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Proceeding paper

Novel Formula as Mosquito Larvicide †

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† Presented at the 2nd International Electronic Conference on Plant Sciences—10th Anniversary of Journal Plants, 1–15 December 2021; Available online: https://iecps2021.sciforum.net/.

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

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

Citation: Hassanein, F.; Awad, O.M.; Harraz, F.M.; Saeed, H.zm; Hussein, A.A. Novel Formula as Mosquito Larvicide. *Plants* **2021**, *10*, x. https://doi.org/10.3390/xxxxx

Academic Editor: Carmen Arena

Published: 15 December 2021

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 or 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]. The pesticides pose a potential risk to humans and unwanted side effects to the environment [5]. About 1 million world-wide deaths and chronic diseases per year were due to the poisoning effect of the pesticide[6]. Natural products represent one of the critical sources of chemical diversity and potential medicinal use [7].

The medicinal importance of natural products that are derived from plants, animals and products of microorganism fermentation has a pharmacological activity in treating different kinds of 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 got its attention because of tripyrrylmethene, 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 aromatherapy effect to cure or prevent diseases, infection and indisposition by means of inhalation in controlling the central nervous system [12]. Also, they have antiparasitic, antibacterial, fungicidal, relaxant, stimulating, and antidepressant effect [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 aimed to study 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 hrs at 28-30◦C, then inoculated in peanut media and kept shaking condition for 48-72 hrs at 28-30◦C and finally subjected to Fermentor, inoculum size was 3%*30 = 90 mL. pH=7, agitation = 400 rpm, aeration was the maximum aeration [17,18], then PDG 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 that 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. Mobile phase: acetonitrile/HPLC water (60:40) and the yield was weighted after putting it in a sterile vail. FT-IR spectrum of the pigment was recorded with a test can Schimadzu FT-IR spectrophotometer at 800-4000nm. The purified pigment was tested by TLC in comparison with the standard PDG.

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]. A FT-IR spectrum of the E.O. was recorded with a Tests can Schimadzu FT-IR spectrophotometer at 800-4000nm. Then its constituents were profiled by GC-MS. E.O. was diluted in diethyl ether and 0.5 L and injected into the gas chromatography (Hewlwett Packard5890)/mass spectrometry (Hewlwett Packard 5989B) (GC–MS) apparatus. The GC column was a 30 m (0.25 mm i.d., film thickness0.25 _m) HP-5MS (5% diphenyl) dimethylpolysiloxane capillary column. The GC conditions were as follows: injector tempera-ture, 240◦C; column temperature, isothermal at 70◦C and held for 2 min, then 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. was determined by 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. On reaching the laboratory of Vector Control and

Pesticide Risks, HIPH, the larvae and the pupae were reared under laboratory conditions. The rearing of the larvae and pupae was done by feeding them on bread meanwhile the adult males were fed on 30% glucose and the adult females were fed on the blood by biting the pigeon. All that was maintained under specific conditions including temperature (26±2ºC), RH (70±5%) and water was replaced every two days.

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

Ten larvae were introduced into conical flask containing 50 mL dechlorinated water and treated with PDG & E.O. in different ppm concentration based on a 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 for the Synergistic Effect of PDG with the E.O., as Mosquito Larvicidal Potential

A combination between 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 didn't show any movement even after pin press, then collected.

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

Started with preparation of 'whole body homogenates. Anticholinesterase Activity kit of Cholinesterase (BTC/DTNB), Biochemical Enterprise[21,22]. Total Protein Activity kit of VITRO SCIENT. Biuret colorimetric endpoint method [23]. Midgut pH medium determination by bromothymol blue dye.

2.7. Stage 7: Probit Analysis Was Used to Determine the LC_{10}, LC_{25} & LC_{50} of PDG and E.O. of T. orientalis Leaves.

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 and that was in agreement to previous studies done by Patil et al., Song et al., and Nakashima, Kurachi, Kato, & 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), had been run from 200-700 nm ranges of wavelengths in UV-Vis spectrophotometer. The maximum absorption peaks had been detected at 540 nm and 541 nm for PNSRR and PNSHR, respectively[26], and that was equivalent to that detected by Kumar and Aparna, 2014 [27].

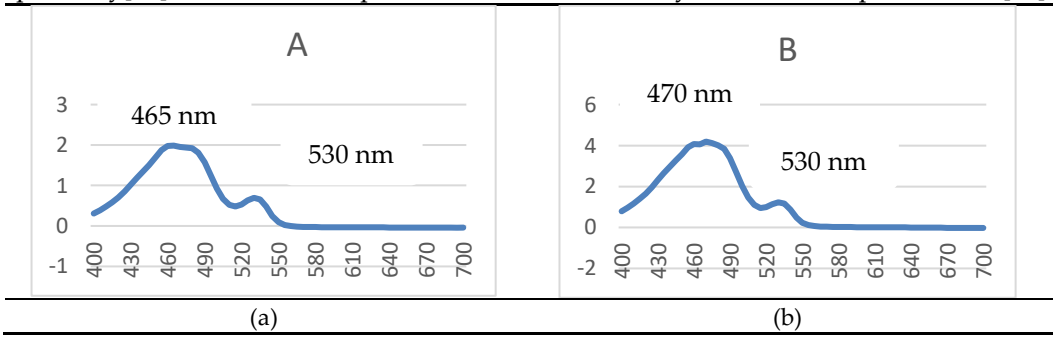


Figure 1. Shows the UV-visible spectrophotometry in the range 400-700 nm for the crude PDG: (**a**) PDG-batch scale; (**b**) PDG-Fermenter (Bioreactor).

The purity of the red pigment was identical to that of the standard PDG by TLC application, (Figure 2). Also, HPLC profiling showed a single peak at 536nm 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 agree to our results Kumar and Aparna, 2014 showed that >95% purity of single peak was observed at R_f > 4mins (4.523 min) [27]. In contrast, Mandal *et al.*, revealed that the R_f was until 4 min, and no sharp peaks have been 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 and that confirms that the tested sample is PDG. Mandal *et al.*, reported that the R_f values of the extracted pigments have been found to be around 0.88 after using methanol, chloroform, and hexane in the ratio of 7:3:1[26].

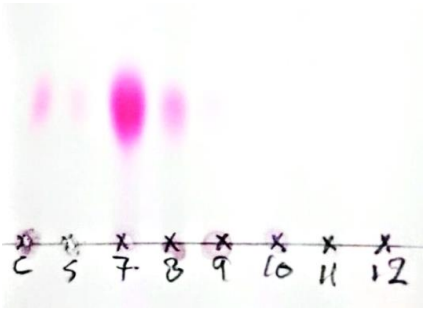
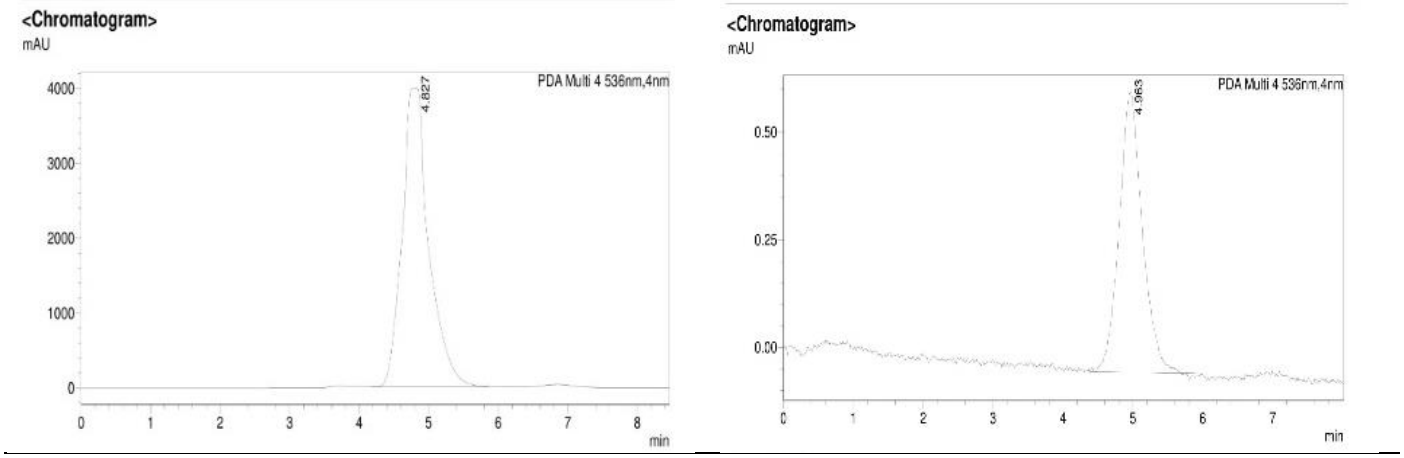


Figure 2. Application of TLC of the purified PDG compared with the standard.



(a) (b)

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}, 3427.0 cm^{-1}, due to the presence of protonated nitrogen and that confirm that the pattern of the red pigment is identical to the standard. Also, FT-IR of the red pigment was matched with that of the standard and that confirms that the red pigment is PDG ($C_{20}H_{25}N_3O$). In agreement to our study, Patil *et al.*, revealed that the absorption was dominated by very strong bands at 2,977 cm^{-1}(aromatic CH) and 1,648 cm^{-1} (aromatic C=C). Also, he reported that PDG exhibits similar absorptions in CHCl3 at 1,630 and 1,602 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 1,648 (C=O), 1,087, 1,047 (C-O and C-N) and 879 cm^{-1}. A strong and broad absorption for NH was evident at νmax 3,403 cm^{-1}. These indicate that this pigments pattern is identical to that of PDG [19].

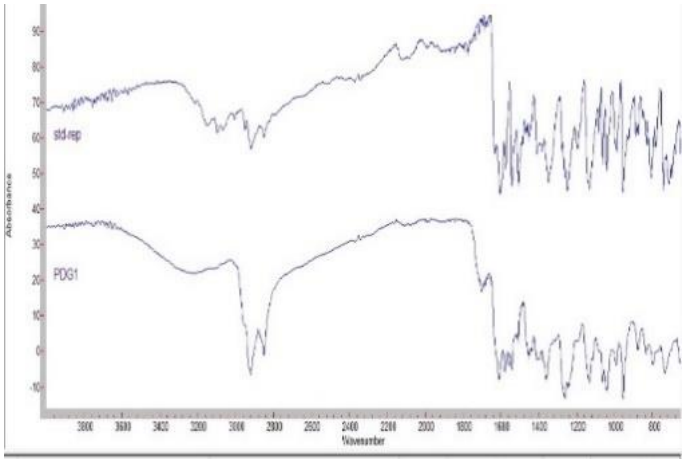


Figure 4. FT-IR Spectra of the purified PDG compared to the standard PDG.

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 3ml E.O. That oil was subjected to FT-IR spectroscopy and revealed different bands with diversity of intensities as shown in Figure 5. The E.O. was characterized by GC-MS and 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 (Table1 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, 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].

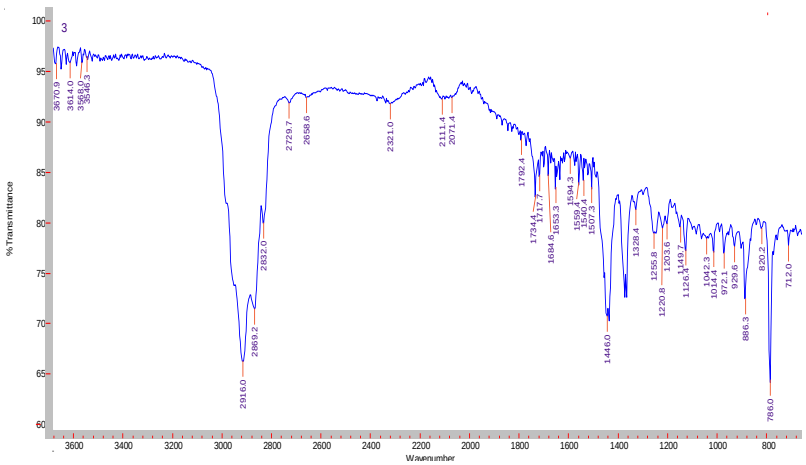


Figure 5. E.O. FT-IR spectrum.

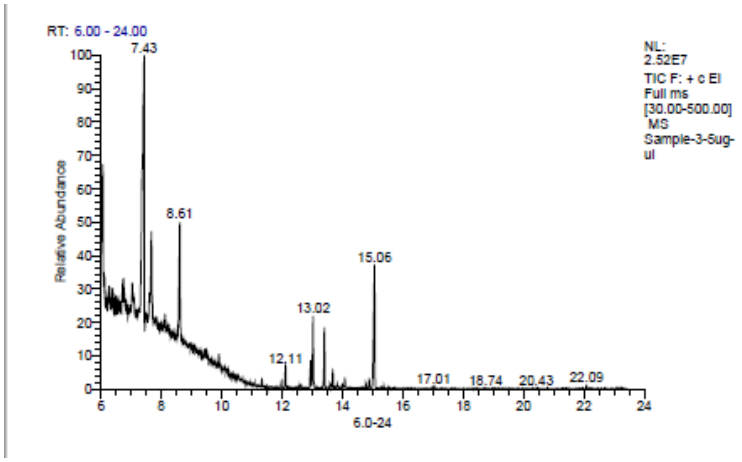


Figure 6. GC-MS analysis for the *E.O.*

In this study, the yield of the EO was 0.3% and that was in agreement that reported by Nickavar *et al.*, [24] where the hydrodistillation of *T. orientalis* leaves gave oils with a yield of 0.25%. 25 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 lower percentage of oxygenated monoterpenes, sesquiterpene hydrocarbons and oxygenated sesquiterpenes 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 were 0.24, 0.48 and 8.87 respectively. Ibrahim et al., [29] repoted that the amount 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 refrative index of the E.O. was 1.482 nD revealing high purity of the E.O., that because its value was in the range of the typical value (1.4785 nD -1.4885 nD) [30], 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 LC_{50} value of PDG (39.5 *ppm*) that 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 μgml^{-1} against 3rd instars of *Ae. Aegypti* and *An. Stephensi* respectively [34].

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, like 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 80ppm from *Platycladus orientalis* oil was sufficient to cause 100% larval mortality against the larvae of *Cx pipiens* after 24h. 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} values against *Cx. pipiens* larvae was 18.60ppm after 24h, hence the authors considered that E.O. as a natural larvicide for mosquito larval control.

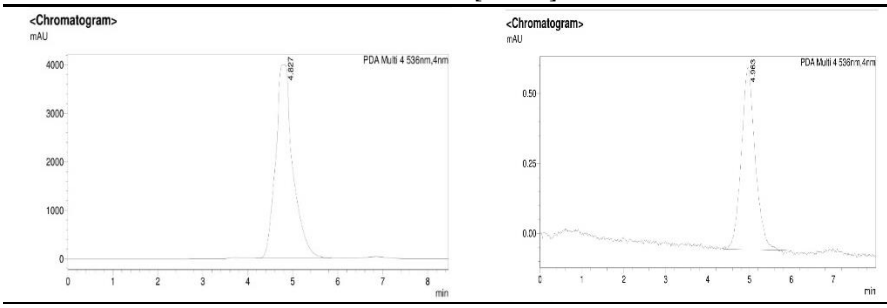
3.4. Investigation for the Combination Effect of PDG with the E.O. as a Mosquito Larvicidal Potential after 24 h

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 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) followed by other combinations with concentrations [37].

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

Table 4 shows that the highest percentage of AChE Arbitry activity 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). Then 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 combination LC_{10} of PDG with LC_{25} and LC_{50} of E.O(0.92% & 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 combination of LC_{10} of PDG with LC_{25} and LC_{50} of E.O (3.47%, 3.13%, 4.13% & 0.91% respectively). Figure 7 shows that the midgut of untreated larvae showed blue color after 12 h incubation in bromothymol dye meanwhile treated larvae showed 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 control among 4th instar larvae of *Ae. Aegypti* [34]. Bromthymol Blue dye is a weak acid and a member of the class of 2,1-benzoxathioles so act as 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 dark blue color by stereomicroscope, in contrast the midgut of treated larvae with PDG, E.O and their combination showed reduction in the pH. In agreement, 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 blue indicating basic condition. That may be attributed the hydrophobicity of natural products including PDG and E.O. and their effect as carbonic anhydrase inhibitoras reported by Zhuang *et al.*[40],

leading to reduction in pH of the midgut that subsequently results in cellular respiration inhibition [41–43].



(a)

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

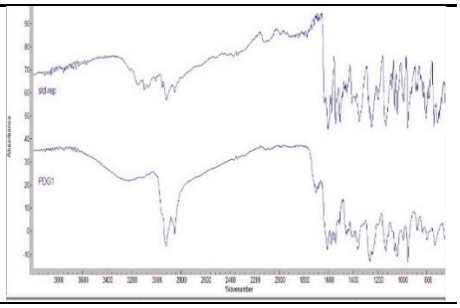


Figure 4: FT-IR Spectra of the purified PDG compared to the standard PDG

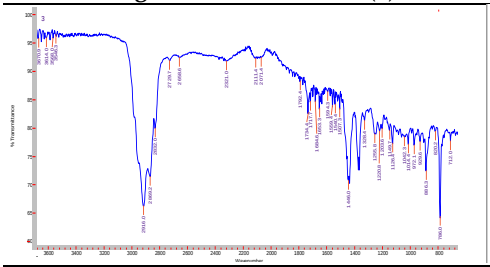


Figure 5: E.O. FT-IR spectrum

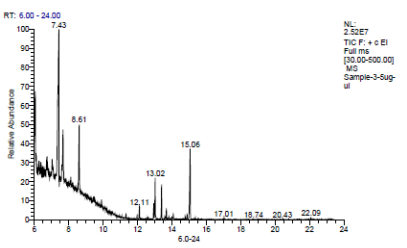


Figure 6: GC-MS analysis for the *E.O*

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%**

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]**	**(χ2)[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 linear correlation coefficient of the median effect plot where R2=1 indicates a perfect conformity. [5] Chi square value. * The experiments were repeated three replicates.

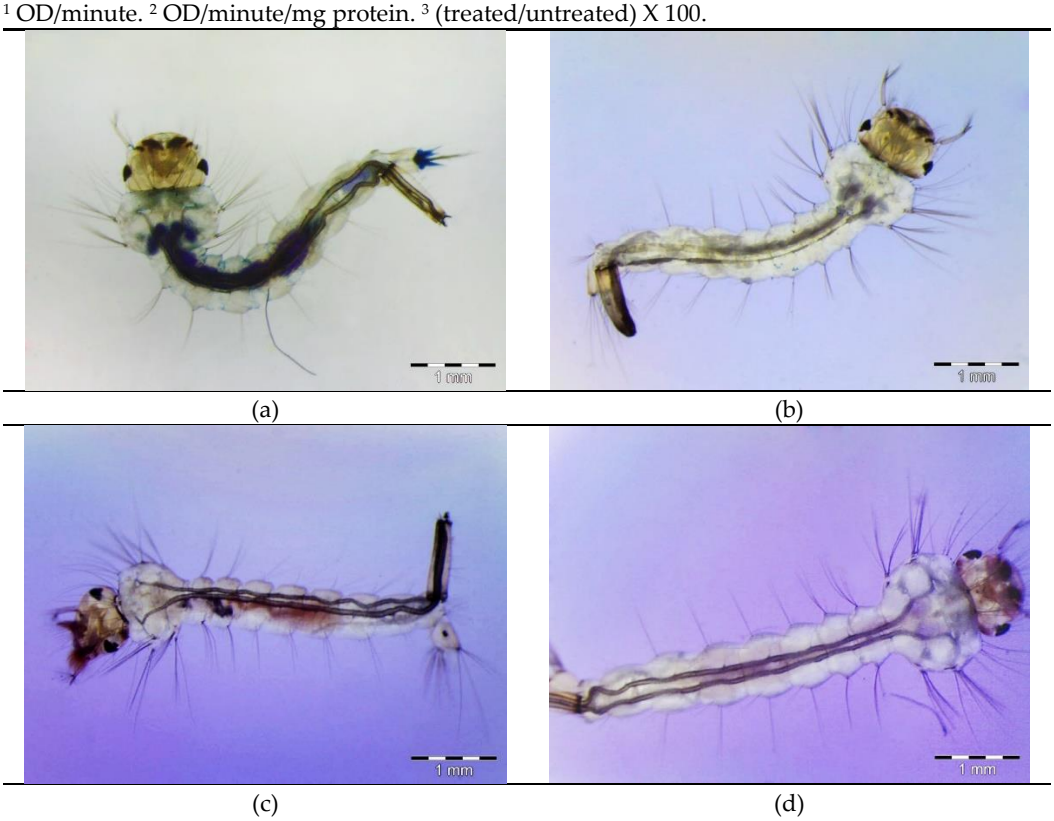
Table 3. Synergistic Larvicidal activity of the LC_{10} of PDG with LC_{25} and LC_{50} of E.O. after 24hrs:.

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

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

Treartment	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) X 100.



(a) (b)

(c) (d)

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; 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 of 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 reduction in the acetylcholine esterase, total protein content and midgut pH as compared to the untreated ones.

Author Contributions: "Conceptualization, A.H. O.M.A., F.M.H.; methodology, F.H., O.M.A., F.M.H., A.H; software, F.H.; PDG Preparation, F.H., A.M.; Essential oil preparation and PDG purification, F.H., F.M.H. ; Softwares, F.H.; Mosquito rearing, F.H., O.M.A.; Dose response bioassay, F.H., O.M.A.; Mode of action, F.H., O.M.A.; resources, F.H..; data curation, F.H., O.M.A, & F.M.H.; writing—original draft preparation, F.H.; writing—review and editing, F.H., O.M.A, & F.M.H.; supervision, A.H., H.S, F.M.H., O.M.A."

Funding: Please add: "This research received no external funding"

Conflicts of Interest: "The authors declare no conflict of interest."

Informed Consent Statement: Not applicable.

Data Availability Statement: Data available on request.

Acknowledgments: In this section, you can acknowledge any support given which is not covered by the author contribution or funding sections. This may include administrative and technical support, or donations in kind (e.g., materials used for experiments).

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