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Journal of Botanical Research
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ARTICLE

Comparative Toxicity of Neem and Peppermint Oils Nano Formulations against *Agrotis ipsilon* (Hufn.) Larvae (Lepidoptera: Noctuidae)

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ARTICLE INFO

Article history
Received: 4 March 2019
Accepted: 19 March 2019
Published Online: 30 April 2019

Keywords:
Agrotis ipsilon larvae
Toxicity
Neem
Pepper mint oil
Nano formulations

ABSTRACT

Applications of nanotechnology in agriculture will result in the development of efficient and potential approaches towards the management of insect pests. The toxicity effects of four essential oils peppermint, thyme, camphor and sage oils were tested against the fourth instar larvae of *Agrotis ipsilon* to select the most effective essential oil to be converted to the nano form. According to the results obtained, peppermint oil was the most toxic compound, which has been used in the present investigation compared with neem oil. The toxicity of bulk and nano- formulations of neem and pepper mint oils were tested against 2nd and 4th instar larvae of *A. ipsilon* under laboratory conditions of 25±2 °C& 65 -70 % R.H.relative humidity The results show that the LC50 value (the concentration used which kill 50% of the tested individuals)of loaded neem or pepper mint were lower (0.62 and 36.47 ppm) compared with neem or pepper mint oil nano-emulsion and bulk neem for the second larval instar. The different formulations of neem are more potent than in case of peppermint oil, as LC50 and LC90 values were significantly lower.

The same trend was found concerning the 4th larval instar. Age of treated larvae had a detrimental effect on the response to the compounds tested. It was noticed that the younger larvae were much more sensitive to the prepared compounds compared to the older ones. The least LC50 value for loaded neem nano-emulsion was 6.68 ppm compared with the highest value for bulk neem oil (16.68 ppm). Also, LC90 values followed the same trend as in case ofLC50. Again, the toxicity of loaded peppermint oil had the most insecticidal activity as expressed by the lowest LC50 value (51.9 ppm) with more insecticidal effect than the bulk(125.43 ppm) or nano-emulsion (85.43 ppm).

The present results indicated that these novel systems could be used in integrated pest management program for *A. ipsilon* control.

1. Introduction

Black cutworm (*Agrotis ipsilon* Hufnagel) is a cosmopolitan pest that affects over 30 important crops, including beans, broccoli, cabbage, carrot, spinach, eggplant, lettuce, potato, tomato, and turnip[1, 2]. Black cutworm larvae feed above ground, and each one can consume over 400 cm^2 of foliage during its development. The terminal and subterminal instar stages account for 80% and 10% of foliage, respectively, and minimal fo-

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 DOI: https://doi.org/10.30564/jrb.v1i1.590

liage loss occurs during the early stages of development. Larvae in the fourth instar stage may sever the stems of young plants, and one larva may sever the stems of several plants in a single night[4].

Insecticides currently used to control *A. ipsilon* infestations are hazardous to humans and animals. Insecticides derived from plants may be a safer alternative [6] and could be used as acost-effective means of pest control for farmers in developing countries if simple extracts can be prepared from readily available plants [5].

Neem (*Azadirachta indica* A. Juss.) is alimonoid-producing species in the mahogany family (MeliaceaeJuss.) and has long been recognized as a source of environment-friendly biopesticides. Extracts from its leaves and seeds act as antifeedants, repellants, and growth disruptors in multiple insect species [8, 9, 10]. They are, however, relatively selective, and neem products have been recommended for many Integrated Pest Management programs [7].

Sharaby and El-Nojiban[11] studied the toxicity of different essential oils against the greasy cut worm. They also added that all tested oils have great effect on growth and development of *A. ipsilon* larvae. Kamaraj et al[12]showed reduction in growth and development of *H. armigera* and *S. litura* larvae when fed exclusively on Neem gum nano formulation (NGNF) treated castor leaves

Although, biotechnological advances existed when botanical material used in plant protection, the major points have been taken in consideration are that of a rapidly degradation and polluting the ecosystem caused by showed prevailing practices. To take the situation it was need to harness innovative approaches towards other solutions such as nanotechnology [13]. Pesticides in nanoparticular form present an attractive solution for this problem. Their effective concentration is expected to be much lower compared to that of bulk materials and they can be formulated without the use of organic solvents. Sodium alginate (Na-Alg) has been used as a controlled release matrix material in medicine [14] and agriculture [15]after cross linking it with calcium chloride and glutaraldehyde. Alginate polysaccharides are identified to be hemocompatible and do not build up in any organs of the human body. Encapsulating nano-particle layers at the emulsion droplet interface may be engineered to increase droplet stability and control of release kinetics. Based on the for mentioned facts, The present investigation aimed to study the effects of three different formulations, bulk, nano-emulsion and loaded nano-emulsion of two main botanical extract, *i.e*. neem extract and peppermint essential oil against *Agrotisipsilon* larvae.

2. Materials and Methods

2.1 Test Insect

Black cutworms *Agrotis ipsilon* (Hufn)were maintained for several generations in rearing units under controlled conditions of 25±2°Cand 65± 5%RH. The adult moths were reared in glass jars measuring 15x25cm. A sucrose solution of 10% concentration was provided for feeding the moths. Females laid their egg masses on black muslins, newly-laid egg masses were collected. The old muslins were replaced by new ones, and the adult moths were provided with fresh feeding solution.

The newly laid eggs were categorized according to their oviposition date and were immediately placed in a suitable container. A small hair brush was used to transfer newly hatched larvae into plastic boxes measuring 25x15cm and containing a suitable amount of clean castor leaves. Larvae at the third instar were separated into individual plastic boxes to prevent cannibalism. Pupae were collected from the larval containers and were transferred to containers of sawdust.

2.2 Tested Plant Extract and Essential Oils

Neem, peppermint (*Mentha x pipreta* L.), camphor (*Cinnamomum camphora* (L.) J. Presl.), thyme (*Thymus vulgaris* L.), and sage (*Salvia officinalis* L.) oils were investigated. Neem oil (0.03% azadirachtin) derived from neem seed kernel extract was obtained from Dr. Kleeberg in Lahnaw, Germany. Peppermint oil with a menthol content of 26%was derived from peppermint (*Menthax pipreta* L.) using the method described by Guenther (1961) [16]. The remaining essential oils were obtained from the l Oil extraction unit at the National Research Centre in Cairo, Egypt.

3. Preliminary Screening Tests of Essential Oils against *A. ipsilon* Larvae

Bioassays were carried out using peppermint, thyme, camphor, and sage essential oils on fourth instar *A. ipsilon* larvae, and the most effective oil was used for the present investigation. Larvae were fed100g of a semi-synthetic diet as described in Shorey and Hale (1965)[17]. The diet was prepared using 500 g kidney beans, 30 g agar, 65g yeast, 3g sorbic acid, 5 g benzoic acid, 10ml formalin, and 10 g ascorbic acid. The kidney beans and agar were autoclaved in 600ml of distilled water and were then ground with the other components, except ascorbic acid, which incorporated with the prepared media after it had cooled to the appropriate temperature. Each essential oil was included in a series of increasing

DOI: https://doi.org/10.30564/jrb.v1i1.590

concentrations in order to calculate LC_{50} values. Concentrations of 0.12, 0.25, 0.5 and 1% were incorporated as aqueous dilutions into 100 g of the semi-synthetic diet. This procedure was carried out immediately before gelling in order to avoid decomposition. Media treated with distilled water and a 100 µl of Tween 80 was used as control. The selected larvae were tested using four replicates per concentration per essential oil with ten larvae in each replicate. Each replicate was housed in a glass tube 10 cm in length and was fedm 1 g of the treated diet.

The larvae were incubated at 25 ± 2 °C and 65-70% RH. Larval mortality was recorded daily for 4 days after treatment and compared with the control larvae. The mortality percentage was corrected using Abbott's formula[18]. The logarithmic (?) relationship between the oil concentrations and larvae mortality were plotted and the LC_{50} values were calculated using Ld-p line program according to Finney (1971) [19].

The results of the primary screening tests were used to select peppermint oil, in addition to the neem extract, for continued experimental work.

4. Preparation of Nano-formulations

4.1 Nano-emulsions Preparation

Emulsions of neem or peppermint oil, Tween 80, and distilled water were prepared using a modification of the method described by Jerobin et al. (2012) [20]. Neem oil was diluted with distilled water to a ratio of 1:1 (oil to water), and peppermint oil was diluted with distilled water to a ratio of 2: 1 (oil to water). Two percent of Tween 80 was added as an emulsifier. The emulsion was then sonicated for 30 minutes using an ultrasonic cleaner set (model WUC-DO3H 290W)set at 60 Hz. It was then resonicated for 1 minute using a high energy ultrasonication probe (model VCX750)set to 750W and 20 kHz, and it was then resonicated again for 30 minute by the ultrasonic cleaner set under cooling conditions [21].

4.2 Preparation of Loaded Nano-emulsions

Alginate nanocapsules were prepared using oil in water (o/w) emulsifications, followed by cross linking using calcium chloride and solvent removal (in case of using solvent),using modified versions of the methods described by[22 , 23].Sodium alginate solution (3%, w/v) was prepared by dissolution in distilled water at 50˚C for 45 min. Tested oils were diluted by distilled water using Tween 80 as an emulsifier with mechanical stirring for 10 min. Briefly, sodium alginate o/w emulsion was made by drop wise dispersion of diluted oil into appropriate volume of alginate solution (1 : 1 oil to alginate in case of neem oil and 1 : 2 in case of pepper mint oil under continuous mechanical stirring at room temperature. The emulsion thus formed were sonicated for 30 min using ultrasonic cleaner set, model WUC-DO3H 290 W and 60 Hz and then sonicated for 2min using a high energyultra sonication probe model VCX 750, 750 W, 20 kHz). An appropriate volume of $CaCl_2$ (2:10 $CaCl_2$ to alginate, respectively) was then added into the resulting emulsion and stirred for an additional 30 min and sonicated as mentioned previews. Nano- capsules were obtained as dispersion in aqueous solution

In another experiment, pepper mint oil was tested with diluting by ethanol as a solvent instead of water and was removed after equilibration under reduced pressure at 40–45˚C for 20 min.

5. Bioassay Tests

Bioassays tests were carried out on second and fourth instar *A. ipsilon* larvae. Samples of 100g semi-synthetic diet previously described by[17] were treated. All prepared formulations were incorporated into the diet as aqueous dilutions at the desired concentrations during the preparation of the diet. Series of concentrations of each formulation were used to calculate the LC_{50} values. Such procedure was carried out just before gelling in order to avoid decomposition of the used materials. Media treated with distilled water mixed with 100 µl of Tween 80 was used as control. All concentrations were prepared according to the active ingredient content in each formulation.

In case of 2^{nd} instar larvae, 5g of treated semi-synthetic diet for each concentration was added in plastic cups of 120ml in capacity. Serial concentrations of bulk neem oil containing 1.5, 3, 6, 9 and 12 ppm azadirachtin were prepared. Neem oil nano-emulsion and loaded neem oil nano- emulsion were prepared by concentrations of 0.75, 1.5, 3 and 6 ppm azadirachtin. Ten 2^{nd} instar larvae were then transferred to each cup with four replicates, of 10 larvae /replicate.

Bioassay tests of pepper mint oil were carried out using serial concentrations of pepper mint oil containing 30, 60, 90 and 120 ppm menthol for bulk oil. For nano-emulsion and loaded nano- emulsion formulations, serial concentrations of 5, 10, 20, 30, and 40 ppm were added to the targeted medium. Ten of 2^{nd} instar larvae were then transferred to glass tubes with four replicates, of 10 larvae /replicate.

In case of 4^{th} instars larvae, glass tubes of 10 cm height were used. One piece weighted 1g of treated diet was cut by the cork borer and was added in each glass

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tube and each larva was transferred individually to each tube with four replicates, of 10 larvae /replicate. Bulk neem oil was tested in concentrations of 6, 12, 18 and 24 ppm azadirachtin. Neem nano- emulsion and loaded nano-emulsion were tested in concentrations of 3, 4.5, 6, 7.5 and 9 ppm. Bulk pepper mint oil was prepared in the concentrations of 30, 60, 120 and 280 ppm menthol. Nano- emulsion and loaded nano- emulsion were prepared in concentrations of 30, 60, 90 and 120 ppm menthol.

Those cups and glass tubes were incubated at 25 ± 2 ℃ and 65 -70% R.H. Larval mortality was recorded daily during 4 days after treatment and adjusted for control experiment and the mortality percentage was corrected using Abbott's formula[18. Concentrations mortality regression lines were plotted in form of log/probit relation and the LC_{50} values were calculated using Ld-p line program according to[19].

6. Statistical Analysis

Data were analyzed using one-way analysis of variance (ANOVA) using SPSS software (Tukey test). A value of $p < 0.05$ was considered statistically significant.

7. Results and Discussion

7.1 Preliminary Bioassay Tests of Essential Oils against *A. ipsilon* Larvae

In order to determine the most effective essential oil to be converted to the nano form, the toxicity effect of four essential oils Pepper mint, Thymus, Camphor, and Sage oil were tested against the 4th instars larvae of *A. ipsilon*. Their toxicity against the 4th instar larvae are given in Table 1. It was shown that pepper mint was the most effective essential oil and thymus oil was the least effective oil. LC_{50} values were 0.45%, 0.60%, 0.73% and 0.86 % for pepper mint, camphor, sage and thyme, respectively after 96hr post treatment. The toxicity index values were 100, 75.00, 61.64 and 52.33%, respectively. The relative potency values were 1.91, 1.43, 1.18 and 1, respectively. The present findings agreed with that recorded by [11]. They arranged the toxicity values of some oils based on LC_{50} values tested against *A. ipsilon* in descending orders as follows garlic, mint, Cumin, carawaya and parsley. As shown the second toxic essential oil was the mint oil. They added that LC_{50} value of mint oil was 0.032% as a contact poison on the larvae. LC_{50} for larvae as a stomach poison and on pupal stage nearly equal they were 0.160 and 0.148%, respectively.

Table 1. LC_{50} and LC_{90}values of different essential oils on *A. ipsilon*4th instars larvae after 96h post treatment

Essential Oils	LC_{50} ml (Fudicial limits)	LC_{90} ml (Fudicial limits)	Slope	Regression	Toxicity index	Relative potency
Pepper mint	0.45 (0.37-0.54)	1.20 (0.91-1.86)	3.01±0.41	0.99	100	1.91
Thyme	0.86 (0.73-1.05)	2.34 (1.76-3.69	2.96±0.40	0.98	52.33	1
Camphor	0.60 (0.50-0.70)	1.44 (1.15-2.05)	3.33±0.42	0.98	75.00	1.43
Sage	0.73 (0.60-0.93)	1.87 (1.33-3.44)	3.12±0.50	0.97	61.64	1.18

7.2 Insecticidal Activity of Neem NanoFormulations against the 2nd Instar Larvae of *A. ipsilon*

Data represented in Table 2 reveal that loaded nano-emulsion from neem oil when tested on the 2*nd* instar larvae hadthe highest insecticidal activity as expressed by lowest LC_{50} value while the bulk crude oil presented the highestLC_{50} value. The LC_{50} values were 0.62, 2.36 and 4.38 ppm for neem loaded nano-emulsion, nano-emulsion and bulk crude oil, respectively. Also LC_{90} values behaved the same activity order as they were 4.86, 5.70 and 8.57ppm, respectively. . Slope values were 1.43, 3.34 and 4.40 with regression coefficient 0.99, 0.98 and 0.99 and toxicity index 100, 26.27 and 14.16.

7.3 Insecticidal Activity of Pepper Mint Oil Nano Formulations against the 2nd Instar Larvae of *A. ipsilon*

Data in Table 2 show descending order of pepper mint oil nano formulations on the 2*nd* instar larvae based on their LC_{50} values as they were bulk oil,nano-emulsion and loaded nano-emulsion represented by 55.77, 43.25 and 36.47 ppm, respectively. LC_{90} values were 163.58, 79.72 and 107.20 ppm, for the three formulations respectively. The relative potency was 1.53 fold for loaded nano-emulsion and 1.29 fold for nano -emulsion more than bulk oil. Slope values were 2.74, 4.83 and 2.74, for bulk, nano-emulsion and loaded nano-emulsion, respectively. Also, toxicity index values were 65.39, 84.32 and 100 for the same order shown previously. The regression coefficient values were 0.99 for both bulk and nano-emulsion, and was 0.97 for loaded nano-emulsion.

Nano-emulsions of pesticidal active ingredients (AIs) have often been suggested to increase the uptake of the AIs, but supporting data in the context of plant protection products remains scarce[24]. The same authors reported

DOI: https://doi.org/10.30564/jrb.v1i1.590

that a series of nano-emulsions of neem oil decreased the LC_{50} (the concentration required to achieve 50% mortality) with decreasing of droplet size, which was interpreted as indicating an increased uptake of smaller droplets. In another study, the efficacy of a nano-emulsion of permethrin presented in [25]was significantly higher than that of the pure AI, which was again interpreted as indicating an increased uptake of the nano-formulated. In a study mentioned by [26], it was shown that the effects on non-target organisms (*i.e*., soil bacteria and plants) were reduced, but the reasons for the different effects on target and non-target organisms have yet to be elucidated. Unfortunately, no comparisons were carried out with commercial formulations. [25]agreed with the present findings that nano-permethrin was more potent in its larvicidal effect against *C. quinquefasciatus* than the bulk form of permethrin. The LC_{50} of nano-permethrin (0.117mg/L) was found to be more effective compared to bulk permethrin (0.715 mg/L). 100% mortality was recorded within 6h for nano permethrin treated samples. But for bulk permethrin treated samples 100% mortality was not observed even after 24h of exposure period.

7.4 Insecticidal Activity of the Different Nano-Formulations from Neem against the 4thInstar Larvae of *A. ipsilon*

Age of treated larvae had a detrimental effect on the response to the compounds tested. It was noticed that the younger larvae were much more sensitive to the prepared compounds compared to the older ones. That was clear in Table 3 showing the LC_{50} values of such preparations on 4thinstar larvae of *Agrotis ipsilon*. The least LC_{50} value for loaded neem nano-emulsion represented by 6.68 ppm followed bynano-emulsion that was 10.82 ppm and the highest LC_{50} value was 16.68 ppm for bulk neem oil. Also, LC_{90} values were 10.15, 14.19 and 34.74 ppm. Relative potency of loaded nano-emulsion was 2.50 fold and nano-emulsion was 1.54 fold more thanbulk oil. In addition, slope values were 4.02, 10.89 and 7.01 and regression was 0.91, 0.99 and 0.98 for bulk, nano-emulsion and loaded nano-emulsion, respectively and toxicity index was 40.05, 61.74 and 100, respectively.

7.5 Toxicity Effect of the DifferentNano-formulations from PepperMintOil on the 4thInstarLarvae of *A. ipsilon*

Similar but less pronounced toxic effects were recorded in studies carried out with the 4th larval instar. Loaded nano-emulsion from peppermint had maximum insecticidal activity as expressed by the lowest LC_{50} value (51.90 ppm) with more insecticidal effective 2.42 fold than the bulk oil followed bynano-emulsion (85.43 ppm) with relative potency 1.47 fold than the bulk oil which showed the highest LC_{50} value (125.43 ppm) (Table 3). LC_{90} values were 89.75, 253.09 and 334.80 ppm, respectively. Slope values were 5.39, 2.72 and 3.01. Toxicity index were 100, 60.75 and 41.38. Regression values were 0.96, 0.99 and 0.99 for loaded nano-emulsion, nano emulsion and bulk respectively.

[27]reported that larvicidal and repellent activities of essential oils have been attributed to their major monoterpenic constituents. The rapid action against some pests is indicative of a neurotoxic mode of action related to the monoterpenes substances. Also, there is evidence effect on acetylcholinestrase and octopamingeric system in insects as confirmed by [28, 29].Generally essential oils and thei rcomponent have been considered safe than other plant derived chemicals like azadirachtin, rotenone or pyrethrum[30]. This could be attributed to existing detoxifying metabolism pathways and bio rational mode of action of monoterpenoids as reported by[31].[32]mentioned that the rapid action of essential oils against some pests

Table 2. LC_{50}, LC_{90} of the neem and peppermint oil nano-formulations against 2nd instar larvae of A. ipsilon

Tested plant extract or Essential oil	Formulation	LC_{50} (ppm) (Fudicial limits)	LC_{90} (ppm) (Fudicial limits)	Slope	Regression	Toxicity index	Relative Potency
Neem	Bulk	4.38 (3.81 - 5.01)	8.57 (7.21 - 11.05)	4.40 ± 0.56	0.99	14.16	1
	Nano emulsion	2.36 (2.03 - 2.90)	5.70 (4.18 - 10.56)	3.34 ± 0.60	0.98	26.27	1.86
	Loaded nano emulsion	0.62 (0.33 - 0.89	4.86 (2.91 - 14.93)	1.43± 0.31	0.99	100	7.06
Mentol	Bulk	55.77 (46.33 - 69.05)	163.58 (118.21 - 287.21)	2.74± 0.42	0.99	65.39	1
	Nano emulsion	43.25 (38.12 - 49.33)	79.72 (67.02-103.85)	4.83 ± 0.63	0.99	84.32	1.29
	Loaded nano emulsion	36.47 (29.72-46.17)	107.20 (77.58 - 177.74)	2.74± 0.36	0.97	100	1.53

Notes: LC50 and LC90 values were calculated and expressed as ppm active ingredient (azadirachtin in neem and menthol in peppermint)

 DOI: https://doi.org/10.30564/jrb.v1i1.590

Table 3. LC_{50}, LC_{90} of the neem and peppermint oil nano-formulations against 4th instar larvae of A. ipsilon

Tested plant extract or Essential oil	Formulation	LC_{50} (ppm) (Fudicial limits)	LC_{90} (ppm) (Fudicial limits)	Slope	Regression	Toxicity index	Relative Potency
Neem	Bulk	16.68 (13.99 - 20.95)	34.74 (25.70 - 76.16)	4.02 ± 0.98	0.91	40.05	1
	Nano emulsion	10.82 (10.18 - 11.46)	14.19 (13.15 - 16.07)	10.89 ± 1.65	0.99	61.74	1.54
	Loaded nano emulsion	6.68 (6.11 - 7.36)	10.15 (8.90 - 12.66)	7.01 ± 1.07	0.98	100	2.50
Mentol	Bulk	125.43 (104.76-152.25)	334.80 (253.81- 520.44)	3.01 ± 0.41	0.99	41.38	1
	Nano emulsion	85.43 (66.20-109.86)	253.09 (177.25 - 510.50)	2.72 ± 0.52	0.99	60.75	1.47
	Loaded nano emulsion	51.90 (44.28 – 60.05)	89.75 (74.89 – 123.94)	5.39 ± 0.97	0.96	100	2.42

Notes: LC_{50} and LC_{90} values were calculated and expressed as ppm active ingredient (azadirachtin in neem and menthol in peppermint oil

is an indication of a neurotoxic mode of action related to monoterpenes substances. Larvicidal and repellent activities of essential oils have been attributed to their major monoterpenic constituents. Also, there is evidence effect on acetylcholinestrase and octopamingeric system in insects as confirmed by[28, 29]. The obtained results show that different nano formulations of the two tested botanical oils have significant effects on the toxicity of the 2nd and 4th larval instars of *A. ipsilon*. Neem nano-formulations were more potent in its larvicidal effect against *A. ipsilon* than peppermint nano-formulations.

However, more information is needed before these toxic effects can be fully explained and understood in Integrated Pest Management (IPM) programs using this plant extract and essential oils.

Acknowledgment: The authors appreciate the role of the National Research Centre in the financial support offered for this investigation.

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 DOI: https://doi.org/10.30564/jrb.v1i1.590