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Control of *Spodoptera littoralis* (Boisd.) Larvae Using Bioactive Chromone Derivative Isolated from *Ruta angustifolia* (Pers.)

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ABSTRACT

The present work is the first isolation of a chromone derivative, 2-(2-methoxyphenylethyl)-4H-chromen-4-one from the acetonic extract of *Ruta angustifolia* leaves using thin-layer chromatography and then identified by infrared and mass spectrometry. Results showed that 2-(2-methoxyphenylethyl)-4H-chromen-4-one possessed larvicidal activity against the fourth instar larvae of *S. littoralis*, which fed on treated leaves under laboratory conditions. LC_{50} values indicated abnormalities in the epithelial cells, muscles and microvilli using a transmission electron microscope in treated compared to untreated larvae. Moreover, a significant reduction was recorded in both the chitinase enzyme and total lipid content compared to the control. Overall the findings of the study suggest that the use of 2-(2-methoxyphenylethyl)-4H-chromen-4-one can act as a new insecticidal agent for controlling the cotton leaf worm (*S. littoralis*).

Key words : *Spodoptera littoralis*, *Ruta angustifolia*, larvicidal activity, 2-(2-methoxyphenylethyl)-4H-chromen-4-one, enzymes

INTRODUCTION

One of the most destructive agricultural pests in tropical and sub-tropical regions is the cotton leafworm, *Spodoptera littoralis* (Boisduval) (Lepidoptera : Noctuidae), also known as the African cotton leafworm, tobacco cutworm, or Egyptian cotton leaf worm. This species is extremely polyphagous, feeding on many host plants covering more than 40 families, containing over 87 economically important plant species that undermine the quality and quantity of crop yields. The European and Mediterranean Plant Protection Organization (EPPO) has registered *S. littoralis* as an A2 quarantine pest (pests are locally present in the EPPO region) since the pest can even spread to the temperate zones through the international transport of vegetables and ornamental plants (EPPO/OEPP, 2015). The majority of synthetic pesticides currently marketed to control this species have adverse effects on human health and the environment. Such products can also destroy natural enemies, enabling an exponential amplification of pest populations and building up the resistance phenomenon (Naqqash *et al.,* 2016; Benelli *et al.,* 2019). There is an immediate need for alternative compounds with different modes of action to control the target pest to solve certain phenomena. This understanding has recently generated worldwide interest in developing safer insecticides and alternative methods, including the review of plant derivatives against insect species of agricultural significance. Considerable attention is paid to natural products to avoid the disadvantages of insecticide use, as they would be non-hazardous, easy to use, specific in their action, and safer for the ecosystem and could reduce insect prohibition. Researchers looked at the use of plant extracts as toxicants, repellents, synergists, growth regulators, and antifeedants for cotton leafworm, *S. littoralis,* and such alternatives may differ depending on the physiology of the pest and the mechanisms of resistance development. The Rutaceae family includes several aromatic plants, primarily in tropical regions, and is now cultivated in many parts of the world. It is often considered indigenous in South Europe and North Africa (Chen *et al.,* 2021). *Ruta angustifolia* Pers. (Sapindales : Rutaceae) a traditional medicine for liver disease and jaundice, containing coumarin, flavonoid and alkaloid compounds, represents this family (Wahyuni *et al.,* 2014). It is also used in cancer treatment by the Chinese community in Malaysia and Singapore (Jaime, 2018). Chromone derivatives are naturally occurring compounds ubiquitous within the plant kingdom and

therefore found in representative amounts in regular human food. These phytochemicals possess various biological activities, such as anti-inflammatory, antimicrobial, antiviral, antitumor and anticancer activities, mainly due to their well-recognized antioxidant properties (Amen *et al.,* 2021). The current study is the first attempt to isolate a chromone derivative, 2-(2-methoxyphenylethyl)-4H-chromen-4-one from the acetonic extract of *R. angustifolia* and to evaluate its insecticidal activity against *S. littoralis* larvae to suppress the damage via ultrastructure and biochemical studies.

MATERIALS AND METHODS

Seeds of *R. angustifolia* (Pers.) were obtained from the Department of Horticulture, Faculty of Agriculture, Zagazig University, Egypt; then, they were planted in a private property (350 m^2) in local gardens at Abu Hammad, Sharqia Governorate, Egypt. Leaves of *R. angustifolia* (Pers.) were collected at the flowering stage (blossoming periods) and kept in a well-dried place for further extraction.

The freshly collected leaves were washed with distilled water and left in the shade to dry for 10 days at normal room temperature. After drying, the leaves were pounded into smaller particles using a mortar and pestle and then blended to powder. The powder was kept in sealed containers and kept at room temperature until required. The chemicals and solvents used in this research were purchased from Al-Gomhoria Co., Sharqia Governorate, Egypt.

R. angustifolia leaves were extracted at room temperature using acetone as the solvent. A 1000 g sample of powder was soaked in 2000 ml of acetone for 72 h. The combined extract was filtered using a muslin cloth and Whitman No. 1 filter paper on the Buchner funnel. The extract was concentrated by a rotary evaporator (Model 349/2, Corning Limited). The crude was weighed and kept in a deep freezer until use.

Acetonic extract of *R. angustifolia* was separated using preparative thin-layer chromatography (TLC) (20 × 20 cm). Acetonic extract of *R. angustifolia* yielded eight main bands on TLC. The isolated compound of *R. angustifolia* 2-(2-methoxyphenylethyl)-4H-chromen-4-one was subjected to infrared spectrometric (IR) analysis and mass spectrometric (MS) analysis to determine the chemical structure of the isolated compound.

A laboratory strain of cotton leafworm, *S. littoralis*, from egg masses reared for more than 30 generations away from any insecticidal contamination at the division of Cotton Leafworm Department, Plant Protection Research Institute, Sharqia Branch, Sharqia Governorate, Egypt, under constant conditions of 27±2°C and 65±5% R. H., was obtained in the present investigation.

The toxicity experiment was performed using the leaf dip technique to estimate the LC_{50} of 2-(2-methoxyphenylethyl)-4H-chromen-4-one. Four tested concentrations (2, 3, 5 and 9%) were used. Newly molted fourth instar larvae of *S. littoralis* were starved for 4 h before the treatment to clear their digestive tract and guarantee fast ingestion of treated leaves. Three cm diameter disks of fresh castor bean leaves *Ricinus communis* L. were dipped for 10 seconds in the tested concentrations and acetone only (as a negative control). The treated leaf disks were left to dry and then offered to the starved larvae as one disk for one larva (10 disks/10 larvae). Ten replicates were subjected to each treatment and control. Larvae were left feeding for 48 h and then supplied with untreated leaves. The mortality rates were recorded after 72 h of treatments and corrected to the control (acetone only) value to avoid any effect caused by the solvent.

Ultrastructure sections were made for the cuticle and muscles for the fourth instar larvae after three days of treating with LC_{50} of 2-(2-methoxyphenylethyl)-4H-chromen-4-one compared to controls treated with acetone. Stained sections were examined with a JEOL 1010 Transmission Electron Microscope at the Regional Center for Mycology and Biotechnology, Al-Azhar University, Egypt.

After 72 h of treatment with LC_{50} of 2-(2-methoxyphenylethyl)-4H-chromen-4-one, healthy individuals of the laboratory fourth instar larvae of *S. littoralis* were starved for 4 h, as well as untreated ones (using acetone only). All groups were homogenized in distilled water (50 mg/1 mg) using chilled glass Teflon tissue. The homogenate samples were centrifuged to remove the deposits. The deposits were used to determine total lipids. Supernatants were referred to as enzyme extracts. All the samples were transferred to

cleaned screw-capped tubes and stored frozen at -20°C until use for the biochemical assays. Three replicates were used for each assay. The activities of the chitinase enzyme and the content of total lipids were determined.

Chitinase (EC 3.2.1.14) activity was determined using 3,5-dinitrosalicylic acid reagent to determine the free aldehyde groups of hexoamines liberated upon chitin digestion. The total lipids were estimated using kits from Diamond Diagnostics.

The corrected mortality percentages were calculated using the computed percentage of mortalities versus corresponding concentrations (Probit analysis) to estimate LC values of 2-(2-methoxyphenylethyl)-4H-chromen-4-one. The data of biochemical and biological parameters were statistically analyzed for significant differences between the control and treated groups using the Student's T-test. All values were expressed as means±standard error.

RESULTS AND DISCUSSION

The compound was isolated from the crude acetonic extract and identified using an IR and MS analyses with the help of the mass bank of North America. Identifications were made by comparing spectra in the Wiley 275.1 and NIST 98 (National Institute of Standard and Technology, Gaithersburg, MD, USA) libraries (Fig. 1). The tested compound occurred as a colourless resin and showed Rf. 0.387 on TLC using the solvent system chloroform or petroleum ether in the ratio 3 : 2. The IR spectrum displayed a broad transmission peak at 1730.81/cm stretching for protons linked

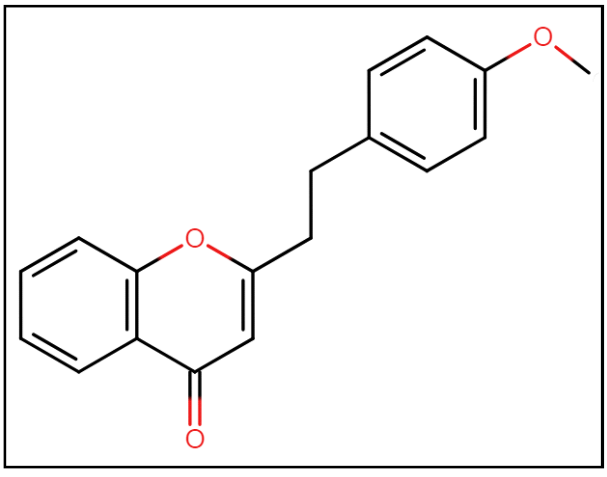

Fig. 1. Structural formula of 2-(2-methoxyphenylethyl)-4H-chromen-4-one.

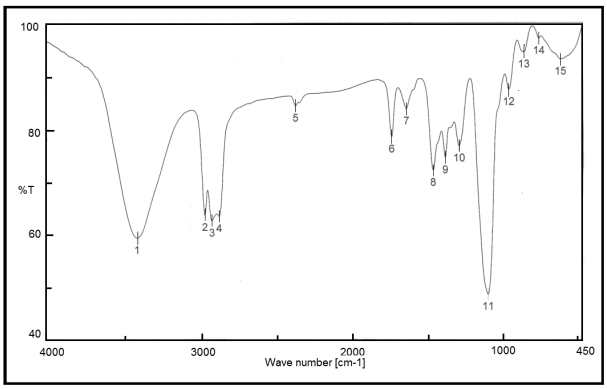

Fig. 2. Infrared of 2-(2-methoxyphenylethyl)-4H-chromen-4-one.

to sp3 carbons (H–C). Additionally, showed absorption at 2924.52/cm, indicating the aliphatic structure (C–C) and weak at 1632.45/cm showed (C=C) double pound (Fig. 2). The MS spectrum (Fig. 3) showed molecular ion signature in the range of 50–630 *m/z*. The mass spectrum showed parent ion at 280 *m/z* and other important fragments at 279 *m/z* (M-1), indicating an isotope peak occurring when losing the OH group from the chromone moiety at the peak of 262 *m/z*. The methoxyphenethyl residue peak at 149 *m/z* was the base fragment related to146 *m/z* indicating the chromone moiety. The peak at 121 *m/z* was due to the neutral loss of C=C from chromone residue. Confirmation of structure and mass similarity were compared to the library using mass bank-data base and found typical for the structure of 2-(2-methoxyphenylethyl)-4H-chromen-4-one. The LC_{50} values were 8.01% for the fourth instar larvae of *S. littoralis* fed on treated leaves with 2-(2-methoxyphenylethyl)-4H-chromen-4-one (Table 1).

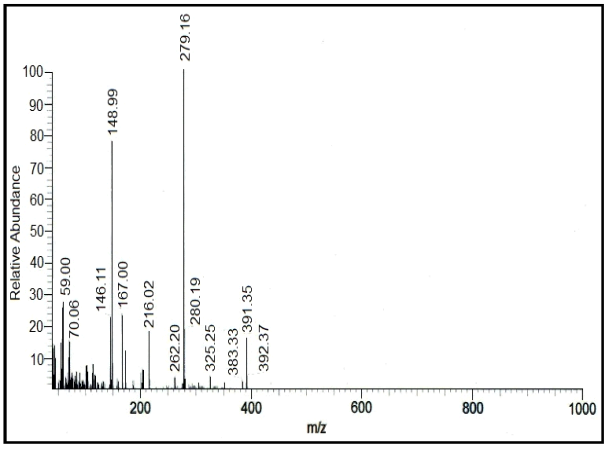

Fig. 3. Mass spectrum of 2-(2-methoxyphenylethyl)-4H-chromen-4-one.

Table 1. Susceptibility of the fourth *S. littoralis* larval instar to the isolated compound from *R. angustifolia* acetonic extract

Treatment	LC_{50}% (LCL-UCL)	LC_{90}% (LCL-UCL)	Slope
[2-[2-(4-methoxyphenyl) ethyl]chromen-4-one]	8.01 (4.59-56.7)	276.6 (44.9-488.67)	0.83±0.26

LCL–Lower confidence limit and UCL–Upper confidence limit. LC_{50}–Lethal concentration that kills 50% of insects. LC_{90}–Lethal concentration that kills 90% of insects.

The compound 2-(2-methoxyphenylethyl)-4H-chromen-4-one ($C_{18}H_{16}O_3$) isolated from *R. angustifolia* was identified by an infrared spectrophotometer and a mass spectrometer. So far, this was the first time that 2-(2-methoxyphenylethyl)-4H-chromen-4-one had been isolated from the acetonic extract of *R. angustifolia* leaves. Meanwhile, this compound had been identified and isolated from other plants with toxic effects, but not from *R. angustifolia*. For instance, three new chromones including 2-phenoxychromone, 6,8-di-C-methylcapillarisin were isolated from methanolic extract of *Pimeneta dioica* leaves (Doyle *et al.,* 2018).

Based on the LC_{50} and LC_{90} values, 2-(2-methoxyphenylethyl)-4H-chromen-4-one had a toxicological effect against the fourth instar larvae of *S. littoralis* after 72 h of treatment. The toxicity of this compound might be attributed to its different activities. Its unique structures and varied pharmacological activities might provide important new leads for the discovery of drugs with novel mechanisms of action. Potential therapeutic indications were cytotoxic, ostrogenic and anti-ostrogenic effects (Doyle *et al.,* 2018). Moreover, the presence of a phenyl substituent at C-2 in the chromone skeleton increased the insect antifeedants and insecticidal activity against the peach potato aphid, *Myzus persicae* (Sulzer) (Stompor *et al.,* 2015).

TEM examinations showed many pathological changes in midgut epithelial cells (columnar, calceiform and regenerative), microvilli and muscles of the *S. littoralis* fourth instar larvae that were treated with LC_{50} of the isolated chromone compound, 2-(2-methoxyphenylethyl)-4H-chromen-4-one, compared to the positive control (acetone).

The typical control midgut lining consisted of a single layer of epithelial cells resting on the basement membrane. Beneath this, the external layer of longitudinal muscle and the internal layer of circular fibers. The epithelium comprised three distinct cell types : columnar cells, the regenerative cells and calceiform cells between them. The latter cells had a distal opening internal groove with a clear cytoplasm and basal nucleus. The interstitial or regenerative cells were generally very small and triangular with a basophilic cytoplasm and a spherical, central nucleus (Fig. 4A). The midgut high columnar cells were the largest cell groups and a more common nucleus with dispersed chromatin (Fig. 5A). Deformations caused by the application of 2-(2-methoxyphenylethyl)-4H-chromen-4-one on the *S. littoralis* fourth instar larvae included fragmentation of the muscle fibers under the columnar cells, separation of the epithelial cell from the basement membrane and accumulation of the nucleus chromatin, vacuolization in the cytoplasm of calceiform cells (Fig. 4B), the appearance of aggregations of secretory electron-dense granules (Fig. 5B) and high vacuolization inside the epithelium due to cytoplasmic and micro-organelles degradation (Fig. 5C).

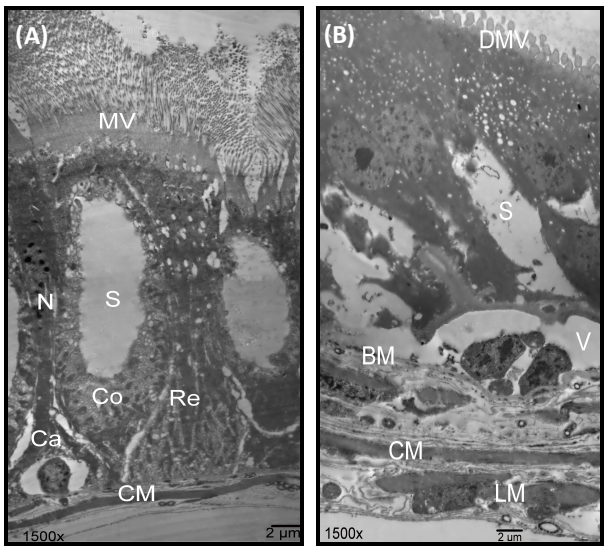

Fig. 4. Electron micrograph illustrating the transverse section in midgut epithelial cells of *S. littoralis* fourth instar larvae [1500X]. (A) +ve control of the fourth larvae of *S. littoralis*. (B) Fourth larvae of *S. littoralis* treated with 2-(2-methoxyphenylethyl)-4H-chromen-4-one [BM–Basement membrane, Ca–Calceiform cell, CM–Circular muscle, Co–Columnar cell, MV–Microvilli, DMV–Disintegrated microvilli, DN–Disintegrated nucleus, LM–Longitudinal muscle, Re–Regenerative cell, N–Nucleus, S–Secretions and V–Vacuoles].

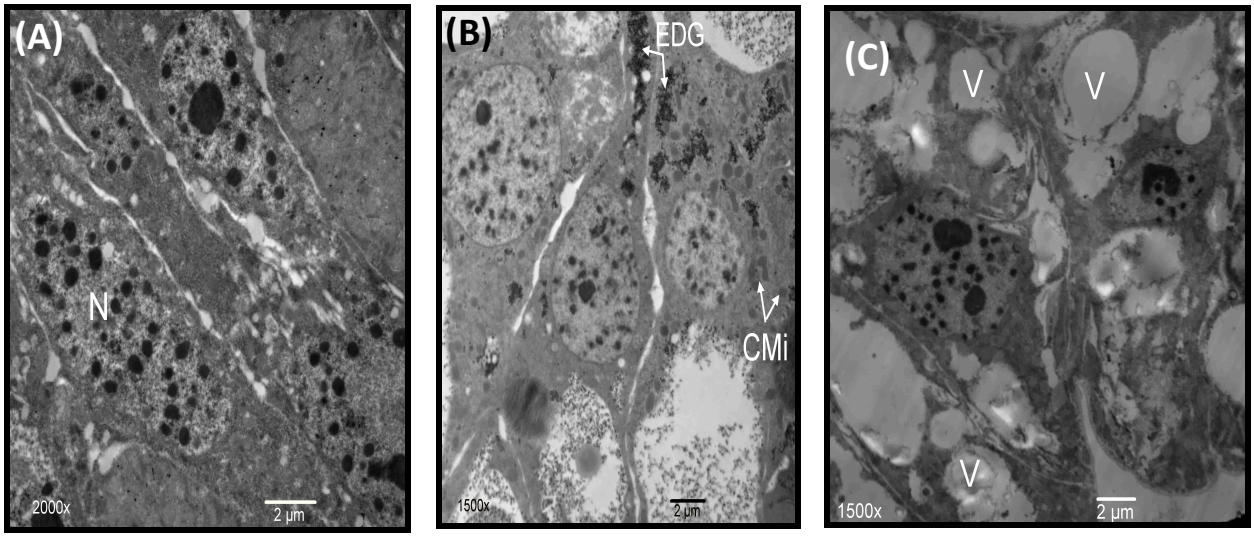

Fig. 5. Electron micrograph illustrating transverse section in the epithelial cells of *S. littoralis* fourth instar larvae [1500X]. (A) Control +ve of the fourth larvae of *S. littoralis*. (B & C) Fourth larvae of *S. littoralis* treated with 2-(2-methoxyphenylethyl)-4H-chromen-4-one [CMi–Condensed mitochondria, N–Nucleus, EDG–Electron-dense granules and V–Vacuoles].

TEM examinations of the apical plasma membrane of the epithelial cells showed that the digestive cells were heavily folded. Usually, these cells, regular in microvilli, had grooved edges or microvilosities, which were responsible for increasing the surface area (Fig. 4A). Many deformations due to the most effective extract 2-(2-methoxyphenylethyl)-4H-chromen-4-one revealed cytoplasmic fragmentation in microvilli (Fig. 5B).

The transverse section in muscle cells clarified that in the normal muscle of untreated larvae, a typically greater number of mitochondria were present, and these tend to be distributed in association with the contractile filaments (Fig. 6A). Treatment with 2-(2-methoxyphenylethyl)-4H-chromen-4-one resulted in strong condensation of mitochondria with different

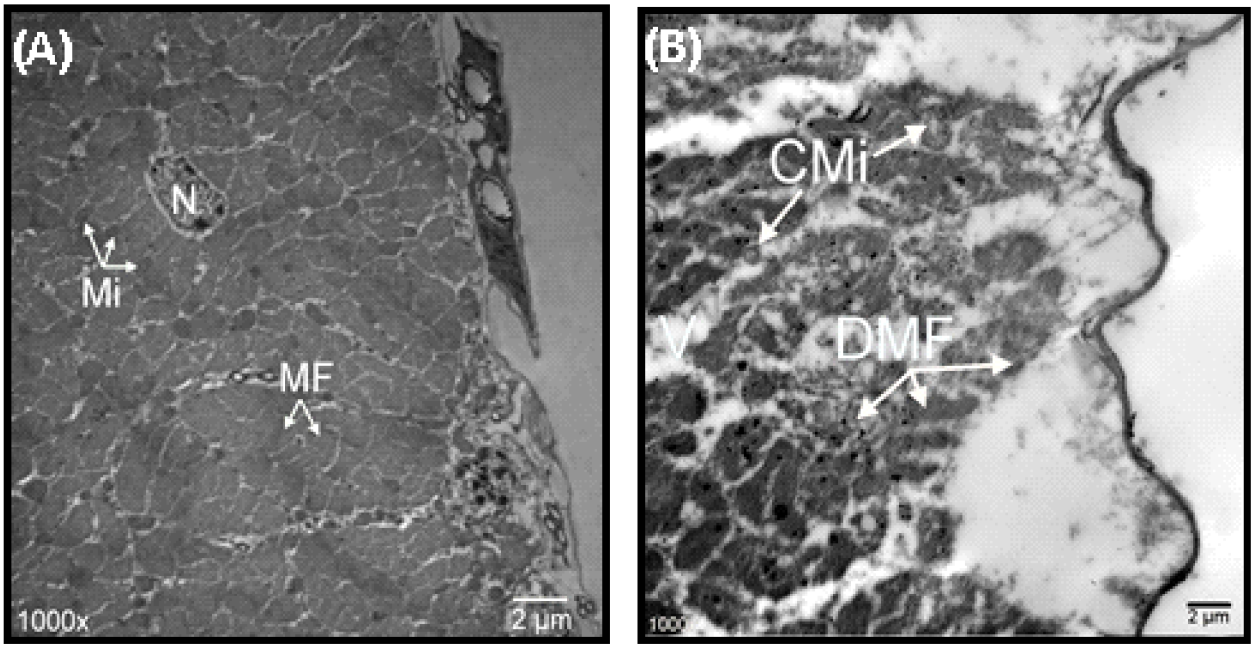

Fig. 6. Electron micrograph illustrating the transverse section in longitudinal muscles of *S. littoralis* fourth instar larvae [1500X]. (A) +ve control of the fourth larvae of *S. littoralis*. (B) Treated with 2-(2-methoxyphenylethyl)-4H-chromen-4-one [CMi–Condensed mitochondria, DMF–Degenerated muscle fiber, Mi–Mitochondria, MF–Muscle fiber, N–Nucleus and V–Vacuoles].

arrangements, while the degradation of myofilaments became obvious (Fig. 6B).

Ultrastructure studies were done using TEM and confirmed the role of the newly isolated compound 2-(2-methoxyphenylethyl)-4H-chromen-4-one in damaging some internal structures i. e. cuticle, muscles, epithelial cells, microvilli and peritrophic membrane. This tested compound was characterized by a mode of action on the cuticle similar to juvenile hormone compounds. Additionally, their activities resembled those of insect growth regulator compounds such as disturbance and shrinking of microfilaments. The latter were grouped into masses and separated by vacuoles and fissures between running chitin microfilaments, so that the cuticle lost its elasticity.

The toxicity of this extracted compound was due to its non-polar and long-chain carbon atoms. This compound possessed permeability through the non-polar cell membrane; thus, they may accumulate within the cells and cause metabolic disturbances similar to the long-chain fatty acids (Suryani *et al.*, 2020). Khedr *et al.* (2015) also evaluated the ultrastructure changes in the fourth larval instar cuticle of *S .littoralis*, such as the cuticle being edges and separation of the endocuticle from the epicuticle, after applying the acetonic extract of *Ipomea carnea* compared to a traditional insecticide (chlorpyrifos). The microvilli lined the inner edge of the epithelial cells and enhanced the absorption rate. These microvilli were continuously renewed from regenerative cells; the most obvious ultra-structural change noticed in the epithelium of the midgut was the disruption of the microvilli, thus the appearance of vacuolization; this was consistent with the observations of Shaurub *et al.* (2020), who noticed histopathological changes in *S. littoralis* midgut larvae treated with garlic and lemon essential oils as vacuolization, necrosis, and destruction of epithelial cells and their boundaries. Several authors observed similar histological alterations in many insects. Ponsankar *et al.* (2018) revealed damages in the midgut epithelium of *S. litura* larvae treated with *Citrullus colocynthis* L.; midgut columnar cell vacuolization, microvilli damages, undulations along the midgut producing flattening of the cells, and growth in micro villosities. Suryani *et al.* (2020) noticed alternation in *S. littoralis*, such as degeneration in the peritrophic matrix and the epithelial lining of the midgut.

The contraction of the muscle depended on ATP generated by the mitochondria. The most prominent role of mitochondria was to produce the energy currency for mechanical work. The intermediate association of mitochondria with the myofilaments was to diffuse chemical energy from the mitochondria to the muscle fibers (Shaurub *et al.,* 2020). In the current study, the extracted components application of larvae caused alterations in myofilaments striation, which may be due to mitochondrial deformations, leading to blocking the energy supply to the muscle needed for mechanical activities. Many types of research were consistent with these results when extracted components were applied to *S. littoralis* larvae. The histological damage in muscles of *S. littoralis* larvae caused by *Hyptis brevipes* of the methanol extract was shown by Sakr (2014).

Results in Table 2 show that the tested compound significantly reduced the chitinase activity (58.33±0.88) compared to the control that recorded 64.00±1.00 µg.N-acet./g.b.w./min. That 2-(2-methoxyphenylethyl)-4H-chromen-4-one had a significant decrease in total lipid content of 1404.00±72.52 µg/ml compared to the control (2825.33±93.32 µg/ml).

Table 2. Changes in chitinase enzyme and content of total lipids of the fourth instar larvae of *S. littoralis* treated with 2-[2-(4-methoxyphenyl)ethyl]chromen-4-one

Treatment	Chitinase (µg. N-acet./g.b.w./min)	Total lipid (µg/ml)
Control	64.00±1.00[a]	2825.33±93.32[a]
2-[2-(4-methoxyphenyl) ethyl]chromen-4-one	58.33±0.88[b]	1404.00±72.52[b]
P	0.0006	0.0002
T	4.25	12.02

Each datum represents the mean of three replicates.
Data expressed as mean±standard error (SE).
Means under each variety having different superscripts in the same row denote a significant difference ($P < 0.05$).

Biochemical considerations in creatures exposed to toxicants were considered a sensitive guide to monitoring disturbances inside the organism. Biochemical parameters were vital diagnostic tools to estimate the effects of stressors. Chitin was a component of the cell walls of fungi and exoskeleton elements of some animals (including worms and arthropods).

Chitinase is generally found in organisms that either need to reshape their chitin or dissolve and digest the chitin of insects. Data indicated that 2-[2-(4-methoxyphenyl)ethyl]chromen-4-one caused a decrease in chitinase activities as compared to the control. In this context, a marked decrease in chitinase enzyme activity was affected by the secondary metabolites (B-sitosterol I and stigmasterol II) of the petroleum ether extract of *Colocasia esculanta* (L.) leaves against the fourth instar larvae of *S. littoralis* relative to the control (Abaza and Gaber, 2017). Similarly, Sabry (2018) evaluated the efficacy of the *Taxodium distichum* (L.) ethanol extract fruits and the chitin synthesis inhibiting insecticide, lufenuron on the fourth instar of larvae *S. littoralis*. The reduction in some carbohydrases and chitinase could be attributed to *Taxodium distichum* ethanol extract.

The biological significances of lipids included storage of energy, metabolic demands, taking part in the constitution of different body parts, signalling and functioning as essential structural components of the cell membrane. The results showed a decrease in total lipid levels in larvae treated with 2-(2phenethyl chromone) 3methoxy compared to the control. This decrease in lipid contents may be due to the decline in lipid synthesizing capacity or may be due to an increase in the hydrolysis of lipids to combat the stress conditions (Toprak, 2020). Furthermore, these results were supported by El-lakwah (2018), who recorded a decrease in total lipid and total protein compared to the control of the second and fourth instar larvae of *S. littoralis* treated with *Ocimum sanctum* L. leaves extract.

CONCLUSION

The results demonstrated for the first time the isolate 2-(2-methoxyphenylethyl)-4H-chromen -4-one from the acetonic extract of *R. angustifolia*. Furthermore, this study established strong evidence of the larvicidal action of 2-(2-methoxyphenylethyl)-4H-chromen-4-one against *S. littoralis* larvae, one of the widespread and harmful lepidopteran. Thus, it offered the possible use of new promising natural insecticides in the area of pest control. However, further screening studies should be conducted on both *S. littoralis* and other lepidopteran pests as well as field application.

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