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Increasing the efficacy of the cotton leaf worm *Spodoptera littoralis* nucleopolyhedrosis virus using certain essential oils

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Abstract

Baculoviruses inactivation by UV sunlight is considered one of the most important factors that reduce their potential use in insect management programs. The three antioxidant essential oils, marjoram, orange, and tea tree were tested as protective additives to the multiple nucleopolyhedrosis virus (*SpliMNPV*) against UV. The results revealed that the orange oil was promising for virus protection, while the tea tree oil and marjoram oil did not protect the virus. The results also showed that the pathogenicity of the combination treatment of *SpliMNPV* and orange oil was increased drastically by adding the LC_{50} (1.0%) concentration of the oil to the virus concentrations. Consequently, the LC_{50} of the mixture was reduced to 5.3×10^3 PIB/ml compared to 3.2×10^5 PIB/ml concentration of virus alone. Concomitantly, the ovicidal and larvicidal effects of orange oils were determined. The results suggested that the combination treatment of orange oil and *SpliMNPV* could be used as an effective method for the cotton leaf worm, *Spodoptera littoralis* (Boisd.), management.

Keywords: *SpliMNPV*, Adjuvant agent, UV irradiation, Essential plant oils, Protective additives

Background

Baculoviruses are one of the most environmental safe bio-pesticides for lepidopteran pests. They are unique of the viruses groups that exclusively infect insect pests and often are highly specific at species levels causing epizootics in the populations of target insects. (Sayed and El-Helaly 2018). The cotton leaf worm *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae) multiple nucleopolyhedrosis virus (*SpliMNPV*) is one of the Baculovirus groups that is specific for *S. littoralis* and can be used effectively in its management. However, it is severely affected by the UV radiation of sunlight, which leads to pyrimidine dimer formation in the viral DNA chains and consequently reduces the *SpliMNPV* persistence in the field (Jones et al. 1993 and Yoon et al. 2000). Despite many trials that have been attempted to protect the viral activity, the search for new effective technique in this

respect is continuously required. Shapiro et al. (2009b) tested 13 plant phenolics for protecting *Spodoptera exigua* (Hubner) nucleopolyhedrovirus (*SeMNPV*). Moreover, 20 naturally derived antioxidants were suggested for *SpliMNPV* protection (El-Helaly et al. 2013). Evidently, sub-lethal concentrations of insecticides could increase the efficacy of NPV pathogenicity and act as adjuvants for virus infectivity, for instance Neem (Shapiro et al. 1994), thiamethoxame and imidacloprid (Trang and Chaudhari 2002), and Spinosad (Jackson et al. 2014). In this context, it can be suggested that the use of an agent to protect the virus degradation by UV light that has insecticidal activity as well as being environmentally safe may make the bio-control strategy of *S. littoralis* more effective and promising. Recently, the essential plant oils have been shown a broad spectrum of actions to insect responses, as many of such oils have been defined as antioxidants with different degrees (Miguel et al., 2010); additionally, they may possess low to high anti-arthropods action against insect pests with a low risk to mammalian (Tripathi et al. 2009 and

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Regnault-Roger et al. 2012). The three essential plant oils, orange, tea tree, and marjoram, have been provided as antioxidant substrates (Bakkali et al. 2008). Interestingly, the toxic activities of orange oil was reported by Raina et al. (2007) on Subterranean termites. Moreover, the plant tea oil was recommended as larvicide of *Sitophilus zeamais* (Mot.) (Liao et al. 2016). Furthermore, the marjoram proved to be effective against *Sitophilus oryzae* (Linn.) and *Tribolium castaneum* (Herbst) (Padin et al. 2000).

The present trial aimed to evaluate the use of three commercial plant oils, orange, tea tree, and marjoram, as adjuvants to *Spli*NPV activity for lepidopteran pest control.

Materials and methods

Insect, virus, and material

The cotton leaf worm, *Spodoptera littoralis* (Boisduval), was established at the laboratory conditions of $25 \pm 2^\circ\text{C}$ and $65 \pm 5\%$ R.H. Newly hatched larvae were reared on semi-artificial diet (Shorey and Hale 1965). Pupae were collected in plastic cups until adult emergence. Adults were transferred into a chimney glass provided with sugar syrup 10% and tissue for laying eggs.

Original Egyptian local isolate of *S. littoralis* multiple nucleopolyhedrovirus (*Spli*MNPV) by Abul Nasr (1956) was used in all/separate experiments. The pathogenicity of *Spli*MNPV was evaluated on the second instar larvae of *S. littoralis*. Eight different virus concentrations 0.0, 1×10^3 , 5×10^3 , 1×10^4 , 5×10^4 , 1×10^5 , 5×10^5 , 1×10^6 BIPs/ml (polyhedral inclusion bodies/ml) were prepared from the stock concentration (2.3×10^9 PIB/ml), and it was diluted in distilled water. The tested additives orange, marjoram and tea tree oils were provided from the local market (Haraz Group Co., LTD). Bioassays of the three essential oils were carried out using different concentrations and prepared in distilled water and Tween 80.

UV protection and prolonging viral activity

The three tested oils, at 5% concentration, were added to *Spli*MNPV 1×10^8 (concentration of LC_{90-95} to neonate larvae). Larvae were exposed to ultraviolet as an artificial source of light (Utalix; 23 sun UV, Germany). This light was fixed in a hood with 50 cm distance away from glass Petri dishes containing virus with different additives. Treatments were at different exposure periods (zero time, 30, 60, 90, and 120 min). Collected dishes were washed by distilled water and then collected virus was poured placed on the surface of a semi-artificial diet in 5 replicates. Each replicate was provided with 10 newly hatched larvae and observed till death or pupation (Shapiro et al. 2008).

Bioassay experiments

The impact of the tested additives for virus protection experiments was tested. The toxicities of either orange oil alone or in combination with *Spli*NPV on the eggs and larvae of *S. littoralis* were determined, while the tea tree and marjoram oils were eliminated for additional further experiments.

Impact of orange oil odor on egg stage

The direct effect of oil odor on eggs was also investigated. A small piece of cotton was fully saturated with different orange oil concentrations (1, 2, 3, 4, and 5%). Each concentration was represented in 15 plastic cups, in three groups. Each group contains five replicates: in the first group, the cotton saturated with the concentration was removed after 24 h, then 48 and 72 h in the second and third groups, respectively. These eggs were observed for hatching and viability on semi-artificial diet up to fifth day of larvae. Larvae were counted and the death percentage was calculated.

Impact of orange oil on the larval stage

The sensitivity of the neonate, second and fourth larval instars, to different oil concentrations (1, 2, 3, 4, and 5%) were conducted. One milliliter of each concentration was provided to the surface of poured semi-artificial diet in plastic cups ($3 \times 3 \times 6$ cm), following the diet surface contamination technique. The process was repeated five times for each tested instar. Larvae were observed till pupation and mortality rates were recorded.

Combination treatments

The concentration–mortality responses of the either mixture (*Spli*MNPV and orange oil) or individual treatments were carried out following the diet surface contamination technique (Cisneros et al. 2002). The combination treatments (*Spli*MNPV and orange oil) were conducted using a mixture of *Spli*MNPV concentrations with the LC_{25} of orange oil and also vice versa, following the procedure of the methods mentioned above. The mortality responses of the larvae were recorded daily in each treatment. Evaluation of the combined treatment compared to the single ones was identified according to (Mansour et al. 1966) the following: Co-toxicity factor (%) = $(\% \text{ Observed mortality} - \% \text{ Expected mortality}) / \% \text{ Expected mortality}$, where the factor (-20 to $+20$) is additive, $+20$ or more is potentiation, and -20 or more is antagonism.

Biological parameters

The impact of orange oil concentrations on the biological parameters of treated *S. littoralis* second instar larvae was evaluated. Larval and pupal durations were recorded. The percentages of pupation, pupal

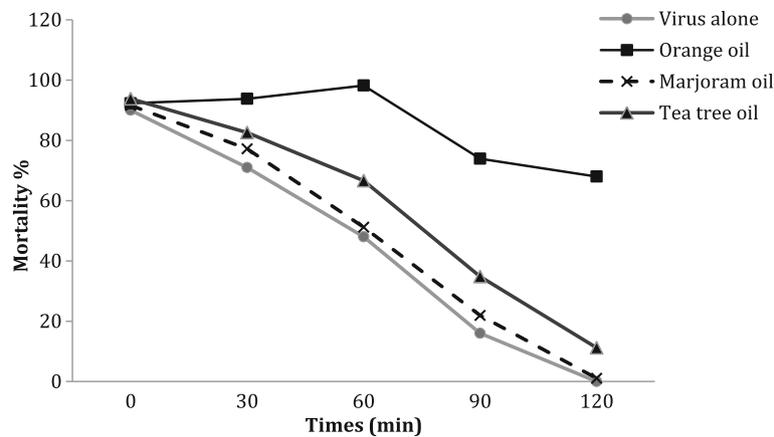


Fig. 1 Effect of UV irradiation on either *SpliMNPV* or in combination with orange, marjoram, and tea tree oils

malformation, and adult emergence were observed. Moreover, the adult longevity was determined. Each treatment was replicated five times, and every replicates contained 50 individuals.

Statistical analysis

The percentages of original activity remaining (% OAR) were carried out according to Muro and Paul (1985) and were calculated as the formula of Sun's model at each UV exposure time (Sun et al. 2004). The results were collected and analyzed according to Ignoffo and Batzer (1971) with the following formula: % OAR = $100 \times e^{-r \times t}$; 95% confidence limits of r as relative rate of inactivation ($h - 1$) were compared between LdMNPV - 45/0 and - 27/0. The half-life of each isolate was calculated from the following formula: $\tau_{1/2} = \ln(2)/r$, and its 95% confidence limit were compared to reveal significant differences. The mortality responses of several treatments in either applying *SpliMNPV* or plant oils were tested using the Probit analysis; slope and LC_{50} were calculated according to LDP line ([http:// www.ehabsoft.com](http://www.ehabsoft.com)). Statistical 12.0, Sigma Plot 12.5, and Graph Pad Prism 5 software were used. The data of biological studies were analyzed by analysis of variance (ANOVA) and the means were analyzed using Tukey's multiple range test ($P = 0.01$) (Steel and Torrie 1960).

Results and discussion

Impact of tested oils on UV protection

Inactivation of *SpliMNPV* was shown at 30-min exposure time either alone or combined with tea tree and marjoram oils, where the mortality percentages were significantly decreased as the UV exposure times increased. Orange oil was the highly effective in prolonging *SpliMNPV* activity Fig. 1. Meanwhile, the mortality percentages at 120-min UV exposure time were drastically lower (0.0 and 1.07) in *SpliMNPV* alone and

SpliMNPV + marjoram oil, respectively, than in *SpliMNPV* + tea tree oil and *SpliMNPV* + orange oil (11.2 and 68%, respectively). The data of original activity remaining (% OAR) in Table 1 demonstrated that the combination of *SpliMNPV* with orange oil was higher % OAR (73.67%) than in the combinations with tea tree oil, marjoram oil, and *SpliMNPV* alone (11.93, 1.16, and 0.0% OAR, respectively) at 120-min UV exposure time. The loss of the potency of the *SpliMNPV* either alone or with both tea tree and marjoram oils was associated with the theoretical of the direct effects of UV light on viral DNA (Ignoffo et al. 1989 and Douki et al. 1999) and/or on polyhedral protein surrounding the viral DNA (Ignoffo et al. 1977 and Eischeid and Linden 2011). The variations of *SpliMNPV* activation by tested antioxidant oils are in line with those investigated by Shapiro et al. (2009a) for using plant extracts against UV radiation to protect *S. exigua* nucleopolyhedrovirus (*SeMNPV*). They ranged those natural products from weak to excellent. Despite the water-derived material of green tea recommended as a highly effective protectant (El-Helaly et al. 2013), the tea tree oil in the present study was ineffective for viral protection. Furthermore, the marjoram oil had a weak effect as a protectant for viral inactivation; however, it was described as a highly

Table 1 (% OAR of the effect of orange, marjoram, and tea tree oils as protective additives to *Spodoptera littoralis* nucleopolyhedrovirus

Experimental time/min	<i>SpliMNPV</i>	<i>SpliMNPV</i> + essential oils		
		Orange	Marjoram	Tea tree
Zero time	–	–	–	–
30 min	79.33	100.00	84.34	87.96
60 min	53.33	96.64	55.96	64.52
90 min	18.01	80.07	23.95	37.06
120 min	0.00	73.67	1.16	11.93

Table 2 Percentages of unhatched *Spodoptera littoralis* eggs in three exposure periods to different concentrations of orange odor

Experimental time/hours	Orange odor concentrations (%)					
	0.0	1.0	2.0	3.0	4.0	5.0
24	3.0 a	4.23 b	5.21 a	7.24 b	8.25 b	10.24 b
48	2.9 a	5.27 b	8.24 b	9.78 ab	9.77 b	12.37 b
72	2.8 a	14.25 a	16.24 c	17.28 a	20.21 a	30.74 a
LSD	0.24	5.9	4.41	8.38	7.18	12.019
P	0.0342	0.016	0.000	0.0072	0.0004	0.003

effective antioxidant agent (Hossain et al. 2012), for which it could be suggested that the plant essential oils may have distinct characteristics as protectant agents against UV radiation. In contrast, obtained results revealed that orange oil had a high effect on virus tolerance against UV. This effect may be due to two high actions of orange oil: its antioxidant activity (Jayaprakash et al. 2008) and toxicity properties (Tripathi et al. 2009). From the aforementioned results, it can be concluded that the orange oil was a distinct protective additive much more than both the tea tree and marjoram oils, which were negative additives.

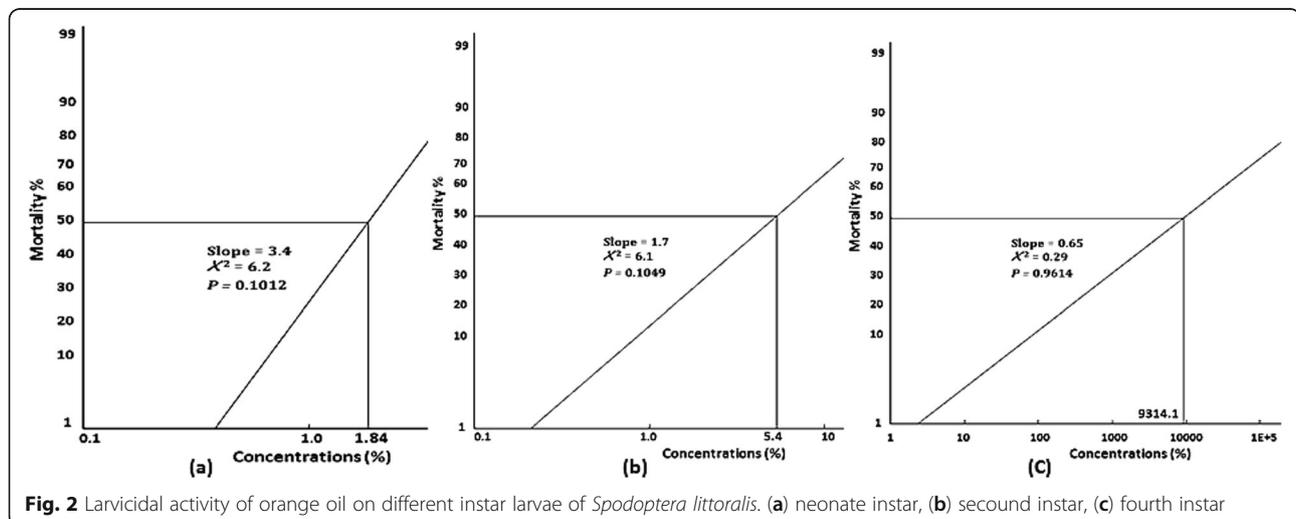
Ovicidal activity of orange oils

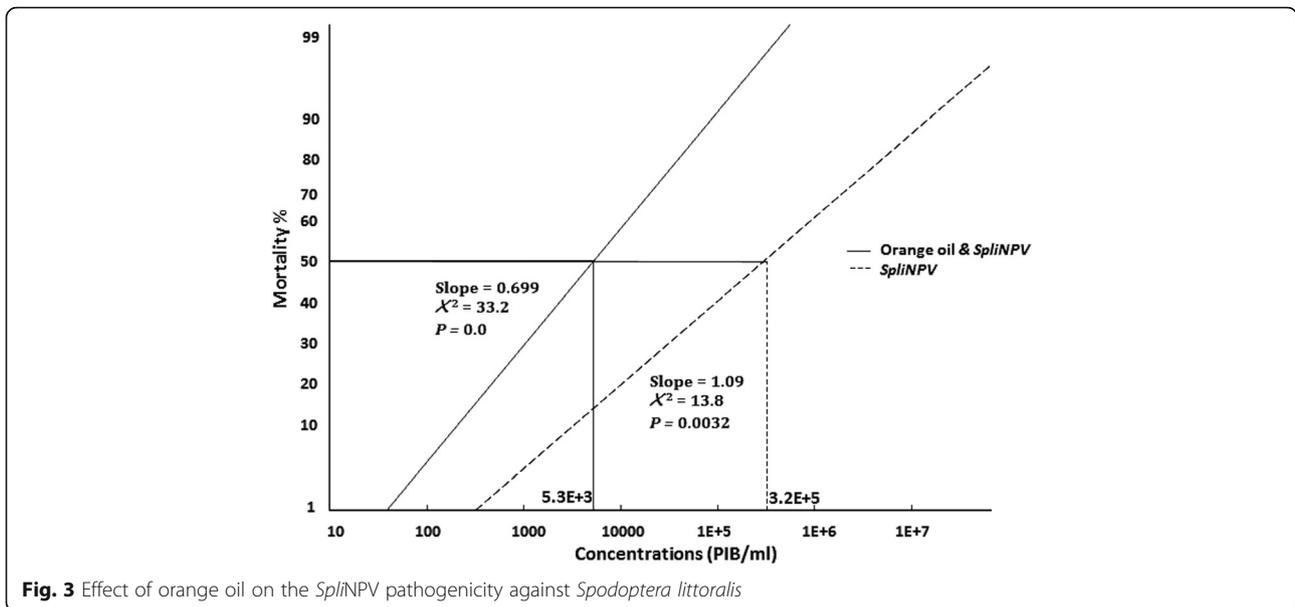
Reverse relationships were noticed in egg hatching with both exposure periods and orange oil odor concentrations (Table 2). The highest percentage (30.7%) of unhatched eggs was observed at the longest exposure time (72 h) and the highest concentration (5%) of orange oil odor, while the lowest percentage (4.23%) was provided in the case of 1% concentration and 24-h exposure time. The obtained results indicated that the percentages of unhatched eggs of 72-h exposure periods were increased

significantly than the 24 and 48 h, while the percentages of 48 h were insignificantly increased than the 24-h exposure time at all concentration treatments. The low ovicidal activity of orange oil was contradicted to that of Phasomkusolsil and Soonwera (2012) who found high mortality rates (56.8, 53.6, and 76.3%) in the egg of the mosquitos species, *Aedes aegypti*, *Anopheles dirus*, and *Culex quinquefasciatus*, respectively, after exposure to 5% orange (*Citrus sinensis*) oil concentration at 3-h exposure time. The ovicidal activity of other plant oils has been attempted on various insect pests; for instance, *Allium sativum* failed the *S. litura* eggs to hatch (Gurusubramanian and Krishna, 1996) and the cinnamon, clove, and cardamom oils, which achieved 100% inhibition of *Tribalium castaneum* eggs at 5.769 mg/l air concentration and 24-h exposure period (Mondal and Khalequzaman 2009). Moreover, *T. confusum* and *Ephestia kuehniella* eggs were completely unhatched by cumin and anise oils treatments (Isikber et al. 2009). The ovicidal action of oils may be due to one or more of the following mechanisms: oxygen depletion for embryonic respiration, biochemical processes, hormonal suppressions, and/or blocking of the micropyle region of the egg chorion (Tunc et al. 2000).

Larvicidal activity of orange oils

The sensitivity of the neonat and the second and fourth larval instars against orange oil was obviously different, as the LC_{50} (9314.1) of the fourth instar larvae was drastically higher than those calculated (5.4 and 1.84) of the second and neonate larval instars, respectively (Fig. 2). The obtained results revealed that the fourth instar larvae were tolerant to orange oil, while the neonate ones were more sensitive than the second instar larvae in response to orange oil concentrations. The toxicity effect





of orange oil upon insect pest may be due to the high percentage of limonene component (92–96%) in its constituents. Limonene component was reported as a toxic agent against some insect pests and usually used as insecticide to control ectoparasites of pet animals (Raina et al. 2007). The findings of the present study coincide with those recorded on mosquitos concerning larvicidal activity of orange oil (Murugan et al. 2012). Similarly, the orange oil was reported as a toxic agent on stored product insect pests (Don-Pedro 1996) *Ceratitis capitata* (Wied.) (Papachristos et al. 2009) and *Musca domestica* (Linn.) (Tarelli et al. 2009).

Combination treatment of orange oil and *SpliMNPV*

The mortality response of *SpliMNPV* was increased drastically by adding the LC₂₅ (1%) of orange oil, where the LC₅₀ of the mixture was reduced to 5.3×10^3 PIB/ml concentration than 3.2×10^5 PIB/ml concentration of the virus alone (Fig. 3). The co-toxicity factor, calculated in this study, revealed that the mixture of 1.0% orange oil and *SpliMNPV* 10^3 , 10^4 , 10^5 , 10^6 PIB/ml concentrations were 75, 75.8, 45, and 38%, respectively. These percentages indicated a potentiation effect. On the other hand, when adding the sub-lethal concentration of *SpliMNPV* (1×10^3 PIB/ml) to the orange oils, the

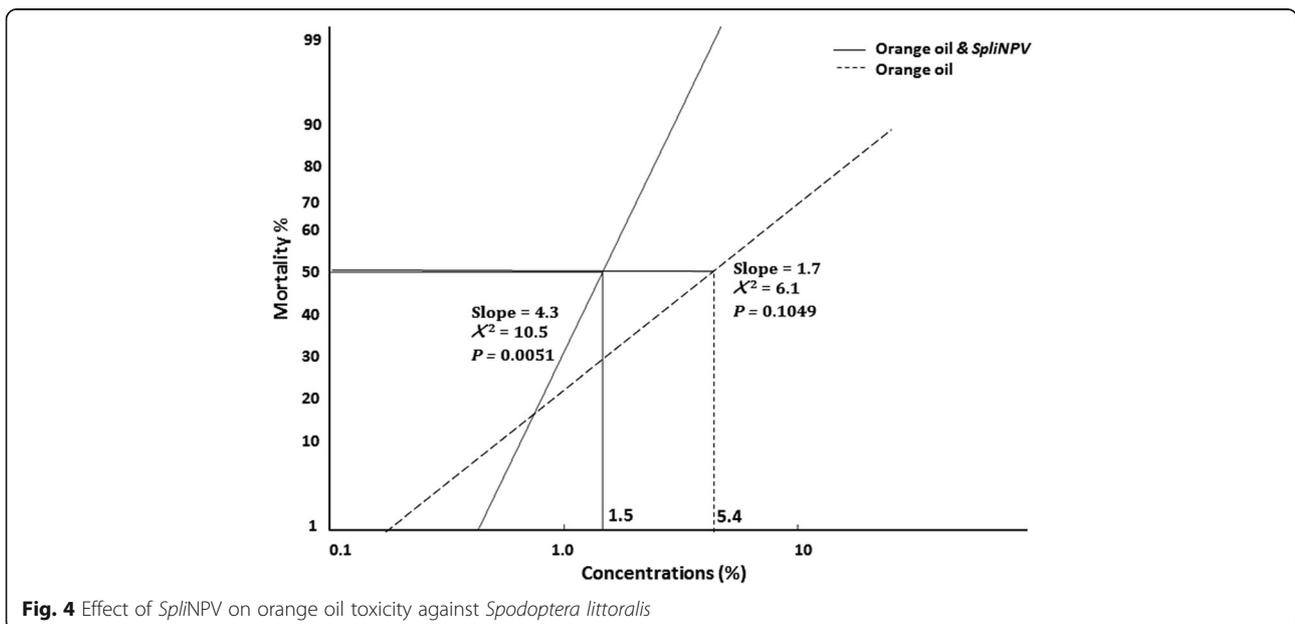


Table 3 Effect of orange oil concentrations on certain reproductive biology of *Spodoptera littoralis* treated as second instar larvae

Concentration (%)	(Avg.) Larval duration	(%) Pupation	(%) Malform-ation	(Avg.) Pupal duration	(%) Adult emergence	(Avg.) adult longevity
0.0	20.4 d	85.0 a	1.0 c	14.6 a	85.8 a	10.2 ab
1.0	27.4 b	84.1 a	1.2 c	15.2 a	86.2 a	9.8 b
2.0	29.3 bc	84.0 a	1.4 c	14.8 a	85.6 a	9.8 b
3.0	26.9 bc	80.2 b	1.8 c	15.0 a	81.0 b	10.3 ab
4.0	28.0 c	76.6 c	6.8 b	14.4 a	80.8 b	11.2 a
5.0	34.0 a	70.2 d	9.4 a	16.2 a	75.4 c	11.4 a
LSD	1.99	2.52	1.48	1.48	2.24	0.968
P	0.00	0.000	0.000	0.0345	0.000	0.0001

mortality percentages were increased by low LC₅₀ (1.5%) than the LC₅₀ (5.4%) of orange oil alone (Fig. 4). The co-toxicity factor of this combination treatment indicated a potentiation effect. The calculated co-toxicity factors were 53, 62, and 72% at the orange oil concentrations of 1, 2, and 3%, respectively with the mixture of 1×10^3 PIB/ml *SpliMNPV* concentrations. The obtained results of orange oil as a synergistic agent of NPV infectivity may be referred to the direct effect of orange oil on the cell membrane permeability, and/or on the hormonal insects and the immunity system (Tripathi et al. 2009). The positive relationship of the *SpliMNPV* pathogenicity and orange oil is in line with several investigations of increasing the efficacy of NPV infectivity in combinations with different adjunct substances, such as *Chlorantrani liprole* with *Helicoverpa armigera* NPV (*HaSNPV*) (Wakil et al. 2012), Azadirachtin with *SpliMNPV* (Shaurub et al. 2014), and Spinosad with *Ag MNPV* (Jackson et al. 2014).

Effect of orange oil on reproductive biology

It is obvious that the larval stage was the most sensitive one than the other stages (pupae and adults), where it became too long as a latent effect of orange oil treatments (Table 3). Meanwhile, the averages of larval duration were significantly longer (27.4, 29.3, 26.9, 28.0, and 34.00 days) in 1, 2, 3, 4, and 5% orange oil concentrations, respectively, than in the control treatment (20.4 days). These results demonstrated that the larvae were very sensitive to the orange oil treatments. This prolongation of larval period may help for increasing the efficacy of Baculovirus activity. Nonetheless, the malformations of pupae resulted from the treated larvae were significantly higher in 4 and 5% concentrations than in 0, 1, 2, and 3% concentrations. As depicted in the same table, there were insignificant differences in the pupal durations among the concentration treatments. Moreover, the data revealed that both pupal and adult emergence percentages were significantly increased at 3, 4, and 5% concentrations than at 0, 1, and 2% concentrations. The aforementioned results revealed that the

orange oil had a negative impact on some biological properties at all tested concentrations. This influence on reproductive biology of treated larvae may be due to the direct action of orange oil on the physiological balance and hormonal changes of the insect. These results are in accordance with Bakkali et al. (2008) and Sharifi-Rad et al. (2017) who reported negative impacts of essential plant oils on insect biological parameters which suppress the population of target pest.

Conclusions

The activity of the entomopathogenic agent *SpliMNPV* by using plant essential oil toxicity actions was studied. Orange oil was excellent for virus protection against UV irradiation, while the tea tree oil was weak and the marjoram oil has negative effect. The orange oil was highly toxic against *S. littoralis* larvae either alone or in combination with *SpliMNPV*. Using the combination of Baculovirus and orange oils may help in developing the lepidopteran pest management strategy.

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Authors' contributions

Authors HE and AE carried out the bioassay study. AE conducted the isolation and propagation of the Baculovirus and prepared the virus suspension. AE and WS carried out the biological studies and statistical analysis. HE, AE, and WS contributed in the experimental design and writing of the manuscript. All authors read and approved the final manuscript.

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The authors declare that they have no objection to the availability of data and materials.

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Consent for publication

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Competing interests

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