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Enhancing the efficacy of the biopesticide *Beauveria bassiana* by adding chitosan to its secondary metabolites

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Abstract
The aim of the present study is to improve the insecticidal activity of the entomopathogenic *Beauveria bassiana* by adding Chitosan to its secondary metabolites. The secondary metabolites were extracted from the broth culture of *B. bassiana* by ethyl acetate solvent. Chitosan was extracted from shrimp shells via three steps; demineralization; deproteinization and de acetylation. The joint action between secondary metabolites and chitosan was determined by calculating the synergistic ratio and co-toxicity coefficient against the cotton leaf worm *Spodoptera littoralis* as an economic insect pest. Also, the effect of both compounds and their mixture on the larval growth and feeding were studied. The results indicated that the mixture of both compounds was more toxic than the individual compound. Likewise, the toxicity of secondary metabolites was the highest compared with chitosan effect. The obtained results from synergistic ratio and co-toxicity coefficient formula revealed to presence the synergism and potentiation between secondary metabolites and chitosan. In addition, larval growth and consumed diet were decreased in case of the mixture of secondary metabolites and chitosan more than individual case. Finally, this study recommended that using chitosan as an additive substance increase the efficiency of secondary metabolites of *B. bassiana* against the cotton leafworm *S. littoralis*. Also, the mixture of both extracts considered an effective insecticide and safe for the environment.

Keywords: joint action; insecticidal activity; antifeedant; growth inhibition; *Spodoptera littoralis*

Introduction
The secondary metabolites of some entomopathogens can be played an effective role in integrated pest management programs (IPM) against many insect pests. Abdullah (2019a) [2] found that the secondary metabolites of *Beauveria bassiana* contain some compounds that were more toxic against cotton leafworm *Spodoptera littoralis.* Also, Abdullah, *et al.*, (2014) [4] Studied the effect of bacterial cells and their culture filtrate of *Bacillus thuringiensis* and *Bacillus subtilis* against cotton aphid (*Aphis gossypii*) and tomato leaf miner (*Tuta absoluta*). They found that high mortality percentage was caused by the culture filtrate of both bacterial strains. Gurulingappa, *et al.*, (2011) [14].
Mentioned that metabolites of *Beauveria bassiana* and *Lecanicillium lecanii* significantly increased mortality of *Aphis gossypii* and reduced its fecundity. Similarly, *Lecanicillium lecanii* was used to control whiteflies and aphids (Wang, *et al.*, 2000 and 2007) [23, 34]. The toxicity of secondary metabolites can be increased against the insects by adding synergist agent such as chitosan substance.

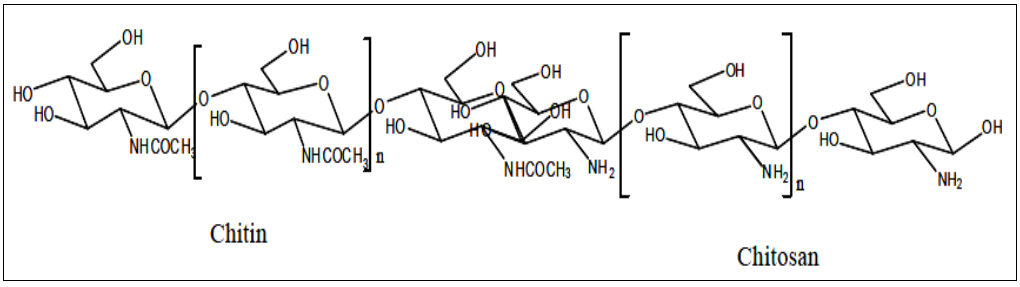

Fig 1: Chitin and chitosan structure as described by Pokhrel, *et al.* (2015) [20].

Chitosan is a natural substance that is extracted from the waste of the marine food processing industry through three processes; demineralization by HCl, deproteinization by NaOH and deacetylation by concentrate NaOH as described by Kumar and Ravi (2017) [16]. Badawy and El-Aswad (2012) [5] mentioned that chitosan is a copolymer of β-(1, 4)-2-acetamido-2-deoxy-d-glucopyranosyl and β-(1, 4)-2-amino-2-deoxy-d-glucopyranosyl units. Also, chitosan is a natural polymer which contains reactive groups as amino (NH_3) and hydroxyl (-OH) groups. As shown in Figure 2, these reactive groups make chitosan able to binding with other substances where the nitrogen is a donor of electron pairs also (-OH) groups can to be participate in sorption (Varma, *et al.* 2004 and Wang, *et al.* 2004) [21, 25]. In addition Dutta *et al.* (2004) mentioned that chitosan is a linear polyamine and has chelating ability for several

transitional metal ions. Chitin and chitosan structures are shown in Figure 1 as described by Pokhrel, *et al.* (2015) [20]. Majeti (2000) and Hench (1998) mentioned that chitosan has many functional properties as ability to form films, chelate metal ions, polyoxysalt formation and optical structural characteristics. Chitosan has high biodegradability, antimicrobial properties and it is non-toxic to vertebrate and humans so it finds several of applications. It is used in agriculture, biomedical industries, food industry, environmental pollution control and so on (Cheba, 2011) [8]. In this study, chitosan was used to improve the efficiency of secondary metabolites which were extracted from *Beauveria bassiana* broth culture to obtain safe pesticide and more toxic against the cotton leafworm *Spodoptera littoralis.* Where, *S. littoralis* widely distributed all over the world and it is polyphagous pest caused more economic damages to several crops.

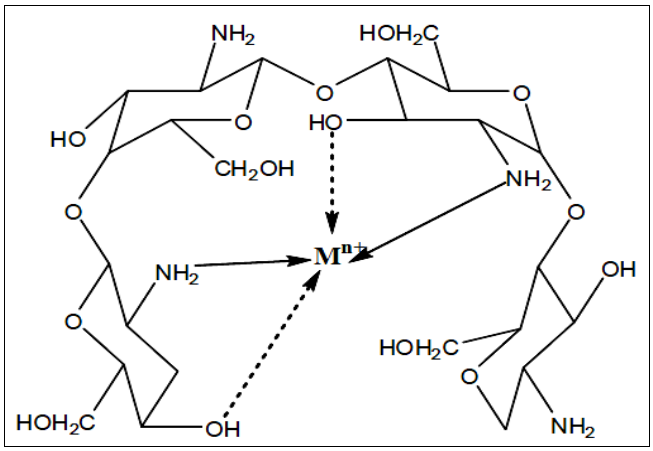

Fig 2: Mechanism of chitosan binding with metal according to Wang *et al.* (2004) [25]

Materials and Methods

Chitosan extraction

Shrimp shells were collected and washed by hot tap water several times. Chitosan was prepared from shrimp shells by three steps, a) De-mineralization by adding 150 ml of 2M HCl to 10 grams of shrimp shells and 150 rpm steering 2 hours at room temperature. Then remove the HCl and wash the shrimp shells by tap water 5 times and one time by hot distilled water and then the shrimp shells were dried at 80 ºC. b) De-proteinization by adding 200 ml of 2M NaOH to 10 grams of de- mineralization shrimp shells and 150 rpm steering 4 hours at 50 ºC. Then remove the NaOH and wash the shrimp shells as described in the first step. The end product is chitin. c) De-acetylation by adding 50 ml of NaOH 50% to 1 gram of chitin (The obtained product from previous step) and steer the mixture 150 rpm at 100 ºC to 5 hours. Then remove the NaOH and wash the chitosan as described in the previous steps and dry it at 60 ºC. The obtained chitosan was weighed and dissolved in 1% acetic acid to prepare the serial concentrations to use them in the bioassay test. This procedure was conducted as described by Varun, *et al.* (2017) [22] and Benhabiles, *et al.* (2012) [7].

Fungus strain

Beauveria bassiana strain was isolated locally from collected insect samples of Dakahliah governorate, Egypt. It was identified by sequencing of ITS gene region as Amplified from genomic DNA using the primer pairs ITS1 (5'-TCCGTAGGGTGAACCTGCGG-3') and ITS4 (5' TCCTCCGCTTATTGATATGC-3') according to White *et al.* (1990) by using PCR. The results of data base of BLAST program on national center for biotechnology information (NCBI) web site https://blast.ncbi.nlm.nih.gov/Blast.cgi refer to that the isolated fungus is *Beauveria bassiana* with probability identification 91.71% and accession number JQ861946.1. The fungus was cultured on potato dextrose agar plates medium and maintained at 5 ºC in the refrigerator system.

Extraction of secondary metabolites from Beauveria bassiana broth culture

The secondary metabolites were extracted from broth culture of *B. bassiana* as mentioned by Abdullah (2019a) [2]. The fungus was grown in potato dextrose broth medium at 25 ºC for 21 days in shaking incubator. After the incubation period, ethyl acetate solvent was added to the broth culture (0.5:1) and homogenized very well. The mixture was filtered to remove mycelium and spores then put in separating funnel and leave it two hours. The mixture will be separated into two layers, the secondary metabolites are in the ethyl acetate layer in the upper. This layer was separated and put in a rotary system under heating to remove the ethyl acetate solvent and obtained the crude extract of the secondary metabolites. Then, the crud extract was weighted to prepare the serial concentrations to use them in the bioassay test.

The Insect pest

Laboratory strain of cotton leafworm *Spodoptera littoralis* was obtained from Plant Protection Research Institute, Agriculture Research Center, Egypt. It was reared on castor leaves as described by El-Sharkawy and Abdullah (2020) [10] to obtain the 2nd and 4th instar larvae and use them in the bioassay test.

Bioassay test

Six serially concentrations from the secondary metabolites of *B. bassiana*, chitosan, and their mixture were prepared as part per million (500, 1000, 2000, 3000, 4000, 5000 ppm). Twenty larvae of the *S. littoralis* were put in plastic cans (2nd and 4th instar larvae separately) without feeding and covered by the muslin for about two hours before the treatment. Then the clean castor leaves disks were dipped in the prepared concentrations of compounds for 30 seconds and allow to air dry. Then the treated leaves were presented to larvae. Each treatment was repeated five times. The control treatment was prepared by the mixture between water and the solvents of secondary metabolites and chitosan. Dead larvae of the 2nd and 4th instar were recorded after 48 hours post-treatment. Mortality percentage were corrected by Abbott's formula (Abbott 1925) [1]. Lethal concentrations, slope values, and 95% confidence limit were calculated by Finney's probit analysis (Finney 1971) [12]. The synergistic ratio between the secondary metabolites and chitosan was estimated by the mentioned formula by Feng (1984) [11].

$$SR = \frac{\text{Toxicity in } LC_{50} \text{ of insecticide alone}}{\text{Toxicity in } LC_{50} \text{ of insecticide and synergist mixture}}$$

Where: SR is the synergistic ratio. If the SR value was more than one the action will be synergism but if the SR value was less than one the action will be antagonism. Also, if the SR value was equal one the action will be additive.

Joint action

The lethal concentrates of secondary metabolites (Sm) and chitosan (Ch) were calculated by Finney's probit analysis. Mixtures from these concentrations of Ch and Sm were prepared as follow: ($LC_{12.5}$ Ch: LC_{25} Sm), ($LC_{12.5}$ Ch: LC_{50} Sm), (LC_{25} Ch: LC_{25} Sm) and (LC_{25} Ch: LC_{50} Sm). Twenty 2nd instar larvae of *S. littoralis* were put in plastic cups before treatment for two hours. Clean disks of castor leaves were dipped in each mixture for 30 seconds and moved to air dry. Then the treated castor leaves were put in larvae cups and covered by the muslin. The control treatment was by untreated disks of castor leaves. Each treatment was replicated five times. The mortality was recorded after two days post-treatment and corrected by Abbott's formula. The joint action between secondary metabolites and chitosan was estimated by calculate the co-toxicity co-efficient as the described formulation by Mansour *et al*., (1966) [18].

$$\text{Co-toxicity co-efficient} = \frac{\text{Observed mortality\%} - \text{Expected mortality\%}}{\text{Expected mortality \%}} \text{ X } 100$$

Where: From -20 to +20 Additive; ≥ +20 Potentiation; ≤ -20 Antagonism

Growth inhibition and antifeeding test

Three concentrations (1000, 2000, 3000 ppm) were prepared from secondary metabolites and chitosan and their mixture. Ten larvae from the third instar of *S. littoralis* were weighted and put in plastic cans. Disks from castor leaves were prepared and dipped in the concentrations solution for 30 second and allow to air dry. After the leaves drying, they were weighted and presented to larvae. After three days post treatment, the larvae and castor leaves in each treatment were weighted to calculate the consumed diet and the gained weight of larvae. Growth inhibition and antifeeding percentages were calculates as formula described by Badawy and El-Aswad (2012) [5].

$$\text{Growth inhibition \%} = \frac{\text{Weight gained in control larvae - Weight gained in treated larvae}}{\text{Weight gained in control larvae}} \text{ X } 100$$

$$\text{Antifeedant \%} = \frac{\text{Weight of diet consumed in control - Weight of diet consumed in treatment}}{\text{Weight of diet consumed in control}} \text{ X } 100$$

Results

Toxicity of secondary metabolites of B. bassiana, chitosan, and their mixture against cotton leafworm S. littoralis

The data presented in Tables 1 & 2 and Figure 3 show the larvicidal activity of secondary metabolites, chitosan, and their mixture against 2nd and 4th instar larvae of cotton leafworm *S. littoralis.* It is clearly that the mixture was more toxic compared with secondary metabolites or chitosan separately. The mortality percentage of 2nd and 4th instar larvae reached to 86% and 71%, respectively in case of the mixture. Also, the mixture toxicity at LC_{50} was 1138 and 2214 ppm, respectively. On the other hand, the results indicated that the secondary metabolites was more toxic compared with chitosan against 2nd and 4th instar larvae. The mortality percentage of 2nd and 4th instar larvae were 74% and 61% respectively in case of secondary metabolites, also its toxicity were 2379 and 3492 ppm, respectively at LC_{50}. But the chitosan recorded low mortality percentage reached to 57% and 48 % against 2nd and 4th instar larvae, respectively also, low toxicity at LC_{50} were 3657 and 5710 ppm, respectively.

Table 1: Mortality percentage of cotton leafworm *S. littoralis* after 48 hours post-treatment by secondary metabolites, chitosan, and their mixture.

Instar larvae	Compounds	Mortality % at different concentrations (ppm)					
		500	1000	2000	3000	4000	5000
2nd instar larvae	Secondary metabolites	23	37	46	58	65	74
	Chitosan	17	27	35	47	52	57
	Mixture	37	43	50	77	81	86
4th instar larvae	Secondary metabolites	10	21	35	46	52	61
	Chitosan	7	10	23	33	41	48
	Mixture	23	29	48	57	63	71

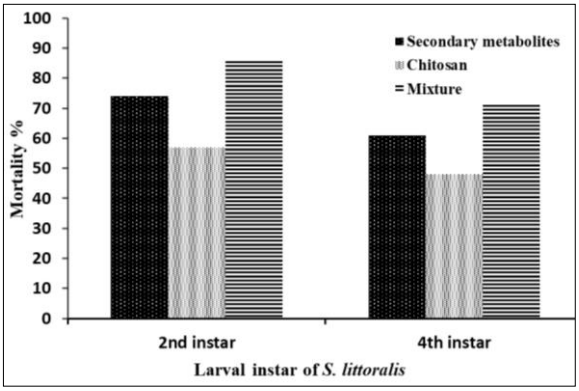

Fig 3: Mortality percentage of 2nd and 4th instar larvae of *S. littoralis* after 48 hours treatment by secondary metabolites, chitosan and their mixture at 5000 ppm.

Table 2: The lethal concentrate values of secondary metabolites, chitosan, and their mixture against the 2nd and 4th instar larvae of *S. littoralis* using leaf dipping technique after 48 hours post-treatment.

Tested compound	LC values	2nd instar larvae			4th instar larvae		
		PPM	Confidence limit 95% (ppm)	Slope ± SE	PPM	Confidence limit 95% (ppm)	Slope ± SE
Secondary metabolites	LC_{25}	590	380 - 789	1.29 ± 0.15	1225	976-1513	1.51 ± 0.17
	LC_{50}	2379	1612 - 3360		3492	2939-4336	
	LC_{90}	19046	12018-39443		24406	15452-49460	
Chitosan	LC_{25}	922	609 -1206	1.12 ± 0.16	2075	1709-2460	1.53 ± 0.19
	LC_{50}	3657	2908 - 5031		5710	4556-7974	
	LC_{90}	50150	24563-175015		39065	22163-98662	
Mixture	LC_{25}	390	84-467	1.45 ± 0.15	652	425-866	1.27 ± 0.15
	LC_{50}	1138	522-1619		2214	1830-2702	
	LC_{90}	8703	7256-39765		22564	13722-50130	

Joint action between secondary metabolites and chitosan against S. littoralis

To investigate the joint toxic action of secondary metabolites and chitosan against the 2nd and 4th instar larvae of cotton leafworm *S. littoralis*. The synergistic ratio and co-toxicity co-efficient were calculated as shown in Tables 3 and 4. The results in Table 3 show that the values of synergistic ratio were more than one at both compounds in case of 2nd and 4th instar larvae these refer to synergistic effect between secondary metabolites and chitosan. Also, the data in Table 4 illustrate the presence of the potentiation effect between both compounds against the 2nd instar larvae where the values of the co-toxicity factor were more than twenty which refers to the potentiation effect.

Table 3: Synergistic effect of chitosan to the insecticidal activity of secondary metabolites of *B. bassiana* against *S. littoralis* after 48 hours post-treatment.

	2nd instar larvae			4th instar larvae		
	Secondary metabolites	Chitosan	Mixture	Secondary metabolites	Chitosan	Mix
LC_{50} (ppm)	2379	3657	2000	3492	5710	2214
Synergistic ratio	1.19	1.83	--	1.58	2.58	--

Synergistic ratio = LC50 of pesticide alone/ LC50 of pesticide + synergist = 1 additive; < 1 antagonism; > synergism

Table 4: The co-toxicity co-efficient of secondary metabolites and chitosan against 2nd instar larvae of *S. littoralis* after 48 hours post-treatment.

Tested compound		Expected mortality%		Observed mortality %		Co-toxicity factor	
Secondary metabolites		LC_{25}	LC_{50}	LC_{25}	LC_{50}	LC_{25}	LC_{50}
Chitosan	$LC_{12.5}$	37.5	62.5	45.2	78.6	20.53	25.75
	LC_{25}	50	75	61.5	95.3	23	27.06

Co-toxicity co-efficient = (Observed mortality% − Expected mortality %/Expected mortality %) × 100 From -20 to +20 Additive; ≥ +20 Potentiation; ≤ -20 Antagonism

Growth inhibition and antifeeding

According to the data in Table 5 and Figure 4 clearly reducing gained weight of larvae at all treatments compared with the control. Also, the presented data show that the growth inhibition percentage of larvae increased in case of the mixture treatment more than the secondary metabolites and chitosan separately. Also, growth inhibition percentage increased in treated larvae by secondary metabolites more than chitosan.

On the other hand, the antifeedant data were supportive of the growth inhibition data. Where, antifeedant percentage was found high in case of the mixture treatment compared with the secondary metabolites and chitosan separately. Also, the presented results illustrate that the consumed diet was less in case of secondary metabolites compared with chitosan treatment.

Table 5: Effect of secondary metabolites, chitosan and their mixture on growth inhibition and antifeedant percentages of the third instar larvae of *S. littoralis*.

Treatment	Concentration ppm	Larval weight gained after 3 days	Growth inhibition% after 3 days	Weight of diet consumed after 3 days	Antifeedant % after 3 days
Control		0.88	00	2.25	00
Secondary metabolites	1000	0.50	43.18	1.93	14.22
	2000	0.43	51.14	1.41	37.33
	3000	0.28	68.18	1.21	45.92
Chitosan	1000	0.71	19.31	1.90	15.40
	2000	0.67	23.86	1.79	20.29
	3000	0.60	31.81	1.72	23.55
Mixture	1000	0.35	60.22	1.50	33.18
	2000	0.25	71.59	1.15	48.59
	3000	0.17	80.68	0.57	74.51

Growth inhibition % = ((Larval weight gained in the control -Larval weight gained in the treatment)/Larval weight gained in the control) X 100Antifeedant %= ((Weight of diet consumed in control- Weight of diet consumed in treatment)/ Weight of diet consumed in control) X 100

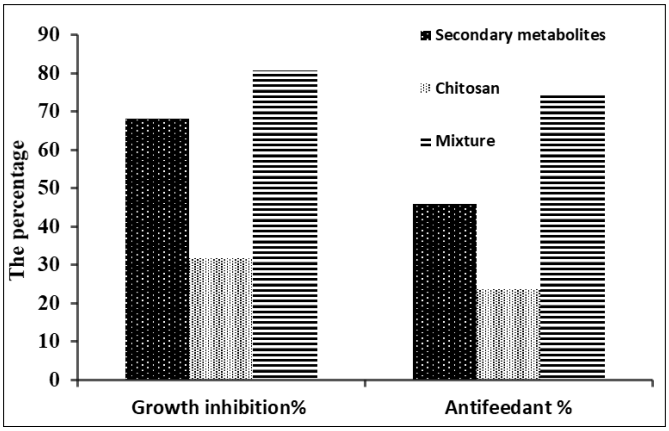

Fig 4: Effect of secondary metabolites, chitosan and their mixture at 3000 ppm on growth inhibition and antifeedant percentages of the 3rd instar larvae of *S. littoralis* after 3 days.

Discussion

In this study, chitosan was used to improve the efficiency of secondary metabolites which were extracted from broth culture of *Beauveria bassiana* to be more toxic against cotton leafworm *Spodoptera littoralis. B. bassiana* is the famous organism in biological pest control but it can't live in unsuitable environments such as high or low temperature degree and the dry media as well as the antagonism between the fungus and many chemical pesticides (Abdullah, 2019b) [3]. So, many researchers extracted the secondary metabolites from the broth culture of *B. bassiana* and used them as a biopesticides as mentioned by Abdullah (2019a) [2]. Fox and Howlett

(2008) found that the secreted metabolites from entomopathogenic fungi cultures were the source of bioactive compounds such as terpenes, fatty acids, non-ribosomal peptides, polyketides and polyketide-peptide hybrid metabolites. In the same trend, Bandani *et al.* (2000) [6] and Molnar *et al*. (2010) [19] mentioned that the secondary metabolites which were secreted from entomopathogens have insecticidal and antifeedant properties. Gas chromatography-mass spectrometry analysis was conducted to Identified the compounds which were found in crude extract of *B. bassiana* secondary metabolites by Abdullah, (2019a) [2]. Who found that some compounds have insecticidal activity and antifeedant as n-Hexadecanoic acid; 9, 12-Octadecadienoic acid, methyl ester, (E,E)-; Hexadecanoic acid, methyl ester; 7,10-Octadecadienoic acid, methyl ester and trans-13-Octadecenoic acid; Tetradecanoic acid, 12-methyl-, methyl ester. The obtained results in this study indicated that the secondary metabolites of *B. bassiana* have an insecticidal activity and antifeeding properties against *S. littoralis* as well as they caused larval growth inhibition as shown in Tables 1, 2 and 5 also Figures 3 and 4.

Chitosan is a biopolymer has a number of variety applications due to its properties such as; solubility, high sticky in the acetic solution, non-toxic to mammalians, it has reactive amino (-NH_2) and hydroxyl (-OH) groups, high biodegradability and antimicrobial properties. These properties make it suitable for several applications as biomedical industries, pest control, food industry, environmental pollution control and others (Pokhrel *et al.* 2015) [20]. Also, Badawy and El-Aswad (2012) [5] studied the toxicity of different concentrations from chitosan against cotton leafworm *Spodoptera littoralis* and oleander aphid *Aphis nerii*. They found that using chitosan alone led to low toxicity against *S. littoralis* reached 50% mortality but when mix it with Ni metal became more toxic where the mortality was 93.3% after 7 days post treatment. In the present study the efficiency of secondary metabolites was increased in case of the mixture with chitosan more than using it alone. Where, the synergistic ratio and co-toxicity coefficient values indicted synergism and potentiation effect of chitosan to secondary metabolites as shown in Tables 3&4, also the toxicity of secondary metabolites was increased in case of mixture more than the separately case. Due to the presence of active groups as amino (-NH_2) and hydroxyl (-OH) groups in chitosan structure, this facilitated its binding to many substances and increase its biological activity. So, Chitosan has the best chelating properties among natural polymers (Varma, *et al*. 2004) [21]. Finally, the present study recommended adding chitosan to the secondary metabolites of *B. bassiana* for increasing its insecticidal activity against the cotton leafworm *S. littoralis* and obtained safe insecticide also use this combination as an alternative to chemical pesticides to reduce environmental pollution.

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