International Journal of Plant & Soil Science

34(3): 41-49, 2022; Article no.IJPSS.83361
ISSN: 2320-7035

Assessing the Single and Combined Toxicity of Chlorantraniliprole with *Bacillus thuringiensis* against Maize Fall Armyworm *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae) under Laboratory Conditions

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/IJPSS/2022/v34i330845

Open Peer Review History:
This journal follows the Advanced Open Peer Review policy. Identity of the Reviewers, Editor(s) and additional Reviewers, peer review comments, different versions of the manuscript, comments of the editors, etc are available here: https://www.sdiarticle5.com/review-history/83361

Original Research Article

Received 07 December 2021
Accepted 13 February 2022
Published 14 February 2022

ABSTRACT

Use of synthetic insecticides for the management of fall armyworm (FAW) *Spodoptera frugiperda* (J. E. Smith) for a longer period will led to development of insecticide resistance. Identification of an eco-friendly synergistic agent to enhance the toxicity potential and reduced pesticide use as well become mandatory in due process. Hence the present study was formulated to find the single and combined toxicity of chlorantraniliprole and *Bacillus thuringiensis* (*Bt*) against the 2nd and 3rd larval instars of *S. frugiperda*. Single toxicity of chlorantraniliprole against 2nd and 3rd larval instars were

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0.87 and 1.52 ppm (LC_{25}); 4.08 and 6.50 ppm (LC_{50}), respectively. With respect to *Bt,* single toxicity against 2nd and 3rd larval instars were 474.39 and 693.48 ppm (LC_{25}); 1008.62 and 1228.62 ppm (LC_{50}), respectively. Combination effect of chlorantraniliprole with *Bt* revealed that 2nd instar of FAW showed supplemental synergism at LC_{50} of chlorantraniliprole + LC_{25} of *Bt*. In the case of LC_{50} of chlorantraniliprole + LC_{50} of *Bt*, LC_{25} of chlorantraniliprole + LC_{50} of *Bt* and LC_{25} of chlorantraniliprole + LC_{50} of *Bt* combinations, they showed sub additive synergism. In 3rd instar larvae, the combined toxicity results were similar for all the combinations of chlorantraniliprole + *Bt* except LC_{25} of chlorantraniliprole + LC_{50} of *Bt* where it showed an antagonistic synergism. Activity of Carboxyl Esterase (CarE), Mixed Function Oxidase (MFO) and Glutathione-S-Transferase (GST) were found to be lesser in chlorantraniliprole LC_{50} + Bt LC_{25} combinations than single toxicity treatments. Therefore, combined use of chlorantraniliprole with *Bt* at LC_{50} of chlorantraniliprole + LC_{25} of *Bt* had supplemental synergism on fall armyworm under laboratory condition.

Keywords: Chlorantraniliprole; Bacillus thuringiensis; Spodoptera frugiperda; combined toxicity; synergism; maize.

1. INTRODUCTION

Maize is the third most important food crop next to rice and wheat in India. Fall armyworm (FAW), *Spodoptera frugiperda* (J. E. Smith) is a cosmopolitan insect native to the tropical and subtropical regions of Americas [1,2]. Larvae of *S. frugiperda* are known to feed on more than 353 plant species [3]. Larvae feed on all growth stages of maize and cause significant yield losses. In India, its incidence was first noticed at the farmer's field in Chikkaballapur, Karnataka on maize [4]. They recorded more than 70% of crop damage. Further, it was reported in Tamil Nadu, Kerala, Karnataka, Maharashtra and Madhya Pradesh in India. In India other than maize, *S. frugiperda* damage was recorded in sugarcane [5], pearl millet, finger millet, sorghum [6], paddy [7], ginger [8], barnyard millet [9], para grass, guinea grass, fodder maize, green amaranth [10], sugar beet [11] and sunflower [12]. Faster spread in geographical distribution and wider host adaptability make FAW a promising pest in India. For the immediate control of FAW menace, farmers rely highly on synthetic pesticides than other management measures. Use of pesticide is inevitable in the current scenario of FAW management. This may led to development of insecticide resistance in FAW. Therefore, it is necessary to use pesticides in a manner that will address both resistance and residue in due course of time. At this juncture, identification of a biosynergistic agent in the form of *Bt* can be a possible ecofriendly alternative for toxicity potentiation and reduced pesticide use as well. Studies also provided valuable information about the combined use of entomopathogenic microorganisms and chemical pesticides [13-15]. Besides, no recent studies reported the combined use or compatibility of new molecules with *Bt.* Keeping this in view, the present investigation was aimed at improving the toxicity of chlorantraniliprole through addition of *Bacillus thuringiensis* (*Bt*) in combination for the effective management of FAW.

2. MATERIALS AND METHODS

2.1 Insect Culture

S. frugiperda culture was maintained at the FAW lab, Department of Agricultural Entomology, Tamil Nadu Agricultural University, Coimbatore. Adults were provided with potted maize seedlings for oviposition. Then the plants were removed from the oviposition cage and transferred to an insect-proof cage to let the eggs hatch and larvae develop. The continuous supply of FAW culture was maintained using semi-synthetic artificial diet [16]. The 2nd and 3rd instar larvae were used for all bioassays.

2.2 Bioassay

Bioassays were conducted using newly moulted 2nd and 3rd instar larvae of *S. frugiperda* obtained from laboratory cultures using leaf disc bioassay method with slight modification [17]. Newly moulted 2nd and 3rd instar larvae were pre-starved for 4 h before bioassay. Each treatments consisted six concentrations of selected insecticides and an untreated control. Leaves were treated with different concentrations of insecticides and shade dried for 1 h. Pre-starved larvae were individually placed into the six well culture plates poured with 1% agar, maintained at 28±1℃ and 12:12 h (light: dark). Mortality data were recorded at 24, 48 and 72 hours after treatment (HAT).

2.3 Classification of Synergism[18]

2.3.1 Independent synergism (= independent action with zero correlation)

A system of two components acting independently and not interfering with each other. If P_M is the probability of death by microorganism taken alone, and P_I the corresponding value for the insecticide, the probability of death by the combined action is $P_{I+M} = P_I + P_M(1-P_I)$ or if the corresponding values of mortality in % are used: $\mathbf{M_{I+M} = M_I + M_M(1-M_I/100)}$.

2.3.2 Subadditive synergism

A system of two components which together produce an effect greater than independent synergism but less than the algebraic sum of two single effects. A weak potentiating effect is necessary to produce such a result.

2.3.3 Supplement synergism

A system of two components which together produce an effect greater than the algebraic sum of single effects $\mathbf{(M_{I+M} > M_I + M_M)}$.

2.3.4 Potentiating synergism

A system of a component "A" causing the effect M_A and a synergist ("S") which alone cause no effect (M_S=0), but which in combination produce an effect which is significantly greater than M_A. This type of synergism may be found when non-lethal concentrations of an insecticide are combined with a microorganism.

2.4 Detoxifying Enzymes assay

It was carried out to study the induction of detoxifying enzymes such as carboxyl esterase (CE), mixed function oxidase (MFO) and Glutathione-S-transferase (GST) in *S. frugiperda* larvae after treating with different insecticide combinations. Untreated larvae were maintained as control. The experiment was replicated three times, each with ten larvae. The surviving larvae were used for enzyme analysis.

2.4.1 Enzyme homogenate preparation

The larvae surviving after treatment as described earlier was used for enzyme homogenate preparation. larvae was weighed and homogenized in ice-cold 20 mM phosphate buffer (pH 8.0) containing 0.2 per cent triton X-100 using pre-chilled pestle and mortar. Five ml of phosphate buffer was used for extraction. The homogenate was centrifuged at 15,000 rpm for 10 minutes and the supernatant collected served as an enzyme source for the assay.

2.4.2 Protein estimation [19]

To one ml of enzyme extract, 5 ml of Bradford reagent was added and allowed for colour development. The absorbance was read at 595 nm. Using the standard graph, quantity of protein in the enzyme extract was calculated.

2.4.3 Carboxylesterase (CarE) assay [20]

Five ml of the working substrate solution (α naphthyl acetate) was mixed with 1ml of enzyme homogenate. After 30 minutes of incubation at room temperature, 1ml of coupling reagent (l% fast blue B salt: 5% sodiumlauryl sulphate = 2: 5, v/v) was added. A red colour developed immediately, which changed to fairly stable blue colour, was measured at 600 nm. Specific activity (SA) of the enzyme was estimated using the formula, which was expressed as n moles of α-naphthol released $minute^{-1}$ $mg\ of\ protein^{-1}$.

2.4.4 Mixed function oxidases (MFO) assay [20]

To 500 µl of enzyme source, 500 µl of tris buffer (pH 7.8) and 20 µl of p-nitroanisole were added. To this 50 µl of NADPH was added in dark at room temperature and kept for 30 minutes. The reaction was stopped by adding 0.5 ml of sodium hydroxide. The reaction mixture was centrifuged at 10,000 rpm for 30 minutes. The absorbance of supernatant was determined at 400 nm. The specific activity (SA) of the enzyme was calculated using the formula and expressed as n moles of p-nitrophenol released $minute^{-1}$ mg of $protein^{-1}$.

2.4.5 Glutathione-S-transferase assay [20]

The total GST activity was determined using CDNB (1-chloro 2,4-dinitrobenzene) and glutathione reduced as substrates 0.1M Glutathione reduced and 0.1M CDNB substrate was prepared in pure ethanol. Potassium phosphate buffer (20 mM) of pH 8.0 was used in assaying GST. Activity of GST was analysed by addition of 0.1 ml of glutathione reduced, 0.1 ml CDNB and 0.1 ml gut homogenate solution to

final volume of 3 ml. The enzymatic reaction was monitored for the optical absorbance increase at wavelength 340 nm at 37°C for 10 min at 1 min interval in the spectrophotometer. Specific GST activity was calculated and articulated in n moles min^{-1} mg $protein^{-1}$.

2.5 Statistical Analysis

Probit analysis was done to calculate LC_{25} LC_{50}, LC_{90} using SPSS. The log concentration probit (LCP) lines were drawn by plotting log concentrations on X-axis and probits on Y-axis. The response of test insect populations was studied at different concentrations of the test insecticides. The combined toxicity of chlorantraniliprole with *Bt* was studied by combining the different lethal concentrations (LC_{25} and LC_{50}) at different proportions [21]. Mortality was corrected by Abbott's formula [22] for each Probit regression analysis.

3. RESULTS

From probit analysis (Table 1), it was found that LC_{25} of chlorantraniliprole for 2nd and 3rd instar of FAW were 0.87 ppm and 1.52 ppm respectively. LC_{25} of *Bt* for 2nd and 3rd instar of FAW were 474.39 ppm and 693.48 ppm respectively. LC_{50} of chlorantraniliprole for 2nd and 3rd instar of FAW was 4.08 ppm and 6.50 ppm respectively. LC_{50} of *Bt* for 2nd and 3rd instar of FAW were 1008.62 ppm and 1228.62 ppm respectively. Results on combination effect of chlorantraniliprole with *Bt* revealed that 2nd instar of FAW showed supplemental synergism at LC_{50} of chlorantraniliprole + LC_{25} of *Bt* (Table 2) as the resulted mortality was higher than their single effect and their independent synergism. In case of LC_{50} of chlorantraniliprole + LC_{50} of *Bt*, LC_{25} of chlorantraniliprole + LC_{50} of *Bt* and LC_{25} of chlorantraniliprole + LC_{50} of *Bt* combinations, they gave sub additive synergism as the mortality was higher than independent synergism but lesser than their sum of single effect. With respect to of 3rd instar larvae, the combined toxicity results were similar for all the combinations of chlorantraniliprole + *Bt* except chlorantraniliprole LC_{25} + *Bt* LC_{50} which showed an antagonistic synergism.

Levels of detoxifying enzymes were assessed in 2nd and 3rd instar larva of *S. frugiperda* treated with different combinations of chlorantraniliprole with *Bacillus thuringiensis*. Carboxylesterase (CarE), mixed function oxidases (MFO) and Glutathione-S-transferase were the three detoxifying enzymes assessed in the present study. Comparing the single and insecticide combination treatments CarE activity was found to be lesser in chlorantraniliprole LC_{50} + Bt LC_{25} (3.76 fold) followed by chlorantraniliprole LC_{25} + Bt LC_{50} (4.09 fold) than the CH_{50} (7.39 fold), chlorantraniliprole (6.42 fold) in relation to control in second instar larvae. Similar trend was observed in third instar larvae also. The MFO activity was also found be lesser in insecticide combination treatments chlorantraniliprole LC_{50} + Bt LC_{25} (1.06 fold) followed by chlorantraniliprole LC_{25} + Bt LC_{50} (1.22 fold) than single action treatments CH_{50} (1.72 fold), CH_{25} (1.62 fold) in the second instar larvae. As like preceding enzyme activities, GST activity was also low in insecticide combination treatment in chlorantraniliprole LC_{50} + Bt LC_{25} (1.01 fold) followed by chlorantraniliprole LC_{25} + Bt LC_{50} (1.13 fold) than the chlorantraniliprole LC_{50} (1.87 fold), chlorantraniliprole LC_{25} (1.78 fold) in relation to control in second instar larvae.

4. DISCUSSION

Combinations of insecticides play a meaningful role compared to single insecticide because they have multiple modes of action. Hence, chemical mixtures may be effective in management of lepidopteran pests [23]. Our results suggested synergistic, additive, and antagonistic effects

Table 1. Toxicity of tested insecticides against 2nd and 3rd instar larvae of *S. frugiperda*

Instar	Insecticide	LC_{25} ppm	LC_{50} ppm	Slope	χ2
2 nd instar	Chlorantraniliprole	0.87 (0.373 – 2.007)	4.08 (2.582 – 6.432)	1.0017	0.1261
	B. thuringiensis	474.39 (349.57 – 643.76)	1008.62 (800.31 – 1271.14)	1.9799	5.0485
3 rd instar	Chlorantraniliprole	1.52 (0.79 – 2.91)	6.50 (4.28 – 9.89)	1.0748	0.8848
	B. thuringiensis	693.48 (549.57 – 743.76)	1228.62 (1137.31 – 1371.14)	1.9799	5.2185

Table 2. Interactive effects of chlorantraniliprole (Ch) + *Bacillus thuringiensis* (*Bt*) on 2nd instar larvae of *S. frugiperda*

Treatments	2nd instar			
	Mortality (%)	Sum of two single effect	Independent synergism	Synergism
Ch LC_{25}	28.57	-	-	-
Ch LC_{50}	52.38	-	-	-
Bt LC_{25}	23.81	-	-	-
Bt LC_{50}	47.62	-	-	-
Ch LC_{50} + *Bt* LC_{50}	76.19	100.00	75.06	Subadditive synergism
Ch LC_{50} + *Bt* LC_{25}	80.95	76.19	63.72	Supplement synergism
Ch LC_{25} + *Bt* LC_{50}	66.67	76.19	62.59	Subadditive synergism
Ch LC_{25} + *Bt* LC_{25}	47.62	52.38	45.58	Subadditive synergism
Control	0.00	-	-	-

Table 3. Interactive effects of chlorantraniliprole (Ch) + *Bacillus thuringiensis* (*Bt*) on 3rd instar larvae of *S. frugiperda*

Treatments	3 rd instar			
	Mortality (%)	Sum of two single effect	Independent synergism	Synergism
Ch LC_{25}	26.32	-	-	-
Ch LC_{50}	52.63	-	-	-
Bt LC_{25}	21.05	-	-	-
Bt LC_{50}	42.11	-	-	-
Ch LC_{50} + *Bt* LC_{50}	73.68	89.47	69.53	Subadditive synergism
Ch LC_{50} + *Bt* LC_{25}	78.95	63.16	55.68	Supplement synergism
Ch LC_{25} + *Bt* LC_{50}	63.16	63.16	54.29	Subadditive synergism
Ch LC_{25} + *Bt* LC_{25}	36.84	36.84	33.52	Antagonistic effect
Control	0.00	-	-	-

among the combinations of insecticides and *Bt* used against *S. frugiperda*. According to Burges, Hussey [18], supplemental synergism was considered as significant as the mortality caused by combined toxicity is higher than sum of two single effect and their independent synergism. Mixtures can be advantageous compared to individual constituents, because they may have different modes of action and may delay the development of resistance [24]. *S. frugiperda* larvae showed subadditive or supplement synergism in combination treatments of Ch+Bt because the chemical insecticides act as stressors and make the larvae more susceptible to Bt. The stressed insects generally seem to be more susceptible to pathogens [25]. Koppenhöfer and Kaya (1996) accounted similar kind of interaction between entomopathogenic nematodes and Bt. Whereas Ansari et al*.* [26] reported synergistic action between *Metarhizium anisopliae* and entomopathogenic nematodes. The mixture of *Bt* + (*Bt* + chlorantraniliprole) (1:1:1; LC50:LC50) produced a synergistic activity compared to other combinations in the field-original highly resistant strain of *P. xylostella* [23].

It is speculated that detoxification enzymes, such as glutathione S-transferase, Mixed Function Oxidase and carboxyl-esterase, play an essential role in the metabolism of carbamates, pyrethroids, and novel insecticides in numerous insects [27,28]. Our enzyme assays showed that there was a high level of GST activity in larvae exposed to chlorantraliniprole alone compared to the CHBt combinations. This significant correlation between GST activity and chlorantraniliprole suggests that the enzymes contribute to the detoxification of chlorantraniliprole. Hu et al*.* [29] and Nehare et al*.* [30] reported increased GST activity in *P. xylostella* treated with exogenous combinations of pesticides, such as indoxacarb, acephate, and chlorantraniliprole. Furthermore, a positive correlation with GST activity was found in response to spinosad insecticide also [31-33]. Similarly, carboxyl-esterase (CarE) activity was also more in chlorantraniliprole alone treated larva of *S. frugiperda* than the combinations.

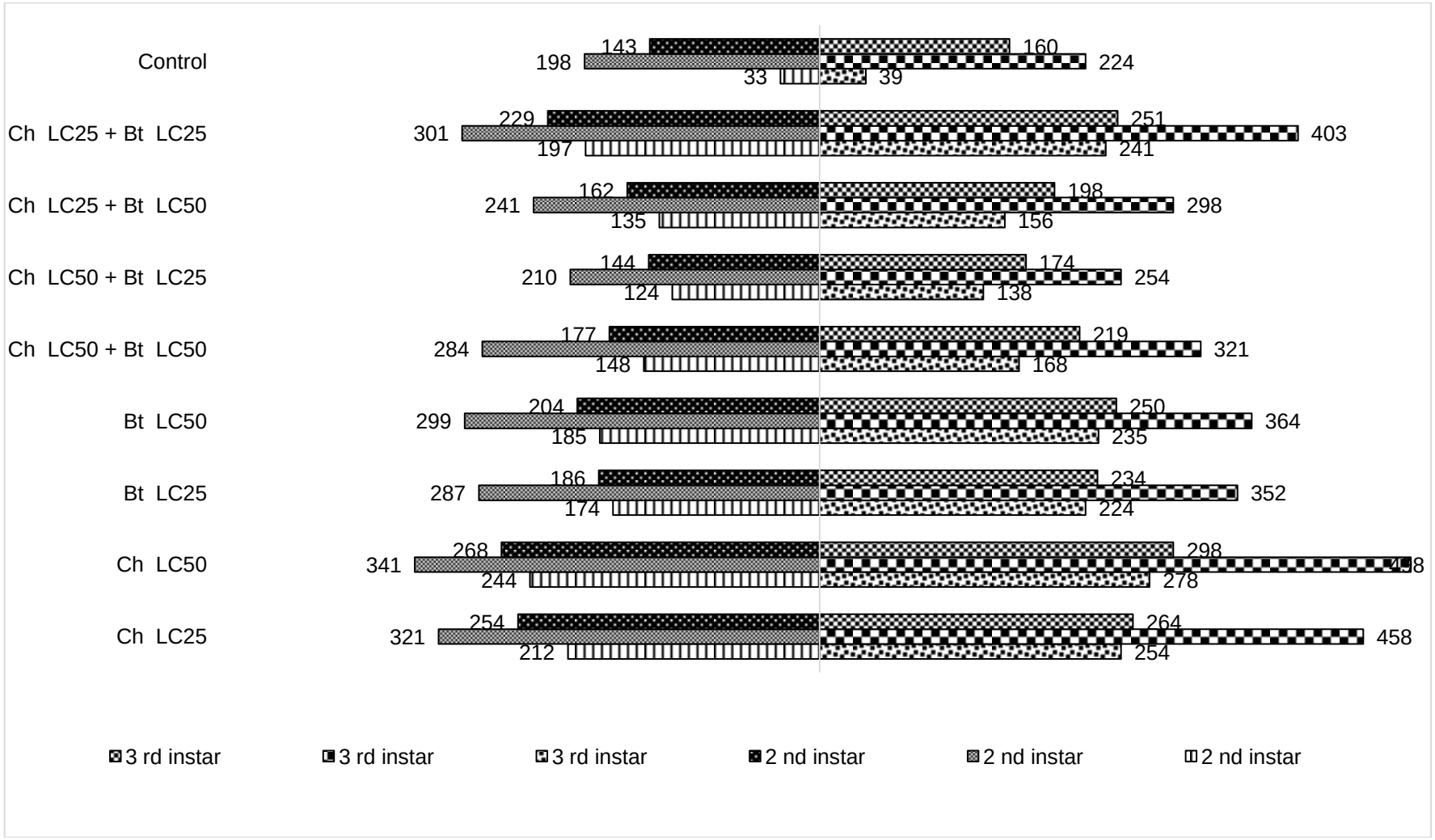

Fig. 1. Levels of detoxifying enzymes in Chlorantraniliprole (Ch) + *Bacillus thuringiensis* (*Bt*) treated 2nd and 3 rd instar larvae of *S. frugiperda*

CarE has also been directly correlated with resistance to chlorantraniliprole and abamectin resistance in *P. xylostella* [34].

5. CONCLUSION

The synergistic combinations may represent a first step towards the utilization of Bt products and insecticides that will considerably delay resistance. Therefore, combination of chlorantraniliprole with *Bacillus thuringiensis* at LC_{50} of chlorantraniliprole + LC_{25} of *Bt*. ratio had supplemental synergism on fall armyworm under laboratory condition.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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