Isolation and characterisation of nematicidal compound, leolorin C, from *Leonotis leonurus* acetone leaf extract

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Compounds studied

Leolorin C.

List of abbreviations

DCM, dichloromethane; EC_{50}, half maximal effective concentration; EtOAc, Ethyl acetate; Hex, hexane; INT, iodonitrotetrazolium violet; MIC, minimum inhibitory concentration; NMR, nuclear magnetic resonance; PPN, plant parasitic nematodes; RKN, root knot nematodes; SD, standard deviation; TLC, thin layer chromatography; UPLC-QTOF-MS, ultra performance liquid chromatography-quadrupole time of flight mass spectrometry;

ABSTRACT

Ethnopharmacological relevance: *Leonotis leonurus* (L.) R.Br. (Lamiaceae) is a perennial shrub native to South Africa used to treat various diseases including digestive tract problems, intestinal worms and constipation.

Aim of the study: The aim was to isolate and characterise nematicidal compounds from leaves of *L. leonurus.*

Materials and methods: Bioassay-guided fractionation was carried out using the free-living nematode *Caenorhabditis elegans* as a model organism. Structural elucidation of the purified compound was carried out using NMR spectroscopic analyses and UPLC-QTOF-MS. The fractions and the isolated compound were tested for nematicidal activity on motility of plant-parasitic *Meloidogyne incognita* juveniles (J2s) and J2 hatch inhibition. Further screening was done to determine the minimum inhibitory concentration (MIC) of the fractions against bacterial phytopathogens and cytotoxicity against Vero kidney cells.

Results: Leoleorin C isolated from *L. leonurus* had moderate activity against *C. elegans* juveniles (34%) but was not active against J2 motility and J2 hatch of *M. incognita*. Thus, activity against the free-living *C. elegans* did not correspond with efficacy against plant-parasitic nematodes. Leoleorin C was not active against the tested bacterial phytopathogens, but some activity was observed in the bioautography assay against *Clavibacter michiganensis* subsp. *michiganensis*, the organism causing bacterial canker in tomatoes. The plant extract, fractions and leolorin C were relatively non-toxic to Vero cells with LC_{50} values greater than 0.01 mg/mL.

Conclusion: The crude extract of *L. leonurus* and fractions may be useful in developing complementary treatments for controlling nematodes and phytopathogens. This study

does not support the use of free-living nematodes as a model to isolate anti-parasitic compounds from plants.

Keywords: *Leonotis leonurus*; anthelmintic; antimicrobial; cytotoxicity; leoleorin C; *Caenorhabditis elegans*

1. Introduction

Plant pathogens, insects and weeds are major constraints to current agricultural production, causing considerable crop losses worldwide (Malandrakis et al., 2019). Pesticides are substances used in agriculture for controlling soilborne pathogens, and have been universally considered as the most efficient, quick, easy and inexpensive solution (Aktar et al., 2009; Sulaiman et al., 2019). However, excessive and unsystematic application of agrochemical inputs, in particularly pesticides and fertilizers, has been found to possess a threat to the environment and humans which is a major problem in promoting sustainable agriculture (Zhang et al., 2018).

Root-knot nematodes (RKN), or *Meloidogyne* spp., are exceptional pathogens of plants which are difficult to control due to their wide host range and high reproductive rates (He et al., 2020). *Meloidogyne incognita* Kofoid and White (Chitwood) is a major plant-parasitic RKN affecting the quantity and quality of annual and perennial crops (Arshad et al., 2020). *Meloidogyne* infections affect translocation of water and nutrients in the root system, and some phytopathogens such as bacteria and fungi may enter the xylem and disrupt the movement of water, thus causing extensive damage to the crop (Jahr et al., 1999). In this study we selected several phytopathogens which affect the health of tomatoes, some of which have been reported to cause major destruction following mechanical injury caused by nematodes. Bacterial diseases caused by *Clavibacter, Xanthomonas* and *Ralstonia* species are a major concern in crop production, resulting in significant economic loss.

The undesired results due to pesticides have necessitated the search for alternative methods for nematode control, hence the use of medicinal plants. Natural product-based compounds have been reported to be non-persistent under field conditions as they are transformed by light, oxygen or microorganisms into less toxic products over time (Nguyen et al., 2013). Their structural complexity, comprising higher molecular weights and greater proportions of oxygen and hydrogen, allow them to be more easily degraded, thus assisting in minimizing toxicity to the environment (Duke et al., 2000).

Leonotis leonurus (L) R.Br. is a perennial shrub from the Lamiaceae family. It is native in South Africa (He et al., 2012) and is commonly called wild dagga or lion's ear in English (van Wyk and Gericke, 2000). In traditional medicine, the plant has been used to treat various diseases including digestive tract problems, intestinal worms and constipation (Watt and Breyer-Brandwijk, 1962; Van Wyk et al., 1997). In a survey conducted by Maphosa and Masika (2010), the plant was reported to be used by farmers to treat helminthosis in goats. The anthelmintic activity of the plant was reported by Maphosa et al. (2010), where the water extract had an EC_{50} value of 1.25 mg/mL in both egg hatch and larval development inhibition assays against *Haemonchus contortus.* McGaw et al. (2000) reported slight activity against the free-living nematode, *Caenorhabditis elegans*. According to the report of Nsuala et al. (2015), more than 50 compounds including terpenoids, and particularly labdane diterpenes, have been reported in *L. leonurus,* with marrubiin being a major constituent.

To the best of our knowledge, *L. leonurus* has not been explored for managing PPN. In previous research, the leaf water extract of *L. leonurus* showed promising activity in terms of reducing the motility of J2 against *M. incognita* and promising activity against juveniles of *C. elegans,* and was not toxic to mammalian Vero cells (Makhubu, 2021). Hence, this study aimed to isolate nematicidal compounds from *L. leonurus* using *Caenorhabditis*

elegans as a test organism in bioassay-guided isolation. *Caenorhabditis elegans* was selected for the present study as a model due to the advantages it provides in the laboratory, such as its short life cycle and small body size, as well as its high sensitivity to toxins and toxicants, which enable testing of various samples (Leung et al., 2008; Wang et al., 2021). In addition to the ease of working with this species, in contrast to parasitic nematodes, is the existence of reports providing leads to development of anthelmintic drugs through understanding their mode of action and mechanisms of resistance using *C. elegans* (Holden-Dye and Walker, 2014).

2. Materials and methods

2.1 Plant collection and bulk extraction

The fresh leaves of *Leonotis leonurus* (L.) R. Br. were collected at the Manie van der Schijff Botanical Garden, University of Pretoria next to the Plant Sciences Complex (25° 45'20,65716" S, 28° 14'8,29464" E) on 25 October 2018 in the spring season. A voucher specimen (PRU 0125287) was deposited in the H.G.W.J. Schweickerdt Herbarium, University of Pretoria. The plant was authenticated by Ms Magda Nel (taxonomist) from the University of Pretoria, Plant Sciences Complex, H.G.W.J. Schweickerdt Herbarium. The leaf material of *L. leonurus* was air dried at room temperature (25˚C) in a well-ventilated room and ground to a fine powder (through a sieve with pore size 1 mm) using a Macsalab mill (Model 200 LAB, Eriez, Bramley, Johannesburg, South Africa). Ground plant material (530 g) was exhaustively extracted in 9L of acetone at room temperature for 6 days and filtered through Whatman No. 1 filter paper. The 9L volume was used to exhaustively extract the plant material. The filtrates were concentrated using a Büchi rotary

evaporator (R-200, Labotec, Johannesburg, South Africa) at 45˚C and dried. This resulted in a mass of 46.62 g of crude extract.

2.2 Liquid/liquid fractionation

The acetone crude extract (46.62 g) was suspended in 800 mL water: acetone (90%). The dissolved extract was sequentially partitioned with *n*-hexane (3 x 900 mL), dichloromethane (3 x 900 mL), ethyl acetate (3 x 900 mL) and *n*-butanol (3 x 625 mL). All fractions obtained, including the final aqueous fractions, were concentrated. The solvent fractions (*n*-hexane, dichloromethane, ethyl acetate and *n*-butanol) were concentrated to dryness using a rotary evaporator while the water fraction was dried in an open Petri dish under flowing cold air. All the obtained fractions were assayed for nematicidal activity in terms of inhibition of *M. incognita* J2 motility and J2 hatching, and inhibition of *C. elegans* motility. Antibacterial activity was investigated against selected bacterial phytopathogens, and cytotoxicity was determined against Vero cells.

2.3 Bioassay-guided isolation of bioactive compounds

The isolation of the bioactive compounds was carried out using the dichloromethane fraction (**Fig. 1**), which was the most active fraction against *M. incognita*. This was carried out by column chromatography using silica gel as the stationary phase. A total mass of 258.66 g of silica gel (Macherey-Nagel, Germany) with particle size 0.063-0.2 mm was packed in a dry column. Thirty (30) g of the dichloromethane fraction was dissolved in hexane/ethyl acetate/dichloromethane (Hex/EtOAc/DCM) (1:1:0.5) made up to 200 mL, and this was mixed with silica gel and allowed to dry under cold air. The dried dichloromethane fraction was then loaded on the column. Varying ratios of ethyl acetate and *n*-hexane of approximately 1 000 mL (v/v) starting from 30% ethyl acetate and 70% *n*-

hexane were used as eluent to give different fractions, gradually increasing the ethyl acetate concentration to 100%. Similar fractions based on thin layer chromatography (TLC) analysis indicating common compounds were combined to give 28 fractions. Fractions with good yield (mass above 1 g) were screened for anthelmintic activity against *C. elegans* following the method described in section 2.4.2 to determine the effective concentration.

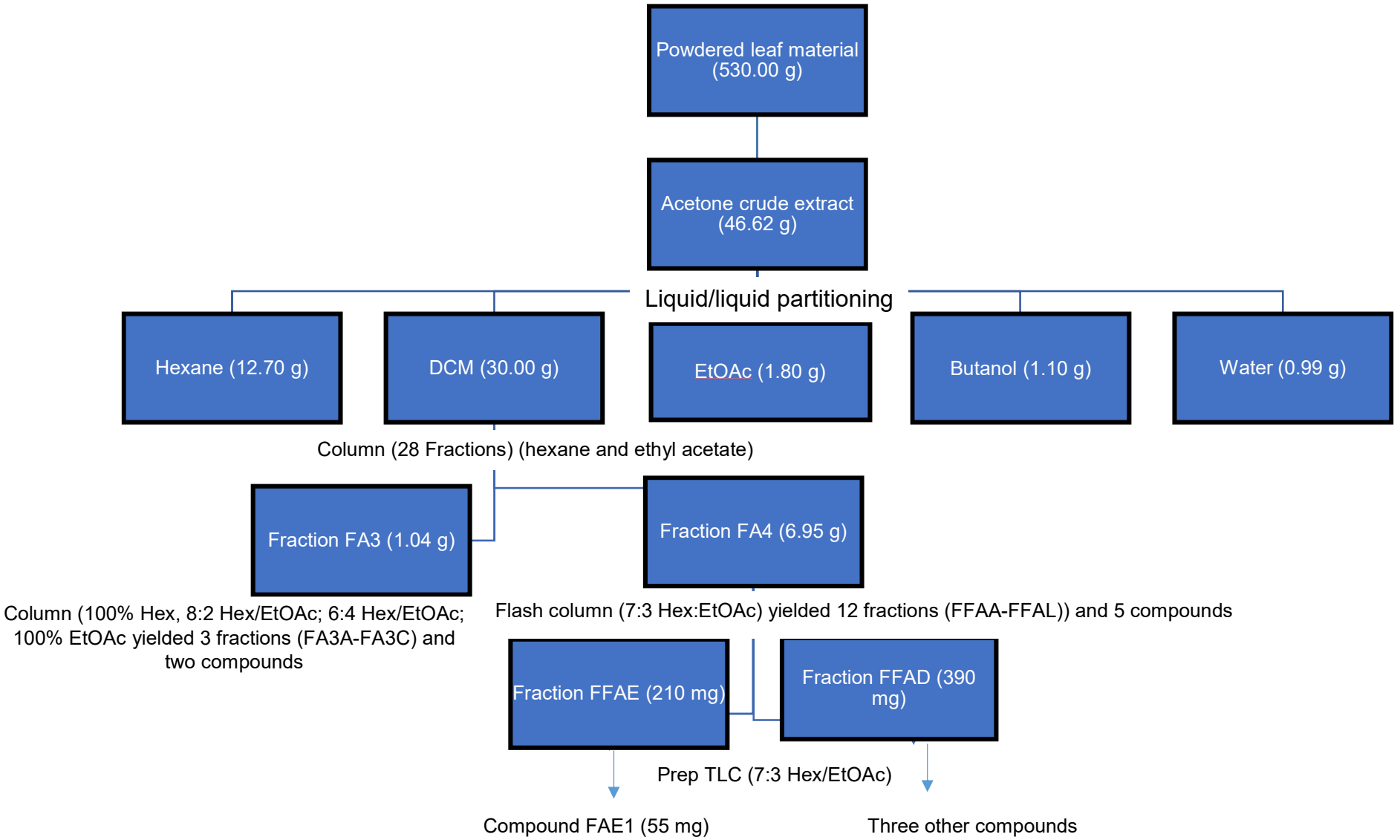


Fig. 1. Flow diagram of isolation process for *Leonotis leonurus* leaf material

The collected fractions were analysed using TLC with Hex/EtOAc (70:30) as the eluent solvent system. Fraction FA4 (6.95 g) was further purified using flash column chromatography employing Hex/EtOAc (70:30) and subsequently 100% EtOAc, which yielded 12 fractions (FFAA-FFAL). The fraction FFAE (210 mg) was further purified using preparative TLC with Hex/EtOAc (70:30) to give compound FAE1 (leoleorin C).

TLC analysis of compounds was conducted, and the plates were allowed to dry before visualisation under ultraviolet light (254 and 360 nm, Camac universal UV lamp TL-600, Muttenz, Switzerland). The plates were sprayed with 1% vanillin-sulphuric acid solution (0.1g vanillin, 28 mL MeOH, and 1 mL sulphuric acid) for further chromogenic visualisation of compounds. The structure of the isolated compound was elucidated using Nuclear Magnetic Resonance (NMR) (Bruker Advance III, Germany) (600 and 400 MHz) spectroscopy, and UPLC-QTOF-MS using a Waters Acquity™ UPLC instrument coupled to a Waters Synapt G2 high definition MS (HDMS). The system was operated with MassLynxTM (version 4.1) software (Waters Inc., Milford, Massachusetts, USA) at the Department of Chemistry, University of Pretoria, South Africa. NMR is a qualitative technique used for structural elucidation and to assess purity of samples (Fuloria and Fuloria, 2013). The UPLC-QTOF-MS assisted in structure elucidation by providing accurate mass and fragmentation patterns of the isolated compounds, and analysis of the crude extract and fraction. In NMR analysis, samples were dissolved in deuterated chloroform ($CDCl_3$) while for liquid chromatography-mass spectrometry (LC–MS), UPLC grade acetonitrile and methanol were used for dissolving the compound. The 1-dimensional (1D) NMR (^{1}H and ^{13}C) spectra were compared with the literature data to conclusively confirm the structures.

2.4 Nematode experiments

2.4.1 Meloidogyne incognita *experiment*

2.4.1.1. Preparation of Meloidogyne incognita *inoculum*

A population of *M. incognita* race 2, confirmed by SCAR-PCR (Zijlstra, 2000; Fourie et al., 2001) was obtained from the ARC-Grain Crops Institute, Potchefstroom, South Africa and cultured at ARC-Tropical and Subtropical Crops, Nelspruit, South Africa. Nematode eggs and infective second-stage juveniles (J2) were extracted from the tomato roots where they were cultured. This was done by gently removing the soil from the roots under running tap water. The roots were then chopped into 1 cm pieces and agitated for 4 min in 300 mL of a 1% NaOCl solution to release the eggs and J2 from the plant tissues (Hussey and Barker, 1973; Hussey and Boerma, 1981). The suspension was poured through a set of nested sieves with apertures from top to bottom of 150, 53, 38, 25 and 20 µm. Eggs held in the 38 µm aperture sieve were washed with distilled water and collected for the J2 hatch inhibition experiment. For the J2 motility experiment, unhatched J2 (contained in the 25 µm sieve) were incubated at 25˚C for 7 days. After incubation, hatched J2 were washed from the 25 µm sieve with distilled water and collected in the 20 µm aperture sieves.

2.4.1.2. Meloidogyne incognita *juvenile (J2) motility and J2 hatch inhibition*

The J2 motility and J2 hatch assays were carried out to determine the nematicidal effect of the fractions and compound against PPN (Khosa, 2013). Modification of the J2 hatch assay was done following the method described by Nguyen et al. (2018). Approximately 38 000 J2 in 10 mL water were used to carry out the motility assay and the same number of eggs was also used for the J2 hatch experiment with continuous agitation every 5 s. A total volume of 100 µL was obtained in both experiments by adding 10 µL of fractions or compound and 90 µL water containing 100 ± 20 J2 for juvenile motility, and 100 ± 20 eggs

for the J2 hatch assay. Plates were agitated for proper mixing and incubated at 22˚C in the dark. After 24, 48 and 72 h, the total number of motile and immotile J2 in each well were counted using an inverted compound microscope at 400 x magnification. For the J2 hatch assay, plates were incubated in an incubator at 25 ± 2°C and the number of hatched J2 was counted after 7, 10 and 14 days using an inverted compound microscope at 400 x magnification. Distilled water was used as the negative control for water fractions while 10% DMSO (used to solubilise the organic fractions and compounds) was used as the control for the organic solvents. As positive control, 2 mg/mL of salicylic acid (Sigma-Aldrich, USA) was used.

The experiment for J2 motility and J2 hatch against *M. incognita* was done in triplicate and repeated twice. Calculations were done as follows:

Percentage immotility was calculated using Abbott's formula (Abbott, 1925):

Immotility (%) = [(immotility percentage in treatment - immotility percentage in untreated control) / (100-immotility percentage in untreated control)] ×100.

The percentage of unhatched J2 in the hatch inhibition assay was calculated according to the following formula (Nguyen et al., 2013):

Hatch inhibition (%) = [(percentage unhatched J2 in untreated control - percentage unhatched J2 in treatment) / percentage unhatched J2 in untreated control] ×100.

2.4.2 Motility assay using Caenorhabditis elegans

Anthelmintic activity of fractions and compounds was assayed using the free-living nematode *C. elegans* var. Bristol (N2), which was cultured on nematode growth (NG) agar seeded with *Escherichia coli*, according to the method of Brenner (1974). The fractions were tested at concentrations of 1, 0.5 and 0.25 mg/mL following the protocol of Rasoanaivo and Ratsimamanga-Urverg (1993), as modified by McGaw et al. (2000). About

100 nematodes (7 to 10-day-old cultures) in M9 buffer (Brenner, 1974) were incubated with different fractions or compound for 24 and 48 h at 25°C in the dark. The anthelmintic drug levamisole (Sigma-Aldrich, USA, 5 and 10 µg/mL) was used as a positive control. Using an inverted microscope, nematodes were counted and determined as motile or non-motile. Motility of hatched juveniles was defined as any unprovoked movement within a 5 s interval (Skantar et al., 2005) and they were counted after 24 and 48 h. Nematodes were considered motile when they exhibited any movement from either the head or the tail, or pharyngeal movements and as non-motile when there was no tail, head or pharyngeal movements during 5 s of observation.

Percentage immotility for *C. elegans* was calculated using Abbott's formula (Abbott, 1925) as follows:

Immotility (%) = [(immotility percentage in treatment - immotility percentage in untreated control)/ (100-immotility percentage in untreated control)] ×100.

2.5MTT assay

The cytotoxic effects of the acetone crude extract, fractions and the isolated compound on the viability of African green monkey kidney cells was investigated using the tetrazolium based colorimetric MTT assay described by Mosmann (1983). The cells were grown in Minimal Essential Medium (MEM) supplemented with 0.1% gentamicin (Virbac) and 5% foetal calf serum (Highveld Biological). Cells obtained from a subconfluent culture were centrifuged at 200 x g for 5 min and were resuspended in MEM growth medium to 5 x 10^4 cells/mL. Briefly, 200 µL of cell suspension were added to each well of columns 2 to 12 and the same volume (200 µL) of MEM was placed in the first column containing no cells to serve as the blank. The plates were incubated for 24 h at 37ºC in a 5% CO_2 incubator to allow the cells to attach and reach the exponential phase of growth before treatment.

The spent medium was removed and replaced with 100 µL fresh MEM, followed by addition of 100 µL sample diluted to the appropriate concentration in MEM. The acetone crude extract was re-dissolved in acetone, while fractions and the isolated compound were re-dissolved in 10% DMSO to give a stock concentration of 100 mg/mL for the extract and fractions, and 20 mg/mL for the compound. The extract and fractions were diluted in serum-free MEM to an initial concentration of 1 mg/mL and further dilutions were prepared in MEM. The compound concentration was adjusted to 0.2 mg/mL in serum-free MEM and further dilutions were prepared in the same medium. The plates containing treatments and positive control (doxorubicin chloride, Pfizer Laboratories) were incubated for 48 h at 37°C in a 5% CO_2 incubator.

After incubation, the MEM from all the wells was removed and cells were washed with 200 µL of phosphate buffered saline (PBS, Whitehead Scientific), followed by addition of 200 µL of MEM and 30 µL MTT (Sigma, stock solution of 5 mg/mL in PBS). The plates were further incubated for 4 h at 37°C. After incubation with MTT, the medium was removed and 50 µL of DMSO was added to each well and the plates were shaken gently to dissolve the MTT formazan crystals. Then the amount of MTT reduction was measured immediately by detecting absorbance in a microplate reader (BioTek Synergy) at a wavelength of 570 nm and a reference wavelength of 630 nm. The experiment was repeated three times and samples were tested in triplicate.

The LC_{50} (concentration resulting in 50% reduction of absorbance) values were determined using linear regression and presented as arithmetic mean values ± standard error of mean. Percentage cell viability was calculated using the following formula:

Percentage cell viability (%) = [(mean absorbance of sample/mean absorbance of control)] X100.

2.6 Antibacterial activity

Five pathogenic bacterial species: *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) BD 1377, *Xanthomonas vesicatoria* BD 1349, *Xanthomonas perforans* BD 1346, *Ralstonia solanacearum* CBD 261, *Ralstonia pseudosolanacearum* BD 1443. These strains had previously been isolated from diseased tomato plants and were stored in the Plant Pathogenic and Plant Protecting Bacteria Culture Collection (verified by T. Goszczynska) of the ARC-Plant Protection Research (ARC-PPR), Roodeplaat, South Africa.

Purified colonies of *Ralstonia*, *Xanthomonas* and *Cmm* were inoculated into Mueller Hinton broth (Beef infusions solids 2 g/l; Starch 1.5 g/l: Casein hydrolysate 17.5 g/l at pH 7.4 ±0.2) and incubated at 28±2°C for 24 h in an orbital shaker incubator with the latter incubated for seven days. *Xanthomonas perforans* was incubated at 28±2°C for 10 days.

2.6.1 Minimum inhibitory concentration (MIC)

A serial microplate dilution method described by Eloff (1998a) was used to determine the minimal inhibitory concentrations of the acetone extract and fractions against bacterial pathogens. The acetone extract was re-dissolved in acetone while the fractions were re-dissolved in 10% DMSO to give a concentration of 10 mg/mL. Inoculum suspension from overnight cultures was prepared by diluting the bacterial colonies with fresh Müller Hinton broth to give a final concentration of McFarland standard No 1 (equivalent to 3.0 x 10^8 cfu/mL). Sterile water (100 µL) was dispensed in each well of a 96-well microtitre plate, and extract and fractions (100 µL) were serially diluted two-fold down the columns. A 100 µL aliquot of bacterial culture was added to each well resulting in concentrations ranging from 2 500 µg/mL to 19.5 µg/mL for fractions and 1 000 µg/mL to 7.8 µg/mL for the compound. A similar two-fold serial dilution of streptomycin (Sigma-Aldrich, USA) (starting

concentration 2 000 µg/mL) was used as a positive control. The solvents (acetone and 10% DMSO) were used as negative controls. Plates were incubated for 24 h at 28°C in a humidified atmosphere. To measure growth, p-iodonitrotetrazolium violet (INT) (Sigma-Aldrich, USA; 40 µL of a 0.2 mg/mL solution in sterile water) was added to each well and incubated further for 30 min. The minimal inhibitory concentration was recorded where clear zones indicated inhibition of the test bacteria by the samples. The reduction of INT to red formazan indicated bacterial growth. Experiments were done in triplicate and repeated two times. Results are presented as the mean MIC value ±SD.

2.6.2 TLC bioautography

To determine the number of active antimicrobial compounds in the extract and fractions, as well as activity of the isolated compound on the selected bacterial strains, a qualitative method using TLC bioautography was carried out. The eluent solvent used for isolation was used to develop plates for bioautograms. The developed TLC plates were sprayed with concentrated bacterial suspensions containing either *Ralstonia, Xanthomonas* or *Cmm* culture to a final concentration of McFarland standard No 1 (equivalent to 3 x 10^8 cfu/mL) cultured at 28°C until they were wet. The moist plates were then allowed to dry and incubated at 37°C in a closed plastic humidified sterile container for 24 h to allow the bacteria to grow on the plates. After incubation, the plates were sprayed with 2 mg/mL of freshly prepared INT in sterile distilled water and incubated further for 1-2 h. White areas indicated where reduction of INT to the coloured formazan did not take place due to the presence of compounds that inhibited the growth of the test bacteria.

Statistical analysis

Experimental results were expressed as percentage for J2 hatch, motility against J2 of *M. incognita*, and motility for *C. elegans* juveniles. Inhibition and motility results were

subjected to analysis of variance (ANOVA) using SPSS version 27.0 (IBM Corp., New York, USA) followed by Tukey's HSD test calculated at $p \leq 0.05$.

3. Results and discussion

3.1 Isolation of leoleorin C

Bioassay-guided fractionation of the dichloromethane fraction of the acetone extract of *L. leonurus* led to the isolation of seven compounds. However, due to inadequate amounts of most of the compounds, only leoleorin C was characterised and tested (^{1}H, ^{13}C NMR and mass analysis in Appendix 1). Acetone was used for extraction because of its miscibility with polar and non-polar solvents, and low toxicity against test organisms (Eloff, 1998b). In preliminary studies (Makhubu, 2021), the acetone crude extract of *L. leonurus* showed promising activity against the motility of *M. incognita* juveniles (J2) and motility of *C. elegans* juveniles. Hence, acetone was the solvent of choice to extract the powdered leaves of *L. leonurus* for bulk extraction for isolation of active compounds.

Leoleorin C **(Fig. 2)** was characterised by comparison with NMR data reported in the literature as shown in **Table 1** (Naidoo et al., 2011). Leoleorin C (compound FAE1) was obtained as white needles and appeared as one purple spot on the TLC plate (retention factor of 0.29) after spraying with vanillin. The accurate mass obtained from the UPLC-MS spectra showed a pseudo molecular ion at *m/z* 359.2283 $[M+Na]^{+}$ in the ESI^{+} (positive mode) which confirmed the molecular formula of $C_{20}H_{32}O_4$. Several compounds were also detected in the acetone and dichloromethane fractions analysed with UPLC-MS. The detected compounds agree with the findings of several researchers that the leaf of the plant is highly concentrated in labdane diterpenes (Kaplan and Rivett, 1968; McKenzie et al., 2006; Naidoo et al., 2011; Maleita et al., 2012; Wu et al., 2013). The major labdane diterpenoids were also identified in the acetone extract and dichloromethane fraction (**Fig.**

3) as leolorin F, compound X, marrubiin and leoleorin B with the help of LC-MS. However, these compounds share similar molecular weights with other labdane diterpenoids as follows: [leoleorin G ($C_{20}H_{28}O_4$, 332.1988), premarrubiin ($C_{20}H_{28}O_4$, 332.3392), marrubiin ($C_{20}H_{28}O_4$, 332.43392), leoleorin J (13ξ-hydroxylabd-5(6), 8(9)-dien-7-on-16, 15-olide) $C_{20}H_{28}O_4$, 332.2066)]; [compound x ($C_{20}H_{28}O_5$, 348.438), EDD ($C_{20}H_{28}O_5$, 348.2009), leoleorin J [$C_{20}H_{28}O_5$1(4α-hydroxy-9α, 13α-epoxylabd-5(6)-en-7-on-16, 15-olide), 348.2015]; [leoleorin C ($C_{20}H_{32}O_4$, 336.465680), leoleorin E ($C_{20}H_{34}O_4$, 338.48156), leoleorin F ($C_{20}H_{34}O_4$, 338.48156) and leoleorin J ($C_{20}H_{34}O_4$, 338.48156)].

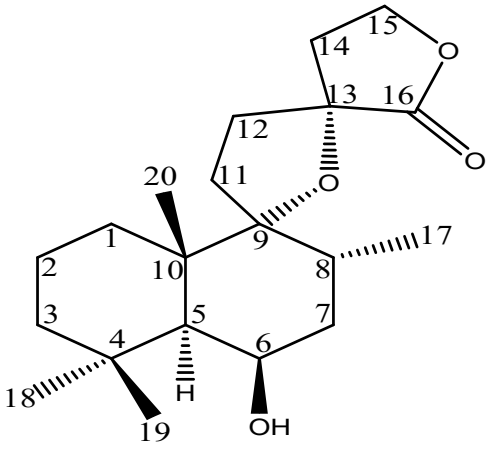


Fig. 2. Structure of the isolated leoleorin C (FAE1) from *Leonotis leonurus*

Table 1. ^{1}H and ^{13}C NMR chemical shifts of leoleorin C ($CDCl_3$)

	*Isolated		#Literature	
Position	**^{1}H δ(ppm)**	**^{13}C δ(ppm)**	**^{1}H δ(ppm)**	**^{13}Cδ(ppm)**
17	0.89 (3H,d, *J*=6.8)	17.78	0.85 (3H,d, *J*=6.0)	17.8
2	1.55 (1H); 1.50 (1H)	18.7	1.55 (1H); 1.50 (1H)	18.7
20	1.27 (3H, s)	20.7	1.25 (3H, s)	20.7
19	1.21 (3H, s)	24.9	1.18 (3H, s)	24.9
11	2.32 (1H); 1.80 (1H)	29.7	2.35 (1H); 1.80 (1H)	29.6
8	2.29 (1H, d, *J*=3.0)	30.6	2.29 (1H, d, *J*=3.0)	30.6
18	0.97 (3H, s)	33.4	0.95 (3H, s)	33.3
12	2.15 (1H); 2.10 (1H)	33.5	2.15 (1H); 2.10 (1H)	33.5
1	1.58 (1H); 1.31 (1H)	34.0	1.58 (1H); 1.30 (1H)	34.0
4		34.4		34.4
14	2.41 (1H); 2.20 (1H, m)	37.2	2.40 (1H, dd, *J* = 6.0, 13.0); 2.20 (1H, dd, *J* = 3.5, 13.0)	37.2
7	1.72 (1H, ddd, *J* = 2.7, 3.0, 13.6) 14.0) 1.40 (1H, ddd, *J* = 2.7, 3.0, 13.6)	40.8	1.70 (1H, ddd, *J* = 2.5, 3.0, 14.0) 14.0) 1.40 (1H, ddd, *J* = 2.5, 3.0)	40.7
10		43.0		43.0
3	1.26 (1H); 1.18 (1H)	44.0	1.26 (1H); 1.18 (1H, *J* = 4.5)	44.0
5	1.35 (1H, d, *J*=2.7)	49.7	1.31 (1H, d, *J*=3.0)	49.7
15	4.40 (1H); 4.30 (1H, brd)	65.4	4.38 (1H) 4.18 (1H, dd, *J* = 3.5, 6.0)	65.5
6	4.21 (1H, m)	68.1	4.31 (1H, ddd, *J*=2.5; 2.5; 2.5)	68.0
13		83.3		83.3
9		95.5		95.5
16		177.1		177.1

*Data obtained in $CDCl_3$, ^{13}C and ^{1}H (600 and 400 MHz); # Data obtained in $CDCl_3$, ^{13}C and ^{1}H (600 MHz)

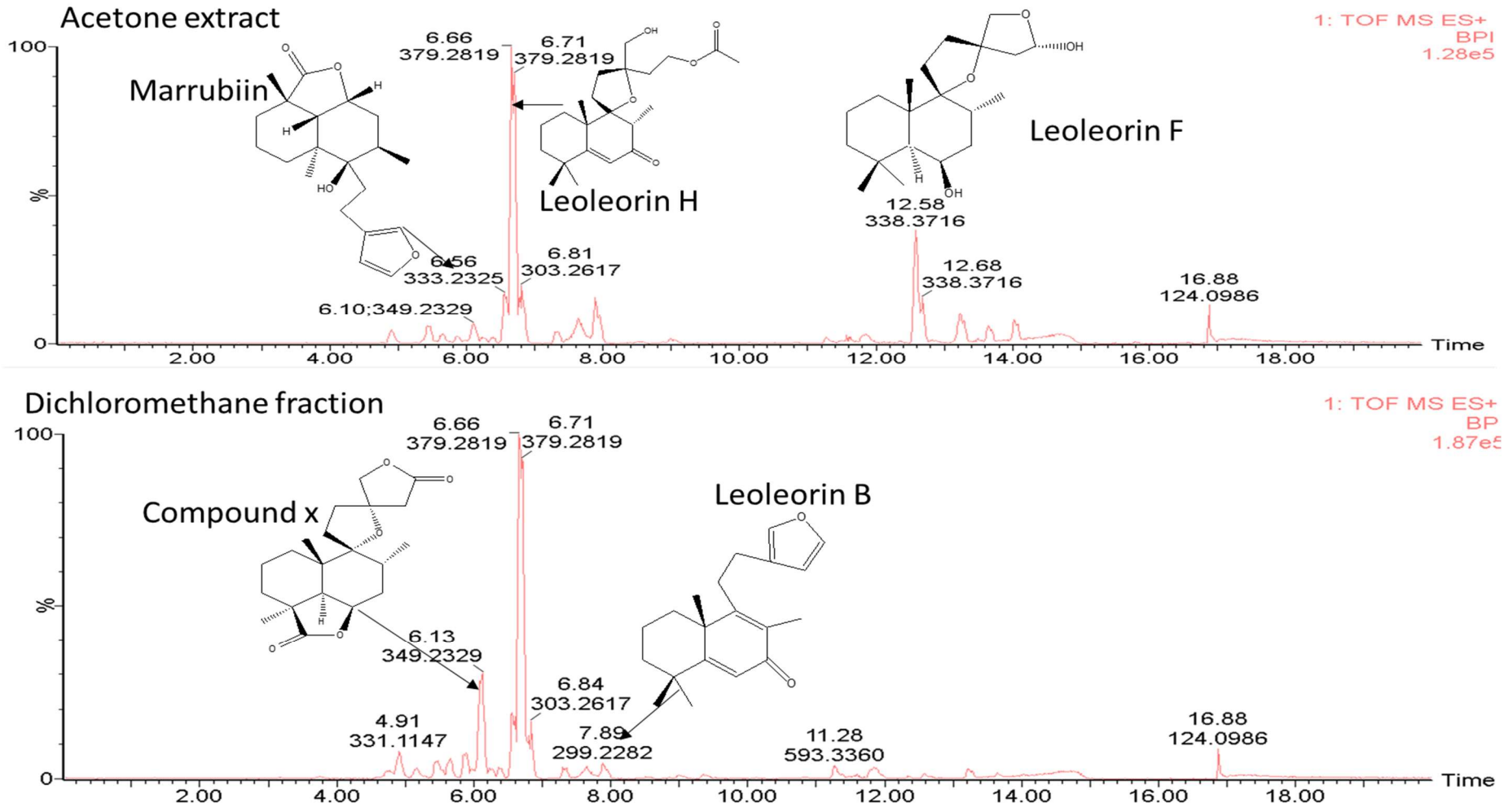
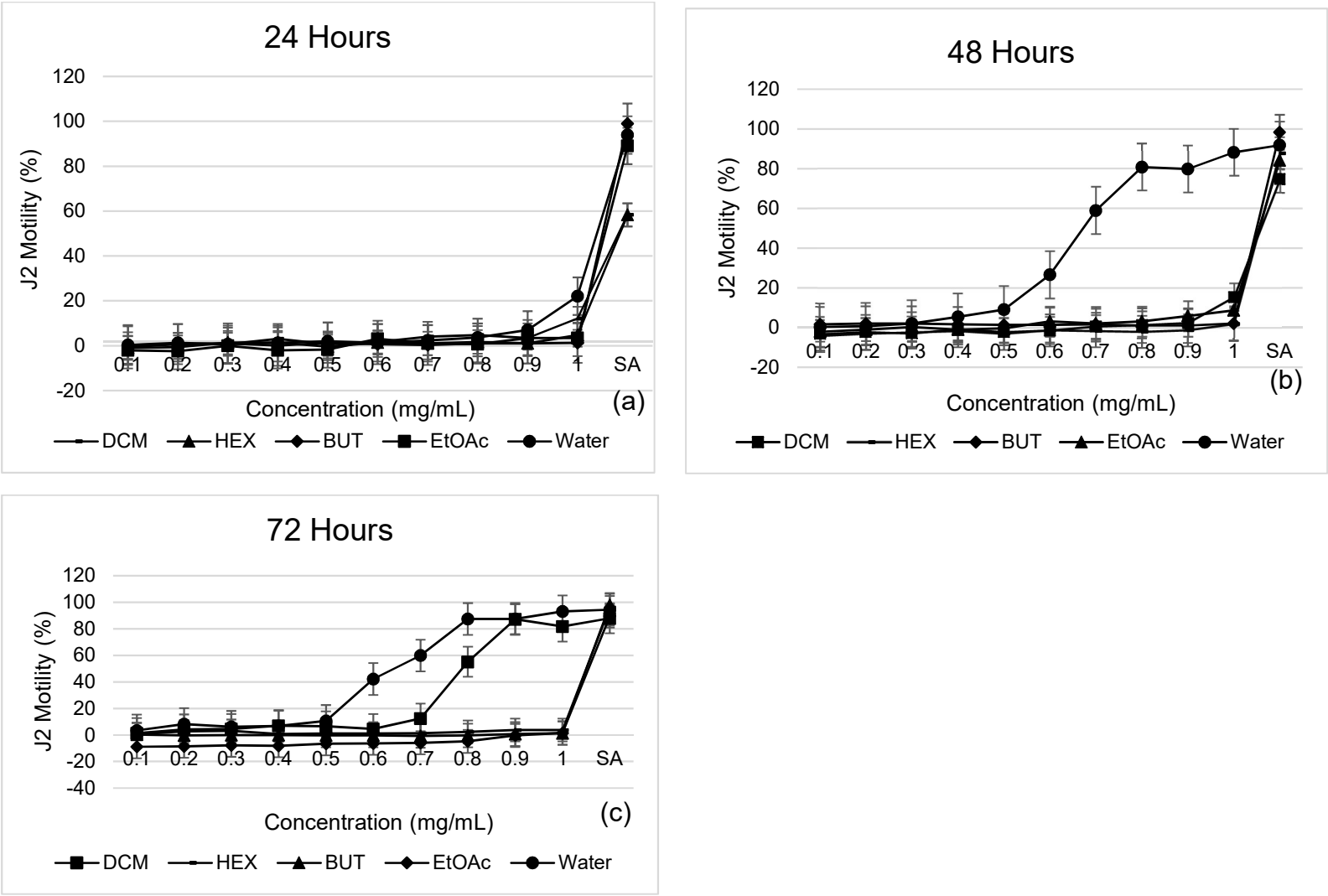


Fig. 3. LC-MS metabolite profiles of acetone extract and dichloromethane fraction of *Leonotis leonurus*

3.2 Nematicidal activity against Meloidogyne incognita

Leonotis leonurus has a wide range of traditional uses, and nematicidal activity of the dichloromethane fraction against *M. incognita* prompted isolation of the constituents that might be responsible for the activity using *C. elegans* as a model. The results showing the effect of fractions against motility of *M. incognita* second-stage juveniles (J2) and the dichloromethane fraction on J2 hatch inhibition are shown in **Fig. 4**. The activity is expected to increase with further purification and the compounds are anticipated to display higher activity compared to the crude extracts and fraction as they would be more concentrated. In the present study, that was not the case since most fractions did not have enhanced activity. Weak activity was demonstrated by the ethyl acetate, *n*-butanol and *n*-hexane fractions with motility not significantly different from each other ($p>0.05$). A high percentage of non-motile juveniles was recorded ($p<0.05$) in the water fraction followed by the DCM fraction. The water fraction had notable activity after 48 h at 0.6 mg/mL, whereas at 1 mg/mL more than 90% J2 immotility was recorded ($p<0.05$) **(Fig. 4b)**. The dichloromethane fraction showed good activity after 72 h at 0.8 mg/mL with 55% inhibition, and hence isolation was continued using this fraction **(Fig. 4c)**. Other fractions were not active against *M. incognita* J2 motility, even at a high concentration of 1 mg/mL during all incubation time periods with immotility less than 5%.



DCM-dichloromethane; HEX-hexane, BUT-Butanol, EtOAc-Ethyl acetate

Fig. 4. Effect of *Leonotis leonurus* fractions against motility of *M. incognita* J2 exposed at 24 h (a), 48 h (b) and 72 h (c). Difference in motility at the 0.05 level of confidence determined according to Tukey's HSD test.

The results showing the effect of the dichloromethane fraction on *M. incognita* J2 hatch inhibition is shown in **Fig. 5**. Inhibition increased with time for the dichloromethane fraction with inhibition of J2 hatching greater than 60% after 10 and 14 days. After 7 days, some juveniles hatched but further incubation resulted in high inhibition.

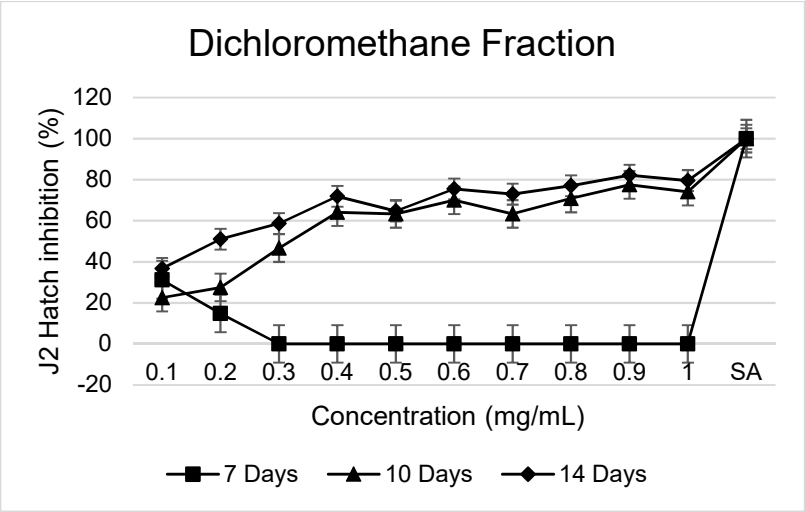


Fig. 5. J2 Hatch inhibition of dichloromethane fraction exposed at 7, 10 and 14 days against *M. incognita.* Difference in J2 hatching at the 0.05 level of confidence reported according to Tukey's HSD test.

The effects of leoleorin C on the J2 hatch and J2 motility is shown in **Fig. 6a** and **Fig. 6b** respectively. Leoleorin C was found to have stimulating effects on J2 of *M. incognita* hatch at all concentrations rather than being inhibitory and did not have any effects on the motility of J2s.

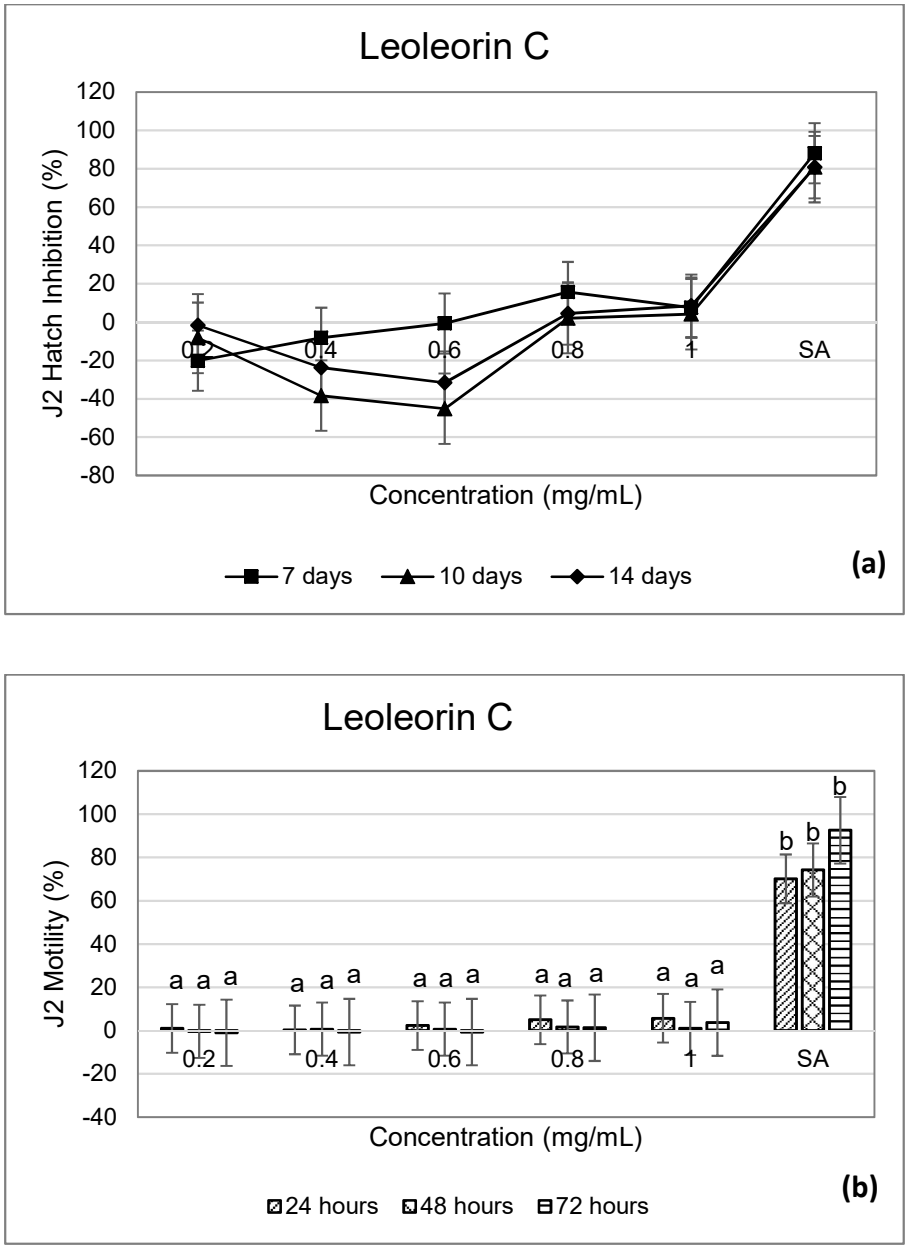


Fig. 6. Effect of leoleorin C against J2 hatch (a) and J2 motility (b) of *M. incognita*. Means followed by a common letter are not significantly different by the Tukey HSD test at the 0.05 level of confidence.

3.3 Motility against Caenorhabditis elegans

The safety and the effect of the acetone crude extract, fractions and the isolated leoleorin C against motility of *C. elegans* are shown in **Table 2**. Good activity was displayed by the sub-fractions against *C. elegans* juveniles compared to the dichloromethane fraction and

acetone crude extract. Fractions FFAD and FFAE demonstrated good activity against *C. elegans* juveniles at the lowest concentration with motility of 80.38 and 91.35%, respectively. Leoleorin C was obtained from fraction FFAE but exhibited only moderate activity. Therefore, leoleorin C is not nematicidal on its own, but compounds in the active fraction are most likely working in synergy. All fractions, the crude extract and the isolated compound leoleorin C were relatively non-toxic to the mammalian Vero cells with all the LC_{50} values being above 0.01 mg/mL compared to doxorubicin, the positive control, with an LC_{50} of 0.01 mg/mL.

Table 2. Cytotoxicity and anthelmintic activity of *L. leonurus* crude extract, fractions and isolated compound on *C. elegans* juveniles exposed at 0.25, 0.5 and 1 mg/mL

Sample	Cytotoxicity (mg/mL)	Concentration (mg/mL)	% Motility	
			24 h	48 h
Acetone crude extract	0.072±0.006	0.5	60.36±3.17	22.17±5.11
		1.0	60.02±2.98	29.72±4.98
Dichloromethane fraction	0.020±0.000	0.5	N/A	44.00±3.14
		1.0	30.40±2.10	76.40±3.11
Fraction FA4	0.117±0.005	0.5	71.53±1.07	N/A
		1.0	82.21±2.01	N/A
Fraction FA3	0.340±0.027	0.5	42.59±3.02	62.01±2.98
		1.0	65.40±2.11	72.11±1.99
Fraction FFAD	0.169±0.016	0.5	59.37±1.04	**80.38**±1.01
		0.25	47.86±3.26	79.11±0.36
Fraction FFAE	0.094±0.003	0.5	58.24±1.48	**91.35**±0.23
		0.25	11.96±2.01	56.96±2.18
Leoleorin C	0.422±0.036	0.5	44.61±2.03	34.08±1.15
Levamisole		5 µg/mL	42.59±1.01	53.37±0.14
		10 µg/mL	51.71±1.23	58.65±1.95

N/A represents where the number of nematodes following incubation was higher than in the blank (% motility could not be calculated) (mean±SE). Doxorubicin hydrochloride was

used as positive control for cytotoxicity and had LC_{50} of 0.0102±0.0025 mg/mL. LC_{50} values are presented as mean±SE obtained from cell viability. Difference in J2 hatching at the 0.05 level of confidence was determined according to Tukey's HSD test.

3.4 Antimicrobial activity

MIC values less than 100 µg/mL are considered to be pharmacologically significant for crude extracts and fractions (Eloff, 2004). Kuete (2010) classified significant activity of the antimicrobial plants as MIC less than 10 µg/mL, moderate as 10 < MIC ≤ 100 µg/mL, and low or negligible as MIC > 100 µg/mL. Fractions and the acetone extract had low to poor antibacterial activity with MIC values ranging from 117-2 500 µg/mL against all the tested bacterial phytopathogens **(Table 3)**. The lack of activity of the fractions was not surprising since the acetone crude extract did not exhibit good antibacterial activity, however, it was expected that activity might increase following fractionation.

Table 3. Minimum inhibitory concentration (MIC) values (µg/mL) of the *Leonotis leonurus* acetone leaf extract and fractions against test bacterial strains

Extract and fractions	**Bacterial isolates**				
	R. pseudosolanacearum	***R. solanacearum***	***C. m. michiganensis***	***X. perforans***	***X. vesicatoria***
Acetone extract	469±221	469±221	938±441	469±221	235±111
n-Hexane	2 500±0.00	1 250±0.00	1 250±0.00	>2 500±0.00	1 407±155
n-Butanol	>2 500±0.00	2 500±0.00	1 563±133	2 500±0.00	>2 500±0.00
Ethyl acetate	469±221	313±0.00	313±0.00	625±0.00	469±221
Dichloromethane	313±0.00	469±221	391±332	625±0.00	313±0.00
Water	>2 500±0.00	2 500±0.00	1 250±0.00	2 500±0.00	>2 500±0.00
Fraction FA4	>2 500±0.00	313±0.00	117±55	2 500±0.00	1 875±883
Streptomycin	12±0.00	19.5±0.00	10±0.00	10±0.00	10±0.00

MIC values are recorded as mean±SD.

In contrast, all fractions and the crude extract showed good activity against all the phytopathogens when assayed using TLC bioautography (results not shown). Fraction FA4 had better activity with MIC of 117 µg/mL against *Cmm* compared to other strains, and good activity was observed in bioautography where different compounds showed inhibition. Leoleorin C did not inhibit the growth of all the tested bacteria when evaluated using bioautography except for *Cmm* where it showed promising activity **(Fig. 7)**. The inactivity of leoleorin C was also observed when it was tested against *Mycobacterium tuberculosis* (Naidoo et al., 2011). *Clavibacter michiganensis* subsp. *michiganensis* causes bacterial wilt and canker in tomato and is one of the most important bacterial disease of tomatoes (Eichenlaub et al., 2007; Waleron et al., 2011). Plants infected at their early life stages develop systemic infections that affect fruit quality and yield, and eventually this leads to plant death (Nandi et al., 2018). Inhibition of compounds against this bacterium and other phytopathogens will assist in developing broad-spectrum antimicrobial agents for diseases infecting crops, particularly tomato.

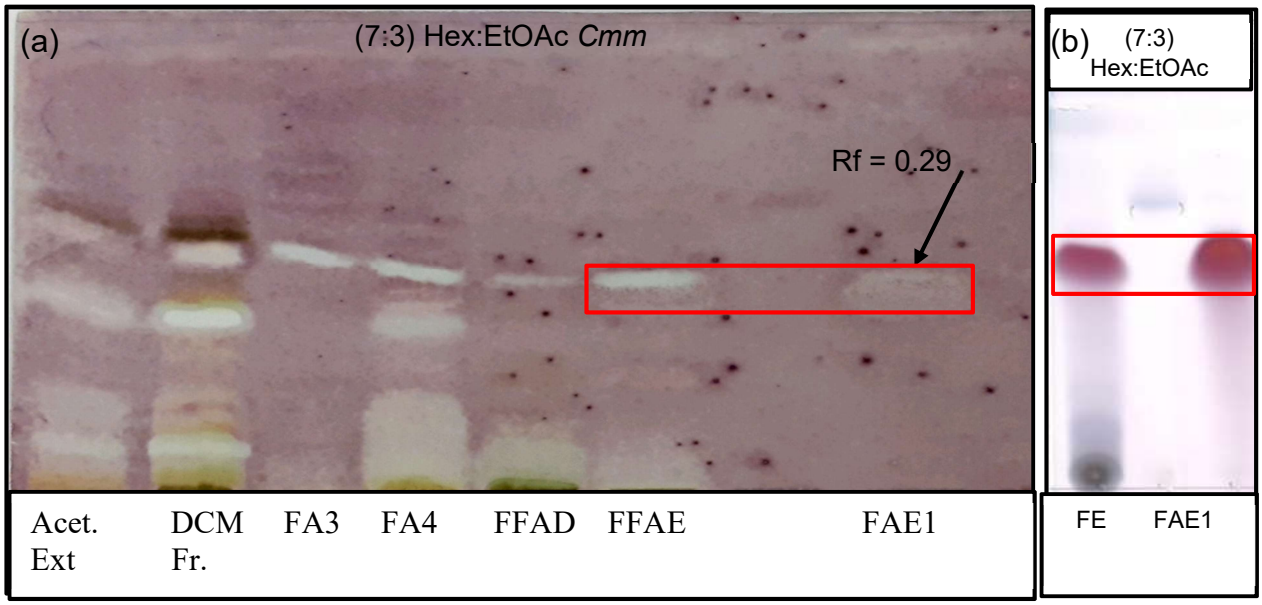


Fig. 7 Fractions and the isolated compounds from *L. leonurus* eluted in 7:3 Hex:EtOAc. TLC plate sprayed with *Cmm* (a) and TLC plate sprayed with vanillin (b)

Highlighted in the red box is the compound leoleorin C (FAE1) sprayed with *Cmm* showing inhibition (a), and TLC plate sprayed with vanillin showing the fraction FFAE where the isolated leoleorin C was obtained (b). Acet = acetone, Fr = fraction, DCM = dichloromethane fraction.

Conclusion

Bioassay-guided fractionation of the dichloromethane fraction led to isolation of leoleorin C. This compound has previously been isolated from the plant, but activity against *M. incognita*, *C. elegans*, selected phytopathogens and cytotoxicity against Vero cells is reported for the first time in this study. Fractions showed good activity against motility of C. *elegans* juveniles and moderate activity against J2 motility of *M. incognita*. Leoleorin C also had moderate activity against *C. elegans*, but only weak activity against *M. incognita*. Thus, activity against the free-living *C. elegans* could not translate to activity against the plant-parasitic nematode with both the compounds and the fractions. Antibacterial activity against phytopathogens using the broth dilution method was weak, but better activity was noted when using the bioautography method. Leoleorin C was not active against all the tested phytopathogens, but only had promising activity against *Cmm.* A combination of compounds from this plant are most likely working in synergy. There is great potential to develop products from *L. leonurus* leaves as alternatives for controlling both nematodes and phytopathogens of important crop species such as tomatoes. Further work is needed to identify which compounds in the leaves of *L. leonurus* are responsible for activity against the parasitic nematode *M. incognita*.

Acknowledgments

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Conflict of interest

The authors declare that they have no conflict of interest.

Author contributions

FNM conducted the experiments, SMM assisted with cytotoxicity assays and GF assisted with the isolation and identification experiments. MCK and LJM supervised the work and provided facilities and funding. All authors edited and approved the final manuscript.

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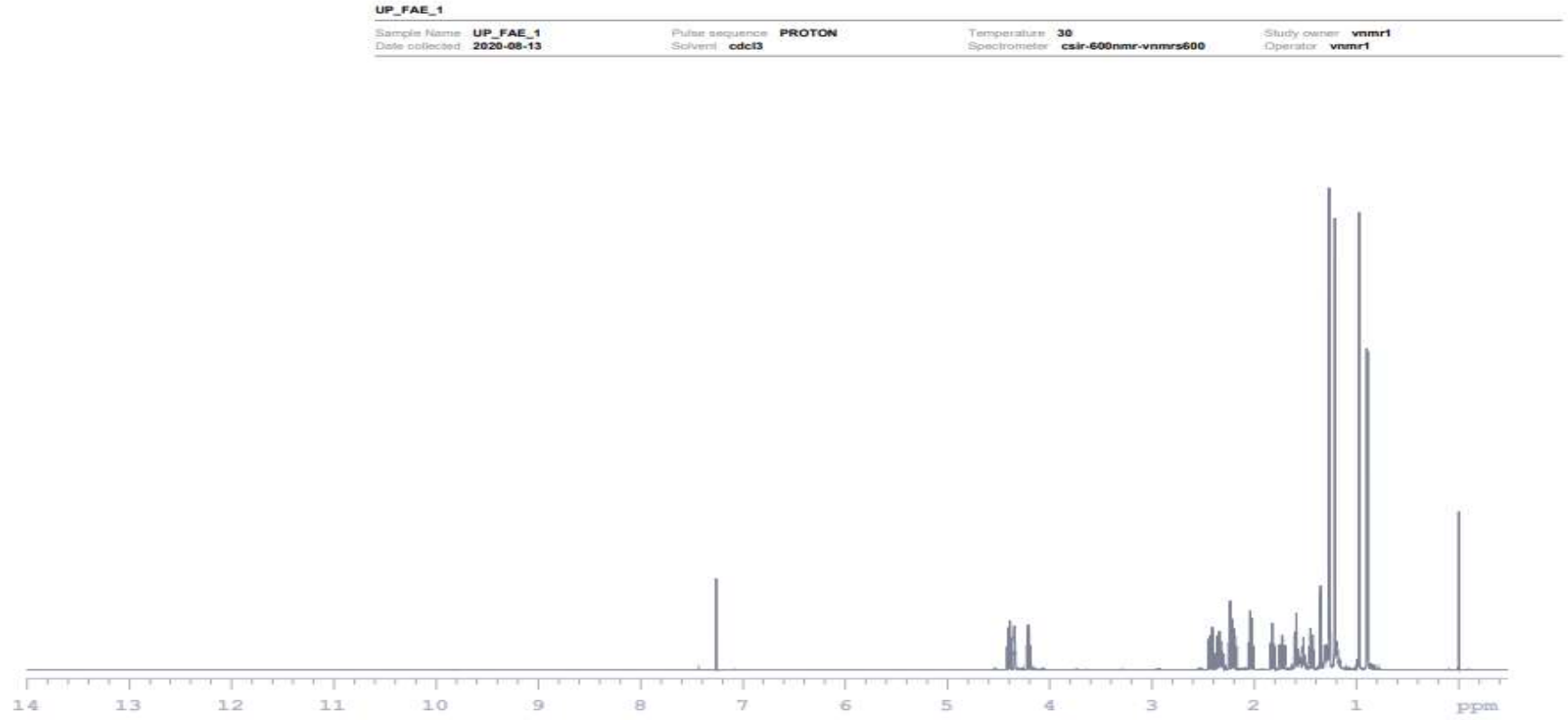
Appendix 1

1H-NMR spectrum expansion. Data obtained in CDCl3 using 600 MHz



UP_FAE_1□

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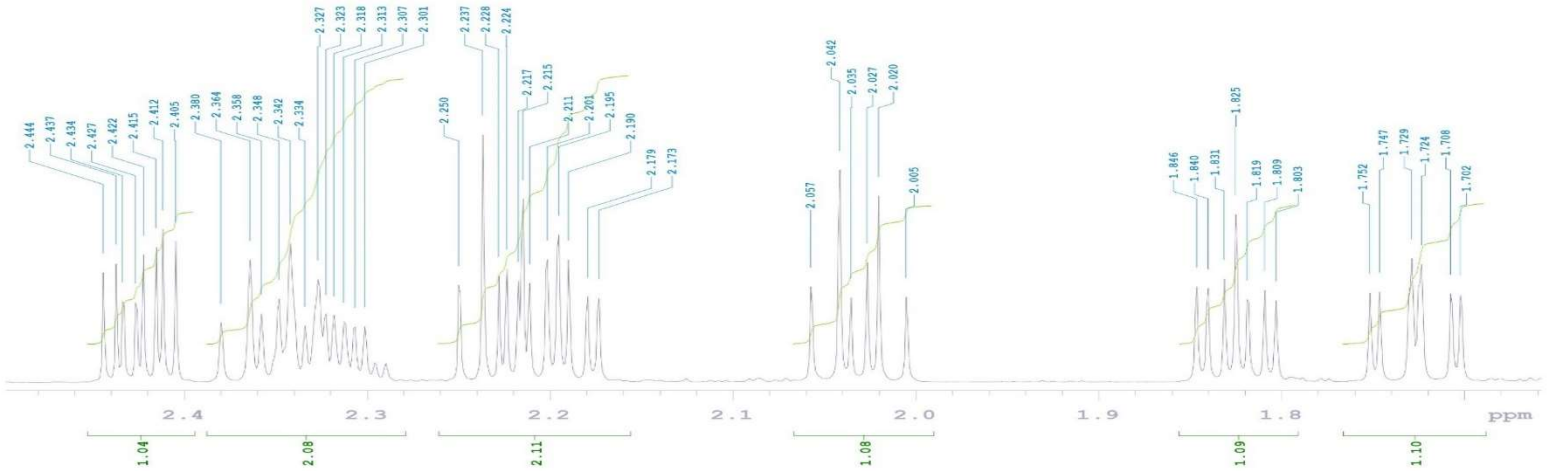

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Plot date 2020-08-14

UP_FAE_1

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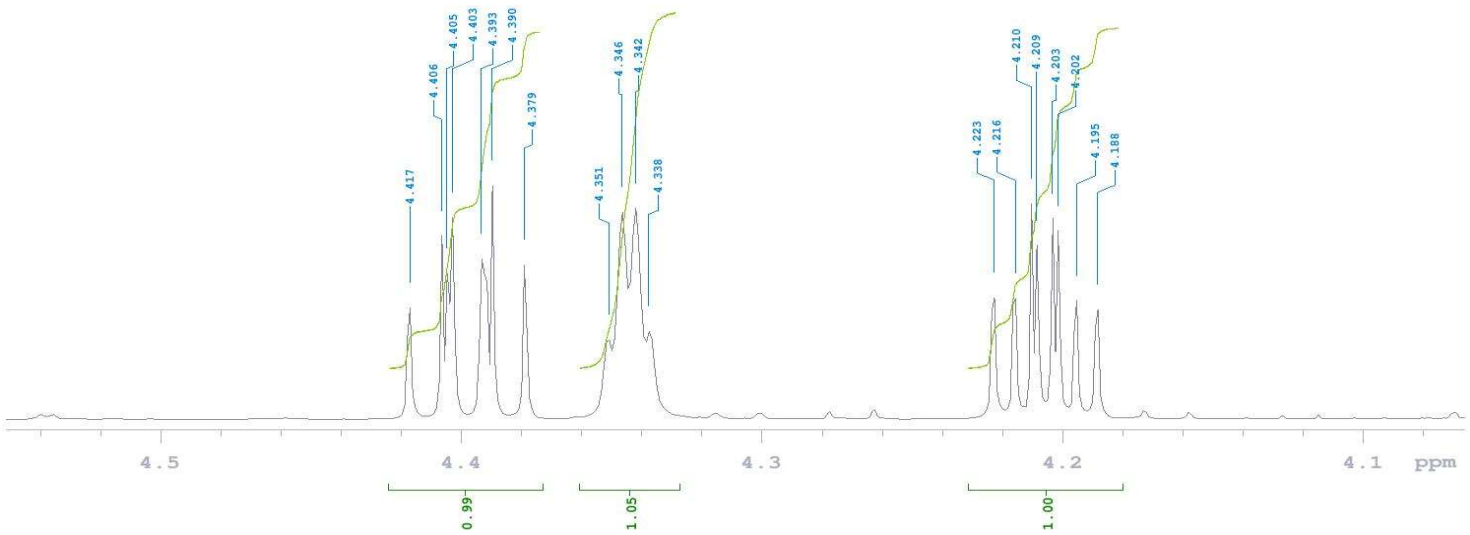

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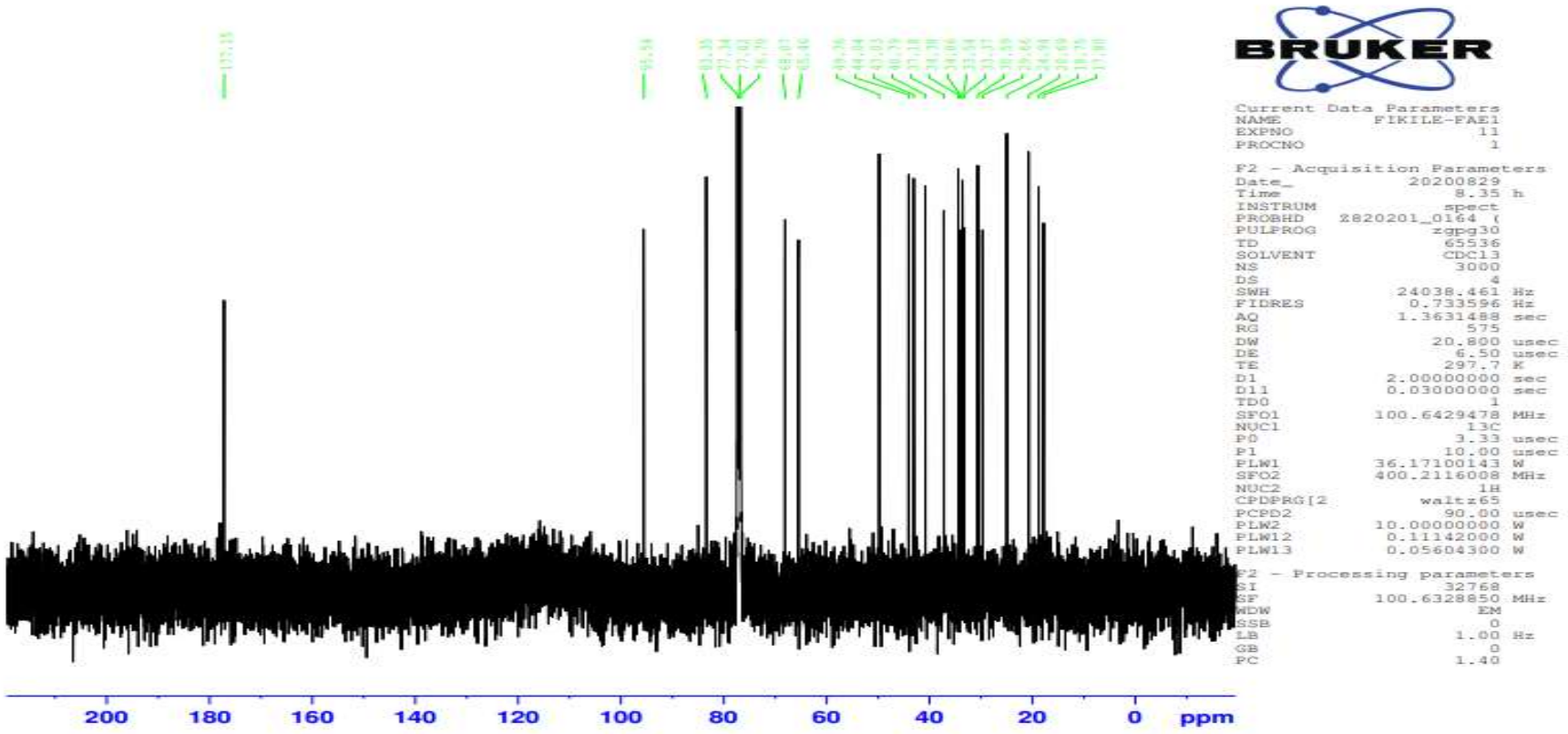
^{13}C-NMR spectrum. Data obtained in CDCl3 using 400 MHz



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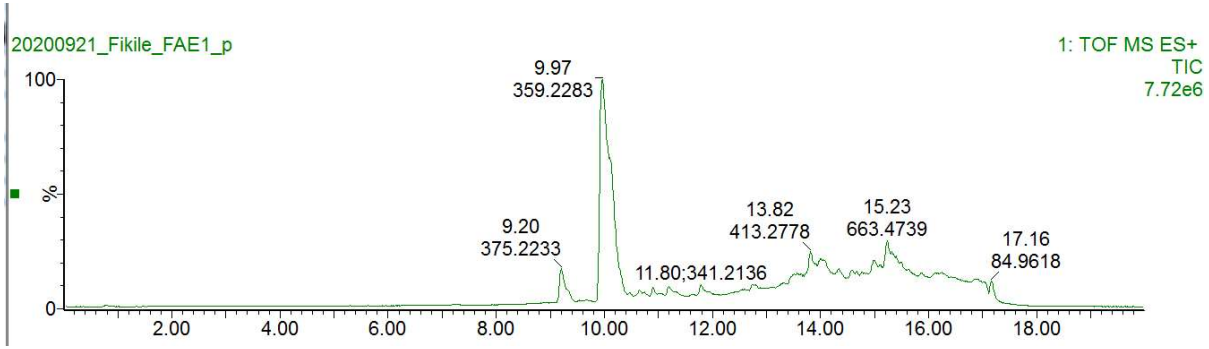
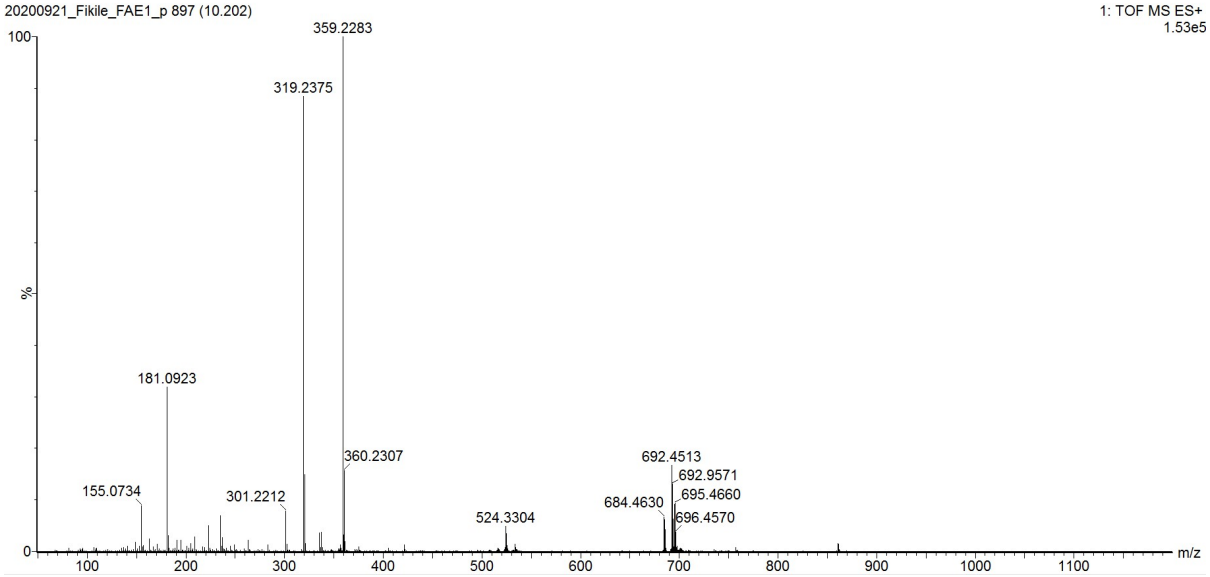
Leoleorin C Mass Analysis





HRTOFMS spectrum (ESI^+)

Single Mass Analysis
Tolerance = 3.0 mDa / DBE: min = -1.5, max = 50.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3
Monoisotopic Mass, Even Electron Ions
141 formula(e) evaluated with 2 results within limits (up to 10 closest results for each mass)
Elements Used:

Mass	Calc. Mass	mDa	PPM	DBE	Formula	i-FIT	i-FIT Norm	Fit Conf %	Frag. Mass	Fragment details	C	H	O	Na
359.2199	359.2198	0.1	0.3	4.5	C20 H32 O4 Na	23...	0.002	99.81			20	32	4	1
	359.2222	-2.3	-6.4	7.5	C22 H31 O4	23...	6.244	0.19			22	31	4	