Effect of *Citrus sinensis* peel extract containing Ag-TiO$_2$ nanocomposite on the percent mortality of *Aedes aegypti* larvae

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Abstract. To date, no efficient method exists for controlling *Aedes aegypti*, the vector of the dengue virus, and there is no definitive treatment for dengue fever. As synthetic insecticides can induce vector resistance, alternative vector controls comprising secondary plant metabolites and nano composites, such as Ag-TiO$_2$, have been investigated. The purpose of this study was to evaluate the effectiveness of *Citrus sinensis* peel alcoholic extract containing Ag-TiO$_2$ on the percent mortality of *A. aegypti* larvae. This experimental study included a control group and the following three treatment groups: 1) *C. sinensis* peel extract alone at five different concentrations (100–500 ppm), 2) Ag-TiO$_2$ nanocomposite alone at five different concentrations (5–25 ppm), and 3) *C. sinensis* peel extract and Ag-TiO$_2$ mixture. Significant positive correlations were observed between Ag-TiO$_2$ concentrations and the percent mortality of *A. aegypti* larvae ($R = 0.823$, $P < 0.05$). Our findings indicate that the addition of Ag-TiO$_2$ increases the larvicidal effect of *C. sinensis* peel extract.

1. Introduction
Dengue fever is an arbovirus infection transmitted to humans by infected *Aedes aegypti* and *A. albopictus* mosquitos. In 2016, the Directorate General of Disease Prevention and Management, Ministry of Health, Republic of Indonesia, has reported the incidence of dengue fever cases in Indonesia to be 77,96/100,000 population and case fatality rate to be 0.79% (1,585 deaths) [1].
According to World Health Organization (WHO), no specific treatment is available for dengue fever, and supportive treatment is generally provided to date. In severe cases, dengue fever can cause abdominal pain; mucosal bleeding; liver enlargement; hematocrit increase accompanied by platelets decrease and other signs that accompany plasma leakage, resulting in shock; or fluid accumulation, resulting in respiratory disorder [2]. Owing to these life-threatening complications, prevention efforts are crucial to reduce the incidence of dengue fever.

Prevention efforts against dengue fever transmission involve vector eradication. The Bureau of Communication and Community Service, Ministry of Health, Republic of Indonesia, recommends the eradication of breeding mosquitos as the most effective method to suppress the incidence of dengue fever. This involves the drainage and closure of mosquito breeding sites in the water reservoirs then sprinkling with larvicides, and recycling unused goods [3].

The main vector responsible for the spread of dengue virus spread is A. aegypti [4]. Neurotoxic synthetic insecticides, such as carbamates, organochlorines, organophosphates, and pyrethroids, have been globally used for vector control. However, resistance to these four insecticides has been detected in America, Africa, and Asia [5]. In 2013, Hardjanti et al. [6] have discovered A. aegypti resistance to organophosphates in Pulo Gadung, Jakarta, Indonesia [6]. Moreover, in 2009–2010, another study has reported vector resistance in Surabaya; in this study, the mortality rate was reported to be 22%–60% [7]. Larval resistance develops through the increased activity of detoxification enzyme, detoxification geneover-transcription or V10161Kdrgene mutation [4].

The use of alternative insecticides derived from plants has been extensively studied. Plants are a rich source of bioactive compounds that can be used for developing environmentally friendly vector control agents. Citrus plants, commonly used in food processing, beverages, and as a flavor enhancer, have demonstrated mosquitoicidal properties [8]. Additionally, the efficacy of *Citrus sinensis* (sweet orange) as a larvicide against A. aegypti has been investigated [8–10]. Murugan et al. [9] have confirmed that a petroleum ether extract of *C. sinensis* peel, which contains secondary metabolites, such as alkaloids, flavonoids, and terpenoids [10], exerted a larvicidal effect against *A. aegypti* (LD50, 342.45 ppm for instar III larvae and 436.93 ppm for instar IV larvae) [9].

With rapid advances in the area of nanoparticle science, larvicides, such as silver nano particles (AgNPs), are commonly being used. Reportedly, AgNPs bio synthesized using *Bacillus thuringiensis* (Bt-AgNPs) show larvicidal effects against *A. aegypti* larvae of instar III and IV [11]. Other nanoparticles, such as titanium dioxide (*TiO₂*), have also been reported to exert larvicidal effects against *A. aegypti* and *Culex quinquefasciatus* larvae [12].

Because many larvae have developed resistance to *C. sinensis* peel extract, we aimed to determine whether its larvicidal effect can be enhanced by the addition of an Ag ion nanocomposite.

2. Methods

This research has been approved by the Health Research Ethics Committe, Faculty of Medicine, Universitas Indonesia-Cipto Mangunkusumo Hospital (certificate number 1059, issued October 23, 2017). The experimental study was conducted in the laboratory of the Department of Parasitology, Faculty of Medicine, University of Indonesia, Jakarta, Indonesia, from September to December 2017. *C. sinensis* fruit was obtained from the Balitjestro plantation run by the Ministry of Agriculture, Republic of Indonesia, East Java. The fruit bore a green peel, and the base weight was 1.355 g.

2.1 Extract preparation

For preparing the *C. sinensis* peel extract, first, the fruit peels were thoroughly dried. Then, the dried peel was ground to to a powder with a blender. The resultant powder was dissolved in 70% alcohol using a magnetic stirrer for 15 min at 5,000 rpm. After standing for 24 h at room temperature, the solution was filtered, and the filtrate was kept a side. The solvent in the filtrate was removed using a vacuum dryer. Finally, a highly concentrated *C. sinensis* peel extract was obtained.
2.2. Nanocomposite manufacture

The nanocomposite was manufactured using a photo-assisted deposition method. The net weight of the Ag-TiO₂ nanocomposite obtained was 1.9 g, with an Ag concentration of 1%. The Ag-TiO₂ nanocomposite was gray, and it turned purple when exposed to the air.

2.3. Breeding of Aedes aegypti larvae

*A. aegypti* eggs deposited on a filter paper were obtained from the Department of Entomology, Bogor Agricultural Institute, West Java. There were approximately 1,500 black eggs on each filter paper. The filter papers were soaked in 1,500 ml water for approximately 3–5 days to allow the eggs to hatch. Sufficient nutrition was provided to the hatched larvae in the form of pellets. Instar III and IV larvae that actively moved when stimulated were selected for the larvicidal bioassay.

2.4. Larvicidal bioassay

The appropriate number of samples to be included in the larvicidal bioassay was calculated according to the WHO guideline [13]. The experiment was repeated four times at each concentration, with 25 larvae on each repetition. A negative control group was treated with aquades only, and the three treatment groups were treated with either five different concentrations of *C. sinensis* extract alone (100, 200, 300, 400, and 500 ppm), Ag-TiO₂ nanocomposite alone (5, 10, 15, 20 and 25 ppm), or a mixture of *C. sinensis* extract and Ag-TiO₂ nanocomposite. Thus, the total number of larvae required for this experiment was 4 × 16 × 25 = 1,600 larvae. The nanocomposite-containing extract group was treated with *C. sinensis* peel extract at concentrations same as the extract-alone group mixed with the optimum concentration of Ag-TiO₂ nanocomposite, as determined on the basis of the results obtained from the nanocomposite-alone group.

2.5. Observation of larvae morphology

Larval morphology was observed using a binocular dissecting microscope. Color change; damage to the thorax, abdomen, or anal segments; and the number of abdominal hair on the dead larvae were recorded.

2.6. Data analysis

SPSS version 20 statistical software was used for data analysis. First, the Shapiro–Wilk normality test was performed on the control and the treatment groups. If the results indicated a normal data distribution, a one-way ANOVA parametric test was performed. If the data distribution was not normal, a Kruskal–Wallis nonparametric test was performed. Then, *post hoc* tests were conducted to determine the differences among the five concentration groups. A linear regression test was used to determine the correlation between each concentration and percent larval mortality. Finally, a probit regression test was performed to determine the LD₅₀ and LD₉₀ concentrations.

3. Results

3.1. Larvicidal bioassay of the extract-alone group

No dead larvae were observed in the negative control group for the first 48 h of observation. In the extract-alone group, the observed mortality rate at 6 h for all *C. sinensis* concentrations was low (0.6%). At 24 h, most larvae treated with the highest concentration (500 ppm) died. However, the overall larval mortality for the extract-alone group at 24 h was relatively low (2%).

All *C. sinensis* peel extract concentrations showed a weak but positive correlation with percent larval mortality at 24 h and 48 h of observation (R = 0.508, R = 0.422, respectively), indicating that percent larval mortality there increased with increase in extract concentration although at a small extent. LD₅₀ at 24 h was 2.171 ppm (Figure 1 and 2). The morphological changes in the extract-alone group varied. Up to 90% of larvae exhibited a paler color and damage to the abdomen or thorax, which are typical characteristics of larvae releasing their body sheaths.
3.2. Larvicidal bioassay of the nanocomposite-alone group
In the nanocomposite-alone group, the larvae treated with high nanocomposite concentrations (20 ppm and 25 ppm) began to die at 1 h. At 2 h, larval mortality was >50% at 20 and 25 ppm. At 4 h, larval mortality was 100% at 20 ppm. However, larvae mortality at 4 h was also observed at the lowest concentration of 5 ppm. At 8 h, 100% larvae died for all nanocomposite concentrations. At 4 h, all Ag-TiO$_2$ nanocomposite concentrations showed a strong positive correlation with percent larval mortality (R = 0.907), indicating a dose-dependent effect (Figure 3). At 4 h, LD$_{50}$ was at 11.4 ppm and LD$_{90}$ was 19.64 ppm. At 6 h, all Ag-TiO$_2$ nanocomposite concentrations showed a weak but positive correlation with percent larval mortality (R = 0.574) (Figure 4). At 6 h, LD$_{50}$ at 6 h was 4.0 ppm and LD$_{90}$ was 8.54 ppm.

In the nanocomposite-alone group, the larvae exhibited a darker color, and 90% larvae suffered abdominal damage, which are typical morphological changes observed in Ag-TiO$_2$ nanocomposite treatment and results from bending of the abdomen due to external damage, giving the appearance of a constriction. Additionally, larvae were observed with breaks in their upper gastrointestinal tract.

3.3. Larvicidal bioassay of the nanocomposite-containing extract group
In the nanocomposite-containing extract group, percent larval mortality was observed at five different $C.~sinensis$ extract concentrations to which 25 ppm Ag-TiO$_2$ nanocomposite was added. The optimal nanocomposite concentration of 25 ppm was determined according to the results obtained from the nanocomposite-alone group.

In the nanocomposite-containing extract group, the larvae began to die at 6 h for all $C.~sinensis$ concentrations except at the highest extract concentration (500 ppm), at which total larval mortality was only 1%. At 6 h, the larval mortality appeared to sequentially decrease for the extract concentrations of 200 to 500 ppm. At 12 h, the mortality rate significantly increased compared with that at 6 h. The larvae mortality at 12 h was 90% concentrations for of 400 and 500 ppm, reaching 100% at 24 h. At 6 h, all Ag-TiO$_2$ nanocomposite concentrations showed a weak negative correlation percent larval mortality (R = 0.163). Thus, the probit regression test to determine the LD$_{50}$ at 6 h was not. At 12 h, all concentrations of $C.~sinensis$ peel extract containing Ag-TiO$_2$ showed a weak but positive correlation with percent larval mortality (R = 0.181) (Figure 5). The probit regression analysis that was performed at 12 h observation determined an LD$_{50}$ and LD$_{90}$ of 16.49 and 489.68 ppm, respectively.

3.4. Summary of larvicidal bioassay results at 6 h
Table 1 summarizes the results of percent larval mortality at 6 h of the extract-alone group, the larvae treated with 25 ppm of nanocomposites, and the group treated with nanocomposite-containing extract, all of which showed a difference in mortality at 6 h. The nanocomposite-containing extract group showed higher mortality than the extract-alone group.

| Treatment                                      | $C.~sinensis$ concentration (ppm) | Total larval mortality (%) (minimum–maximum) |
|-----------------------------------------------|----------------------------------|---------------------------------------------|
| $C.~sinensis$ peel extract                    | 100                              | 0                                           |
|                                               | 200                              | 1 (0–1)                                     |
|                                               | 300                              | 1 (0–1)                                     |
|                                               | 400                              | 0                                           |
|                                               | 500                              | 1 (0–1)                                     |
| Ag-TiO$_2$ nanocomposite                      | 25                               | 100                                         |
| $C.~sinensis$ peel extract + Ag-TiO$_2$ nanocomposite (25 ppm) | 100                              | 4 (0–2)                                     |
|                                               | 200                              | 13 (1–8)                                    |
|                                               | 300                              | 10 (0–5)                                    |
|                                               | 400                              | 9 (0–6)                                     |
|                                               | 500                              | 1 (0–1)                                     |
Figure 1. Correlation of *Citrus sinensis* peel extract concentration with percent larval mortality at 24 h

Figure 2. Correlation of *Citrus sinensis* peel extract concentration with percent larval mortality at 48 h
Figure 3. Correlation of Ag-TiO$_2$ nanocomposite concentration with percent larval mortality at 4 h

Figure 4. Correlation of Ag-TiO$_2$ nanocomposite concentration with percent larval mortality at 6 h
4. Discussion

Dengue fever is caused by a dengue virus infection. *A. aegypti* is the main vector of this arbovirus. Because dengue fever is potentially fatal, efforts need to be made toward efficient vector eradication. The use of alternative plant-derived insecticides has been extensively studied owing to the development of vector resistance to synthetic insecticides in many countries, including Indonesia [6, 7].

This study observed that at 6 h, *A. aegypti* larvae treated with *C. sinensis* peel extract in alcohol demonstrated only 0.6% total mortality. Larval mortality at 24 h was relatively variable, supporting WHO’s argument that a valid bioassay of larval mortality should use 24–48 h of observational data [13].

According to Muruganet al [9], at 24 h, percent mortality of larvae treated with *C. sinensis* peel extract in petroleum ether varied between 23% and 70% at concentrations of 100 and 500 ppm, respectively [9]. This is in contrast to our results, which demonstrated a much lower percentage of larval mortality (2%). This disparity in mortality rates could be due to differences in insolvent usage while preparing the *C. sinensis* extract stock solution. We used aquades for preparing stock solutions, while Murugan et al. used acetone [9]. The dissolution of the stock solution would be improved if dissolved with the same solution as the immersion solution because the polarity of the solution would remain unchanged. Aquades shows the polarity index of 10.2, whereas acetone and methanol show the polarity index of 5.1.

Reportedly, *C. sinensis* peel extract with solvent contains active alkaloids, carbohydrates, glycosides, tannins, phenolics, saponins, triterpenoids, and flavonoids [14], which exhibit insecticidal properties [11, 15]. Alkaloids slow larvae movement by interfering with nerve impulse transmission [15]. We demonstrated that higher extract concentrations slowed movements in a greater number of larvae. Saponins disrupt the oxygen supply to larvae, thereby compromising their growth.

For the extract-alone group, LD$_{50}$ at 24 h was 2.171 ppm, which was much lower than the values reported in other studies; Kumar et al. have reported LD$_{50}$ values of 39.51 and 55.58 ppm with *C.
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observed.

extract group, which displayed the same correlation direction at both observation points; this finding is consistent with that reported by Banu et al. who have investigated the effect of Ag nanoparticles, as well as by Thandapani et al. who have investigated the effect of TiO$_2$nanohybrid [11,12]. Both these studies have reported that the percent larval mortality increased with increasing concentrations of Ag nanoparticles and TiO$_2$nanohybrid, respectively. Furthermore, in these studies, not all larvae died at 24 h of Ag and TiO$_2$ treatments. In contrast, we observed 100% larval mortality at 8 h for all nanocomposite concentrations in the nanocomposite-alone group, which is significantly different, being three times faster than the previously reported time. However, the lethal concentration of Ag-TiO$_2$ nanocomposite cannot be compared with that reported in others owing to difference in observation time when performing the probit regression test. The shorter time to mortality observed in our study may have been caused by an increase in the photocatalytic activity of Ag-TiO$_2$. A previous study has observed that the photocatalytic activity of Ag-TiO$_2$ increased compared with that of TiO$_2$ nanoparticles alone. Furthermore, Ag-TiO$_2$nanocompositewith AgNO$_3$ precursors has been reported to demonstrate higher photoactivity compared with Ag-TiO$_2$ nanocomposite with other precursors [16]. We suspect that because of this property, the Ag-TiO$_2$ nanocomposite exerted a faster larvicidal effect than Ag nanoparticles or TiO$_2$nanoparticles alone.

In the nanocomposite-alone group, larvae were observed with breaks in their upper gastrointestinal tract. Such changes could be induced by the small size of the nanocomposite, which contains Ag nanoparticles approximately 6–20 nm in length. Because the epithelial cells of the larval midgut are larger (up to several micrometer [17]), the relatively smaller size of the nanocomposite could allow for easier penetration into midgut epithelial cells.

Our study is the first to demonstrate the larvicidal effects of an Ag-TiO$_2$ nanocomposite-containing extract of C. sinensis peel. To activate the photocatalytic properties of the Ag-TiO$_2$, UV light exposure is required. Thus, the nanocomposite-alone and the nanocomposite-containing extract groups were exposed to UV light.

The direction of the correlation of Ag-TiO$_2$ nanocomposite concentration with percent larval mortality at 6 h and 12 h exhibited different patterns in all groups except the nanocomposite-alone group, which displayed the same correlation direction at both observation points; this may have been caused through several mechanisms. First, the increased turbidity of the solution with increasing extract concentration might have blocked the UV light required to catalyze the Ag-TiO$_2$ nanocomposite. Second, the Ag-TiO$_2$ nanocomposite might have reacted with the active substances contained in the extract. Finally, the strength of the UV radiation applied to the nanocomposite-containing extract group might have weakened because this group was the last to be treated and observed.

The morphological characteristics appeared to be a mix of the typical characteristics of C. sinensis extract and Ag-TiO$_2$ nanocomposite treatments. Many larvae had released their body sheath, and many displayed a bent abdominal segmental though the anal segment of the larvae appeared intact. The bending of the abdominal segment might be attributed to the same mechanism as mentioned for that in the nanocomposite-alone group above.
5. Conclusion
The addition of Ag-TiO$_2$ to _C. Sinensis_ peel extract increased its larvicidal effect against _A. aegypti_. Changes in larval morphology included the darkening color, release of the body sheath, abdominal damage, and abdominal bending.

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