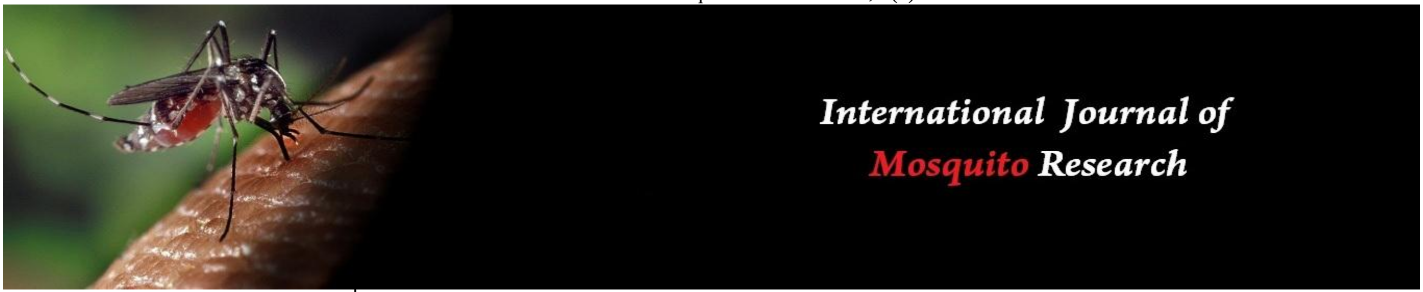

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Photosensitizing effects of certain xanthene dyes on *Culex pipiens* larvae (Diptera- Culicidae)

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Abstract
The response of the fourth instar larvae of *Culex pipiens* to three photosensitizing dyes (Rose Bengal, phloxine B and rhodamine B) was investigated under laboratory conditions. The mortality percentages increased as the concentrations and exposure times of all tested dyes increased. As judged by a comparison of median lethal concentration values, Rose Bengal was the most effective dye followed by phloxine B, then rhodamine B. Treatment fourth instar larvae with different concentrations of three tested dyes induced morphological changes and aberrations in larval stage. In the current study, based on median lethal time values, Rose Bengal had the lowest time values and fastest action compared to other tested dyes. Results also revealed that the addition of non- toxic concentrations of both tween 20 and tween 60 to median lethal concentration of Rose Bengal showed no significant changes and didn't appear synergism to their efficacies compared to Rose Bengal used alone.

Keywords: photosensitizing dyes, rose bengal, phloxine B, rhodamine B, tween 20, tween 60

Introduction
The *Culex pipiens* is one of the most distributing vectors of pathogens for humans. Mosquitoes (Diptera: Culicidae) species are vectors responsible for the transmission of various infectious diseases with medical and veterinary importance including filariasis, malaria, and arboviruses [1]. The best counteractive action of mosquito borne diseases is accomplished by decreasing the mosquito population in any of the different life cycle stages by using larvicidal substances. Currently, some problems are caused by the multiple usages of chemical insecticides and reported with respect to the persistence and increase of non- biodegradable chemicals in the environment, the biological enlargement through the food chain, the toxic effect to human health and to non- target organisms, and the increase of insecticide resistance [2]. It is now supposed that photo-insecticides may provide the main of a new generation of pesticide [3]. Photosensitivity insecticide has attracted increasing attention as a new type of highly efficient and environment friendly pesticide to be used to control the pest due to its rapid photo degradation in the visible light [4]. The mechanism for photodynamic activity has been described [5]. Photosensitization including light, a photosensitizer, and oxygen is a probably damaging event in biological systems. This creates several reactive oxygen species (ROS) that are able of damaging several subcellular structures [6]. Xanthene dyes are one of the main classes of photodynamic sensitizers have been most extensively studied as pesticide [4]. Xanthene derivatives and other photosensitizers undergo rapid activation when exposed to light, leading to the formation of singlet oxygen and superoxide anions. Therefore, when insects ingest a photosensitizer and are then exposed to light their detoxifying systems, and they consequently, die. Xanthene dyes represent a family of photosensitizers that have been extensively tested as photo-insecticides on several dipteran adults [5]. Different halogenated xanthenes as Rose Bengal, phloxine B and rhodamine B have proven to be effective photo-insecticides against at least two-dozen insect species. Diptera are particularly susceptible to the photodynamic action of dyes [4]. The current study aimed to evaluate the efficiency of three different xanthene dyes in controlling 4th larval instar of *Culex pipiens* during photosensitization process and referring to synergistic effect of some adjuvants on photosensitizer effectiveness.

Materials and Methods
Insect culture
The wild species of immature stages of *Culex pipiens* mosquito were collected from drainage at El-prince estate in Al-Qalyubia governorate. Mosquitoes colony were transferred into laboratory. All the stock cultures were reared for at least eight generations in rearing room under controlled laboratory conditions at temperature 27±3°C, relative humidity 60- 70% and for photoperiods 12-12 light-dark regime. Larvae fed on ground dried bread and dried brewer's yeast (2:1). Insects were reared corresponded to designed technique [7].

Selected photoactive dyes and additives
Three photosensitizers were used to evaluate their toxicity against 4th larval instar of *Culex pipiens*, Rose Bengal, phloxine B & rhodamine B were obtained from Alfa company in Benha neared faculty of Science as commercial powder from Egyptian international center for import.

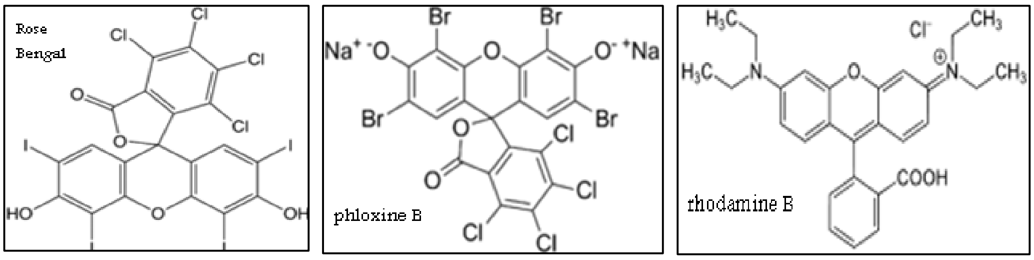


Fig 1: Chemical structure of Rose Bengal, phloxine B & rhodamine B

Preparation of photoactive dye concentrations
Six concentrations of three dyes (Rose Bengal (RB), phloxine B (PhB) and rhodamine B (RhB)) were prepared in dechlorinated water (3×10^{-5}, 5×10^{-5}, 8×10^{-5}, 1×10^{-4}, 1×10^{-3} and 1×10^{-2} M) to test the suscepility of the fourth instar larvae. A group of 100 larvae was used for each test, and every dilution had 5 replicates. Each replicate contains 20 larvae. Twenty-five ml of each concentration was added to glassed beaker (250 ml). Larvae of *Culex pipiens* were separated from the culture and placed in glassed beakers. They allowed starving for eight hours [8], then they transferred to other glass beakers containing prepared photosensitizers with previous larval diet for treated samples and only larval diet for control samples, the beakers were taken out doors where exposure to sunlight and mortality was recorded every half an hour for 5 hours of directed subjecting to sunlight. The recorded mortality was averaged to establish a regression lined using log probit scales representing concentrations versus percentage of mortality [9]. Toxicity index developed was employed for the direct comparison of the tested dyes. Relative potency values were measured corresponded to the designed method [10].

Certain additives
These adjuvants were tween 20 and tween 60 were obtained from Alfa company in Benha neared faculty of Science as liquid that used against 4th larval instar of *Culex pipiens*. Groups of larvae were treated with various concentrations of additives (1×10^{-5}, 1×10^{-4}, 1×10^{-3} and 1×10^{-2} M) with median lethal concentration of a most effective photosensitizer as in previous preparations. Other groups of larvae were treated with the same concentrations of additives but without a photosensitizer to serve as control. Averaged mortality was recorded after sunlight exposure.

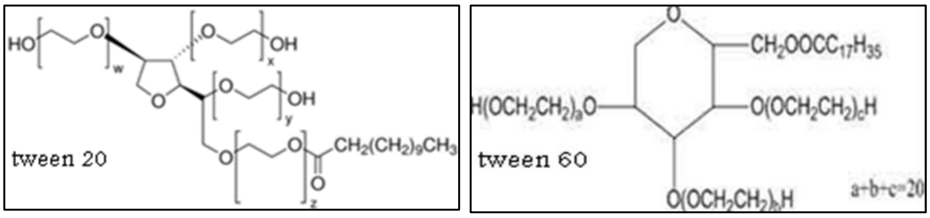


Fig 2: Chemical structure of tween® 20 & tween® 60

Statistical analysis
Data achieved were examined using one- way analysis of variance (ANOVA) by SPSS program [11]. Moreover, the LC_{50}, LT^{50} values calculated using probit analysis [12]. The obtained data from certain additives were manipulated statistically with SPSS using Independent- Samples T- test at $P < 0.05$.

Results
Screening of the susceptibility of mosquito *Culex pipiens* larvae to the selected photosensitizer compounds
Three selected dyes had toxic effects against 4th instar larvae. The mortality rate was directly dependent on concentrations in all selected dyes, i.e., percent mortality in larvae was significantly increased with increasing of concentrations. The maximum toxic effect was showed in treated larvae with Rose Bengal (RB) (100%) at concentration 1×10^{-2}M, followed by phloxine B (PhB) (94%), then rhodamine B (RhB) (89%) at the highest concentration.

Probit analysis in a table (1) revealed that the LC_{50} after 5 hours post- exposure was 6.62×10^{-5} M for (RB), 1.07×10^{-4} M for (PhB) and 1.41×10^{-4} M for (RhB). Results also showed LC_{95} of tested dyes on mosquito larvae, which were 2.7×10^{-4} M for (RB), 3.99×10^{-3} M for (PhB) and 1.06×10^{-2} M for (RhB). The results indicated that Rose Bengal was the most effective dye while a rhodamine B was the least effective one. Data in Table (1) and Fig. (1) showed also the toxicity index based on LC_{50} of Rose Bengal was 100% and were 61.87 and 46.95% for phloxine B and a rhodamine B, respectively. The relative potency of Rose Bengal was nearly 2.13 times more potent than phloxine B and a rhodamine B.

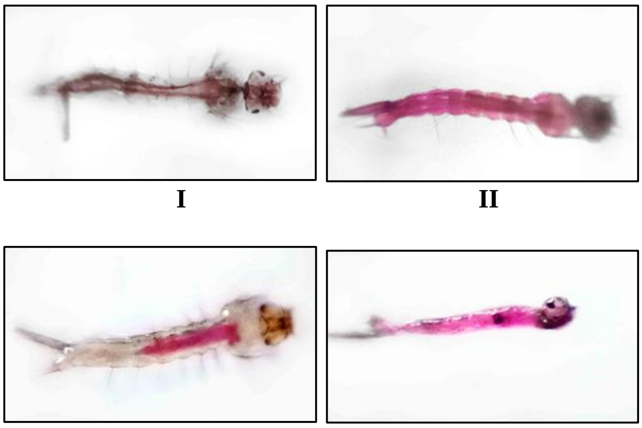
Table 1: Effect of different concentrations of Rose Bengal, phloxine B and rhodamine B on percentage mortality and (LC_{50}), (LC_{95}), Toxicity index and Relative potency against *Culex pipiens* larvae exposed to direct sunlight for 5 hours.

Photosensitizing compounds	Concentration (Molar)	Percentage mortality (%)	LC50 confidence limits (Molar)	LC95 confidence limits (Molar)	Slope	Toxicity index	Relative potency
Rose Bengal (RhB)	3×10^{-5}	17	6.62×10^{-5} (7.4×10^{-5}-5.92×10^{-5})	2.7×10^{-4} (3.9×10^{-4}-1.89×10^{-4})	2.687±0.1025	100	2.13
	5×10^{-5}	34					
	8×10^{-5}	53					
	1×10^{-4}	80					
	1×10^{-3}	99					
	1×10^{-2}	100					
	0.0 (control)	0.0					
phloxine B (PhB)	3×10^{-5}	15	1.07×10^{-4} (1.3755×10^{-4} - 8.3×10^{-5})	3.99×10^{-3} (7.95×10^{-3}-2.02×10^{-3})	1.046±8.97×10-3	61.87	1.32
	5×10^{-5}	31					
	8×10^{-5}	48					
	1×10^{-4}	67					
	1×10^{-3}	88					
	1×10^{-2}	94					
	0.0 (control)	0.0					
rhodamine B (RhB)	3×10^{-5}	13	1.41×10^{-4} (1.56×10^{-4}-1.88×10^{-4})	1.06×10^{-2} (4.68×10^{-3}-2.42×10^{-2})	0.88± 6.28×10-3	46.95	1
	5×10^{-5}	30					
	8×10^{-5}	43					
	1×10^{-4}	65					
	1×10^{-3}	83					
	1×10^{-2}	89					
	0.0 (control)	0.0					

Morphological abnormalities

Plates (1, 2, 3 & 4): Normal 4th larval instar in Plate (1) and morphological abnormalities in *Culex pipiens* larvae after treatment with Rose Bengal showing: a colored thorax and abdomen or small part of it by pink color of dye as in Plate (2), (II & III), become thinner with unchitinized abdominal cuticle as in (I, VI &IV) especially at high concentrations; folded and collapsing abdomen as in (V). After larval treatment with a rhodamine B showing: colored head, thorax and abdomen or thorax only by violet color of dye as in Plate (3) from (I to V), In larvae treated by phloxine B, all body of larvae become red and deformed pupae are also noticed as in Plate (4), (I & II).

Plate 1: Normal fourth larval instar

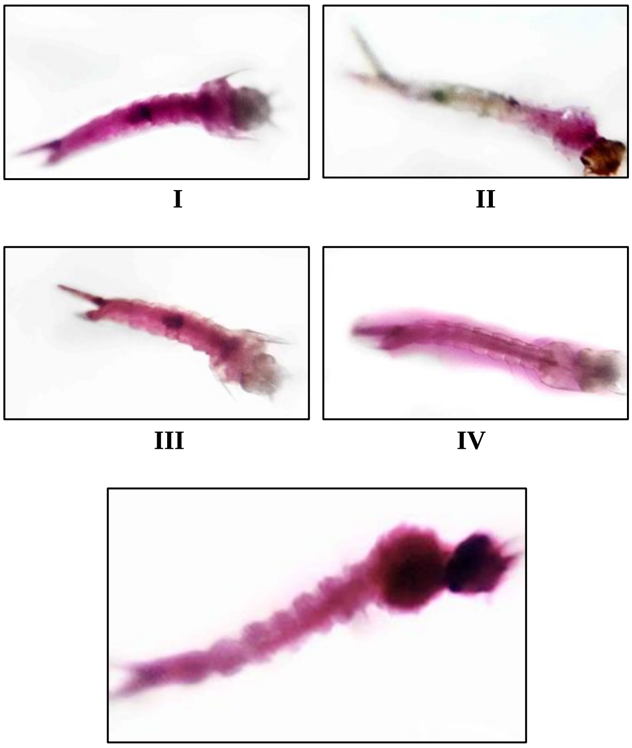


I II

III IV

V VI

Plate 2: Treated larvae with Rose Bengal

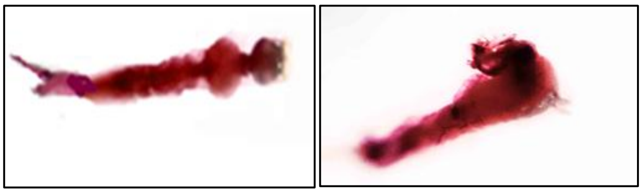


I II

III IV

V

Plate 3: Treated larvae with rhodamine B



I II

Plate 4: Treated larvae with phloxine B

Photodynamic effect of three dyes to control 4th larval instar of *Culex pipiens*

Results obtained in Table (2) showed that the mean number of dead larvae treated with the lowest concentration 3×10^{-5} M of Rose Bengal was 1.00±0.447 after 3 hours of sunlight exposure, then the mean number of dead larvae was slightly increased to 3.40±1.077 after 5 hours. While at the highest concentration of Rose Bengal 1×10^{-2} M after one hour, the mean number of larval mortality was increased significantly to 3.60±0.600, representing 18% larval mortality at ($P<0.05$). As the time of light exposure increased, the mortality percent increased significantly to reach to maximum larval mortality 20.00±0.00 which representing 100% larval mortality after 5 hours of sunlight exposure. In the same way, the mean number of dead larvae treated with phloxine B at the lowest concentration 3×10^{-5} M was significantly increased to reach 3.00±0.837 after 5 hours. At highest concentration of phloxine B 1×10^{-2} M caused 2.20±0.583 after 1 hr and after 3 hours significantly increased to 11.00±0.316 until reach to 18.80±0.200 of larval mortality after 5 hours of sunlight exposure. In addition, the mean number of dead larvae treated with the lowest concentration of rhodamine B 3×10^{-5} M showed significantly increased in mean of larval mortality to reach to 2.60±0.400 after 5 hours. At highest concentration of rhodamine B 1×10^{-2} M caused 1.20±0.200 larval mortality after 1 hour of light exposure. The mean number of dead larvae was significantly increased ($P<0.05$) from 9.80±0.490 after 3 hours to the maximum percentage of larval mortality (89%) after 5 hours of light exposure.

From Table (2) The LT_{50} values of the concentration (5×10^{-5} M) of Rose Bengal, phloxine B and rhodamine B were 5.87, 6.05 and 6.02 hours, respectively. But the LT_{50} values of the highest concentration (1×10^{-2} M) were 1.56, 2.40 and 2.73 hours for Rose Bengal, phloxine B and rhodamine B, respectively. This indicated that Rose Bengal was the most effective dye among tested dyes.

Table 2: Photodynamic effect of Rose Bengal, phloxine B and rhodamine B on 4th instar larvae of *Culex pipiens*.

Sun light exposure periods (hours)	**Concentrations of Rose Bengal Mean no. of dead larvae ± SE (% of mortality)**						
	Control	**3×10^{-5}(%)**	**5×10^{-5}**	**8×10^{-5}**	**1×10^{-4}**	**1×10^{-3}**	**1×10^{-2}**
1	0.00±0.0	0.00±0.00^a (0%)	0.00±0.00^a (0%)	0.00±0.00^a (0%)	0.80±0.374^a (4%)	1.60±0.400^a (8%)	3.60±0.600^a (5%)
3	0.00±0.0	1.00±0.447^a (5%)	2.00±0.447^b (10%)	4.40±0.510^b (22%)	7.60±0.927^b (38%)	10.20±0.735^b (51%)	17.00±0.316^b (80%)
5	0.00±0.0	3.40±1.077^b (17%)	6.80±0.663^c (34%)	10.60±0.245^c (53%)	16.00±0.316^c (80%)	19.80±0.200^c (99%)	20.00±0.000^c (100%)
LT_{50} (hrs)	-	7.82	5.87	4.78	3.33	2.46	1.56
Sun light exposure periods (hours)	**Concentrations of phloxine B Mean no. of dead larvae ± SE**						
	Control	3×10^{-5}	5×10^{-5}	8×10^{-5}	1×10^{-4}	1×10^{-3}	1×10^{-2}
1	0.00±0.0	0.00±0.00^a (0%)	0.00±0.00^a (0%)	0.00±0.00^a (0%)	1.60±0.812^a (8%)	2.00±0.316^a (10%)	2.20±0.583^a (11%)
3	0.00±0.0	0.00±0.00^a (0%)	2.00±0.707^b (10%)	4.00±0.447^b (20%)	8.00±1.378^b (40%)	9.20±1.158^b (46%)	11.00±0.316^b (55%)
5	0.00±0.0	3.00±0.837^b (15%)	6.20±2.458^c (31%)	9.60±0.510^c (48%)	13.40±0.872^c (67%)	17.60±0.510^c (88%)	18.80±0.200^c (94%)
LT_5(hrs)	-	7.44	6.05	4.84	3.55	2.82	2.40
Sun light exposure periods (hours)	**Concentrations of rhodamine B Mean no. of dead larvae ± SE**						
	Control	3×10^{-5}	5×10^{-5}	8×10^{-5}	1×10^{-4}	1×10^{-3}	1×10^{-2}
1	0.00±0.0	0.00±0.00^a (0%)	0.00±0.00^a (0%)	0.00±0.00^a (0%)	0.00±0.00^a (0%)	0.20±0.200^a (1%)	1.20±0.200^a (5%)
3	0.00±0.0	0.00±0.00^a (0%)	2.40±0.510^b (12%)	4.40±0.927^b (22%)	7.60±1.208^b (38%)	9.60±1.36^b (48%)	9.80±0.490^b (49%)
5	0.00±0.0	2.60±0.400^b (13%)	6.00±0.316^c (30%)	8.60±1.289^c (43%)	13.00±0.316^c (65%)	16.60±0.510^c (83%)	17.80±0.200^c (89%)
LT_5(hrs)	-	6.95	6.02	4.84	3.66	2.94	2.73

Each value represents mean of 5 replicates± SE, "each replicate contain 20 larvae". Mean±SE followed by same letters are not significantly different in the same column ($P>0.05$) (F-test).

Effect of certain additives on the activity of dyes

In this experiment two adjuvants tween 20 and tween 60 were tested for their effects on Rose Bengal against 4th instar larvae of *Culex pipiens*. Groups of *Culex* larvae treated with LC_{50} of Rose Bengal (6.62×10^{-5} M) and different concentrations of each adjuvant as (1×10^{-5}, 1×10^{-4}, 1×10^{-3} and 1×10^{-2} M). Other groups of larvae were treated with the same concentrations of each adjuvant without Rose Bengal to serve as control. We observed that tween 20 was more effective than tween 60 when added with LC_{50} of Rose Bengal at 1×10^{-3} M, but the effect of LC_{50} of Rose Bengal alone gave higher larval mortality than using adjuvant with Rose Bengal as shown in Fig. (3& 4).

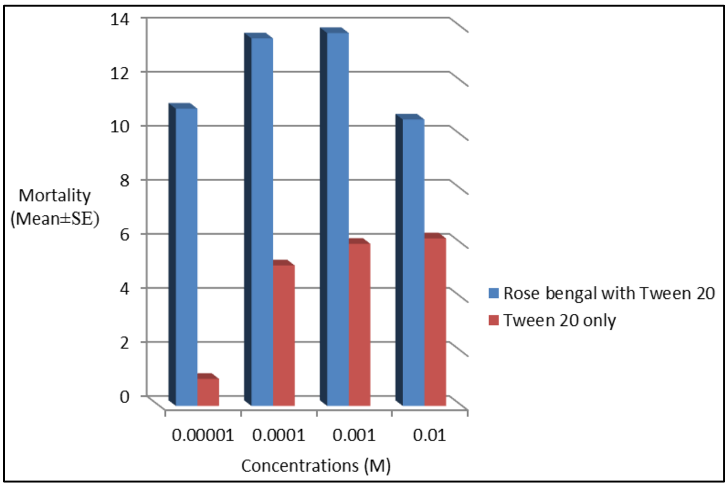


Fig 3: Effect of tween 20 on phototoxicity of Rose Bengal (LC_{50}) on 4th instar larvae of *Culex pipiens*.

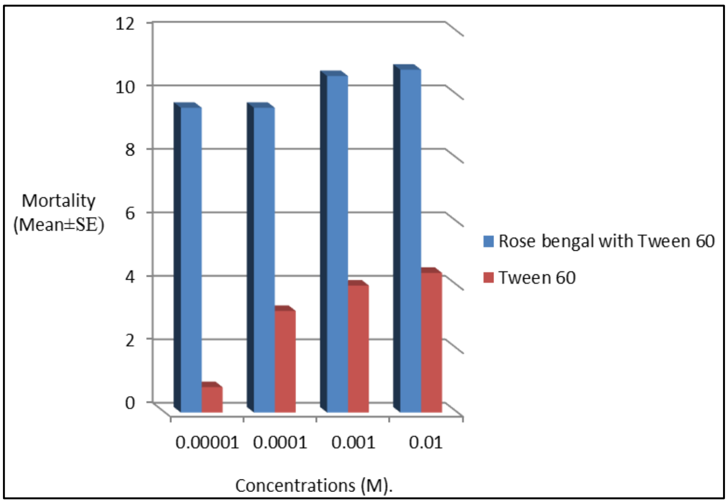


Fig 4: Effect of tween 60 on phototoxicity of Rose Bengal (LC_{50}) on 4th larval instar of *Culex pipiens*.

Discussion

Several studies indicated that photosensitizer compounds represent a possible alternative to traditional chemical compounds [13, 14]. Photosensitizers are capable of absorbing sunlight and transferring the energy to oxygen molecules to form a toxic form called singlet oxygen ($^1O_2$) and damage biological substrate by photosensitized oxidation [15].

Screening of the susceptibility of the mosquito, *Culex pipiens* larvae on the selected photosensitizer compounds.

As revealed from the obtained results, Rose Bengal was the most effective dye while a rhodamine B was the least effective one. In agreement with the present results; the efficiency of Rose Bengal against *Culex pipiens* and other insects has been detected by several authors as against 4th instar larvae of *Aedes aegypti* (L.), *Anopheles stephensi* (Liston) and *Culex quinquefasciatus* Say [16] and, against adult house fly [17].

Rose Bengal from results caused high mortality of *Culex pipiens* larvae at high concentration; similarly, a significant increase in the mortality of *Culex* larvae treated with hematoporphyrin as a result of the high oxidative stress caused by photosensitizer effect [18]. The actions of other photosensitizers including porphyrins caused identical results against *Aedes aegypti* [19]. Also, time of starvation before treatment with dyes necessary to facilitate increasing feeding and accelerating dye entry into the body tissues, and ensured uniform feeding rates by larvae so in this assay we exposed larvae for starvation for 8 hours before treatment [8].

The obtained results indicated that toxicity greatly varied according to the chemical structure nature of treated dyes that the dyes with the greater number of the halogen atom substituents yield greater toxicity. Therefore, the halogen atoms amplify the reactions [5]. The amplification is due to enhanced intersystem crossing from 1st excited singlet state to the 1st excited triplet state of the dye, allowing for more efficient interaction with oxygen molecules, where Rose Bengal has the greatest number of halogen atoms (4 iodine and 4 chlorine atoms), phloxine B has (4 bromine and 4 chlorine atoms) and rhodamine B has the lowest number of halogen atoms (one chlorine atom) but substitution of xanthene dyes with heavier atoms made the dyes more toxic (Iodine > Bromine) so phloxine B was weaker than Rose Bengal [20].

This results may be also due to that the singlet oxygen quantum yield of Rose Bengal is 0.76, phloxine B is 0.59 and rhodamine B is 0.55. The later reference proved that Rose Bengal considered an efficient generator of cytotoxic singlet oxygen upon photo- activation [21].

Toxicological studies with probit analysis revealed that the LC_{50} after 5 hrs for Rose Bengal < phloxine B < rhodamine B, so it is well known that, the slope value is a very important feature of regression line, since it helps to predict the reduction in pest population [22]. The slope value as presented in results

showed the different toxicity levels among insecticides.
The more gradient of linear graphs, means the more toxic to insecticides, while the less gradient of linear graphs, the less toxic of insecticides due to the big change in concentrations (X-axis) but small number of insect mortality (Y- axis).
The sensitivity of 4th instar larvae was reported by toxicity index which was a mean for comparing the relative toxicity of insecticides [23]. Rose Bengal was taken as standard compound and given the arbitrary index value as 100 units and this might be due to a much faster metabolism of this dye. Also, the relative potency of Rose Bengal in previous results appeared nearly 2.13 times more potent than phloxine B and rhodamine B.

Morphological abnormalities
Treatment of 4th instar larvae with different concentrations of three tested dyes, Rose Bengal, phloxine B and rhodamine B exhibited morphological aberrations including appearance of thin and unchitinized abdominal cuticle, this view may be extended to our data where it may result from anti-feedant effect by photosensitizer action and coloration of all parts of larval body with dyes especially digestive tract. Similar results were described on mosquito larvae *Culex quinquefasciatus* Say [16], they recorded that the treated larvae with Rose Bengal caused strong coloration of larvae. These results also were in accordance with the finding that used noval haematoporphyrin dye (HPF) against *Aedes caspius* larvae, and they come back their results to the adhering HPF particles to the internal lining of alimentary canal [24]. This means that the larvae couldn´t release the amount of dye even after long time.

Photodynamic effect of three tested dyes on 4th instar larvae of *Culex pipiens*.
It was apparent in the early studies that the light source was critical to success of the dye as an efficacious insect control agent. Natural sunlight is probably generally more efficient than both artificial one due to intense enough and contains photons of proper wavelength to be readily absorbed by dyes [25] so that it is a very efficient light source for causing toxicity. For halogenated xanthene dyes as tested dyes, maximum absorbance is in 540- 560 nm range [26].
This result agreed with some authors who found that the effect of hematoporphyrin dye on Culex pipiens larvae survival in sunny seasons was more efficient than in other seasons [27]. Similar results were obtained against *Musca domestica* [28].
As revealed from obtained results the efficacy of photosensitizers dependent on the concentration of tested dyes and also on time of exposure to sunlight after treatment as the concentration of the dye and time of sunlight exposure increased, the percentage of larval mortality increased. The lowest concentration of Rose Bengal, phloxine B and rhodamine B (3×10^{-5} M) caused 17, 15 and 13% mortality, respectively after 5 hours of sunlight exposure, while at highest concentration of previous tested dyes (1×10^{-2} M) caused 100, 94 and 89% larval mortality, respectively after same previous time of sunlight exposure. Similar observations were recorded against black cutworm larvae who said that Rose Bengal at concentration 5×10^{-3} M resulted in the highest level of mortality, followed by phloxine B and erythrosine B and LT_{50} values decreased with increasing concentration [25].
This may be attributed to accumulation number of photons needed to kill 50% of population decreased as the intensity increased. This would indicate that there is a regenerative capacity within the insect that is more efficiently overcome by photodynamic action as the light intensity increased. Photosensitization including light, photosensitizer, and oxygen is a probably damaging occurrence in biological systems. This creates various reactive oxygen species (ROS) such as singlet oxygen, hydrogen peroxide, superoxide and hydroperoxyl or hydroxyl radicals that are capable of damaging various sub-lethal structures and molecules [6]. The efficiency of photodynamic sensitizers as insecticidal agents is affected by a variety of experimental parameters and the first factor being photosensitizer concentration. The photo-insecticidal effect steadily increased with increasing the concentration of the photosensitizer, the second factor was the duration of the post treatment light exposure time. There an inverse relationship with the concentration and the exposure time when increased the concentration this led to decrease exposure time to sunlight. The rate of the photosensitized killing of pests appeared to increase with prolongation of post treatment exposure to light [29].
In this context photolysis and quantum yield, which determine the energy efficiency of the photodynamic action represent the most important factors related to photosensitivity and phototoxicity [30].

Effect of certain additives on the activity of dyes
In the present study, tween 20 was more effective than tween 60 when mixed with LC_{50} of Rose Bengal against *Culex pipiens* larvae but at the highest concentration of tween 20 (1×10^{-2} M), the mean larval mortality decreased. These results proved that addition of tween 20 to Rose Bengal at high concentration led to discourage action of each other, also results appeared effect of LC_{50} of dye alone was higher than addition adjuvant with dye against *Culex pipiens* larvae. In a similar manner, when several commercial surfactants as Hyper- Active, kinetic and tween 60 at 1% added to 0.01% phloxine B led to reduce citrus thrips mortality, whereas tween 60 and Dyne- Amic at 0.25% caused no effect after addition with phloxine B [31]. Disappearance of synergistic effect on efficiency of Rose Bengal against *Culex* larvae after addition some adjuvants attributed to Rose Bengal is anionic dye and tween 20 & 60 are nonionic surfactant, the interaction between the anionic dye and a nonionic surfactant has been shown to be hydrophobic in nature with the expanse of interaction rising with increasing hydrophobicity of alkyl side-chain on dyes [32]. This previous reference indicator to insolubility of mixture of both dye and surfactant with water in which mosquitoes live and consequently, appearance of difficulties in feeding behavior so toxicity of dye may be decreased or not affected.

Conclusions
Conclusively, our results revealed that the tested dye, Rose Bengal showed pronouncing insecticidal activities against mosquitoes in both concentration and sunlight exposure time. Based on LC_{50} values, Rose Bengal exhibited the highest toxicity against *Culex pipiens* larvae. Also, results showed that the addition of some surfactants to tested dye exhibited no effect as application of dye alone which give high effectiveness against *Culex pipiens*. Generally, Rose Bengal could be used in pest control management strategies as alternative to traditional insecticides in controlling pest to decrease costs and positively affect environment and human health.

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