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ORIGINAL ARTICLE

Proof of concept: could snake venoms be a potential source of bioactive compounds for control of mould growth and mycotoxin production

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Significance and Impact of this Study: In all, 10 different snake venoms were examined as new sources of natural crop protection compounds. Using a Bioscreen C rapid assay optical density approach, it was possible to screen and identify snake venoms and fractions which could inhibit growth and/or mycotoxin production by five different mycotoxigenic filamentous fungi. This is a promising source of natural bioactive compounds for future agro-industry applications.

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Abstract

The objective was to screen 10 snake venoms for their efficacy to control growth and mycotoxin production by important mycotoxigenic fungi including *Aspergillus flavus*, *Aspergillus westerdijkiae*, *Penicillium verrucosum*, *Fusarium graminearum* and *F. langsethiae*. The Bioscreen C rapid assay system was used. The venoms from the Viperidae snake family delayed growth of some of the test fungi, especially *F. graminearum* and *F. langsethiae* and sometimes *A. flavus*. Some were also able to reduce mycotoxin production. The two most potent crude snake venoms (*Naja nigricollis* and *N. siamensis;* 41 and 43 fractions, respectively) were further fractionated and 83/84 of these fractions were able to reduce mycotoxin production by >90% in two of the mycotoxigenic fungi examined. This study suggests that there may be significant potential for the identification of novel fungistatic/fungicidal bioactive compounds as preservatives of raw and processed food commodities post-harvest from such snake venoms.

Introduction

There is a worldwide trend to reduce the number of antifungal chemical compounds which can be used for crop protection uses because of their health and environmental impacts (EPA 2020; European commission 2020). The food and feed industry is thus increasingly seeking alternative sources of natural antifungal compounds with potential for controlling the growth of food-borne pathogens.

For cereals, the key contaminating toxigenic fungal pathogens predominantly come from the *Aspergillus*, *Fusarium* and *Penicillium* genera. They are particularly important as they produce mycotoxins including aflatoxins, trichothecenes and ochratoxin A. The presence of these compounds in food products is strictly regulated worldwide (European Union 2006; Wu and Khlangwiset 2010). Research has been focused on screening alternative sources of naturally produced antimicrobial compounds from extreme environments, micro-organisms and plant extracts, especially essential oils (Aldred *et al.* 2008; Bluma *et al.* 2008; Verheecke *et al.* 2014; El Khoury *et al.* 2016; Lahoum *et al.* 2016; Mylona *et al.* 2019; Mohd Danial *et al.* 2020; García-Díaz *et al.* 2020). However, there have been few new lead compounds which have been found for use in food and feed chains to reduce mould growth and mycotoxin contamination, without impacting on the organoleptic properties of the final product.

Snake venoms have recently become an increasing source of antimicrobial compounds (San *et al.* 2010; De Oliveira Junior *et al.* 2013). However, such studies of

their antimicrobial capacity have predominantly focused on controlling bacterial growth.

The aim of this study was to assess the potential of 10 crude snake venoms to control (i) fungal growth and (ii) the mycotoxin production by five major mycotoxigenic fungal species including *Aspergillus flavus* (aflatoxins; AFB_1 and AFB_2), *Aspergillus westerdijkiae* and *Penicillium verrucosum* (ochratoxin A; OTA), *Fusarium graminearum* (nivalenol; NIV) and *F. langsethiae* (T-2), using the high-throughput screening technique for filamentous fungi using the BioScreen C (Medina *et al.* 2012). The two most interesting snake venoms were further fractionated, and the efficacy of their fractions also tested.

Results and discussion

Impact of crude snake venoms on fungal growth

Figure 1 shows three examples of the effect of different concentrations of specific crude venom extracts of *Naja siamensis* on temporal growth of *A. flavus*, *F. graminearum* and *F. langsethiae*. This shows that the effect against different mycotoxigenic fungi varies. Overall, the growth of each test species was impacted by at least 2 out of the 10 crude venoms examined. Depending on the crude venom, the minimum inhibitory concentration observed ranged from the highest concentration (10% crude extract) to the lowest tested (0·04%).

Comparisons between treatments were done using the time to detection (TTD) using the temporal growth datasets. TTD is described as the necessary time for fungal growth to reach a specific OD value. Table 1 shows the effect of the 10 venoms on the TTD of the five mycotoxigenic fungi screened. The TTD data represented include the minimum crude extract concentration showing an increase in TTD >25%. Of the fungi tested, both *F. graminearum* and *F. langsethiae* were the most sensitive species to the venoms screened, with 5 and 7 out of the 10 tested resulting in a significant increase in the TTD (see Table 1). The crude venoms from *Naja nigricollis* and *N. siamensis* were able to completely inhibit fungal growth of the *Fusarium* species at all concentrations examined. *Naja nigricollis* crude venom was also very effective in inhibiting the growth of *A. westerdijkiae*.

The results showed that *Aspergillus* and *Penicillium* had a different sensitivity to the snake venoms screened when compared to *Fusarium* sp. Thus, a higher crude venom concentration was needed to reduce growth of the former species, while much lower concentrations were required for the latter *Fusarium* species. These results are in accordance with Bound *et al.* (2016) that observed a reduced efficacy of 2,3-dideoxyglucosides of terpenes, phenols and alcohols for the control of fungal growth when comparing

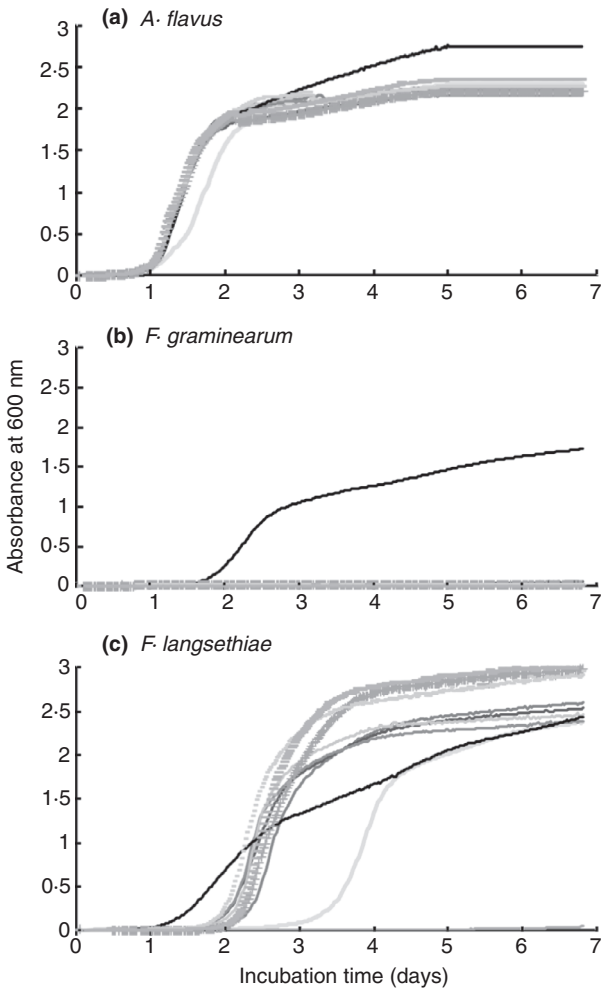

Figure 1 Impact of different concentration of *Naja siamensis* crude extract on (a) *Aspergillus flavus*, (b) *Fusarium graminearum* and (c) *F. langsethiae* growth. Legend: 10%, 5%, 2·50%, 1·25%, 0·63%, 0·31%, 0·16%, 0·08%, 0·04% and control.

Aspergillus and *Fusarium* sp. Further analysis of the snake venoms composition is required to confirm the nature of the compounds involved in control of growth and/or mycotoxin production. A potential source of antifungal compounds from snake venoms could be antimicrobial peptides already extracted from snake venom and which have become an increasing source of therapeutical solutions for control of human pathogens including *Candida albicans* (Kang *et al.* 2017). These peptides have shown encouraging results for inhibiting *A. flavus* growth and aflatoxin production (Huang *et al.* 1997).

Effect of crude snake venoms on mycotoxin production

The impact of the 10 crude venoms on mycotoxin production by the five mycotoxigenic species is also shown

Table 1 Effect of different concentrations of snake venom on fungal growth

		Fungal species									
		A. flavus		*A. westerdijkiae*		*P. verrucosum*		*F. graminearum*		*F. langsethiae*	
Snake family	Snake species	TTD 25%	Aflatoxins	TTD 25%	OTA	TTD 25%	OTA	TTD 25%	NIV	TTD 25%	T-2 toxin
Elapidae	*Dendroaspis viridis*	NI	↑ (×2·85)	NI	=	NI	↓ (×0·18)	NI	=	10%	=
	Naja naja	NI	↓ (×0·08)	NI	=	NI	↓ (×0·00)	NI	↓ (×0·20)	NI	=
	Naja nigricollis	NI	↓ (×0·00)	0·04%	=	NI	↓ (×0·22)	0·04%	↓ (×0·43)	0·04%	=
	Naja siamensis	NI	↓ (×0·18)	0·16%	=	NI	↓ (×0·00)	0·04%	↓ (×0·03)	2·5%	=
	Oxyuranus scutellatus canni	NI	↓ (×0·03)	NI	↑ (×2·27)	NI	↑ (×56·07)	NI	=	NI	=
Viperidae	*Bothrops asper*	NI	↓ (×0·08)	NI	=	10%	↓ (×0·22)	10%	↓ (×0·12)	5%	=
	Calloselasma rhodostoma	10%	↓ (×0·03)	NI	=	10%	↓ (×0·00)	10%	↓ (×0·04)	NI	↑ (×6·18)
	Crotalus basiliscus	10%	NA	NI	=	10%	↓ (×0·34)	NI	↓ (×0·01)	5%	↓ (×0·00)
	Bitis gabonica rhinoceros	10%	NA	NI	=	NI	↓ (×0·22)	NI	↓ (×0·12)	5%	=
	Vipera xanthina	NI	NA	NI	=	10%	↓ (×0·00)	10%	=	10%	=

NI: no impact on fungal growth; aflatoxins: sum of Aflatoxin B_1 and B_2; OTA: ochratoxin A; NIV: nivalenol; =variation lower than twofold compared to control. ↑ or ↓: variation higher/lower than twofold compared to control when treated with the undiluted crude extract at 10%; NA: not analysed. time to detection (TTD) 25%: lowest concentration of snake venom by which the TTD is at least 25% longer than the control.

in Table 1. The production of AFB_1 and AFB_2 was generally reduced by the application of the crude venoms. The response to exposure varied from 0·18-fold of the control mycotoxin production remaining to complete inhibition by the *N. nigricollis* crude venom.

Ochratoxin A production was either unaffected or reduced by the exposure to the crude venoms. The exception was the impact of *Oxyranus scutellatus canni* venom where OTA production by both *A. westerdijkiae* and *P. verrucosum* was actually enhanced by 2·3-fold and 56·1-fold, respectively. Indeed, for *A. westerdijkiae*, no crude extract was able to reduce OTA production by more than 2-fold. However, for *P. verrucosum*, the OTA production was reduced by 9 of the 10 crude venoms used. The crude venoms from *N. siamensis*, *Calloselasma rhodostoma* and *Vipera xanthina* were the most effective, completely inhibiting OTA production by *P. verrucosum*.

Fusarium graminearum only produced NIV under the conditions tested. The production was generally reduced by 7 of the 10 crude snake venoms examined. The most effective venoms were those from *N. siamensis* and *Crotalus basiliscus* with very low non-inhibitory concentrations (NIC; × 0·03, × 0·01-fold, respectively).

The production of T-2 toxin by *F. langsethiae* was similar in the presence of 8 of the 10 tested crude venoms. However, the crude venom of *C. basiliscus* was the most interesting, as it was able to completely inhibit T-2 production. No HT-2 was produced by this strain in these experiments.

Our results showed that some mycotoxins produced by *Fusarium* (Trichothecenes A and B) were not produced at all, especially DON, 3-AcDON, 15-AcDON nor HT-2. It is possible that longer incubation times would be needed for production and quantification of these toxins. Previous studies suggest that trichothecene production may be more likely after 6–10 days of incubation *in vitro* in different media (Hope *et al.* 2005; Duverger *et al.* 2011; Ferruz *et al.* 2016). In the present experiments, NIV was produced by *F. graminearum*. Usually DON and NIV production parallel each other. The presence of NIV only may be related to the medium composition used and the timeframe of the experiments. Previously, Hope and Magan (2003) showed that under some environmental conditions, NIV was likely to be produced earlier and at higher levels than DON.

Overall, crude extracts of the venoms of *N. nigricollis* and *N. siamensis* had the widest range of efficacy against the mycotoxigenic fungi examined. Consequently, these two crude extracts were further investigated in more detail.

Efficacy of *Naja nigricollis* and *N. siamensis* venom fractions for controlling fungal growth

Table 2 summarizes the number of fractions extracted from *N. nigricollis* and *N. siamensis* (total of 41 and 43 fractions, respectively) which led to a reduction of fungal growth. *Fusarium langsethiae* was the most affected mycotoxigenic species with more than half of all the fractions (47/84) extracted from both venoms leading to a reduction in fungal growth. For the *Aspergillus* and *Penicillium* species examined, 1–3 fractions were found to be very effective. Overall, 13 fractions (8 from *N. nigricollis* and 5 from *N. siamensis*) reduced growth of two or more tested mycotoxigenic species, especially the *Fusarium* sp. examined.

Table 2 Number of venom fractions impacting fungal growth and/or mycotoxins production of key mycotoxigenic fungi

	Naja nigricollis			*Naja siamensis*		
Fungal species	TTD >25% increase	Mycotoxin reduction (>90%)	TTD 25% and mycotoxin reduction (>90%)	TTD> 25% increase	Mycotoxin reduction (>90%)	TTD 25% and mycotoxin reduction (>90%)
Aspergillus flavus	0	36	0	2	32	2
Aspergillus westerdijkiae	3	27	2	0	32	0
Penicillium verrucosum	1	40	1	0	40	0
Fusarium graminearum	8	21	1	5	29	2
Fusarium langsethiae	25	21	15	22	25	17
Two or more the fungi above impacted	8	41	1*	5	42	2*

Number represented is the number of fractions able to increase the time to detection (TTD) by at least 25% and/or reduction of mycotoxin production by >90%.

*This number includes only the values with the TTD and the mycotoxins reduction occurring on the same fungi for at least two fungi.

Impact of *Naja nigricollis* and *N. siamensis* fractions on mycotoxin production

The impact of the numbers of fractions which resulted in control of mycotoxin production is summarized in Fig. 2. Overall, 41–93% of the fractions were able to reduce mycotoxins production by >95% when compared to the control production, depending on the species examined.

Species which produced OTA, *P. verrucosum* and *A. westerdijkiae*, appeared to be the most sensitive, with 93 and 69% of the fractions leading to >95% inhibition of OTA production.

The control of AFB_1/B_2 production by *A. flavus* was variable. Thus, 55% reduced AFBs production by >95, 13·75% between 90 and 95%, 30% between 90 and 0%. Interestingly, 1·25% enhanced production by up to 160%. A similar profile was observed for the two trichothecenes producers with slightly (41–46%) lesser proportion inhibiting mycotoxin production by >95%, when compared to AFBs production.

Table 2 summarizes the impact of the tested fractions on both TTD and mycotoxins production independently and when pooled together. *Fusarium langsethiae* was the most sensitive fungus based on both TTD and T-2 production by the fractions of snake venoms (*N. nigricollis* and *N. siamensis*) with 32 fractions inhibiting growth and toxin production.

The two crude snake venoms with the best efficacy (*N. nigricollis* and *N. siamensis*) were examined in more detail by screening the different fractions. This showed that only 13/84 fractions inhibited fungal growth by two or more fungi. However, 83/84 fractions were able to reduce mycotoxins production by >90%. Regarding fungal growth, a number of studies have shown a low percentage of extract fractions, especially from natural antifungal sources such as plant extracts or actinobacteria, were effective at controlling growth of fungal pathogens (Lahoum *et al.* 2016; Nalubega *et al.* 2016; Dikhoba *et al.* 2019). However, many of these studies did not examine impacts on mycotoxin production. For mycotoxigenic fungi, it is important to consider efficacy in controlling mycotoxin production. It may be that the fractions are able to interfere with the gene clusters involved in the biosynthesis of these mycotoxins, resulting in inhibition. More studies are needed at the physiological and molecular level to understand these effects on the control of mycotoxin biosynthesis.

For the first time, snake venoms were screened as a source of natural compounds for control of growth and toxin production by key mycotoxigenic species. This has shown that the venom extracts of Elapidae and Viperidae family either delayed growth or reduced mycotoxin production >95% by *A. flavus*, *A. westerdijkiae*, *P. verrucosum*, *F. graminearum* and *F. langsethiae*. Further fractionation of two snake venoms (*N. nigricollis* and *N. siamensis*) showed very good efficacy with 83/84 fractions having >90% reduction of mycotoxin synthesis by these fungal species. Potential thus exists for exploiting such extracts for the development of lead bioactive compounds for crop protection applications in food and feed chains.

Materials and methods

Fungal strains

Strains of five mycotoxigenic fungal species were used in this study. These were *A. flavus* (NRRL 3357, an AFB_1/B_2 producer), *P. verrucosum* (OTA 11, a OTA producer), *A.*

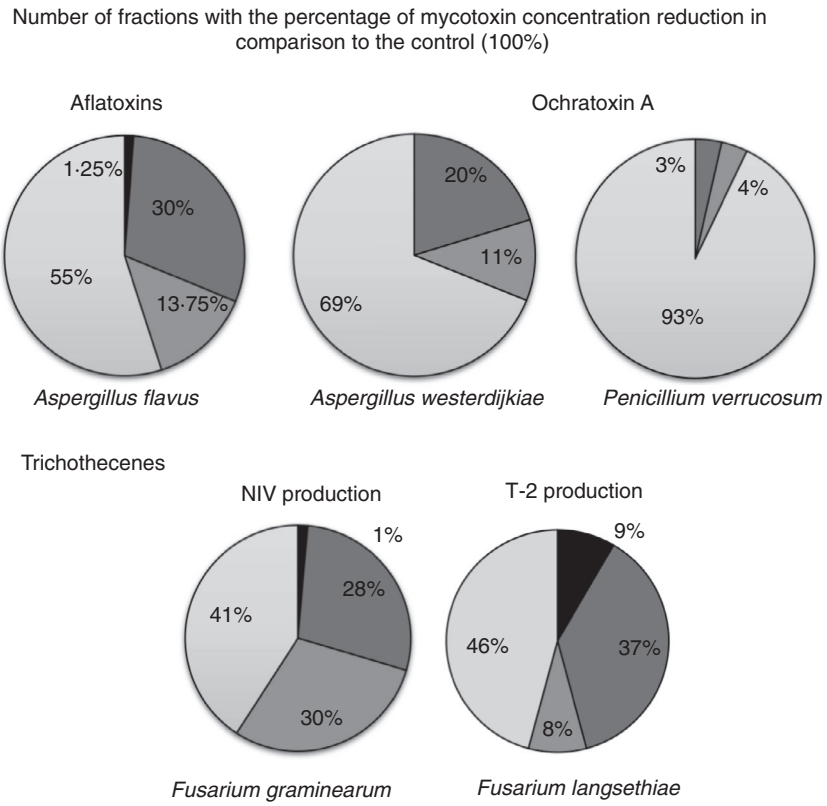

Figure 2 Pie plot summarizing the percentage of mycotoxins produced in the presence of the *Naja nigricollis* and *N. siamensis* crude extracts fractions. Impacts are divided depending on the mycotoxins: (a) aflatoxins production by *Aspergillus flavus*, (b) ochratoxin A production by *A. westerdijkiae* and *P. verrucosum*, (c) Trichothecenes by *Fusarium graminearum* and *F. langsethiae* (■ (<0%), ■ (0%<x<90%), ■ (90%<x<95%), □ (>95%)).

westerdijkiae (IBT 23971, OTA producer), *F. langsethiae* (UK oats, 2004/54, a T-2 producer) and *F. graminearum* (FgB (L1-2/2D), NIV producer) (Medina and Magan 2010; Nierman *et al.* 2016; Mohd Danial *et al.* 2020).

Culture media and inoculation

The strains were subcultured on malt extract agar at 25°C for 7 days in the dark to allow sporulation. The spores were harvested using a surface-sterilized loop in a safety cabinet and placed in 9 ml of sterile water containing 0·01% Tween 80 solution. This was mixed well and the spore concentration determined with a haemocytometer. This was then diluted as required to obtain a final spore concentration of 10^5 spores per ml for subsequent use in the Bioscreen C wells.

Semisolid yeast extract sucrose (YES) medium containing 20 g l^{-1} of yeast extract (Sigma Aldrich, Steinheim, Germany), 150 g l^{-1} of sucrose (Fisher Scientific, Basingstoke, UK), 0·5 g l^{-1} of magnesium sulphate (Fisher Scientific) and 0·125% w/v of agar (n° 3, Oxoid, Fisher Scientific) was used as the medium in the Bioscreen C 100 well titre plate assays.

Snake venoms and snake venom fractions

The crude snake venoms used in these experiments are listed in Table 1 and were provided by Venomtech Ltd (Kent, UK). First, 10 crude venoms belonging to two different snake families: Elipidae and Viperidae and with origins in different continents were tested. In a second stage, the crude venoms from *N. nigricollis* and *N. siamensis* were fractioned by Venomtech using RP-HPLC (McCullough *et al.* 2018) and 41 and 43 fractions, were obtained, respectively.

Screening of efficacy for growth inhibition and mycotoxin control using the Bioscreen C

The Bioscreen C uses 2 × 100 well microtitre plates (Bioscreen C Microbiological Growth Analyser; Labsystems, Helsinki, Finland). A stock of YES medium was inoculated with the previously prepared spore suspensions to obtain a final concentration of 10^5 spores per ml. 150 µl of these media was then loaded into the entire 100-well plate. For inoculation, another 150 µl of YES medium and 10% of each crude venom or each venom fraction

were loaded into the wells corresponding to the first column. Then half-dilutions of the crude venoms or the fractions were performed using a multi-channel pipette until a dilution of 1/128 was achieved for each row. The plates were sealed and incubated for 10 days at 25°C. The Bioscreen C automatically controls the temperature.

This system, as previously described by Medina *et al.* (2012), allows the optical density (OD; 600 nm) to be measured automatically every 30 min and compiled and stored via the Easy Bioscreen Experiment software (EZExperiment) provided. The raw datasets obtained from the Bioscreen C were subjected to two further steps before analysis to correct the different background signals. The determination of the TTD at an OD of 0·5 was calculated using a Microsoft® Excel® template that uses linear interpolation between successive OD readings to do the calculations and was kindly provided by Dr R.J.W Lambert (Bidlas *et al.* 2008). An increase in the TTD can be correlated with a reduction in fungal growth (Medina *et al.* 2012).

Mycotoxin analyses

After the 10-day experimental period, the samples were harvested and placed in 2 ml Eppendorfs, labelled and stored at −20°C until analysis. AFB_1/B_2 (AFBs) quantification was done using a method described by Medina and Magan (2012) with some modifications. 500 µl of chloroform was used as the extraction solvent. For the extractions of OTA and NIV and T-2 toxins, the first step of extraction consisted of the addition of an equal volume (150 µl) of warm distilled water containing 4% agar. Each sample was mixed and left to rest until the agar had solidified. For *P. verrucosum* and *A. westerdijkiae*, OTA was extracted by the addition of 500 µl of methanol and shaking for 2 h at 150 rev min^{-1}. The solution was filtered (PTFE, 0·22 µm) prior to analysis by HPLC-FLD using the method of Medina *et al.* (2004). For type B Trichothecenes (15-Acetyldeoxynivalenol; 15-AcDON, 3-Acetyldeoxynivalenol; 3-AcDON, Deoxynivalenol; DON and Nivalenol; NIV), 500 µl of methanol : water (80 : 20 v/v) was added to each Eppendorf tube. For T-2 and HT-2 analyses, 500 µl of acetonitrile : water (84 : 16 v/v) was added, then left 2 h under agitation at 150 rev min^{-1}. The samples were then analysed by HPLC-DAD using the method described by Medina *et al.* (2010).

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Authors' contribution

CV-V: HPLC and BioScreen C full data analysis and interpretation, writing original, supervision and submission; JM: Lab experiments; EG-C: Practical data analysis and writing review; NM: Writing, reviewing and editing, supervision and AM: Experimental design, concept, supervision, writing, reviewing and funding acquisition.

Conflict of Interest

The authors have no conflict of interest to declare.

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