ENTOMOLOGY

Potential of neem oil extract® against Palmetto weevil larvae, *Rhynchophorus cruentatus* Fabricius (Coleoptera: Curculionidae) and its impact on some detoxification enzymes

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Abstract

Palmetto weevil, *Rhynchophorus cruentatus* (Fabricius) (Coleoptera: Curculionidae), is considered the giant weevil in North America (Weissling & Giblin-Davis, 1997). It is a severe pest of palm trees, especially cabbage palms *(Sabal palmetto)*. Larval stages feed heavily on the internal palm soft tissues causing total palm loss (Hunsberger *et al*., 2000). We know that reports about controlling this pest are very scarce. This research project focuses, for the first time, on controlling *Rhynchophorus cruentatus* via plant-based insecticides. This study examined the potential of using neem oil extract®, a commercial product, against the third-instar larvae of palmetto weevil (*R. cruentatus*). Impacts of neem oil extract® on mortality, larval weight, the activity of detoxification enzymes, and thei gene expression levels were examined. Neem oil extract® manifested dose-dependent larvicidal activity against the third-instar larvae of *R. cruentatus*. Investigations revealed higher mortality and reduction in weight 24 hours post-treatment. LC_{50} and LC_{90} values were estimated 24 h post-treatment to be 12.04% and 26.48%, respectively. Biochemical analysis revealed increasing activities of three detoxification enzymes (Acetylcholinesterase, Glutathione S-transferase, and Superoxide dismutase) in the third-instar larvae after 8 h of treatment with LC_{50}. A significant elevation in the expression levels of detoxification genes (Acetylcholinesterase, Glutathione S-transferase, Cytochrome P450, and Superoxide dismutase) was recorded in the treated larva. Our findings help to underline the detoxification mechanisms of *R. cruentatus* larva against neem oil extract® at both biochemical and molecular levels. Thus, neem oil extract® had a lethal potential against third-instar larvae of *R. cruentatus* and is suggested as a safe bioinsecticide that may be used in IPM of palm trees as an alternative to chemical insecticides.

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Introduction

Palmetto weevil, *Rhynchophorus cruentatus*) (Fabricius) (Coleoptera: Curculionidae), is considered the giant weevil in North America (Weissling & Giblin-Davis, 1997). It is native to Florida but has been found as far as south Texas to the west and South Carolina to the north (Thomas, 2010). It is represented as a concealed tissue borer to different palm trees, including cabbage palms *(Sabal palmetto)*, Canary Island date palms (*Phoenix canariensis*), Latan palms (*Latania sp*.), royal palms (*Roystonea sp.), Washingtonia sp.,* Bismarck palms (*Bismarckia nobilis*), and coconut palm (*Cocos nucifera* Linnaeus) (Weissling & Giblin-Davis, 1997). The infestations cannot be detected in the early stages leading to difficulties in treatment. Larvae are the most dan-

gerous stage; they penetrate deep into the crown and the stem using their long rostrums, making tunnels, feeding on soft tissues, and disrupting the palm's vascular system, causing trunk weakness and total loss of the palms. For generations, all developmental stages remain inside the palm trees (Hunsberger *et al*., 2000).

Controlling *R. cruentatus* depended mainly on chemical insecticides (Giblin-davis & Howard, 1988). Previous laboratory investigations were conducted to detect the impact of different commercially available pesticides in controlling palmetto weevil adults (Giblin-Davis & Howard, 1989). The following insecticides were tested: Lindane, chlorpyrifos, propoxur, dimethoate, and methomyl. The tested insecticides effectively killed the weevils compared to the control, except methomyl (Giblin-Davis & Howard, 1989). Recently, chemical control has become undesirable since chemicals lead to harmful effects on the environment and evolution of pest resistance against insecticides (Isman, 2006) and because of the cryptic habit of insects as well. Thus, safer and more effective control approaches should be explored.

Recently, bio-insecticides have become essential to insect management programs. Many biological control agents have been explored, including predators, parasitoids, entomopathogenic bacteria, fungi, nematodes, viruses, and botanical extracts (Kumar *et al*., 2022). Botanical-based insecticides have many bioactive compounds that can substitute synthetic insecticides (Mady *et al*., 2021). Different botanical extracts are involved for several purposes; they work as repellents and antifeedants and are toxic to specific organisms (Yan *et al*., 2021). Botanical insecticides have many advantages and offer adequate substitutes for synthetic insecticides in the field where the preservation of the beneficial organisms and environment is critical. They possess low mammalian toxicity and no side effects on plant growth, representing the least environmental pollution and health hazards (Isman, 2006).

Different investigations have evaluated the toxicity of plant extracts against many coleopteran species. Abbas *et al*. (2022) demonstrated the lethal impact of vitex and rosemary plant extracts on *Tribolium castaneum* (the flour beetle). Halliru and Suleiman (2022) confirmed the lethal effect of balsam spurge, the henna tree, and white eye plant extracts against S*itophilus zeamais* (The maize weevil). The insecticidal activity of various plant extracts, including sea ambrosia, French cotton, curcuma, black pepper, onion-garlic mixture, basil, thyme, clove, marjoram, and citronella grass, have been demonstrated against *Rhynchophorus ferrugineus* (Salama and Ismail, 2007; Hussain *et al*., 2017; Al-Shuraym *et al*., 2020; Reyad *et al*., 2020; Darrag *et al*., 2021; Mady *et al*., 2021; Yan *et al*., 2021; Al Dawsari and Alam, 2022).

The Neem tree, *Azadirachta indica* A. Juss (Family: Meliaceae), is reported with several bioactive components, including phenols, steroids, ketones, carotenoids, and triterpenoids that have been extracted from its different parts and are known with their several effects against multiple insect species (Rehimi *et al*., 2011). Neem oil, extracted from neem seeds, is known for its insecticidal activity and can be effective against phytopathogens (Isman, 2006). It is considered a repellent and antifeedant, and it delays larval development and sterilizes adults (Hummel *et al*., 2011). Azadirachtin is the most important ingredient because of its insecticidal properties, low potential for developing resistance and non-toxic to mammals, fishes, and pollinators (Valizadeh *et al*., 2013). Neem-based products entered the marketplace and became commercially available many years ago in the United States (Isman, 2006).

Neem-based products have an essential role in controlling different insect species (Trisyono and Whalon, 1999; Weathersbee and Tang, 2002; Barnard and Xue, 2004; Pavela *et al*., 2009; Rehimi *et al*., 2011; Wondafrash *et al*., 2012; Senthil-Nathan, 2013; Valizadeh *et al*., 2013; Halder *et al*., 2017; Schneider *et al*., 2017; Yadav *et al*., 2022). The lethal effect of neem-based products (Neem cake, Neem leaf, Neem oil) was confirmed against the 2nd and 3rd larval instars of the rhinoceros beetle, *Oryctes rhinoceros* Linnaeus (Manivasagam *et al*., 2022). Different investigations have evaluated the insecticidal properties of neem plant extracts against *Rhynchophorus ferrugineus* (Bream *et al*., 2001; Nassar and Abdullah, 2001; Mohamed *et al*., 2003; El-Bokl *et al*., 2010; Merghem and Mohamed, 2017; Hamadah, 2019; Hajjar *et al*., 2021).

According to previous studies, there is no provided data about the effectiveness of neem oil extract on the mortality levels of *R. cruentatus*. There is a shortage of literature dealing with the impact of this insecticide on the *R. cruentatus* at biochemical and molecular levels. After applying this biopesticide, the biochemical and physiological changes inside the larval body will help understand its defense mechanisms. Providing more of this information would help discover new effective control methods leading to establishing suitable plans for the integrated management of this pest.

Thus, in this investigation, we decided to test the potential of neem oil extract®, a commercial product, against *R. cruentatus*, for the first time. We aimed to detect effective methods for controlling *R. cruentatus* third-instar larvae depending on plant-based products with non-hazard effects on humans. Also, we aimed to underline the detoxification mechanism against the toxicity of neem oil extract® by exploring its impact on detoxification enzymes at biochemical and molecular levels.

Materials and Methods

Insect samples

R. cruentatus adults were obtained from infested palm trees at Kathryn Abbey Hanna Park, Jacksonville, FL, USA (30°22'18.59"N - 81°24'23.39"W). Adult weevils were collected using food-baited aggregation pheromone (cruentol) traps (Weissling *et al*., 1994). Traps were set up in the park's forested area, specifically, the maritime hammock habitat, where no pesticides were applied. Then, they were reared in a controlled rearing room at the Entomology Department, University of Georgia, USA. The rearing technique is a modified method from the previous work by Giblin-Davis *et al*. (1989), as pineapples were replaced with apples, which are cheaper and available in local stores. Also, the fecundity of palmetto weevils was studied when using apples as an egg-laying substrate by Weissling and Giblin-Davis (1994). An essential outcome of this study was that adult weevils had 8 times more fecundity when using apples instead of pineapples. A colony of palmetto weevils was maintained under laboratory conditions (26±2°C, 65±5% RH and, 16:8 LD) in covered perforated plastic jars and provided with apple slices as a primary food source and egg-laying substrate. Eggs were collected daily; hatched larvae were harvested, transferred to separate plastic jars, and provided with apple slices as a food source. In addition, sugarcane fibers were added to the late larval instars to help them construct the cocoons and complete the life cycle. Food was replaced every three days.

Young larvae from the third instar were collected and separated into groups for the bioassays. Choosing the young larval instars as a target for this study is to interrupt the weevil's life cycle earlier, therefore, overcome infestation in its early stages. Since the first and second instars are very small and difficult to handle, the third instar is the most suitable stage, with a suitable size for applying the bioassay.

Toxicity bioassay

Oral toxicity bioassay was conducted using neem oil extract® concentrate, a commercial product purchased from (chemisco®), against the third-instar larvae of *R. cruentatus* to evaluate its toxicity. Based on preliminary laboratory toxicity bioassays, serial concentrations of neem oil extract® were prepared and applied by the food-dipping method, as a 10-gm apple slice was dipped in the desired concentration of neem oil extract for 5 min, followed by air drying.

Dose-response recordings were conducted using eight concentrations from neem oil extract® dissolved in distilled water: 5%, 8%, 10%, 12%, 15%, 20%, 25%, and 30%. Distilled water was used to treat the control group. Each larva was fed singly on the treated apple slices under laboratory conditions (26±2°C, 65±5% RH, and 16:8 LD). This bioassay was repeated three times with five individuals for each treatment. The physical responses of the treated *R. cruentatus* larvae were observed and compared with the untreated ones. Larval mortality was recorded 24 hours post-treatment.

Impact of neem oil extract® on larval weight

Neem oil extract® impact on larval weight was investigated by applying LC_{50} and LC_{90} concentrations on the third-instar larvae, in addition to a control group. Each treatment had five larvae with three replicates. Each larva was fed separately on a 10-gm apple slice dipped in the desired concentration for 5 min. Larval weight was recorded using analytical weight balance at the beginning of the experiment, 24 h, and 48 h post-treatment.

Biochemical analysis

Enzyme preparation

Five samples from third-instar larvae were treated with LC_{50} of neem oil extract® and then frozen immobilized 8 h post-treatment. Another five untreated samples were prepared and used as controls. Each larva was dissected in ice-cold distilled water, and then whole guts were removed and homogenized separately in 0.1 M potassium phosphate buffer (pH=7), then centrifuged for 15 min at 10,000 rpm at 4°C. The supernatants were moved out to new Eppendorf tubes and kept at -80°C until used for the enzyme activity determination.

Acetylcholinesterase activity (AChE) assay

The activity of AChE was determined using Amplite™ Colorimetric Acetylcholinesterase Assay Kit (Cat. No. 1400) (AAT Bioquest, Inc., Sunnyvale, CA) as reported in the instructions provided by the manufacturer. The kit used 2,4-Dinitrothiocyanatebenzen (DTNB) to quantify the thiolcholine resulting from the hydrolysis of acetylthiolcholine by AChE. The absorption rate is directly proportional to the formation of thiolcholine, thus the AchE activity. The absorbance values were read by a microplate reader at 410±5 nm.

Glutathione S-transferase activity (GST) assay

The GST activity was determined using QuantiChrom™ Glutathione S-transferase Assay Kit (Cat. No. DGST001) (BioAssay Systems, Hayward, CA, USA) as per the producer's guide. Per as Habig method (Habig *et al*., 1974), the kit principle depends on the reaction between 1-chloro-2,4-dinitrobenzene ((CDNB), GST substrate) and Glutathione (GSH). The GST enzyme enhanced GS-DNB synthesis, resulting in a dinitrophenyl thioether that can be detected using a microplate reader at 340 nm. Absorbance intensity was detected every minute over 10 minutes. The absorbance intensity is proportional to the GST activity.

Superoxide dismutase (SOD) assay

SOD activity was detected using Amplite™ Colorimetric Superoxide Dismutase (SOD) Assay Kit (Cat. No. 11305) (AAT Bioquest, Inc., Sunnyvale, CA, USA) as reported on the instructions provided by the manufacturer. The assay can be conducted in a convenient 96-well microtiter-plate format as xanthine oxidase (XO) can convert xanthine to superoxide radical ions, uric acid, and hydrogen peroxide. Superoxide ions react with ReadiView™ SOD560 to produce a compound that absorbs around 560 nm. SOD can inhibit the reaction of ReadiView™ SOD560 with superoxide, therefore reducing the absorption values at 560 nm. The SOD activity is directly proportional to the reduction in the absorption of ReadiView™ SOD560 at 560 nm (Sun *et al*., 1988).

Total protein estimation

Protein amount was determined depending on Bradford's method (Bradford, 1976), using a total protein assay kit (Bio-Rad Laboratories, Inc.) with BSA used as a standard. In this method, body proteins react with an alkaline copper solution, producing a purplish-blue complex, which absorbs at 595 nm. Absorption value has a direct relation to the whole-body protein amount.

Quantification of detoxification genes using qRT-PCR

RNA extraction and cDNA construction

Two groups of third-instar *R. cruentatus* larvae were used for total RNA isolation. One group was the control, including three untreated larvae, and another group of three treated larvae with the LC_{50} was the treated group. After 8 h of treatment, larvae were dissected in saline to isolate guts, and then liquid nitrogen was used to grind gut samples using a mortar. Total RNA was extracted from the guts of six larvae (third instar) using TRIzol reagent (Ambion, Life Technologies, Carlsbad, CA, USA; cat no. 15596). Total RNA amount was detected with NanoDrop™ Spectrophotometer (N-1000). Each larva's total RNA was reverse-transcribed independently according to the protocol of Invitrogen™ SuperScript™ III-RT Kit (Life Technologies, Carlsbad, CA, USA; cat no. 18080044).

qRT-PCR

Constructed cDNA was used as a template for quantifying target genes expression using particular conserved primers (Table 1) manufactured by Integrated DNA Technologies, Inc. (NC, USA) as per the producer's guide of the IQ SYBR® Green Supermix kit (Bio-Rad Laboratories, Inc.). Conserved primers were designed based on Primaclade, a web-based application (Gadberry *et al*., 2005). Bio-Rad CFX Connect™ Real-Time PCR detection system instrument was used to perform this experiment with 20 µl total sample volume. The amplification thermal cycle conditions were: 2 min at 95°C for the beginning cycle (initial denaturation), followed by 40 PCR cycles of denaturation at 95°C for 10s and annealing and extension at 60°C for 30s. After amplification, to eliminate primer dimer possible formation, melting curves ranging from 65°C for 0.5s and 95°C for 5s were included. Three technical replicates were prepared from each sample of the three samples. The resulting values were compared with the controls' values by relative fold expression. The $^{\Delta\Delta}$Ct method was used to analyze data, considering PCR efficiency (Livak & Schmittgen, 2001). Ribosomal protein and β-Actin were chosen as the housekeeping genes based on previous reports from other coleopterans (Tang *et al*., 2017; Chen *et al*., 2020; Pinheiro & Siegfried, 2020). The control relative fold expression of each gene was set to one.

Statistical analysis

All mortalities were counted and corrected, as Abbot's formula (Abbott, 1925), then expressed in percent. LC_{50} and LC_{90} were estimated using the probit analysis method (Finney, 1948). Differences among groups were significant at (P-value≤0.05). Means of larval weight were compared using analysis of variance (ANOVA). A post-hoc test (Bonferroni corrected) revealed significant differences among means.

Absorption data of all tested enzymes were recorded, and enzymatic activities were calculated according to the manufacturer's guide and presented as means ± SD. Each enzyme-specific activity in 1 mg Protein was calculated. A *t*-test was performed to analyze significant differences between means (P<0.01).

Gene's relative fold expression values were presented as geometric means ± SD. Significant differences among means were analyzed using *t*-test and one-way ANOVA followed by Post-hoc (Bonferroni corrected) (P≤0.01).

Results

Dose mortality response

Neem oil extract® had a significant lethal effect on the third-instar larvae of *R. cruentatus* ($F_{1,6}$=40.87, P=0.00069). Serial concentrations of neem oil extract® have observed mortality rates ranging from 7% to 100% 24 hours after treatment. The control group exhibited no larval mortality. The observed mortality percentages were (7%, 20%, 47%, 53%, 60%, 80%, 87% and 100%) at the concentrations of (5%, 8%, 10%, 12%, 15%, 20%, 25%, and 30%), respectively (Figure 1). The lethal effect of neem oil extract was concentration-dependent and increased gradually as the concentration was raised. The highest lethal effect (100% mortality) was observed at a concentration (30%) after 24 h of treatment. As shown in Table 2, the LC_{50} and LC_{90} values were calculated 24 h after treatments, equal to 12.03 % and 26.48%, respectively.

Impact on larval weight

Laboratory observations recorded that the larvae were paralyzed and stopped feeding directly after the treatment with LC_{50} and LC_{90} of neem oil extract®. So, as shown in Table 3, larval weight was dramatically reduced in the treated larvae compared to the control larvae, 24 h ($F_{2,42}$=26.496, P<0.0001) and 48 h ($F_{2,42}$=45.38, P<0.0001) post-treatment with LC_{50} and LC_{90}.

The third-instar larvae, treated with LC_{50}, showed significant weight loss with weight reduction percentage equals to (19.3%) and (37.9%), 24 h and 48 h post-treatment, respectively, compared to

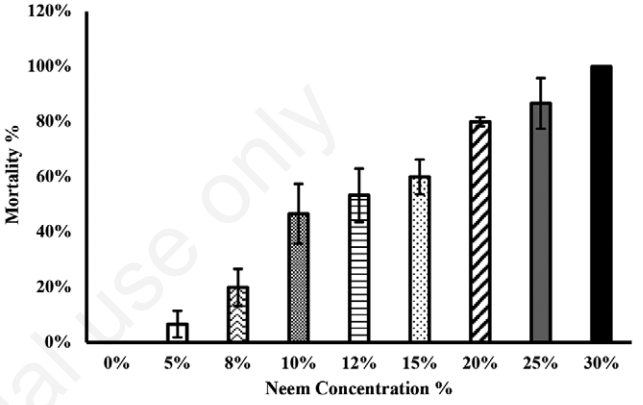

Figure 1. Larval mortality (%) of *R. cruentatus* third-instar larvae, 24 h after exposure to different concentrations of neem oil extract®.

Table 1. Detoxification genes conserved primer sequences from *R. cruentatus* third-instar larvae, used for qRT-PCR expression analysis.

Target gene	Product size	Forward (5`-3`)	Reverse (5`-3`)
Beta-Actin	146 bp	GATGTTGCGGCTCTTGTCGT	GAGTCCTTTTGACCCATACC
Ribosomal protein	137 bp	GGAATGAGAGGTGCATTTGG	AACTTGAACTTGGCACGCCT
Cytochrome P450	235 bp	ARTTCGATCCAGAACGTTTYTC	AAAAYCCTCCTTCWGCAGTGT
Acetylcholinesterase	157 bp	AAYCCAATYCCWACWCCWAACG	CYAAAGGGTAKAGTTTYTCCCAYTC
Glutathione s-transferase	186 bp	TKWAGMTAAATCCKKCTCATA	TGAAKASMGTWCCACAGTCRAAA
Superoxide dismutase	211 bp	GACCACAAGYKGCTRTAGCTGTTTT	TAYGGRTTAAARTGTCCTCCY

Table 2. The LC50 and LC_{90} values, confidence limit (%95), and regression slope at 24 h post-treatment with neem oil extract® in *R. cruentatus* third-instar larvae.

Treatment	LC_{50} (95% CL)	LC_{90} (95% CL)	X^2	df	Slope ± SE
Neem oil extract®	12.04 (10.04-14.14)	26.48 (21.16-39.33)	0.9236	7	3.74 ± 0.63

Table 3. Mean weight ± SD (g) of *R. cruentatus* third-instar larvae after neem oil extract® treatment.

Treatment	Initial weight (g)	Weight (g) at 1 DAT	Weight (g) at 2 DAT
Control	0.1294±0.038[a]	0.1426±0.045[a]	0.1579±0.051[a]
LC_{50}	0.0908±0.008[c]	0.0733±0.0108[c]	0.0564±0.013[c]
LC_{90}	0.1015±0.012[b]	0.0872±0.013[b]	0.0685±0.016[b]

[a,b,c]Means within a column marked by different letters have significant differences at P<0.01, One ANOVA followed by Post-hoc (Bonferroni corrected). DAT, days after treatment.

their initial weight ($F_{2,42}$=37.223, P<0.0001). The third-instar larvae, treated with LC_{90}, showed significant weight reduction percentage equals (14.6%) and (32.5%), 24 h, and 48 h post-treatment, respectively, compared to their initial weight ($F_{2,42}$ = 22.453, P<0.0001). On the contrary, control larvae continued to feed normally, showing a non-significant weight increase, and gaining a weight percentage equals to (10.2%) and (22%) after 24 h and 48 h, respectively, compared with their starting weight ($F_{2,42}$=1.512, P=0.2).

Detoxification enzyme activities

The effect of neem oil extract® on three detoxification enzyme activities in *R. cruentatus* larva is described in Table 4. As explicated in the table, the amount of the total protein in the treated group had a non-significant increase (P>0.05) compared to the control group.

By measuring three detoxifying enzymes, Acetylcholinesterase (AChE), glutathione S-transferase (GST), and superoxide dismutase (SOD) activity level, it was found that the LC_{50} of neem insecticide highly significantly increased the acetylcholinesterase activity (P<0.0001) in the treated larvae. Similarly, GST activity increased in a highly significant manner with LC_{50} concentration (P<0.0001). During this investigation, the SOD activity level in LC_{50}-treated larvae was significantly greater than the SOD activity in the control group (P<0.01). AChE activity level in the treated larvae showed the most significant increase, followed by GST activity. Results showed that SOD activity level was the lowest significant among the tested detoxification enzymes.

The specific activity of each enzyme was calculated in each 1 mg protein. Results also showed significant increases in AChE, GST, and SOD specific activity levels in the treated larvae compared to controls, as illustrated in Figure 2.

Relative expression of detoxification genes using qRT-PCR

LC_{50} treatment of neem oil extract® promoted expression levels of detoxification genes such as Acetylcholinesterase (AchE), glutathione S-transferase (GST), cytochrome P450 (CYP), and superoxide dismutase (SOD) in the third-instar larvae of *R. cruentatus*. The expression of detoxification genes 8 h post-treatment with neem oil extract® showed significant differences ($F_{3,8}$=395.08, P<0.001) (Figure 3). Among the tested genes, AChE was the highest expressed gene with 17.04-fold, followed by GST gene expression with 5.72-fold. Both CYP and SOD showed the lowest expressed genes with 2.48 and 2.38-fold, respectively. All tested genes had significantly more expression in the treated samples than in the control, as shown in Figure 4.

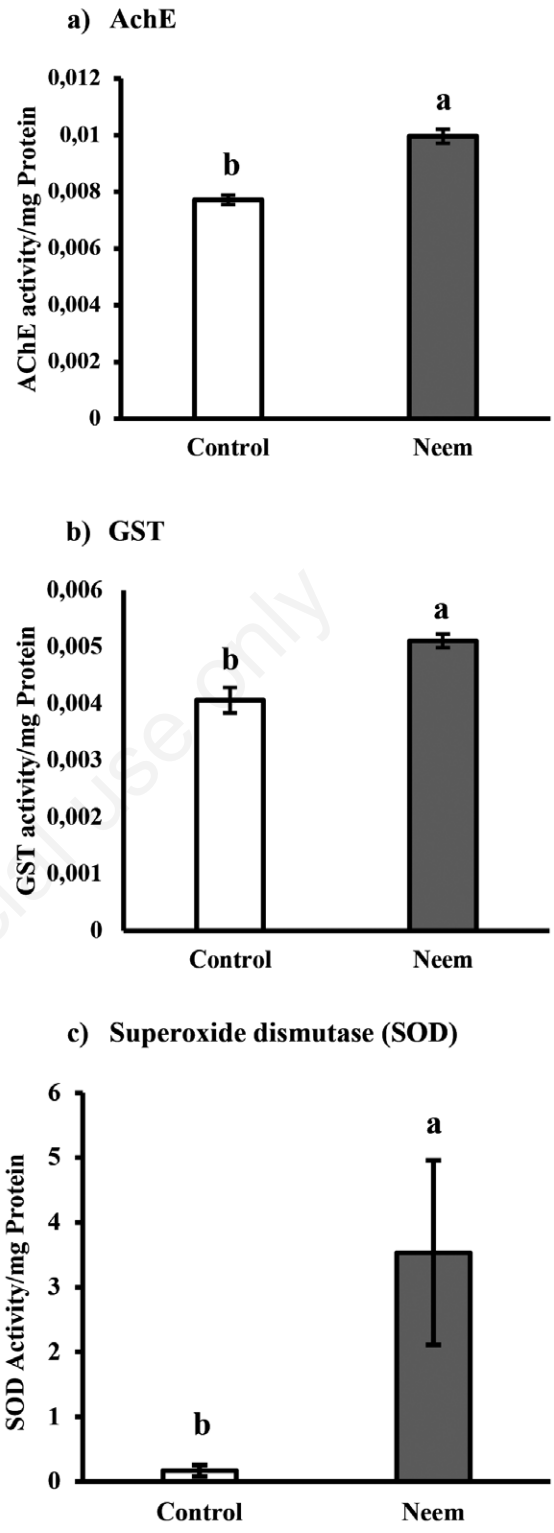

Figure 2. Detoxification enzymes specific activity in third instar *R. cruentatus* larvae after exposure to a control or neem oil extract®. a) AChE, b) GST, and c) SOD. Columns represent the means (±SD) of five samples with two technical replicates. Different letters refer significant differences from the control group (*t*-test, P<0.01).

Table 4. Biochemical effects of neem oil extract® on some detoxification enzymes activities of *R. cruentatus* third instar larvae.

Enzyme	Control	Treated (LC_{50})
Acetylcholinesterase (AchE) (mU/mL)	267.54±5.818[b]	382.92±9.338[a]
Glutathione S transferase (GST) (U/L)	140.934±7.766[b]	196.60±4.656[a]
Superoxide dismutase (SOD) (U/mL)	5.891±3.028[b]	135.826±54.751[a]
Total Protein mg/mL	34.65±4.58199[a]	38.44±3.645[a]

Data were represented as means (±SD) of five samples with two technical replicates. [a,b,c]Means ± SD marked by different letters in the same row have significant differences at P<0.01 using the t-test.

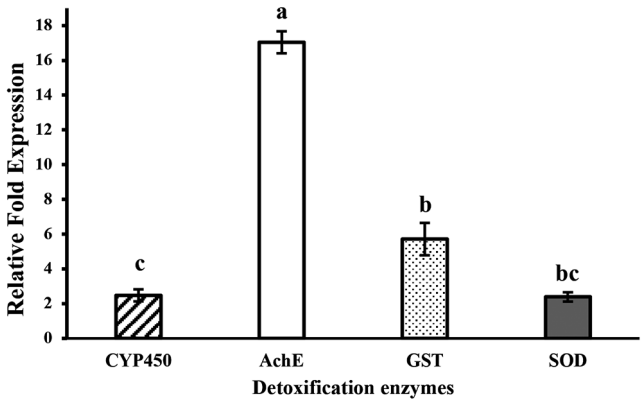

Figure 3. The expression levels of detoxification genes of the third-instar larvae of *R. cruentatus* treated with LC_{50} neem oil extract®, 8 h post-treatment by qRT-PCR. Bars (Geometric means ± SD) marked by a different letter(s) are statistically different (One-way ANOVA followed by a post-hoc test, Bonferroni corrected), (P≤0.001).

Discussion

The genus *Rhynchophorus* is considered to have ten described species of weevils that infest different types of palm trees. One of these weevils is *Rhynchophorus cruentatus* (palmetto weevil), which causes considerable damage to newly transplanted and stressed palms (Weissling & Giblin-Davis, 1997). Recently, there has been an observed increase in palms killed by *R. cruentatus*, and the infestation is distributed to reach palms at local palm nurseries and public parks in North Florida. Control strategies for this weevil mainly depend on systematic neonicotinoid insecticides that can be used for curative and preventative treatments, but persistence is necessary (Weissling & Giblin-Davis, 1997). Concerning environmental control and integrated pest management (IPM), botanical extractions having insecticidal, antifeedant, and repellent properties are desirable substitutes for chemical insecticides (Isman, 2000).

According to the literature, neem-based products have been applied against different insect species (Hamadah, 2019; Hajjar *et al*., 2021; Manivasagam *et al*., 2022; Yadav *et al*., 2022); however, there is no data about the effectiveness of botanical insecticide, neem oil extract against *R. cruentatus*. Thus, our goal of this study is to focus on controlling *R. cruentatus* via neem oil extract®, a commercial product, and investigating its impact on mortality, lar-

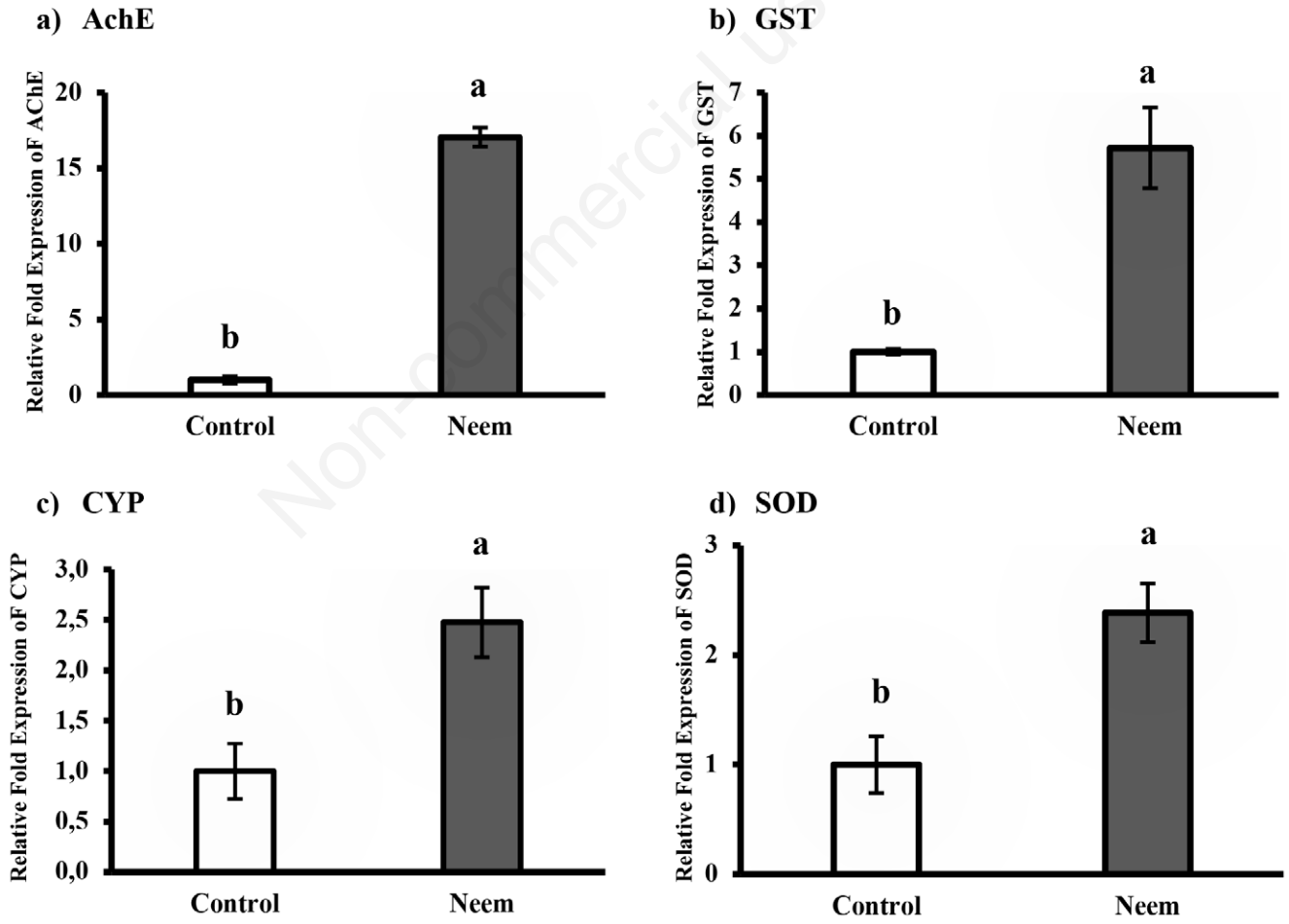

Figure 4. The expression levels of detoxification genes in the third-instar larvae of *R. cruentatus* treated with LC_{50} neem oil extract®, 8 h post-treatment using qRT-PCR, (a) AChE, (b) GST, (c) CYP, and (d) SOD. For each gene, the cycle threshold (Ct) values in the treated larvae were normalized using the controls' values and turned into absolute values as relative fold expressions using $2^{-\Delta\Delta Ct}$. Bars (Geometric means ± SD) marked with different letters refer to significant differences from the control group (t-test, P≤0.01).

val weight, the activity of detoxification enzymes, and detoxification gene expression levels. For the first time, this study has reported that neem oil extract® had various effects on the third-instar larvae of *R. cruentatus*. It induced mortality, larval weight loss, enhanced detoxification enzymes' activity, and increased expression of detoxification genes.

Depending on our results, the third-instar larvae of *R.* cruentatus are susceptible to commercial neem oil extract® that caused mortality after 24 h of treatment, proving its insecticidal activities. Neem oil extract® showed acute toxicity, imparting LC_{50} (12.04%) and LC_{90} (26.48%) against *R.* cruentatus third-instar larvae. Several studies reported the lethal impact of neem tree extracts against several insects. As illustrated in literature, Azadirachtin, the most effective component in neem oil, had a toxic effect on the fourth day-old larva of *Rhynchophorus ferrugineus* when applied topically (Nassar & Abdullah, 2001). A similar study mentioned that the neem oil toxic potential had been investigated in *R. ferrugineus* pre-pupal larvae (El-Bokl *et al*., 2010), showing its ability to disturb insect reproduction, growth, and development. Ethanolic neem extract proved its lethal potential against adults and larvae of *R. ferrugineus* (Merghem & Mohamed, 2017). Hamadah (2019) studied the disturbing effect of NeemAzal on the fat body and the hemolymph enzymatic activities in *R. ferrugineus* larvae. The repellency effect of neem extract against *R. ferrugineus* has been studied and confirmed by Hajjar *et al*. (2021).

A commercial neem-based product, Neemix, could successfully reduce the survival rate and weight gain in *Diaprepes abbreviates* (the root weevil) (Weathersbee & Tang, 2002) and *Leptinotarsa decemlineata* (Colorado potato beetle) (Trisyono & Whalon, 1999). The efficacy of neem-based insecticide, Achook® against elm leaf beetles (*Xanthogaleruca luteola*) has been well demonstrated, indicating a toxic effect against elm leaf beetles (Valizadeh *et al*., 2013). These findings reported that the LC_{50} equals 3.3 ppm 48 h post-treatment, which is much lower than our LC_{50} (12.04%) 24 h post-treatment. Manivasagam *et al*. (2022) confirmed the lethal effect of neem-based products (Neem cake, Neem leaf, Neem oil) against the 2nd and 3rd larval instars of the rhinoceros beetle. The efficacy of NeemAzal in decreasing *Dasineura brassicae* (brassica pod midge) field infestations was proved (Pavela *et al*., 2009). In the case of lepidopterans, NeemAzal proved its insecticidal activity by causing mortality among different life stages of *Spodoptera littoralis* (the Egyptian cotton leafworm) (Ghoneim *et al*., 2000). Previous research reported that neem oil commercial formulations have a toxic potential against adults and pupa of *Diatraea saccharalis Fabricius* (the sugarcane borer) and reduce further development (Schneider *et al*., 2017). Vijayneem (neem-based insecticide) recorded 100% mortality to *Spodoptera litura* (Fab) larvae 10 days after treatment (Radhika *et al*., 2018). In lepidopterans such as *Helicoverpa armigera* and *Spodoptera litura*, neem gum extract and neem gum nano-formulation were assessed. Both formulations showed toxicity and significant effects on antifeedant activities against both insects (Kamaraj *et al*., 2018).

The outcomes of our study demonstrated that neem oil extract significantly reduced the body weight of third-instar larvae of *R. cruentatus* after treatment with LC_{50} and LC_{90} compared to the controls. Similar data mentioned the antifeedant activity of neem-based insecticide Achook® against elm leaf beetles (*Xanthogaleruca luteola*) that can inhibit insect growth (Valizadeh *et al*., 2013). In a like manner, another study demonstrated that African cotton bollworm (*Helicoverpa armigera*) larval weight had been reduced significantly after the treatment with neem plant (*Azadirachta indica*) (Wondafrash *et al*., 2012). The enhanced larval weight loss of treated *R. cruentatus* third-instar larvae unveils that neem has antifeedant effects, which is compatible with previous reports on different insects (Trisyono & Whalon, 1999; Weathersbee & Tang, 2002; Mohamed *et al*., 2003).

The current investigation assessed the activity of detoxification enzymes (AchE, GST, and SOD) of the third-instar larvae fed on apple slices mixed with neem oil extract®. Acetylcholinesterase (AChE) is a vital member of Esterases (ESTs), which is essential for nerve response and is involved in detoxification by hydrolyzing the ester bond (Hemingway & Karunaratne, 1998). AChE breaks down the acetylcholine, producing choline and acetic acid through hydrolytic activity. Glutathione S-transferase (GST) is an enzyme that has a principal role in detoxification. It protects cells against toxicants by stimulating the attachment of glutathione to the compounds' electrophilic and hydrophobic sites. So, they are involved in organophosphorus metabolism, and their activity is induced by xenobiotics, such as plant defense allelochemicals. GST also plays a critical role in insecticide resistance (Vanhaelen *et al*., 2001). Superoxide dismutases (SODs) are antioxidant factors that can oxidize reactive oxygen species. They catalyze the dismutation of superoxide, a reactive oxygen species in cells, into hydrogen peroxide and oxygen. They are considered an essential antioxidant defense against superoxide radicals (Bolter & Chefurka, 1990).

In the current research, AChE, GST, and SOD activities were significantly elevated in the treated larvae compared to the controls. These outcomes reveal that these enzymes have an essential role in detoxifying toxins. Previous investigations are consistent with our data as general esterase and GST activities were significantly increased in elm leaf beetles (*Xanthogaleruca luteola*) that were treated with Achook®, a pesticide derived from neem (Valizadeh *et al*., 2013) and also increased in *Glyphodes pylolais* larvae after *Artemisia annua* extract treatment (Khosravi *et al*., 2011). Investigations of the hemolymph of *R. ferrugineus* larva detected a significate elevation of esterase enzymes after treatment with *Beauveria bassiana* (Didair *et al*., 2018). Lufenuron led to a significant increase in α and β esterase activity in the 6th instar larva of *R. ferrugineus* (El-Sobki and Ali, 2020). *Spodoptera litura* larvae showed an increased level of esterase enzymes after treatments with monocrotophos + Vijayneem (neem-based insecticide) (Radhika *et al*., 2018).

Compatible with our findings, El-Sobki and Ali (2020) recorded a significant elevation of GST activity in the 6th instar larva of *R. ferrugineus* after exposure to 3 different insecticides (chlorfluazuron, hexaflumuron, and lufenuron). D- camphor, an active compound extracted from the camphor tree, increased the activity of the GST enzyme in *Pagiophloeus tsushimanus* weevil larva and was accompanied by the enhancement of GST genes (Li *et al*., 2022). Also, GST activity was induced after treatment with *Brassicaceae* secondary metabolites in *Myzus persicae, Trichoplusia ni* (Hübner), *Anticarsia gemmatalis* (Hübner), and *Heliothis virescens* (Fabricius) (Vanhaelen *et al*., 2001). AChE and GST enzyme activities were measured in *R. ferrugineus* larva after infection with *Beauveria bassiana*; results showed high activities of these enzymes in the infected larva compared with uninfected larvae (Ahmed *et al*., 2021). Yuan *et al*. (2020) reported that activity levels of GST and CYP enzymes were higher in *Hyphantria cunea* treated larva with tannic acid compared to 24- the control.

In corresponding with our results, Bamidele *et al*. (2013) observed a significant increase in SOD activity in *R. phoenicis* (The African palm weevil) larvae after exposure to DDVP (2, 2-dichlorovinyl dimethyl phosphate). Studying the infection of *Galleria mellonella* with entomopathogenic nematodes revealed that SOD activity increased in the infected larvae 12 h post-infection (Zółtowska *et al*., 2006). Also, larvae of *Lymantria dispar* showed high activity of SOD after exposure to carvacrol (Chen *et al*., 2021).

In contrast with our findings, AChE, GST, and SOD activities

were significantly inhibited in *R. ferrugineus* after exposure to different pesticides such as methomyl, chlorpyrifos, and spinosad (Ahmed & El-Sobki, 2021). Hexaflumuron was reported to decrease α and β esterase activity in the 6th instar larva of *R. ferrugineus* (El-Sobki and Ali, 2020). The AChE enzyme level decreased in *Hyphantria cunea* larva treated with tannic acid (Yuan *et al*., 2020). The neem oil extract did not affect AChE activity levels in the treated house flies but could reduce the GST enzyme activity (Soyelu *et al*., 2020). GST enzyme activity was reduced in *Spodoptera littoralis* larva after treatment with the Lc_{50} of different essential oils, including garlic, eucalyptus, lavender, and peppermint (Ibrahim and Abd El-Kareem, 2018). Fungal infection of Asian citrus psyllid showed that GST and SOD enzyme activities significantly decreased in infected insects 5 days after treatment (Qasim *et al*., 2021).

The amount of total protein was measured, and results revealed a non-significant rise in the treated larvae compared with the untreated larvae. Our results have differed from previous observations in elm leaf beetles (*Xanthogaleruca luteola*) that were treated with Achook®; a commercial insecticide derived from neem (Valizadeh *et al*., 2013) and *Glyphodes pyloalis* larvae after *Artemisia annua* extract treatment (Khosravi *et al*., 2011), detecting a significant reduction in total protein amount.

In terms of the physiological effects, neem oil extract® promoted a major increase in the expression levels of detoxification genes in the treated larvae. Our study outcomes revealed that neem oil extract® was able to induce four detoxification gene expression levels of third-instar *R. cruentatus* larvae, including, AchE, GST, CYP, and SOD. Among all the investigated genes, according to our data, AChE was the highest expressed gene with approximately a 17.04-fold increase; this is agreed with the observations of Hussain *et al*. (2017), who indicated that piperine significantly stimulated esterase expression levels by a 15-fold increase in *R. ferrugineus* larvae. Contrary to our findings, the esterase expression level remained very low in *R. ferrugineus* larvae treated with phenylpropanoids (AlJabr *et al*., 2017).

Data presented in this study showed that neem oil extract® significantly stimulated GST expression, resulting in a 5.72-fold increase. Promoted expression of the GST gene was also detected in resistant *R. ferrugineus* populations fed on an artificial diet supplemented with cypermethrin and ethion (Al-Ayedh *et al*., 2016). Our findings are in harmony with the previous report on *R. ferrugineus* larvae treated with piperine and black pepper extracts and exhibited high expression levels of GST (Hussain *et al*., 2017). Similarly, high GST expression levels were detected in corn bug (*Eurygaster integriceps* Puton) after treatment with *Artemisia annua* Linnaeus (Zibaee & Bandani, 2010). GST gene level was over-expressed in *Chironomus riparius* after treatment with silver ions and silver nanoparticles (Nair *et al*., 2013). Yu *et al*. (2022) detected upregulation in 11 genes of GST compared to the downregulation of 9 GST genes in *Spodoptera frugiperda* larva when treated with neem derivative (azadirachtin). Su *et al*. (2021) found that 13 GST genes were significantly over-expressed in the abamectin-treated larva of *Grapholita molesta*; however, cytochrome P450 genes were not affected.

The present laboratory experiments show that cytochrome P450 was overexpressed approximately 2.48-fold in the treated third-instar palmetto weevil larvae fed on apple slices mixed with neem oil extract. Our results are compatible with previous findings that demonstrated that cytochrome P450 gene expression was stimulated in *Helicoverpa armigera* Hübner after treatment with sub-lethal concentrations of cantharidin mixed with diet (Rashid *et al*., 2013). Increasing GST and CYP enzyme activities in *Helicoverpa armigera* larva accompanied by upregulation of the CYP gene after exposure to 4 different plant volatiles was reported (Wu *et al*., 2021). The phenylpropanoids enhanced expression levels of both GST and CYP in the tenth-instar larvae of *R. ferrugineus* (AlJabr *et al*., 2017). According to Al-Harbi *et al*. (2021), cytochrome P450 gene expression was upregulated in *Sitophilus oryzae* larvae after exposure to lavender essential oil. Yu *et al*. (2022) detected upregulation in 29 genes of CYP compared to the downregulation of 24 CYP genes in *Spodoptera frugiperda* larva when treated with neem derivative (azadirachtin).Tremendous expression of cytochrome P450 (35-fold) was detected in the eight-larval instar of *R. ferrugineus* fed on diets mixed with piperine (Hussain *et al*., 2017). Similar outcomes were observed in variegated cutworm fed on peppermint leaves as cytochrome P450 activity was promoted to be 45-fold (Yu *et al*., 1979). On the contrary, AlJabr *et al*. (2017) found that coumarin targeted CYP and GST genes, silencing their expression levels and leading to a low LD_{50} value.

In contrast with our findings, Esterase, GST, and CYP expression levels were decreased in *R. ferrugineus* eight-instar larvae after treatment with onion-garlic mixture extract (Al-Shuraym *et al*., 2020). Our results showed a similar stimulation in SOD expression level with a 2.38-fold increase from the control. However, CYP and SOD expression levels remained significantly lower than AchE and GST. SOD gene level was over-expressed in *Chironomus riparius* after treatment with silver ions; however, no significant change was detected when applying silver nanoparticles (Nair *et al*., 2013). On the other hand, Qasim *et al*. (2021) found that fungal infection of Asian citrus psyllid led to the downregulation of CYP and SOD gene expression levels in infected insects 5 days after treatment.

Enhanced expression of these detoxification genes is well documented as a defense mechanism against toxicants (Rashid *et al*., 2013). Our investigations do not reveal any inhibition in the activities of the tested detoxification enzymes and their gene expression levels in the treated larvae, proposing their engagement in the insecticide detoxification and defense mechanism of *R. cruentatus* larvae. Also, this high detoxification gene expression level perhaps explains the obtained high LC_{50} value (12.04%). Finally, the outcomes of this study show that neem oil extract® serves as an effective and safer bioinsecticide and promises its ability to control *R. cruentatus* larvae.

Conclusions

In conclusion, the current study demonstrates the efficacy of using commercial neem oil extract® to control the third-instar larvae of *R. cruentatus* by proving its significant insecticidal effects. It induces larval weight loss and has significant physiological impacts. Tracing the detoxification enzymes' activities and gene expression levels provide perceptions of the detoxification mechanism of *R. cruentatus*. This project is the first to show how neem oil extract® can be an efficient plant-based insecticide against palmetto weevils *(R. cruentatus)* under laboratory conditions. Further investigations should be conducted to determine its impact in the field.

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