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Research Article

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New insights on the utilization of ultrasonicated mustard seed cake: chemical composition and antagonistic potential for root-knot nematode, *Meloidogyne javanica*

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Abstract: This study focused, for the first time, on the effect of ultrasonic features on the extraction efficiency of secondary metabolites in mustard seed cake (MSC). The nematostatic potential of sonicated seed cake was examined against the second-stage juveniles (J2s) of root-knot nematode, *Meloidogyne javanica*. The results show that a 35 ppm (parts per million) concentration of a sonicated extract (SE) sample of MSC caused 65% J2s mortality at 18 h exposure period in vitro. It also significantly suppressed the root-knot index (RKI=0.94) in tomato roots. The lethal concentration values for SE were 51.76, 29.79, and 13.34 ppm, respectively, at 6, 12, and 18 h of the exposure period, and the lethal concentration values for the non-sonicated extract (NSE) sample were 116.95, 76.38, and 55.59 ppm, respectively, at similar exposure time. Sinapine and gluconapin were identified as the major compounds in ultrasonic-assisted MSC. Because of the high extraction efficiency of metabolites in the SE, all treatments of SE were shown to be antagonistic to J2s. Thus, this study of ultrasonication activity-based profiling of MSC may help generate target-based compounds at a scale relevant to the control of disease caused by nematodes in economic crops.

Key words: Plant-parasitic nematode; Plant by-product; Sono-extraction; Liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS); Natural compound; Nematode management

1 Introduction

The *Meloidogyne* genus (root-knot nematode) has a worldwide distribution and is the cause of severe crop losses worldwide (Wesemael et al., 2011). Of 70 known *Meloidogyne* spp., only *Meloidogyne javanica*, *Meloidogyne incognita*, *Meloidogyne arenaria*, and *Meloidogyne hapla* are of major economic importance. The severity of damage caused by *Meloidogyne* spp. is often species-specific and varies depending on plant species, crop rotation, season, and soil type (Potter and Olthof, 1993). The key and most common symptom of infected plants is the appearance of root galls within their root systems, in which the *Meloidogyne* spp. are embedded.

The infected host plants are thus severely affected, and the plants become susceptible to wilting, growth reduction, and infection by other plant pathogens. Effective management methods to combat root-knot nematodes include synthetic nematicides, which is the preferred method for farmers in most countries (Whitehead, 1997). However, these synthetic nematicides harm our natural environment and are especially harmful to non-target organisms of the host plants. In recent years, a new environmentally friendly direction based on plant by-products has been developed as an option to achieve sustainability in agriculture (Rahman and Somers, 2005; Valdes et al., 2012). Advances in sustainability science, including the recent development of coupled plant by-product sono-extraction, provide a new context for integrating knowledge about compound extraction. In addition, the selection of plant

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species and their by-products is crucial. An improperly selected plant species/by-product reduces or harms populations of beneficial soil microorganisms that prey on plant-parasitic nematodes (Henderson et al., 2009). In this context, identifying plant species and their by-products that act as antinematodal agents and the elucidating of their mechanism of action might serve the purpose of controlling nematode infection.

Brassicaceous crops and their by-products, such as seed cake, although often utilized for nematode control, host a wide range of plant-parasitic nematodes, including *Meloidogyne* spp. (Rahman and Somers, 2005; Valdes et al., 2012; Fourie et al., 2016). The plant by-products resulting from oil extraction are largely defatted seed cakes, with 32.02 Mt produced in 2019 in the European Union alone (FEDIOL, 2020). Thus, its disposal and its evaluation as a by-product are a global challenge. Recently, oil cakes have become an attractive source for producing bioactive substances such as phenolic and flavonoid compounds (Ramachandran et al., 2007; Teh and Birch, 2014). Similarly, the biocidal compounds released from the seed cake of *Brassica* spp. were found to be toxic to soil-borne diseases and pests (Rahman and Somers, 2005; de Corato et al., 2015). Secondary metabolites of seed cake are enclosed in rigid and insoluble structures such as vacuoles, lipoprotein bilayers, lignin, hull, and cell walls (Corrales et al., 2008). So the choice of extraction method is critical. For sufficient adoption, techniques and tools are needed to resolve research challenges for filling gaps regarding promising secondary metabolites for nematode management strategies. Our research interest has concentrated on increasing the extraction efficiency for secondary metabolites of mustard seed cake (MSC) for further use.

Ultrasonication is one of several rapidly emerging techniques devised to enhance quality and processing, minimize the use of organic solvents, and maximize the bioavailability of organic products (Knorr et al., 2011; Rincón et al., 2013). The representative range for the frequency that is generally used in these ultrasonic techniques lies between 20 kHz and 500 MHz (Yusaf and Al-Juboori, 2014). During the extraction process, a considerable part of the energy of the ultrasonic waves converts into thermal energy. Ultrasonic waves pass through a conductive medium while putting pressure on the medium. Ultrasound ranging from 20 to 100 kHz is used in chemically critical systems in which chemical and physical changes are required, as it can cause cavitation (Pilli et al., 2011). A significant advantage of this technique over other tools that are used to characterize materials is that it is a non-invasive, non-destructive, economic, eco-friendly, and sustainable technology and can operate in concentrated and solid materials (Teh and Birch, 2014). Potential applications of ultrasonic techniques have been reported and discussed to understand changes of content as well as extraction capacity of secondary metabolites, in particular phenolics, polyphenol content, and antioxidants within the extracted samples of various seed cakes undergoing ultrasonic treatment (Teh and Birch, 2014; Khalili and Dinani, 2018). When ultrasonic treatment is applied to a liquid, ultrasonic waves are tied with a cyclic succession of expansion (rarefaction) and compression phases imparted by mechanical vibration (Tang, 2003). This technique has high efficiency and low extraction time in accelerating promising natural compounds (Picó, 2013).

In this study, experiments were conducted to evaluate the nematostatic potential of ultrasonic-assisted MSC against the second-stage juveniles (J2s) of *M. javanica* and to assess the potential strength of sonicated extract (SE) samples when used in various experimental strategies. In addition, we characterized the composition of secondary metabolites, including nematostatic compounds, in the SE samples by liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS). We also investigated the morphological variation of ultrasonic-assisted MSC by scanning electron microscopy (SEM). The current method can be applied as a sustainable approach to enhancing secondary metabolite extraction efficiency in MSC extract.

2 Materials and methods

2.1 Materials and reagents

Defatted cake of *Brassica campestris* L. (mustard) was obtained from the Oil Seed Extractions Mill of Aligarh, India. Air-dried seed cake was ground with a blender (Black & Decker BXBL6001IN, USA) to produce the powder. The dried powder was then passed through a 300-mesh-size sieve, placed in a plastic bag, and stored in an airtight container at 4 °C prior to analysis. Methanol and acetone of analytical grade were purchased from Sisco Research Laboratories

(SRL) Pvt. Ltd., India. Ultra-pure water was obtained from the Thermo Scientific Barnstead Ultrapure Water System (Gen Pure Pro UV-TOC/UF, Sweden).

2.2 Ultrasonic bath treatment of mustard seed cake

MSC powder (10 g) was prepared with 100 mL of the solvent mixture in a glass beaker. The solvent mixture composition was prepared with methanol, acetone, and water in a 7:7:6 volume ratio and then loaded in a digital ultrasonic bath (Labman Scientific Instruments Pvt. Ltd., India. The digital ultrasonic bath was set at 40 kHz and 100 W of power at room temperature for 35 min. The filtrates were then placed in a centrifuge at 3000 r/min for 15 min, and the supernatant was collected in an amber reagent bottle before sample preparation for analysis. This sample was termed sonicated extract (SE).

2.3 Non-ultrasonic bath treatment of mustard seed cake

MSC (10 g) prepared in 100 mL of mixed solvent (methanol, acetone, water in a 7:7:6 volume ratio). The mixture was stirred in a conical flask with a magnetic stirrer at room temperature for 35 min. Filtrates were then placed in the centrifuge at 3000 r/min for 15 min, and the supernatant was collected in an amber reagent bottle before sample preparation for analysis. This sample was termed non-sonicated extract (NSE).

2.4 SEM of sonicated extract

The morphological alterations in the extracted samples were analyzed using SEM (JSM 6510 LV, Jeol, Tokyo, Japan). Dry filtrates were obtained by heating with a burner for approximately 1 min, and the precipitates were scratched out in a 2-mL Eppendorf tube for SEM analysis. The dried filtrates were carefully placed on a clean aluminium stub which had double-sided tape and was gold-coated (JEOL JFC-1600 fine auto coater) with a layer about 14 nm thick. After this, the aluminium stubs were loaded into the SEM for imaging.

2.5 Sample preparation

The collected supernatants of both SE and NSE were further filtered separately via a syringe filter of 0.22 μm diameter (Millipore, Germany). The filtrates were diluted with methanol to the final working concentration for analysis. The sample solutions were stored at −20 °C until use and vortexed before injection into the LC-ESI-MS system for analysis. A mere 0.50-μL aliquot was found to be sufficient for injecting into the LC-ESI-MS system.

2.6 LC-MS analysis

The analysis of the MSC extract was performed on an accelerated ultra-performance liquid chromatography (UPLC) system equipped with a pump, autosampler, and photodiode array (PDA) detector. A tandem quadrupole detector (TQD) triple quadrupole ESI ion trap mass spectrometer was coupled to the LC system (Waters, Milford, MA, USA). The UPLC column used was an Accucore C18 column (150 mm×2.1 mm, 2.6 μm). Dual-mode (±) LC-ESI-MS experiments were performed after injecting 0.50 μL extract with the autosampler. Water acidified with 0.1% (volume fraction) formic acid (Eluent A) and acetonitrile acidified with 0.1% (volume fraction) formic acid (Eluent B) were used as solvent at a constant flow rate of 0.250 μL/min. The column temperature was maintained at 30 °C. The elution gradient used was: 0 min (5% B), 0–1 min (5% B), 1–6 min (5% to 30% B), 6–12 min (30% to 60% B), 12–16 min (60% B), 16–20 min (60% to 80% B), 20–24 min (80% B), 24–26 min (80% to 5% B), and 26–30 min (5% B).

MS analysis was performed on a TQD triple quadrupole equipped with an ESI-MS probe coupled to UPLC. Mass spectra were recorded in a mass-to-charge ratio (m/z) range of 150–2000 Da, and data were acquired and processed using the MassLynx™ software (Waters Corporation). Accurate masses of selected metabolites were observed and compared with their exact mass (theoretical mass) values manually. The m/z values of identified compounds with retention time are listed in Tables 1 and 2.

2.7 Isolation, culture amplification, and identification of *M. javanica*

The *M. javanica* (root-knot nematode isolate) inoculum was produced on susceptible eggplant plants. Nematode egg masses were extracted from the infected roots. The egg masses were then placed on a sieve (8.5 cm in diameter), which was already layered with tissue papers over glass Petri plates (10 cm in diameter) at 25 °C to obtain J2s as inoculum. J2s were collected from the bottom of the Petri plate, and those that emerged from 2 to 5 d were stored at 9 °C. Aliquots of the J2s suspensions were placed in growth chambers

for 8 h before inoculation to acclimatize J2s to the testing temperatures.

SEM was used for species identification. An adult female of *M. javanica* was dissected from the infected eggplant root, and the perineal pattern was prepared according to the technique previously described by de O. Abrantes and de A. Santos (1989). The adult female was placed in a few drops of lactic acid (45%) on a transparent glass slide. The posterior portion with cuticular markings surrounding the vulva and anus of the female was cut using a sharp blade to obtain the perineal pattern. The cut section was then transferred onto another glass slide, and a drop of lactic acid (45%, volume fraction) was placed on a round coverslip, which was rimmed with a thick ring of glycerol. A few drops of formalin (2%, volume fraction) were then added to the glycerol every 2–3 min to wash out the lactic acid. The perineal patterns were allowed to dry in a glass desiccator at room temperature. Before SEM analysis, the coverslip was detached from the glass slide, mounted onto an SEM stub with double-sided adhesive tape, and coated with 14 nm of gold. It was then examined in the SEM (JSM 6510 LV, Jeol, Tokyo, Japan). The surface morphology of the perineal pattern of *M. javanica* was reviewed from the SEM images (Fig. 1). The key features of the perineal pattern were observed. These were the presence of a low dorsal arch, smooth striae, a tail whorl often distinct, a distinct lateral field, and demarcation from the striae by more or less parallel lines.

2.8 J2s mortality and J2s mobility recover tests

For the mortality test, 200 J2s were incubated in small Petri plates of 5 cm in diameter, containing 2 mL of SE and NSE samples extracted separately. The mortality test was exposed to varying concentrations of extracted samples, namely, 5, 15, 25, and 35 ppm (parts per million). J2s kept in Petri plates filled with only 2 mL distilled water (DW) were considered as control. J2s were washed three times with water and re-suspended in a counting dish, and both mobile and dead J2s were recorded at a 6, 12, and 18 h interval

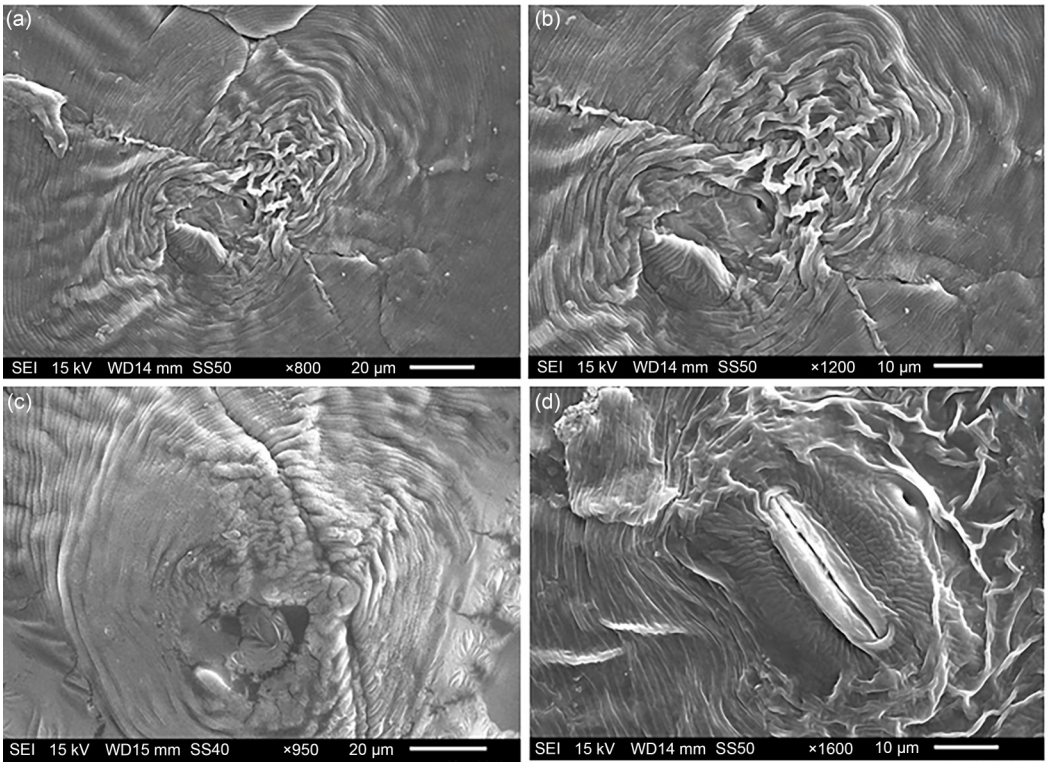

Fig. 1 Scanning electron microscopy (SEM) images of the perineal pattern of *M. javanica*, which show a rounded to flattened dorsal arch and conspicuous lateral lines that separated the dorsal and ventral regions of the patterns. The SEM images were obtained and analyzed at ×800 (a) and ×1200 (b) magnification levels. (c) A close view of the distinct lateral line in a perineal pattern distinguishes this species from other *Meloidogyne* spp. (d) An inner area was marked by coarsely broken striae and contained the vulva and anus.

from the incubation period. J2s without detectable movement were considered dead. The mortality test was performed with four replicates. Subsequently, a J2s mobility recovery test was also performed. The recovery strength of J2s was analyzed after 18 h of incubation in 35 ppm of SE samples. To confirm recovery strength, J2s were washed three times and re-suspended in water to check for movement. Mobile and immobile J2s were counted at 1, 3, 5, and 7 d to examine their capability to recover mobility. Maximum immobility (65%) was observed on the first day after incubation, but a significant recovery in J2s mobility was also found at 3 d. Furthermore, the percentage of immobile J2s (40%) at 5 d after 18 h of incubation in the SE sample (35 ppm) was not significantly different from that in a similar suspension at 7 d, indicating that J2s cannot recover further. These data collectively indicate that an SE solution (35 ppm) has a toxic effect on J2s (*M. javanica*) mobility.

2.9 Root-knot index assessment-pot study

Bare root-dip treated tomato seedlings (two weeks old) in a 35-ppm extract of MSC were transplanted into 15-cm-diameter clay pots filled with 1.5 kg autoclaved soil mixtures (sand and organic matter). Before transplanting, part of the root was dipped in 35 ppm of SE and NSE separately for a 30-min duration. Three days after the transplantation, about 2500 J2s of *M. javanica* were root inoculated into each tomato plant. The tomato seedlings without J2s treatment were considered as control. Post-transplantation, each tomato seedling was irrigated with water regularly to maintain moisture in the rhizospheric soil. Four replicates were set up for each treatment. Sixty days later, the pot experiment was terminated for assessment of root-knot index (RKI) severity in the tomatoes. The RKI was calculated using the standard scale method of 0 to 5, where 0=no galls, 1=1 to 2 galls, 2=3 to 10 galls, 3=11 to 30 galls, 4=31 to 100 galls, and 5=more than 100 galls (Taylor and Sasser, 1978).

2.10 Data analysis

Statistical data analysis was carried out with the open-source software R Version 2.14.1. The significance of differences among treatments was determined by Duncan's multiple range test (DMRT). According to DMRT at $P \leqslant 0.05$, means depicted by the same letter are not significantly different. Dose-response probabilistic (Probit) models were used on J2s mortality data to determine the lethal concentration of MSC filtrates that kill J2s of *M. javanica* by 50% (LC_{50}).

3 Results

3.1 SEM of the mustard seed cake extract

SEM images provided important foundational observations to acquire morphological alterations in the target samples. The SEM images were studied closely to observe the surface morphology. Fig. 2 shows the SEM images of the SE and NSE at different magnification levels. Figs. 2a–2d show pictures of an NSE sample (control set-up). These images represent smooth surfaces without pores or fissures and intact surface structures, firmly bonded to each other. In comparison, pictures of an SE depict uneven and disrupted microstructures (Figs. 2e–2h).

3.2 Ultrasonication impacts on the production of secondary metabolites and identified mass profiles

By taking advantage of these ultrasonic treatments, we identified 17 secondary metabolites, including nematostatic compounds, in the extract of MSC. The LC-ESI-MS spectra of SE are presented in Figs. 3 and 4. These compounds were identified as 3-methylsulfinyl propyl isothiocyanate, sinapine, 1-methoxybrassitin, 4-hydroxybenzyl glucosinolates, patuletin 3-gentiobioside, isorhamnetin 3,7-di-*O*-β-glucopyranoside, rutalexin, benzeneacetaldehyde, 5-methylthiopentylglucosinolate, campesterol, coumestrol, methyl oleate, 4-hydroxyglucobrassicin, gluconapin, Se-methylselenocysteine, methoxybrassinin, and indole-3-acetonitrile, and their corresponding peak ion masses were found at *m/z* 163.1, 310.2, 496.2 $[M+2H]^+$, 458.2 $[M+CH_3OH+H]^+$, 656.2, 682.3 $[M+ACN+H]^+$, 274.3 $[M+ACN+H]^+$, 162.2 $[M+ACN+H]^+$, 477.1 $[M+ACN+H]^+$, 400.1, 301.2 $[M+CH_3OH+H]^+$, 338.3 $[M+ACN+H]^+$, 885.5 $[M+ACN+2H]^+$, 372.1 $[M-H]^-$, 545.1 $[3M-H]^-$, 265.2 $[M-H]^-$, and 311.1 $[2M-H]^-$, respectively (Figs. 3 and 4). Among identified compounds, sinapine was found as the most abundant compound, followed by gluconapin, with a relative percentage of peak area of 34.5% and 32.2%, respectively (Tables 1 and 2). The percent peak areas of all identified compounds of the SE sample compared with those of the NSE sample are illustrated in Tables 1 and 2. For example, the peak areas of identified compounds in SE

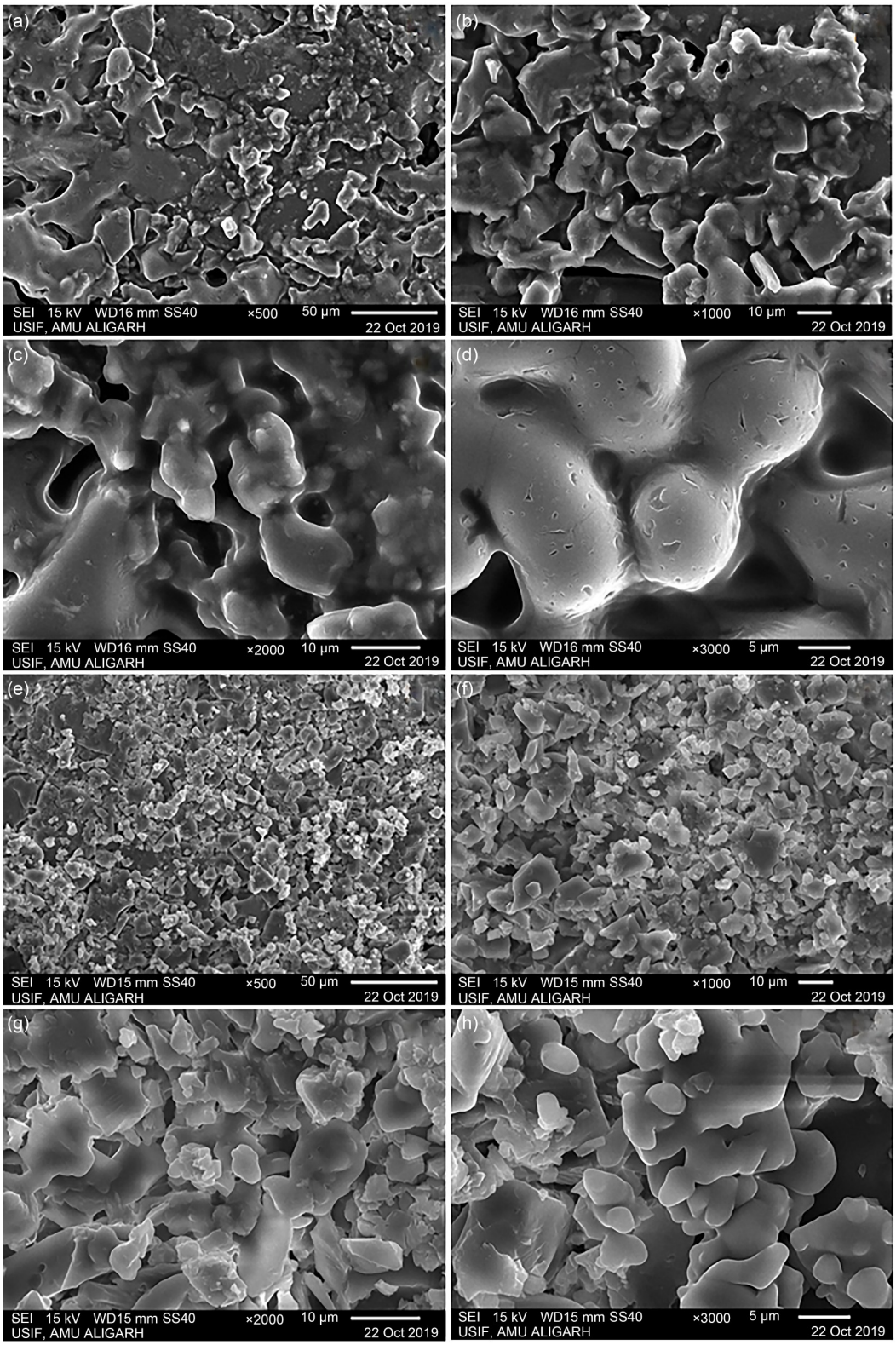

Fig. 2 Scanning electron microscopy (SEM) images of mustard seed cake (MSC) at ×500 (a, e), ×1000 (b, f), ×2000 (c, g), and ×3000 (d, h) magnification levels. (a–d) The surface morphology of a non-sonicated extract (NSE) sample; (e–h) The surface morphology of a sonicated extract (SE) sample. The images of the SE sample showing significant morphological alterations on the surface, which occurred via ultrasonication.

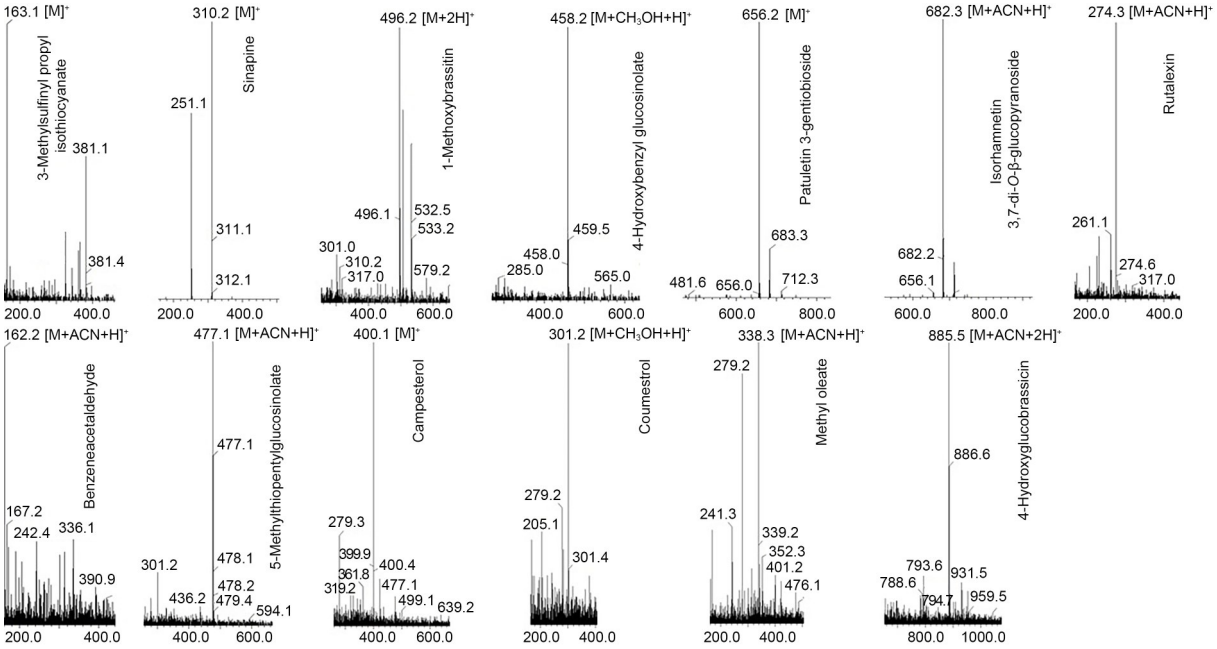

Fig. 3 LC-ESI-MS spectra of the SE samples acquired in positive ion mode. The mass peaks below *m*/*z* 1000 were selected for the identification of secondary metabolites. The compounds with mass peak ions are apparent: 3-methylsulfinyl propyl isothiocyanate, *m*/*z* 163.1 at RT=1.14 min; sinapine, *m*/*z* 310.2 at RT=4.76 min; 1-methoxybrassitin, *m*/*z* 496.2 $[M+2H]^+$ at RT=5.71 min; 4-hydroxybenzyl glucosinolates, *m*/*z* 458.2 $[M+CH_3OH+H]^+$ at RT=6.77 min; patuletin 3-gentiobioside, *m*/*z* 656.2 at RT=7.86 min; isorhamnetin 3,7-di-*O*-β-glucopyranoside, *m*/*z* 682.3 $[M+ACN+H]^+$ at RT=8.03 min; rutalexin, *m*/*z* 274.3 $[M+ACN+H]^+$ at RT=11.44 min; benzeneacetaldehyde, *m*/*z* 162.2 $[M+ACN+H]^+$ at RT=14.20 min; 5-methylthiopentylglucosinolate, *m*/*z* 477.1 $[M+ACN+H]^+$ at RT=14.72 min; campesterol, *m*/*z* 400.1 at RT=17.10 min; coumestrol, *m*/*z* 301.2 $[M+CH_3OH+H]^+$ at RT=18.50 min; methyl oleate, *m*/*z* 338.3 $[M+ACN+H]^+$ at RT=22.22 min; 4-hydroxyglucobrassicin, *m*/*z* 885.5 $[M+ACN+2H]^+$ at RT=26.07 min. LC-ESI-MS: liquid chromatography-electrospray ionization-mass spectrometry; SE: sonicated extract; RT: retention time.

were 13.0% (isorhamnetin 3,7-di-*O*-β-glucopyranoside), 8.4% (5-methylthiopentylglucosinolate), 8.2% (patuletin 3-gentiobioside), 7.8% (rutalexin), and 6.2% (3-methylsulfinyl propyl isothiocyanate) at retention time of 8.03, 14.72, 7.86, 11.44, and 1.14 min, respectively, in positive mode of LC-ESI-MS. In negative mode, we also noted the percent peak areas as 32.2% (gluconapin), 16.5% (methoxybrassinin), 8.2% (Se-methylselenocysteine), and 2% (indole-3-acetonitrile), respectively, at retention time of 1.47, 14.12, 6.17, and 19.92 min. At the same retention time and instrumental set-up, the percent areas of peak ions were observed to be lower in the NSE than in the SE. Thus, it is worth considering that the identified compounds in SE show a higher percentage of peak area.

3.3 In vitro nematostatic effects of sonicated extract and non-sonicated extract samples on J2s mortality

Table 3 shows the variation in the rate of J2s death in the aqueous concentration of extract at different incubation periods. We observed that J2s mortality was nil in water (control). In the parallel study, the mortality rate of J2s was highest (65.00%) at 18-h incubation period in a 35-ppm concentration of SE, while the least mortality (6.25%) was seen in 5-ppm NSE. The pattern of J2s death found was in direct proportion to the concentration strength and incubation period in both types of extract samples. All the concentrations of both SE and NSE showed an impact on J2s mortality; however, the mortality rate of J2s was much higher in different concentrations of SE.

3.4 LC_{50} of sonicated extract and non-sonicated extract samples against J2s of *M. javanica*

The LC_{50} value was determined for mortality in J2s after immersion in SE and NSE samples at three different durations of exposure: 6, 12, and 18 h. The LC_{50} values of SE at 6, 12, and 18 h of exposure duration were 51.76, 29.79, and 13.34 ppm, respectively, for J2s mortality, while for NSE, the respective LC_{50} values

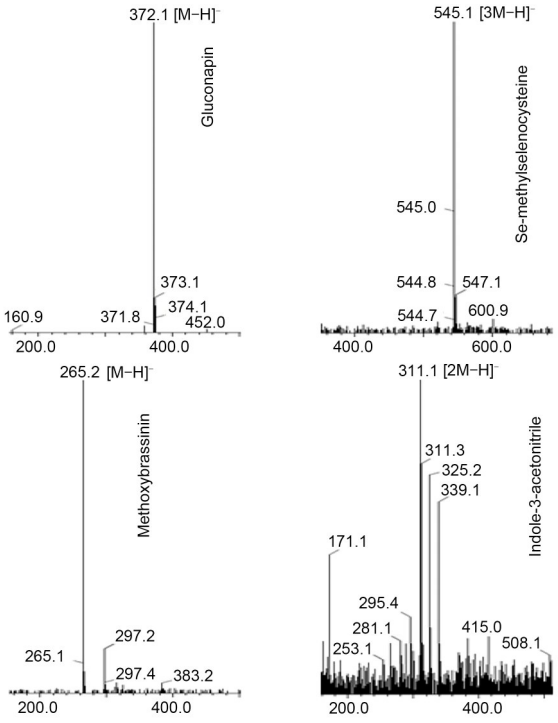

Fig. 4 LC-ESI-MS spectra of the SE sample acquired in negative ion mode. The mass peaks below *m*/*z* 1000 were selected for the identification of compounds. The compounds with mass peak ions are apparent: gluconapin, *m*/*z* 372.1 [M−H]⁻ at RT=1.47 min; Se-methylselenocysteine, *m*/*z* 545.1 [3M−H]⁻ at RT=6.17 min; methoxybrassinin, *m*/*z* 265.2 [M−H]⁻ at RT=14.12 min; indole-3-acetonitrile, *m*/*z* 311.1 [2M−H]⁻ at RT=19.92 min. LC-ESI-MS: liquid chromatography-electrospray ionization-mass spectrometry; SE: sonicated extract; RT: retention time.

were 116.95, 76.38, and 55.59 ppm at a similar exposure time. This result indicates that the value of LC_{50} was higher to kill half of the J2s population during the short exposure time, while for a longer duration of exposure, the obtained value of LC_{50} was lower (Table 4). The LC_{50} response of SE was observed to be most effective in J2s mortality compared to the tested LC_{50} response of NSE at a similar duration of exposures (Table 4). Probit analysis was performed to obtain LC_{50} with 95% confidence limits.

3.5 In vitro reversibility test of J2s

This test was conducted to understand the recovery activity of paralyzed J2s of *M. javanica* in the SE sample. The J2s incubated in SE were found incapable of movement on the first day after transfer to the water. Still, mobility in J2s significantly increased when J2s were maintained in water for a week. The data of percent paralyzed J2s are shown in Fig. 5 at the 1st, 3rd, 5th, and 7th days. However, we observed that mobility in J2s did not return to levels comparable to the control at the end of the week. The results indicated that the SE could contain a high enough capacity of nematostatic compounds to kill the J2s. In this study, we also noted that if the incubation period of treatment is considered short, then treated J2s, after washing in water, would recover from paralysis within a week or two.

Table 1 Secondary metabolites in the extracted sample of mustard seed cake identified by LC-ESI-MS in positive mode

RT (min)	Secondary nematostatic compound	Molecular ion mass (*m*/*z*)	Adduct molecular ion mass (*m*/*z*)	Area (%)	
				SE	NSE
1.14	3-Methylsulfinyl propyl isothiocyanate	163.01		6.2	6.0
4.76	Sinapine	310.16		34.5	26.3
5.71	1-Methoxybrassitin	250.07	496.2 $[M+2H]^+$	3.6	PND
6.77	4-Hydroxybenzyl glucosinolate	425.04	458.2 $[M+CH_3OH+H]^+$	2.2	0.9
7.86	Patuletin 3-gentiobioside	656.15		8.2	6.5
8.03	Isorhamnetin 3,7-di-*O*-β-glucopyranoside	640.16	682.3 $[M+ACN+H]^+$	13	PND
11.44	Rutalexin	232.03	274.3 $[M+ACN+H]^+$	7.8	5.3
14.20	Benzeneacetaldehyde	120.05	162.2 $[M+ACN+H]^+$	2.2	2.0
14.72	5-Methylthiopentylglucosinolate	435.06	477.1 $[M+ACN+H]^+$	8.4	6.3
17.10	Campesterol	400.37		2.8	2.8
18.50	Coumestrol	268.03	301.2 $[M+CH_3OH+H]^+$	3.7	2.0
22.22	Methyl oleate	296.27	338.3 $[M+ACN+H]^+$	2.6	2.5
26.07	4-Hydroxyglucobrassicin	464.05	885.5 $[M+ACN+2H]^+$	4.8	2.3

RT: retention time; ACN: acetonitrile; PND: peak not determined; SE: sonicated extract; NSE: non-sonicated extract; *m*/*z*: mass-to-charge ratio, considered as the mass; LC-ESI-MS: liquid chromatography-electrospray ionization-mass spectrometry.

Table 2 Secondary metabolites in the extracted sample of mustard seed cake identified by LC-ESI-MS in negative mode

RT (min)	Secondary nematostatic compound	Molecular ion mass (*m*/*z*)	Adduct molecular ion mass (*m*/*z*)	Area (%)	
				SE	NSE
1.47	Gluconapin	373.05	372.1 $[M-H]^-$	32.2	25.4
6.17	Se-Methylselenocysteine	182.97	545.1 $[3M-H]^-$	8.2	PND
14.12	Methoxybrassinin	266.05	265.2 $[M-H]^-$	16.5	12.3
19.92	Indole-3-acetonitrile	156.06	311.1 $[2M-H]^-$	2.0	1.8

ACN: acetonitrile; PND: peak not determined; RT: retention time; SE: sonicated extract; NSE: non-sonicated extract; *m*/*z*: mass-to-charge ratio, considered as the mass; LC-ESI-MS: liquid chromatography-electrospray ionization-mass spectrometry.

Table 3 In vitro nematostatic impact of MSC samples on the J2s of *M. javanica* after a 6, 12, and 18 h incubation periods

Treatment	Incubation period (h)	Mortality of J2s nematodes (%)				
		5 ppm	15 ppm	25 ppm	35 ppm	DW
SE	6	13.75±1.49^d	22.00±1.29^c	33.50±1.70^b	45.50±1.93^a	0
	12	21.75±1.37^d	32.25±1.49^c	42.25±1.49^b	56.75±2.01^a	0
	18	31.50±1.04^d	42.00±1.47^c	51.75±1.75^b	65.00±1.58^a	0
NSE	6	6.25±0.85^d	13.75±1.10^c	22.25±1.49^b	30.75±1.25^a	0
	12	9.50±1.04^d	18.00±1.29^c	27.00±1.68^b	37.50±1.70^a	0
	18	12.25±1.10^d	23.25±1.49^c	33.50±1.70^b	43.00±1.47^a	0

Values expressed as mean±standard error of four measurements, with different superscript letters within groups, are significantly different ($P \leqslant 0.05$). MSC: mustard seed cake; SE: sonicated extract; NSE: non-sonicated extract; J2s: the second stage juveniles; ppm: parts per million; DW: distilled water.

Table 4 LC_{50} of MSC extract necessary for mortality in J2s of *M. javanica* as determined by Probit analysis

Treatment	LC_{50} with 95% confidence limit (ppm)		
	6 h	12 h	18 h
SE	51.76	29.79	13.34
NSE	116.95	76.38	55.59

LC_{50}: lethal concentration of MSC filtrates that kill J2s of *M. javanica* by 50%; MSC: mustard seed cake; J2s: the second stage juveniles; SE: sonicated extract; NSE: non-sonicated extract; ppm: parts per million; Probit: probabilistic.

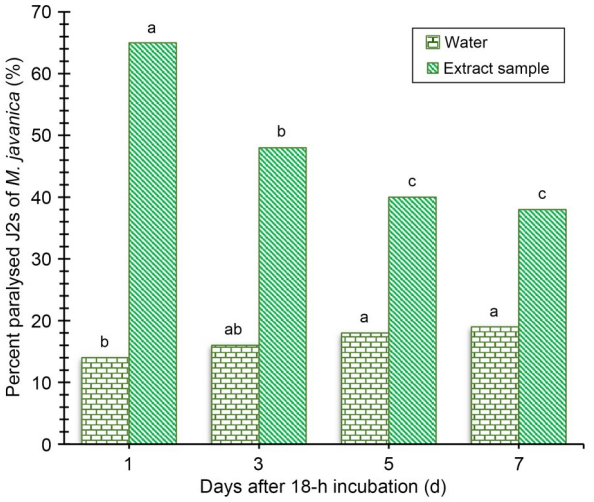

Fig. 5 In vitro reversibility test of paralyzed J2s of *M. javanica*. J2s were transferred to water after 18-h incubation in 35 ppm of the SE sample to determine reversibility. Data with the same letters for water or extract sample are not significantly different (P>0.05). J2s: the second stage juveniles; ppm: parts per million; SE: sonicated extract.

3.6 Root-knot index assessment in tomato roots

In a pot experiment, J2s-infected roots of tomato plants were analyzed. Specifically, the RKI was considered as the basis for analysis. We observed that the roots were severely knotted in the experiment's control set-up, while fewer and smaller root knots were developed in the root dip-treated tomato plants with SE and NSE. The RKIs of the control plants, NSE-treated plants (35-ppm), and SE-treated plants (35-ppm) were 4.65, 1.50, and 0.94, respectively (Table 5). We also noted that the RKI in plants treated with SE was significantly reduced with increased concentrations. Various levels of NSE also limited the RKI, although less effectively. Interestingly, an SE of 35-ppm concentration used as a root-dip treatment significantly ($P \leqslant 0.05$) controlled root-knot infection in tomato roots (Table 5).

Table 5 Effects of different concentrations of SE and NSE on root-knot-producing nematode, *M. javanica*, in a pot experiment

Treatment	Concentration (ppm)	RKI
SE	5	3.070±0.047[c]
	15	2.710±0.031[b]
	25	1.850±0.064[e]
	35	0.940±0.027[g]
NSE	5	3.850±0.028[b]
	15	3.070±0.047[c]
	25	2.650±0.064[d]
	35	1.500±0.129[f]
Control		4.650±0.064[a]

The RKI data of tomato plants are shown as mean±standard error (n=4). Means with the same letter are not significantly different (P> 0.05) at the probability level, according to Duncan's multiple range test. RKI: root-knot index; SE: sonicated extract; NSE: non-sonicated extract; ppm: parts per million.

4 Discussion

Natural compounds are generally enclosed in rigid and insoluble structures such as vacuoles, bilayers, lignin, and cell walls (Corrales et al., 2009). These factors have prevented the extraction of secondary metabolite compounds from seed cakes by straightforward extraction methods. This is why we used the ultrasonication technique to enhance the extraction efficiency of secondary nematostatic compounds. Seventeen secondary metabolites, including nematostatic compounds, were identified in the SE sample. The ultrasonic features assisted in releasing the metabolites in SE. Similar observations have also been reported in other seed cake samples, such as defatted hemp, flax, and canola seed cakes (Teh and Birch, 2014). The effectiveness of ultrasound power was observed in enhancing polyphenol extraction. Willis (2017) reported approximately 400 000 extant species of vascular plants on earth, which are the source of hundreds of thousands of metabolites whose utility has not been well explored. Ultrasound technology will undoubtedly augment their role as a sustainable approach, having the additional capacity to extract an enormous repertoire of specialized (or secondary metabolite) compounds (Alseekh and Fernie, 2018).

In vitro results demonstrated that when the concentrations of extract of 5, 15, 25, and 35 ppm were tested individually against J2s of *M. javanica*, a precise compelling level of extract strength was observed. These results are the first to demonstrate the contact nematostatic potential of the SE of MSC. The mortality of J2s was severe when exposed to SE because of the high extraction efficiency of compounds in the samples via ultrasonic treatment. The results are in accordance with earlier reports (Corrales et al., 2009; Ntalli and Caboni, 2012; Dahlin and Hallmann, 2020), which reported that mustard plants and their by-products possess antinematode and antimicrobial properties. In the literature, plant by-products belonging to the family Brassicaceae are probably the most cited due to the presence of isothiocyanate and related hydrolysis products, which include a broad spectrum of biocidal activities (Matthiessen and Kirkegaard, 2006; Agerbirk and Olsen, 2012; Ntalli and Caboni, 2012; Dahlin and Hallmann, 2020). Sinapine, a choline ester of sinapic acid, is one of the significant phenolic choline esters and occurs at high concentrations in Brassicaceae seeds (Zhou et al., 2005), which was also detected in this study as a significant compound. The antinematode properties of this phenolic compound have also been reported by Vaganan et al. (2014). The most considerable concentration was found to be 35 ppm with strong killing properties (LC_{50}=51.76, 29.79, and 13.34 ppm at 6, 12, and 18 h exposure, respectively (Table 4)). Thus, the collective activity of SE makes MSC likely to be a more promising plant-based natural agent acting against nematodes.

Pot experiment results demonstrate that the impacts of all SE treatments were significant compared to the NSE when applied as a bare-root dip treatment in tomato roots that were exhibited (Table 5). The mustard plants contain glucosinolate compounds and their derivatives, including isothiocyanates. These compounds have a marked anesthetic effect on nematodes (Ntalli and Caboni, 2012; Dahlin and Hallmann, 2020). Our results are strongly in agreement with previous reports of the active suppression of nematodes in soil amended with plant by-products of the family Brassicaceae (Matthiessen and Kirkegaard, 2006). A single mustard plant can contain several different types of glucosinolates. The types and quantities of GSLs are highly variable among the species and even among the cultivars of mustard (Kruger et al., 2013). These nematostatic compounds are generally safe in our ecosystem (Ntalli and Caboni, 2012).

5 Conclusions

We determined the strength of the MSC extract solution, which can be strongly stimulated by ultrasound features and has shown potent antagonistic activity to J2s of *M. javanica*. The nematostatic action was due to the high extraction efficiency of nematostatic compounds in the SE sample. A significant alteration was also observed in SEM images on the surface of ultrasonic-assisted MSC, which might be due to the phenomenon of the microstreaming effect. Sonication treatment induces a microstreaming effect and can enhance mass transfer produced on cavitational bubble collapse. This, in turn, results in the cell wall destruction of plant tissues, thus providing better contact and interactions of solvents in and out of the plant materials. Indeed, ultrasonic features supported the extraction efficiency in the target sample. Because of these features, secondary metabolites were significantly enhanced in the SE, which caused J2s mortality, paralysis, and reduced infectivity in tomato plants. Sinapine and gluconapin were found as major compounds in the SE. Therefore, ultrasonically treated plant by-products can be explored for agricultural applications as an effective strategy for enhancing the extractability of natural compounds. They could efficiently play a significant role in preventing nematode disease.

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Author contributions

Hera NADEEM and Faheem AHMAD conceived and designed the experiments, interpreted the results, and drafted the manuscript. Hera NADEEM performed the experiments and analyzed the data. Pieter MALAN, Amir KHAN, Mohd ASIF, Mansoor AHMAD SIDDIQUI, and Simon TUHAFENI ANGOMBE commented and critically revised the manuscript. All authors have read and approved the final manuscript. All authors have full access to data in the study and take responsibility for the integrity and security of the data.

Compliance with ethics guidelines

Hera NADEEM, Pieter MALAN, Amir KHAN, Mohd ASIF, Mansoor AHMAD SIDDIQUI, Simon TUHAFENI ANGOMBE, and Faheem AHMAD declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

References

Agerbirk N, Olsen CE, 2012. Glucosinolate structures in evolution. *Phytochemistry*, 77:16-45. https://doi.org/10.1016/j.phytochem.2012.02.005

Alseekh S, Fernie AR, 2018. Metabolomics 20 years on: what have we learned and what hurdles remain? *Plant J*, 94(6):933-942. https://doi.org/10.1111/tpj.13950

Corrales M, Toepfl S, Butz P, et al., 2008. Extraction of anthocyanins from grape by-products assisted by ultrasonics, high hydrostatic pressure or pulsed electric fields: a comparison. *Innov Food Sci Emerg*, 9(1):85-91. https://doi.org/10.1016/j.ifset.2007.06.002

Corrales M, Han JH, Tauscher B, 2009. Antimicrobial properties of grape seed extracts and their effectiveness after incorporation into pea starch films. *Int J Food Sci Technol*, 44(2):425-433. https://doi.org/10.1111/j.1365-2621.2008.01790.x

Dahlin P, Hallmann J, 2020. New insights on the role of allyl isothiocyanate in controlling the root knot nematode, *Meloidogyne hapla*. *Plants*, 9(5):603. https://doi.org/10.3390/plants9050603

de Corato U, Pane C, Bruno GL, et al., 2015. Co-products from a biofuel production chain in crop disease management: a review. *Crop Prot*, 68:12-26. https://doi.org/10.1016/j.cropro.2014.10.025

de O Abrantes IM, de A Santos MSN, 1989. A technique for preparing perineal patterns of root-knot nematodes for scanning electron microscopy. *J Nematol*, 21(1):138-139.

FEDIOL, 2020. The EU oil and protein meal industry, world production data for plant oils and fatty acids. http://www.fediol.eu

Fourie H, Ahuja P, Lammers J, et al., 2016. Brassicaceae-based management strategies as an alternative to combat nematode pests: a synopsis. *Crop Prot*, 80:21-41. https://doi.org/10.1016/j.cropro.2015.10.026

Henderson DR, Riga E, Ramirez RA, et al., 2009. Mustard biofumigation disrupts biological control by *Steinernema* spp. nematodes in the soil. *Biol Control*, 48(3):316-322. https://doi.org/10.1016/j.biocontrol.2008.12.004

Khalili F, Dinani ST, 2018. Extraction of phenolic compounds from olive-waste cake using ultrasonic process. *J Food Meas Charact*, 12(2):974-981. https://doi.org/10.1007/s11694-017-9712-1

Knorr D, Froehling A, Jaeger H, et al., 2011. Emerging technologies in food processing. *Annu Rev Food Sci Technol*, 2:203-235. https://doi.org/10.1146/annurev.food.102308.124129.

Kruger DHM, Fourie JC, Malan AP, 2013. Cover crops with biofumigation properties for the suppression of plant-parasitic nematodes: a review. *South Afr J Enol Vitic*, 34(2):287-295. https://doi.org/10.21548/34-2-1107

Matthiessen JN, Kirkegaard JA, 2006. Biofumigation and enhanced biodegradation: opportunity and challenge in

soilborne pest and disease management. *Crit Rev Plant Sci*, 25(3):235-265. https://doi.org/10.1080/07352680600611543

Ntalli NG, Caboni P, 2012. Botanical nematicides: a review. *J Agric Food Chem*, 60(40):9929-9940. https://doi.org/10.1021/jf303107j

Picó Y, 2013. Ultrasound-assisted extraction for food and environmental samples. *TrAC Trends Anal Chem*, 43:84-99. https://doi.org/10.1016/j.trac.2012.12.005

Pilli S, Bhunia P, Yan S, et al., 2011. Ultrasonic pretreatment of sludge: a review. *Ultrason Sonochem*, 18(1):1-18. https://doi.org/10.1016/j.ultsonch.2010.02.014

Potter JW, Olthof THA, 1993. Nematode pests of vegetable crops. *In*: Evans K, Trudgill DL, Webser JM (Eds.), Plant-Parasitic Nematodes in Temperate Agriculture. CAB International, Wallingford, UK, p.171-207.

Rahman L, Somers T, 2005. Suppression of root knot nematode (*Meloidogyne javanica*) after incorporation of Indian mustard cv. Nemfix as green manure and seed meal in vineyards. *Australas Plant Pathol*, 34(1):77-83. https://doi.org/10.1071/AP04081

Ramachandran S, Singh SK, Larroche C, et al., 2007. Oil cakes, and their biotechnological applications—a review. *Biores Technol*, 98(10):2000-2009. https://doi.org/10.1016/j.biortech.2006.08.002

Rincón B, Bujalance L, Fermoso FG, et al., 2013. Biochemical methane potential of two-phase olive mill solid waste: influence of thermal pretreatment on the process kinetic. *Biores Technol*, 140:249-255. https://doi.org/10.1016/j.biortech.2013.04.090

Tang WZ, 2003. Physicochemical Treatment of Hazardous Wastes. CRC Press, Boca Raton, the US, p.439. https://doi.org/10.1201/9780203506219

Taylor AL, Sasser JN, 1978. Biology, Identification and Control of Root-knot Nematodes (*Meloidogyne* Species). North Carolina State University, *Raleigh*, USA, p.111.

Teh SS, Birch EJ, 2014. Effect of ultrasonic treatment on the polyphenol content and antioxidant capacity of extract from defatted hemp, flax and canola seed cakes. *Ultrason Sonochem*, 21(1):346-353. https://doi.org/10.1016/j.ultsonch.2013.08.002

Vaganan MM, Ravi I, Nandakumar A, et al., 2014. Phenylpropanoid enzymes, phenolic polymers and metabolites as chemical defenses to infection of *Pratylenchus coffeae* in roots of resistant and susceptible bananas (*Musa* spp.). *Indian J Exp Biol*, 52:252-260.

Valdes Y, Viaene N, Moens M, 2012. Effects of yellow mustard amendments on the soil nematode community in a potato field with focus on *Globodera rostochiensis*. *Appl Soil Ecol*, 59:39-47. https://doi.org/10.1016/j.apsoil.2012.03.011

Wesemael W, Viaene N, Moens M, 2011. Root-knotnematodes (*Meloidogyne* spp.) in Europe. *Nematology*, 13(1):3-16. https://doi.org/10.1163/138855410X526831

Whitehead AG, 1997. Plant Nematode Control. CAB International, Wallingford, UK.

Willis KJ, 2017. State of the World's Plants 2017. Royal Botanic Gardens, Kew, London.

Yusaf T, Al-Juboori RA, 2014. Alternative methods of microorganism disruption for agricultural applications. *App Energy*, 114:909-923. https://doi.org/10.1016/j.apenergy.2013.08.085

Zhou H, Huang YX, Hoshi T, et al., 2005. Electrochemistry of sinapine and its detection in medicinal plants. *Anal Bioanal Chem*, 382(4):1196-1201. https://doi.org/10.1007/s00216-005-3241-1