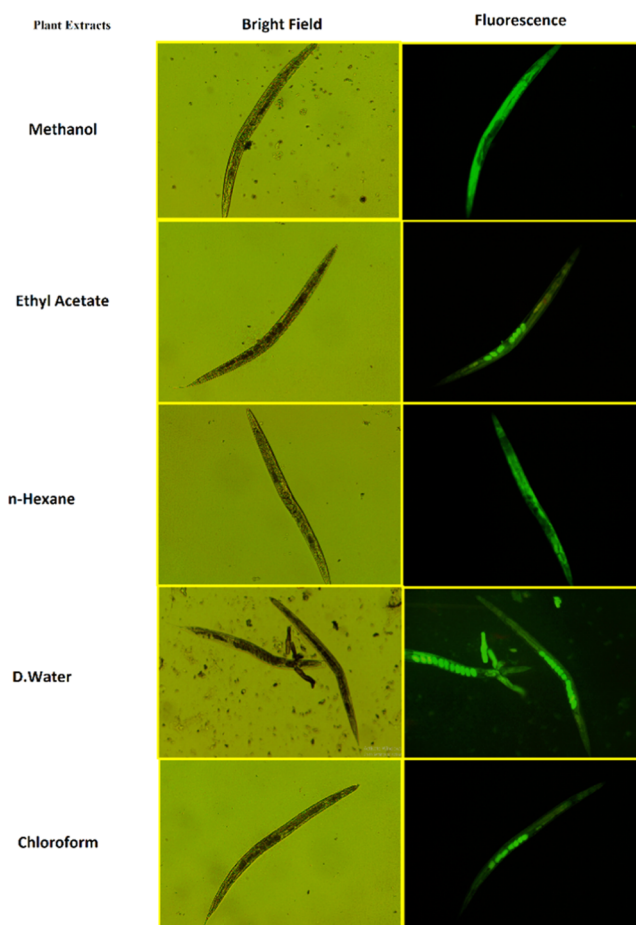
uterus (eggs) only. The D-water extract displays the fluorescence of green color in the uterus (eggs) and yellowish green color in gonadal cells. The chloroform extract displays the fluorescence of green color in the uterus (eggs) and gonadal cells only. In the case of *M. arvensis,* methanol and ethyl acetate extracts showed high apoptosis.

S. nigrum and *M. arvensis* leaf extracts contained many bioactive chemical compounds (phytochemicals and primary and secondary metabolites) and became a cause of apoptosis, leading to cell death in different developmental organs of *C. elegans.* In the present investigation, fluorescence microscopy provided intriguing information regarding the effect of *S. nigrum* and *M. arvensis* leaf extracts on *C. elegans* gonadal cells, muscular cells, and uterus (eggs) leading to cell death. Sajid and Azim also obtained such fluorescence microscopic results while studying the effect of honey on *C. elegans* intestinal cells and gonadal cells.[16]

3.4. Screening Analysis. Six transgenic worm strains (GFP and RFP reporter gene strains) were used to screen out the effect of *S. nigrum* and *M. arvensis* leaf extracts on *C. elegans* stress genes. The results of both plants are shown in Figures 5 and 6 on each gene after 24 h of incubation at 20 °C.

Table 5. Effect of *S. nigrum* and *M. arvensis* Extracts on Egg Hatching in % at 1 mg/mL

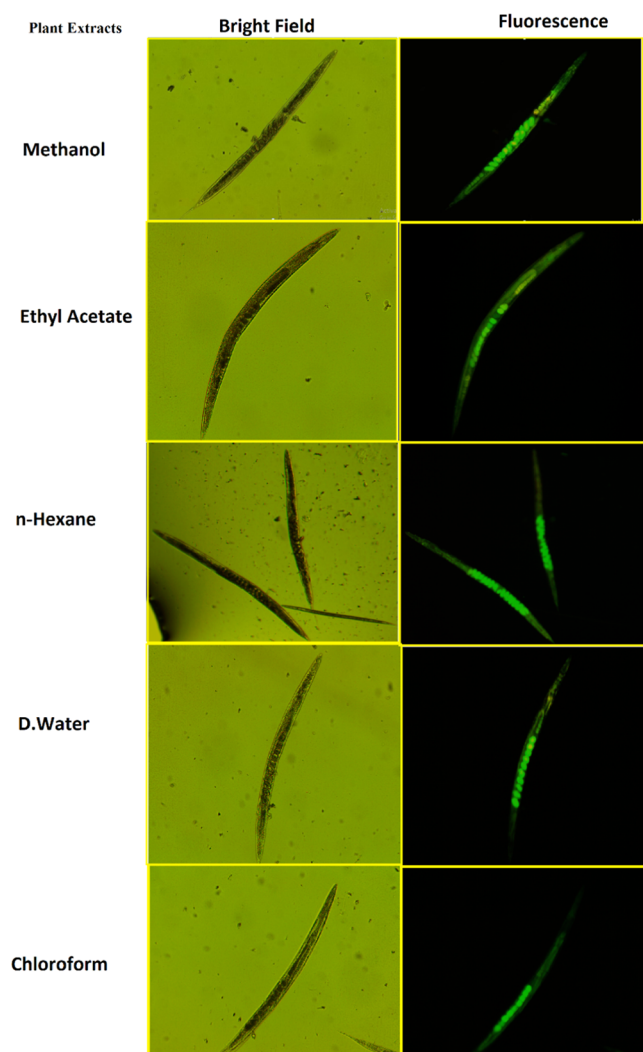| plants	methanol	ethyl acetate	*n*-hexane	D-water	chloroform	≤1% DMSO	ivermectin 100 μg/mL
S. nigrum	67.43 ± 4.23	45.72 ± 3.96	39.20 ± 2.09	52.41 ± 3.58	58.45 ± 3.59	4.15 ± 1.69	100 ± 0.00
M. arvensis	64.65 ± 3.99	79.45 ± 4.05	51.87 ± 4.78	27.47 ± 2.08	34.93 ± 2.62	3.84 ± 1.49	100 ± 0.00

Figure 4. Fluorescence microscopy of *M. arvensis* leaf extracts.

Fluorescence-indicated expression of gene details is mentioned below.

3.4.1. Solanum nigrum. Oxidative stress reporter, gst-4 was expressed by all five extracts (methanol, ethyl acetate, *n*-hexane, D-water, and chloroform). Endoplasmic reticulum stress reporter hsp-4 was expressed only by *n*-hexane extracts. Mitochondrial stress reporter hsp-6 was not expressed by any extract. Heat shock stress reporter hsp-16.2 was expressed by *n*-hexane, D-water, and chloroform only. Osmotic stress reporter gpdh-1 was expressed by all five extracts. Metal RNA stress reporter mtl-2 was not expressed by any extract. All standards expressed their specific stress reporter maximum, as depicted in Figure 5.

3.4.2. Mentha arvensis. Oxidative stress reporter gst-4 was expressed by all five extracts (methanol, ethyl acetate, *n*-hexane, D-water, and chloroform). Endoplasmic reticulum stress reporter hsp-4 was expressed only in *n*-hexane extract. Mitochondrial stress reporter hsp-6 was expressed by methanol extract only. Heat shock stress reporter hsp-16.2 was expressed by all five extracts. Osmotic stress reporter gpdh-1 was expressed by methanol, *n*-hexane, and D-water extracts. Metal RNA stress reporter mlt-2 was not expressed by any extract, as depicted in Figure 6.

This is the only screening technique to screen out the plant extract, whether it expressed the genes or not. Further, these were quantitatively confirmed by qRT-PCR, because qRT-PCR is very sensitive and showed very minute expression accurately.

3.5. Gene Expression Analysis. Gene expression analysis was carried out through qRT-PCR to highlight the gene in normal and affected *C. elegans*. Expression of about six *C. elegans* genes was done to determine biochemical pathways and molecular functions affected by *S. nigrum* and *M. arvensis* leaf extracts (0.312 mg/mL) after 3 h of incubation at 20 °C. Selection of these genes was based on their use as stress response genes. The genes included gst-4, hsp-4, hsp-6, hsp-16.2, gpdh-1, and mlt-2. The gene that showed expression of a fold change of ≥2.0 in biological triplicates was considered as up- or downregulated.

Figure 7 shows *S. nigrum* gene expression results. Results showed that the **gst-4** gene expressed by all five extracts, that is, methanol, ethyl acetate, *n*-hexane, D-water, and chloroform extracts with fold-change values around 33.78 ± 3.87, 13.28 ± 1.62, 28.16 ± 2.00, 15.03 ± 0.88, and 23.35 ± 3.41, respectively. The **hsp-4** gene expressed by only *n*-hexane extract was around 3.82 ± 0.64 fold change. It is worth noting that the **hsp-6** gene was not expressed by any extract. The **hsp-16.2** gene expressed by *n*-hexane, D-water, and chloroform extracts showed 4.26 ± 1.17, 7.23 ± 0.13, and 6.38 ± 0.70 fold-change values, respectively. The **gpdh-1** gene was expressed by all five extracts (methanol, 27.55 ± 2.69 fold change; ethyl acetate, 13.8 ± 1.26 fold change; *n*-hexane, 12.62 ± 2.51 fold change; D-water, 14.38 ± 1.66 fold change; and chloroform, 8.76 ± 1.70 fold change). The **mtl-2** gene was not expressed by any extract. The expression results of all these six genes are given in comparison with normal *C. elegans* genes.

Figure 8 shows *M. arvensis* gene expression results. The **gst-4** gene was expressed by all five extracts (methanol, ethyl acetate, *n*-hexane, D-water, and chloroform extracts showed 42.91 ± 2.31, 10.54 ± 0.79, 20.58 ± 0.75, 29.54 ± 2.46, and 8.88 ± 1.57 fold-change values, respectively). The **hsp-4** gene was expressed by methanol, ethyl acetate, *n*-hexane, and chloroform extracts, whose fold-change values were around 2.98 ± 0.64, 2.91 ± 0.48, 3.21 ± 0.39, and 2.93 ± 0.51, respectively. It is worth noting that **hsp-6** gene was expressed by methanol and ethyl acetate extracts with 9.35 ± 1.01 and 2.62 ± 0.14 fold-change values, respectively. The **hsp-16.2** gene was expressed by all five extracts with 21.21 ± 2.39, 8.10 ± 1.02, 9.15 ± 1.00, 12.56 ± 2.03, and 3.39 ± 0.81 fold-change values, respectively. The **gpdh-1** gene was expressed by extracts of methanol (6.63 ± 1.10 fold change), *n*-hexane (7.57 ± 0.68 fold change), and D-water (9.06 ± 1.09 fold change). The **mtl-2** gene was not expressed by any extract. The expression results of all these six genes are presented in comparison with normal *C. elegans* genes.

The qRT-PCR results showed that the expression of gst-4, gpdh-1, and hsp-16.2 gene transcripts was highly up-regulated by both plant leaf extracts, while that of hsp-4 and hsp-6 gene transcripts was up-regulated by few extracts. Gene gst-4 encodes glutathione S-transferase and is involved in glutathione transferase activity and in the glutathione metabolic process. Gene gpdh-1 encodes glycerol-3-phosphate dehydrogenase and is involved in glycerol-3-phosphate dehydrogenase activity, NADH oxidation, glycerol-3-phosphate metabolic process, and glycerol-3-phosphate dehydrogenase complex. Gene hsp-16.2 encodes heat shock protein and performed

Plant Fraction	gst-4	hsp-4	hsp-6	hsp-16.2	gpdh-1	mtl-2
Methanol	3.38				2.91	
Ethyl Acetate	1.85	0.25			1.96	
N-Hexane	3.04	0.69		0.97	1.77	
Chloroform	2.67			1.34	1.53	
D.Water	2.19			1.47	2.01	
Standards	4	4	4	4	4	4

0-5 % Fluorescence	
5-25 % Fluorescence	1
25-50 % Fluorescence	2
50-75 % Fluorescence	3
75-100 % Fluorescence	4

C.elegan strain	Gene	Standards Used	Used as Reporter
VP596	gst-4	Acrylamide 5 mM	Dedoxification Response reporter
SJ4005	hsp-4	thapsigargin 100 µM	Endoplasmic reticulum stress
SJ4100	hsp-6	Paraqute 1mM	Mitichondrial Stress
QV65	hsp-16.2	2 hrs Heat at 37 degree	Heat shock stress
VP604	gpdh-1	NaCl 250 µM	Osmotic Stress
CL2122	mtl-2	CdCl2 100 µM	Matel RNA Stress

Figure 5. Screening of stress genes in transgenic *C. elegans* using *S. nigrum* leaf extracts.

Plant Fraction	gst-4	hsp-4	hsp-6	hsp-16.2	gpdh-1	mtl-2
Methanol	3.79	0.18	1.46	2.58	1.27	
Ethyl Acetate	1.72	0.19	0.25	1.35	0.15	
N-Hexane	2.44	0.42		1.58	1.22	
Chloroform	1.39	0.23		0.69	0.15	
D.Water	2.98			1.81	1.45	
Standards	4	4	4	4	4	4

0-5 % Fluorescence	
5-25 % Fluorescence	1
25-50 % Fluorescence	2
50-75 % Fluorescence	3
75-100 % Fluorescence	4

C.elegan strain	Gene	Standards Used	Used as Reporter
VP596	gst-4	Acrylamide 5 mM	Dedoxification Response reporter
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SJ4100	hsp-6	Paraqute 1mM	Mitichondrial Stress
QV65	hsp-16.2	2 hrs Heat at 37 degree	Heat shock stress
VP604	gpdh-1	NaCl 250 µM	Osmotic Stress
CL2122	mtl-2	CdCl2 100 µM	Matel RNA Stress

Figure 6. Screening of stress genes in transgenic *C. elegans* using *M. arvensis* leaf extracts.

unfolded protein binding activity that responds to heat that occurred in the cytoplasm. Gene *hsp*-4 enables RNA polymerase II-specific DNA-binding transcription factor binding activity and is involved in the endoplasmic reticulum unfolded protein response. Gene *hsp*-6 performed misfolded protein binding activity, ATP binding activity, unfolded protein binding activity, and mitochondrial unfolded protein response. All these genes that are affected by both plant extracts might be due to the presence of bioactive compounds and phytochemicals, as discussed earlier (see Section 3.1 for plant extract nematicidal activity). This genomic study provides information about the changes that occurred in gene expression which further may help to find out or discover biochemical pathways and their molecular functions affected by exposure to medicinal plant extracts. It may also assist in predicting the mechanism of action of bioactive compounds and phytochemicals present in the plant extract within the phylum Nematoda conserved pathways; they may be good candidates for novel broad-scale anthelmintic drug(s).

4. CONCLUSIONS

The overall results of the study showed that *S. nigrum* chloroform extract and *M. arvensis* methanol extract exhibited excellent nematicidal potential. Both plant extracts showed more potent anti-egg hatching potential, that is, at 1 mg/mL. Fluorescence microscopy analysis showed an apoptotic effect
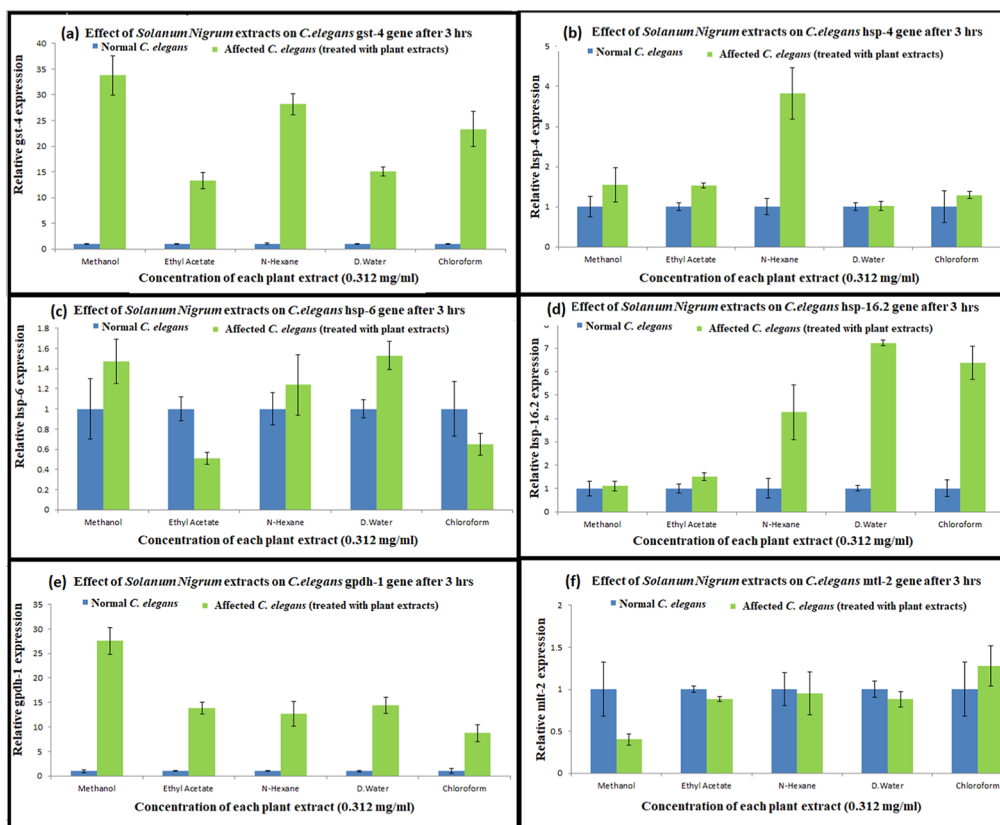
Figure 7. Quantitative RT-PCR analysis of *C. elegans* stress genes treated with *S. nigrum* leaf extracts: (a) gst-4, (b) hsp-4, (c) hsp-6, (d) hsp-16.2, (e) gpdh-1, and (f) mtl-2.

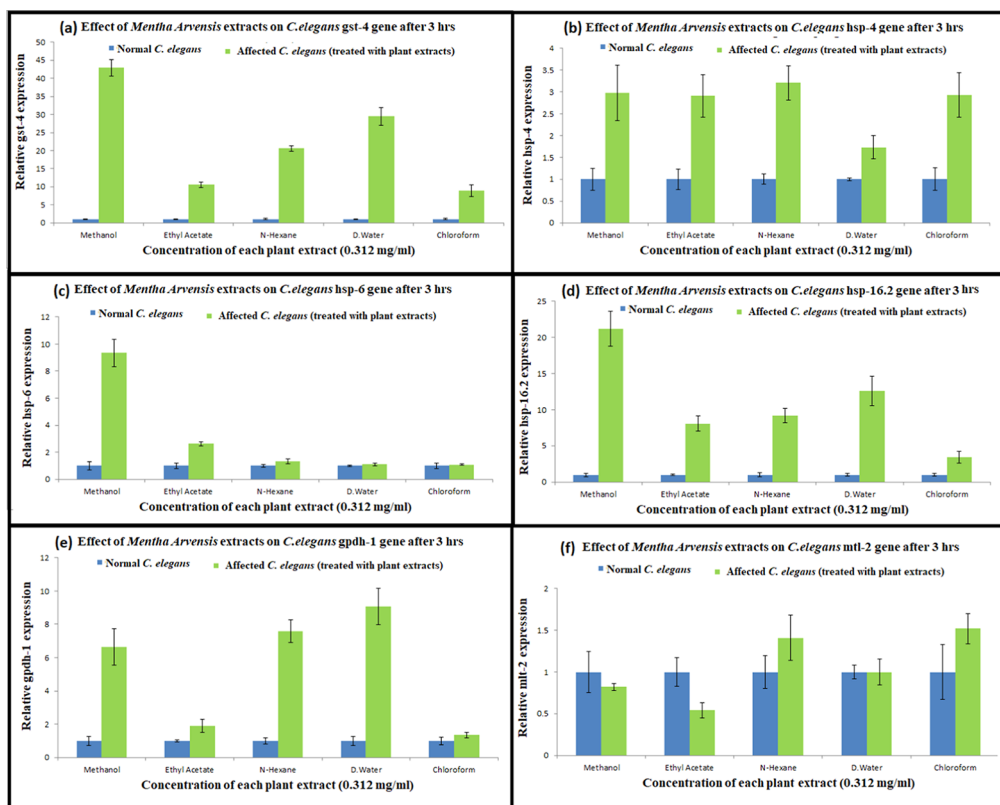**Figure 8.** Quantitative RT-PCR analysis of *C. elegans* stress genes treated with *M. arvensis* leaf extracts: (a) gst-4, (b) hsp-4, (c) hsp-6, (d) hsp-16.2, (e) gpdh-1, and (f) mtl-2.

in gonadal cells, muscle cells, and uterus (eggs) caused by *S. nigrum* and *M. arvensis* leaf extracts. Quantitative RT-PCR analysis showed that all extracts of *S. nigrum* and *M. arvensis* expressed the *gst*-4, *hsp*-16.2, and *gpdh*-1 genes, while *hsp*-4 and *hsp*-6 were expressed by some extracts. From the results, it can be concluded that both plants can be used as broad-scale anthelmintic drug(s) to control parasitic nematode infections.

■ AUTHOR INFORMATION

Corresponding Authors

Muhammad Sajid − *Department of Biochemistry, Hazara University, Mansehra 21300 Khyber Pakhtunkhwa, Pakistan*; Email: sajid931@hotmail.com

Wajid Rehman − *Department of Chemistry, Hazara University, Mansehra 21300 Khyber Pakhtunkhwa, Pakistan*; ⓘ orcid.org/0000-0003-0128-0377; Email: sono_waj@yahoo.com

Authors

Naqeeb Ullah Khan − *Department of Biochemistry, Hazara University, Mansehra 21300 Khyber Pakhtunkhwa, Pakistan*

Saira Bibi − *Department of Chemistry, Hazara University, Mansehra 21300 Khyber Pakhtunkhwa, Pakistan*

Mohammed M. Alanazi − *Department of Pharmaceutical Chemistry, College of Pharmacy, King Saud University, Riyadh 11451, Saudi Arabia*

Magda H. Abdellatif − *Department of Chemistry, College of Sciences, Taif University, Taif 21944, Saudi Arabia*

Complete contact information is available at:
https://pubs.acs.org/10.1021/acsomega.2c08124

Notes

The authors declare no competing financial interest.

■ REFERENCES

(1) Hotez, P. J.; Alvarado, M.; Basáñez, M. G.; Bolliger, I.; Bourne, R.; Boussinesq, M.; Naghavi, M. The global burden of disease study 2010: interpretation and implications for the neglected tropical diseases. *PLoS Neglected Trop. Dis.* **2014**, *8*, No. e2865.

(2) Bethony, J.; Brooker, S.; Albonico, M.; Geiger, S. M.; Loukas, A.; Diemert, D.; Hotez, P. J. Soil-transmitted helminth infections: ascariasis, trichuriasis, and hookworm. *Lancet* **2006**, *367*, 1521−1532.

(3) Abad, P.; Gouzy, J.; Aury, J. M.; Castagnone-Sereno, P.; Danchin, E. G.; Deleury, E.; Wincker, P. Genome sequence of the metazoan plant-parasitic nematode Meloidogyne incognita. *Nat. Biotechnol.* **2008**, *26*, 909−915.

(4) Kappagoda, S.; Singh, U.; Blackburn, B. G. Antiparasitic therapy. *Mayo Clin. Proc.* **2011**, *86*, 561−583.

(5) Scott, I.; Pomroy, W. E.; Kenyon, P. R.; Smith, G.; Adlington, B.; Moss, A. Lack of efficacy of monepantel against Teladorsagia

circumcincta and Trichostrongylus colubriformis. *Vet. Parasitol.* **2013**, *198*, 166−171.

(6) Oka, Y.; Nacar, S.; Putievsky, E.; Ravid, U.; Yaniv, Z.; Spiegel, Y. Nematicidal activity of essential oils and their components against the root-knot nematode. *Phytopathology* **2000**, *90*, 710−715.

(7) Shinwari, Z. K. Medicinal plants research in Pakistan. *J. Med. Plants Res.* **2010**, *4*, 161−176.

(8) Newman, D. J.; Cragg, G. M. Natural products as sources of new drugs over the last 25 years. *J. Nat. Prod.* **2007**, *70*, 461−477.

(9) Kumar, A.; Baruah, A.; Tomioka, M.; Iino, Y.; Kalita, M. C.; Khan, M. Caenorhabditis elegans: a model to understand host-microbe interactions. *Cell. Mol. Life Sci.* **2020**, *77*, 1229−1249.

(10) Seydoux, G. The P Granules of C. elegans: A Genetic Model for the Study of RNA-Protein Condensates. *J. Mol. Biol.* **2018**, *430*, 4702−4710.

(11) Jones, A. K.; Buckingham, S. D.; Sattelle, D. B. Chemistry-to-gene screens in Caenorhabditis elegans. *Nat. Rev. Drug Discovery* **2005**, *4*, 321−330.

(12) Green, R. J. Antioxidant activity of peanut plant tissues. MS Thesis, North Carolina State University, 2004.

(13) Kianbakht, S.; Jahaniani, F. Evaluation of antibacterial activity of Tribulus terrestris L. growing in Iran. *Iran. J. Pharmacol. Ther.* **2003**, *2*, 22−24.

(14) Siddiqui, S.; Verma, A.; Rather, A. A.; Jabeen, F.; Meghvansi, M. K. Preliminary phytochemicals analysis of some important medicinal and aromatic plants. *Adv. Biol. Res.* **2009**, *3*, 188−195.

(15) Makkar, M. K.; Sharma, S.; Kaur, H. Evaluation of Mentha arvensis essential oil and its major constituents for fungitoxicity. *J. Food Sci. Technol.* **2018**, *55*, 3840−3844.

(16) Sajid, M.; Azim, M. K. Characterization of the nematicidal activity of natural honey. *J. Agric. Food Chem.* **2012**, *60*, 7428−7434.

(17) Hong, L.; Li, G.; Zhou, W.; Wang, X.; Zhang, K. Screening and isolation of a nematicidal sesquiterpene from Magnolia grandiflora L. *Pest Manage. Sci.* **2007**, *63*, 301−305.

(18) Leung, C. K.; Deonarine, A.; Strange, K.; Choe, K. P. High-throughput Screening and Biosensing with Fluorescent C. elegans Strains. *J. Visualized Exp.* **2011**, *51*, 2745.

(19) Adamu, M.; Naidoo, V.; Eloff, J. N. Efficacy and toxicity of thirteen plant leaf acetone extracts used in ethnoveterinary medicine in South Africa on egg hatching and larval development of Haemonchus contortus. *BMC Vet. Res.* **2013**, *9*, 38.

(20) Zhang, J.; Campbell, R. E.; Ting, A. Y.; Tsien, R. Y. Creating new fluorescent probes for cell biology. *Nat. Rev. Mol. Cell Biol.* **2002**, *3*, 906−918.

(21) Miyawaki, A. Green fluorescent protein-like proteins in reef Anthozoa animals. *Cell Struct. Funct.* **2002**, *27*, 343−347.

(22) Dodd, W.; Tang, L.; Lone, J. C.; Wimberly, K.; Wu, C. W.; Consalvo, C.; Wright, J. E.; Pujol, N.; Choe, K. P. A Damage Sensor Associated with the Cuticle Coordinates Three Core Environmental Stress Responses in Caenorhabditis elegans. *Genetics* **2018**, *208*, 1467−1482.

(23) Wong, S. Q.; Jones, A.; Dodd, S.; Grimes, D.; Barclay, J. W.; Marson, A. G.; Cunliffe, V. T.; Burgoyne, R. D.; Sills, G. J.; Morgan, A. A Caenorhabditis elegans assay of seizure-like activity optimised for identifying antiepileptic drugs and their mechanisms of action. *J. Neurosci. Methods* **2018**, *309*, 132−142.

(24) Hu, Q.; D'Amora, D. R.; MacNeil, L. T.; Walhout, A.; Kubiseski, T. J. The Oxidative Stress Response in Caenorhabditis elegans Requires the GATA Transcription Factor ELT-3 and SKN-1/Nrf2. *Genetics* **2017**, *206*, 1909−1922.

(25) Wu, Z.; Senchuk, M. M.; Dues, D. J.; Johnson, B. K.; Cooper, J. F.; Lew, L.; Machiela, E.; Schaar, C. E.; DeJonge, H.; Blackwell, T. K.; Van Raamsdonk, J. M. Mitochondrial unfolded protein response transcription factor ATFS-1 promotes longevity in a long-lived mitochondrial mutant through activation of stress response pathways. *BMC Biol.* **2018**, *16*, 147.

(26) Wu, C. W.; Wang, Y.; Choe, K. P. F-Box Protein XREP-4 Is a New Regulator of the Oxidative Stress Response in Caenorhabditis elegans. *Genetics* **2017**, *206*, 859−871.

(27) Shore, D. E.; Carr, C. E.; Ruvkun, G. Induction of cytoprotective pathways is central to the extension of lifespan conferred by multiple longevity pathways. *PLoS Genet.* **2012**, *8*, No. e1002792.

(28) Bilal, B.; Azim, M. K. Nematicidal activity of 'major royal jelly protein'-containing glycoproteins from Acacia honey. *Exp. Parasitol.* **2018**, *192*, 52−59.

(29) Liu, F.; Yang, Z.; Zheng, X.; Luo, S.; Zhang, K.; Li, G. Nematicidal coumarin from Ficus carica L. *J. Asia-Pac. Entomol.* **2011**, *14*, 79−81.

(30) Li, G. H.; Dang, L. Z.; Hong, L. J.; Zheng, L. J.; Liu, F. F.; Li, L.; Zhang, K. Q. Nematicidal Activity of Honokiol and Magnolol Isolated fromMagnolia tripetala. *J. Phytopathol.* **2009**, *157*, 390−392.

(31) Andrés, M. F.; González-Coloma, A.; Sanz, J.; Burillo, J.; Sainz, P. Nematicidal activity of essential oils: a review. *Phytochem. Rev.* **2012**, *11*, 371−390.

(32) Satyal, P.; Woods, K. E.; Dosoky, N. S.; Neupane, S.; Setzer, W. N. Biological activities and volatile constituents of Aegle marmelos (L.) Corrêa from Nepal. *J. Med. Act. Plants* **2012**, *1*, 114−122.

(33) Chanotiya, C. S.; Pragadheesh, V. S.; Yadav, A.; Gupta, P.; Lal, R. K. Cyclodextrin-based Gas Chromatography and GC/MS methods for determination of chiral pair constituents in mint essential oils. *J. Essent. Oil Res.* **2021**, *33*, 23−31.

(34) Afolayan, A. J.; Bvenura, C. Essential oil composition and phytotoxicity effects of Solanum nigrum L. on seed germination of some vegetables. *Appl. Ecol. Environ. Res.* **2018**, *16*, 553−561.

(35) Chirumamilla, P.; Dharavath, S. B.; Taduri, S. GC-MS profiling and antibacterial activity of Solanum khasianum leaf and root extracts. *Bull. Natl. Res. Cent.* **2022**, *46*, 127.