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Callistemon citrinus (Myrtaceae) methanolic leaf extract: a potent mosquitocidal agent for controlling dengue vector mosquito *Aedes aegypti* (Diptera: Culicidae)

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Abstract
Mosquitoes are the carriers of severe and well-known illnesses such as malaria, arboviral encephalitis, dengue, chikungunya and yellow fever, which cause significant morbidity and mortality in humans and domestic animals around the world. For many decades chemical insecticides are widely used to control mosquitoes. Mosquitoes have developed resistance against these chemical insecticides and also adversely affected the environment. There is an imperative demand to search novel eco-friendly substitutes which are more effective, safe and economical. Plant extracts with proven insecticidal properties are an alternate to these insecticides. The present study was aimed to investigate phytochemicals of the methanolic extract of *C. citrinus*. Bioassay test are carried out using the world health organisation standard method, for testing the efficacy of methanolic leaf extract of *C. citrinus* on *Ae. aegypti* at different stages of development viz I, II, III, IV instars and pupae and mortality was observed after 24 hrs exposure. Young larvae were found to be relatively more susceptible then the older ones. During the larvicidal bioassay with methanolic leaf extract of *C. citrinus* the fourth instar larvae of *Ae. aegypti* were monitored carefully for behavioural modifications. Behavioural observations of the methanolic leaf extract treated larvae revealed excitation and restlessness with persistent and aggressive anal biting behaviour indicating the probable effect of the extract on the neuromuscular system of larvae. Microscopic observations of the dead larvae showed shrunken internal membrane of anal papillae. The larvae of *Ae. aegypti* subjected to methanolic leaf extract treatment exhibited a variety of malformations during the course of their development. The current results were considered promising to proceed in studying the bioactive plants which represent an environmentally sound alternative for the synthetic insecticides.

Keywords: *C. citrinus*, leaf extract, *Aedes aegypti*, larvicidal, pupicidal, behavioural and morphological studies

1. Introduction

Aedes aegypti is a vector of dengue viral infection, dengue hemorrhagic fever dengue shock syndrome, chikungunya and zika virus (ZIKV).At present, no effective vaccine is available for these diseases; therefore, mosquito control is the only way of reducing the incidence of such diseases [1]. The control methods should aim at the weakest link of the life cycle of the mosquito, which is the larval stage. Larviciding is a successful way of reducing mosquito densities in their breeding places before they emerge into adults. During the immature stage, mosquitoes are relatively immobile; remaining more concentrated than they are in the adult stage [2]. Control of mosquito borne diseases is becoming increasingly difficult because of the increasing resistance of mosquitoes to insecticides [3]. Considerable skill was achieved in the control of mosquito borne diseases by utilization of synthetic insecticides and it has also provoked unwanted toxic effect to the environment and human health [4]. Thus, a crucial attention has to be paid for the development of novel, eco-friendly, environmentally safe, biodegradable and low cost natural insecticides [5].

During the last decade, various studies on natural plant products against mosquito vectors indicate them as possible alternatives to synthetic chemical insecticides [6]. Plants produce a broad range of bioactive chemical compounds [7] which are easily biodegradable, target specific, lower bioaccumulation and low or sometimes non-toxic to higher animals [8]. The phytochemicals derived from plant sources possess a complex of chemicals with unique biological activity.

The phytochemicals derived from plant resources can act as larvicidal, ovicidal, oviposition deterrence, growth and reproduction inhibitors, repellents, growth regulation, fecundity suppression, male sterility and smoke toxicity [9, 10]. Some of the plant leaves extracts are tested for their diverse insecticidal properties on the medically important mosquitoes [11-23].

As far as our literature survey is concerned that there was no information available on the larvicidal, behavioural and morphological effects of the leaf extract of the *C. citrinus*. The present study was therefore carried out to evaluate mosquitocidal properties of *C. citrinus* methanolic leaf extract against the vector mosquito, *Ae. aegypti*.

Callistemon citrinus (Linn.) (Myrtaceae) is a slow-growing ornamental shurb that grows to a height of around 10 meters and comprises over 30 species that has medicinal importance [24]. *C. citrinus* is a native to Australia, but is also widely distributed in Asian countries. It is commonly known as crimson bottle brush, red bottle brush or lemon bottle brush and in Tamil it is called as palasu tree because of its spiky inflorescence that resembles a bottle brush. The inflorescence is crimson in colour and cylindrical and flowers are borne in spring and summer. Leaves are lanceolate shaped broadly up to 7.5 cm long with prominent veins. The plant has been used by tribal communities of India for the treatment of gastrointestinal disorders, pain infectious diseases and a local community of lower Himalaya region used it for genitourinary, kidney infection, bleeding ulcer, arthritis, gout, cough, bronchitis, tuberculosis and insecticide effects [25]. Over the years, *C. citrinus* have been extensively analysed scientifically and reported to possess anti-cholinesterase activity [26], wound healing activity [4], hepatoprotective activity [27], cardioprotective activity [28], anti-inflammatory activity [29], antidiabetic activity, hypolipidemic activity, antioxidant activity, nematicidal, larvicidal, and pupicidal activites [30]. *C. citrinus* also used as weed control [31] and as bioindicators for environmental management [32]. The leaves were used to cure respiratory tract infections. The leaf of the plant is used as a tea substitute and it has a refreshing flavor too. The oil from the leaves of *C. citrinus* has been proved to have anti-nociceptive and anti-inflammatory effects in experimental animals [33]. Several flavonoids, saponins, quinones, steroids, terpenoids, tannins, phenolic compounds have been isolated from its leaves [34].

The aim of the present study is therefore to find out:

- Phytochemical analysis of methanolic leaf extract of *C. citrinus*,
- Estimate the toxicity of the methanolic leaf extract of *C. citrinus* on the larvae and pupae of *Ae. aegypti,*
- Impact of methanolic leaf extract of *C. citrinus* on the behaviour of the fourth instar larvae of *Ae. aegypti*,
- Evaluation of methanolic leaf extract of *C. citrinus* on the morphology of larvae and pupae of *Ae. aegypti*.

2. Materials and Methods

2.1 Colonization of *Aedes aegypti*

The eggs of *Ae. aegypti* were collected from National Institute for Communicable Disease (NICD), Mettupalayam, Coimbatore (Dt), Tamil Nadu, India without exposure to any insecticide. The eggs were then brought to the laboratory and transferred to enamel trays containing water and kept for larval hatching. They were hatched, reared and have been still maintained for many generations in the laboratory. The eggs and larvae obtained from this stock were used for different experiment. The larvae were reared in plastic cups. They were daily provided with commercial fish food [35] *ad libitum.* Feeding mosquitoes on human arm for experimental purposes was suggested by [36, 37]. Both females and males were provided with 10% glucose solution as described by [38] on cotton wicks. The cotton was always kept moist with the solution and changed every day. An egg trap (cup) lined with filter paper containing pure water was always placed at a corner of the cage. This arrangement made the collection of eggs easier.

2.2 Collection of plant materials

C. citrinus leaves were collected from Gandhipuram, Coimbatore District, Tamil Nadu, India. The identification of the plant was authentified at BSI (Botanical Survey of India), Coimbatore.

2.3 Preparation of plant extract

The collected leaves brought to the laboratory. The plant leaves were observed carefully for any kind of diseases or infection and if found any, those parts were separated and not used for the experiment. The selected leaves washed with distilled water in order to clean dust or any particle stuck to them. Then the leaves kept for drying under shade at room temperature (27± 2 ºC) for about 2 weeks till they dried completely. The leaves were finely powdered using electric blender. Powdered plant material (100g) was soaked in methanol (1000 ml) in airtight wide mouth bottle and kept for 4 days with periodic shaking. After that, the extract was filtered using Whatman No.1filter paper and kept in Petri dishes for drying at room temperature [39]. Dried extract was used for the preparation of stock solution. 1 g of the concentrated extract of *C. citrinus* leaves was dissolved in 100ml water and kept as stock solution. This stock solution was used to prepare the desired concentrations (from 0.10% to 0.90%) of the extract for exposure of the mosquito larvae.

2.4 Phytochemical analysis of the plant extract

Phytochemical analysis of the plant extract were carried out according to the methodology of [40, 41]. The qualitative phytochemical analysis revealed the presence of different phytochemicals such as carbohydrates, tannins, saponins, flavonoids, quinones, terpenoids, phenols and steroids (Table-1).

Table 1: Qualitative phytochemical analysis of *C. citrinus* methanolic leaf extract.

Phytochemical compounds	Results
Carbohydrates	+
Tannins	+
Saponins	+
Flavonoids	+
Alkaloids	_
Quinones	+
Glycosides	_
Cardiac Glycosides	_
Terpenoids	+
Triterpenoids	_
Phenols	+
Coumarins	_
Steroids	+
Phytosteroids	_
Phlobatannins	_
Anthaquinones	_

(+): Present
(-): Not present

2.5 Larvicidal, Pupicidal bioassay test
Bioassay test was carried out for testing the efficacy of methanolic leaf extract of *C. citrinus* on *Ae. aegypti* at different stages of development viz I, II, III and IV instars and pupae. Instructions of [42] for conducting bioassay experiment with mosquito larvae were carefully followed. The values of LC_{50}/24hrs and their 95% confidence limit of upper confidence limit (UCL) and lower confidence limit (LCL), regression and chi- square values were calculated using probit analysis [43]. The SPSS 17.0 (Statistical Package of Social Sciences) used for statistical analysis.

2.6 Behavioural studies in *Ae. aegypti* larvae treated with methanolic leaf extract of *C. citrinus*
During the larvicidal bioassay with methanolic leaf extract of *C. citrinus* the fourth instar larvae of *Ae. aegypti* were monitored carefully for behavioural modifications. The observations included wriggling speed, movements in different directions, aggregation behaviour, etc. The larval behaviour was recorded and photographed. Similar observations were made in control larvae for comparison with the treated larvae [44, 45].

2.7 Morphological studies in *Ae. aegypti* larvae treated with methanolic leaf extract of *C. citrinus*
After the larvicidal bioassay, the dead larvae and pupae were separated and probed under light microscope for any aberrations in their morphology. Any aberrations observed was recorded, photographed compared with the features observed in the controls [44, 45].

3. Results and Discussion
3.1 Toxicity of methanolic leaf extract of *C. citrinus* to the developmental stages of *Ae. aegypti*
Bioassay test were conducted to find out the toxicity of methanolic leaf extract to I, II, III, IV instars and pupae of the mosquitoes of *Ae. aegypti*. The data were subjected to Finney's method of probit analysis. The results expressed in terms of LC_{50} / 24 hour. LC_{50} / 24 hour values of methanolic leaf extract of *C. citrinus* to I instar larvae was 0.295% (24hrs), and this was found to gradually increase with the age of larvae. Pupae showed the highest resistance to the methanolic leaf extract of *C. citrinus* as evident from the relatively higher LC_{50} / 24 hour values 0.826% (Table 2).

Table 2: LC_{50} / 24 hour values of methanolic leaf extract of *C. citrinus* to the pre-adult stages (I, II, III, IV instar and pupae) of *Aedes aegypti*

Stages of Development (Instars)	Number of larvae / trail	LC_{50}/24 hour (%)	Confidence limit		Regression equation	R value	Slope	Chi-square	Degrees of freedom
			LL (%)	UL (%)					
I	20	0.295	0.249	0.336	y = 185x - 4.5	0.958	220	1.106	3(16.26)
II	20	0.382	0.352	0.427	y = 200x - 26	0.982	205	0.863	3(16.26)
III	20	0.494	0.453	0.548	y = 185x - 42.5	0.992	225	0.902	3(16.26)
IV	20	0.615	0.571	0.654	y = 180x - 58	0.952	190	0.820	3(16.26)
Pupae	20	0.826	0.783	0.876	y = 175x - 88.75	0.934	200	0.815	3(16.26)

Table 3: Impact of the methanolic leaf extract (LC_{50} value) of *C. citrinus* on the behaviour of early fourth instar larvae of *Aedes aegypti*.

Time	Methanolic leaf extract (LC_{50} /24 hrs Value)
0 Min	All larvae exhibited natural and vigorous movements
5 Min	Initial excitation, agitation and restlessness
10 Min	Restlessness
30 Min	(25.000 ± 0.012)a (10.000 ± 0.250)b coiling movements due to aggressive self-biting of their anal forming ring like structure
1 hr	(35.000 ± 0.033)a (20.000 ± 0.120)b (10.000 ± 0.200)c
2 hr	Severe restlessness tremor, convulsions, struggling behaviour followed by paralysis (40.000 ± 0.333)a (30.000 ± 0.222)b (20.000 ± 0.010)c
3 hr	(50.000 ± 0.012)a (22.333 ± 0.323)c
24 hr	(50.000 ± 0.577)c

a = % larvae sank ± SD,
b = %moribund larvae ± SD,
c = % dead larvae ± SD

3.2 Effect of methanolic leaf extract of *C. citrinus* on the behaviour of early fourth instar larvae of *Ae. aegypti*
The early fourth instars of *Ae. aegypti* treated with methanolic leaf extract of *C. citrinus*(LC_{50}/24hrs values 0.615) were carefully observed for their behaviour, the larvae showed a peculiar pattern. Immediately after exposure to the extract, all larvae exhibited natural and vigorous movements (Table 3). However, only 5 minutes of exposure resulted in initial excitation, agitation, and restlessness in the dengue larvae. Gradually, the larvae resorted to swift wriggling movements which persisted for approximately after 30 minutes, 25% larvae started sinking at the bottom of plastic jars. In contrast, remaining larvae exhibited severe restlessness behaviour with aggressive self-biting of their anal papillae with their mouth parts. Consequently, the larvae formed ring-like structure and showed coiling movements (Plate 4a, b, c). After 2 hours exposure of methanolic leaf extract to the larvae showed tremor and convulsions followed by paralysis and 40% of the larvae sank down to bottom, 30% moribund larvae and 14% of larvae dead (Plate 4d, e, f).

When the dead larvae of *Ae. aegypti* were observed after the larvicidal bioassay, the larvae showed morphological aberrations and structural alterations in the anal gills and respiratory siphon on comparison to the control larvae (Plate 3, 3b). The observations clearly established the damaged anal papillae and respiratory siphon. The anal papillae showed shrunken and folded cuticle of the internal membrane (Plate 3c). On the other hand, the external membrane of anal papillae was completely intact with no structural changes.

3.3 Effect of methanolic leaf extract of *C. citrinus* on morphology of *Ae. aegypti*
Morphological abnormalities occurred among the larvae exposed to lethal concentrations of methanolic leaf extract of *C. citrinus.* Pupae survived through treatment frequently showed a variety of changes viz. pre-pupa of *Ae. aegypti*, anterior side larval head is attached, but thoracic region like a pupa and larval case of proceeding instar is attached in the abdominal region (Plate 1a), dwarf, darkly pigmented and damaged cephalothoracic region of *Ae. aegypti* pupa (Plate 1b), demelanized pupa of *Ae. aegypti* with larval case of

proceeding instar attached in the head region (Plate 1c), pupa of *Ae. aegypti* with straight abdomen (Plate 1d), the abdomen of the adult has pupal characteristic with paddles and damaged mouth parts in the anterior region (Plate 2a), not completely emerged adult of *Ae. aegypti* with the abdominal part, wings and legs attached to the pupal skin (Plate 2b).

Botanicals are proved to be efficient bio pesticides not only as crude extract but as solvent extracts also [46]. In addition to that, the crude extracts may be more effective compared to the individual active compounds, due to natural synergism that discourages the development of resistance in the vectors [47]. Insecticidal effects of plant extracts vary not only according to plant species, mosquito species, geographical varieties and parts used, but also due to extraction methodology adapted and the polarity of the solvents used during extraction.

The results showed that the methanolic leaf extract of *C. citrinus* possesses significant larvicidal properties against *Ae. aegypti*. The findings agree with some of the previous reports. the LC_{50}/24 hrs values of ethanolic leaf extract of *Delonix elata* to 1,2, 3, 4 instars larvae of *Ae. aegypti*, were 4.91,5.16, 5.95 and 6.87% [48]; the ethanol leaf extract of *Annona reticulata* was found more effective against *Cx. quinquefasciatus* than *Ae. aegypti* larvae and its LC_{50} values gradually decreased with increased period of exposure. LC_{50} values of the extract recorded after 24 hrs of exposure were 0.502, 2.937, 4.204, 6.224 ppm respectively against 1st-4th larvae of against *Cx. quinquefasciatus* and 6.883, 5.992, 14.57, 19.88 ppm against *Ae. aegypti* larvae [49]; *Annona reticulata* methanol leaf extract was more effective against 3rd instar *Ae. aegypti* larvae with LC_{50}/24hrs values of 95.24ppm respectively and against 3rd instar larvae *An. stephensi* larvae 262.71ppm respectively [50].

The effectiveness of this plant could be attributed to the presence of phytochemical compounds that act as insecticides [51]. *C. citrinus* leaves have furnished carbohydrates, tannins, saponins, flavonoids, quinones, terpenoids, phenols and steroids (Table-1). These compounds were previously reported to have mosquito larvicidal activity [52]. These compounds may jointly (or) independently contribute to larvicidal activity against *Ae. aegypti*. The phytochemicals interfered with proper functioning of mitochondria more specifically at the porton transforming sites [8] and phytochemicals primarily effect the midgut epithelium and secondarily affect the gastric caeca and the malpighian tubules in mosquito larvae [53, 54] have suggested that the saponin molecules interact with the cuticle membrane of the larvae, ultimately disarranging the membrane could be the most probable reason for the larval death. Flavonoids have larvicidal effects due to their mechanism of action to inhibit the respiratory system and disrupt the electron transport process in the larval body thus decreasing ATP production and reducing the use of oxygen by mitochondria. Besides, the tannins work by inactivating the enzymes and proteins in the larvae body [55].

The behavioural observations of the early IV instar larvae of *Ae. aegypti* treated with the methanolic leaf extract of *C. citrinus* revealed excitation, restlessness, and aggressive movements in the larvae. These symptoms being similar to those caused by nerve poisons suggested that the extract of *C. citrinus* could possibly act as cytolysin and had an impact on the neuromuscular system of larvae. Our findings corresponded to those of a few earlier studies [61-65] suggesting the extract acting as nerve poison; though, the aggressive and uncoordinated movements along with other toxic symptoms were observed at relatively different time intervals. Our studies also showed coiling movements in the treated larvae with aggressive anal biting behaviour further enforcing the neurotoxic impact of the extract on *Ae. aegypti* larvae. Similar behavioural observations have been reported by [66] in the larvae of *Ae. aegypti* when assayed with extracts prepared from *Argemone mexicana*. It has been found that the anal papillae of mosquitoes have some role in the regulating electrolyte balance required for the life sustainability [67]. This suggests the cytotoxic effects of *C. citrinus* extract leading to electrolyte imbalance in the anal region causing violent anal biting.

Present investigations also resulted in the structural aberrations in the anal papillae of early IV instars of *Ae. aegypti* when exposed to methanolic leaf extract of *C. citrinus*. The microscopic observations of the anal papillae showed shrunken internal membrane, while the external membranes did not show any modifications. These results are in agreement with the similar internal shrinkage reported by [66] in the anal papillae of *Ae. aegypti* larvae when assayed with variable extracts of *Argemone mexicana*. Likewise destructed anal papillae with shrunken cuticular border have been observed by [68] in *Cx. quinquefasciatus* larvae when treated with ethanolic extract of *Kaempferia galangal*. It has been suggested that the structural deformity in the anal papillae possibly led to anomalous functions which may have led to the disturbance in osmotic and ionic regulation [64]. They also suggested that the disruptive regulatory functions may have caused the mortality of the *Ae. aegypti* larvae.

Similar deformities were found to occur during the development of *Ae. aegypti, Cx. quinquefasciatus* and *An. stephensi* in media treated with hexane, diethyl ether, dichloromethane and ethyl acetate extracts of *Murraya koenigii* leaf [56]; ethanolic, acetone and petroleum ether extracts of leaves from *Cupressus semprevirens* were tested against 3rd instar larvae of *Cx. pipiens*, various degrees of morphogenetic abnormalities were observed in immature and adult stages [57]; the hexane, diethyl ether, dichloromethane and ethyl acetate extracts of *Abutilon indicum* leaf were evaluated for their pupal deformities against *Ae. aegypti*, *Cx. quinquefasciatus* and *An. stephensi* [58, 59] observed that phytoextracts affect larval morphology, resulting in pigmentation and alternations in head and abdomen shape.

These morphological abnormalities are commonly caused by botanical extract and are attributed to result from disturbance to growth regulating hormones [60].

Plate 1: Control pupa of *Ae. aegypti*.

Plate 1a: Pre- pupa of *Ae. aegypti*. Anterior side larval head is attached, but thoracic region like a pupa and larval case of proceeding instar is attached in the abdominal region.

Plate 1b: Dwarf, darkly pigmented and damaged cephalothoracic region of *Ae. aegypti* pupa.

Plate 1c: Demelanized pupa of *Ae. aegypti* with larval case of proceeding instar attached in the head region.

Plate 1d: Pupa of *Ae. aegypti* with straight abdomen.

Plate 2: Control adult of *Ae. aegypti*.

Plate 2a: The abdomen of the adult has pupal characteristic with paddles and damaged mouth parts in the anterior region.

Plate 2b: Not completely emerged adult of *Ae. aegypti* with the abdominal part, wings and legs attached to the pupal skin.

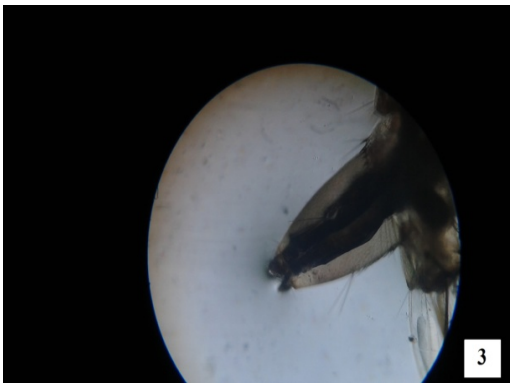

Plate 3: Respiratory siphon of control IV instar larva of *Ae. aegypti*.

Plate 3a: Damaged respiratory siphon of control IV instar larva of *Ae. aegypti* due to treatment of methanolic leaf extract of *C. citrinus.*

Plate 3b: Light microscope view of the anal gills of control IV instar larva of *Ae. aegypti*.

Plate 3c: Light microscope view of the anal gills of IV instar larva of *Ae. aegypti* treated with methanolic leaf extract of *C. citrinus* showing four anal gills with externally normal appearance but internally shrunken structure and folded membrane.

Plate 4: Control IV instar larvae of *Ae. aegypti*.

Plate 4 a, b, c: Larvae treated with methanolic leaf extract of *C. citrinus*; The circled larvae show the aggressive anal gill biting behaviour forming ring shaped structure.

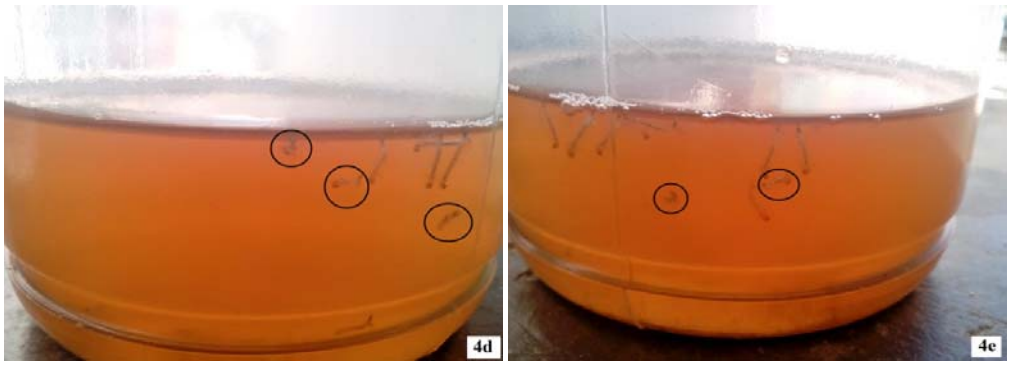

Plate 4 d, e: Moribund larvae of *Ae. aegypti* treated with methanolic leaf extract of *C. citrinus* (in circle).

Plate 4 f: Dead larvae of *Ae. aegypti* treated with methanolic leaf extract of *C. citrinus* (in circle).

4. Conclusion

The findings of the present study suggest that the methanolic leaf extract of the plant *C. citrinus* has significant mosquitocidal activity against the *Ae. aegypti*. The most important aspect of the present finding is that the plant extract are environment friendly and can be used effectively to replace the chemical control measures which are dangerous for the whole biodiversity. Chemical control measures have deteriorated the natural ecosystems and has made immense losses to humans, animals and plant species. In light of obtained results, it is highly recommended that in future chemical control measures should be eliminated as far as possible. Further investigations about the mode of action of the constituent effect on non-target organisms and field evaluation are necessary prior to commercialization.

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