Integrated Control of *Aedes Aegypti* With Parasitic Nematodes, Biological Control Agents, Chemical Insecticides and Plant Essential Oil

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Abstract

Aedes aegypti can transmit dengue fever, yellow fever, Chikungunya fever, Zika virus disease and vector density control is the most effective way to prevent these infectious diseases. However, the extensive use of chemical pesticides has caused a series of problems, such as environmental pollution, killing non-target organisms and so on. In this study, a parasitic nematode, *Romanomermis wuchangensis* was used in the larviciding evaluation of *Ae. aegypti*, while the activity of four chemical insecticides and biological control agents were tested. Besides, *Mentha haplocalyx* essential oil was isolated and its olfactory physiological function with OBP1 protein of *Ae. aegypti* antenna was measured by the prokaryotic expression and fluorescence competitive binding assay. Compared with the control group, *R. wuchangensis* indicated high efficiency and environmental friendliness in the control of *Ae. aegypti.* After the second instar larvae were parasitized, the mortality of two treatment groups exceeded 75%. Compared to control group, the quantitative real-time PCR analysis results demonstrated that *SOD*, *POD* and *CAT* genes had obvious high expression levels in the nematodes parasitic groups. The antioxidant enzyme test results also exhibited obvious difference of SOD, CAT and POD during the nematode parasitic period. Besides, *Bacillus thuringiensis* (Bti) and chemical insecticide experimental results also showed great insecticidal efficacy against mosquito larvae. Five chemical components including Menthol, Pinene, Limonene, Isopulegol and Pulegone were identified from *M. haplocalyx* and exhibited great binding ability with AaegOBP1 protein. Present results illustrated that the integrated application of these various mosquito vector control methods in the future has broad prospects.

Introduction

The mosquito *Aedes aegypti* were considered to be one of the most dangerous medical insects in the world [1] , as they were vectors of several globally important vector-borne diseases, including dengue virus (DENV) [2] , yellow fever virus [3] and chikungunya virus (CHIKV) [4] . In the past 50 years, the dengue epidemic caused more than one billion infections and one million deaths, and the number of cases have increased 30 times without any signs of slowing down [5,6] . Moreover, in Asia and the Americas, the burden of dengue was approximately 1300 disability-adjusted life years (DALYs) per million population, which was similar to the other childhood and tropical diseases in this regions [7] . Since 1978, dengue has been detected for nearly forty years in China and it also occasionally erupts in Guangdong Province every year. Geographically, the dengue outbreaks have gradually expanded from Guangdong, Hainan, and Guangxi in Southern coastal regions of China to other regions including Fujian, Zhejiang and Yunnan Provinces [8] . Due to the outbreak of dengue fever in Guangdong Province in 2014, more than ten thousands of people were infected by the dengue epidemic and the local government spent >$30 million US dollars to control mosquito populations [9] .

Until now, because of no vaccine or specific treatment for dengue fever, many indirect prevention and control measures have been taken, such as population surveillance, disease prevention and rapid outbreak response are used to improve mosquito vector control[1]. The use of insecticides has often been

the only feasible method of disease control. For instance, wide-scale house spraying of DDT during the 1950s to 1960s dramatically reduced malaria incidence in Asia, and the pyrethroids were introduced to Cambodia in the late 1980s for malaria and dengue control[5]. In China, more than 27, 000 kg of pyrethroids were used for ultra-low volume spraying in the dengue control of Guangzhou city in 2014, and a large amounts of temephos and fenthion were also used as larvicides to prevent the mosquito spread[10]. However, the use of chemical pesticides not only polluted the environment but also caused great harm to non-target insects including butterfly, honeybee, bumblebee and other pollinators. The widespread use of chemical pesticides could easily interfere normal physiological behavior of butterfly and bee, such as visiting flowers, foraging, pollination and other ecological function[11,12].

Insect growth regulators (IGRs) have been widely used in pest control in many areas, which were highly effective larviciding agents of mosquito larvae and showed low mammalian toxicity and more safety to most of non-target organisms[13,14]. Pyriproxyfen and S-methoprene were two kinds of effective juvenile hormone mimic with low toxicity to mammals, which could affect the hormonal balance in insects, thus strongly inhibit embryogenesis, metamorphosis and adult formation[15,16]. Diflubenzuron was also a readily available insect growth regulator, which could inhibit chitin synthesis and exhibited the ovicidal and larvicidal properties[17]. Besides, there were also some bioinsecticides used for larvae control, such as the microbial agent *Bacillus thuringiensis* (Bti) has become the most commonly used larvicide worldwide, which can form spores which contain crystals, predominantly comprising one or more Cry and/or Cyt proteins that have potent and specific insecticidal activity[18,19]. Plant-derived essential oils were also an environmentally friendly control agent, especially for repelling adult mosquitoes, indicating that they can be an important supplement for mosquito pest control[20].

In addition, nematodes also played a key role in the field prevention of mosquito pests, such as *Romanomermis iyengari* (Mermithidae) was one of entomopathogenic nematodes which parasitized and killed mosquito larvae[21]. In our laboratory, the mermithid nematode *R. wuchangensis* has also been successfully used as an ecosystem-friendly biocontrol agent for mosquito control. Our previous study indicated that *R. wuchangensis* could infect *C. quinquefasciatus* and *Ae. albopictus*, and the infection rate and fatality rate of *C. quinquefasciatus* reached 49.18% and 100% in the field experiment[29]. The results demonstrated that the control of mosquito by nematodes had the characteristics of environmental friendliness and long-term sustainability, suggesting they maybe widely used in field biological control of mosquito pests.

Reactive oxygen species (ROS) produced by insect cells was the first line of defense against the invasion of insecticides and parasites, including hydrogen peroxide (H_2O_2), free hydroxyl (OH^-) and superoxide anion (O^{2-})[22]. Insects mainly protected themselves by antioxidant enzymes, such as Superoxide dismutase (SOD) could effectively remove O^{2-} and convert it into H_2O_2, Catalase (CAT) and Peroxidase (POD) work together to remove H_2O_2, which these antioxidant enzymes coordinated to regulate ROS in insects to keep them in dynamic balance[23,24]. Early studies have shown that in the process of

parasitizing the host, the death of the host was usually caused by oxidative damage, including *Sarcophagha crassipalpis, Nasonia vitripennis*, *Plutella xylostella*,, *Cotesia plutellae, Tenebrio Molitor* and *Scleroderma guani*, etc[25–27]. However, there was no report about the oxidative damage effect of nematode on the mosquito, though it may play a vital role of biological control of mosquitoes.

In this study, we present statistics on the incidence of dengue fever in China from 2015 to 2018 prior to the novel Coronavirus outbreak. Besides, we compared the control effects of chemical insecticides, IGRs, *B. thuringiensis* and plant essential oil on the larvae and adult of *Ae. aegypti*. The mortality rate of *Ae. aegypti* was observed during parasitized by *R. wuchangensis*, and the activities of SOD, POD and CAT during the lethal period of the host were tested to determine whether oxidative damage was involved, then explore the potential cause of oxidative damage to the host through the content of Malondialdehyde (MDA). Simultaneously, Real-time quantitative PCR were used to investigate the expression patterns of SOD, POD and CAT genes of *Ae. aegypti* larvae under different parasitic period by *R. wuchangensis*. In addition, the effective components of plant essential oil was identified from *Mentha haplocalyx* and chemoecological functional analysis of olfactory protein (OBP1) of *Ae. aegypti* were also investigated.

Materials And Methods

Ethics statement

The *Ae. aegypti* strain was provided by China CDC and reared in the laboratory of Hubei provincial center for disease control and prevention (Wuhan City, China). *Romanomermis wuchangensis* was a parasitic nematode and maintained in our laboratory incubator of Central China Normal University (Wuhan City, China). All animal experiments were carried out in accordance with the experimental guidance and regulations of Hubei CDC. *Mentha haplocalyx* was a common medicinal plant that grown in the experimental field and the extracted plant essential oils were carried out in accordance with relevant guidelines and regulations. All volunteer study in the repellent experiments of mosquito were conducted with the informed/written consent of all subjects and all experimental methods were carried out in accordance with relevant guidelines and regulations. All experimental protocols and animal procedures were also approved by the institutional committee of school of life science at Central China Normal University in China (CCNUIRB).

Statistical analysis of dengue fever cases in China

The data of dengue fever cases in China (excluding Hong Kong, Macao and Taiwan) from 2015 to 2018 were collected through the national infectious disease surveillance and reporting information system of Chinese Center for Disease Control and prevention (China CDC). The distribution of dengue cases in China was drawn by Arcgis 10.5 software. The data of seasonal distribution, population distribution, age and gender distribution and occupation distribution were calculated by Excel 2019.

Animals rearing and chemicals

Mosquito populations were maintained at insectary conditions (26°C±2°C; relative humidity 70%±10% L:D 14:10) and females were fed on mouse blood to complete their gonotrophic cycle. The mouse blood was collected from experimental animal center of Hubei provincial center for disease control and prevention (Wuhan City, China) and were also approved by the Institutional Review Board at Central China Normal University in China (CCNUIRB). Second instar larvae of *Ae. aegypti* was infected by *R. wuchangensis* by the ratio of 1:5 (mosquito: nematode). Then the infected mosquitos were maintained in the Plastic basin. When the nematodes emerged from *Ae. aegypti*, the nematodes of each developmental stage were independently collected and stored at 4°C. Insecticides were used in this study: deltamethrin, permethrin, temephos, propoxur, pyriproxyfen, S-methoprene, diflubenzuron and 7000 ITU/mg *Bacillus thuringiensis* (Bti). All tested insecticides were provided by China CDC.

IGRs tests

50 second instar larvae of *Ae. aegypti* were infected by 250 and 500 *R. wuchangensis* in glass breakers, by the ratio of 1:5 (mosquito: nematode) and 1:10, respectively. No nematodes were added to the control group and each beaker was filled with 50 mL chlorine-free water. Both the control group and treatment group were performed as three replicates and reared in the insectary. After 48 hours, add chlorine-free water to 100 mL. The mosquito larvae were observed every 24 hours and count the death of larvae.

A series of glass breakers (250 mL) were filled with 99.9 mL chlorine-free water, either 0.1 mL stock solution insecticide or 0.1 mL acetone was added. The acetone solution served as a control. For each concentration of the insecticide stock solution, three replicates were performed. Twenty-five third instars of uniform size were exposed to the insecticide solution in each replicate. The glass breakers were covered with medical gauze (to contain emerged adults) and a perforated plastic cap (to minimize evaporation). Emergence was determined after insecticide exposure as complete emergence had occurred in all controls at this time.

Chemical insecticides and Bti bioassays

In order to compare the insecticidal effects of biological insecticides, four representative chemical insecticides (temephos, permethrin, propoxur and deltamethrin) were used for comparative analysis. Chemical insecticides bioassays were set up as described above for the IGRs bioassays, except that mortality was determined after 72 h of exposure, and larvae were considered dead if they were unresponsive to touching with a probe[28]. *Bacillus thuringiensis* was set up as described above except that the solvent is changed from acetone to chlorine-free water.

Repellent activity of plant essential oil against mosquitos

The plant essential oil was isolated from the fresh leaves of *Mentha haplocalyx* by a Clevenger-type apparatus[20,29] and stored at -80°C until use. The chemical composition of this essential oil was analyzed by gas chromatography-mass spectrometry (GC-MS). All *Ae. aegypti* adults were kept in the cage (30 x 30 x 30 cm) and randomly separated into four groups of twenty-five each and were fasting for 1 day before the test. The *M. haplocalyx* essential oil (1.25μl, 2.5μl, 5μl, 10μl, 20μl) were applied on the ventral part of

volunteer forearm and inserted into the cage. The repellent activity of essential oil against *Ae. aegypti* was measured using the method described by Nasrul et al.[30]. The alcohol was conducted as control and each test concentration was repeated three times.

Assays of antioxidant enzyme activities and oxidative damage parameter

A series of glass breakers (250 mL) were filled with 99.9 mL chlorine-free water, either 0.1 mL stock solution temephos (0.003 mg/L, Preliminary experiments indicated this was the maximum sublethal concentration of temephos) or 0.1 mL acetone was added. The acetone solution served as a control. For each of the stock solution, three replicates were performed. 50 third instar larvae of uniform size were exposed to the insecticide solution in each replicate. Then, 50 second instar larvae of *Ae. aegypti* were infected by *R. wuchangensis* in glass breakers, by the ratio of 1:10 (mosquito: nematode). No nematodes were used for the blank control comparison and each beaker was filled with 50 mL chlorine-free water. Both the control group and treatment group were performed as three replicates, reared in the insectary. All larvae in one glass breaker were assembled and homogenized in ice-cold buffer (0.8% NaCl, pH 7.4) in a proportion of 0.1 g body weight to 1 mL of buffer. After the homogenates were centrifuged at 10,000×g for 15 min at 4°C, the supernatant was collected for test. Antioxidant enzyme activities and oxidative damage parameter were measured using a commercially available assay kit (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China).

Real-time quantitative PCR analysis of antioxidant enzyme genes

The *Ae. aegypti* larvae (second instar) was infected by *R. wuchangensis* by the ratio of 1:10 (mosquito: nematode). Expression patterns of three antioxidant enzyme genes (*SOD*, AY745980.1, *POD*, XM_021849339.1, *CAT*, XM_001663550.3) were determined by real-time quantitative PCR analysis. Total RNA was extracted from different treatments with Trizol test kit and six primers (*SOD-YF, SOD-YR, POD-YF, POD-YR, CAT-YF* and *CAT-YR*, *Rps3* gene used as the reference, Table 1) were used to test their relative abundance of three antioxidant enzyme genes in mRNA level. *Actin* gene was used as the reference and real-time quantitative PCR was measured on a Bio-Rad CFX 96 PCR system. Three technical replicates and three biological replicates were conducted to check reproducibility. The experimental process refers to the method in our previous literature and the real-time PCR data was analyzed by the $2^{-\Delta Ct}$ method[31].

Table 1
Primers used in the experiments.

Primer name	Sequence (5'-3')
SOD-YF	AAAGGAAATGAAAGCCGC
SOD-YR	GTGGAACCAATCCCGTAGTA
POD-YF	CGGAGACAAGGAAAACGC
POD-YR	AGATCGACCTCGGGGAT
CAT-YF	AAGAAAACCCCGCTCGCT
CAT-YR	GGCATCCTTCAGGTGAGTAGC
Rps3-YF	GCCTGACATCTTTCTTT
Rps3-YR	CGAGGACATTCTGGGT
OBP1- BamHIF	CGCGGATCCGACGTTACTCCGCGGC
OBP1-Xho1R	CCGCTCGAGTTAAATCAGGAAGTAATGCTTGG

Olfactory recognition of *Ae. aegypti* OBP1 protein and potential repellent ligands from plant essential oil

The *Ae aegypti* olfactory gene *OBP1* (genebank number: AY189223.1) was used for prokaryotic expression and affinity chromatography purification. The target gene was induced by IPTG and expressed in *E. coli* prokaryotic expression system using by their technical methods described by Zhou et al[32]. After purification, the target protein was tested for its binding capacity with chemical ligands with potential repellent activity in *M. haplocalyx* essential oil. The fluorescence competitive binding assay was tested on the fluorescence spectrophotometer Hitachi F-4500 according to our laboratory previous method of Mao et al[31]. The fluorescent probe N-phenyl-1-naphthylamine (1-NPN) and potential repellent ligands from chemical composition of plant essential oil were used to measure their competitive affinities to *Ae. aegypti* OBP1 protein.

Statistical analysis

The mortality rate of tested larvae was analyzed using SPSS 20.0 software. Then the estimation of the lethal and emergence inhibition concentration of 50% confidence limits was obtained. All the data were analyzed by one-way ANOVA with the SPSS 20.0 and the figures were drawn by GraphPad Prism software. The result in the graphs represents the mean values ± SE. Statistical testing of significance was analyzed using Tukey’s multiple range test.

Results

Overview of dengue fever cases

A total of 16936 cases of dengue fever were reported in mainland China from 2015 to 2018, of which 12984 cases were local cases and 4042 were imported cases. The epidemiological characteristics of dengue fever cases were statistically analyzed throughout the year, and the epidemic began to rise in August and reached its peak in September every year. The main imported countries were Myanmar and Cambodia, and the proportion of imported cases showed an increasing trend (Table 2). Local cases were mainly concentrated in Guangdong (6232 cases), Yunnan (4189 cases), Zhejiang (1248 cases), Fujian (995 cases), Hunan (101 cases), Shandong (80 cases), Hainan (14 cases) and Guangxi (10 cases), sporadic local cases also have been reported in Shanghai, Henan and Anhui (Fig. 1). Imported cases were distributed in 57 countries (regions), mainly in Southeast Asia and South Asia. The main imported countries were Myanmar (1305 cases), Cambodia (489 cases), Thailand (381 cases), Malaysia (265 cases), the Philippines (205 cases), Indonesia (169 cases) and Vietnam (167 cases). The main provinces with imported cases were Yunnan (1240 cases), Guangdong (793 cases), Zhejiang (316 cases), Fujian (268 cases), Guangxi (113 cases) and Jiangsu (110 cases) (Fig. 2).

Table 2
Dengue fever cases in mainland China from 2015 to 2018

Year	Local cases	Imported cases	Total cases	Proportion of imported cases	Major imported country(proportion)
2015	3116	742	3858	19%	Myanmar (47%)
2016	1563	485	2048	24%	Myanmar (24%)
2017	4468	1425	5893	24%	Cambodia (49%)
2018	3747	1390	5137	27%	Cambodia (31%)

Dengue cases outbreak typically occur from August to November (89% of the total reported cases), with September being the peak. In China, the ratio of male and female affected by Dengue was 1.14:1, and the age was mainly from 20 to 65 years old (81%). The occupational distribution of reported cases were housework and unemployment (19%), business services (16%), farmers (14%), workers (10%), retirees (9%) and students (7%), accounting for 75% of the total reported cases.

Biological insecticides assays

In biological insecticides assays, the larvicidal activities of *R. wuchangensis* to second instar larvae of *Ae. Aegypti* were shown in figure 3, from the fifth day after infection, the *Ae. Aegypti* larvae gradually appeared dead individuals. In the different affected ratio groups (mosquito: nematode, 1:5 and 1:10), their mortality rate were 75.79% and 96.L00%, respectively. As shown in Table 3, three novel IGRs could obviously control the number of mosquito pests. Among them, the pyriproxyfen was the most toxic

larvicide (EC_{50}=0.000023 mg/L), followed by diflubenzuron (0.001659 mg/L) and S-methoprene (0.0856 mg/L). The efficacy of pyriproxyfen on *Ae. aegypti* larvae was more efficient than the other two IGRs.

Table 3
Insecticidal activity of IGRs to third instar larvae of *Ae. Aegypti*

Insecticide	EC_{50}(95% CI) (mg/L)	χ^2(df)	Slope	n
Pyriproxyfen	0.000023(0.000020-0.000026)	1.786(3)	2.869	450
S-Methoprene	0.0845(0.0744-0.0960)	2.738(3)	2.967	450
Diflubenzuron	0.001659(0.001478-0.001861)	3.169(3)	3.546	450

Chemical insecticides bioassay

Four representative chemical insecticides including temephos, permethrin, propoxur and deltamethrin were tested for their insecticidal efficacy against *Aedes aegypti*. Toxicities of four novel insecticides (temephos, permethrin, propoxur, deltamethrin) to third instar larvae of *Ae. aegypti* were shown in Table 4, and LC_{50} values were statistically analyzed. The deltamethrin was the most effective larvicide (LC_{50}=0.001658 mg/L), followed by permethrin (0.009222 mg/L), temephos (0.009371 mg/L) and propoxur (0.585596 mg/L). The dosage of propoxur was significantly higher than that of the other three insecticides.

Table 4
Toxicity of four chemical insecticides and Bti to third instar larvae of *Ae. Aegypti*

Insecticide	LC_{50}(95% CI) (mg/L)	χ^2(df)	Slope	n
Deltamethrin	0.001658(0.001454-0.001884)	4.039(3)	2.908	450
Permethrin	0.009222(0.007849-0.010903)	3.354(3)	2.078	450
Temephos	0.009371(0.008447-0.010403)	1.558(3)	4.291	450
Propoxur	0.585598(0.520827-0.658832)	1.145(3)	3.416	450
B. thuringiensis	0.000936(0.000842-0.001041)	2.119(3)	4.121	450

Chemical composition identification of *M. haplocalyx* essential oil and repellent activity

A total of 16 compounds in the *M. haplocalyx* leaf essential oil were identified and their abundances were shown in Table 5. Analysis of the essential oil by GC-MS revealed that Menthol, Pinene and

Cyclohexanone were the most abundant three major constituents in the essential oil, consisting 32.87%, 25.04% and 10.95% of the total oil, respectively. Other main compounds in this essential oil contained Limonene (3.83%), Menthyl acetate (2.87%), Longifolene (1.79%), Isopulegol (1.64%) and Pulegone (1.43%). Repellent testing of *M. haplocalyx* essential oil against *Ae. aegypti* indicated its excellent protection at greater doses of 20µl, 10µl and moderate protection at 5µl and 2.5µl. In contrast, the protection time was significantly reduced at the lowest dose 1.25µl. For all the tested groups, the repellent activity gradually increased with increasing concentrations (Fig. 4). Therefore, the essential oil of *M. haplocalyx* may prove useful in the development of mosquito repellents as an effective personal protection measure against mosquito bites.

Table 5
The chemical composition of *M. haplocalyx* essential oil

No.	Compounds	Abundance (%)
1	Pinene	25.04
2	Camphene	0.83
3	3-Octanol	0.31
4	1-methyl-2-(1-methylethyl) Benzene	1.09
5	Limonene	3.83
6	Isopulegol	1.64
7	Cyclohexanone	10.95
8	Menthol	32.87
9	Cyclohexanol	0.63
10	3-Cyclohexene-1-methanol	1.04
11	3-Hexenyl isovalerate	0.46
12	Pulegone	1.43
13	Menthyl acetate	2.87
14	alpha-Longipinene	0.29
15	Longifolene	1.79
16	Caryophyllene	0.55

Antioxidant enzyme activities and oxidative damage parameter

Compared to control group, we have noted the significant rise of MDA content in the larvae after parasitized by *R. wuchangensis* for 1 (p<0.01), 3 (p<0.05), and 5 (p<0.05) days (Fig. 5A). The MDA content in the larvae also significantly increased (p<0.01) after treated by temephos for 12 hours (Fig. 5B). A marked (p<0.05) elevation of SOD activity in *Ae. Aegypti* larvae was recorded when they were parasitized by *R. wuchangensis* for 1 and 5 days. However, the SOD activity was significantly (p<0.01) lower than the control group when they were parasitized by *R. wuchangensis* after 3 days (Fig. 6A). For temephos, SOD activity in *Ae. Aegypti* larvae was significantly increased (p<0.05) after treated for 24 hours (Fig. 6B). Besides, the POD activity was significantly enhanced in *Ae. Aegypti* larvae when parasitized by *R. wuchangensis* after 1 (p<0.05) and 5 (p<0.01) days, but no difference after 3 days (Fig. 7A). Temephos did not affect POD activity in *Ae. Aegypti* larvae (Fig. 7B). And there was no significant difference in CAT activity of *Ae. Aegypti* larvae, when parasitized by *R. wuchangensis* after 1 and 3 days, but after 5 days, there was also a significant (p<0.01) decrease in the treatment group (Fig. 8A). However, there was no significant difference of CAT activity in *Ae. aegypti* larvae after treated by temephos (Fig. 8B).

Real-time quantitative PCR analysis of antioxidant enzyme genes

Fluorescent quantitative PCR was used to detect the expression of antioxidant enzyme genes in different periods after *Ae. aegypti* larvae infected by nematodes. The real-time PCR results indicated that the expression levels of *SOD*, *POD* and *CAT* genes in the experimental group were up-regulated after parasitism (Fig. 9). Moreover, the expression levels of *Ae. aegypti SOD* gene in the experimental groups were significantly up-regulated at day 1, day 3 and day 5 (Fig. 9A) in each stage of nematode parasitization. These results suggesting that *SOD*, *CAT* and *POD* genes may play important roles in the resistance of *Ae. aegypti* to nematode invasion.

Purification of recombinant AaegOBP1 and fluorescence competitive binding activity

The *AaegOBP1* gene was successfully expressed in *E. coli* prokaryotic expression system after IPTG induction and purified by Ni-NTA resin affinity chromatography after ultrasonication. Sodium dodecyl sulfatepolyacrylamide gel electrophoresis (SDS-PAGE) analysis indicated that the AaegOBP1 protein was mainly expressed in supernatant (Fig. 10). The fluorescence competitive binding results demonstrated that AaegOBP1 protein could bind five ligand volatiles from *M. haplocalyx* essential oil, highlighting their great binding ability (Fig. 11). The AaegOBP1 protein showed the best binding affinity to Menthol and Pinene, with *Ki* values (the calculated inhibition constants) of 13.19 μM and 14.55 μM (Table 6). The purified target protein also exhibited significant binding capacity to other volatiles from *M. haplocalyx* essential oil including Limonene, Isopulegol and Pulegone, with the *Ki* values of 15.16 μM, 16.57 μM and 19.75 μM, respectively (Table 6).

Table 6
The binding constants of different ligands with AaegOBP1 protein

No.	Compounds	AaegOBP1	
		IC_{50} (μM)	K_i (μM)
1	Pinene	17.56	14.55
2	Limonene	18.16	15.16
3	Isopulegol	20.74	16.57
4	Cyclohexanone	-	-
5	Menthol	15.83	13.19
6	Pulegone	23.79	19.75
7	Menthyl acetate	-	-
8	Longifolene	-	-

Discussion

Dengue is one of the most common vector-borne diseases and has caused substantial health, society and economic burdens to all stakeholders. *Ae. aegypti* is a major vector for dengue, yellow fever, and chikungunya viruses, these diseases are increasing global public health concerns due to their rapid geographical spread and increasing disease burden[33–35]. With abundant rainfall, suitable for Aedes breeding, and frequent foreign trade, the highest dengue fever cases were reported in Guangdong and Yunnan province in southern areas of China. These two provinces keep close connections with South East Asian countries that are in the DF-endemic regions[36]. At present, chemical insecticides are still the main method to control mosquitoes, followed by insect growth regulators, plant essential oils and biological insecticides. Therefore, the development of effective and eco-friendly mosquito control methods is required in to minimize the negative effects of currently marketed insecticides, including multidrug resistance, environment pollution, killing non-target organisms, etc[37]. In this study, we compare the efficacy of several commonly used biological control agents and chemical insecticides on *Ae*. *Aegypti* larvae.

In addition, *R. wuchangensis* is also an important biological control agent and can specifically parasitize *Aedes* and *Culex* mosquito larvae, which is harmless to non-target organisms and environment. At the ratio of 1:10, the mortality rate of the mosquito larvae can exceed 95%. Ayaba et al. found that 100% L1 stage of *An. gambiae* larvae died within 24 hours after *Romanomermis iyengari* infection, and 100% of both L2 and L3 stage of larvae died within 7 days[38]. Our laboratory nematode *R. wuchangensis* also showed similar lethal efficiency to the *R. iyengari* against mosquito larvae. Except for nematodes, *Bacillus thuringiensis* (Bti) as an important microbial insecticide were also used to control mosquito

larvae in this study. Actually, all mosquito populations were fully susceptible to Bti, which was currently used both in Brazil and Switzerland[39]. This kind of biological insecticide was only partially used in China because of its high cost. Moreover, it's worth our attention that long term use of Bti was subject to the development of resistance to Bti toxins[40]. Expect for Bti, there were entomopathogenic fungi that produced infective spores (conidia) could attach and penetrate the cuticle of mosquitoes, which released toxins that result in mosquito death[41]. Natural enemies feeding on mosquito larvae and pupae also could play important roles in reducing mosquito populations, such as fish, omnivorous copepods, Several species of copepods, etc[42].

In addition to biological control agents, chemical repellent agents and insecticides are also used to control *Aedes aegypti*, *Aedes albopictus* and other mosquito pests in the field. Temephos is the most widely used larvicide for mosquito control, which is often applied to prevent *Ae. Aegypti* larvae in the household containers[43]. Based on present result, the LC_{50} was 0.009371 mg/L, and all of the larvae were dead as the concentration of 0.032 mg/L, suggesting that *Ae. aegypti* larvae are susceptible to temephos and it can be used to quickly control an outbreak of *Ae. aegypti* larvae. Similar to this study, in Argentina and French, the LC_{50} of temephos to different regions of *Ae. Aegypti* populations were between 0.00294 mg/L-0.01017 mg/L[44] and 0.008 mg/L-0.265 mg/L[45]. Moreover, the increased MDA content and decreased activities of antioxidative enzymes also suggested that the consumption of ROS depleted and inhibited the antioxidative enzymes activities, leading to increased *Ae. aegypti* larvae mortality. Present results demonstrated that the efficacy of temephos and pyrethroids were much higher than that of propoxur, highlighting that these highly effective and less toxic chemical insecticides can be used to control *Aedes* mosquitoes in different areas of the world.

In addition to control of mosquito larvae, different concentrations of plant essential oil can obviously repel adult mosquitoes, which is helpful for the prevention of mosquito pests. In present study, we also further explore the olfactory protein (OBP1) of *Ae. aegypti* and performed fluorescence competitive binding experiments with ligand compounds with higher content in plant essential oil. Fluorescence competitive binding analysis suggested that AaegOBP1 protein have strong binding ability to Menthol, Pinene, Limonene, Isopulegol and Pulegone from *Mentha haplocalyx*. These results indicated that AaegOBP1 protein could specifically distinguish the volatile odor molecules of plant essential oil through the recognition of different ligand compounds, involving in the olfactory repellent behavior of adult *Ae. aegypti*. Shi et al. reported that AsinOBP1 protein of Anopheles sinensis can bind Diethyltoluamide (DEET), which provides important reference value for further exploring the olfactory mechanism of mosquito pests[46]. In the subsequent experiments, protein homology modeling, molecular docking and site-directed mutation analysis of AaegOBP1 protein will also be used to further confirm repellent molecular mechanism of *Ae. aegypti*.

In conclusion, we assume that parasitism has the potential to generate oxidative stress in *Ae. Aegypti* larvae. During the infection, oxidative stress may be a metabolic adaptation and induce oxidative stress in host insects' antioxidant response, thus improving the survival ability of insects to infection. However,

when the balance is destroyed, the oxidative damage caused by ROS will increase the mortality of infected insects. With the widespread use of insecticides, the problem of insecticide resistance is becoming increasingly prominent. We recommend that multiple insecticides should be mixed-use and used in rotation, to slowing down the insecticide resistance. Obviously, the nematode *R. wuchangensis* is an efficient and environmentally friendly biological larvicide, highlighting it may play an important role in the prevention and control of many mosquito pests in the future.

Declarations

Acknowledgments

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Author Contributions

J.F.X., H.A. and G.X.W. initiated the project, conceived and designed the experiments. J.F.X. and H.A. wrote the manuscript. J.F.X., H.Z., R.M. and C.X.L. performed the biological and chemical insecticides tests, antioxidant enzyme activities assay, real-time quantitative PCR, prokaryotic expression, fluorescence competitive binding assay and data analysis. All authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

Competing interests

The authors declare no competing interests.

References

1. Santos, D.M., Rodriguez, C., Lagunes, A., Tejeda, M., Silva, G. Differential susceptibility of spinosad against eggs, larval instars, pupae, and female of *Aedes aegypti*. *South Entomol*. 41(4), 1111-1120 (2016).
2. Simmons, C.P., *et al*. Therapeutics for dengue: recommendations for design and conduct of early-phase clinical trials. *PloS Neglect Trop D*. 6(9), e1752 (2012).
3. Dinair, C.L. *et al*. Potential risk of re-emergence of urban transmission of yellow fever virus in Brazil facilitated by competent *Aedes Sci. Rep*. 7(1), 4848 (2017).
4. Isabelle, L.G., Antoine, N., Sylvie, C., Christine, P., Xavierde, L. Chikungunya in the Americas. *Lancet*. 383(9916), 8-14 (2014).

5. Wang, F.G. *et al*. Cartography of odor chemicals in the dengue vector mosquito (*Aedes aegypti* Diptera/Culicidae). *Sci. Rep*. 9(1), 8510 (2019).
6. Bhatt, S. *et al*. The global distribution and burden of dengue. *Nature*. 496, 504-507 (2013).
7. Gubler, D.J. & Meltzer, M. Impact of dengue/dengue hemorrhagic fever on the developing world. *Virus Res.* 53, 35-70 (1999).
8. Wu J. Y., Lun Z. R., James A. A., Chen X. G. Dengue Fever in mainland China. Amer Soc Trop Med & Hygiene. 83(3), 664-71(2010).
9. Meng, F.X., Wang, Y.G., Feng, L. & Liu, Q.Y. Review on dengue prevention and control and mosquito integrated management in China. Chin. *Vector Biol. & Control*. 26, 4-10 (2015).
10. Wang, Y.G. *et al*. A survey of insecticide resistance in *Aedes albopictus* (Diptera: Culicidae) during a 2014 dengue fever outbreak in Guangzhou, China. *Econ. Entomol.* 110(1), 239-244 (2017).
11. Williamson, S.M., Willis, S.J. & Wright, G.A. Exposure to neonicotinoids influences the motor function of adult worker honeybees. *Ecotoxicology*. 23, 1409-1418 (2014).
12. Xue, J., Tan, C., Zhang, X., Feng, B. & Xia, S. Fabrication of epigallocatechin-3-gallate nanocarrier based on glycosylated casein: stability and interaction mechanism. *Agric. Food Chem.* 62, 4677-4684 (2014).
13. Vythilingam, I., Luz, B.M., Hanni, R., Beng, T.S. & Huat, T.C. Laboratory and field evaluation of the insect growth regulator pyriproxyfen (SUMILARV 0.5G) against dengue vectors. *Am. Mosq. Control Assoc.* 21(3), 296-300 (2005).
14. Mulla, M.S. The future of insect growth regulators in vector control. *Am. Mosq. Control Assoc*. 11(2), 269-273(1995).
15. Baker, S.L. & Yan, N.D. Accumulated organic debris in catch basins improves the efficacy of S-methoprene against mosquitoes in Toronto, Ontario, Canada. *Am. Mosq. Control Assoc*. 26, 172-182(2010).
16. Stockwell, P.J. *et al*. A field evaluation of four larval mosquito control methods in urban catch basins. *Am. Mosq. Control Assoc*. 22(4), 666-671 (2006).
17. Graf, J.F. The role of insect growth regulators in arthropod control. *Parasitology Today*. 9(12), 471-474 (1993).
18. Rowe, G.E., Margaritis, A. & Wei, N. Specific oxygen uptake rate variations during batch fermentation of *Bacillus thuringiensis* subspecies *kurstaki* HD-1. *Biotechnol Progr*. 19(5), 1439-1443 (2008).
19. Sanahuja, G. *et al*. *Bacillus thuringiensis*: a century of research, development and commercial applications. *Plant Biotechnol J*. 9(3), 283-300 (2015).
20. Sumitha, K.V. & Thoppil, J.E. Larvicidal efficacy and chemical constituents of *gratissimum* L. (Lamiaceae) essential oil against *Aedes albopictus* Skuse (Diptera: Culicidae). *Parasitol Res*. 115(2): 673-680 (2016).
21. Abagli, A.Z., Alavo, T.B.C., Perez-Pacheco, R. & Platzer, E.G. Efficacy of the mermithid nematode, *Romanomermis iyengari*, for the biocontrol of *Anopheles gambiae*, the major malaria vector in sub-

Saharan Africa. *Parasites & Vectors.* 12, 253 (2019).

22. Lushchak, V.I. Environmentally induced oxidative stress in aquatic animals. *Aquat Toxicol*. 101(1), 13-30 (2011).
23. Felton, G.W. & Summers, C.B. Antioxidant systems in insects. *Arch Insect Biochem*. 29(2), 187-97 (1995).
24. Federico, A. *et al*. Mitochondria, oxidative stress and neurodegeneration. *Neurol. Sci*. 322(1-2), 254-262 (2012).
25. Danneels, E.L. *et al*. Early changes in the pupal transcriptome of the flesh fly *Sarcophagha crassipalpis* to parasitization by the ectoparasitic wasp, *Nasonia vitripennis*. *Insect Biochem Molec*. 43(12), 1189-1200 (2013).
26. Takeda, T., Nakamatsu, Y. & Tanaka, T. Parasitization by *Cotesia plutellae* enhances detoxifying enzyme activity in *Plutella xylostella*. *Pestic Biochem Phys*. 86(1), 15-22 (2006).
27. Zhu, J.Y., Ze, S.Z., Stanley, D.W. & Yang, B. Parasitization by *Scleroderma guani* influences expression of superoxide dismutase genes in *Tenebrio molitor*. *Arch Insect Biochem*. 87(1), 40-52 (2014).
28. World Health Organization. 2005. Guidelines for Laboratory and Field Testing of Mosquito Larvicides. Communicable disease control, prevention and eradication, WHO pesticide evaluation scheme. WHO/CDS/WHOPES/GCDPP/1.3. World Health Organization, Geneva, Switzerland.
29. Anonymous. *European Pharmacopoeia*, 3rd edition. Council of Europe, Strasbourg, 121-122 (1996).
30. Nasrul, W., Sriwidodo, Ferry, F. S., Angga, C.N. & Ajeng, N.M. Repellent activity of essential oils from *Cananga odorata Lamk* and *Cymbopogon nardus* on corn Starch-based thixogel. *J Young Pharma*. 10(2): 118-123 (2018).
31. Mao, A.P. *et al*. Sex pheromone recognition and characterization of three pheromone-binding proteins in the legume pod borer, *Maruca vitrata Fabricius* (lepidoptera: crambidae). *Rep*. 6, 34484 (2016).
32. Zhou, J. *et al*. Identification of host-plant volatiles and characterization of two novel general odorant-binding proteins from the legume pod borer, *Maruca vitrata Fabricius* (Lepidoptera: Crambidae). *PLoS ONE*, e0141208 (2015).
33. Hall, A.B. *et al*. A male-determining factor in the mosquito *Aedes aegypti*. *Science*. 348(6240), 1268-1270 (2015).
34. Kraemer, M.U.G. *et al*. The global distribution of the arbovirus vectors *Aedes aegypti* and *albopictus*. *eLife*. 4, e08347 (2015).
35. Benelli, G. *et al*. The recent outbreaks of Zika virus: Mosquito control faces a further challenge. *Asian Pac J Trop Med*. 6(4), 253-258(2016).
36. Fan, J., *et al*. Identifying the high-risk areas and associated meteorological factors of dengue transmission in Guangdong Province, China from 2005 to 2011. *Epidemiol Infect*. 142(3): 634-643 (2014) (In Chinese).
37. Seccacini, E. *et al*. Effectiveness of pyriproxyfen and diflubenzuron formulations as larvicides against *Aedes aegypti*. *Am. Mosq. Control Assoc*. 24(3), 398-403 (2008).

38. Staples, K., Oosthuizen, J. & Lund, M. Effectiveness of S-Methoprene briquets and application method for mosquito control in urban road gullies/catch basins/gully pots in a mediterranean climate: implications for ross river virus transmission. *Am. Mosq. Control Assoc*. 32(3), 203 (2016).
39. Suter, T. *et al*. Insecticide susceptibility of *Aedes albopictus* and *aegypti* from Brazil and the Swiss-Italian border region. *Parasites & Vectors*. 10(1), 431 (2017).
40. Griffin, L.F. & Knight, J.M. A review of the role of fish as biological control agents of disease vector mosquitoes in mangrove forests: reducing human health risks while reducing environmental risk. *Wetl Ecol Manag*. 20(3), 243-252 (2012).
41. Goindin, D. *et al*. Levels of insecticide resistance to deltamethrin, malathion, and temephos, and associated mechanisms in *Aedes aegypti* mosquitoes from the Guadeloupe and Saint Martin islands (French West Indies). *Infect Dis Poverty*. 6 (2017).
42. Ayorinde, A., Oboh, B., Oduola, A. & Otubanjo, O. The insecticide susceptibility status of *Aedes aegypti* (Diptera: Culicidae) in farm and nonfarm sites of lagos state, nigeria. *Insect Sci*. 15(1), 75 (2015).
43. Thongwat, D. & Bunchu, N. Susceptibility to temephos, permethrin and deltamethrin of *Aedes aegypti* (Diptera: Culicidae) from Muang district, Phitsanulok Province, Thailand. *Asian Pac J Trop Med*. 14-18 (2015).
44. Lushchak, V.I. Environmentally induced oxidative stress in aquatic animals. *Aquat Toxicol*. 101(1), 13-30 (2011).
45. Cadet, J., Sage, E. & Douki, T. Ultraviolet radiation-mediated damage to cellular DNA. *Mutation Research*. 571(1-2), 3-17 (2005).
46. Shi, Z. P. *et al*. Binding characteristics of the odorant binding protein AsinOBP1 of Anopheles sinensis (Diptera: Culicidae) with the mosquito repellent DEET. *Acta Entomologica Sinica*. 61(1), 139-148 (2018).

Figures

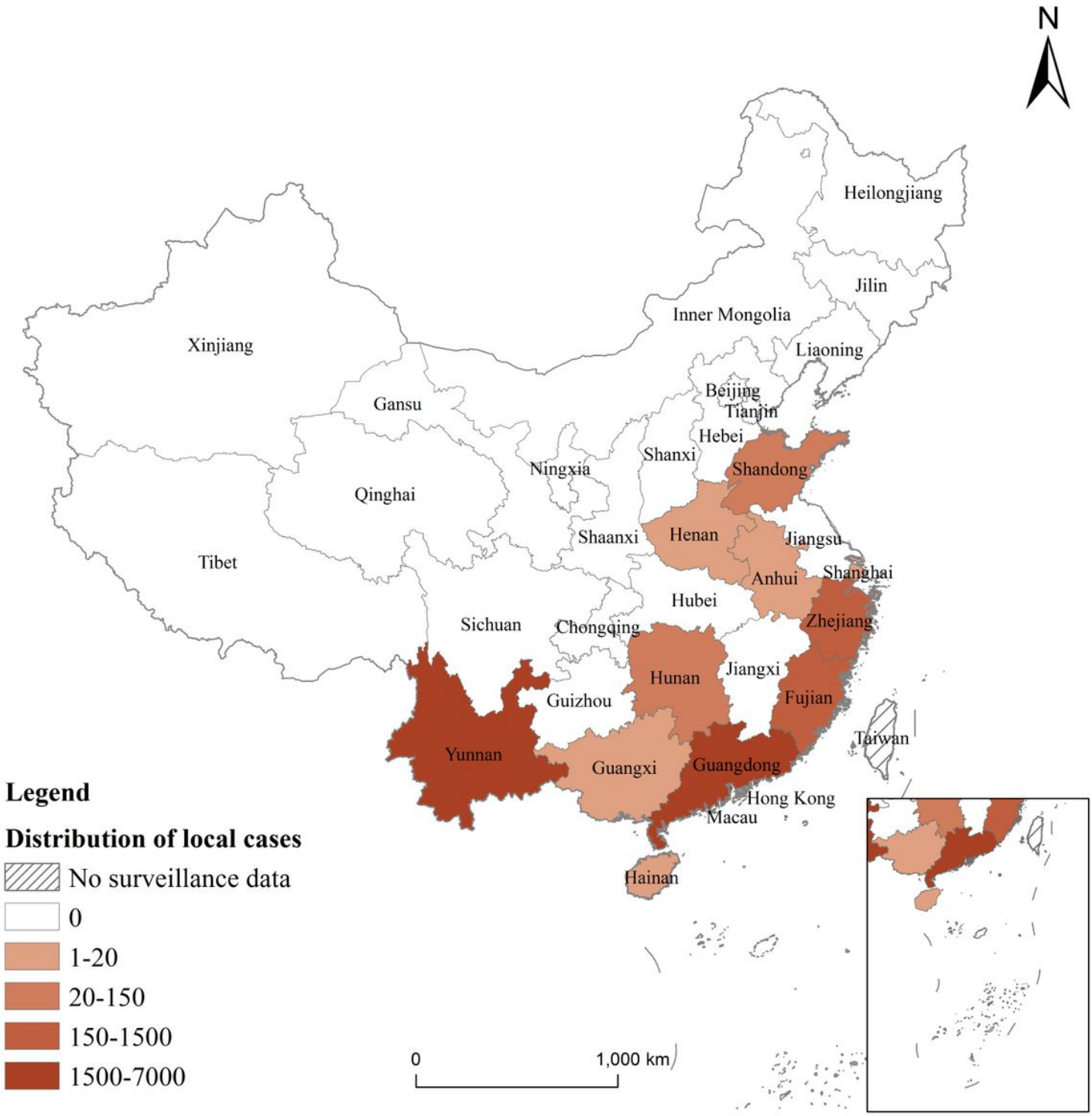

Figure 1

Geographic distribution of local dengue cases in mainland China from 2015 to 2018.

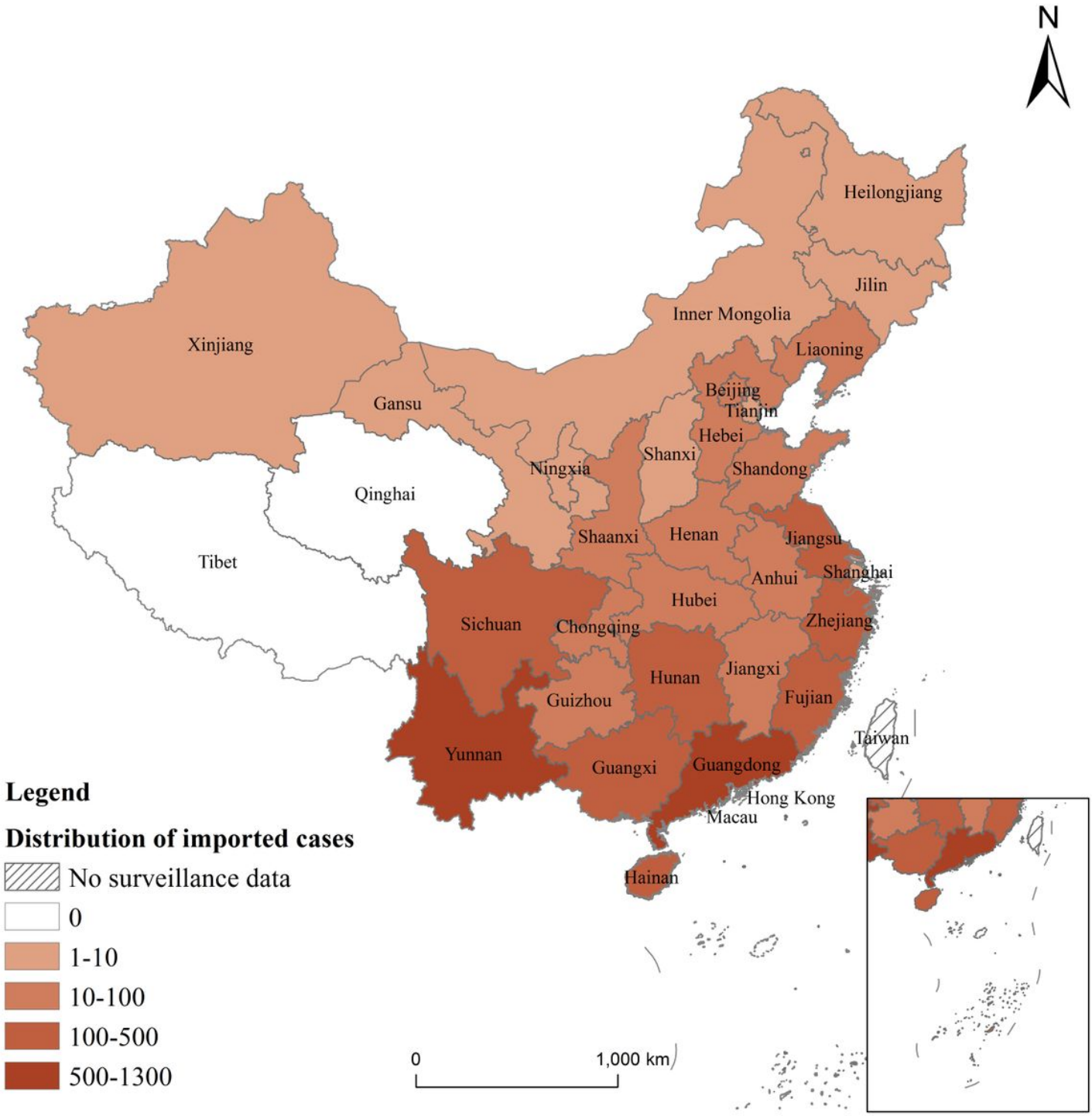

Figure 2

Geographic distribution of imported dengue cases in mainland China from 2015 to 2018.

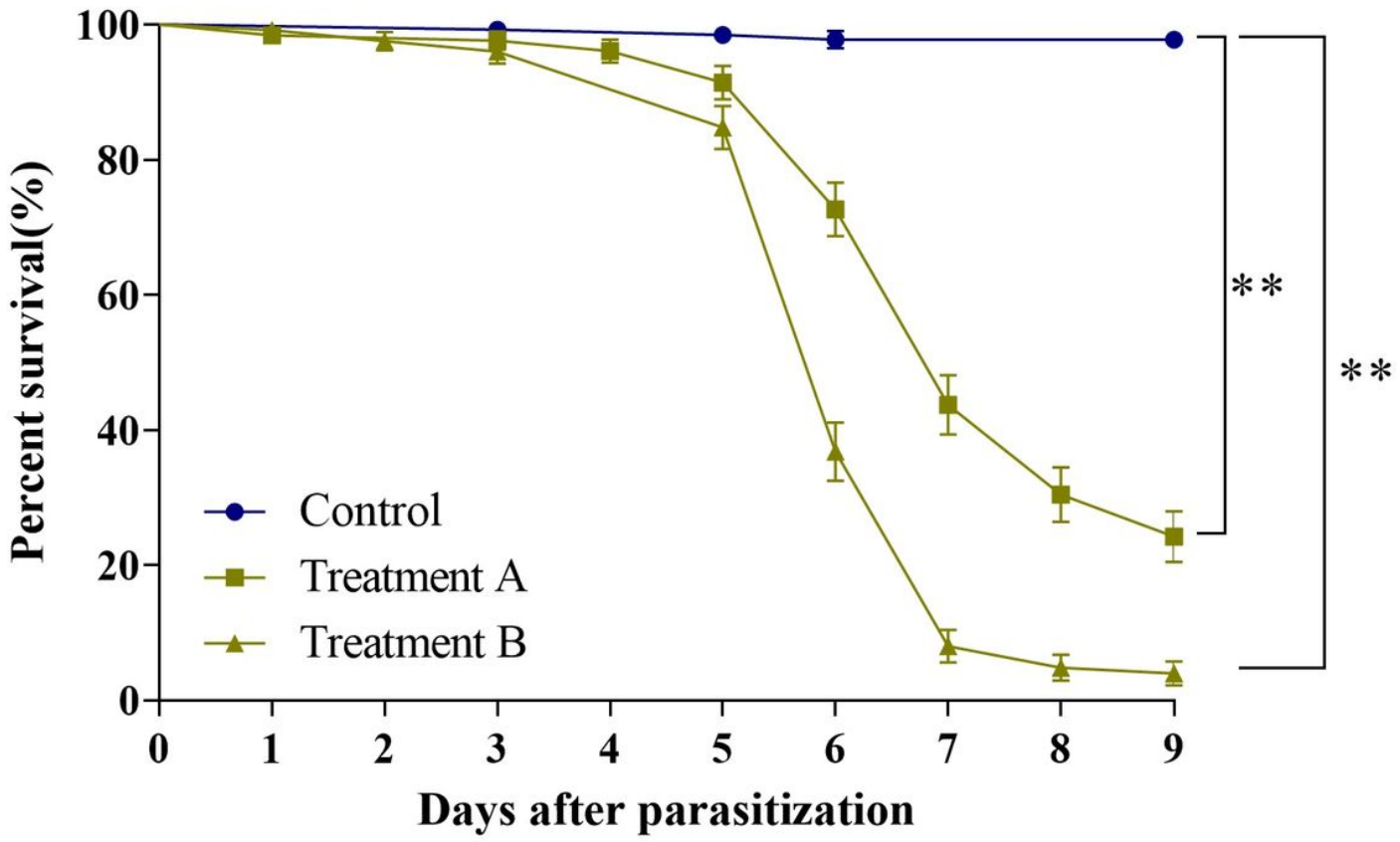

Figure 3

Insecticidal activity of *R. wuchangensis* to second instar larvae of *Ae. aegypti*. Treatment A, 1:5 (mosquito: nematode), treatment B, 1:10 (mosquito: nematode).

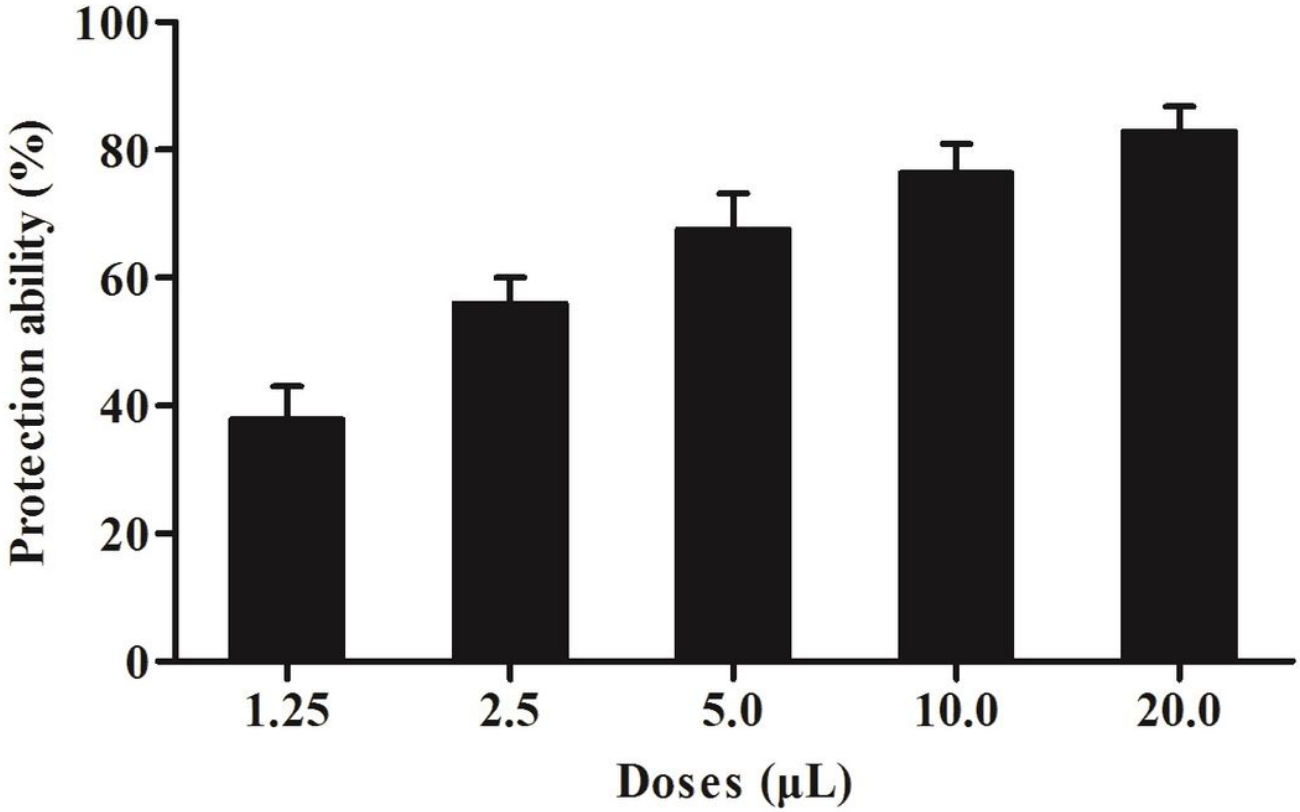

Figure 4

Mosquito repellent activity of essential oil from *M. haplocalyx* against *Ae. Aegypti* adults.

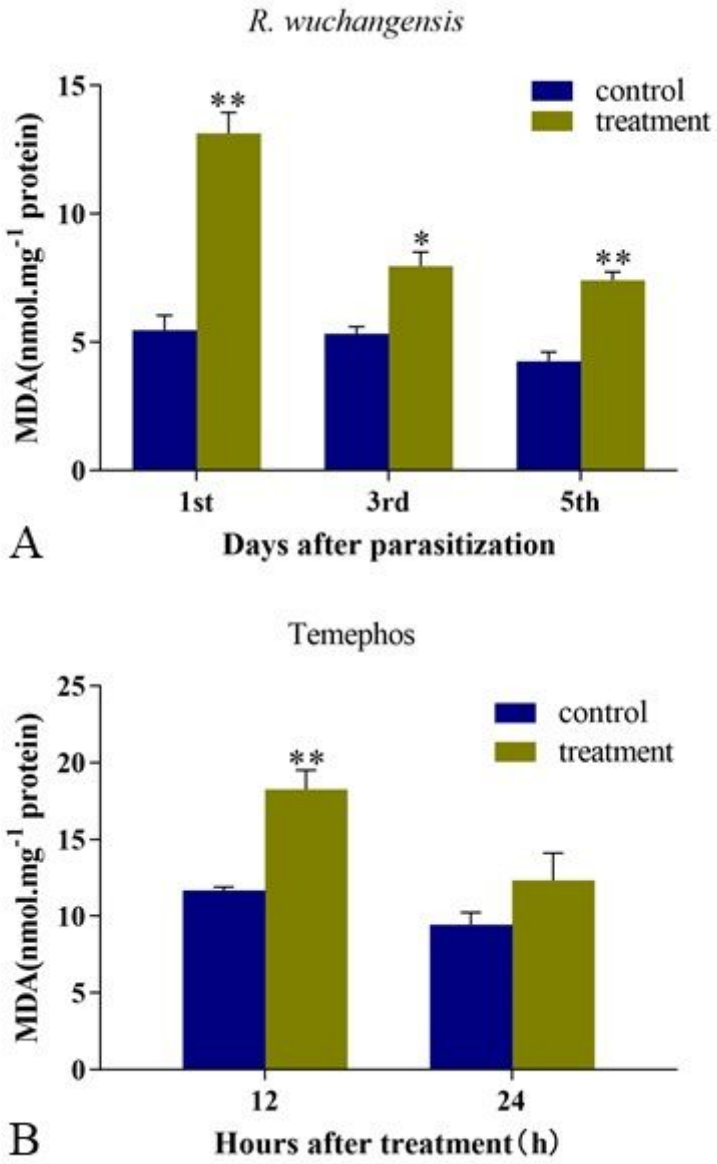

Figure 5

Effects of *R. wuchangensis* parasitization (A) and temephos treatment (B) on MDA of *Ae. aegypti* larvae for different time. Values are mean±SE. Asterisk designates statistically significant difference between control and treatment (*p<0.05; **p<0.01).

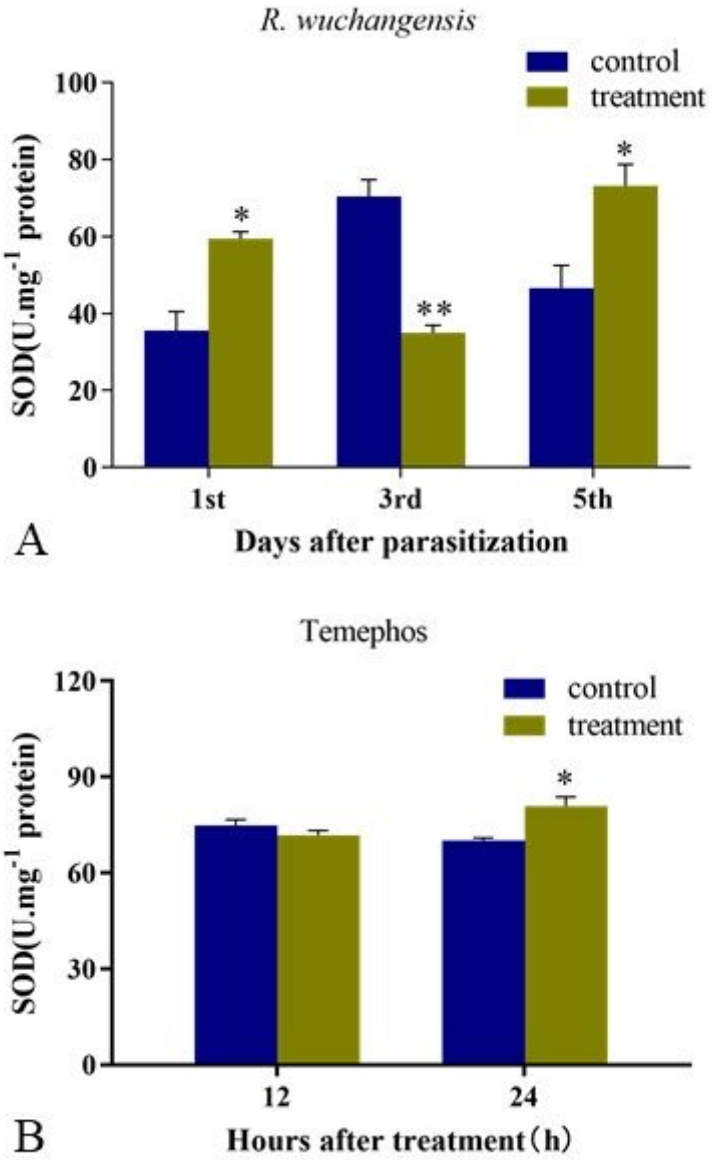

Figure 6

Effects of *R. wuchangensis* parasitization (A) and temephos treatment (B) on SOD activities of *Ae. aegypti* larvae for different time. Values are mean±SE. Asterisk designates statistically significant difference between control and treatment (*p<0.05; **p<0.01).

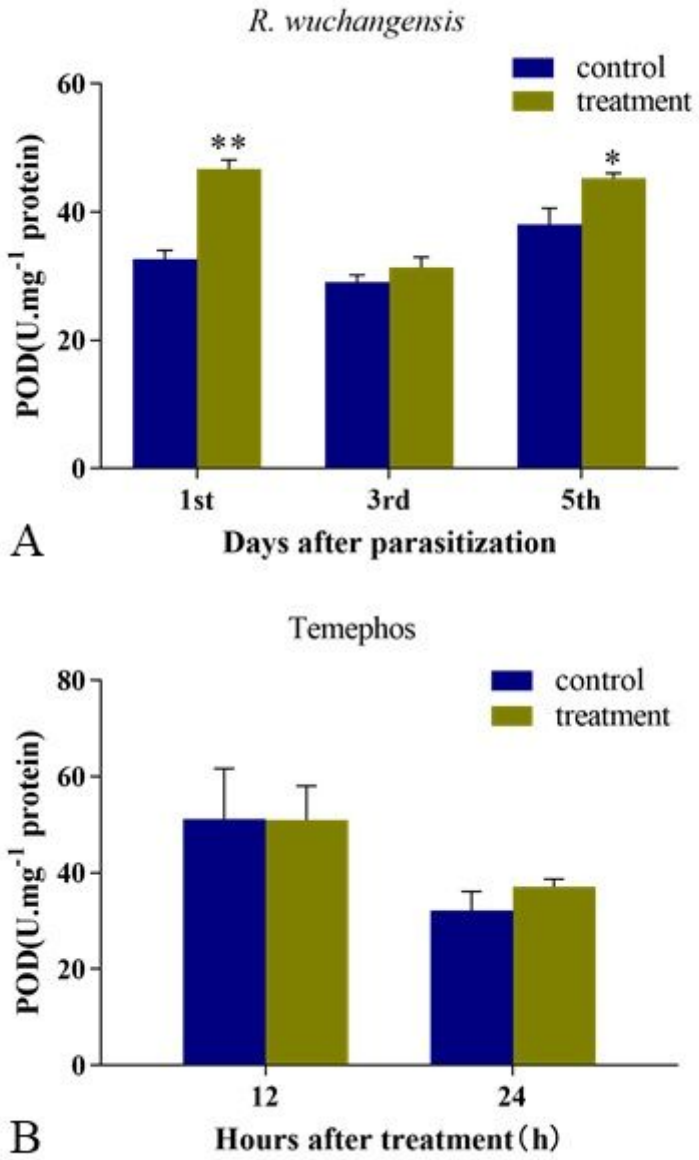

Figure 7

Effects of *R. wuchangensis* parasitization (A) and temephos treatment (B) on POD activities of *Ae. aegypti* larvae for different time. Values are mean±SE. Asterisk designates statistically significant difference between control and treatment (*p<0.05; **p<0.01).

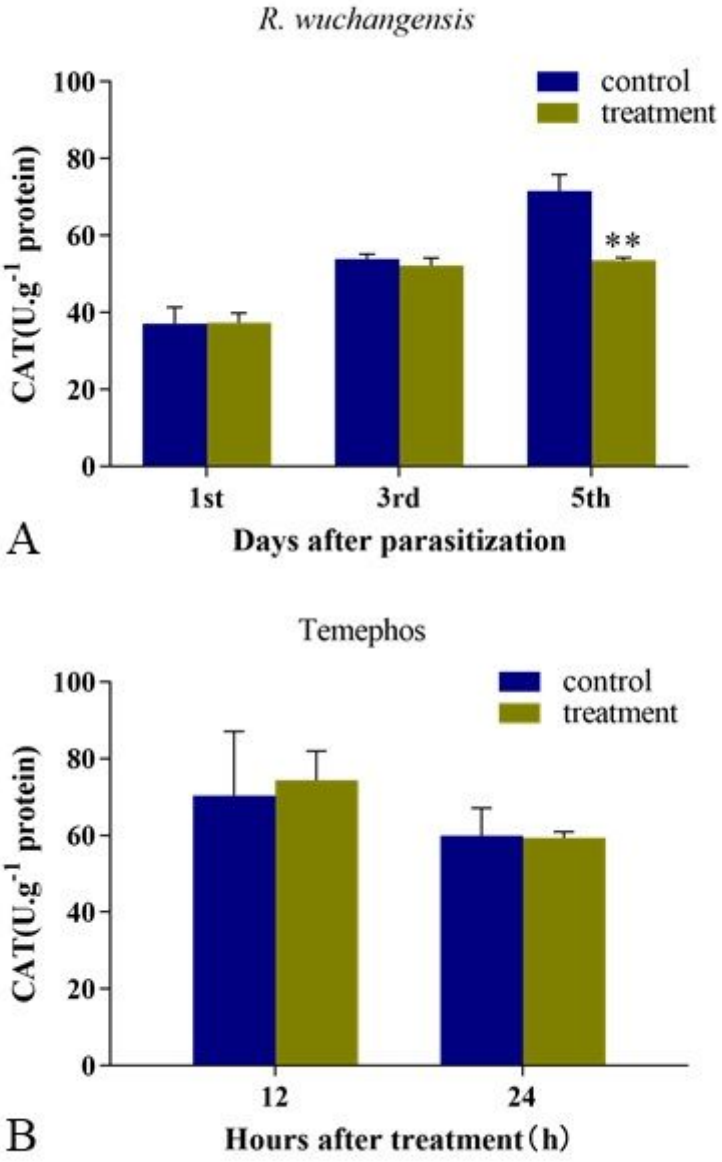

Figure 8

Effects of *R. wuchangensis* parasitization (A) and temephos treatment (B) on CAT activities of *Ae. aegypti* larvae for different time. Values are mean±SE. Asterisk designates statistically significant difference between control and treatment (*p<0.05; **p<0.01).

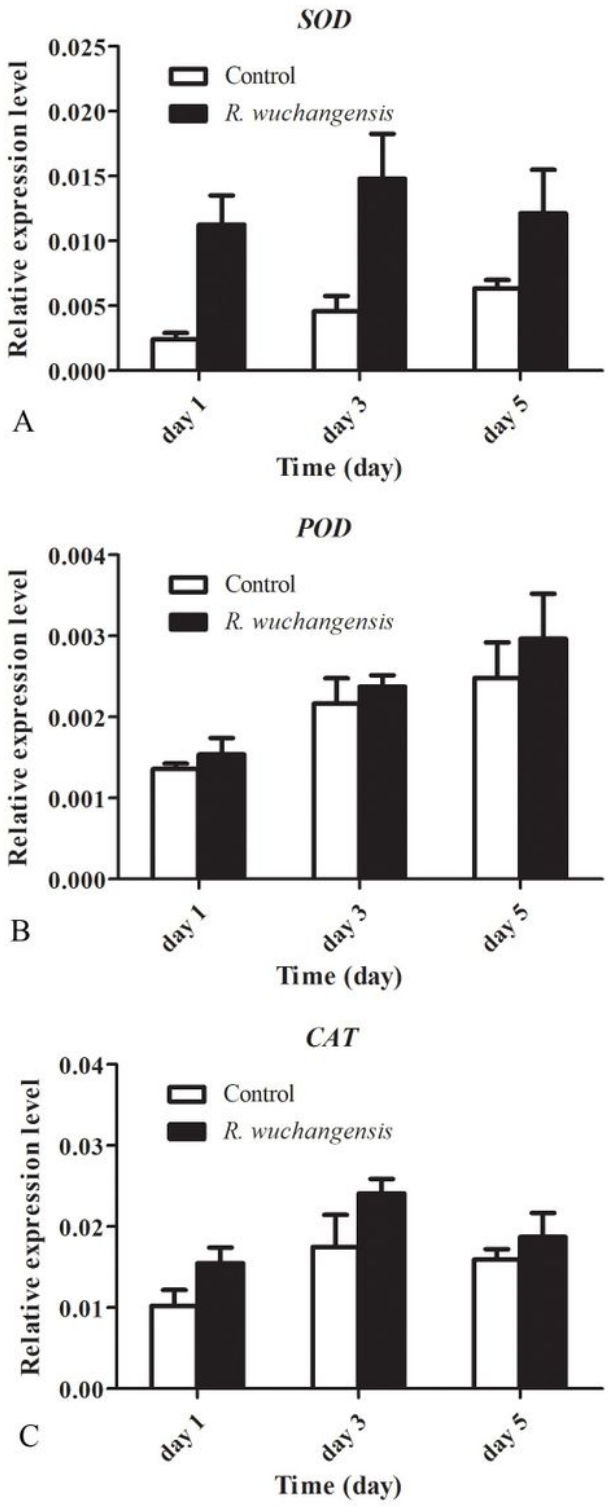

Figure 9

Real-time quantitative PCR analysis of antioxidant enzyme genes. A, *SOD*, B, *POD*, C, *CAT*.

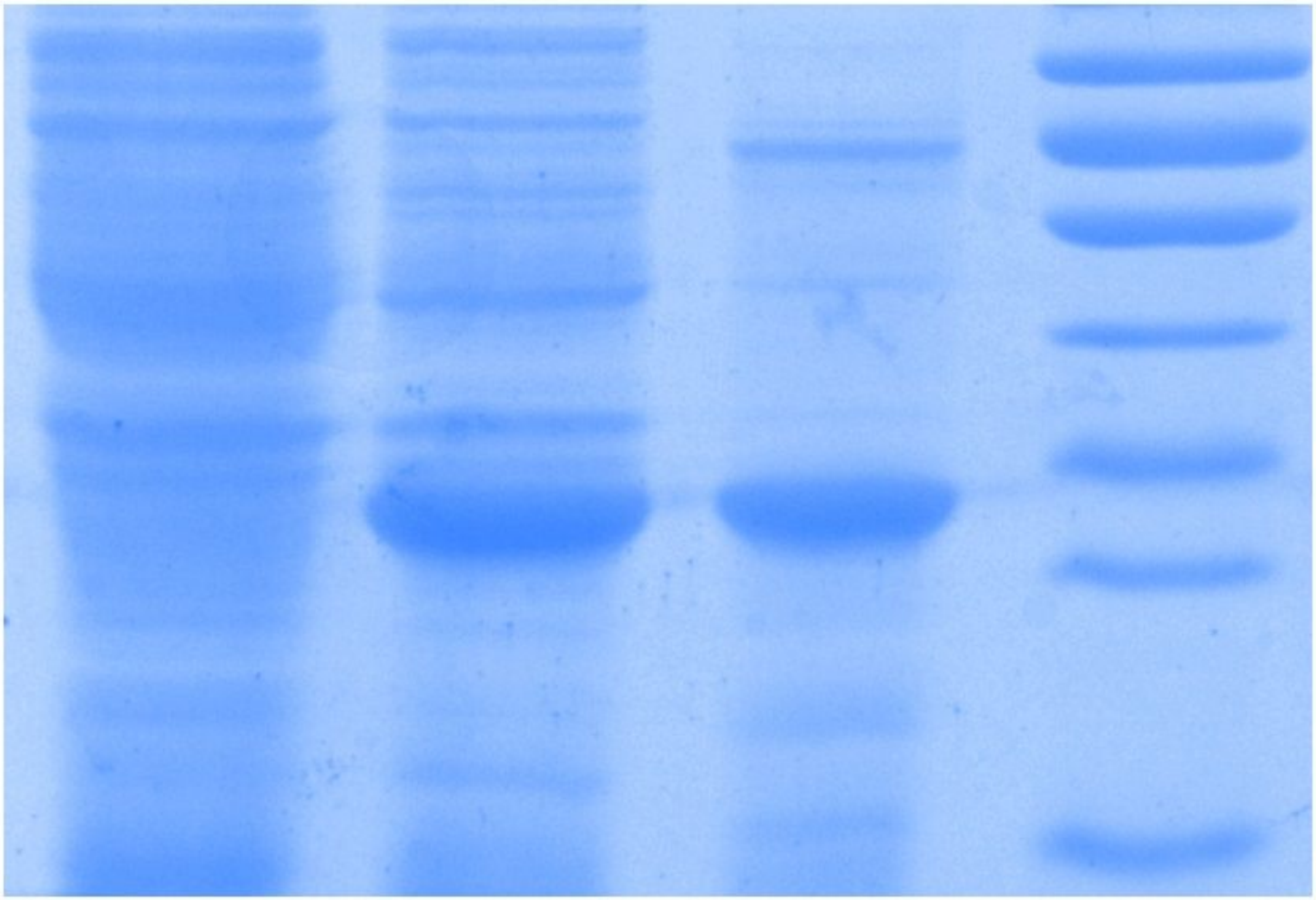

Figure 10

SDS-PAGE electrophoretic analysis of *Ae. aegypti* OBP1 protein. Lane 1 - noninduced *E. coli* OBP1, Lane 2 - induced *E. coli* OBP1, Lane 3 - purified OBP1, Lane 4 - Marker protein.

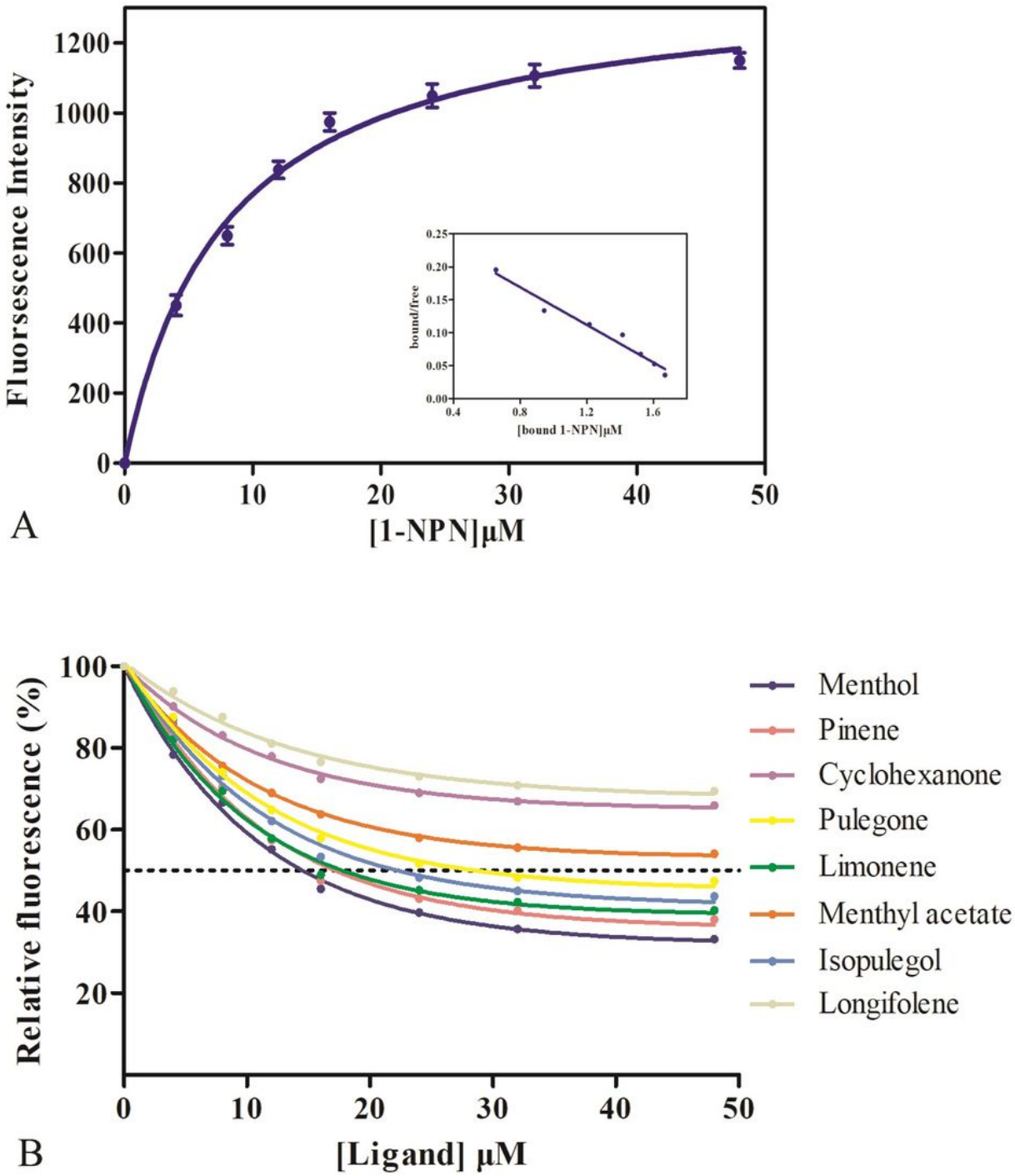

Figure 11

Ligand-binding experiments. (A) Binding curve and relative Scatchard plot. (B) Competitive binding curves of repellent ligands to AaegOBP1.

Supplementary Files

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