Insecticidal efficacy and possibility of Combretum trifoliatum Vent . (Myrtales: Combretaceae) extracts in controlling Spodoptera frugiperda (J.E. Smith) (Lepidoptera: Noctuidae)

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Research Article

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Insecticidal efficacy and possibility of Combretum trifoliatum Vent. (Myrtales: Combretaceae) extracts in controlling Spodoptera frugiperda (J.E. Smith) (Lepidoptera: Noctuidae)

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Abstract

BACKGROUND: The fall armyworm *Spodoptera frugiperda* (J.E. Smith), is an important pest of agronomical crops. It is interesting to discover secondary metabolites in plants that are environmentally safer than synthetic pesticides. For this purpose, *Combretum trifoliatum* crude extract and its isolated compounds were investigated for their insecticidal activities against *S. frugiperda*.

RESULTS: The median lethal dose (LD_{50}) was evaluated in the second-instar larvae using the topical application method. The isolated compounds, apigenin and camphor, demonstrated a highly toxic effect on larvae at a lower LD_{50} dose than crude extract. Moreover, when the larvae were exposed to crude extract concentrations, the development to pupa and adult stages was reduced by more than 50%. The ovicidal toxicity was examined using a hand sprayer. The extract concentration 5, 10, and 20 µg/egg significantly decreased the egg hatchability. In addition, crude extract showed a significant difference in inhibiting acetylcholinesterase (AChE) activity while crude extract and camphor showed significant inhibitory effects on carboxylesterase (CE) and glutathione-*S*-transferase (GST) activities.

CONCLUSION: The crude ethanol extract of *Combretum trifoliatum* was toxic to *S. frugiperda* in terms of larval mortality, negatively affecting biological parameters, and decreasing egg hatchability. Additionally, the activities of cholinergic and detoxifying enzymes were affected by crude extract and its isolated compounds. These results highlight that *Combretum trifoliatum* might be efficient as a bioinsecticide to control *S. frugiperda*.

Keywords: *Combretum trifoliatum* Vent.; fall armyworm; 5,7-dihydroxy-2-(4-hydroxyphenyl)chromen-4-one; 1,7,7-trimethylbicyclo[2.2.1]heptan-2-one; bioinsecticides; detoxification enzymes

1 INTRODUCTION

The fall armyworm *Spodoptera frugiperda* (J.E. Smith), a polyphagous insect pest, is a member of the order Lepidoptera. It is native to tropical and subtropical areas in the Americas. Since 2016, it has spread on the African continent, and recently, it has been found in Asia and all of southeast Asia, including Thailand.[1–4] The damage caused by the larval stage has been reported in several cultivated economic plant species, principally Poaceae, Asteraceae, and Fabaceae. All plant organs are consumed, especially leaves, stems, and reproductive organs, causing significant losses in product yields.[5,6] The most commonly used technique to control this pest in the current agricultural era is the use of synthetic insecticides, the five main families being pyrethroids, neonicotinoids, pyrroles, quinolines and spinosyns.[7] These substances can be toxic to the environment, for example cypermethrin, a pyrethroid has been shown to be harmful to fish and aquatic invertebrates.[8–11] Moreover, the application of synthetic pyrethroids has increased *S. frugiperda* larvae resistance in both field and laboratory populations.[12–14] To reduce the risk of synthetic insecticide usage, Integrated pest management (IPM) also known as integrated pest control, is a broad-based approach that integrates both chemical and non-chemical practices for economic control of pests, such as

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the use of botanical insecticides or genetically modified plants, which contain the endospore from *Bacillus thuringiensis* (*Bt*) and produce *Bt* toxins to be resistant to *S. frugiperda* in corn and cotton crops.[15–17] Natural products made from plants have been widely used and strongly exploited for their insecticidal activities because they generally affect the target pest and eventually closely related organisms while showing low toxicity and rapid degradation. They are therefore good candidates for biopesticide development in the context of IPM.[18,19]

Comparable to synthetic insecticides, several plant extracts have demonstrated insecticidal efficacy. For instance, the extracts obtained from *Ocimum canum* (Lamiaceae), *Ocimum sanctum* (Lamiaceae), *Rhinacanthus nasutus* (Acanthaceae),[20] *Curculigo orchioides* (Hypoxidaceae), *Evolulus alsinoides* (Convolvulaceae), *Phyllanthus debilis* (Phyllanthaceae),[21] *Acorus calamus* (Acoraceae), *Piper retrofractum* (Piperaceae),[22] *Cymbopogon citratus* (Poaceae), *Lippia javanica* (Verbenaceae), *Nicotiana tabacum* (Solanaceae)[23] caused lethal effects on *Spodoptera* spp.

Combretum trifoliatum Vent. belongs to the family Combretaceae originating from Indo-China is also found in southeast Asia to Australia.[24] In Thailand, 13 species were found throughout tropical, subtropical and coastal regions.[25] Many classes of secondary metabolites have been found in the genus *Combretum*, including triterpenes, terpenoids, flavonoids, phenols, steroids, lignans, and miscellaneous compounds.[26–28] The usage of plants of Combretaceae family is widely experienced for therapeutic purposes, including antiviral, antibacterial, antifungal, antihelmintic activity. For agricultural purposes, a few studies reported their molluscicidal activity, particularly insecticidal activity, such as repelling *Cryptotermes brevis* (Walker) (Isoptera: Kalotermitidae) feeding.[29,30] Moreover, the leaf extracts of *Terminalia catappa* L. and *Conocarpus erectus* L. had repellent and antifeedant effects on the stored product pest, *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae).[27,31]

However, there are few research areas which are reported for insecticidal activity from Combretaceae plant on Lepidopteran species. For example, Feng *et al.*[32] reported the *Quisqualis indica* L. ethanolic extract had contact toxic effect on *Spodoptera exigua*.

Considering the qualities mentioned earlier, *Combretum trifoliatum* is a promising candidate plant to characterize novel secondary metabolites. Therefore, we investigated the ovicidal toxicity, contact toxicity, cholinergic enzyme and detoxification enzyme activities, as well as effects on some biological parameters, using ethanolic extracts on *S. frugiperda* larvae under laboratory conditions.

2 MATERIALS AND METHODS

2.1 Plant material collection and extract preparation

For general experimental procedures, column chromatography (CC) used silica gel 60 (70-230 mesh; Merck Millipore, Darmstadt, Germany). The Prep-TLC used Kieselgel 60 PF254 (0.5 mm; Merck Millipore).

The leaves and twigs of *Combretum trifoliatum* were collected from the Nakhonsawan campus, Mahidol University, Nakhonsawan, Thailand. The plant was identified by Plant varieties protection office, Bangkok, Thailand. The voucher specimen (BKF No. 203356) was deposited at the Bangkok herbarium.

Air-dried and finely powdered leaves and twigs of *Combretum trifoliatum* (1 kg) were extracted with ethanol (20 L × 5 days × 5 times) at room temperature to produce crude ethanol extracts (34.13 g). The crude ethanol was isolated by silica gel No. 7734 (500 g) (ethyl acetate, hexane and methanol–ethyl acetate and methanol solvent gradient elution) to yield fractions A_1–A_5. Fraction A_3 (2.06 g) yielded camphor (331.41 mg) after Si-gel CC (dichloromethane–hexane solvent gradient elution), followed by recrystallization with methanol–dichloromethane. A_4 (4.11 g) has a yellow solid and recrystallized by methanol to give apigenin (21.13 mg). All solvents commercial grade used for extraction and separation were purchased from Merck Thailand, distilled within the boiling point range before use.

All pure compounds were verified by their physical properties and spectroscopic data, as found in the literature.[33,34]

2.2 Insect rearing

The initial colony of *S. frugiperda* was obtained from the National Center for Engineering and Biotechnology, Bangkok, Thailand. Insects were reared under laboratory conditions in the Animal Toxicology and Physiology Specialty Research Unit (ATPSRU), Department of Zoology, Faculty of Science, Kasetsart University, Bangkok, Thailand. The larvae were continuously maintained on an artificial diet (mixture of mung bean, instant yeast, agar, wheat germ, methyl paraben (Sigma-Aldrich, Singapore), sorbic acid (Sigma-Aldrich, St Louis, MO, USA), L-Ascorbic acid (Sigma-Aldrich, St Louis, MO, USA), mixed vitamin solution (Sigma-Aldrich, Singapore), 40% formaldehyde solution (Ajax Finechem, Scoresby, VIC, Australia), Moxipharm™ amoxicillin solution and distilled water) and adults were fed an absorbent cotton soaked with 20% honey solution. All stages of *S. frugiperda* were maintained in the environmental chamber (MLR 352H; Panasonic Corp., Gunma, Japan) at 27 °C with 70% relative humidity (RH) and 16 h:8 h (light/dark) photoperiod.[22]

All experimental procedures in this research were performed with the approval of an appropriate Animal Ethics committee of Kasetsart University (ACKU64-SCI-020).

2.3 Contact toxicity bioassay

For the contact toxicity test, *S. frugiperda* second-instar larvae (4-day old after hatching) were treated by topical application.[22] The negative control treatment was treated with acetone (Loba Chemie, India) mixed with 5% dimethyl sulfoxide (DMSO) (Merck KGaA, Darmstadt, Germany). The treatment doses consisted of *Combretum trifoliatum* crude extract and the isolated compounds, including apigenin and camphor serial diluted solutions at doses of 1.25, 2.5, 5, 10, and 20 µg/larva and range of 0.001 to 0.2 µg/larva for positive control, cypermethrin 35% EC commercial grade (Cycatak™) were prepared in acetone mixed with 5% DMSO. Each dose (1 µL) was applied to the insect's thorax using an Eppendorf™ micropipette. Six replicates (environmental chambers) were used for each treatment, with ten larvae per environmental chamber (MLR 352H; Panasonic Corp.) (total number of larvae per treatment = 60). After treatment, larvae were fed an artificial diet (replaced daily) and maintained at 27 °C with 70% RH and 16 h:8 h (light/dark) photoperiod. For the post-treatment, larval mortality was recorded at 24 and 48 h and the probit analysis was conducted to calculate the lethal dose-mortality, median lethal dose (LD_{50}) and sublethal dose (LD_{30}) using StatPlus program for Windows, version 2017 (AnalystSoft Inc., Canada).

2.4 Growth inhibition bioassay

Second-instar (4-day old after hatching) *S. frugiperda* larvae were treated with serial dilution doses of 1.25, 2.5, 5, 10, and 20 µg/larva of *Combretum trifoliatum* crude extract. The negative control was treated with acetone mixed with 5% DMSO and 0.0047 µg/larva (LD_{50}) for positive control, cypermethrin 35% EC commercial grade (Cycatak™). In each dose, 1 µL of solution was applied on

the thorax of each larvae using an Eppendorf™ micropipette. Six replicates, with ten larvae per replicate were performed per treatment (total number of larvae per treatment = 60, five treatments).

After treated, the larvae that survived to each treatment were individually reared, fed on artificial diet, and replaced daily. Then, reared in an environmental chamber following conditions, 27 °C with 70% RH and 16 h:8 h (light/dark) photoperiod until emerge to adult.[22] Pupal weight was measured after complete formation on an analytical balance (XSR205DU; Mettler Toledo, Greifensee, Switzerland). The biological parameters evaluated were duration of larval stage, percentage of pupation, pupal weight, duration of pupal stage and adult emergence.[35]

2.5 Ovicidal toxicity bioassay

To examine the ovicidal activity, *S. frugiperda* egg masses at 1–2 days old were clipped from the oviposition site and the egg masses hair covering was removed using a fine brush for this experiment. To determine the percentage of hatchability, single or multiple egg masses were selected to certify 250 eggs in each replicate (total number of eggs per treatment = 750). In the experiment, acetone mixed with 5% DMSO was used as the negative control group, *Combretum trifoliatum* crude extract serially diluted solutions at doses of 1.25, 2.5, 5, 10, and 20 µg/egg were used as treatment groups and cypermethrin 35% EC commercial grade (Cycatak™) in a concentration of 0.0047 µg/egg (LD_{50}) was used as the positive control. Egg masses were placed in a Petri dish, then sprayed with 1 mL of each solution by hand sprayer and were then transferred to an environmental chamber following conditions, 27 °C with 70% RH and 16 h:8 h (light/dark) photoperiod until hatching. The experiment was conducted in completely randomized design (CRD) with three replicates per treatment. The observations on the number of eggs hatched in each egg mass and the hatching larvae were recorded daily in each treatment under stereo microscope (Stemi 508; Zeiss, Oberkochen, Germany) using ZEN 2 core imaging software. Afterwards, the hatched neonates were counted and the percentage of egg hatchability was calculated.[36]

2.6 Enzyme bioassays

2.6.1 *Enzyme extraction method*

To measure the enzyme activities of acetylcholinesterase (AChE), carboxylesterase (CE) and glutathione-*S*-transferase (GST) within tissues, *S. frugiperda* second-instar larvae were treated with LD_{30} values of *Combretum trifoliatum* crude extract and isolated compounds, including apigenin and camphor, as the treatment group. However, the negative control was acetone mixed with 5% DMSO and the positive control was LD_{30} of cypermethrin 35% EC commercial grade (Cycatak™). Fifteen surviving *S. frugiperda* second-instar larvae after 24 and 48 h of LD_{30} were subjected to *in vivo* assays.[37,38] Fifteen surviving larvae were pooled and ground in a cold homogenized buffer, 100 mM phosphate buffer (pH 7.2) mixed with 1% Triton™ X-100 (Sigma-Aldrich, Singapore) and then centrifuged at 12000 rpm for 5 min at 4 °C using an refrigerated centrifuge (1248R; Gyrozen) and compared to the negative control group. The supernatants were then collected separately and stored at −20 °C.

2.6.2 *Acetylcholinesterase (AChE) activity*

The AChE activity analysis method was modified from Ellman *et al.*[39] The mixtures containing potassium phosphate buffer (100 mM, pH 7.2) was mixed with 50 µL supernatant and incubated at 30 °C for 30 min. Then TpS solution [10 mM DTNB, 0.1 mM EDTA, 10 mM ASCh and potassium phosphate buffer (100 mM, pH 7.2) (Sigma-Aldrich, Singapore)] were added. The activity was measured at a wavelength of 412 nm by kinetic mode using a BioTek PowerWave XS 340 microplate reader (Bio-Tek, Winooski, VT, USA). Three biological replicates per treatment were estimated.

2.6.3 *Carboxylesterase (CE) activity*

The CE activity was analyzed by a *p*-nitrophenyl acetate (*p*NPA; Sigma-Aldrich, Singapore) assay modified from Bullangpoti *et al.*[38] Briefly, 40 µL enzyme solution was mixed with the phosphate buffer (50 mM, pH 7.4), then 10 mM *p*NPA was added. Enzyme activity was measured by kinetic mode at 410 nm at 37 °C using a BioTek PowerWave XS 340 microplate reader. The CE activity was determined using an extinction coefficient of 176.4705 for *p*NPA. Three biological replicates were estimated per treatment.

2.6.4 *Glutathione-S-transferase (GST) activity*

To determine the GST activity, the method was modified from Oppenoorth *et al.*[40] Potassium phosphate buffer (50 mM, pH 7.4) was mixed with glutathione solution and 150 mM 1-chloro-2,-4-dinitrobenzene (CDNB) solution (Sigma-Aldrich, Singapore). The activity of the reaction was measured at 37 °C and 340 nm with a BioTek PowerWave XS 340 microplate reader. The GST activity was determined using an extinction coefficient of 0.0096 for CDNB. Three biological replicates were estimated per treatment.

2.7 Statistical analysis

The data were subjected to Probit analysis to calculate the LD_{50} using the StatPlus program for Windows, version 2017 (AnalystSoft Inc.). The analyses of the biological parameters (duration of larval stage, percentage of pupation, pupal weight, duration of pupal stage and adult emergence), ovicidal activity, cholinergic enzyme and detoxification enzyme activities were subjected to one-way analysis of variance (ANOVA). Means were compared with the negative control treatment by the Dunnett's post-test at $P < 0.05$. Statistical analysis was performed with IBM SPSS Statistics for MacOS, version 29.0.1.0 (IBM Corp., Armonk, NY, USA).

3 RESULTS

3.1 Isolated compound

The physical properties of the melting points were recorded on a digital Electrothermal IA 9000 series melting point apparatus (Rochford, ESS. Great Britain). Ultraviolet (UV) spectra were measured in ethanol or methanol with a JASCO 530 spectrometer (Jasco, Tokyo, Japan). The infrared (IR) spectra were recorded on a Perkin Elmer 2000 FTIR spectrometer (Perkin Elmer, Waltham, MA, USA); major bands were recorded in wavenumber (ν_{max}; in cm^{-1}). The proton (^1H) nuclear magnetic resonance (NMR), carbon-13 (^{13}C)-NMR, DEPT (distortionless enhancement by polarization transfer), and two-dimensional (2D) NMR spectra were recorded on a Bruker Ascend™ 400 MHz or Bruker AVANCE 500 MHz (Bruker, Karlsruhe, Germany). The chemical shifts were reported in part per million (δ, ppm) using tetramethylsilane (TMS) as an internal standard. Deuterated chloroform for NMR were purchased from Sigma-Aldrich, Taufkirchen, Germany. The electron ionization mass spectrometry (EI-MS) results were recorded on a Thermo Finnigan Polaris Q mass spectrometer

(Scientific Inc., Markham, ON, Canada) at 70 eV (probe). Finally, High-resolution time-of-flight mass spectrometry (HR-TOF-MS) results were recorded on a Bruker Micromass model VQ-TOF model (Bruker, San Jose, USA).

After purification, two pure compounds were detected based on melting points and spectral analysis data as described:

Camphor (Fig. 1(A))[33]: colorless needle, melting point (m.p.) = 172–175 °C (reff 176–177 °C); IR (KBr) ν_{max} 2958, 1738, 1447, 1391, 1303, 1276, 1045; HIMS ESI calculate for 175.1097 [M + Na^+] ($C_{10}H_{16}ONa$ found 175.1911); ^{1}H-NMR ($CDCl_3$, 400 Hz) δ 2.36 (1H, ddd, J = 18.0, 4.5, 3.3, H-3) 2.10 (1H, t, J = 4.5 H-4), 1.96 (1H, m, H-5a), 1.69 (1H, dd, J = 13.1, 4.1 H-6a), 1.41 (1H, m, H-6b), 1.35 (1H, m, H-5b), 0.97 (3H, s, CH_3-9), 0.92 (3H, s, CH_3-10), 0.85 (3H, s, CH_3-8); ^{13}C-NMR ($CDCl_3$, 100 Hz) δ 58.04 (C-1), 220.95 (C-2), 43.57 (C-3), 43.33 (C-4), 27.25 (C-5), 30.12 (C-6), 47.09 (C-7), 19.92 (CH_3-8) 19.29 (CH_3-9), 9.34 (CH_3-10).

Apigenin (Fig. 1(B))[34]: yellow solid, m.p. = 320–321 °C (reff 324–325 °C); IR (KBr) ν_{max} 3291, 1651, 1608, 1354; HIMS ESI calculate for 293.2093 [M + Na^+] ($C_{15}H_{10}O_5Na$ found 293.2100); ^{1}H-NMR (DMSO-d_6,400 Hz) δ 12.94 (OH-4), 7.89 (2H, m, H-2′, H-6′), 6.90 (2H, m, H-3′, H-5′), 6.75 (1H, s, H-3), 6.45 (1H, d, J = 2.0 Hz, H-6, 6.17 (1H, d, J = 2.0 Hz, H-8); ^{13}C-NMR (DMSO-d_6, 100 Hz) δ 163.85 (C-2), 102.90 (C-3), 181.81 (C-4), 161.22 (C-5), 98.93 (C-6), 164.23 (C-7), 94.06 (C-8), 157.40 (C-4a) 103.76 (C-8a), 121.26 (C-1′), 128.54 (C-2′, C-6′), 116.06 (C-3′, C-5′) 161.50 (C-4′).

3.2 Contact toxicity

The contact toxicity of the *Combretum trifoliatum* extract was evaluated using a topical application. The LD_{50} values of this extract and cypermethrin are shown in Table 1. Second-instar larvae mortality rate demonstrated that the *Combretum trifoliatum* isolated compounds, camphor showed the highest toxicity, with an LD_{50} value of 2.490 ± 0.533 µg/larva (mean ± standard error); followed by apigenin with an LD_{50} of 3.449 ± 0.567 µg/larva, crude extract with an LD_{50} of 6.081 ± 0.614 µg/larva. However, cypermethrin used as a positive control was highly toxic with an LD_{50} value of 0.0021 ± 0.0009 µg/larva after 48 h exposure.

3.3 Growth inhibition

The growth inhibition of the *Combretum trifoliatum* extract was evaluated with *S. frugiperda* second-instar larvae by topical application. The biological parameters, including duration of larval stage, percentage of pupation, pupal weight, duration of pupal stage, and adult emergence. Moreover, abnormalities during every developmental stage were observed.

The results in Table 2 show the toxicity effect on the biological parameters of *S. frugiperda*. Duration of larval stage was similar between larvae treated with 1.25, 2.5, and 5 µg/larva of *Combretum trifoliatum* extract and negative control group. The doses of 10 and 20 µg/larva were significantly increased compared to the control (F = 6.479, df = 6, 181 P < 0.001, where the degree of

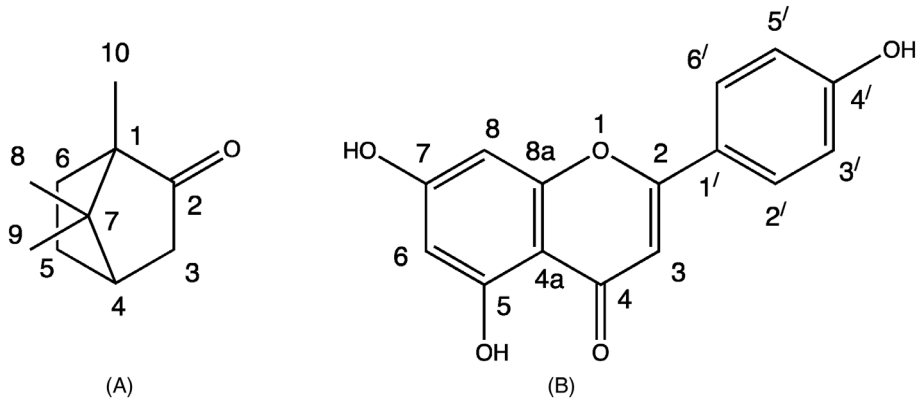

Figure 1. Pure compounds isolated from *Combretum trifoliatum* Vent. as (A) camphor and (B) apigenin.

Table 1. Toxicity of *Combretum trifoliatum* ethanolic crude extract and its active compounds (µg/larva) against second-instar of *Spodoptera frugiperda* larvae at 24 and 48 h by topical application (mean ± standard error)

Treatment	Time (h)	LD_{50} (µg/larva)	LD_{50} SE	χ^2	df	LD_{50} LCL	LD_{50} UCL	Slope ± SE	*P*-value
Combretum trifoliatum crude extract	24	8.351	0.725	4.598	3	7.117	10.000	1.817 ± 0.164	0.204
	48	6.081	0.614	3.939	3	5.023	7.456	1.416 ± 0.148	0.268
Apigenin	24	4.030	0.456	15.173	3	2.917	5.211	2.226 ± 0.270	0.010
	48	3.449	0.567	21.884	3	2.092	4.851	1.997 ± 0.310	0.001
Camphor	24	3.078	0.535	10.273	3	1.462	6.481	2.080 ± 0.491	0.006
	48	2.490	0.533	12.771	3	0.998	6.212	1.983 ± 0.548	0.002
Cypermethrin (positive control)	24	0.0047	0.0016	3.145	10	0.0019	0.0087	1.135 ± 0.238	0.002
	48	0.0021	0.0009	2.185	10	0.0007	0.0048	0.762 ± 0.124	0.001

Abbreviations: LD_{50}, lethal dose at which half the population is killed. The active ingredient concentration is expressed in parts per million (ppm); SE, standard error; χ^2, chi square; df, degrees of freedom; LCL, lower confidence limit; UCL, upper confidence limit.

Table 2. Biological parameters of *Spodoptera frugiperda* larvae development treated with *Combretum trifoliatum* ethanolic crude extract [mean ± standard error (SE)]

Treatment dose (µg/larva)	Duration of larval stage (days ± SE)	Percentage of pupation (% ± SE)	Pupal weight (mg ± SE)	Duration of pupal stage (days ± SE)	Adult emergence (% ± SE)
Negative control *Combretum trifoliatum* crude extract	17.780 ± 1.69	86.667 ± 4.22	218.152 ± 3.67	6.500 ± 0.12	80.000 ± 3.65
1.25	18.280 ± 1.44	60.000 ± 4.47†	218.633 ± 4.38	6.219 ± 0.14	53.333 ± 4.22†
2.5	18.300 ± 1.47	50.000 ± 5.16†	226.459 ± 8.51	6.600 ± 0.20	41.667 ± 4.01†
5	17.357 ± 1.52	46.667 ± 5.58†	214.024 ± 3.42	6.286 ± 0.17	35.000 ± 6.71†
10	19.000 ± 1.35†	21.667 ± 3.07†	215.747 ± 8.33	7.333 ± 0.33†	15.000 ± 2.24†
20	19.750 ± 1.67†	13.333 ± 2.11†	222.094 ± 9.34	8.000 ± 0.45†	8.333 ± 3.07†
Cypermethrin (0.0047)	16.696 ± 1.40†	38.333 ± 3.07†	213.488 ± 6.78	6.278 ± 0.23	30.000 ± 3.65†
	$F = 6.479$ df = 6, 181 $P < 0.001$	$F = 35.132$ df = 6, 35 $P < 0.001$	$F = 0.582$ df = 6, 181 $P = 0.744$	$F = 4.914$ df = 6, 151 $P < 0.001$	$F = 33.966$ df = 6, 35 $P < 0.001$

† Statistically significant differences compared to the negative control group (Dunnett's test $P < 0.05$).

freedom of the denominator = 181). Moreover, multiple morphological defects by *Combretum trifoliatum* extract were observed. In larval-pupal intermediates, the larval body contracted and pupa covered larval exuviae caused incomplete pupation. Exposure to ethanolic extract significantly reduced the percentage of pupation compared to control ($F = 35.132$, df = 6, 35 $P < 0.001$, where the degree of freedom of the denominator = 35). The highest success rate of development from larvae to pupae was observed at 1.25 µg/larva. In contrast, pupal weight was not significantly different ($F = 0.582$, df = 6, 181 $P = 0.744$, where the degree of freedom of the denominator = 181). Duration of pupal stage were significantly different in the 10 and 20 µg/larva of *Combretum trifoliatum* treated group ($F = 4.914$, df = 6, 151 $P < 0.001$, where the degree of freedom of the denominator = 151). The extract produced pupae abnormality during pupal-adult intermediates, abnormal pupae with malformed head and thoracic appendages and incomplete eclosion adultoid from pupal cuticle. Interestingly, there was a dose response to adult emergence of *S. frugiperda*. Adult emergence was significantly reduced ($F = 33.966$, df = 6, 35 $P < 0.001$, where the degree of freedom of the denominator = 35), the median percentage of emerged adult was lower than 50% when concentration higher than 2.5 µg/larva were exposed at second-instar larvae. In the adult stage abnormal characters were observed, in particular reduced thoracic body and malformed wings.

3.4 Ovicidal toxicity

Combretum trifoliatum crude extract was also evaluated for ovicidal activity at concentrations ranging from 1.25 to 20 µg/egg. The mean percentage of hatched eggs of the control was over 95%. As indicated in Table 3, the percentages of hatched eggs treated with different concentrations of crude extract were significantly lower than for the control when extract were administrated in the range of 5, 10 and 20 µg/egg. The highest concentration treated (20 µg/egg) resulted in the lowest percentage of hatched eggs (59.200 ± 6.557%). Likewise, hatchable eggs was observed in all treatment groups. Except those treated with 20 µg/egg crude extract and cypermethrin as a positive control ($F = 14.988$, df = 6, 14 $P < 0.001$, where the degree of freedom of the denominator = 14).

Table 3. Ovicidal toxicity of the *Combretum trifoliatum* ethanolic crude extract on *Spodoptera frugiperda* eggs [mean ± standard error (SE)]

Treatment dose (µg/egg)	Total eggs (number)	Egg hatchability (% ± SE)
Negative control *Combretum trifoliatum* crude extract	750	96.933 ± 3.512
1.25	750	82.267 ± 5.508
2.5	750	79.867 ± 2.517
5	750	66.133 ± 7.572†
10	750	63.200 ± 7.211†
20	750	59.200 ± 6.557†
Cypermethrin (0.0047)	750	47.867 ± 4.933† ($F = 14.988$, df = 6, 14, $P < 0.001$)

† Statistically significant differences compared to the negative control group (Dunnett's test $P < 0.05$).

3.5 Effects on cholinergic and detoxification enzyme activities

Table 4 summarizes the cholinergic and detoxification enzyme activities using the different bioassays.

3.5.1 *Acetylcholinesterase (AChE)*

Treatment with crude *Combretum trifoliatum* extract and cypermethrin showed significant inhibition at 24 h ($F = 1254.203$, df = 4, 55 $P < 0.001$, where the degree of freedom of the denominator = 55) and 48 h ($F = 131.226$, df = 4, 55 $P < 0.001$, where the degree of freedom of the denominator = 55) post-treatment. Conversely, Apigenin treatment showed significant induction at 24 and 48 h post-treatment (Dunnett's test $P < 0.05$). However, camphor treatment also induced a significant inhibition at 24 h but not at 48 h.

3.5.2 *Carboxylesterase (CE)*

All isolated compounds inhibited the CE activity. Crude extract, apigenin, camphor and cypermethrin induced a significant reduction in the CE activity at 24 h ($F = 261.492$, df = 4, 55 $P < 0.001$,

Table 4. Cholinergic enzyme and detoxification enzyme activities of second-instar *Spodoptera frugiperda* larvae at 24 and 48 h after treatment with LD_{30} of *Combretum trifoliatum* ethanolic crude extract and isolated compounds [mean ± standard error (SE)]

Treatments (µg/larva)	Acetylcholinesterase[†] (CF)		Activity		Carboxylesterase[‡] (CF)		Activity		Glutathione-*S*-transferase[§] (CF)		Activity	
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
Negative control	3.778 ± 0.053	7.622 ± 0.066	—	—	13.675 ± 0.215	20.732 ± 1.860	—	—	0.129 ± 0.004	0.133 ± 0.004	—	—
Crude extract (4.299)	2.665 ± 0.027* (1.42)	3.812 ± 0.378* (1.99)	Inhibition	Inhibition	4.495 ± 0.133* (3.04)	11.274 ± 0.504* (1.84)	Inhibition	Inhibition	0.086 ± 0.003* (1.5)	0.118 ± 0.003 (1.13)	Inhibition	No effect
Apigenin (2.345)	6.621 ± 0.064* (0.57)	9.172 ± 0.098* (0.83)	Induction	Induction	12.059 ± 0.319* (1.13)	18.027 ± 0.538 (1.15)	Inhibition	No effect	0.114 ± 0.004 (1.13)	0.122 ± 0.007 (1.09)	No effect	No effect
Camphor (1.724)	4.178 ± 0.046* (0.90)	7.539 ± 0.104 (1.01)	Induction	No effect	8.331 ± 0.282* (1.64)	14.576 ± 0.522* (1.42)	Inhibition	Inhibition	0.102 ± 0.006* (1.26)	0.096 ± 0.002* (1.4)	Inhibition	Inhibition
Cypermethrin (0.0016)	2.591 ± 0.029* (1.46)	3.183 ± 0.306* (2.39)	Inhibition	Inhibition	4.347 ± 0.321* (3.15)	9.628 ± 0.438* (2.15)	Inhibition	Inhibition	0.079 ± 0.004* (1.63)	0.104 ± 0.004* (1.28)	Inhibition	Inhibition
	$F = 1254.203$ df = 4, 55 $P < 0.001$	$F = 131.226$ df = 4, 55 $P < 0.001$			$F = 261.492$ df = 4, 55 $P < 0.001$	$F = 23.734$ df = 4, 55 $P < 0.001$			$F = 24.123$ df = 4, 55 $P < 0.001$	$F = 12.035$ df = 4, 55 $P < 0.001$		

Note: CF is the correlation factor (enzyme activity of control treated group/enzyme activity of treatment treated group).
*Statistically significant differences compared to the negative control group (Dunnett's test $P < 0.05$).
[†] Acetylcholinesterase activity ± SE (nM acetylcholine hydrolyzed/min/mg).
[‡] Carboxylesterase activity ± SE (nM *p*-nitrophenol/min/mg).
[§] Glutathione-*S*-transferase ± SE (1-chloro-2,4-dinitrobenzene conjugated product/min/mg).

where the degree of freedom of the denominator = 55) and 48 h ($F = 23.734$, df = 4, 55 $P < 0.001$, where the degree of freedom of the denominator = 55) as determined by the correlation factor at 24 h of 3.04, 1.13, 1.64 and 3.15 respectively. Apigenin did not affect the CE activity at 48 h.

3.5.3 *Glutathione-S-transferase (GST)*

Most of the treatments, including crude extract, camphor and cypermethrin reduced the GST activity, whereas apigenin treatment did not significantly decrease this enzyme activity at 24 h ($F = 24.123$, df = 4, 55 $P < 0.001$, where the degree of freedom of the denominator = 55) and 48 h ($F = 12.035$, df = 4, 55 $P < 0.001$, where the degree of freedom of the denominator = 55).

4 DISCUSSION

Spodoptera frugiperda (J.E. Smith) is a highly invasive polyphagous pest. It has infested and seriously damaged a variety of plants, causing economic losses.[41] To control this pest, we are concerned about biological traits, such as a short life cycle, high fecundity, and high resistance to a range of insecticides. Therefore, the aim of this study was to investigate the potential use of *Combretum trifoliatum* extracts for the development of an insect pest control strategy by assessing the toxicity of extract throughout the entire life-cycle of *S. frugiperda*. In particular, we investigated ovicidal toxicity, larvicidal toxicity, toxicity to biological parameters, and enzymatic activity.

Plants produce a variety of secondary metabolites as herbivore defense mechanisms. The composition of plant secondary metabolites is complex and different in each plant's organ and tissue.[42] *Combretum trifoliatum* is a member of the family Combretaceae, a large family of herbs, shrubs and trees. The members in this family have been used as medicinal plants.[43] Several secondary metabolites are bioactive compounds with important roles and have been evaluated for antibacterial, antifungal, antiparasitic, and molluscicidal activities as well as many medicinal activities.[44–47] A previous study on other Combretaceae plants has shown that these plants can be used as alternative medicines and inhibit the survival of protozoan parasites.[48] Moreover, the crude leaf extracts from *Terminalia catappa* showed strong repellent properties against stored grain pests[27] and the study from Yuan *et al.*[49] found tannic acid isolated from Combretaceae plants also significantly reduced nutritional parameters, growth rate, and inhibited the AChE activity of *Hyphantria cunea* (Drury) larvae. The study from Feng *et al.*[32] reported the toxicity of *Q. indica* L. had an insecticidal activity against Lepidopteran, *S. exigua* in China. Meanwhile, the insecticidal effects of *Combretum trifoliatum* have not been reported.

According to the results of our phytochemical analyses, ethanolic extracts of the *Combretum trifoliatum* leaves and twigs contain the flavonoids compound apigenin and the terpenoids compound camphor. The compounds detected within the crude leaf extracts of *Combretum erythrophyllum* belong to many classes such as alkaloids. The most commonly isolated alkaloids include quinine, morphine, caffeine, strychnine, and cinchonine. Moreover, glycosides and phenolic compounds including tannins, flavonoids, and combretastatin were present.[50–52] Miaffo *et al.*[53] showed that the extract of *Combretum molle* twigs contains chemical compounds such as flavonoids, saponins, terpenoid, and tannins. Analysis of leaves and stem bark extract of *Combretum leprosum* Mart. showed the presence of flavonoids, steroids, tannins, phenols, saponins, and triterpenes.[54] Our results and several studies therefore indicate that various classes of compounds are present in the *Combretum* species, including flavonoids and terpenoids.

Insect control targets the larval stage because newly hatched larvae feed on host plants. It needs high energy, protein, and amino acids from plants for development and reproduction, it greedily feeds on host plant and causes significant plant damage.[55,56] Cannibalism in this species is related to higher population density, temperature, or lower plant nutrition. Interestingly, some researchers reared 40 *S. frugiperda* larvae in a box with an artificial diet, and they found that the high nutritional value of artificial diets reduced larval cannibalism.[57] To evaluate the contact toxicity in our research, we found that the LD_{50} for larvae treated with isolated extracts were lower than LD_{50} of crude extract. There are several studies indicating that phytochemicals from *O. canum* (Lamiaceae), *O. sanctum* (Lamiaceae), *Rhinacanthus nasutus* (Acanthaceae), *Abrus precatorius* (Fabaceae), *Murraya koenigii* (Rutaceae), *Curculigo orchioides* (Hypoxidaceae), *Evolulus alsinoides* (Convolvulaceae), *Phyllanthus debilis* (Phyllanthaceae), *Swertia corymbosa* (Gentianaceae), *Zanthoxylum limonella* (Rutaceae), *Acorus calamus* (Acoraceae), *Piper retrofractum* (Piperaceae), *Hymenoxys robusta* (Asteraceae) and *Alpinia galanga* (Zingiberaceae) had bioefficacy in protecting agricultural products from other *Spodoptera* species.[20–22,58,59]

The effective LD_{50} value of camphor, an isolated extract, caused a higher mortality than the crude extract. Similarly, many studies reported that the pure extract, α-thujone from *Hyptis marrubioides* Epling, linalool and 1,8-cineole from *Ocimum basilicum* L. had more toxic effects on *S. frugiperda* larvae.[60,61] The insecticidal efficacy of camphor has been reported in various studies against stored-product pests, including the pure camphor compound demonstrating contact and fumigant toxicity against *Sitophilus oryzae* L. and *Rhyzopertha dominica* (F.) after 24 h of exposure at 0.1 µL/720 mL.[62] D-Camphor extracted from *Cinnamomum camphora* L. also exhibited contact toxicity against *Tribolium castaneum* and *Lasioderma serricorne* (F.) with LD_{50} value of 13.44 µg/adult and > 50.0 µg/adult, respectively.[63] Another report demonstrated that fumigation of camphor showed toxicity on *Solenopsis invicta* minor and major workers that exhibited decreased feeding rate when fumigated at the LC_{50} value of 1.91 and 5.59 µg/mL, respectively.[64]

However, side effects of botanical insecticides still occurred and caused sublethal effects, such as physiological and biological effects, which include effects on development, adult longevity and fecundity.[8,65] The results in Table 2 showed that the duration of larval stage and duration of pupal stage were significantly increased in the groups of 10 and 20 µg/larva, while pupal weight showed no significant difference. Similarly, the groups exposed to *Combretum trifoliatum* extract showed that percentage of pupation, and adult emergence significantly decreased. Our results are similar to those of Silva-Aguayo *et al.*[66] and Cruz *et al.*[67] who studied the biological parameters of *S. frugiperda* exposed to *Peumus boldus* Molina or *Piper hispidinervum* and *Syzygium aromaticum* L. extracts. Their results showed sublethal effects such as an increase in the duration of larval stage and duration of pupal stage. In the same way, their extracts also showed a decrease in the percentage of pupation especially reduced adult emergence. Our findings indicated that some of the larvae survived after 48 h of exposure, which could be explained by the xenobiotic molecule being eliminated by a detoxifying mechanism.[68] Furthermore, each extract has a distinct effectiveness. The chemical

structure with higher lipophilicity can penetrate easily into the insect's integument, and the extract has the potential to affect mortality and biological parameters. Because most botanical compounds degrade easily, environmental degradation of each extract also affects efficiency.[35]

Topical application of *Combretum trifoliatum* ethanolic extract exhibited toxic effects on *S. frugiperda* from the second-instar larvae to the adult stage. In this study an increase in mortality associated with the increase in extract concentration was observed, given that the effects of the extract rely on both dose and exposure time. In our study, the topical applications were applied using an Eppendorf™ micropipette, instead of a Hamilton™ microsyringe. Although, the microsyringe presents greater precision in the size of the drop, the micropipette is one of the principal devices for transferring liquid in the topical application method to the insect, and it can deliver a low-viscosity solution.[69]

Insecticidal activities have been reported in other Combretaceae plants, as antifeedant for *Locusta migratoria*.[70] Among the biological parameters, average numbers of eggs in the group treated with *Combretum trifoliatum* extract continue to decrease. Similar results were reported by Usha Rani *et al*.,[27] as the leaf extract of *Terminalia catappa* was shown to efficiently control the stored product pests, *Sitophilus oryzae*, *Rhyzopertha dominica*, *Tribolium castaneum* and *Callosobruchus chinensis* L. This crude extract can reduce the ovipositional preferences. Having different effects on insects, such as reduced progeny production, feeding deterrent activity, and repellance. Moreover, *Q. indica* ethanolic extract is shown to be toxic on contact to the *S. exigua*. The percentage of mortality shown is 10%, 16.67%, and 16.67% in 24, 48, and 72 h. after *S. exigua* third-instar larvae received 10 mg/mL extract.[32] These experimental results enhanced our knowledge of the efficiency of *Combretum* species extracts in agricultural insect control.

Several studies reported the efficacy of plant extracts for controlling *Spodoptera* insects, especially in the larvae stage, but few studies have looked at an earlier stage, the egg. After adult mating success, the number of eggs laid reached 2000 eggs during female life cycle, thus it is necessary to investigate the *S. frugiperda* eggs.[71,72] Baltaci *et al*.[73] reported that the susceptibility of an insect egg to a toxic material varied according to age. Younger eggs (1-day old eggs) were more susceptible than older eggs (3 to 5 days old eggs). Therefore, egg masses aged 1–2 days were used in our study.

Our results indicated that *Combretum trifoliatum* crude extract can cause ovicidal toxicity with reduced egg hatchability from 82.3% to 59.2% after exposure to concentrations of 1.25 to 20 µg/egg of *Combretum trifoliatum* crude extract. Our findings are similar to other active ingredients that showed larvicidal hatchability of *Spodoptera litura* (F.) and *S. exigua* eggs and to some stored-product pests, *Euvrilletta peltata* (Harris) and *Lyctus brunneus* (Stephens)[73,74] after dipped with *Pogostemon cablin* (Blanco) Benth or pogostone.[75] However, *Combretum trifoliatum* crude extract (20 µg/egg) showed 59.2% hatching which was less effective than *Zanthoxylum armatum* extract on *S. litura*.[75,76] It is possible that the multilayer structure of eggs, covered by a mucous envelope and several layers of chorion is efficient in protecting eggs not only from xenobiotics but also parasitoids.[77]

Insecticides are frequently used to manage pests, however this strategy results in the development of pest resistance and has harmful effects on non-target organisms. The synthetic pyrethroids, including cypermethrin, were first isolated from *Chrysanthemum cinerariaefolium*. It is known to influence both the peripheral and central nervous systems, causing hyperactivity and paralysis by activating sodium channels in neuronal membranes.[78,79] AChE is a crucial enzyme that regulates the neurotransmitter by hydrolyzing acetylcholine (ACh) to choline and acetate at cholinergic synapses.[80] Our results in Table 4 indicated that an ethanolic crude extract of *Combretum trifoliatum* can inhibit AChE activity, with exception of the groups treated with apigenin or camphor alone, which induced the AChE activity. It is possible that the differences in AChE activity for each compound are caused by insect resistance, different binding mechanisms, and the AChE structure in each insect species.[81,82] The results are similar to previous studies that reported the inhibition of AChE activity by plant extracts including *Gutierrezia microcephala* A. Gray against *S. frugiperda*,[83] *Artemisia nakaii* Pamp. against *S. litura*,[84] and *Piper ribesioides* against *S. exigua*.[85]

Detoxification mechanisms are one of the most common mechanisms by which insects become insecticide resistant in the *Spodoptera* genus.[68] After xenobiotics (e.g., plant secondary metabolites and insecticides) penetrate through the cuticle to the cell, it is functionalized by the detoxification enzymes. Phase I enzymes (P450 and CE) play an important role to increase xenobiotic polarity by catalyzing oxidation reactions, hydrolysis and reduction. Then, these xenobiotics are conjugated with endogenous molecules to small hydrophilic molecules by a group of transferases which are phase II enzymes (e.g., GST and UDP-glycosyltransferase) and finally, during phase III, the less toxic metabolites excreted out of the cell by ATP-binding cassette transporters. Our findings showed that ethanolic crude extract and most of its isolated compounds inhibited CE and GST activity in *S. frugiperda* (Table 4). Noticeably, both the crude extract and the isolated compounds, including camphor inhibited CE activity more significantly and for a longer period. Conversely, only camphor inhibited GST activity for up to 48 h, but crude extract inhibited more efficiently. According to several studies, plant defenses generally rely on a combination of compounds rather than a single compound. Therefore, the synergistic effects of compound mixtures or plant essential oils and their compounds could be employed to manage insecticide resistance by inhibiting insect detoxification enzymes.[22,78]

5 CONCLUSION

Our results have shown that compounds isolated from *Combretum trifoliatum* as well as the ethanolic crude extract have an insecticidal effect on *S. frugiperda*. The crude extract resulted in a reduction in percentage of pupation and adult emergence. It also acts by decreasing egg hatching and by inhibiting certain enzyme activities, notably AChE, CE and GST. This crude extract could be developed as an alternative approach to synthetic insecticides and used in IPM programs.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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