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The Potency of Abamectin Formulations against the Pine Wood Nematode, *Bursaphelenchus xylophilus*

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Abamectin offers great protection against *Bursaphelenchus xylophilus*, a well-known devastating pathogen of pine tree stands. Trunk injection of nematicides is currently the most preferred method of control. This study aimed to evaluate the potency of the commonly used formulations of abamectin against *B. xylophilus*. Twenty-one formulations of abamectin were evaluated by comparing their sublethal toxicities and reproduction inhibition potentials against *B. xylophilus*. Nematodes were treated with diluted formulation concentrations in multi-well culture plates. And, populations pre-exposed to pre-determined concentrations of the formulations were inoculated onto *Botrytis cinerea* culture, and in pine twig cuttings. Potency was contrastingly different among formulations, with LC_{95} of 0.00285 and 0.39462 mg/ml for the most, and the least potent formulation, respectively. Paralysis generally occurred at an application dose of 0.06 µg/ml or higher, and formulations with high sublethal toxicities caused significant paralysis levels at the tested doses, albeit the variations. Nematode reproduction was evident at lower doses of 0.00053-0.0006 µg/ml both on *Botrytis cinerea* and pine twigs, with significant variations among formulations. Thus, the study highlighted the inconsistencies in the potency of similar product formulations with the same active ingredient concentration against the target organism, and the need to analyze the potential antagonistic effects of the additives used in formulations.

Keywords **:** efficacy, nematicide, sublethal toxicity, trunk injection

Avermectins represent a group of closely related 16-membered macrocyclic lactones with a great diversity of functionalities (Cvetovich et al., 1994). They are derived from natural fermentation of a soil microbe *Streptomyces avermitilis*; and are among the most potent broad-spectrum naturally occurring anthelmintic, miticidal, and insecticidal compounds widely applied as pesticides in agricultural production (Jansson et al., 1997; Osman et al., 2020; Yoon et al., 2004). These compounds and their derivatives are known to act as neurotoxins by interfering with the normal function of gamma-aminobutyric acid (Jansson et al., 1997; Lasota and Dybas, 1991; Zhang et al., 2020). Abamectin is one of the avermectins that has been successfully commercialized in crop protection as an extremely potent insecticidal and (or) nematicidal agent (Khalil, 2013; Radwan et al., 2019). It has been mostly applied as a foliar spray in insect pest management systems, and soil applications in control of the notorious root-knot

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and other plant-parasitic nematodes (Qiao et al., 2012). And in recent years, abamectin has been assessed and subsequently applied as a trunk injection agent against the pine wood nematode (PWN) (*Bursaphelenchus xylophilus*) as a strategy for the management of the pine wilt disease (PWD) (Choi et al., 2022; Lee et al., 2009, 2016, 2021; Rajasekharan et al., 2017).

The PWN, *B. xylophilus*, is a well-known destructive pathogen of economic importance in pine forest ecosystems, especially in Asian countries and Europe (Kikuchi et al., 2011). The nematode is responsible for an epidemic of PWD on susceptible pine tree varieties in specific countries, including Japan, China, Korea, Portugal, and Spain (Baojun and Qouli, 1989; Kosaka et al., 2001; Proença et al., 2010; Shin, 2008). In Korea, significant pine tree damage and wood yield losses caused by the nematode continue to be recorded since the first record of the disease in 1988 in the southern part of the country (Choi and Moon, 1989; Kwon et al., 2011). The disease is now known to have gradually spread from southern region to several areas in the northern part of the country, with an estimated economic loss of about $6.5 million recorded in the last decade (Kim et al., 2020). The nematode is normally conveyed between host trees by its insect vector (*Monochamus* spp.). Control measures are normally designed to target the PWN and its vector. Physical removal and burning of nematode-infected trees, fumigation of infected wood and aerial application of insecticides are some of the common control methods that have been applied in the past years (Shin, 2008). However, in 2005, abamectin and its derivative (emamectin benzoate) were tested and registered for trunk injection use. The compounds are currently the most preferred preventive nematicidal agents applied against the nematodes proliferating in live pine trees (Bi et al., 2015; Kong et al., 2006; Kwon et al., 2021). These chemicals are known to offer a persistent nematicidal effect and are proven to be more active than other available conventional trunk-injection agents (Lee et al., 2009, 2021; Takai et al., 2000, 2004).

With the increasing demand for abamectin in the control of PWN and other pests or pathogens, more chemical companies continue to make use of the biotechnological advances in recent years to specialize in improved overproduction of the compound using unique biotechnological processes, intricacies, and applications, often producing formulations of similar strength in terms of active ingredient concentration. In recent years, there has also been an increase in production of mixed formulation compounds to target more than one pest and (or) pathogen. Abamectin is being produced as a single chemical compound formulation or in combination with other insecticides such as sulfoxaflor, dinotefuran, acetamiprid, including its derivative, emamectin benzoate, among others. However, studies on the toxic effect levels of pesticide compounds are mostly done on pure active ingredient (Beggel et al., 2010; Cox and Surgan, 2006). But chemical companies use specific unique inert ingredients in addition to the unique biotechnological processes to produce the final formulation products that are deemed ready for agricultural use. The non-uniformity of inert ingredients, coupled with the non-mandatory disclosure of their actual content may significantly influence the efficacy of the final product. This may lead to production of final formulations possessing substantially altered or different potency (Beggel et al., 2010; Schmuck et al., 1994). In our recent study on the efficacy of emamectin formulations against the PWN (Lee et al., 2023), we uncovered the significant disparities in the performance of various formulations of similar active ingredient strength against the PWN. In the current study, the potency of various abamectin formulations against the PWN is equally evaluated by determining their sublethal toxicity levels, and the potential to inhibit nematode reproduction after pre-exposure of the nematode populations.

Materials and Methods

Nematode population. The *Bursaphelenchus xylophilus* strain used in the current study was extracted from infected pinewood sample (*Pinus densiflora*), taken from Gumi area, Gyeongsangbuk-do Province, Republic of Korea in 2021. The nematodes (The juvenile and adult stages) were extracted from the infected wood chips using the Baermann funnel method (Jenkins, 1964), and were subsequently maintained on *Botrytis cinerea* (Kishi, 1995). Briefly, the extracted PWN isolates were maintained on a non-sporulating strain of *Botrytis cinerea* grown on potato dextrose agar at 25°C as described by Kishi (1995) and Takemoto (2008).

Chemical formulations. Seventeen abamectin single compound (1 dispersible concentrate [DC] and 16 emulsifiable concentrates [EC] with a 1.8% active ingredient concentration) and four mixed compound formulations (abamectin 1.8%-sulfoxaflor soluble concentrate [SL], abamectin 1.8%-dinotefuran microemulsion concentrate [ME], abamectin 1.6%-acetamiprid [ME], and abamectin 1.6%-emamectin benzoate [ME]) used in this study were procured from the Korean local markets and(or) their respective pro-

ducing companies. The formulations were assigned blind-case codes from A to U to enable anonymous and impartial empirical analysis of compound performance and presentation of results (Table 1).

Quantification of avermectins B1a and B1b in the formulations. Avermectins B1a and B1b were quantified by a modified method of Rural Development Administration guidance for the pesticide quality inspection method (Rural Development Administration, 2022). Briefly, the abamectin content was calculated with the sum of avermectin B1a and B1b. Abamectin standard was purchased as an analytical-standard grade from HPC Standard GmbH (Gohrisch, Germany); it was a mixture of avermectin B1a and B1b, and the ratios were 97.9% and 2.1%, respectively. The calibration solution was prepared in 10.0-500 μg/ml of abamectin with acetonitrile, and the linearity (R^2) was 1.0000 for the total avermectins. The commercial pesticides were diluted for the quantitative analysis with acetonitrile, then filtered with a syringe filter for the UHPLC analysis (Ultimate 3000, Thermo Fisher Scientific Inc., Waltham, MA, USA). C18 column (4.6 × 250 mm, 5 μm, Agilent Technologies Inc., Santa Clara, CA, USA) was used for the separation, and the avermectins were detected at 245 nm. All the analyses were performed with three replications. The quantity of avermectin was expressed to the sum of avermectin B1a and B1b (Table 1).

Sublethal toxicity test. The potency of the various formulations of abamectin was evaluated by determining their sublethal toxicity. Appropriate test range of each formulation or group of formulations was ascertained by performing preliminary tests at various dilutions. The formulations were subsequently grouped into four different test ranges, following the preliminary test results. These included: 0.00254-0.00847, 0.00254-0.20084, 0.00338-0.10203, and 0.0847-0.4684 mg/ml. Serial dilutions of the test formulations were prepared with distilled water to give seven different test concentrations within the selected test range. The lowest dilution was the amount of the active ingredient in the formulation capable of causing 1-10% mortality, and the highest value represented the concentration at which 90-100% mortality could be registered after a 24-h treatment of the formulation against the nematodes. A 0.5 ml nematode suspension containing 100 nematodes was prepared through homogenization. Briefly, nematode suspension was homogenized by adding distilled water to a subsample of the nematode population, before blowing air through the diluted suspension several times using a pipette (Van Bezooijen, 2006). A 0.5 ml nematode suspension containing 100 nematodes (a mixture of mainly 3rd, 4th stage juveniles and adults) was subsequently filled in each well of a 12 multi-well culture plate (SPL Life Sciences Co., Ltd., Pocheon, Korea), and an equal volume of test compound in selected varying dilutions was added. The multi-well culture plates were wrapped with aluminum foil and kept at 25ºC in the growth chamber (Han Baek HB 303 DH-0, Han Baek, Bucheon, Korea). The experiment was terminated after 24 h, and the number of live and dead nematodes was determined under a Nikon SM2 1000 microscope (Tokyo, Japan). Nematodes were considered dead when no response or movement was detected after several repeated touches with a nematode-picking needle. The test experiment comprised four replicates for each selected compound concentration, and was repeated twice.

Nematode paralysis test. Nematode paralysis tests were performed to assess the effect of the various formulations of abamectin at lower concentration. Serial dilutions of the formulations were prepared to give five different concentration test levels within the range between 0.006-0.6 μg/ml, as described above. The lowest dilution was the concentration capable of causing 1-10% paralysis of the test nematode population, and the highest value was the amount of the active ingredient at which 90-100% paralysis of the test population could be registered after 24-h treatment. The experiment was set up the same way as described above in the sublethal toxicity test. The multi-well culture plates (SPL Life Sciences Co., Ltd.) were wrapped with aluminum foil and kept at 25ºC in the growth chamber (Han Baek HB 303 DH-0). Paralyzed nematodes were counted under a Nikon SM2 1000 microscope after 24 h. The experiments were set up with four replicates for each tested concentration, and were repeated twice. Nematodes were considered paralyzed when no motion was detected but could respond after being prodded severally with a nematode-picking needle.

Reproduction inhibition test. The effect of the formulations on nematode reproduction was investigated in two bioassay studies. In the first bioassay, PWN populations (mixture of all stages and sex) were exposed to the selected varying concentrations of the chemical formulations (0.6, 0.06, 0.006, and 0.0006 μg/ml for abamectin 1.8% formulations, and 0.53, 0.053, 0.0053, and 0.00053 μg/ml for abamectin 1.6% formulations) for 24 h at 25°C, as described by Cheng et al. (2017). The nematodes were subsequently washed three times with sterilized water in a 10-ml centrifuge tube to get rid of the treated chemical, and homogenized to the appropriate nematode numbers needed

for experimentation. A population of 100 nematodes was inoculated onto a uniform *Botrytis cinerea* culture in a Petri dish. The experiment was replicated four times for each chemical concentration and was repeated twice. Fresh untreated nematodes were used in the control set up. The inoculated culture plates were kept at 25ºC in the growth chamber (Han Baek HB 303 DH-0) for 10 days. Nematodes were recovered from all the Petri dish contents using the Baermann funnel method. Counting of the final populations was performed under a Nikon SM2 1000 microscope, and the nematode reproduction factor (Pf/Pi [Pf, final nematode population; Pi, initial nematode population]) was calculated for each formulation.

The second bioassay was conducted in a similar way as described above but, in pine tree twigs according to the method of Shin et al. (2015). Nematodes were pre-exposed to the selected varying concentrations as described above before being inoculated in pine twigs. Briefly, twenty-centimeter-long fresh twigs were cut from live *Pinus densiflora* tree stands in Gumi area, Gyeongsangbuk-do Province, Republic of Korea. The twig-cuttings were sealed at both ends with paraffin to curtail moisture loss and eventual rapid drying. Small holes (diameter × depth, 0.7 × 0.5 cm) were drilled in the middle of the twig cutting and cotton wool was inserted to serve as a source of infection after nematode injection. A population of 1,000 nematodes was injected into the twigs through the cotton wool. The area of inoculation was carefully sealed off with parafilm and subsequently wrapped with aluminum foil before transferring the twig cuttings into the growth chamber at 25ºC (Han Baek HB 303 DH-0). Untreated nematodes were inoculated in the control. The treatments were replicated four times and repeated twice as noted above, and the experiment was terminated after 30 days. Nematode populations were extracted from the twigs by cutting twig portions of 5 cm from the treatment point in both directions into small pieces before suspending them in the Baermann funnel. Nematode populations were enumerated under Nikon SM2 1000 microscope, and the nematode reproduction factor Pf/Pi) (Pf, final nematode population; Pi, initial nematode population) was calculated for each formulation.

Data analysis. Data were tested for homogeneity of variance and subsequently subjected to analysis of variance using SAS statistical package version 9.4 (SAS Institute Inc., Cary, NC, USA). The lethal concentration values ($LC_{10, 20, 50, 90,}$ and $_{95}$) were determined using probit analysis. There were no statistical differences between the two repetitions in nematode reproduction and paralysis data. Therefore, all replications were used in analysis (n = 8 replications). Treatment means of nematode populations and rates of paralysis were subjected to analysis of variance according to the general linear model procedure and were compared using Tukey's honestly significant difference at $P \leq 0.05$. The reproduction factors (Pf/Pi) of populations recovered from each treatment were calculated in Microsoft Excel (Microsoft Corporation, Richmond, WA, USA).

Results

Quantified avermectin concentration and sublethal toxicity. The analysis of the actual concentration of avermectins in the tested formulations showed some deviations in the quantified concentrations when compared with the indicated concentrations on the respective product labels (Table 1). However, the potency of the formulations was

Table 1. List of abamectin compound formulations tested against *Bursaphelenchus xylophilus*

Code	Active ingredient (%)[a]	Quantified concentration (%)
A	Abamectin EC 1.8	2.00
B	Abamectin EC 1.8	2.06
C	Abamectin EC 1.8	2.14
D	Abamectin DC 1.8	1.36
E	Abamectin EC 1.8	1.81
F	Abamectin EC 1.8	2.05
G	Abamectin EC 1.8	2.09
H	Abamectin EC 1.8	1.98
I	Abamectin EC 1.8	1.32
J	Abamectin EC 1.8	2.08
K	Abamectin EC 1.8	1.93
L	Abamectin EC 1.8	1.79
M	Abamectin EC 1.8	2.16
N	Abamectin EC 1.8	2.03
O	Abamectin EC 1.8	2.14
P	Abamectin EC 1.8	2.18
Q	Abamectin EC 1.8	2.05
R	Abamectin + Acetamiprid ME 1.6+7	2.24
S	Abamectin + Dinotefuran ME 1.8+8	1.44
T	Abamectin + Emamectin ME 1.6+2.5	1.15
U	Abamectin + Sulfoxaflor SL 1.8+4.2	2.09

[a]EC, emulsifiable concentrate; DC, dispersible concentrate; ME, microemulsion concentrate; SL, soluble concentrate. Quantified concentration represents the actual quantity of abamectin measured in the current study. It is expressed to the sum of emamectin B1a and B1b (n = 3).

Table 2. The toxicity of abamectin formulations against *Bursaphelenchus xylophilus*

Code[a]	Lethal concentration (95% FL) (mg/ml)				
	LC_{10}	LC_{20}	LC_{50}	LC_{90}	LC_{95}
A	0.24895 (0.24426-0.25299)	0.26124 (0.25741-0.26461)	0.28647 (0.28363-0.28935)	0.32966 (0.32444-0.33592)	0.34304 (0.33659-0.35092)
B	0.01041 (0.00992-0.01078)	0.01106 (0.01066-0.01137)	0.01242 (0.01215-0.01267)	0.01482 (0.01437-0.01543)	0.01558 (0.01502-0.01637)
C	0.03136 (0.02631-0.03488)	0.0353 (0.03084-0.03848)	0.04429 (0.04109-0.04721)	0.06256 (0.05755-0.07124)	0.06899 (0.06245-0.08116)
D	0.23355 (0.2288-0.23765)	0.24536 (0.24142-0.24883)	0.26964 (0.26671-0.27254)	0.3113 (0.30636-0.3172)	0.32425 (0.31814-0.33165)
E	0.22823 (0.22222-0.23331)	0.24096 (0.23595-0.24529)	0.26731 (0.26362-0.27096)	0.31309 (0.30695-0.3206)	0.32744 (0.31983-0.33695)
F	0.22823 (0.22222-0.23331)	0.24096 (0.23595-0.24529)	0.26731 (0.26362-0.27096)	0.31309 (0.30695-0.3206)	0.32744 (0.31983-0.33695)
G	0.21329 (0.20976-0.21651)	0.22698 (0.22399-0.22973)	0.25566 (0.25348-0.2578)	0.30646 (0.30293-0.31038)	0.32261 (0.31815-0.32764)
H	0.22052 (0.21664-0.22404)	0.23574 (0.23249-0.23872)	0.26784 (0.26544-0.27022)	0.32533 (0.32088-0.33034)	0.34377 (0.33812-0.35021)
I	0.02149 (0.01298-0.0303)	0.03095 (0.02037-0.04122)	0.06218 (0.04798-0.07473)	0.17987 (0.16109-0.20299)	0.24308 (0.21437-0.28544)
J	0.01089 (0.01057-0.01116)	0.01153 (0.01127-0.01176)	0.01286 (0.01267-0.01306)	0.01519 (0.01484-0.01564)	0.01592 (0.01548-0.0165)
K	0.111 (0.10028-0.12056)	0.13431 (0.124-0.14351)	0.19344 (0.1844-0.20216)	0.33713 (0.31769-0.36181)	0.39462 (0.3672-0.43074)
L	0.22915 (0.22352-0.23396)	0.24204 (0.23734-0.24615)	0.26876 (0.26526-0.27221)	0.31521 (0.30937-0.32227)	0.32979 (0.32254-0.33871)
M	0.2277 (0.22224-0.23251)	0.24517 (0.24067-0.24921)	0.2824 (0.2789-0.28597)	0.35023 (0.3427-0.3592)	0.37227 (0.36269-0.38383)
N	0.01686 (0.01676-0.01696)	0.01746 (0.01737-0.01754)	0.01866 (0.01859-0.01873)	0.02064 (0.0205-0.0208)	0.02124 (0.02107-0.02144)
O	0.00916 (0.0086-0.00962)	0.01013 (0.00966-0.01053)	0.01229 (0.01196-0.01263)	0.01649 (0.01578-0.01747)	0.01793 (0.01699-0.01925)
P	0.27784 (0.273996-0.281294)	0.292184 (0.289024-0.295081)	0.32172 (0.31919-0.324299)	0.372532 (0.367646-0.378142)	0.388344 (0.382313-0.395345)
Q	0.00988 (0.00974-0.01)	0.01052 (0.01041-0.01063)	0.01187 (0.01177-0.01197)	0.01426 (0.01409-0.01445)	0.01502 (0.01481-0.01526)
R	0.04703 (0.04545-0.04852)	0.05617 (0.05471-0.05757)	0.07892 (0.07745-0.08043)	0.13243 (0.12814-0.1373)	0.15337 (0.14744-0.16016)
S	0.285811 (0.283264-0.288111)	0.29652 (0.294489-0.298379)	0.318139 (0.316713-0.319563)	0.354122 (0.351402-0.357181)	0.365045 (0.361664-0.36888)
T	0.02262 (0.02214-0.02306)	0.02477 (0.02436-0.02515)	0.02946 (0.02911-0.02983)	0.03838 (0.03757-0.0393)	0.04137 (0.04034-0.04256)
U	0.0021 (0.00208-0.00212)	0.0022 (0.00219-0.00222)	0.0024 (0.00239-0.00242)	0.00275 (0.00273-0.00277)	0.00285 (0.00283-0.00288)

[a]A-Q: Single compound formulations (abamectin); R-U: Mixed compound formulations (abamectin + a pesticide). Lethal concentrations ($LC_{10, 20, 50, 90,}$ and $_{95}$ [mg/ml]) data were calculated after 24 h (n = 8).

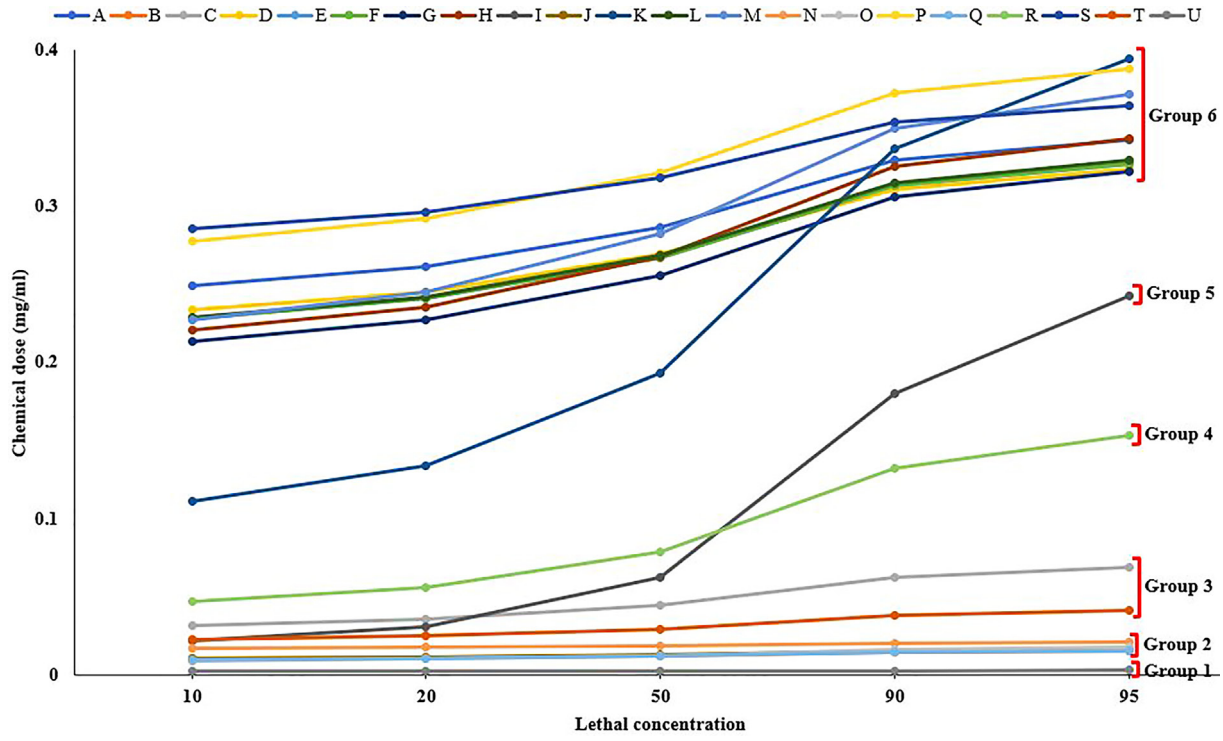

Fig. 1. The potency of abamectin formulations against *Bursaphelenchus xylophilus* after 24-h treatment.

not dependent on the recorded deviations. For instance, the quantified abamectin concentrations of the least potent formulations (A, D-H, K-M, P, and S) were relatively higher than the indicated concentrations on the respective product labels, except for formulation D and S (Table 1).

Generally, mortality of *B. xylophilus* consistently increased with increase in concentration in all the tested formulations. The lethal concentration values ($LC_{10,\ 20,\ 50,\ 90,}$ and $_{95}$) were significantly different among the formulations despite the uniformity of the indicated active ingredient concentrations (Table 2). Abamectin formulations conformed to six groups based on relative similarities in sublethal toxicities (Fig. 1). Group 1 comprised only one formulation (formulation U) (abamectin + sulfoxaflor mixed compound), and was the most effective, with LC_{95} of 0.00285 mg/ml. Five abamectin single compound formulations (B, J, N, O, and Q) constituted the second group, with LC_{95} range of 0.01502-0.02124 mg/ml. Group 3 was constituted by one mixed compound and one single compound formulation (T and C, with LC_{95} of 0.04137 and 0.06899 mg/ml, respectively). Group 4 and 5 were constituted by one compound formulation each (R and I, with LC_{95} of 0.15337 and 0.24308 mg/ml, respectively). Group 6 was constituted by the least potent formulations (A, D-H, K-M, P, and S, with LC_{95} values ranging between 0.32261 mg/ml [in formulation G] and 0.39462 mg/ml [in formulation K]), as shown in Table 2 and Fig. 1. Group 6 was dominated by single abamectin compound formulations, except for a mixed formulation S with LC_{95} of 0.365045 mg/ml.

Paralysis test. Significant variations in rates of paralysis were recorded at the varying concentrations of abamectin formulations (Table 3). The highest rates of nematode paralysis were evident at the highest exposure dose of 0.6 µg/ml, and there were significant differences among the formulations ($F = 134.86$, $df = 21$, $P < 0.0001$). Contrary to the six groups recorded in toxicity test, abamectin formulations conformed to seven groups based on relative similarities in rates of nematode paralysis. Group 1 comprised four formulations: A, B, D, and Q, causing significant rates of paralysis at the highest application dose of 0.6 µg/ml (98-100%). Formulation B was the most effective, causing 100% paralysis of the treated nematodes (Table 3). Group 2 was constituted by formulations; E, F, H, and I, with rates of paralysis ranging between 89.4 and 91.7%. Groups 3 and 4 were each constituted by only one formulation (formulations U and C with rates of nematode paralysis of 83.5 and 71.2%, respectively) (Table 3). Formulations G, M, and P were grouped together in group 5 with a similar rate of nematode paralysis (75.6-75.8%). Group 6 was con-

Table 3. Comparison of paralysis rates induced by abamectin formulations against *Bursaphelenchus xylophilus*

Code[a]	Paralysis rate (%)				
	0.6 μg/ml	0.06 μg/ml	0.015 μg/ml	0.0075 μg/ml	0.006 μg/ml
A	98.6 ± 0.9 a	85.7 ± 6.1 dc	82.6 ± 3.2 b	43.7 ± 10.9 b	8.7 ± 6.6 bc
B	100 ± 0 a	99.4 ± 1.3 a	92.3 ± 3.7 a	3.6 ± 1.8 ij	2 ± 2.2 cd
C	71.2 ± 5.4 de	64.7 ± 13.9 g	27.6 ± 8.2 hi	6.8 ± 3.3 ij	1.9 ± 2.4 cd
D	98.2 ± 1.3 a	97.1 ± 1.8 ab	77.6 ± 12.1 bc	9.6 ± 4.3 hij	0 ± 0 d
E	91.7 ± 5.3 b	79.8 ± 10.7 de	56.6 ± 20.2 ef	13.1 ± 8.7 ghi	6 ± 6.3 cd
F	89.4 ± 4.3 b	89.4 ± 2.9 bc	80 ± 3.9 bc	14.9 ± 5.6 fghi	0.7 ± 0.8 d
G	75.8 ± 4.2 d	63.1 ± 6.8 g	45 ± 17 g	6.8 ± 8.8 ij	4.1 ± 2.5 cd
H	90.8 ± 1.3 b	79.8 ± 3.5 de	64.5 ± 9 de	30.1 ± 28.7 cde	4.2 ± 3.7 cd
I	90.1 ± 2.1 b	81.2 ± 3.5 de	69.7 ± 11.1 cd	65 ± 16.7 a	16.4 ± 14.4 a
J	69.9 ± 6 efg	62.2 ± 6.6 g	55.5 ± 6.5 efg	8.2 ± 4.6 ij	1.3 ± 1.3 cd
K	65.1 ± 12 fgh	64.8 ± 13.8 g	49.4 ± 14.5 fg	24.3 ± 17.1 def	16.4 ± 16.2 a
L	68.5 ± 6.5 ef	64.9 ± 4.4 g	16.4 ± 5.9 j	0.8 ± 0.8 j	0.1 ± 0.4 d
M	75.8 ± 6.2 d	68.3 ± 7.6 fg	45.7 ± 5 g	22.7 ± 5.7 defg	7.6 ± 5.2 cd
N	67.5 ± 9.9 efg	66.4 ± 13.5 g	53.2 ± 16.8 fg	32.7 ± 14. 1cd	20.8 ± 16.5 a
O	62.9 ± 2.9 gh	49.9 ± 11.7 h	22.2 ± 6.6 hij	12.7 ± 4.3 ghi	3.3 ± 2.8 cd
P	75.6 ± 3.4 d	67.1 ± 5 fg	31.5 ± 6.5 h	14.2 ± 6.3 fghi	2.3 ± 1.1 cd
Q	97.9 ± 0.9 a	93.5 ± 2.5 abc	76 ± 3.4 bc	47 ± 5.5 b	18.4 ± 2.2 a
R	65.8 ± 8.1 efg	53.6 ± 10.7 h	32.8 ± 12.9 h	20.3 ± 7.3 efgh	0.9 ± 0.8 d
S	59.9 ± 6.3 h	47.9 ± 6 h	19.8 ± 4.6 ij	7.5 ± 5.7 ij	0.9 ± 1.1 d
T	50.7 ± 5.8 i	35.9 ± 7.5 i	28.4 ± 5.3 hi	10.5 ± 2.6 hij	1.1 ± 1.3 d
U	83.5 ± 3.7 c	74.7 ± 3 ef	53.2 ± 6 fg	37.6 ± 6 bc	14.3 ± 2.6 ab
Control	0 ± 0 j	0 ± 0 j	0 ± 0 k	0 ± 0 j	0 ± 0 d

Values are presented as mean ± standard deviation.
[a]Formulation R and T contain abamectin 1.6% (application rates: 0.53, 0.053, 0.01325, 0.0066, and 0.0053). Paralysis rate data were analyzed after 24 h. Mean values followed by the same letters indicate similar groups (Tukey's honestly significant difference, $P < 0.05$) ($n = 8$).

stituted by formulations J, K, L, N, O, R, and S, despite the differences within the group (rates of nematode paralysis = 60-70%; ($F = 1.53$, $df = 6$, $P = 0.1886$). Formulation T (group 7) caused the least rate of nematode paralysis (50.7% at the highest application dose of 0.6 μg/ml).

A general similar trend was observed at a lower dose of 0.06 μg/ml. Group 1, 2, and 3 formulations (A, B, D, E, F, H, I, Q, and U) maintained significantly high nematode paralysis rate, ranging between 74.7% (in U) to 99.4% (in B) ($F = 66.86$, $df = 21$, $P < 0.0001$). Nematode paralysis rates of ≤ 68% were recorded in all other formulations at the same application dose. At the application dose of 0.015 μg/ml, only formulations A, B, D, F, and Q showed a significantly sustained paralysis effect (76-92%) ($F = 51.21$, $df = 21$, $P < 0.0001$). In general, most formulations with high sublethal toxicities caused significant paralysis levels at the tested application doses. Disparities were however evident at lower application doses of 0.0075 and 0.006 μg/ml (Table 3). For instance, at 0.0075 μg/ml, only formulation I showed a sustained high paralysis rate of 65%. All other formulations caused paralysis rates of less than 50% (1-47%). Notably, with the exception of abamectin 1.8%-sulfoxaflor mixed formulation, all other abamectin-insecticide mixed formulations showed low rates of paralysis even at the maximum tested doses (50-60% at maximum tested dose of 0.6 μg/ml in formulation S, and 0.53 μg/ml in R and T) (Table 3).

Reproduction inhibition test

Reproduction inhibition on *Botrytis cinerea*. Treatment with the selected concentrations of abamectin formulations inhibited reproduction of *B. xylophilus* on *Botrytis cinerea*, despite the recorded variations among the formulations. Among the 1.8% abamectin formulations, no nematode reproduction was recorded in populations pre-exposed to high concentrations (0.6 and 0.06 μg/ml) (reproduction factor = 0: data not shown). Similarly, the 1.6% abamectin

formulations also completely inhibited reproduction at high application doses of 0.53 and 0.053 µg/ml. Low reproduction rates were evident at a 0.006 µg/ml application dose. Inhibition of reproduction was more pronounced in populations treated with formulations A-C, F, J, N, O, Q, and U (reproduction factor range = 0.36-12.46) (Fig. 2A). The two formulations containing 1.6% abamectin (R and T) were also effective at reproduction inhibition at an application dose of 0.0053 µg/ml (reproductive factor: 3.2-8.0). The highest inhibitive effect was recorded in treatment with a mixed abamectin-insecticide formulation U (abamectin 1.8% + sulfoxaflor), with a reproduction factor of as low as 0.36). Modest reproduction was evident in treatments with formulations D, E, G, H, L, and S (reproduction factor range = 19.6-59.6). The highest reproduction was recorded in populations treated with formulations I, K, and P (reproduction factor: 125.5, 207.8, and 219.2, respectively). The nematode numbers in all the chemical treatments were significantly lower than in control (reproduction factor = 755.9) ($F = 163.40$, $df = 21$, $P < 0.0001$).

At the least tested dose of 0.0006 µg/mL among the 1.8% abamectin formulations, high reproduction rates were recorded, and significant variations were more evident among the formulations (Fig. 2B). Formulations A-C, J, N, Q, and U yielded relatively lower reproduction rates than other formulations (reproduction factor range: 70.1-208.2 vs. 239.6-725). Mixed formulations R and T (1.6% abamectin) also yielded low reproduction rates at the lowest dose of 0.00053 µg/ml (reproductive factor = 178.6-262.2). The lowest reproduction was recorded in abamectin-sulfoxaflor mixed formulation (reproduction factor = 70.1) and the highest reproduction occurred in populations treated with a single abamectin compound formulation I (reproduction factor = 725). Nematode numbers in all abamectin treatments were significantly lower than populations recovered in the control ($F = 14.79$, $df = 21$, $P < 0.0001$).

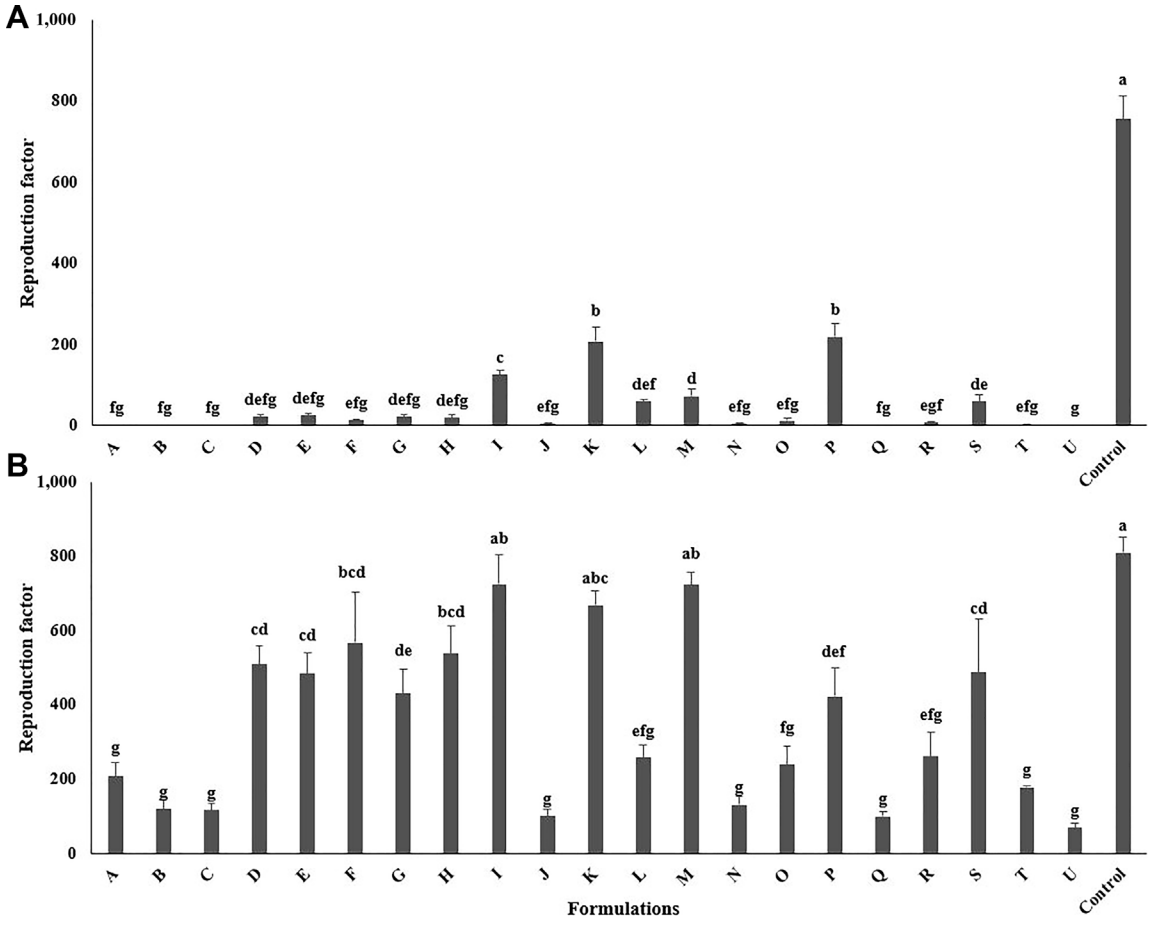

Fig. 2. The effect of abamectin formulations on reproduction of *Bursaphelenchus xylophilus* on *Botrytis cinerea* after 10 days of treatment; (A) treated with a 0.006 µg/ml dose; (B) treated with a 0.0006 µg/ml dose. Error bars indicate the standard error of the means. Bars with the same letter are not significantly different (Tukey's honestly significant difference, $P < 0.05$). Reproduction factor = Pf/Pi (Pf, final nematode population; Pi, initial nematode population).

Reproduction inhibition in twig cuttings. Significant inhibition of nematode reproduction in twig cuttings was evident at all the tested concentrations of abamectin. Reproduction was also completely inhibited in populations pre-exposed to higher concentrations of all formulations (0.6 and 0.06 µg/ml for 1.8% formulations and 0.53 and 0.053 µg/ml rates for the 1.6% abamectin formulations) (reproduction factor = 0: data not shown). At a 0.006 µg/ml treatment dose, the highest reproduction inhibition was recorded in treatments with formulations Q (a single compound formulation) and U (a mixed abamectin-sulfoxaflor formulation), with reproduction factors of 0.31 and 0.34, respectively (Fig. 3A). Significant inhibition was also recorded in several formulations (B-G, J, and L-P) (reproduction factor range = 0.46-1.71). Formulations R and T (1.6% abamectin) also significantly reduced reproduction at a lower comparable dose of 0.0053 µg/ml (reproduction factor: 0.74 and 2.6). Formulations A, H, I, K, and S were less effective at inhibiting nematode reproduction (reproduction factor range = 2.16 and 3.64). The recovered nematode numbers in all formulation treatments were significantly lower than the control (reproduction factor = 11.4) (F = 315.23, df = 21, $P < 0.0001$).

At the lowest tested doses of 0.0006 and 0.00053 µg/ml (for the 1.6% abamectin formulations) a relatively similar trend among treatments was evident, albeit the variations (Fig. 3B). Formulations B-D, F, J, L-O, Q, and U were the most effective formulations, with a reproduction factor range of 0.84-2.79 (Fig. 3B). R and T were less effective, with reproduction factors of 7.6 and 4.8, respectively). Low reproduction inhibition was recorded in formulations A, E, G-I, K, P, and S (reproduction factor range = 5.90-10.12). However, the recovered nematode numbers in all formulation treatments were also significantly lower than the con-

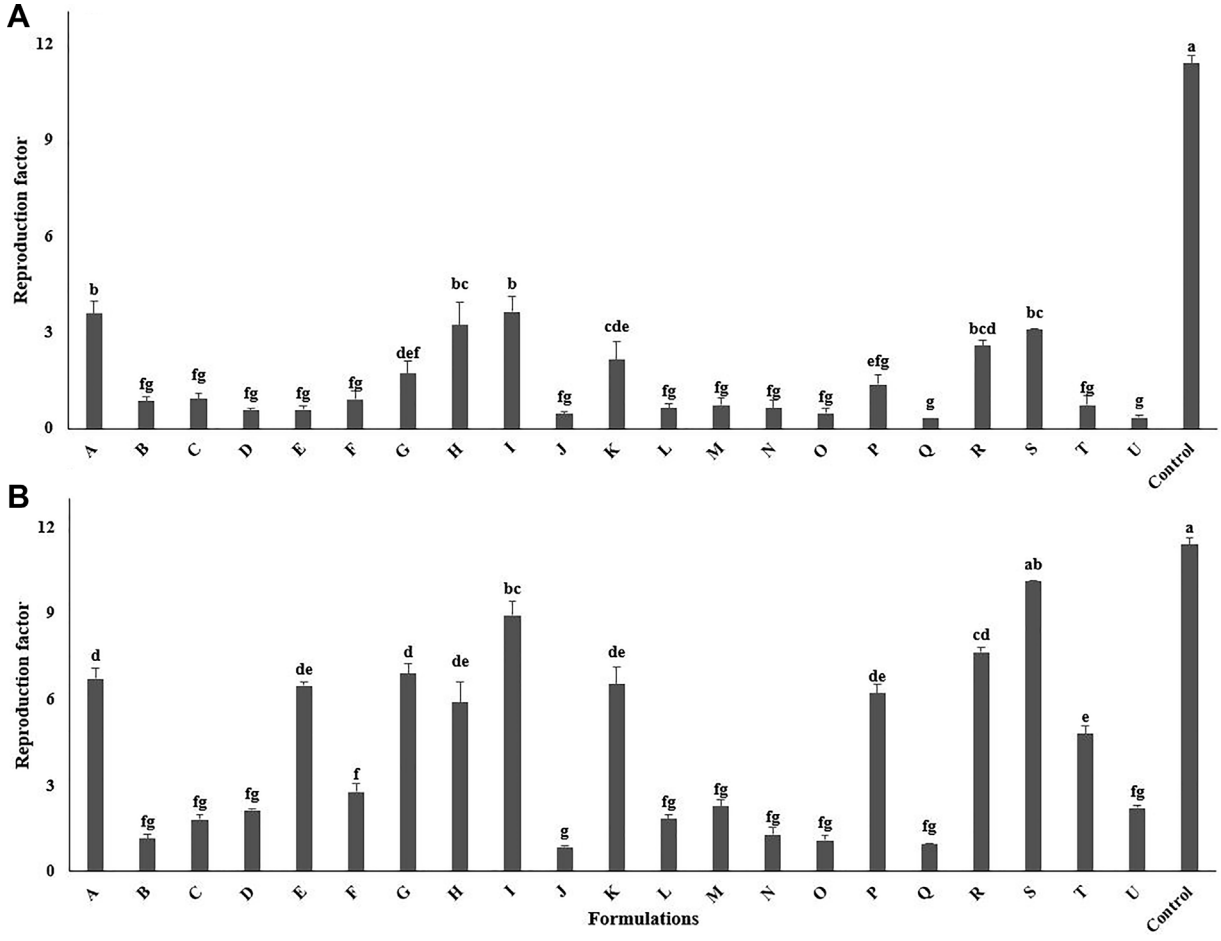

Fig. 3. The effect of abamectin formulations on reproduction of *Bursaphelenchus xylophilus* in pine twigs after 30 days of treatment; (A) treated with a 0.006 µg/ml dose; (B) treated with a 0.0006 µg/ml dose. Error bars indicate the standard error of the means. Bars with the same letter are not significantly different (Tukey's honestly significant difference, $P < 0.05$). Reproduction factor = Pf/Pi (Pf, final nematode population; Pi, initial nematode population).

trol (reproduction factor = 11.4) (F = 235.88, df = 21, P < 0.0001).

Discussion

Our results emphasize the nematicidal efficacy, and the reproduction suppressive effect of abamectin against *B. xylophilus*. Despite the variations, all formulations demonstrated sustained negative effect on the nematode populations. These findings are in agreement with what has been previously reported against the PWN and other notorious plant-parasitic nematodes like root-knot, and cyst nematodes (Cabrera et al., 2009; Liu et al., 2020; Radwan et al., 2019). For example, Rajasekharan et al. (2017) reported a dose-dependent mortality in *B. xylophilus*, with maximum inhibition recorded at 50 µg/ml. At an application dose of 10 µg/ml, abamectin markedly reduced *B. xylophilus* egg hatching by 44%. Cheng et al. (2017) reported an LC_{50} of 1.36 µg/ml after a 12-h treatment of *B. xylophilus*. Radwan et al. (2019) reported that exposing infective juveniles of *Meloidogyne incognita* to 100 mg/l abamectin concentration caused a 71% mortality. Cabrera et al. (2009) reported that abamectin seed treatment at concentrations ranging between 0.3 and 1 mg a.s./seed was highly effective against root-knot nematodes in tomato, with a retained efficacy in the soil of at least 8 weeks. Similar results were reported by Becker et al. (2003) and Abawi et al. (2003). Importantly, along with its derivative emamectin benzoate, the two macrocyclic lactones are potent trunk injection compounds for the control of the PWN, and their control efficacy is touted to last more than 2 years in treated pine stands (Kwon et al., 2021; Shin, 2008; Sousa et al., 2013). The unique mode of action, coupled with high efficacy reduces the possibilities of developing resistance against these compounds and the risks of cross-resistance within avermectins (Zhang et al., 2020); though some level of cross-resistance has already been reported in other target pests like *Tetranychus urticae* (Xue et al., 2020).

Abamectin and emamectin benzoate are therefore highly efficient, broad-spectrum macrocyclic lactones with proven potency against *B. xylophilus* (Lee et al., 2009; Shin, 2008). However, despite the reported efficacy, our results also highlight a formulation dependent potency. There were consistent disparities in the efficacy of formulations. Group 1 formulation, specifically a mixture of abamectin and sulfoxaflor was shown to be about five times more potent compared to the most effective single abamectin compound formulation. And when compared with the least effective formulation among the tested compounds, abamectin-sulfoxaflor mixed formulation was remarkably over hundred times more potent (LC_{50} = 0.00285 vs. 0.39462 mg/ml) despite the similarity in abamectin content (1.8%). Similar results detailing significant disparities in the performance of various formulations of emamectin benzoate against the PWN have already been reported (Lee et al., 2023).

It is important to note that in addition to the principle active ingredient, commercial pesticide formulations, including abamectin and emamectin benzoate, are invariably constituted by cocktails of chemicals, normally referred to as other (inert) ingredients. These “other ingredients” may not be single ingredients but a combination of several compounds. For example, solubilizers are deemed to be a crucial part of abamectin and emamectin benzoate formulations, as they improve the transportation and bioavailability of the chemical-active ingredient in live pine tree stands (Matsuura, 1984; Takai et al., 2001). And all these additives interact to bring about the final chemical complexity in the final product formulation. This may significantly influence the efficacy of the final product. Currently, toxicity effect and health-related risk assessment of pesticides focuses almost exclusively on the active ingredient (Mesnage and Antoniou, 2018). However, the significant proportion of the “inert ingredients” in formulations may interact and alter the toxicity of the active ingredient(s) (Beggel et al., 2010).

Some diluents have potentially significant toxicologic effects on non-target organisms, and are potential phytotoxicants. Unfortunately, there seems to be no uniformity in the type of inert ingredients to be used in similar commercial formulations, with the choice being dependent on the preference of the producer. Our results demonstrate that there might be potential antagonistic effects of the non-disclosed “other ingredients” on the potency of abamectin. Different ingredients present in a given formulation are regulated differently depending on their bioactivity. However, some are even unregulated, owing to the perceived inertness in the sense of devoid pesticide activity. Yet, various studies continue to reveal that these supposedly “inactive” diluents can substantially alter the performance of the active ingredient, with possibilities of the final product becoming either more toxic or less effective than the principle regulated active ingredients in the formulation (Cox and Surgan, 2006; Mesnage and Antoniou, 2018; Mesnage et al., 2013; Schmuck et al., 1994).

In the recent past, numerous examples of inert-ingredient related effects on pesticide potency have been widely reported. For instance, Mayer and Ellersieck (1986) compared the potency of 161 technical grade

compounds to their marketed formulations and showed that toxicity was not affected in 57%, increased in 32%, and decreased in 11% of the cases. Padula et al. (2012) demonstrated that Azadieno, a product formulation of amitraz induced statistically significant genotoxic effect at lower concentrations than active ingredient amitraz alone. Li et al. (2015) also showed that the cytotoxicity of chlorfluazuron in Tn5B1-4 cells could be reduced by PEG6000. Also, Nagy et al. (2020), identified eight studies that demonstrated reduced toxicity of product formulations in relation to their active ingredient. The disparities were attributed to potential antagonistic effect between the constituents. Our results partly agree with such findings and highlight the inconsistencies in the potency of similar product formulations with the same active ingredient concentration against the target organism. In the analysis of the actual concentration of avermectins in all the tested formulations, the quantified concentrations (B1a and B1b) showed some deviations from the indicated quantities on the respective labels. However, the potency was not directly dependent on the recorded deviations. For instance, 9 of the 11 least potent formulations in group 6 yielded relatively higher concentrations of avermectins than the indicated amounts on their respective product labels.

In conclusion, much attention is currently given to the biotechnological overproduction, and structural diversification of avermectin B1 to produce other related effective derivatives (Pitterna et al., 2009; Zhuo et al., 2014). This has seen producers specialize in production of avermectins and their derivatives using unique intricacies. Differences in intricacies may have a significant influence on the performance of the final product formulations. Therefore, there is need to analyze the potential antagonistic effects which may arise as result of the large number of other ingredients or additives that are being used in formulations. This can be achieved through rigorous testing of the additives, complementary to the toxicity studies of the active ingredients in formulations.

Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

Acknowledgments

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