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Proceeding Paper

Novel Formula as Mosquito Larvicide †

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Abstract: Background: Natural products derived from plants and secondary metabolites from microorganisms are promising in the discovery of synthetic analogs with improved efficacy, potency, and safety. Our study attempts to examine the effect of a new formula as a mosquito larvicide. Methods: Isolation and characterization of prodigiosin and essential oil from *Thuja orientalis* and purification of PDG. The dose response bioassay, the synergistic effect, and the mode of action are investigated for each preparation. Results: The treatment of the 3rd larva stage of *Cx. pipiens* reveals that the LC_{50} of PDG and *T. orientalis* leaves' E.O are 39.5 ± 0.341 ppm and 102.9 ± 0.46 ppm, respectively, after 24 h. The combination of LC10 of PDG with LC25 and LC50 of the E.O. shows a synergistic effect resulting in 33.3% and 100% death, respectively. Individual and combination treatment show a reduction in the activity of acetylcholine esterase, total protein, and AChE specific gravity as compared to the untreated 3rd larva stage of *Cx. pipiens*. PDG and E.O. result in a reduction in midgut pH leading to cellular respiration inhibition as compared to untreaded larvae that show alkaline medium. Conclusions: PDG and the *T. orientails* leaves' oil combination show a promising synergistic potency against the 3rd larva stage of *Cx. pipiens*.

Keywords: *Culex pipiens*; essential oil; larvae; leaves; prodigiosin; *Thuja orientalis*

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1. Introduction

Vector-borne diseases account for more than 17% of all infections, causing more than 1 million deaths annually [1]. Mosquitoes are responsible for the transmission of many medically important pathogens such as viruses, bacteria, and parasites, which cause serious diseases such as malaria, dengue, West Nile virus, yellow fever, encephalitis, filariasis, and Zika fever [2,3]. Mosquito-borne diseases can be prevented by several methods, including chemical and biological techniques, as well as genetic control, environmental management, and personal protection [4]. Pesticides pose a potential risk to humans and unwanted side effects to the environment [5]. Annually, approximately 1 million deaths worldwide and diagnoses of chronic diseases are due to the poisoning effect of pesticides [6]. Natural products represent one of the critical sources of chemical diversity and potential medicinal use [7].

Natural products derived from plants, animals, and microorganism fermentation have medicinal importance and pharmacological activity in treating different diseases [8,9]. Prodigiosin (PDG) is one of the most studied bioactive pigments of microbial origin normally produced by *Serratia marcescens* (SM), Pseudomonas magnesorubra, Vibrio psychroerythrous, and other bacteria. SM, a Gram-negative Entero bactericeae has received

attention because of triarylmethane, a naturally occurring dark red pigment [10]. Prodigiosin revealed a broad range of inhibitory activities against many bacterial, fungal, and protozoan species [11]. In addition, essential oils have an aromatherapy effect to cure or prevent diseases, infection, and indisposition by means of inhalation in controlling the central nervous system [12]. Furthermore, they have anti-parasitic, antibacterial, fungicidal, relaxant, stimulating, and antidepressant effects [13]. *Thuja orientails* (*T. orientails*) exhibits extensive biological activities including anticancer, antiepileptic, anti-inflammatory, antibacterial, antifungal activities, hair growth-promoting, antiviral, antiallergic, antioxidant, and molluscicidal [14–16]. Therefore, the present work aims to study a novel formula as mosquito larvicidal.

2. Materials and Methods

2.1. Stage 1: Preparation, Characterization, Purification, and Identification of Prodigiosin

Under aseptic conditions, *S. marcescens* was inoculated and incubated at shaking conditions for 24 h at 28–30 °C; then, it was inoculated in peanut media and kept in shaking condition for 48–72 h at 28–30 °C. It was finally subjected to Fermentor, with inoculum size 3%*30 = 90 mL. Furthermore, pH = 7, agitation = 400 rpm, and aeration was the maximum aeration [17,18]; then, PDG was extracted later by alkaline medium. The crude PDG was purified through n-hexane: ethyl acetate (2:1) as a solvent. The yield was identified by UV-visible spectrophotometry in the range 400–700 nm in absolute ethanol to find the maximum absorption spectra against methanol as a blank [19]. Then, the pigment was purified by preparative HPLC using C18 column (2.5 × 10 cm) with a flow rate of 0.8 mL/min and an injection volume of 10 µL. The mobile phase used was acetonitrile/HPLC water (60:40). Then the FT-IR spectrum of the pigment was recorded with a test case Shimadzu FT-IR spectrophotometer at 800–4000 nm. Finally, application of the purified PDG fractions and the standard on TLC.

2.2. Stage 2: Preparation and Characterization of the Essential Oil Isolated from T. orientalis

Fresh leaves of *T. orientalis* were collected from Anotoniadis Botanical garden in Alexandria, Egypt in August 2019. The plants were authenticated by Dr. Hesham Ali, Antoniadis Research Center, and specimens were deposited at the Herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Alexandria University. Essential oil (E.O.) was prepared by water-steam distillation [20]. An FT-IR spectrum of the E.O. has been recorded with a Tests can Shimadzu FT-IR spectrophotometer at 800–4000 nm. Then, its constituents were profiled by GC-MS. E.O. was diluted in diethyl ether and 0.5 L was injected into the gas chromatography (GC–MS) apparatus. The GC column was a 30 m (0.25 mm i.d., film thickness 0.25 _m) HP-5MS (5% diphenyl) dimethylpolysiloxane capillary column. The GC conditions were as follows: injector temperature, 240 °C; column temperature, isothermal at 70 °C and held for 2 min; then, it was programmed to 280 °C at 6∘C/min and held at this temperature for 2 min; ion source temperature, 200 °C; detector temperature, 300 °C. Helium was used as the carrier gas at the rate of 1 mL/min. The effluent of the GC column was introduced directly into the ion source of the MS. Spectra were obtained in the EI mode with 70 eV ionization energy. The sector mass analyzer was set to scan from 40 to 400 amu for 5 s. The oil components were identified by comparison of their retention indices and mass spectra with the NIST Mass Spectral Library and the refractive index (RI) of the crude E.O. This was determined by a refractometer.

2.3. Stage 3: The Fourth Stage: Maintaining the Mosquito by Rearing the Culture of Culex Pipiens

Larvae and pupae of *Cx pipiens* were purchased two times from the Institute of Medical Insects in El-Dokki, Cairo-Egypt. Upon reaching the laboratory of Vector Control and Pesticide Risks, HIPH, the larvae, and the pupae were reared under laboratory conditions. The rearing of the aquatic stages was on bread. That of the adult males was on glucose 30%, and the females on blood meals from pigeons. All that was maintained under specific

conditions including temperature (26 ± 2 °C) and RH (70 ± 5%), and water was replaced every two days.

2.4. Stage 4: The Fifth Stage: Dose-Response Bioassay Separately PDG and E.O. as Mosquito Potential

Ten larvae were introduced into a conical flask containing 50 mL dechlorinated water and treated with PDG and E.O. in different ppm concentrations based on preliminary screening results (0, 20, 30, 40, 50, and 60, and 0, 25, 50, 75, 100, and 150 for E.O).

2.5. Stage 5: Investigation of the Synergistic Effect of PDG with the E.O. as Mosquito Larvicidal Potential

A combination of LC_{10} from PDG with LC_{25} and LC_{50} from the E.O. was applied and replicated three times. The mortality of the larvae was recorded after 24 h. The larvae were observed for any movement and considered as dead when they did not show any movement even after pin press; they were then collected.

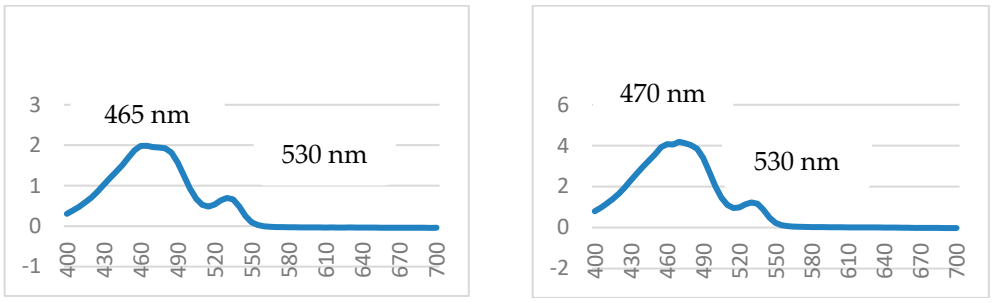
2.6. Stage 6: The Seventh Stage: Investigating the Mode of Action of PDG and E.O. for Mosquito Larvicidal Potential

The AChE was determined using the anticholinesterase activity Kit of Cholinesterase (BTC/DTNB), Biochemical Enterprise [21,22]. Total protein was determined using the Kit of VITRO SCIENT and Biuret colorimetric endpoint method [23]. Finally, the midgut pH medium and bromothymol blue dye, respectively.

3. Results and Discussion

3.1. Isolation, Purification, and Characterization of PDG from SM

Figure 1 shows that the crude PDG was identified by UV-visible spectrophotometry in the range 700–400 nm and the maximum UV absorbance was observed at 530 nm for both batch scale and bioreactor samples; this was in agreement with previous studies done by Patil et al., Song et al., and Nakashima, Kurachi, Kato, and Oda [19,24,25] for purified PDG from *Serratia* sp. KH −95. Higher absorption rates were detected for purified red pigment extracted from solutions of two bacteria strains (PNSRR and PNSHR), which had been run in the range of 200 to 700 nm. The maximum absorption peaks were detected at 540 nm and 541 nm for PNSRR and PNSHR, respectively [26]; that was equivalent to what was detected by Kumar and Aparna, 2014 [27].



(**a**) (**b**)

Figure 1. Shows the UV-visible spectrophotometry in the range 400–700 nm for the crude PDG: (**a**) PDG batch scale; (**b**) PDG fermenter (bioreactor).

The purity of the red pigment was identical to that of the standard PDG by TLC application (Figure 2). In addition, HPLC profiling showed a single peak at 536 nm for both the column chromatography purified pigment and the standard PDG, and retention times (R_f) were 4.827 min and 4.963 min, respectively (Figure 3). In agreement with our results, Kumar and Aparna, 2014 showed that >95% purity of a single peak was observed at R_f > 4 min (4.523 min) [27]. In contrast, Mandal et al. revealed that the R_f went until

4 min, and no sharp peaks were observed after 4 min [26]. The application of TLC for the identification of the purified red pigment in the present study revealed that the best mobile phase was n-hexan:ethyl acetate (*v*/*v*) (2:1) and the R_f of the tested sample was equal to that of the standard; this confirms that the tested sample is PDG. Mandal et al. reported that the R_f values of the extracted pigments were found to be approximately 0.88 after using methanol, chloroform, and hexane in the ratio of 7:3:1 [26].

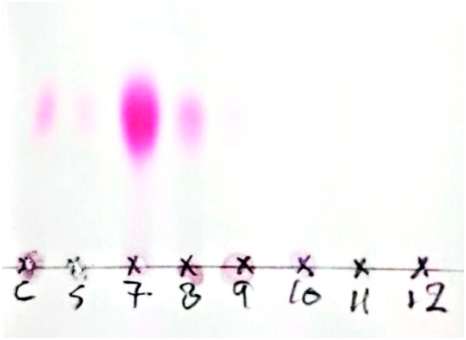


Figure 2. Application of TLC of the purified PDG compared with the standard.

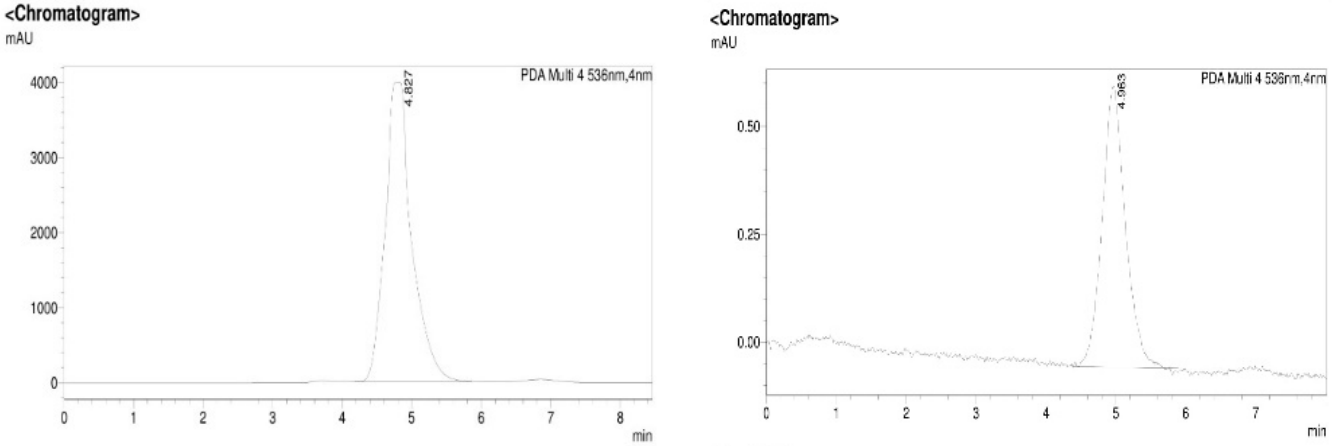


Figure 3. HPLC for the: (**a**) Purified red pigment; (**b**) Standard PDG.

Figure 4 shows that the FT-IR absorption in ethanol for the studied red pigment was dominated by very strong bands at 2921.5 cm^{-1} and 2851.1 cm^{-1} (aromatic CH). The fingerprint region for the red pigment was characterized by medium intensity bands at νmax 1609.3 cm^{-1}, 1362.4 cm^{-1}, 1265.1 cm^{-1}, 1040.9 cm^{-1}, and 955.1 cm^{-1}. In contrast, lower intensity bands were observed at νmax 2373.9 cm^{-1}, 2341.99 cm^{-1}, 2292.8 cm^{-1}, and 2166.9 cm^{-1} (alkyl C-H). A broad peak pyrrole was observed at 3105.841 cm^{-1}, 3274.8 cm^{-1}, and 3427.0 cm^{-1}, due to the presence of protonated nitrogen; this confirms that the pattern of the red pigment is identical to the standard. In addition, the FT-IR of the red pigment was matched with that of the standard and this confirms that the red pigment is PDG ($C_{20}H_{25}N_3O$). In agreement with our study, Patil et al. revealed that the absorption was dominated by very strong bands at 2977 cm^{-1}(aromatic CH) and 1648 cm^{-1} (aromatic C=C). Furthermore, he reported that PDG exhibits similar absorptions in CHCl3 at 1630 and 1602 cm^{-1}, except that the relative intensities are reversed and the first band is possibly a pyrrolenine (C=N). In addition, the fingerprint region for the red pigment was characterized by medium intensity bands at νmax 1648 (C=O), 1087, 1047 (C-O and C-N), and 879 cm^{-1}. A strong and broad absorption for NH was evident at νmax 3403 cm^{-1}. This indicates that this pigment pattern is identical to that of PDG [19].

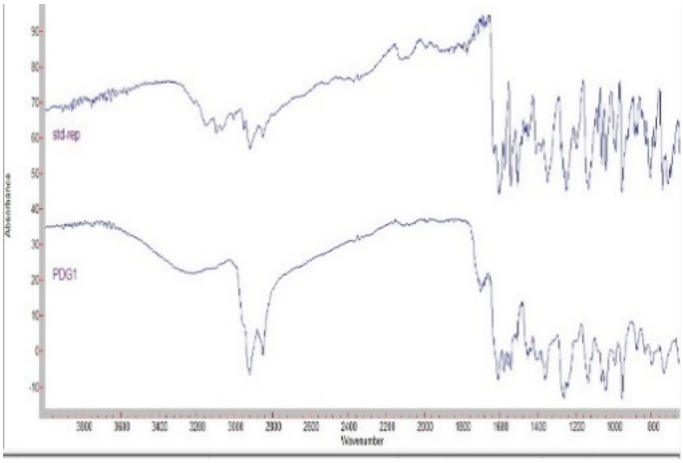


Figure 4. FT-IR spectra of the purified PDG compared to the standard PDG.

3.2. Preparation and Characterization of E.O. from Fresh Leaves of T. orientalis

Water-steam distillation of 750 gm from fresh leaves of *T. orientalis* revealed 3 mL of E.O. That oil was subjected to FT-IR spectroscopy and it revealed different bands with a diversity of intensities, as shown in Figure 5. The E.O. was characterized by GC-MS and it identified 25 constituents. The representative GC-MS revealed that the total chemical composition of E.O. of *T. orientalis* is 97.04%, as shown in Table 1 and Figure 6. FT-IR absorption in ethanol was dominated by very strong bands at 2916.0 cm^{-1}, 2869.2 cm^{-1}, and 2832.0 cm^{-1} (aliphatic CH). The fingerprint region was characterized by medium intensity bands at νmax (1445.0 cm^{-1}, 1220.8 cm^{-1}, and 786.0 cm^{-1}) (C=C). In contrast, a lower intensity band was observed at νmax (1734.4 cm^{-1}) (C=O). In 2014, Al-Ammar recorded that the infrared spectrum of *Thuja* showed the presence of the following groups: –OH and/or –NH2 (3346.27 cm^{-1}) broadband, –CH aliphatic (2665 cm^{-1}), C=O at (1708 cm^{-1}), C=C double bond at (1612 cm^{-1}), –NH (1535.23 cm^{-1}), C– O–C (1078.13 cm^{-1} and 1029.92 cm^{-1}), C–O or –COOH (1448.14 cm^{-1}), and –OCH3 (1195.78 cm^{-1}) [28].

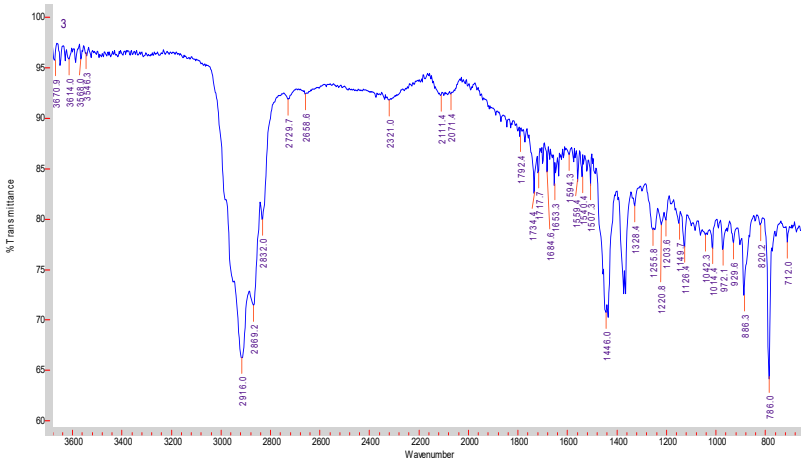


Figure 5. E.O. FT-IR spectrum.

Table 1. GC-MS identification for the constituents of the essential oils.

Monoterpene Hydrocarbons				Oxygenated Monoterpene			
Peak	**RT**	**Constituents**	**%**	**Peak**	**RT**	**Constituents**	**%**
1	6.1	α-Pinene	17.19	8	8.61	p-Menth-2-en-1-ol	9.21
2	6.27	α-Fenchene	1.69	9	9.45	Camphor	0.49
3	6.81	α- Phellandrene	3.94	10	9.51	Citronellal	0.32
4	7.04	β -Myrcene	3.21	11	10.11	α-Terpineol	0.29
5	7.37	3-Carene	30.26	12	10.56	Citronellol	0.26
6	7.65	D-Limonene	7.72	13	11.32	iso- Bornyl acetate	0.37
7	9.9	α-Terpinene	0.97	14	12.11	α-Terpinyl acetate	1.14
Total			64.98	Total			12.08
Sesquiterpene Hydrocarbons				**Oxygenated Sesquiterpene**			
Peak	**RT**	**Constituents**	**%**	**Peak**	**RT**	**Constituents**	**%**
15	12.44	α-Copaene	0.88	23	14.77	Caryophyllene oxide	0.24
16	12.92	Cedrene	0.26	24	14.88	α-Acorenol	0.48
17	12.94	Di-epi- α-Cedrene	1.4	25	15.02	Cedrol	8.87
18	12.98	Caryophyllene	3.67	Total			9.59
19	13.37	α-Humulene	2.87	Total Monoterpene hydrocarbons%			64.98
20	13.59	α-Muurolene	0.49	Total Oxygenated Monoterpene%			12.08
21	13.82	Gurjunene	0.29	Total Sesquiterpene hydrocarbons%			10.39
22	14.08	β -Cubebene	0.53	Total Oxygenated Sesquiterpene%			9.59
Total			10.39	Total			97.04%

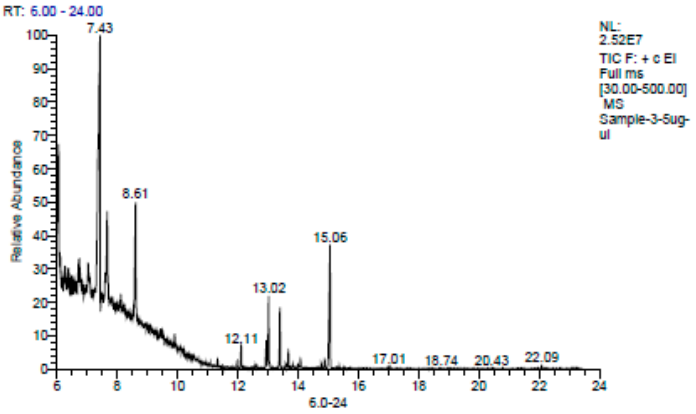


Figure 6. GC-MS analysis for the E.O.

In this study, the yield of the EO was 0.3% and that was in agreement with what was reported by Nickavar et al. [24], where the hydrodistillation of *T. orientalis* leaves gave oils with a yield of 0.25%. Twenty-five compounds of the studied EO were identified by GC-MS, revealing that the total chemical composition of E.O. is 97.04%, constituting 64.98% of monoterpene hydrocarbons, followed by a lower percentage of oxygenated monoterpenes, sesquiterpene hydrocarbons, and oxygenated sesquiterpenes, which were 12.08%, 10.39%, and 9.59%, respectively. The percentages of the main monoterpene hydrocarbons were 3-carene 30.26, α-pinene 17.19, α-phellandrene 3.94, β-Myrcene 3.21, D-Limonene 7.72, α-Fenchene 1.69, and α-terpinene 0.97. The percentages of the main oxygenated monoterpenes were p -Menth-2-en-1-ol 9.21, α-Terpinyl acetate 1.14, Camphor 0.49, α-Terpineol 0.29, Citronellol 0.26, Citronellal 0.32, and iso-Bornyl acetate 0.37. The percentages of the main sesquiterpene hydrocarbons were caryophyllene 3.67, α-Humulene 2.87, Di-epi- α-Cedrene 1.4, α-Copaene o.88, β-Cubebene 0.53, α-Muurolene 0.49, Gurjunene 0.29, and Cedrene 0.26. The main oxygenated sesquiterpenes were Caryophyllene oxide, α-Acorenol, and cedrol, which were 0.24, 0.48, and 8.87, respectively. Ibrahim et al. [29] reported that the percent-

ages of oxygenated compounds, hydrocarbons monoterpenes, and sesquiterpenes in the *T. orientalis* leaves' oil were 29.85%, 44.74%, and 24.35%, respectively. The major components were α - pinene (21.83%), benzyl benzoate (19.12%), caryophyllene (12.07%), and α - cedrol (6.86%). The refractive index of the E.O. was 1.482 nD, revealing high purity of the E.O. This is because its value was in the range of the typical value (1.4785 nD–1.4885 nD) [30]; the higher value (1.5) was detected in 2022 by Rehman et al. [31].

3.3. Dose Response Bioassay Separately of the Preparations

The result of log probit analysis (95% confidence level) recorded that the LC_{50} value of PDG (39.5ppm) showed a high larvicidal rate after 24 as compared to the E.O. (102.9 ppm) (Table 2). In 2002, Metacycloprodigiosin hydrochloride and bafilomycin A1 revealed a significant antimalarial activity; meanwhile, spectinabilin moderately inhibited the proliferation of *P. falciparum* K1 [32]. Jeon et al. [33] reported that the larvicidal activities of leaf oils prepared from *T. orientalis* were significantly higher than those of stem, fruit, and seed oils against 4th-instar larvae of *Ae. aegypti* and *Cx. pipiens* pallens. Leaf oils of *T. orientalis* leaves show promise as activity natural larvicides against *Ae. aegypti* and *Cx. pipiens* pallens. In India (2015), pure PDG showed LC_{50} values 15.6 ± 1.48 and 24.7 ± 1.47 μg mL^{-1} against 3rd instars of *Ae. Aegypti* and *An. Stephensi*, respectively [34].

Table 2. Larvicidal activity of the studied preparations after 24 h against the 3rd larval stage of *Cx. pipiens*.

Larvicide	LC50/(ppm) [1]	95% Confidence Limits		Slope [2] ± SE	Intercept [3] ± SE	(R2) [4]	(χ^2) [5]
		Lower	Upper				
PDG	39.5	29.7	52.5	2.9	0.321	0.946	0.924
EO	102.9	69.9	153.3	2.172	0.623	0.839	0.532

[1] The concentration causing 50% mortality [2] Slope of the concentration-mortality regression line ± standard error. [3] Intercept of the regression line ± SE. [4] The conformity parameter for goodness of fit to the median-effect principle (MEP) of the mass action law. It is a linear correlation coefficient of the median effect plot where R2 = 1 indicates a perfect conformity. [5] Chi square value.

In 2016, it was reported that the larvicidal properties of *Plectranthus barbatus* leaves EO (40, 80, 120, 160, and 200 μg/mL) and their components, such as eugenol, α-pinene, and β-caryophyllene (12–100 μg/mL each) were measured using WHO protocol. EO displayed considerable larvicidal properties with LC_{50} values of 94.3 μg/mL for *Cx tritaeniorhynchus*. The three main components (eugenol, α-pinene, and β-caryophyllene) demonstrated potent larvicidal properties (LC50 = 30.8, 36.8, and 48.2 μg/mL, respectively) [35]. Two years later, Sanei-Dehkordi et al. [36] investigated that the dosage of 80 ppm from *Platycladus orientalis* oil was sufficient to cause 100% larval mortality against the larvae of *Cx pipiens* after 24 h. Forty-six components in leaves of *P. orientalis* were identified. The major components were α-Pinene (20.17%), 3-Carene (14%), and Cedrol (9.51%). The LC_{50} value against *Cx. pipiens* larvae was 18.60 ppm after 24 h; hence, the authors considered E.O. as a natural larvicide for mosquito larval control.

3.4. Investigation of the Combination Effect of PDG with the E.O. as a Mosquito Larvicidal Potential after 24 h

Table 3 shows that the combination between LC_{10} PDG and LC_{50} of E.O. had a high synergistic effect on the mortality rate after 24 h compared with its combination with LC_{25} E.O. (100% and 33.3%, respectively). *Clerodendrum inerme* showed the highest toxicity when tested individually at 24 h against early 4th instar mosquito larvae, *Aedes aegypti*. In contrast, *G. sepium* showed low toxicity (LC_{50} = 292 ppm and LC_{50} = 564 ppm, respectively). The maximum synergistic activities were found in the combination extracts of *Vitex negundo* with *Pongamia glabra* (LC_{50} = 191.73 ppm). These results are significantly more effective than the combination extract ratio of *C. inerme* with *P. glabra* (LC_{50} = 195.02 ppm) and *Gliricidia sepium* with *P. glabra* (LC_{50} = 328.72 ppm) [37].

Table 3. Synergistic larvicidal activity of the LC_{10} of PDG with LC_{25} and LC_{50} of E.O. after 24 h.

Sample	LC10 of PDG with LC25 of Oil	LC10 of PDG with LC50 of Oil
% Death	33.3	100

3.5. Investigating the Mode of Action of PDG and E.O. for Mosquito Larvicidal Potential

Table 4 shows that the highest percentage of AChE arbitrary activity by unit/gm tissue was (5.8%) among untreated 3rd larvae of *Cx. pipiens*, followed by the treated ones with E.O. and PDG (3.5% and 2.5%, respectively). Next were those treated with combination LC_{10} of PDG with LC_{25} and LC_{50} of E.O. (3.8% and 3.0%, respectively). Concerning the total protein in mg/gm tissue, the untreated larvae showed a high percentage (1.32%) compared with E.O. (1.12%), followed by those treated with PDG (0.72%) and those treated with a combination of LC_{10} of PDG with LC_{25} and LC_{50} of E.O (0.92% and 0.52%, respectively). Regarding the AChE arbitrary specific activity, the untreated larvae showed the highest rate (4.39%) as compared to PDG, EO, and treated ones with a combination of LC_{10} of PDG with LC_{25} and LC_{50} of E.O (3.47%, 3.13%, 4.13%, and 0.91%, respectively). Figure 7 shows that the midgut of untreated larvae showed a blue color after 12 h incubation in bromothymol dye; meanwhile, treated larvae showed a yellowish color, indicating reduction in the pH medium. PDG causes reduction in the AChE and the total protein content of the treated larvae. AChE breaks down the neurotransmitter Ach at the synaptic cleft so that the nerve impulse can be transported across the gap. Neurotransmitters must be cleaned immediately after the message is passed, and if not, it causes paralysis [38]. Larvicidal activities of leaf oils prepared from *T. orientalis* were significantly higher than those of stem, fruit, and seed oils against 4th-instar larvae of *Ae. aegypti* and *Cx. pipiens* pallens. Leaf oils of *T. orientalis* leaves show promise as activity natural larvicides against *Ae. aegypti* and *Cx. pipiens* pallens [33]. Purified PDG caused reduction in the activity of AChE and total protein content of the treated larvae by 70% and 43.4%, respectively, compared to the control among the 4th-instar larvae of *Ae. Aegypti* [34]. Bromothymol blue dye is a weak acid and a member of the class of 2,1-benzoxathioles, so it acts as a pH indicator. This reagent is blue in alkaline media, green in neutral media, and yellow in acidic media [39]. In the present study, untreated larvae showed a dark blue color by a stereomicroscope, in contrast to the midgut of the treated larvae with PDG, E.O, and their combination, which showed a reduction in the pH. In agreement with this, Suryawanshi et al. [34] reported that the larval midgut of PDG treated larvae showed a greenish yellow color, suggesting acidic pH; in contrast, untreated larvae showed up as blue, indicating the basic condition. As reported by Zhuang et al. [40], this may be attributed to the hydrophobicity of natural products including PDG and E.O. and their effect as carbonic anhydrase inhibitors, leading to a reduction in pH of the midgut that subsequently results in cellular respiration inhibition [41–43].

Table 4. Biochemical effect of PDG and essential oil of *T.orientalis'* leaves on AChE activity extracted from *Cx. pipiens* larvae.

Treatment	AChE Arbitrary Activity unit/gm Tissue [1] (%) [3]	Total Protein in mg/gm Tissue (%) [3]	AChE Arbitrary Specific Activity [2] (%) [3]
Untreated	5.8	1.32	4.39
PDG LC_{50}	2.5	0.72	3.47
EO LC_{50}	3.5	1.12	3.13
PDG LC_{10} + EO LC_{25}	3.8	0.92	4.13
PDG LC_{10} + EO LC_{50}	3.2	0.52	0.91

[1] OD/minute. [2] OD/minute/mg protein. [3] (treated/untreated) 100.

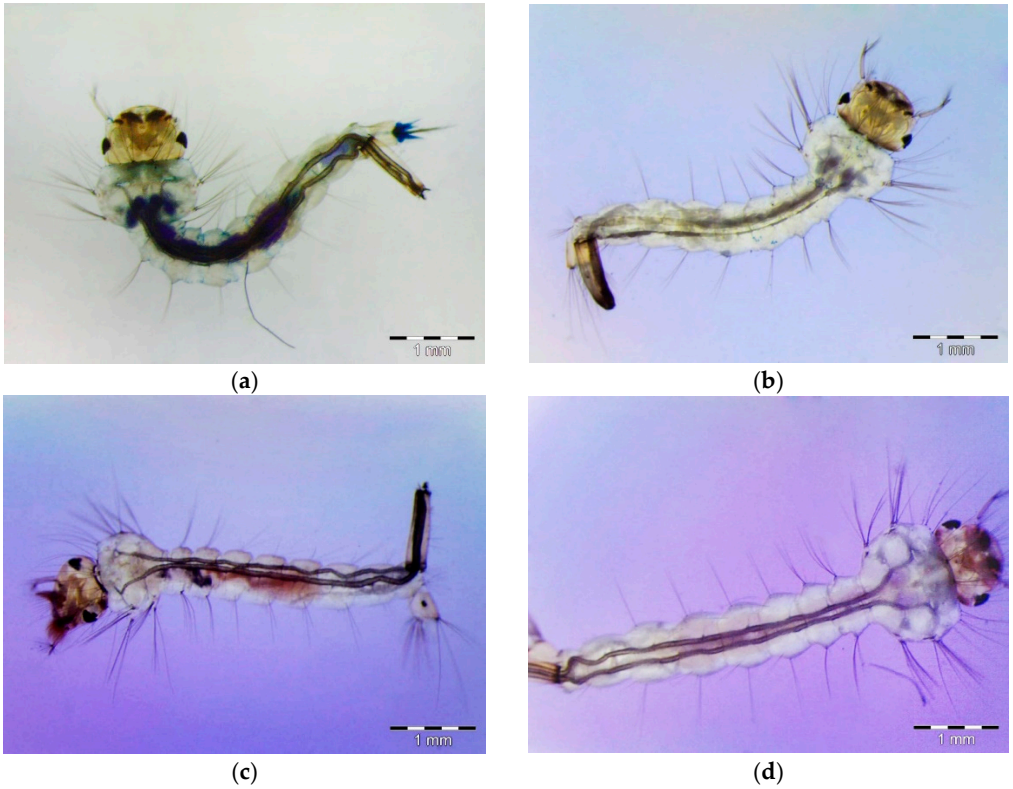


Figure 7. Shows midgut pH of untreated and treated 3rd larval stage of *Cx. pipiens* by using Bromothymol blue dye (1.6 X): (**a**) untreated larvae showed alkaline pH midgut; (**b**) PDG treated larvae showed reduction in pH midgut; (**c**) E.O. treated larvae showed reduction in pH midgut; (**d**) PDG and E.O treated larvae showed severe reduction in pH midgut.

4. Conclusions

High LC_{50} was observed in essential oils' *Thuja orientalis* leaves. The combination between LC_{10} of prodigiosin and LC_{50} of *T. orientalis* leaves showed the highest synergistic effect (100%). The treated 3rd larval *Cx. pipiens* showed a reduction in the acetylcholine esterase, total protein content, and midgut pH as compared to the untreated ones.

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