Density-dependent growth response and sensitivity of *Meloidogyne incognita* to *Maerua angolensis* and *Tabernaemontana elegans*: Card Model

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Data will be provided upon request

Abstract

Curve-fitting Allelochemical Response Dosage (CARD) computer-based model has proven to be a valuable tool in the definition of dosage response relationships between organisms and various plant extracts. Its use in the explanation of the relationship between *Maerua angolensis* and *Tabernaemontana elegans* concentrations and *Meloidogyne incognita* second stage juvenile (J2) hatch and mortality has not been reported. The objective of this study was to use the CARD model in the determination of *M. incognita* sensitivity, density-dependent growth (DDG) patterns and minimum inhibition concentrations of *M. angolensis* and *T. elegans* plant extracts on juvenile hatch and mortality. The CARD model quantified concentration ranges of the two plant extracts that could stimulate (D_m-R_h), saturate (R_h-D_0) and inhibit (D_0-D_{100}), J2 hatch and mortality. *Meloidogyne incognita* J2 hatch was moderately sensitive to *M. angolensis* and *T. elegans* plant extracts with average sensitivity ranking of 20 and 19 units, respectively. J2 mortalities were moderately sensitive to *T. elegans* average sensitivity ranking of 15 units whereas, highly sensitive to *M. angolensis* at 2 units. The minimum juvenile hatch inhibition and lethal concentrations for the two extracts were comparable and very low at 0.07; 0.06 $mg.mL^{-1}$ and 0.13; 094 $mg.mL^{-1}$, respectively. In conclusion, the low minimum concentrations for *M. incognita* J2 hatch inhibition and lethal concentrations of *M. angolensis* and *T. elegans* plant extracts and moderate sensitivity values provide substantial evidence of high potency of the two extracts in the potential management of *M. incognita*.

Key words: CARD model; *Maerua angolensis;* medicinal plant; phytonematicides; sensitivity index; *Tabernaemontana elegans*

Introduction

Mathematical modelling has had an extensive use in crop protection, especially in the development of integrated pest management strategies (Nayak et al. 2018; Walczak 2011). Most of these models focussed on disease risk assessments (Gutsche & Rossburg 1997) and impact of pests on yield (Donatelli et al. 2017; Herbert et al. 1992) with few developed to predict the impact of synthetic pesticide applications and threshold levels for spraying on the environment and crop being protected (Fisker et al. 2007; Walczak 2011). There are currently limited models developed for quick preliminary evaluation of phytopesticide efficacy (Liu et al. 2003) even though they have been proven to be viable alternatives to synthetic chemicals in pest management (Mashela et al. 2015).

Liu et al. (2003) developed a computer-based model that assessed the response of an organism when exposed to varying concentrations of allelochemicals. The model has been adapted for use in phytonematicide dosage trials (Dube 2016; Mafeo 2012; Mashela et al. 2015; Pelinganga 2013). The initial adoption of the model was focused on quantifying all stages of the density-dependent growth (DDG) patterns of different crop plants to increasing concentrations of phytonematicides, values which were later used to determine a concentration of a phytonematicide which stimulates plant growth, while suppressing population densities of the target pest (Mafeo 2012; Pelinganga 2013). Dube (2016) demonstrated that the model also has potential in *in vitro* trials of phytonematicides for quick screening through establishment of toxicity and minimum inhibition concentration.

Curve-fitting Allelochemical Response Dosage (CARD) model is based on mathematical model of curvilinear regression equations. The model uses several generated biological indices to formulate a density-dependent growth response of organism to allelochemicals and various other variables (Liu et al. 2003). The two most used biological indices of the model are those that characterises the organism's response towards

allelochemicals in a DDG pattern and sensitivity index (k) (Mashela et al. 2015). The density-dependent response patterns are critical in the determination of the optimum and/or minimum inhibition concentration of allelochemicals in pest management (Dube 2016) while the *k*-value has been extensively used as a measure of phytotoxic plant response (Mashela et al. 2015; Pelinganga et al. 2013) and toxicity/efficacy of the allelochemicals to the pest (Dube et al. 2018). Usually, *k*-values start from zero and increase as discrete numbers when the sensitivity of the test organism to the allelochemical decreased (Liu et al. 2003).

Maerua angolensis and *Tabernaemontana elegans* plant extracts are used in traditional medicine for the treatment of various domestic animal and human ailments (Khosa 2013; Wink & Van Wyk 2008) and have shown potential in glasshouse, microplot and field trials in controlling *Meloidogyne* on tomato (Khosa 2013). Root-knot nematodes (*Meloidogyne*), on the other hand, are the most damaging nematode pests that parasitize crops (Jones *et al*., 2013). Information on the use of the CARD model to explain the relationship between the concentrations of the two plant extracts and plant-parasitic nematodes is not available. The objective of this study was to use the CARD model in the determination of *Meloidogyne incognita* sensitivity, DDG patterns and minimum inhibition concentrations of *M. angolensis* and *T. elegans* plant extracts on juvenile hatch and mortality.

Materials and Methods

Collection of plant material and preparation of powdered leaf meals

Leaves of *M. angolensis* and *T. elegans* were collected from traditional healers in the Limpopo Province. The botanical origin of the leaf samples were identified and confirmed by a botanist from the South African National Biodiversity Institute (SANBI, Tshwane) and assigned voucher specimen numbers: *M. angolensis* 3112000/PRE099594-0 and *T. elegans*

6603000/PRE0812589-0. The leaf parts were chopped into 5 cm pieces and oven-dried for 4 days at 52 °C prior to grinding in a Wiley mill and passing the material through a 1-mm-aperture sieve (Makkar 1999). Milled crude plant materials of 25 kg were bulk-stored in marked, air-tight glass containers at room temperature in the dark until used in the experiments.

Five hundred gram of crude leaf meal of *M. angolensis* and *T. elegans*, were separately and independently left to soak in 1 L de-ionized water for 24 hours at room temperature. The extracts were filtered and the filtrates were freeze-dried to remove the water from the material.

Preparation of stock solutions

Ten stock solutions were prepared for each of the plant extract. Using an analytical balance, measures from 10 mg to 100 mg in increments of 10 mg were made from each extract and placed separately into sterile 1.5- ml Eppendorf tubes. This was followed by adding a solution of pluronic gel and de-ionized water to fill each tube to the 1 ml mark, after which the tubes were tightly closed. The extracts were brought into suspension in each tube by thoroughly shaking it for 45 minutes. The stock solutions were stored at -20 °C in a freezer so that the stability of the extracts would not be affected.

Preparation of nematode inoculum

A population of *M. incognita* race 2, of which the identification was confirmed by sequence-characterised amplified regions-polymerase chain reaction (SCAR-PCR) (Fourie et al. 2001; Zijlstra et al. 2000), was obtained from the ARC-Grain Crops Institute, Potchefstroom, SA, and multiplied for a period of 2 months in glasshouse on susceptible tomato (*Solanum lycopersicum* L.) plants cv. 'Floradade'.

Dark-brown coloured egg masses of *M. incognita* were obtained from infected tomato plants, placed in 1% NaOCl solution and shaken for 30 s to surface-sterilise them and remove the gelatin matrix. The sterilised eggs were thorough rinsed in distilled water before used in J2 hatch bioassay.

Freshly-hatched J2 were obtained by transferring surface-sterilised eggs in Petri dishes containing 10 mL distilled water and placed in an incubator at 25 ± 2 °C. J2 that hatched during the first 24 h were discarded with J2 that hatched during the subsequent 48 h used in the mortality bioassay (Dube & Mashela 2016).

Meloidogyne incognita second-stage juvenile mortality and hatch assays

In vitro trials were initiated under laboratory conditions at the Biosciences Unit at the Council for Scientific and Industrial Research (CSIR) in Pretoria, South Africa (approx. 25°44'47.96" S, 28°16'47.01" E).

Second-stage juvenile mortality assays: The concentrations of *M. angolensis* and *T. elegans* extracts were tested for nematode J2 mortality at ten different concentrations (0.1-1.0 mg ml^{-1}) each in 96-well test plates. This was achieved as follows: A suspension of 20ml pure pluronic gel and de-ionized water was put into 50- ml Eppendorf tubes. This was followed by adding a 10- ml suspension of J2 of *M. incognita* race 2 in water containing approximately 35 000 J2. The suspension was thoroughly shaken manually for 5 s. From this a 90- µl suspension containing 100 ± 20 J2 was transferred to the 96-well plate using a pipette. Ten microlitre extract of each concentration was subsequently put in each well plate in a specific direction and order. Thus, the final volume in each well was 100 µl. Treatments were arranged in a randomised complete block design (RCBD), replicated four times.

The ten treatment concentrations of each plant plus two controls of pure pluronic gel + deionized water solution and 1.8 mg.mL^{-1} salicylic acid dissolved in MeOH were assessed. Each well plate was sealed tightly with plastic wrapping to avoid any cross-well contamination and shaken for 10 minutes at 1 000 revolutions per minute (RPM) on an Orbit 1000 (Labnet, Edison, USA) laboratory shaker. The number of living and dead J2 were determined prior to the onset of each experiment using an inverted compound microscope (Carl Zeiss, Jena Germany) set at 10X and 32X magnification. The plates were incubated at 22 °C in the dark and the number of dead J2 per well plate was counted at 24, 48 and 72 hours after the onset of experiment. Two independent trials were conducted for each experiment.

Second-stage juvenile hatch assays: The exposure of eggs to different extracts and concentrations of *M. angolensis* and *T. elegans* were done with 100 ± 20 eggs in 96-well plates. Ten concentrations (0.1-1.0 mg mL^{-1}) of each plant extract plus two controls of pure pluronic gel + deionized water solution and 1.8 mg.mL^{-1} salicylic acid dissolved in MeOH were arranged in RCBD with four replications. Every treatment was randomly assigned to four rows (replicate blocks). After having sealed each well plate tightly with parafilm to avoid any cross-well contamination, each well plate was shaken for 10 minutes at 1 000 RPM using an Orbit 1000 (Labnet, Edison, USA) laboratory shaker. The number of hatched J2 were determined prior to the onset of each experiment using an inverted compound microscope (Carl Zeiss, Jena Germany) set at 10X and 32X magnification. The plates were incubated at 22 °C in the dark and the number of hatched J2 per well plate were counted at 7, 14 and 21 days after the onset of experiment. Two independent trials were conducted for each plant extract.

Statistical analysis

Cumulative J2 percentage hatch and mortality were made per treatment after each incubation period. The standardized residuals of each variable were tested for deviations from normality using Shapiro-Wilk's test. Data were transformed using arc-sine (percentage)^{½} prior to analysis of variance using SAS/STAT statistical software (SAS Institute 2008). Fisher's protected t-LSD (least significant difference) was calculated at 5 % level of significance to compare means of significant effects (Snedecor & Cochran 1980). Significant mean exposure period values were subjected to the CARD model (Liu et al. 2003) to generate the J2 hatch and mortality curves using the quadratic equation $Y = b_2x^2 + b_1x + c$, where Y = J2 hatch inhibition or mortality mean value and x = plant extract concentration mean value. Additionally, the CARD-generated biological indices, *viz*., threshold stimulation (D_m), saturation point (R_h), 0% inhibition concentration (D_0), 50% inhibition concentration (D_{50}), 100% inhibition concentration (D_{100}), sensitivity index (k) and coefficient of determination (R^2) (Liu et al. 2003), were summarised. Unless otherwise stated, only treatments that were significant at 5% probability level were discussed.

Results

***Maerua angolensis* and *Tabernaemontana elegans* plant extracts on juvenile hatch**

The CARD model quantified concentration ranges of the two plant extracts that could stimulate (D_m-R_h), saturate (R_h-D_0) and inhibit (D_0-D_{100}), J2 hatch (Table 1). The CARD-generated DDG patterns demonstrated an increase in juvenile hatch inhibition with the increase in concentration of the plant extracts (Figure 1). The DDG patterns were explained by 74, 93 and 97 % of the derived models at 7-, 14- and 21-day exposure periods, respectively, for *M. angolensis* and 87, 98 and 99 %, at the same exposure periods for *T. elegans* (Table 1). The sensitivity of J2 hatch to increasing concentrations of *M. angolensis* and *T. elegans* was moderate at an average sensitivity ranking (k) of 20 and 19 units, espectively (Table 1).

Table 1. CARD generated biological indices of *Meloidogyne incognita* second-stage juvenile hatch to increasing concentrations of *Maerua angolensis* and *Tabernaemontana elegans*.

Biological index	*Maerua angolensis*			*Tabernaemontana elegans*		
	Exposure period (days)			Exposure period (days)		
	7	14	21	7	14	21
Threshold stimulation (D_m)	-	0.265	0.329	0.122	0.169	0.173
Saturation point (R_h)	0.636	1.001	1.136	0.584	0.973	1.001
0% inhibition (D_0)	0.636	1.266	1.465	0.706	1.142	1.174
50% inhibition (D_{50}) 100% inhibition (D_{100}) R^2	0.636	1.266	1.465	0.706	1.142	1.174
	0.636	1.266	1.465	0.706	1.142	1.174
	0.74	0.93	0.97	0.87	0.98	0.99
Sensitivity index (k)	20	20	20	20	17	19
Average sensitivity ranking (k)		20			19	

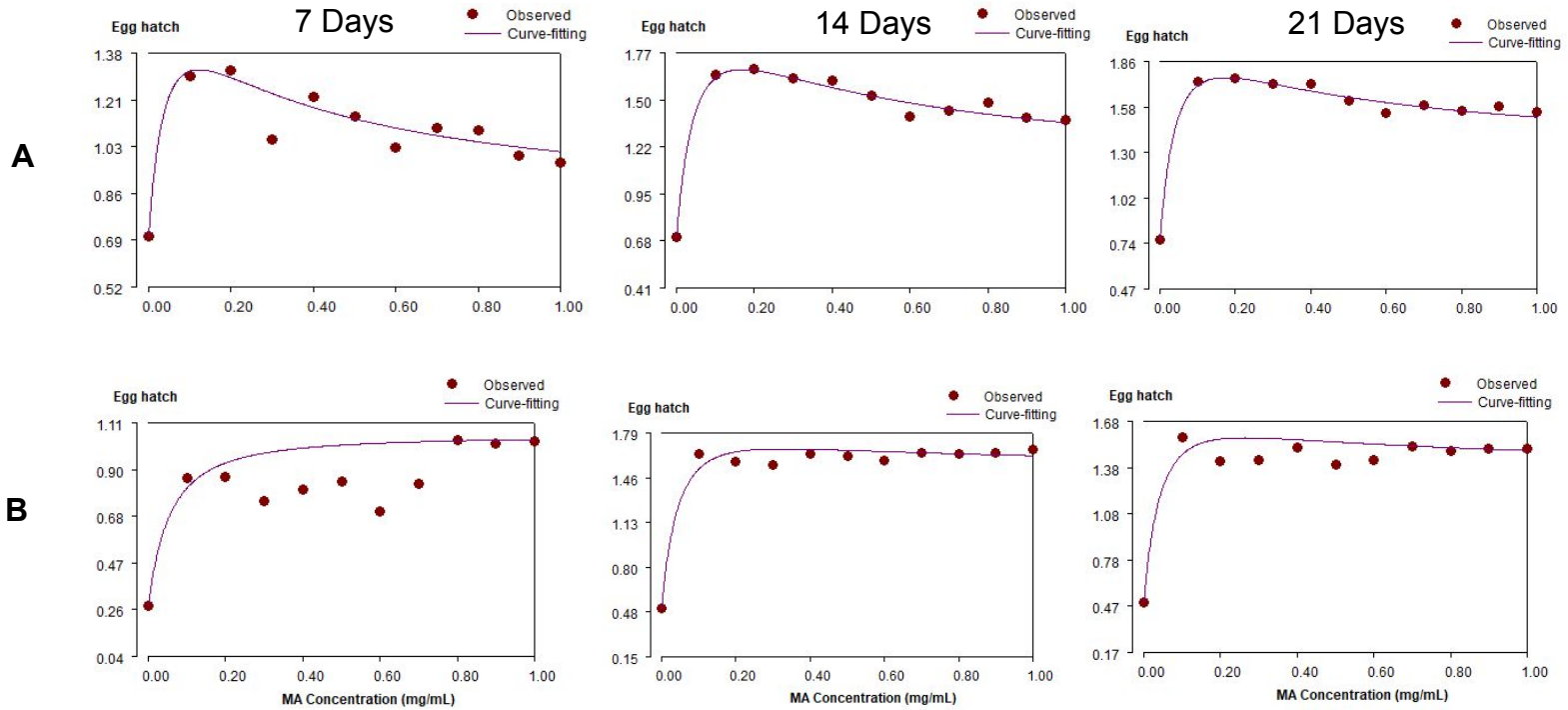

Figure 1. Density-dependent growth patterns of *Meloidogyne incognita* juvenile hatch at 7, 14 and 21 days of incubation in *Tabernaemontana elegans* (A) and *Maerua angolensis* (B).

Table 2. *Maerua angolensis* and *Tabernaemontana elegans* minimum *Meloidogyne incognita* juvenile hatch inhibition concentration at three exposure periods.

	Maerua angolensis		*Tabernaemontana elegans*	
Exposure period (days)	Model	x $(mg.mL^{-1})^z$	Model	x $(mg.mL^{-1})$
7	$y = 0.30 + 11.16x - 48.94x^2$	0.11	$y = 0.71 + 21.09x - 190.37x^2$	0.06
14	$y = 0.52 + 27.16x - 184.20x^2$	0.07	$y = 0.71 + 27.79x - 198.40x^2$	0.07
21	$y = 0.52 + 29.09x - 186.29x^2$	0.08	$y = 0.77 + 30.24x - 228.44x^2$	0.07
Mean	0.08		0.07	

$^z x = -b_1/2b_2$, where $y = b_2x^2 + b_1x + c$.

The minimum juvenile hatch inhibition concentration for the two extracts were comparable at 0.06 and 0.07 mg.mL^{-1} for *M. angolensis* and *T. elegans,* respectively (Table 2).

Maerua angolensis and *Tabernaemontana elegans* plant extracts on juvenile mortality

The effects of the two plant extracts on *M. incognita* juvenile mortality were similar to those of juvenile hatch in terms of the CARD model quantified concentration ranges that could stimulate (D_m-R_h), saturate (R_h-D_0) and inhibit (D_0-D_{100}), J2 mortality (Table 3) and DDG patterns (Figure 2). The DDG patterns were explained by 92, 85 and 96% of the derived models at 24-, 48- and 72-hours exposure periods, respectively for *M. angolensis* and 89, 94 and 70% at the same exposure periods for *T. elegans* (Table 3). Second stage juvenile mortality were highly sensitive to *M. angolensis* with average k-value of 2 units, whereas the sensitivity to *T. elegans* was moderate at k-value of 15 units (Table 3). The minimum juvenile mortality concentration for the two extracts were very low at 0.13 and 0.94 µg.mL^{-1} for *M. angolensis* and *T. elegans,* respectively (Table 4).

Discussion

Maerua angolensis and *Tabernaemontana elegans* plant extracts on *Meloidogyne incognita* juvenile hatch

The nematode suppressive nature of the two plant extracts used in traditional medicine in this study is similar to reports made previously on other plants (Ntalli & Caboni 2012). Several plant extracts used in traditional medicine have demonstrated a measure of bioactivity on nematode J2 hatch (Kalaiselvam & Devaraj, 2011; Javed et al. 2008; Ibrahim et al. 2006). The most studied plant species include, garlic *(Allium sativum* L.*),* chrysanthemum (*Chrysathemum coronarium* L.), fennel (*Foeniculum vulgare* Mill) (Ibrahim et al. 2006),

Table 3. Biological indices of *Meloidogyne incognita* second-stage juvenile mortality to increasing concentrations of *Maerua angolensis* and *Tabernaemontana elegans*.

Biological index	*Maerua angolensis*			*Tabernaemontana elegans*		
	Exposure period (h)			Exposure period (h)		
	24	48	72	24	48	72
Threshold stimulation (D_m)	1.008	0.626	-	-	0.888	0.265
Saturation point (R_h)	0.992	1.124	2.433	0.921	0.886	0.918
0% inhibition (D_0)	15.602	1.750	2.433	0.921	1.774	1.183
50% inhibition (D_{50}) 100% inhibition (D_{100}) R^2	15.821	1.750	2.433	0.921	1.774	1.183
	16.00	1.750	2.433	0.921	1.774	1.183
	0.92	0.85	0.96	0.89	0.94	0.70
Sensitivity index (k)	3	0	3	17	9	20
Average sensitivity ranking (k)	2			15		

Table 4. *Maerua angolensis* and *Tabernaemontana elegans* minimum *Meloidogyne incognita* juvenile mortality concentration at three exposure periods.

Exposure period (h)	*Maerua angolensis*		*Tabernaemontana elegans*	
	Model	x $(mg.mL^{-1})^z$	Model	x $(mg.mL^{-1})$
24	$y = 0.01 + 4.67x - 5.50x^2$	0.42	$y = 0.03 + 13.23x - 47.51x^2$	0.14
48	$y = 0.16 + 3.59x - 2.87x^2$	0.63	$y = 0.12 + 9.52x - 25.59x^2$	0.19
72	$y = 0.52 + 2.75x - 0.78x^2$	1.76	$y = 0.24 + 24.91x - 168.95x^2$	0.07
Mean	0.94		0.13	

$^z x = -b_1/2b_2$, where $y = b_2x^2 + b_1x + c$.

mugwort (*Artemisia vulgaris* L.*)* (Costa et al. 2003), *Azadirachta indica* (Javed et al. 2008), *Inula viscose I. viscose* (Oka et al. 2001), white cedar *(Melia azedarach* L.*),* elderberry (*Sambucus nigra* L.) (Akyazi 2014) and *Tagetes* sp. (Kalaiselvam & Devaraj 2011). In most of these plants, DDG responses have been reported with majority of these observations showing an inverse relationship between J2 hatch and the increasing concentrations (Akyazi 2014; Kalaiselvam & Devaraj 2011; Javed et al. 2008).

Mashela et al. (2015) reported that generally, biological systems respond to extrinsic and intrinsic factors in a DDG patterns, characterised by three growth phases, namely, stimulation, saturation (neutral) and inhibition in support of the theory postulated by Liu et al. (2003). The CARD model was developed to quantify these DDG response patterns of biological entities to increasing concentration of allelochemicals (Liu et al. 2003). The stages of DDG patterns were observed on *M. incognita* J2 hatch, mobility and mortality exposed to increasing concentrations of fermented crude extracts of *Cucumis myriocarpus* and *Cucumis. africanus* (Dube et al. 2018; Dube & Mashela 2017). All three stages of DDG patterns were observed in the current study when *M. incognita* J2 were exposed to *M. angolensis* and *T. elegans* plant extracts (Table 1; Figure 1) supporting previous documentations (Mashela et al. 2015; Liu et al. 2003). The observations in the current study provided evidence for the potential use of the CARD model as previously reported (Mashela et al. 2015).

The higher the sensitivity (k) value of an organism, the higher the tolerance of that organism to allelochemicals, *vice versa* (Liu et al. 2003). In the current trials, J2 hatch was moderately sensitive to both *M. angolensis* and *T. elegans* plant extracts (Table 1). In plant-parasitic nematodes, successful J2 hatch and other behaviours depends on the ability of the chemical concentrations in soil solutions to enter and be detected by chemoreceptors in developing nematode inside eggs (McSorley 2003). The moderate sensitivity effects of the two

plant extracts could be explained in terms of the role played by the layers of the eggs that could have provided some kind of restrictions to the movement of chemicals hence affecting the concentrations to which the nematode inside the egg is exposed to (McSorley 2003).

In the current study, minimum J2 hatch inhibition of *M. angolensis* and *T. elegans* plant extracts were very low at all exposure periods (Table 2) compared to previous studies (Dube & Mashela 2016). The findings contradict the previous report that low minimum inhibition concentrations are associated with high extract sensitivities (Dube 2016). In the current study, minimum J2 hatch inhibition concentrations were low, whereas the sensitivity to the two plant extracts were moderate.

Maerua angolensis and *Tabernaemontana elegans* plant extracts on *Meloidogyne incognita* juvenile mortality

The bioactive properties of *M. angolensis* and *T. elegans* plant extracts used in traditional medicine is similar to previous observations of *in vitro* studies of essential oil from true myrtle (*Myrtus communis* L.) that exhibited high mortalities of *M. incognita* (Archana & Prasad 2014). Various other plant extracts from *Datura. stramonium, A. indica*, *Moringa* species, *Artemisia. vulgaris* and *Allium. sativum* all displayed lethal properties to *Meloidogyne* species (Claudius-Cole et al. 2010; Ibrahim et al. 2006; Nelaballe & Mukkara 2013).

The J2 mortality DDG patterns observed in this study (Figure 2) agreed with previously postulated hypothesis on response of organisms to increasing concentrations of various allelochemicals (Liu et al. 2003). Pure cucurbitacin A and B also exhibited all stages of DDG patterns on *M. incognita* J2 mortality when the CARD model was used (Dube & Mashela 2016), findings similar to current study.

The minimum lethal concentration due to *M. angolensis* and *T. elegans* observed in this study were very low (Table 4) compared to previous studies (Ardakani et al. 2013). Essential

oils at concentrations as high as 250 mg.mL^{-1} could not cause mortality of *M. incognita* J2 (Ardakani et al. 2013) compared to averages of 0.13 and 0.94 mg.mL^{-1} observed in the current study. For pure isothiocyanate, aldehydes and ketones, *M. incognita* J2 mortality were observed at very low concentrations of 5, 15 and 13 µl.L^{-1}, respectively (Ntalli & Caboni 2012) compared to crude plant extracts in the current study.

Even though the *M. incognita* J2 hatch sensitivities to *M. angolensis* and *T. elegans* were both moderate and comparable, the sensitivity of J2 mortality to the two plant extracts differed (Table 3). *Meloidogyne incognita* J2 mortalities were more sensitive to *M. angolensis* than to *T. elegans.*

The difference in effects of the same plant extracts on *M. incognita* eggs and J2 can be explained in terms of the role played by the layers of the eggs that could have provided restrictions to the movement of chemicals into the egg where a developing nematode is found and the properties of the active substances in the extracts to penetrate these layers (Dube 2016). This heightened sensitivity of J2 to plant extracts when compared to eggs has been reported in a number of other studies (Akyazi 2014; Javed et al. 2008).

In conclusion, CARD model was able to generate the DDG patterns, sensitivity values and minimum inhibition concentrations of *M. angolensis* and *T. elegans. Meloidogyne incognita* J2 hatch inhibition and mortality to *M. angolensis* and *T. elegans* observed in this study demonstrated the nematicidal properties of the two products. The low minimum J2 hatch, lethal concentrations, and moderate sensitivity values observed in this study, provides evidence of potentially high potency of the *M. angolensis* and *T. elegans* when compared to other-plant-based compound.

Disclosure statement

No potential conflict of interest was reported by the authors.

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