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博士学位论文

利用生物合成的纳米银颗粒防治白纹伊蚊的方法

Biosynthesis of silver nanoparticles as a modern approach for the management of Asian tiger mosquito *Aedes albopictus*

作者姓名	**Hassan Ahmed Ga'al**
指导教师	**Jianchu Mo**
学科 (专业)	**农业昆虫与害虫防治**
研究方向	**城市昆虫学**
所在学院	**农业与生物技术学院**
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A DISSERTATION FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

In

Entomology and Vector Control

(Nanobiopesticides)

By

Hassan Ahmed Ga'al

Supervised by

Professor Jianchu Mo

Institute of Insect Sciences, College of Agriculture and Biotechnology, Zhejiang University, Hangzhou, P.R. China

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论文作者签名:_____

指导教师签名:_____

论文评阅人 1：双向隐名评审

评阅人 2：双向隐名评审

评阅人 3：双向隐名评审

评阅人 4：双向隐名评审

评阅人 5：双向隐名评审

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Zhejiang University, Hangzhou 310058,
Peoples' Republic of China.

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I hereby declare that this dissertation is my original work carried out under the guidance and supervision of Professor Jianchu Mo, College of Agriculture and Biotechnology, Zhejiang University, Hangzhou, China and has not been submitted for examination to another University or any other institution of higher learning for the award of degree.

Hassan Ahmed Ga'al

Date:

Institute of Insect Sciences,

College of Agriculture and Biotechnology,

Zhejiang University, Hangzhou, P.R. China

Certification

This is to certify that the work presented herein titled **"Biosynthesis of silver nanoparticles as a modern approach for the management of Asian tiger mosquito *Aedes albopictus*"** was carried out and submitted by Mr. Hassan Ahmed Ga'al, candidate for the degree of Doctor of Philosophy (PhD) in Agricultural Entomology and Pest Control, College of Agriculture and Biotechnology, Zhejiang University, under my supervision. The investigation was an original work and the research report has not been submitted earlier for the award of any other degree.

Supervisor ……..…

Professor Jianchu Mo

Date ……2018-06-07……

College of Agriculture and Biotechnology,

Zhejiang University, Hangzhou 310058,

Peoples' Republic of China.

本研究承蒙

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Abstract

Arthropods are highly dangerous vectors of pathogens and parasites, which considered as serious health problems in the increasing world population of humans and animals. The Asian tiger mosquito *Aedes (Stegomyia) albopictus* (Skuse 1894) represent an important vector of several arboviruses to human and animals, including chikungunya, dengue and various encephalitis viruses, it's also competent vector for Zika virus. Current control methods against mosquito vectors basically rely on application of various conventional chemical insecticides. The intensive usage and irregular application of these conventional insecticides to a wide range and unregulated form has created environmental and human health problems.

Nanotechnology is a multidisciplinary field emerged within past decades and playing fundamental role in many fields including agriculture, pharmacology, medicine and health industries. Therefore, the application of nanotechnology as a clean, non-toxic, eco-friendly and operative nanostructured materials in the pest management strategies can overcome the current challenges in this field and vector control. Thus, the concept of this study was to employ silver nanoparticles (AgNPs) as an environmentally friend and effective tool for mosquito vector control. Consequently, the *Aquilaria sinensis* and *Pogostemon cablin* essential oils were used for green synthesis of AgNPs as an innovative tool for vector mosquitoes management. UV–vis spectrophotometry (UV), Fourier transform infrared spectroscopy (FTIR), scanning electron microscopy (SEM), transmission electron microscopy (TEM), X-ray diffraction analysis (XRD) and energy-dispersive X-ray spectroscopy (EDX) were used to confirm the AgNPs formation and their biophysical characterization. Biosynthesized AgNPs were mostly spherical in shape, crystalline in nature with face-centred cubic geometry and their mean size ranged between 15 and 87 nm. The 24-h

exposure of *Aquilaria sinensis* essential oil synthesized (AsEO-AgNPs) and *Pogostemon cablin* essential oil synthesized (PcEO-AgNPs) were highly effective against the larvae (I-IV instar) and pupae of *Aedes albopictus* mosquito, and had LC_{50} values ranging from 0.81 (I) to 1.12 (IV) and LC_{50} values ranging from 0.85 (I) to 1.19 (IV), respectively. Furthermore, histological analysis of the midgut cells of the control and treated larvae exhibited that the epithelial cells and brush border were highly affected by the fabricated AgNPs compared to the essential oils (AsEO and PcEO).

Our study also conducted another experiment for one-step synthesis of AgNPs using plant originated compound Salicylic acid (SA) and its derivative Dinitrosalicylic acd (DNS) and thus, the fabricated AgNPs were evaluated against larvae and pupae of *Ae. albopictus*. The synthesized AgNP by SA and DNS showed high toxic efficiency against *Ae. albopictus* larvae and pupae, the LC_{50} values of SA-AgNPs were 1.2 ppm (I), 1.4 ppm (II), 1.8 ppm (III), 2.0 ppm (IV) and 1.4 ppm (pupae), whereas LC_{50} values of DNS-AgNPs were 1.2 ppm (I), 1.5 ppm (II), 1.8 ppm (III) 2.3 ppm (IV) and 1.4 ppm (pupae). Moreover, the investigations toward the systemic effect of the tested substances on the fourth instar larvae of *Ae. albopictus* was evaluated and the levels of total protein, esterases, acetylcholine esterase, and phosphatase enzymes were found to be significantly decreased as compared with the control. Our results of this experiment highlighted that SA-AgNPs and DNS-AgNPs could be employed as potential tools to control mosquito larvae and pupae populations.

Similarly, larvae and pupae of *Ae. albopictus* were exposed to graduated concentrations of MELs biosurfactant and synthesized AgNPs for 24 h. the maximum toxicity was revealed in the synthesized AgNPs against larvae and pupae of *Ae. albopictus* with LC_{50} values ranging from 0.14μg/mL (I) to 0.38μg/mL (IV) compared to the MELs toxicity which have had LC_{50} values ranging from 45 μg/mL (I) to 100

μg/mL (pupae). Further investigations regarding to the systemic effects exerted by the tested materials against fourth instar larvae of *Ae. albopictus* showed an alterations in the levels of total protein, superoxide dismutase (SOD), peroxidases (POD) and catalase (CAT) enzymes activity. Moreover, the TEM images of the midgut cells of the control and treated larvae explored AgNPs accumulation in the rough endoplasmic reticulum and other cell organelles such as nucleolus and mitochondria.

In concise, our findings in this study explored that the green-mediated synthesis of AgNPs have great promise as larvicidal and pupicidal agents. According to their cost-effectiveness, simplicity in the synthesis process, reducing and stabilizing efficiency as well as their higher environmental safety, the plant and fungi extracts mediated synthesis of AgNPs would be boon for the development of clean, nontoxic and environmentally acceptable insecticide for agricultural pest management and mosquito vector control.

摘要

节肢动物是重要的病原生物和寄生虫载体，其传播的流行疾病给人类和动物种群带来了严重的健康问题。白纹伊蚊是人类和动物虫媒病毒的重要传播媒介，能够传播包括基孔肯雅病、登革热和塞卡病毒在内的多种病毒。长期以来蚊子的防控方法主要依赖于化学杀虫剂，这些杀虫剂的大量的、不规范的应用造成了环境污染以及人类健康等问题。

纳米技术是在过去几十年中出现的一个多学科领域，在农业、药理学和医学等领域发挥着重要作用。将清洁、无毒、环保和有效的纳米结构材料应用于有害生物防治对策中，可以克服当前有害病媒控制方面的诸多问题。因此，该研究的目的就是探究将银纳米颗粒(AgNPs)作为一种有效、环境友好的蚊媒控制工具的可能性。在该研究中，使用白木香精油和广藿香精油作为合成 AgNPs 的基本材料。同时，使用紫外-可见分光光度计(UV)、傅里叶红外光谱仪(FTIR)、扫描电子显微镜(SEM)、透射电子显微镜(TEM)、X 射线衍射分析(XRD)、能量弥散 X 射线谱(EDX) 等方法确认 AgNPs 的形成及其生物-物理特性。在结构方面，生物合成的 AgNPs 大多呈球形，自然结晶，具有面心立方几何形状，其平均尺寸介于 15-87nm 之间。由白木香精油合成的 AsEO-AgNPs 和广藿香精油合成的 PcEO-AgNPs 都能在 24h 后对白纹伊蚊的幼虫和蛹产生有效作用，其 LC_{50} 值区间分别为 0.81 (I)-1.12 (IV)和 0.85 (I)-1.19 (IV)。此外，比较对照组和处理组中幼虫中肠细胞组织发现，与正常精油(AsEO and PcEO)相比，经修饰过的精油 AgNPs 处理的幼虫上皮细胞及纹状缘都发生了较大变化。

此外，该研究还利用植物的化合物水杨酸(SA)和它的衍生物二硝基水杨酸(DNS)进行一步法合成 AgNPs，并通过实验对合成的 AgNPs 对白纹伊蚊幼虫和蛹的毒杀作用进行检测。结果显示利用 SA 和 DNS 合成的 AgNPs 对白纹伊蚊幼虫和蛹具有很强的致毒效应，其中 SA-AgNPs 的 LC_{50} 值分别为 1.2 mg/L (I)、1.4 mg/L (II)、1.8 mg/L (III)、2.0 mg/L (IV) 以及 1.4 mg/L (蛹)，而 DNS-AgNPs 的 LC_{50} 值 1.2 mg/L (I)、1.5 mg/L (II)、1.8 mg/L (III)、 2.3 mg/L (IV) 和 1.4 mg/L (蛹)。此外，通过研究受试物质对四龄幼虫的系统性影响后发现，其总蛋白，酯酶，乙酰胆碱酯酶和磷酸酶的水平和对照相比显著降低。该研究结果进一步证实了 SA-AgNPs 和 DNS-AgNPs 可以作为控制蚊子幼虫和蛹的潜在工具。

此外，通过将白纹伊蚊的幼虫和蛹暴露在浓度渐变的 MEL 生物表面活性剂和合成的 AgNPs 中 24h 发现，AgNPs 和 MEL 对幼虫和蛹的 LC_{50} 值，分别为 0.14 μg/mL (I) 到 0.38 μg/mL (IV)和 45 μg/mL (I)到 100 μg/mL (蛹)。进一步分析受试材料对四龄幼虫的系统影响发现，幼虫总蛋白水平、超氧化物歧化酶(SOD)、过氧化物酶(POD)和过氧化氢酶(CAT)的酶活性都发生了改变。同时，通过比较对照组和处理组中幼虫中肠细胞的 TEM 图，发现 AgNPs 在幼虫粗面内质网和其它细胞器如细胞核和线粒体中积累。

综上所述，该研究发现使用绿色方法合成的 AgNPs 作为害虫杀虫剂具有很大的应用前景。鉴于其合成成本低、程序简易、稳定性高以及对环境污染小等优势，由植物和真菌提取物介导合成的 AgNPs 将成为农业害虫治理和蚊媒控制工作中一种干净、无毒和环境友好的新型杀虫剂。

List of abbreviation

FTIR	Fourier transform infrared spectrometer	**ROS**	Reactive oxygen species
TEM	Transmission Electron Microscopy	**SA**	Salicylic acid
SEM	Scanning Electron Microscopic	**DNS**	Dinitrosalicylic acid
UV-Vis	UV-Visible spectrophotometer	**AsEO**	Aquilaria sinensis essential oil
XRD	X-ray diffractometer	**PcEO**	Pogostemon cablin essential oil
EDX	Energy Dispersive X-ray	**MELs**	Mannosylerythritol lipids
NaOH	Sodium hydroxide	**Ag+**	Silver ion
AgNO3	Silver nitrate	**Ag**	Silver element
AgNPs	Silver nanoparticles	**SPR**	Surface plasmon resonance
NPs	Nanoparticles	**AChE**	Acetylcholine esterase
LCL	Lower confidence limit	**CAT**	Catalase
UCL	Upper confidence limit	**DMSO**	Dimethyl sulfoxide
LC	Lethal concentration	**DTNB**	(5,5'-Dithiobis-(2-nitrobenzoic acid))
DMF	Dimethyl formamide	**SOD**	Superoxide dismutase

Chapter 1. Introduction

Arthropods are highly dangerous vectors of pathogens and parasites, which considered as serious health problems in the increasing world population of humans and animals. Mosquitoes (Diptera: Culicidae) approximately comprising 3500 species are found distributed in worldwide even in the Arctic regions and the main genera which transmit human disease-causing pathogens, such as *Anopheles*, *Aedes*, and *Culex* are mainly found in the tropical, subtropical regions (White et al 2004). Although a few species of the mosquitoes on earth can transmit disease to humans but it represents a key threat for millions of people worldwide. Most female mosquitoes require blood meals from vertebrates including humans to obtain the essential nutrition for eggs production. Some of female mosquitoes are absolutely selective feeders, restricted to one or a few closely related species, while some others can feed in a wide extent manner, varying between mammals, birds, and reptiles (Tolle 2009). The female mosquitoes transmit important diseases, including malaria, dengue, chikungunya virus, Zika virus, yellow fever, Japanese encephalitis, and filariasis into the host animals while injecting saliva which may contain these pathogens and parasites. Moreover, Culicidae transmit serious parasites and pathogens to the horses and dogs, including Eastern equine encephalitis dog heartworm, and West Nile virus (Benelli 2016a). Mosquito-borne viral diseases have been ranked as the most important in the worldwide pandemic potential in 2012 (Masud et al. 2017). WHO reported that the increase of the number and spread of mosquito vectors worldwide are caused by environmental changes, and particularly arboviruses is arising as a critical public health concern. Sadly, a 30-fold increase of cases within the past 50 years has been reported, which caused quite a lot of human mortality and staggering economic costs (WHO 2016). Unfortunately, there is no successful treatment for most of the arboviruses

vectored by mosquitoes, with special reference to chikungunya and dengue viruses. In addition, even for other mosquito borne diseases, such as malaria, there are significant challenges that still preclude their successful management (Mishra et al. 2017). The Asian tiger mosquito *Aedes (Stegomyia) albopictus* (Skuse 1894) originates from tropical regions in the Southeast Asia. It is considered an important vector of several viruses of human and animals, including chikungunya, dengue and various encephalitis viruses, it's also competent vector for Zika virus. In addition to viruses, the *Ae. albopictus* mosquito is an efficient vector of dirofilarial worms (Kreßet al. 2017). The tiger mosquito is one of the most invasive mosquito species in the world. It started to spread globally from its original distribution areas in the Southeast Asia and it has been detected in many new countries within last several decades. The global spread of the tiger mosquitoes has resulted noticeable attention because of its ability to transmit numerous medically important pathogens and parasites, which pose a significant risk to human and animal health, in addition to being a horrible nuisance (Walther et al. 2017).

The mosquito borne diseases burden mentioned above emphasizes the vital role of effective and eco-friendly control strategies. Current control methods against mosquito vectors basically rely on conventional chemical insecticides which is a particular chemically synthesized substance used towards the control of agricultural insect pests and vector borne diseases. The application of these chemical insecticides started since the beginning of the twentieth century, where the humankind is using several forms of insecticides which lead to the accumulation of hazardous chemicals in the environment. These conventional insecticides can be classified into different groups based on their chemical moieties like organochlorines, organophosphates, carbamates and pyrethroids. The intensive usage and irregular application of these conventional

insecticides to a wide range and unregulated form has created environmental and human health problems (Mishra et al. 2017). The higher residual pollution of the synthetic chemical insecticides in the environment is considered primary demerits in their application. Previous studies have shown that the residues of insecticides were found in the air, waterways and soil, therefore the accumulation of these residues in the environment causes eco-toxicity problem (Bhatnagar 2001). Several other strategies towards the vector mosquitoes control also employed such as (i) insecticide treated bed nets, (ii) adult repellents, (iii) biological control agents against mosquito larvae and pupae including fishes, amphibians and copepods (iv) Sterile Insect Technique (SIT), (v) "boosted SIT", (vi) symbiont-based methods and (vii) transgenic mosquitoes (Benelli 2016a). One of the main challenges faced during the vector control practices in the world is the resistance development in the mosquito strains towards the insecticides, as we mentioned above, the heavy usage of the insecticides against agricultural pests and the overuse of treated bed nets against mosquito bites has resulted in the existence of resistance in the mosquito strains. The major resistance has been reported towards the pyrethroids which seems to be a critical threat for mosquito control strategy since it is the only insecticidal class which is currently employed and also the only recommended insecticide for treating bed nets, due to their low toxicity for humans compared to other conventional insecticides (Zaim et al. 2000). Many researchers tried to discover an alternative pest control tools to get rigid resistance challenges towards integrated pest control strategies, and they proposed that, the employment of green-fabricated nanopesticides can be considered as a promising effective tool towards the control of insect pests and mosquito vectors (Walther et al. 2017).

In the recent years, nanotechnology has been revolutionized a wide range of research fields, including medical, veterinary and entomological sciences. It is noteworthy that nanoparticles (NPs) showed high stability under UV-light which prolongs their efficacy, this is because of the high surface of NPs to volume ratio, which leads for outstanding optical and catalytic performances. This was attracted many researchers to apply for them as optimal drug carriers, pest management approaches as well as in liquid and sol-gel environments as bioactive agents with desirable properties (Benelli and Lukehart 2017). Additionally, the green synthetic approach of NPs is beneficial over the other forms of the conventional chemical and physical synthesis methods used in nanotechnology. This refers to that green synthesis of NPs is generally inexpensive, rapid, eco-friendly and can be prepared in normal conditions such as pressure, energy and temperature, without using toxic chemicals (Kumar et al. 2015). In the green synthesis methods, metal, metal oxide, silica, and carbon NPs have been fabricated, employing plant-driven and invertebrate extracts and their selected metabolites/constituents as well as microorganisms' extracts and their metabolites as stabilizing and reducing agents. The concentration of the plant or other biologically extracts, and the concentration of the NPs substrate are key factors which effect the size, shape and the stability of these green synthesized NPs. Different techniques for analyzing NPs size have been used to explore the mean particle sizes of green synthesized NPs (Murugan et al. 2015, Rajan et al. 2015).

Developing suitable methods of formulation and stabilization of nanoinsecticides based on green synthesized NPs and to prolong their persistence in the environment is quite important for effective control strategies against agricultural pests and vector mosquitoes. In this context, the green synthesis of NPs has been proposed as highly promising technique that provide a remarkable increase of pesticide efficacy. One of

the most important advantages of green-mediated nanofabrication, could be pointed out that green synthesis-based routes are simple process and not require costly maintenance procedures in the laboratory (Benelli 2016a).

Objectives of this study

The current study has investigated the mosquitocidal property of green-mediated silver NPs using plant essential oils, plant hormones and fungal extracts against Asian tiger mosquitoes. This study revealed that the green fabricated AgNPs is an efficient and promising tool for pest control management, especially for aquatic stages of mosquitoes. In addition to, the green fabricated AgNPs are cost-effective, eco-friendly, and nontoxic method. Furthermore, the highly stability of AgNPs under UV-light have enhanced their great pesticidal property and its applicability.

The main objectives of this study were as follows:

1. Synthesis and characterization of AgNPs using *Aquilaria sinensis* and *Pogostemon cablin* essential oils as well as salicylic acid and dinitrosalicylic acid as reducing and stabilization agents of AgNPs and the evaluation of mosquitocidal potency of this green mediated AgNPs as an efficient and eco-friendly pest management tool.
2. Synthesis and characterization of AgNPs using glycolipid biosurfactant (Mannosylerythritol lipids) produced from the ustilaginomycetous yeast *Pseudozyma aphidis*, and its insecticidal efficiency evaluation.
3. To investigate the mode of action of AgNPs and its toxicity pathways within the mosquito larvae body.

Chapter 2. Review of literature

2.1 Nanotechnology and its pesticidal efficiency

Nanoscience is an arising and influential discipline of science which have attainable various novel and cost effective yields and applications. Recently, nanotechnology research has been interesting more in medicinal industries, public health sector and pest management (Beyene et al. 2017). Sustainable nanotechnology is promising solutions for the challenges in different health sectors such as medicine and public health sectors. The nanostructure materials are the basic issue for all applications of nanoscience, it is widely accepted in the background of nanotechnology to focus on the units of size, rather than of any other unit of scientific measurement (Husen and Siddiqi 2014). Nanomaterials are unique substances which falling less than 100 nm, although a few sources reported less than 1 μm. it's generally in between the microscopic and mesoscopic. They have absolute physicochemical properties such as magnetic property, catalytic property, optical property, insecticidal property and antimicrobial property at the Nano-level, which gives consequences of superior property in chemical activity, catalytic behavior and biological activity, compared to larger particles of the similar chemical composition (El-Nour et al. 2010). The superior bioavailability of nanomaterials may ensure the greater utilization by the body tissues, and individual cells, compared to the larger units. Nanomaterials with 300 nm size, can be used up by individual cells while nanomaterials below 70 nm can use up by the cells' nuclei, where they can lead detrimental effects (Nasrollahzadeh 2014). Although the approach of the nanotechnology is wide range applied in many fields such as medicinal products and pharmaceutics, human health appliances, industrial fields, biomedical fields, engineering, electronics, and environmental studies, it's less extent application in pest management and vector control (Hamzeh and Sunahara 2013). The classic pest

management and vector control strategies have mainly focused on using a variety of synthetic chemical insecticides including carbamates, pyrethroids, organophosphates, and insect growth regulators as well as microbial insecticides mainly an endotoxin from *Bacillus thuringiensis var. israelensis* (Benelli 2015). Furthermore, removal or reduction of indoor and outdoor mosquito breeding sites and even on large-scale breeding sites, as well as personal mosquito repellents were employed. However, the extensive usage of synthetic insecticides, against mosquito adults and its aquatic stages, has led to occurrence of detrimental effects on the environment and human health, in addition to resistance development of targeted mosquito vectors (Naqqash et al. 2016). Pictorial comparison between the conventional pesticides and nanopesticides are represented in (Figure 1). Thus, novel eco-friendly and safer strategies to manage mosquito vectors are urgently needed. To overcome the above mentioned problems, researchers began to develop environmentally benign methods based on the employment of natural products which are generally considered an eco-friendly resources for synthesis of nanobiomaterials as pesticides.

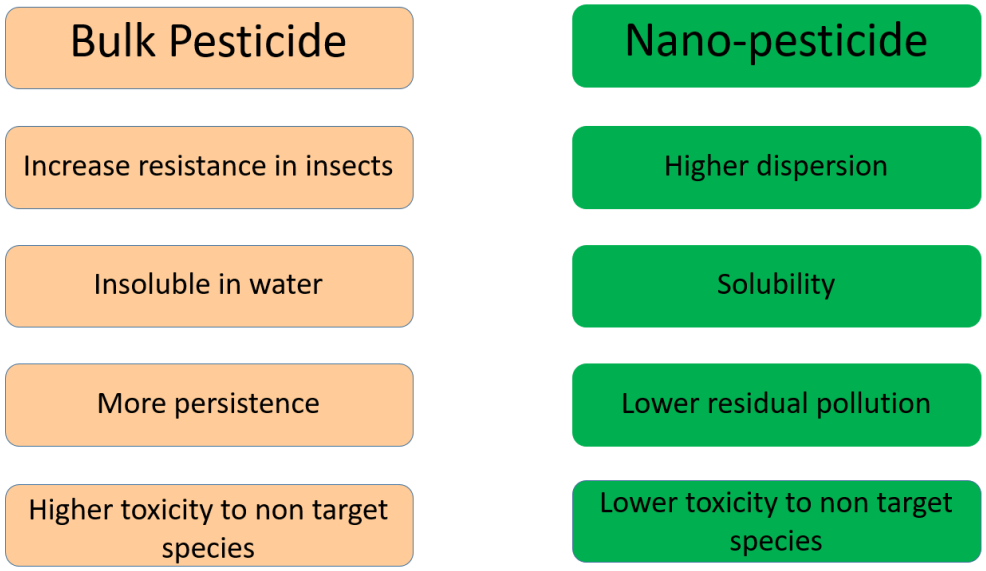

Figure. 1- Pictorial representation of comparative study between the conventional pesticides and nanopesticides (Mishra et al. 2017).

The biologically-mediated synthesis method of NPs is beneficial over physical and chemical routes, since it is cost-effective, single-step, and does not require high temperature, pressure, energy, and also does not use high toxic chemicals (Kumar et al. 2015). Recently, a novel and rapid biological synthesis of metal NPs has been accomplished using considerable number of plants and plant-borne compounds as shown in (Figure 2), as well as large number of bacteria, fungi and viruses (Narayanan and Sakthivel 2010, Rajan et al. 2015).

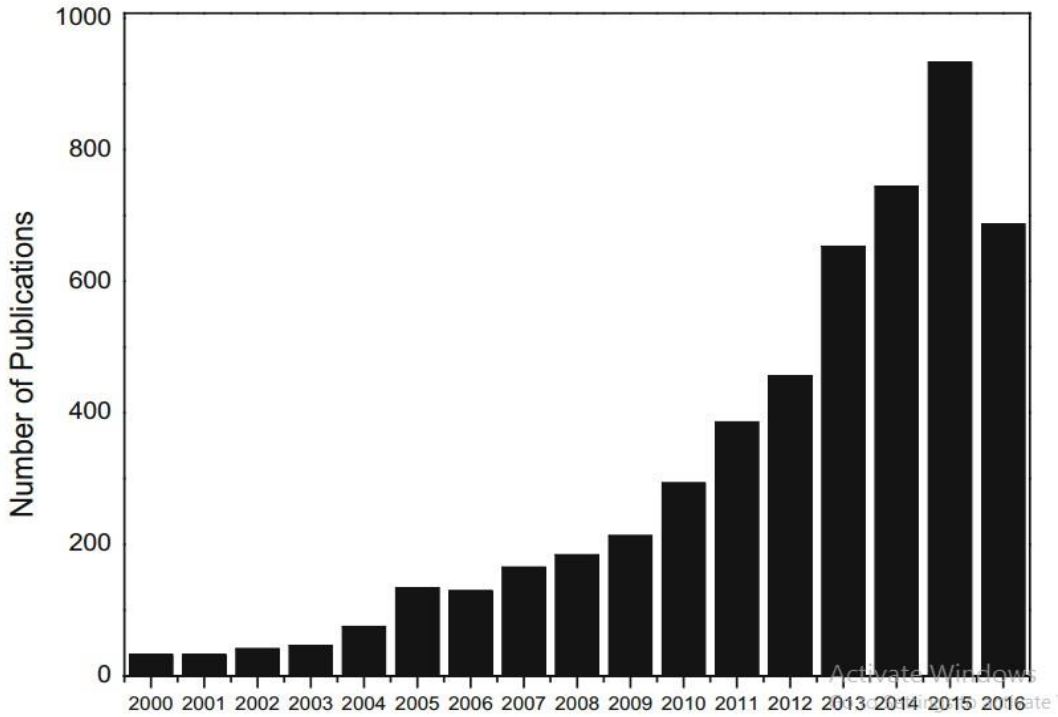

Figure. 2- Annual publications showcasing the green synthesis of metal nanoparticles (Peralta-Videa et al. 2016).

Silver (Ag) element is in one set with Cupper and Gold transition metals. The Ag characterized with high thermal and electrical conductivity, it's soft and lustrous transition element. The medical properties of silver have been experiencing for over 2000 years. It has been used for medical and therapeutic purposes before recognized the microbial infections. In the recent years, the green- synthesized AgNPs reported to have strong antimicrobial and mosquitocidal efficiency (Rawani et al. 2013, Fouad et al. 2017b). The majority studies of the green mediated AgNPs which focused on mosquito larvicidal and pupicidal toxicity were calculated an extremely low LC_{50} (Rawani et al. 2013, Fouad et al. 2017a, Ga'al et al. 2017a, Ga'al et al. 2017b). Therefore green-fabricated AgNPs contributes an ideal approach for mosquito vector control and pest management strategy.

2.2 Synthesis methods of AgNPs

We highlighted in this section, a brief overview on physical, chemical, and biological synthesis of AgNPs. Generally two approaches of metallic NPs synthesis are top-down and bottom-up including, physical, chemical, and biological means. Usual synthesis and most production of AgNPs uses physical and chemical means. For example, the top-down method involves grinding of bulk metals and the stabilization of the formed metallic nanoparticles using colloidal protecting agents. In addintion to, electrochemical methods, decomposition and reduction of metals, as bottom-up method approach (Beyene et al. 2017). Commonly used chemical and physical methods in nanomaterial fabrication and produced NPs'sizes are illustrated in (Figure 3).

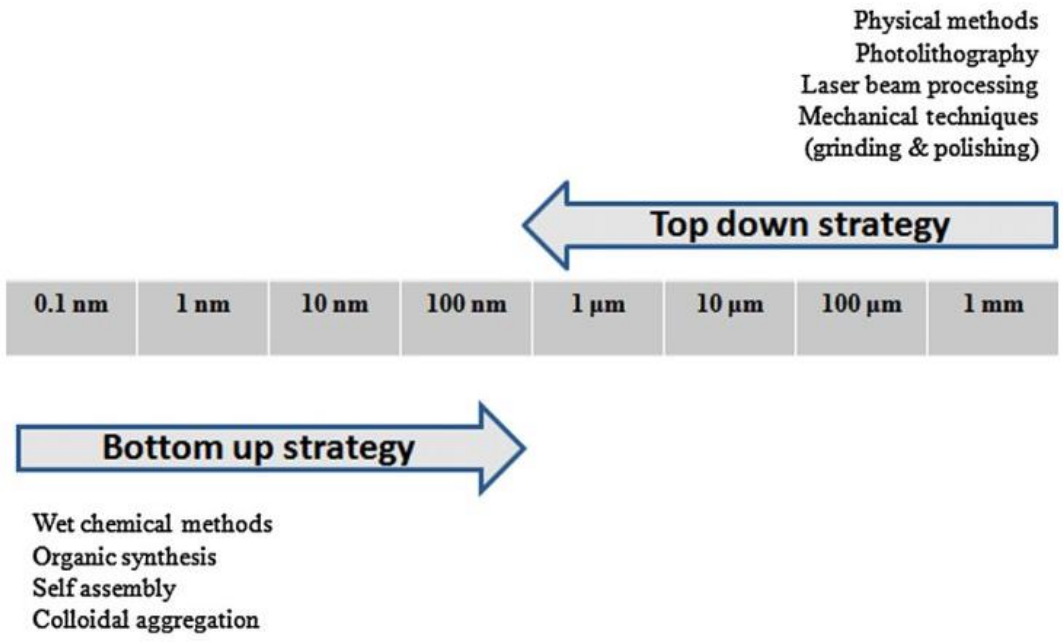

Figure. 3- Common chemical and physical methods for synthesis of nanomaterials (Manikam et al. 2011).

2.2.1 Physical methods of AgNPs synthesis

Evaporation-condensation is an important physical approach which could be done using a tube furnace at atmospheric pressure. The material source within a boat in a center at the furnace is vaporized into a carrier gas. Nanoparticles of Ag have previously been produced using the evaporation/condensation technique (Kruis et al. 2000). Several demerits have been encountered using a tube furnace for the producing of AgNPs, including that tube furnace consumes a lot of energy while the environmental temperature increases around the source material, call for high concentration, takes a large space, and requires a long time to achieve thermal stability (Tarasenko et al. 2006).

Laser ablation of metallic bulk materials is also another approach of physical methods which used for synthesis of AgNPs in solution. The ablation efficiency and the characteristics of the synthesized AgNPs are strongly depend upon many factors such as the wavelength of the laser exposing on the metallic target, the laser fluence, the ablation time term, the time extent of the laser pulses and the potency of liquid medium, with or without employment of surfactants (El-Nour et al. 2010). The synthesis of AgNPs by laser ablation is terminated using surfactant coating solution. Thus the AgNPs synthesized in a solution of high surfactant concentration are smaller than those synthesized in a solution of low surfactant concentration (Kawasaki and Nishimura 2006). The advantages of laser ablation can be pointed out the formation of metal colloids in the absence of chemical reagents in solutions. Therefore, pure and clean metallic colloids can be produced by this method, which will be beneficial for further applications (Iravani et al. 2014).

2.2.2 Chemical methods of AgNPs synthesis

Chemical reduction method is the most applied approaches for the fabrication of stable AgNPs using water or organic reducing agents. Frequently used reducing agents includes sodium citrate, borohydride, polyol process, ascorbate, N,N-dimethylformamide (DMF) elemental hydrogen, Ascorbic acid, poly ethylene glycol, ammonium formate and hydrazine. Silver ions (Ag+) reduction in aqueous media commonly produces a colloidal AgNPs. First, the reduction of different complexes of silver ions ends to the formation of silver atoms and is followed by aggregation into oligomeric clusters. These clusters ultimately lead to the production of colloidal AgNPs (El-Nour et al. 2010). Early studies reported that employ of a strong reducing agents such as sodium borohydride, formed in small and monodispersed particles, while the formation of larger particles was not easy to control. Whereas the use of a weak reducing agents such as citrate, showed slower reduction rate, but the distribution of particles size was difficult to control (Shirtcliffe et al. 1999). The employment of protective agents is very important to stabilize dispersive nanoparticles through the metal nanoparticle preparation process. The protective agents can be bind on or absorbed onto the surface of the nanoparticles, avoiding their aggregation and allowing to dissolve in several solvents. For example, Oliveira and his collagenous (Oliveira et al. 2005), used dodecanethiol as capping agent for preparation of colloidal AgNPs based on Brust procedure. The frequently used stabilizing polymers are poly (ethylene glycol) (PEG), poly (vinylpyrolidone) (PVP), polymethylmethacrylate (PMMA) and poly (methacrylic acid) (PMAA).

The microemulsion procedure which contain two-phase aqueous organic systems is another approach in which can be prepared colloidal AgNPs. The synthesis of AgNPs by this method is based on the spatial separation of metal precursor and

reducing agent in two separate phases. The interaction rate between the reactants (metal precursor phase and the reducing agent phase) is controlled by the moderator phase between the two reactant liquids. The metal agglomeration synthesized at the moderator phase are capped, due to the stabilizer molecules coated the surface of the synthesized metal NPs which occurring in the nonpolar aqueous medium and moved to the organic aqueous medium by the inter phase carrier (Krutyakov et al. 2008). However, several drawbacks of this method have been reported including the employment of highly toxic and harmful organic solvents. Also it's expensive to synthesize AgNPs by this method, because of using large amounts of organic liquids and surfactants in the system (El-Nour et al. 2010).

Furthermore, UV-catalyzed photoreduction is a simple and effective approach to produce AgNPs and AuNPs in the presence of PVP, PAA, citrate, and collagen. For instance, Huang and his colleague fabricated AgNPs through photoreduction of silver nitrate ($AgNO_3$) in layered inorganic suspensions (laponite) which serves as capping agent that prevent NPs from aggregation (Huang and Yang 2008). The source of the UV and the irradiation period are factors effecting on the distribution, size and stability of fabricated AgNPs. Although, the physical and chemical methods are more popular for NP production, but not cost-effective and also the employment of high toxic compounds limit their applications.

2.2.3 Green-synthesis method of AgNPs

Recently, biosynthetic approaches of AgNPs using biological systems such as bacteria, fungi, yeast, invertebrates and plant extracts as a reducing and stabilizing agents have been employed. Thus the green synthesis method could be an alternative approach to chemical and physical methods for the synthesis of AgNP in an eco-friendly manner. This technique has revolutionized in the last several decades because

of its simplicity, nontoxicity, availability of wide range of plants extracts and its metabolites as well as other vast reserves of microorganisms and invertebrates. The reducing and capping agents of AgNPs are widely distributed in the living systems.

2.2.3.1 Plant synthesis of AgNPs

A large number of plant types range from algae to angiosperms have used to fabricate AgNPs. Almost all plant parts including stem, bark, leaves and roots have been used for AgNPs synthesis (Srikar et al. 2016). The traditional medicinally important plants including *Azadirachta indica* (Tripathi et al. 2009), *Tinospora cordifolia* (Anuj and Ishnava 2013), *Boerhaavia diffusa* (Kumar et al. 2014), *Aloe vera* (Chandran et al. 2006), *Catharanthus roseus* (Mukunthan et al. 2011), *Ocimum tenuiflorum* (Patil et al. 2012), *Cocos nucifera* (Roopan et al. 2013), *Terminalia chebula* (Edison and Sethuraman 2012), and *Emblica officinalis* (Ankamwar et al. 2005), have been synthesized colloidal AgNPs. Also common plant spices such as *Cinnamon zeylanicum* (Sathishkumar et al. 2009), *Piper nigrum* (Shukla et al. 2010) were used for the fabrication of AgNPs. Essential oils producing plants such as *Coleus aromaticus* (Vilas et al. 2016b), *Nigella sativa* (Manju et al. 2016) and *Myristica fragrans* (Vilas et al. 2014) also reported to facilitate synthesis of AgNPs. Plant metabolites including proteins (Elumalai et al. 2014), hormones (Ga'al et al. 2017a), and chlorophyll (Shankar et al. 2003), extracted from the plant parts were also found to be acting as reducing and stabilizing agents for fabricated AgNPs. The frequently common procedure for AgNPs synthesis using plant parts usually involves: collection of the interested plant parts, followed by the washing thoroughly two or three times with tap water to remove attached biotic substances, more cleaning with double distilled water are implemented and then shade-dried. The dried plant parts are powdered using electric or domestic blenders. For the preparation of the plant broth, a

desired amount of the plant powder is boiled with double distilled water, followed by filtration using Whitman filter paper number 1, until homogeneous solution is resulted. For the synthesis of AgNPs, several milliliters of the plant extracts are added into the determined amounts of $AgNO_3$ solution and it consequently leads to the reduction of Ag^+ to Ag^0 which can be initially confirmed by measuring the UV–visible spectra of the solution at regular intervals (Govindarajan et al. 2016a).

2.2.3.2 Microbial synthesis of AgNPs

The use of microorganisms as possible nano-factories for the synthesis of NPs such as silver or gold has recently attracted a lot of researcher's interest. This is because the growing need to develop an eco-friendly process for NPs synthesis which does not use detrimental chemicals. Microorganisms play a major role in the remediation of deleterious metal ions into the elemental metal atoms through the reduction process, using enzymes which are produced by the cell activities (Shankar et al. 2016). Bacteria are considered the most abundant microorganisms on the Earth. A large number of bacterial species have been used in nanotechnology as an alternative method for the synthesis of AgNPs. For instance, the genera, Bacillus is widely used for the synthesis of AgNPs, including *Bacillus megaterium* (Banu and Balasubramanian 2015), *Bacillus amyloliquefaciens, Bacillus subtilis* (Fouad et al. 2017b), *Bacillus licheniformis, Bacillus marisflavi, Bacillus flexus* and *Bacillus brevis* (Saravanan et al. 2018), *Bacillus cereus* (Babu and Gunasekaran 2013) Also other bacterial strains such as *Pseudomonas aeruginosa* (Kumar and Mamidyala 2011), *Pseudomonas veronii* (Baker et al. 2015), *Klebsiella pneumoniae, Escherichia coli, Pseudomonas jessinii* (Müller et al. 2016) and *Streptomyces griseoplanus* (Vijayabharathi et al. 2018) were similarly found to be suitable for the green fabrication of AgNPs. These Studies have shown that different bacterial species are able to reduce Ag metal ions into the Ag metal atoms in

both intra- and extracellularly. Independently of the bacterial strains employed as a reducing and stabilizing agents, the majority of the resulting fabricated AgNPs have shown polydispersity. Different conditions of incubation assays such as temperature and pH changes, metal precursor concentration and incubation time have resulted in dramatic variances in the size, shape and number of synthesized particles (Quester et al. 2013). Fungi also considered an important biological sources for the fabrication of AgNPs. This is because it produces a large amount of intracellular and extracellular enzymes which capable to synthesis mono-dispersed AgNPs with well-defined properties. Thus, a wide range of fungal species have been used to synthesize AgNPs. For instance, Shareef J. U. *et al*, used *Penicillium sps* fungus for the extracellular synthesis of AgNPs (Shareef et al. 2017). Also Ma Liang. *et al*, studied the *Penicillium aculeatum* fungus efficiency for extracellular biosynthesis of AgNPs (Ma et al. 2017). Other researchers studied an extracellular biosynthesis of AgNPs using the *Rhizopus stolonifera* fungus (AbdelRahim et al. 2017). Several earlier studies have also described the capability of fungi for the fabrication of AgNPs using various fungal strains including *Fusarium oxysporum* (Ahmad et al. 2003), *Aspergillus fumigatus Fresenius* (Prabhu et al. 2009), *Phanerochaete chrysosporium* (Vigneshwaran et al. 2006), *Aspergillus flavus* (Vigneshwaran et al. 2007), *Aspergillus niger* (Gade et al. 2008), *Fusarium semitectum* (Basavaraja et al. 2008), *Volvariella volvacea* (Philip 2009), *Cladosporium cladosporioides* (Balaji et al. 2009), *Phoma glomerata* (Birla et al. 2009) and *Trichoderma viride* (Fayaz et al. 2010). Fungi are considered an excellent biological source for the synthesis of AgNPs, when compared to bacteria, this is because fungi secretes high amount of metabolites which capable to synthesize a large amount of AgNPs. Other benefits for using fungi as green reducing and stabilizing agents can be concluded that fungi have a high intracellular metal uptake capacities,

also fungi secrete specific enzymes such as reductase which facilitates the biosynthesis of metal nanoparticles such as AgNPs. In addition to that, fungi grow easily over the inorganic substrate surfaces which leads to the metal being distributed as a catalyst, as well as it can be easily cultivated on a large scale by solid substrate fermentation in which large amount of biomass will produced for processing (Hulkoti and Taranath 2014).

Notably, the employment of algae as bio-factory in NPs synthesis has also become one of the prominent fields of research. A wide number of algal species have been utilized for the synthesis of AgNPs as reducing and capping agents. For instance, Neveen Abdel-Raouf and his colleagues employed the *Padina pavonia* (brown algae) for fabrication of AgNPs (Abdel-Raouf et al. 2018). Other researchers used *Jania rubens* and *Sargassum dentifolium* aqueous extracts as a reducing and capping agents for the synthesis of AgNPs (Saber et al. 2017). In addition to, earlier studies reported that polysaccharide extracted from red algae *Gracilaria birdiae* served as potential bio-fabrication agent of AgNPs (de Aragao et al. 2016). However, the algae are well-known for their richness of lipids, proteins, certain vitamins and minerals, also they contain several bioactive molecules such as polysaccharides, and polyphones which can serve effectively as both reducing and capping agents of AgNPs (Hulkoti and Taranath 2014).

2.3 Characterization techniques of AgNPs

Characterization of nanoparticles is key to optimize and control nanoparticles synthesis and its applications. A number of spectroscopic and microscopic techniques are employed to characterize synthesized AgNPs, including UV–vis spectra, FT-IR spectra, TEM microscopy, AFM microscopy, SEM microscopy and XRD patterns. The UV–vis (spectrophotometer) serves as primary technique for initially conformation of the synthesized AgNPs by monitoring the presence of absorption peaks. The vast

majority of studies on AgNPs have recorded the spectra of the UV-vis system within the range 200–800 nm at duration intervals, to confirm the formation of AgNPs. The UV-vis is used to confirm sample formation by showing the surface plasmon resonance (SPR) bands (Ga'al et al. 2017a). Moreover the morphology, particle size and shape characteristics of the AgNPs are determined by microscopic equipments such as SEM, AFM and high resolution TEM. In addition to spectroscopic techniques which can determine the crystallinity property (XRD), particles size distribution (DLS) and the types of active functional groups (FT-IR) are also applied for the characterization of AgNPs (El-Nour et al. 2010).

2.4 AgNPs Applications

Recently, AgNPs have gained remarkable interest for using in a wide range of fields such as textile coatings, electronics, catalysis, optics, medicine, biotechnology, water treatment, food storage and a number of environmental applications. The AgNPs showed potential inhibitory activity against microbial pathogens, which leads to use widely as antimicrobial agents in a number of products. For example, AgNPs are used in recovery of wounds and ulcers, mainly as dressings and creams forms, they are also employed to coat medical equipments such as surgical masks and dentures (Roy et al. 2013). According to the textile industry, sterile hospital clothes possessing AgNPs as antimicrobial agents are employed to prevent or minimize infection with pathogenic bacteria such as *S. aureus* (Durán et al. 2007). The antimicrobial efficiency of AgNPs is probably due to their unique properties like large surface area, which enhance interaction with the microorganisms. They bind to the cell membrane, and then easily penetrate inside the cell thereby interacting with biomolecules and cell organelles which results in interfering the important biological processes of cells. Depending upon their unique chemical properties, intracellularly released AgNPs interact with

antioxidant molecules by their thiol groups such as thioredoxin, superoxide dismutase (SOD) and glutathione (GSH), leading to high oxidative stress, increasing lipid peroxidation, and following apoptotic cell death. The released AgNPs may also translocate to the different organelles, such as the nucleus and mitochondria, and thereby interact with membrane molecules which leads to detrimental effects including DNA damage, genotoxicity, inflammation, mitochondrial dysfunction, altered cell morphology, and subsequently cell death by necrosis or apoptosis (Zhang et al. 2014a). A summary of AgNPs penetration and the cytotoxicity effects is presented in (Figure 4).

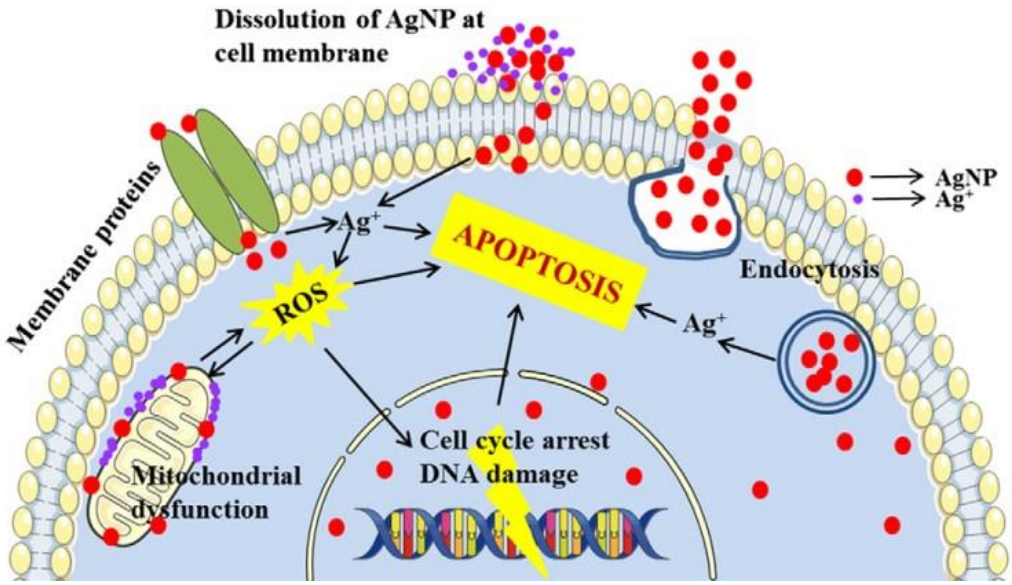

Figure. 4- A summary of AgNPs penetration and the cytotoxicity effects (Zhang et al. 2014a).

2.5 *Aedes albopictus* Mosquitoes

2.5.1 Scientific classification

Kingdom	Animalia
phylum	Arthropoda
Class	Insect
Order	Diptera
Family	Culicidae
Genus	Aedes
Species	Albopictus

2.5.2 Lifespan and characteristics

This mosquito has also common names such as Asian tiger mosquito or forest day mosquito, the entire aquatic or immature cycle (i.e., from egg to adult) can occur in a short time around 7-9 days, and the lifespan for adult mosquitoes is around three weeks. It's characterized by its special black and white stripes in the dorsal and banded legs, adult length range from 2 to 10 mm. The body size variation in the adult mosquitoes results in to the food supply and larval population density within the breeding sites. Generally the medium body size of adult mosquitoes is smaller than 10 mm, this because the seldom optimal of circumstances in the breeding areas (Costanzo et al. 2015). Males are smaller than females almost around 20%, but morphologically are similar to each other. Furthermore, as in all mosquito species, the male's antennae are noticeably bushier in comparison to the females and possesses auditory receptors for detection of the female. The males' maxillary palps are also longer than their proboscises whereas the females' maxillary palps are much shorter. Moreover, the hind

legs tarsus of the males are more silvery than females. However, no other morphological characteristics for differentiation between sexes. However, the surest and easiest way to distinguish Asian tiger mosquito from other *Aedes* speices is a silvery-white line of tight scales starts between the eyes and continues down to the dorsal side of the thorax (Farjana and Tuno 2013).

2.5.3 Ecology and feeding behavior

The *Ae. albopictus* mosquito originated from the tropical and subtropical areas of Southeast Asia; however, in the recent decades, this species has spread in many countries in the world by the transportation of goods and growing international travel (Kamgang et al. 2012). They often rest in dark indoor places like bathrooms and under beds. They are mostly active within the daylight hours for feeding indoors, it's also prefers biting from dawn to dusk. They can bite rapidly which allows it to fly and escape most swat attempts by people. The females of this species also feeds on the birds and other mammals besides humans. During the blood meal mostly is cut-off short without ingestion enough blood for the development of their eggs. Therefore, the tiger mosquitoes bite different hosts over the development period of the egg, which makes them an efficient vector for disease transmitting (Farjana and Tuno 2013). In contrast, the male of the tiger mosquito primarily feeds on nectar. The females of *Ae. albopictus* lays their eggs near to the surface of water, like a stagnant pools. The eggs hatch to larvae when covered it by the raining water. However, all open containers or receptacles holding water will adequate for larval growth, even if they contain a little bit of water. The running water also considered suffice breeding sites for larval development. Their flight is short range (around 200m) so breeding areas are probably near to where this mosquito is available (Kamgang et al. 2012).

2.5.4 *Ae. albopictus* mosquito importance

Recently, this mosquito gained great medical importance in many countries including china, because it closely related to human and animal health, it is flies and feeds typically in the daytime as well as at dusk and dawn. During feeding on blood meal it can transmits several human viruses like chikungunya and dengue viruses, in addition to pathogenic nematodes such as *Dirofilaria (Nochtiella)* in tropical and non-tropical regions, contributing to the global spread of vector borne diseases. Therefore it's highly important to grow up our knowledge about this mosquito such as exploring suitable control techniques against them, determination of its role in disease transmitting, insecticide resistance and its ecological relevance to develop better control strategy and integrated pest management (Ga'al et al. 2017).

Chapter 3. Synthesis, characterization and efficacy of silver nanoparticles against *Aedes albopictus* larvae and pupae

Abstract

Silver nanoparticles have been studied in a wide range of medical and entomological research works due to their eco-friendly aspects. In our study salicylic acid (SA) and its derivative, 3,5-dinitrosalicylic acid (DNS), were used in a one-step synthesis of silver nanoparticles. First, UV–vis absorption spectroscopy was used to detect the formation of silver nanoparticles. Second, the synthesized nanoparticles were characterized using scanning electron microscope, transmission electron microscope; energy-dispersive spectroscopy, X-ray diffraction analysis and Fourier transform infrared spectroscopy. I, II, III and IV Instar larvae and pupae of *Ae. albopictus* were exposed to various concentrations of SA, DNS and synthesized AgNPs for 24h to evaluate the larvicidal and pupicidal effect. In larvicidal bioassay of SA, moderate mortality was observed at 180 ppm against *Ae. albopictus* with LC_{50} values of 83.14, 108.01, 135.40 and 141.12 ppm for instar larvae I, II, III and IV, respectively. Synthesized AgNPs showed highest mortality rate at 12 ppm and the LC_{50} values of SAAgNPs were 1.16 ppm (I), 1.35 ppm (II), 1.74 ppm (III), 1.94 ppm (IV) and 1.36 ppm (pupae). Whereas LC_{50} values of DNSAgNPs were 1.23 ppm (I), 1.49 ppm (II), 1.78 ppm (III) 2.21 ppm (IV) and 1.39 ppm (pupae). Moreover, the investigations toward the systemic effect of the tested substances on the fourth instar larvae of *Ae. albopictus* was evaluated and the levels of total proteins, esterases, acetylcholine esterase, and phosphatase enzymes were found to be significantly decreased as compared with the control. These results highlight that SA-AgNPs and DNS-AgNPs are potential tools to control larval populations of mosquito.

Keywords: *Ae. albopictus*, Salicylic acid, Dinitrosalicylic acid, SAAgNPs, DNSAgNPs, Systemic effect.

3.1 Introduction

Mosquitoes (Diptera: Culicidae) are crucial threat for millions of people and animals worldwide, since they act as vectors for devastating pathogens and parasites of various diseases such as malaria, avian malaria, yellow fever, dengue, Japanese encephalitis, Zika virus, Rift Valley fever, Western equine encephalomyelitis, bancroftian and brugian filariae, canine heart-worm disease (Dirofilaria immitis), and setariosis (Setaria spp.) (Benelli and Mehlhorn 2016, Zahran et al. 2017). Due to its ecological and physiological plasticity, the Asian tiger mosquito *Aedes albopictus* (Skuse) (Diptera, Culicidae), is currently known as the most invasive mosquito in the world, extending its range via shipments of lucky bamboo, moist plants and water containers, and most commonly, the international trade of used tires (Benedict et al. 2007, Bedini et al. 2016). Dengue is the most important mosquito-borne viral disease, with approximately half of the world's population living in dengue-endemic countries, and it is rapidly spreading, the primary vector of dengue is the *Ae. aegypti* mosquito, while the *Ae. albopictus* can also sustain dengue virus transmission in humans (WHO 2016). Current control of mosquito vectors of medical and veterinary importance is mainly based on the use of synthetic insecticides, such as organophosphates and pyrethroids against mosquitoes, microbial control agents, indoor residual spraying such as pyrethroids, organochlorine and carbamates, as well as insecticide-treated bed nets are also employed (Liu et al. 2012, Benelli 2015).

The use of synthetic chemical insecticides to control mosquitoes has produced populations with resistance, and can cause undesired effects on beneficial non-target

organisms (Devine and Furlong 2007). Moreover, these chemicals have negative effects on human health and the environment (Naqqash et al. 2016).

Recently, vector control using plant secondary metabolites is arising as a good alternative because of their eco-friendly properties and high biodegradability (Benelli 2015). Phenolic substances, including simple phenols and phenolic acids, are a major class of phytochemicals that have already demonstrated significant antimicrobial properties (Simoes et al. 2009). Salicylic acid (SA) is a phenolic compound (Figure 5a, b) widely distributed in many plants, especially white willow, meadowsweet and wintergreen. Also, it is a valuable plant hormone that mediates host responses against microbial pathogens (Kumar 2014). It is well known that SA participates in regulating defenses in plants and is predominantly associated with resistance against biotrophic and hemibiotrophic pathogens (Tayeh et al. 2013). Moreover SA has been used to reduce fever and prevent heart attack (Gupta et al. 2013) and it is also a key ingredient in many skin-care products for the treatment of acne, psoriasis, calluses, corns, keratosis pilaris and warts (Mueller et al. 2012). The biocidal potential of SA and its derivatives were earlier elucidated, SA showed the nematicidal effect against larvae of *Meloidogyne* sp. (Abd-alla et al. 2013). Salicylate can inhibit the growth of the corn earworm *Helicoverpa zea* (Li et al. 2002), as well as the growth of the moth larvae *Operophtera brumata* (Ruuhola et al. 2001). The use of plant-borne products for the green synthesis of nanoparticles is a low-cost, single-step, and eco-friendly (Huang et al. 2007).

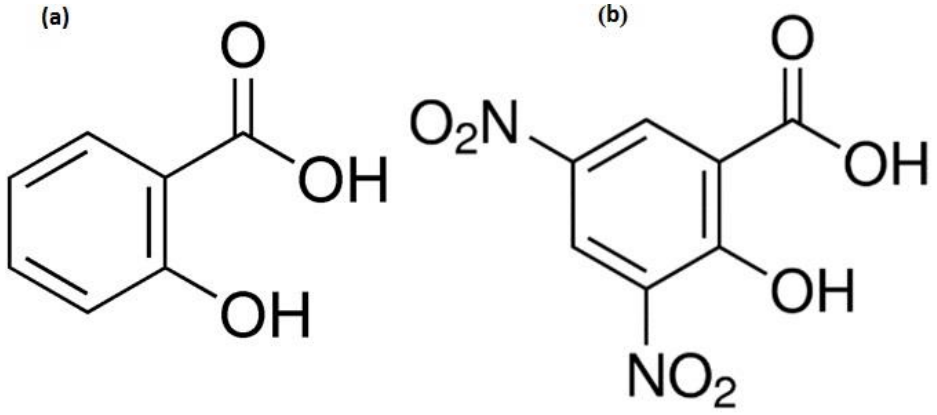

Figure. 5 Chemical structure of (a) salicylic acid, (b) 3, 5-dinitrosalicylic acid.

Nanotechnology has revolutionized a wide range of research fields, including medical and entomological research (Murugan et al. 2016). Biologically synthesized metal nanoparticles showed high effectiveness against mosquito vectors (Suresh et al. 2015). Fungi, bacteria, and plants are commonly used to biologically synthesize nanomaterials (Thakkar et al. 2010, Dar et al. 2013). In the current study, the larvicidal and pupicidal activity of plant-borne chemicals, SA and its derivative DNS as well as salicylic acid-silver nanoparticles (SAAgNPs) and 3,5-dinitrosalicylic acid-silver nanoparticles (DNSAgNPs) were tested for acute toxicity against *Ae. albopictus*. UV–Vis spectrophotometry, Fourier transform infrared (FTIR) spectroscopy, X-ray diffraction (XRD), scanning electron microscopy (SEM), transmission electron microscopy (TEM), and energy-dispersive spectroscopy (EDS) were used to characterize the synthesized AgNPs.

3.2 Materials and methods

3.2.1 Chemicals and reagents

Salicylic acid, 3,5-dinitrosalicylic acid, acetylcholine iodide, 5,5-dithiobis-2-nitro benzoic acid, fast blue salt, α-and β-naphthyl acetate, folin-Ciocalteau reagent were obtained from Sigma-Aldrich Chemicals (St Louis, MO, USA), β-nitrophenyl

phosphate was obtained from Bitech (Shanghai, China), and all other chemicals, and reagents used were of the highest analytical grade purchased from local companies.

3.2.2 Insects rearing

The eggs of *Ae. albopictus* obtained from mosquito colonies were reared in the laboratory of Urban Entomology, Institute of Insect Sciences, Zhejiang University. They were allowed to hatch out under the controlled laboratory conditions at room temperature (RT: 26± 2ºC, and 70–85% relative humidity, RH) with a naturally prevailing photoperiod of 14:10 h (Light/Dark). The larvae were maintained in dechlorinated tap water and were fed with finely ground rat food. The different developmental stages of mosquito larvae and pupae were used for the bioassays.

3.2.3 Preparation of test materials

The SA acid and DNS stock solutions of 1000 ppm were prepared by adding 0.1g of SA or DNS in 100 ml distilled water in glass beakers, and kept in room temperature for further use. For bioassay experiment, graded concentrations (60, 100, 140 and 180 ppm) were prepared through mixing of stock solutions with variable amount of distilled water.

3.2.4 Synthesis of silver nanoparticles

Silver nanoparticles (AgNPs) were synthesized using a phenolic acid reduction method. SA and DNS were utilized as both a reducing and stabilizing agents. In a typical synthesis, SA (4mM) and DNS (2mM) solutions were separately prepared in 18 ml double distilled water. The pH value of the solutions was adjusted to 10.5 using 0.1 M of sodium hydroxide solution, and was dropped slowly into 2 mM (100ml) silver nitrate solution. The reaction mixtures were subsequently heated to 100ºC in boiling water bath for about two hours until the color turned from colorless to brownish red

color for SA and from light green yellow to yellowish red color for DNS. In order to remove the unreacted reductants and the excess silver nitrate ions, AgNP suspensions were centrifuged at 15,000 rpm for 12 min. The supernatant liquid was discarded and the pellet obtained was redispersed in deionized water. The centrifugation process was repeated three times to wash off any absorbed substances on the surface of the silver nanoparticles, and finally stored at 4 ℃ for future use.

3.2.5 Characterization of AgNPs

The synthesized nanoparticles were primarily characterized by UV–vis spectroscopy UV-2550 spectra (Shimadzu, Japan) at a resolution of 1 nm in the range of 200–700 nm. Furthermore, the reaction mixture was subjected to centrifugation at 15,000 rpm for 20 min, and the resulting pellet was dissolved in de-ionized water and filtered through a Millipore filter (0.45 μm), and freeze-dried. An aliquot of this filtrate containing silver nanoparticles was used for scanning electron microscopy (SEM), transmission electron microscopy (TEM), energy-dispersive spectroscopy (EDS), X-ray diffraction (XRD) and Fourier transform infrared (FTIR). The FT-IR analysis was accomplished with the aliquot of reduced silver nanoparticles and recorded under identical conditions in the range of 500–4000 cm^{-1} region at a resolution of 4 cm^{-1} using Fourier transform infrared spectrometer (Vector 22, Bruker, Germany). The quality and formation of compounds were tested using Siemens X-ray diffractometer (XRD) analysis. Where, XRD pattern was measured by drop coated film of dried powder of silver nanoparticles onto glass slides. The operation conditions were at a voltage of 45 keV and a current of 20 mA with Cu-Ka radiation as an X-ray source in the range of 20–80 at the 2h angle. Further, the morphology of the synthesized AgNPs was investigated by SEM using TM-1000 (Hitachi, Japan). Thin film of the sample was prepared on a carbon coated copper grid by simply dropping a very small amount

of the sample on the grid. The instrument was equipped with an EDS to confirm the presence of silver metal.

The film on the SEM grid was then allowed to dry by putting the grid under a mercury lamp for 5 min. The structural characterization of AgNPs was carried out by TEM (JEM-1230, JEOL, Akishima, Japan). The extra sample was removed from carbon-coated copper grid using the cone of a blotting paper and sample was placed on the carbon-coated copper grid to make a thin film of the sample and then it was kept in a grid box sequentially.

3.2.6 Larvicidal and pupicidal bioassays

The larvicidal and pupicidal activity was assessed as per procedure by WHO with some modifications (WHO 1996). Twenty-five *Ae. albopictus* I, II, III, IV larvae instar and pupae were separately introduced in a 250ml beaker containing 100 ml of dechlorinated tap water and exposed for 24 h to dosages of 60, 100, 140 and 180 ppm (SA or DNS) and 1, 4, 8 and 12 ppm (SAAgNP or DNSAgNP). A 0.5mg larval food was provided for each test concentration. Each concentration was replicated four times against all instars and pupae. Control mosquitoes were exposed for 24 h to the corresponding concentration of silver nitrate solution or dechlorinated water. Percentage mortality was calculated as follows:

$$Percentage\ mortality = \left[\frac{number\ of\ dead\ individuals}{number\ of\ treated\ individuals}\right] \times 100$$

3.2.7 Mode of action of AgNPs on vector mosquito

3.2.7.1 Preparation of whole body homogenates

The treated and control fourth instar larvae were washed with double distilled water and the adhering water was completely removed from the body surface by

blotting with tissue paper. The larvae (50 individuals) were pooled and homogenized in Eppendorf tubes (held in crushed ice) using a Teflon hand homogenizer in 1 ml of 0.9% saline for eventual estimation of total proteins, acetylcholinesterase, esterases and phosphatases activity. The whole body homogenates were centrifuged (9000g; 4ºC) for 20 min and the clear supernatants were used for the biochemical analysis. Solution for homogenization and glassware were all kept at 4ºC prior to use, and the homogenates were held on ice until used for various assays.

3.2.7.2 Determination of protein concentration

The proteins in the larval homogenates were first precipitated by 80% ethanol (Subhashini and Ravindranath 1980), and the protein concentration was estimated by the method of Lowery (Lowry et al. 1951).

3.2.7.3 Acetylcholinesterase assays

With minor modifications acetylcholinesterase activity in the whole body homogenates of larvae was spectrophotometrically measured using acetylcholine iodide as a substrate (Ikezawa and Taguchi 1981). Each aliquot of homogenate (200µL) was mixed successively with 200µL of sodium phosphate buffer (100 mM, pH 7.5), 50µL of 10 mM DTNB and 50µL of 12.5 mM acetylcholine iodide. After incubation for 5 min at RT, the optical density of the sample was read at 400 nm against suitable reagent blank.

3.2.7.4 Esterase assay

Carboxyl esterase activity in the larval homogenates was measured by the method of Van Asperen (Asparen 1962) with minor modifications. Briefly, 200µl of control and test larval homogenate were mixed with 2ml of the alpha and beta naphthyl acetate solution, reaction was stand for 30 min at room temperature. After incubation, 500µl of the fast blue SDS reagent was added (22.5 mg fast blue salt in 2.25 ml distilled water

and 5% w/v SDS in 0.2M phosphate buffer) (pH 7.2) and color was allowed to develop for 15 min at RT. The optical density of the sample was measured at 588 nm in the spectrophotometer against the respective reagent blank.

3.2.7.5 Phosphatase assay

The levels of acid and alkaline phosphatases in the larval homogenates were measured by the method of Asakura with minor modifications (Asakura 1978). The acid phosphatase activity was estimated by mixing 100µl of larval homogenate with 400µl of 50 mM sodium acetate buffer (pH 4.0) and 500µl of 15 mM β-nitrophenyl phosphate. For the estimation of alkaline phosphatase activity, 100µl of homogenate was mixed with 400µl of 50 mM Tris–HCl buffer (pH 8.0) and 500µl of 15 mM p-nitrophenyl phosphate. After incubation for 15 min at 37^{o}C in water bath, the enzymatic reaction was stopped by adding 100µl of 0.5N NaOH solution and centrifuged (4000g; 5 min). The absorbency of the resulting clear supernatant from each sample was read at 440 nm against the appropriate reagent blank.

3.2.8 Statistical analysis

SPSS software package 16.0 version was used for all analyses. Data from larvicidal and pupicidal experiments were analyzed by probit analysis, calculating LC_{50} and LC_{90} (Finney 1971). The difference in the levels of various biochemical parameters between control and experimental larvae was tested for statistical significance using mean difference Student's t-test (SPSS software). Chi-square values were calculated using the SPSS software package 16.0 version (SPSS Inc., Chicago, IL). The acceptance level of statistical significance was $p \leq 0.05$ in all instances.

3.3 Results and discussion

3.3.1 Characterization of silver nanoparticles

The silver nanoparticles (AgNPs) were synthesized via reduction of silver nitrate using the SA and DNS. This was a one-step silver nitrate reduction process in which the SA and DNS acted as both the reducing and stabilizing agents. The formation of AgNPs was visually confirmed by the change of reaction mixture colors (figure 6 a, b). When the alkaline solution containing the SA or DNS were mixed with $AgNO_3$ solution under high temperature condition, the reaction mixtures turned from colorless to brownish red color for SA and from light green yellow color to yellowish red color for DNS within 2 hours in boiling water bath. This may due to the excitation of surface Plasmon vibrations of the synthesized AgNPs (Govindarajan and Benelli 2016). In the present study, the de-protonated hydroxyl and carboxylic groups from the SA and DNS made it a stronger complex agent for silver ions in the presence of an alkaline additive (NaOH). The silver ions subsequently oxidized the hydroxyl groups into carbonyl groups in the reduction reaction as the silver ions were simultaneously reduced into the AgNPs. Different studies have recently reported that an alkaline medium is a precondition for synthesis of AgNPs using active reducing components from the bio-sources (Guo et al. 2015).

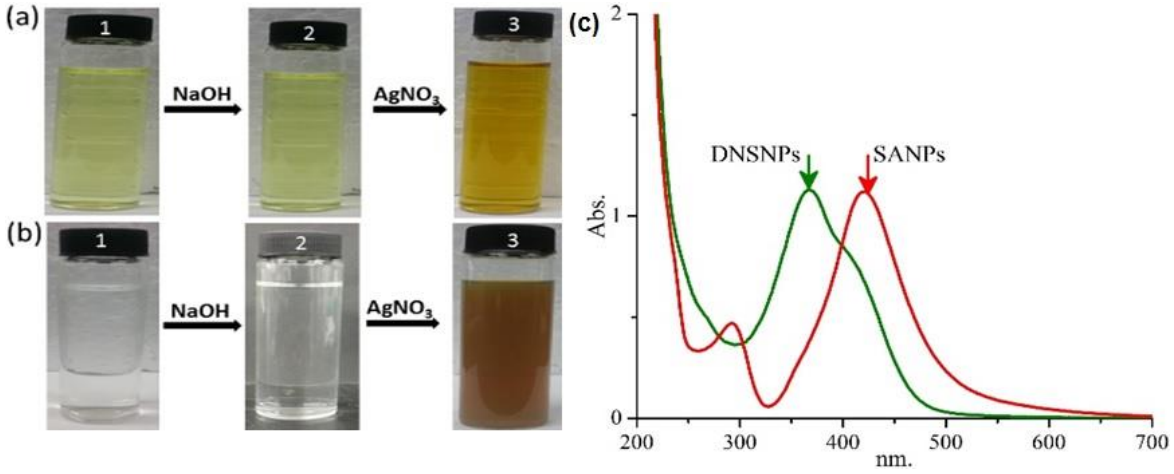

Figure. 6 Preparation process of AgNPs, and UV-vis spectrum. a- (1) aqueous solution containing dinitrosalicylic acid, (2) alkaline solution containing dinitrosalicylic acid, (3) nanoparticles solution. b- (1) aqueous solution containing salicylic acid, (2) alkaline solution containing salicylic acid, (3) nanoparticles solution. c- UV–Vis adsorption spectrum of AgNPs. The maximum absorption peaks of DNS-AgNP and SA-AgNP were 390 and 430 nm respectively after 150 min from the reaction.

The UV–vis absorption spectra from the reaction solutions in the present investigation were monitored in the 200–700 nm range (Figure 6c). The absorption peak was observed at 430 nm for SAAg-NP, due the excitation of the surface plasmon resonance (SPR) to the fabricated AgNPs (Mahyoub et al. 2016). Whereas the wavelength from the maximum absorption peak of DNSAg-NP was more intense at 390 nm, indicating the formation of AgNPs with small size (Suman et al. 2013a). The absorption spectra of spherical nanoparticles showed single SPR peak, while the anisotropic particles could give two or more SPR peaks depending on the shape of the particles. In the present study, symmetry of the nanoparticle was responsible for the number of SPR peaks, and the two synthesized AgNPs showed a single SPR peak revealing spherical shape of AgNPs (Solairaj and Rameshthangam 2016).

Further the SEM analysis of our fabricated AgNPs was performed in order to investigate the morphology and size distribution of AgNPs (Figure 7a). SEM of SAAg-NP and DNSAg-NP showed spherical and cubic structures with a size range of 30 – 60 nm and 35 – 60 nm respectively, which was in agreement with the shape of SPR band in the UV Vis spectrum (AlQahtani et al. 2016).

The EDS pattern of SAAgNPs and DNSAgNPs were shown in (Figure 7b). The strong peaks of silver element was observed at 3 KeV, which indicates that the silver is the major constituent in AgNPs, thus confirming the successful preparation of AgNPs (Suresh et al. 2017).

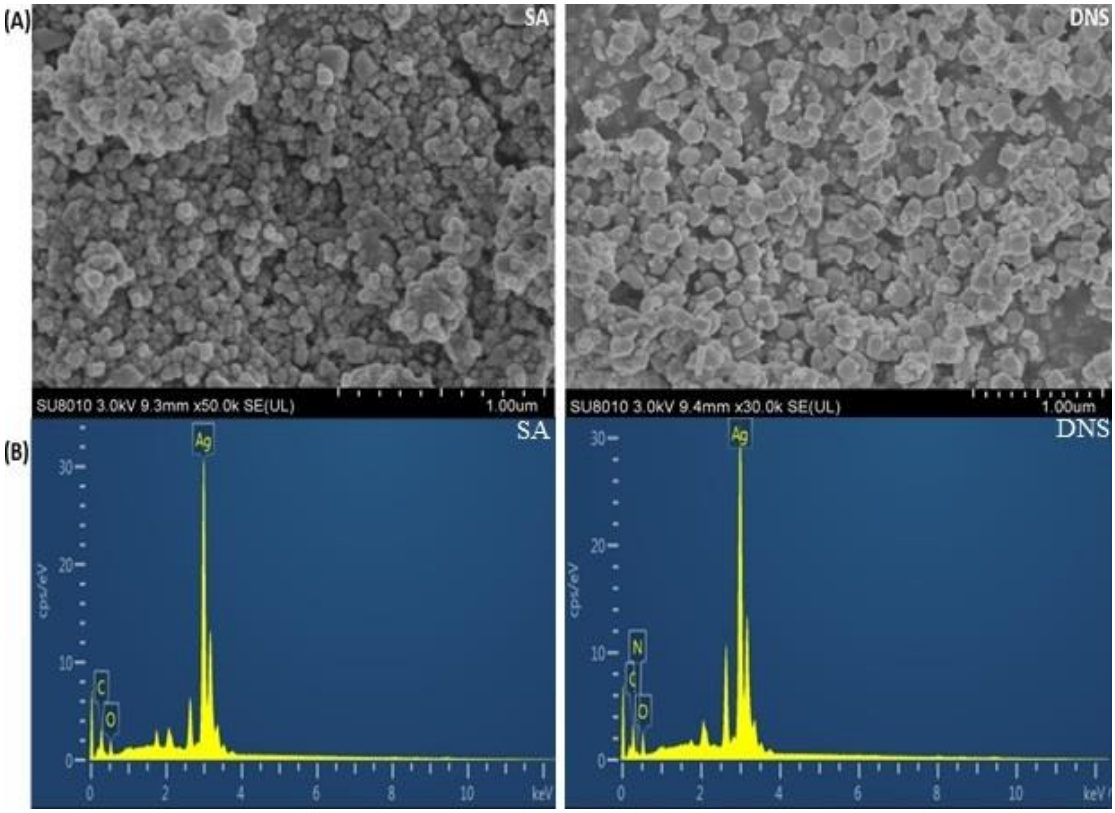

Figure. 7 (a) SEM images of synthesized AgNPs using Salicylic acid (SA) and Dinitrosalicylic acid (DNS). (b) EDX patterns of synthesized AgNPs.

The detailed characterization by transmission electron microscopy (TEM) analysis revealed that the synthesized SAAg-NP (Figure 8a) were quite uniform and had more spherical shape with the average particle size of 35nm. The TEM of DNSAg-NP (Figure 8a) showed that the AgNPs were spherical in shape and has the average particle size of 37 nm. It was also observed that the synthesized AgNPs were bound with thin layer of molecule that might be SA or DNS coating molecules, which act as stabilizing agent, therefore, the particles were poly dispersed without direct contact and stable for long period of time (Govindarajan et al. 2016b).

XRD analysis showed intense peaks at 2 theta values of 38.27, 46.27 64.64, and 77.66 which corresponds the (111), (200), (220), and (311), lattice planes of face-centered cubic structure of silver, respectively (Figure 8b). The sharpening of the peaks clearly indicates that the particles were in the nano-regions (Velayutham and Ramanibai 2016). Thus, XRD highlighted that AgNPs formed by the reduction of $AgNO_3$ with SA and DNS were crystalline in nature (Vimala et al. 2015).

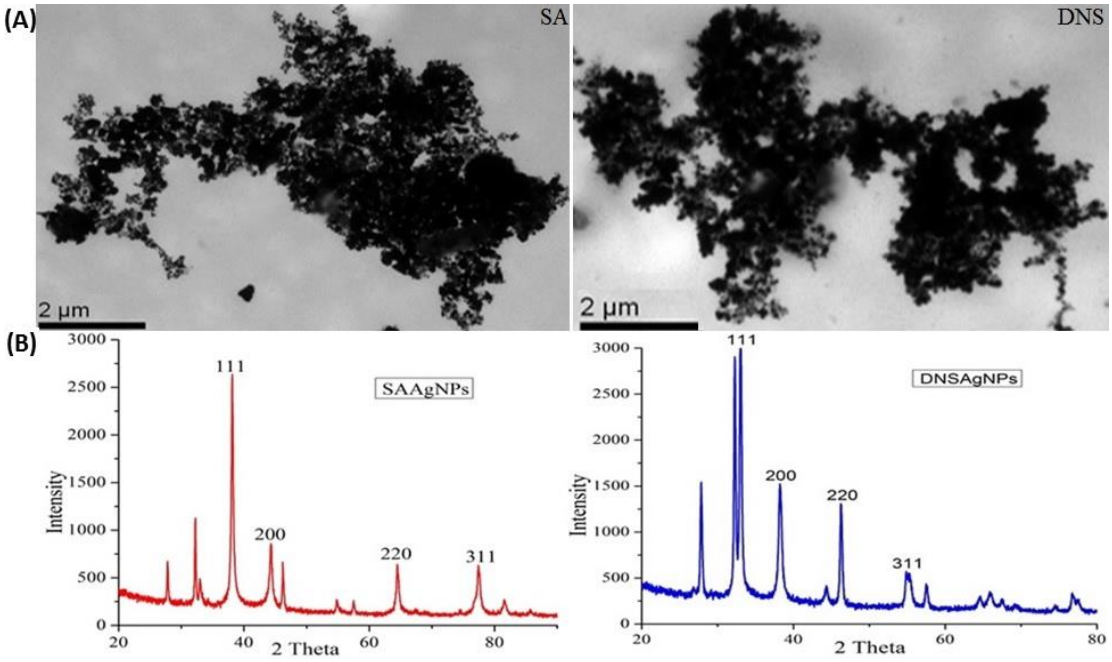

Figure. 8 (a) TEM images of synthesized AgNPs using Salicylic acid (SA) and dinitrosalicylic acid (DNS). (b) XRD patterns of synthesized silver nanoparticles by SA and DNS.

FTIR analysis of the SAAgNP and DNSAgNP was carried out to verify the types of chemical bonds which may be responsible for synthesis and stabilization of AgNP in the mixture. The typical spectra of AgNPs using SA were shown in (Figure 9). The absorption bands due to vibration of chemical molecules at 3412, 1644, 1394, 1057, and 523 cm^{-1} were recorded in the spectrum of wave number between 4,000 and 500 cm^{-1}. The very strong broad absorption peak at 3412 cm^{-1} (O–H stretch and bending); strong and sharp peak at 1644 cm^{-1} (carboxylic acid) in the SAAgNPs indicate the role of SA in the reduction and capping of Ag^{+} to Ag. The presence of peak at 1394 cm^{-1} could be ascribed to aromatic ring stretching vibrations of the SA while the peak at 1057 cm^{-1} assigned to C–O stretching mode in the SAAgNPs. These functional groups might be the responsible for the AgNP synthesis process (Govindarajan et al. 2016a). The IR spectrum of synthesized AgNPs by DNS was shown in (Fig 5.) The spectrum

shows transmittance peaks at 3283, 1651, 1448, 1380, and 552 cm^{-1}. The strong broad absorption peak at 3283 cm^{-1} represents the presence of hydroxyl group (O–H stretch and bending) (Fouad et al. 2017b).

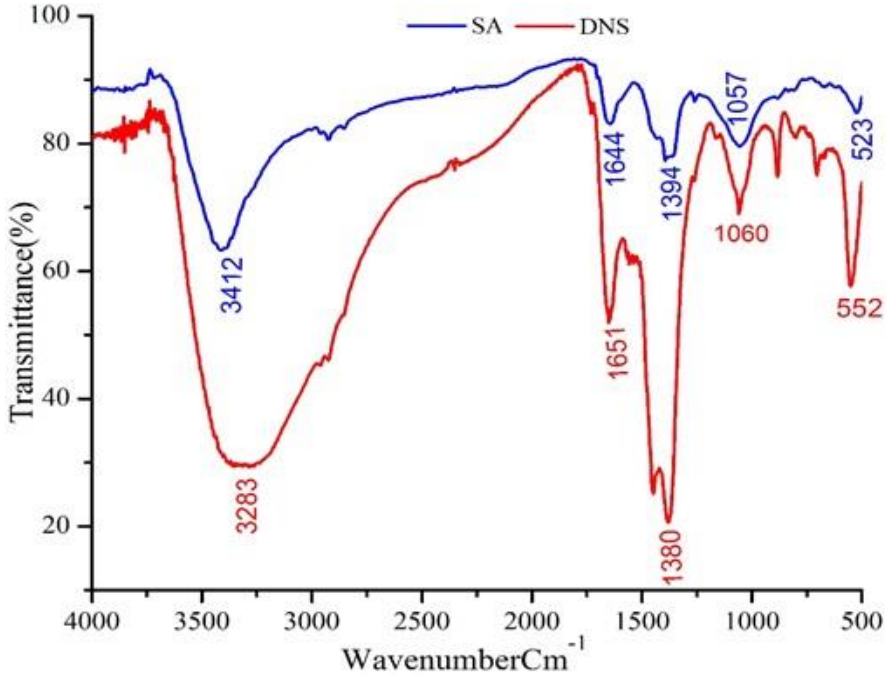

Figure. 9 Fourier transform infrared (FT-IR) spectrum of freeze-dried powder of silver nanoparticles synthesized using Salicylic acid (SA) and Dinitrosalicylic acid (DNS).

The sharp absorption peak close to 1651 cm^{-1} might be linked with (C=O) stretching vibration in the carbonyl groups of DNS (Suresh et al. 2017). The comparison between the two kinds of AgNPs synthesized by SA and DNS shows that there were slight changes in all recorded bands of IR spectrum for the synthesized AgNPs, with the possible reason of the different functional groups of SA and DNS. Our findings are in agreement with AgNP synthesized using *Clerodendrum chinense* (Govindarajan et al. 2016c).

3.3.2 Larvicidal and pupicidal effect of SA, DNS and AgNPs

In laboratory conditions, 24h exposure of SA was toxic against larval instars (I–IV) of *Ae. albopictus*. The LC_{50} values of SA were 83 ppm (I), 108 ppm (II), 135 ppm (III) and 141 ppm (IV) (table 1). A dose-dependent effect was found which in agreement with a number of previously reported plant-borne pesticides (Govindarajan and Benelli 2016, Kovendan et al. 2016, Pavela et al. 2016). The mosquitocidal potential of SA has been scarcely investigated. To the best of our knowledge, (Mondal et al. 2014) reported the mosquito larvicidal effect of SA whereas LC_{50} at 107.12 against (III) instar larvae of *Cx. quinquefasciatus* was recorded after 24h of exposure. On the other hand, the activity of SA in defense response against various pathogenic infections in the plants has been well documented in literature. Singh and his colleagues reported that salicylic acid activated a cascade of events resulting in the inhibition of viral replication and their cell-to-cell and long distance transmission in plants (Singh et al. 2004). In addition, SA was capable of inhibiting completely the fungal germination (da Rocha Neto et al. 2016). Furthermore SA is an effective molecule for the control of postharvest gray mold of grape fruit (Qin et al. 2015). Also, different formulations of SA in emulsifiable concentrate and wettable granules decreased the egg masses and the growth of Root-Knot nematodes *Meloidogyne Spp* (Hagag et al. 2016). On the other hand, our experiment reported that SA is not toxic against *Ae. albopictus* pupae even at higher dosages, also all tested concentrations of DNS against *Ae. albopictus* larvae and pupae were did not cause mortality (table 1).

Table 1. Larval and pupal toxicity of salicylic acid and dinitrosalicylic acid against dengue vector *Ae. albopictus*.

treatments	Stage	LC_{50} (LC_{90}) ppm	95% confidence limit LC_{50} (LC_{90}) ppm		Regression equation	X^2 (d=3)	
			LCL	UCL			p-value
SA	I	86 (306)	72 (233)	98 (501)	Y= -4.37+2.33x	3.70	0.16
	II	108 (467)	92 (314)	125 (1047)	Y= -4.00+2.00x	1.16	0.56
	III	135 (564)	118 (365)	164 (1371)	Y= -4.20+2.00x	0.14	0.93
	IV	141 (606)	122 (383)	174 (1579)	Y= -4.20+2.00x	1.49	0.48
	pupa	-	-	-	-	-	
DNS	-	-	-	-	-	-	

LC_{50} = Lethal concentration that kills 50%. LC_{90} = Lethal concentration that kills 90%. LCL = lower confidence limit. UCL = upper confidence limit. $\chi 2$ = Chi-square value. df = degrees of freedom. - = No mortality.

Notably the AgNP synthesized by SA and DNS were highly toxic against *Ae. albopictus* larvae and pupae, the LC_{50} values of SA-AgNPs were 1.2 ppm (I), 1.4 ppm (II), 1.7 ppm (III), 1.9 ppm (IV) and 1.4 ppm (pupae), whereas LC_{50} values of DNS-AgNPs were 1.2 ppm (I), 1.5 ppm (II), 1.8 ppm (III) 2.2 ppm (IV) and 1.4 ppm (pupae), (Table 2). To the best of our knowledge, this is the first report about the toxicity of AgNPs synthesized by using SA and DNS. In the recent years, the green-synthesized AgNPs showed larvicidal and pupicidal potentials against a number of mosquito vectors (Benelli 2016b). For instance, AgNPs synthesized using *Couroupita guianensis* Aubl (leaf and fruit) extracts were tested against fourth larvae of *Aedes aegypti* with LC_{50} values of 2.1, 2.09 ppm, respectively (Vimala et al. 2015). Another study by Govindarajan and his colleagues highlighted that the *Z. diphylla* - synthesized AgNP were highly effective against *An. subpictus*, *Ae. albopictus* and *Cx. tritaeniorhynchus* larvae, where LC_{50} values were 12.53, 13.42 and 14.61 µg/ ml (Govindarajan et al. 2016a). Later on, K. Kovendan and other researchers reported that the AgNPs synthesized using *Psychotria nilgiriensis* were highly toxic against the *Aedes aegypti* larvae and pupae; LC_{50} were 20.26 µg/ml (I), 24,08 µg/ml (II), 29.37 µg/ml (III), 35.33 µg/ml (IV) and 43.12 µg/ml (pupae) (Kovendan et al. 2016).

Table 2. Larval and pupal toxicity of salicylic acid and dinitrosalicylic acid synthesized silver nanoparticles against dengue vector *Ae. albopictus.*

treatment	stage	LC_{50} (LC_{90}) ppm	95% confidence limit LC_{50} (LC_{90}) ppm		Regression equation	X2 (df=3)	
			LCL	UCL			p-value
SA-AgNP	I	1.2 (3.7)	0.93 (3.04)	1.40 (4.70)	Y= -0.50+4.00x	4.13	0.13
	II	1.4 (4.6)	1.09 (3.78)	1.63 (5.80)	Y= -0.50+3.00x	5.80	0.06
	III	1.8 (5.6)	1.51 (4.66)	2.10 (6.91)	Y= -1.00+3.00x	5.61	0.06
	IV	2.1 (6.9)	1.71 (5.74)	2.40 (8.65)	Y= -1.00+3.33x	3.86	0.15
	pupa	1.4 (3.8)	1.15 (3.15)	1.60 (4.71)	Y= -0.50+3.00x	0.58	0.75
DNS-AgNP	I	1.2 (3.1)	1.05 (2.56)	1.44 (3.87)	Y= -0.50+3.00x	1.21	0.55
	II	1.5 (3.4)	1.29 (2.90)	1.71 (4.26)	Y= -1.00+5.00x	0.57	0.75
	III	1.8 (4.2)	1.55 (3.55)	2.04 (5.05)	Y= -1.00+5.00x	1.98	0.37
	IV	2.3 (6.3)	1.94 (5.33)	2.60 (7.62)	Y= -1.00+4.17x	5.70	0.06
	pupa	1.4 (3.2)	1.19 (2.71)	1.60 (4.04)	Y= -1.00+5.00x	0.60	0.75

LC_{50} = Lethal concentration that kills 50%. LC_{90} = Lethal concentration that kills 90%. LCL = lower confidence limit. UCL = upper confidence limit. $\chi 2$ = Chi-square value. df = degrees of freedom.

3.3.3 Inhibitory activity evaluation of SA, DNS, and synthesized AgNPs on the larval enzymes

The effects of SA, DNS, SAAg-NP and DNSAg-NP on the biochemical constituents (acetylcholine esterase, alpha and beta carboxyl esterase, acid and alkaline phosphotase and total protein concentration) of forth instar larvae of *Ae. albopictus* were analyzed and the results are showed in **(**Figure10). The treated samples with SA showed decrease activity of acetylcholine esterase compared to the control, where the acetylcholine esterase activity values of control and SA treated larvae were 0.70 and 0.42 µM /min/mg protein, respectively. However, the level of acetylcholine esterase was reduced drastically after the treatment with SAAg-NP and DNSAg-NP as compared with the control groups (Figure 10b). AChE involved in the process of catalyzing the hydrolysis of the neurotransmitter ACh at nerve synapses and neuromuscular junctions and their activity was inhibited by the toxicity of tested

compounds. Solairaj and his colleague described the inhibition of acetylcholine esterase activity by α-chitin nanoparticles (CNP) from the shells of *Penaeus monodon Fabricius,* silver nanoparticles (AgNP) and α-chitin/silver nanocomposite after treated against *Ae. Aegypti* (Solairaj and Rameshthangam 2016). Later on researchers illustrated the inhibition of AChE caused by other tested compounds such as Plumbagin (Pradeepa et al. 2016).

As depicted in (Figure 10c) the exposure of SA inhibited α- carboxyl esterase activity in the fourth instar larvae of *Ae. albopictus*, as compared with the control. Wherein its activity decreased from 0.51 to 0.43 µM α-naphthol /min/mg protein. Similarly, the exposure of SA was slightly inhibited the β- carboxyl esterase activity from 0.44 to 0.40 µM β-naphthol /min/mg protein, (Figure 10d). Besides this, we found that α- and β- carboxyl esterase activities were highly inhibited by the SAAg-NP to 0.33 and 0.30 µM α,β-naphthol /min/mg protein respectively, also the α- and β- carboxyl esterase activities were highly decreased by the DNSAg-NP to 0.34 and 0.31 µM α,β-naphthol /min/mg protein respectively. Esterases are the primary enzymes involved in development of resistance mechanisms to chemical insecticides by splitting the carboxylester and phosphodiester bonds. The detoxifying activity of α- and β- carboxylesterase were used as biomarkers in studies with various mosquito vectors (Agra-Neto et al. 2014, Pradeepa et al. 2016, Selin-Rani et al. 2016). Our present study reported that SA, SAAg-NP and DNSAg-NP were able to highly reduce the activity of α- and β- carboxylesterase and the level of detoxifying enzymes are down regulated significantly in the development of *Ae. albopictus* larvae. Similar results were found by Koodalingam *et al* (Koodalingam et al. 2011, Koodalingam et al. 2012) after exposure of the larvae of *Ae. aegypti* to Vectobar and aqueous soapnut kernel extract which decreased the activities of α- and β- carboxyl esterase. Further (Edwin et al.

2016) reported in studies of andrographolide from *Andrographis paniculata* plant against larvae of *Ae. aegypti* which inhibited the level of α- and β- carboxyl esterase activities.

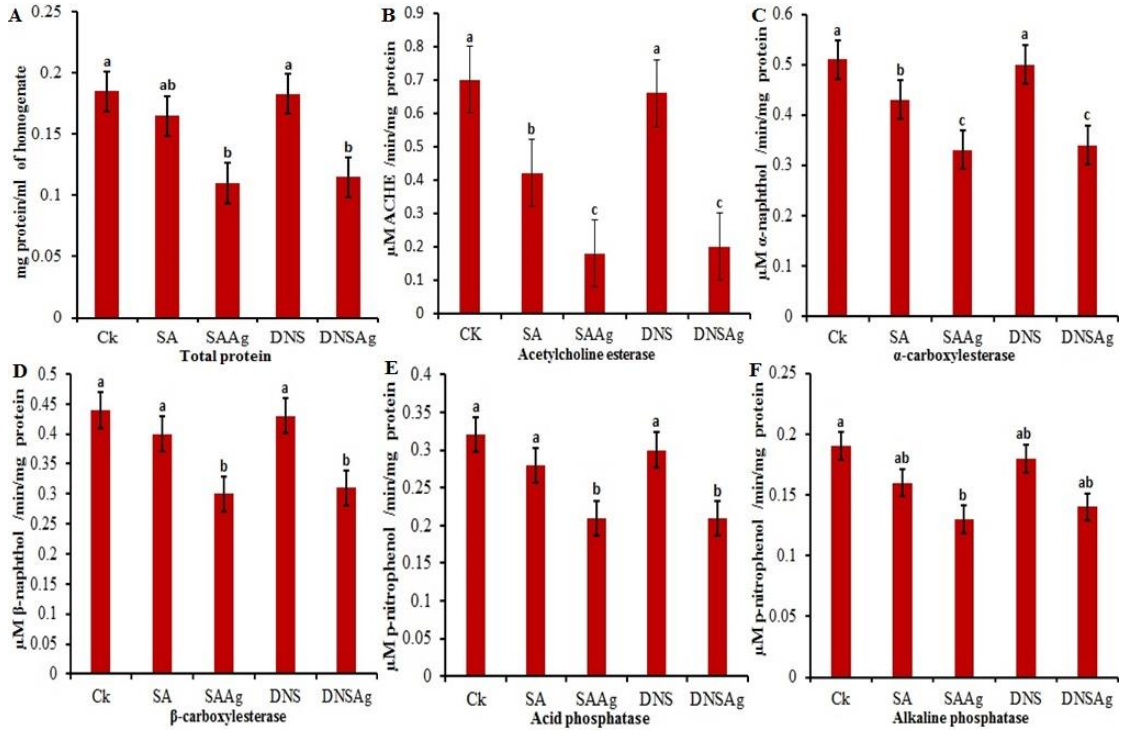

Figure. 10 Impact of SA, DNS, SAAgNP, and DNSAgNP on the enzyme activities and total proteins. (a) total protein concentration, (b) Acetylcholine esterase, (c) α-caboxylesterase, (d) β-carboxylesterase, (e) acid phosphatase and (f) alkaline phosphatase enzyme. Each bar represents mean ± SE of three replicates using different preparations of larval homogenates.

As shown in (Figure 10 e,f) the exposure of fourth instar larvae of *Ae. albopictus* to SA, SAAg-NP and DNSAg-NP resulted in reduction of the level of acid and alkaline phosphatase activities when compared with the control. In the acid phosphatase assay the level of acid phosphatase activity decreased from the value of control 0.32 to values of SA, SAAg-NP and DNSAg-NP 0.28, and 0.21 µM p-nitrophenol /min/mg protein respectively. Whereas in the alkaline phosphatase assay the SA, SAAg-NP and

DNSAg-NP treated fourth instar larvae showed values of 0.16, 0.13 and 0.14 µM p-nitrophenol /min/mg protein respectively compared with the control value 0.19 µM p-nitrophenol /min/mg protein. Acid and alkaline phosphatase plays an important role in the hydrolytic cleavage of phosphoric acid esters and they regulated the acid–alkaline balance (Walter and Schutt 1974). Also, these enzymes are important for many remarkable physiological processes such as metabolism and cellular signaling processes (Nathan et al. 2007). The exposure of larvae to SA revealed moderate inhibition in the level of acid and alkaline phosphotase activity, while the exposure of SAAg-NP and DNSAg-NP to the larvae showed the highest inhibition level of these enzymes. Similar results were found in mosquito larvae exposed to α-chitin nanoparticles (CNP) from the shells of *Penaeus monodon Fabricius,* silver nanoparticles (AgNP) and α-chitin/silver nanocomposite (Solairaj and Rameshthangam 2016). However, the exposure of vectobar to the mosquito larvae showed a decrease in the level of alkaline phosphatase activity, but not acid phosphatase activity whereas it increased the level of acid phosphatase activity (Koodalingam et al. 2012). Furthermore, the exposure of plant secondary metabolites inhibited in the level of acid and alkaline phosphatases in the larvae of *Cnaphalocrocis medinalis* (Nathan et al. 2007). In the present study, the steady decreasing impact of SA, SAAg-NP and DNSAg-NP on the acid and alkaline phosphatase activities might lead the deterioration of various biochemical functions such as metabolism, transfer of energy by ATP, tissue differentiation and growth of the vector mosquito (Koodalingam et al. 2012).

The total protein concentration of larval homogenate exposed to SA, SAAg-NP, DNS and DNSAg-NP were analyzed and compared to the untreated larval homogenate (Figure 10a). The exposure of the larvae to SA was found to be decreasing the total

protein level from the control value of 0.185 to 0.165 mg protein/ml of homogenate, while the exposure of the larvae to SAAg-NP and DNSAg-NP were found to be highly declined and showed value of 0.11 and 0.12 mg protein/mL, respectively. A number of several studies have examined the impact of a various larvicidal products on the protein level in target insect pests. The total protein level of larvae and pupae exposed to kernel extract from the soapnut, *Sapindus emarginatus* was found to be affected (Koodalingam et al. 2011). Similar results were reported in the protein levels of *Ae. aegypti* larvae upon exposure to CNP, AgNP and CNP/AgNP nanocomposite and Bti based product vectobar (Koodalingam et al. 2012, Solairaj and Rameshthangam 2016). In the current study, we observed that the total protein levels reduced upon exposure of SA, SAAg-NP and DNSAg-NP and this might be due to the direct toxic effect on the protein synthetic pathways of the mosquito larvae.

3.4 Conclusion

In conclusion, salicylic acid and its derivative 3, 5- dinitrosalicylic acid was used cost-effectively in one-step synthesis of silver nanoparticles as a reducing and capping agents. The dengue vector mosquito was highly susceptible to the fabricated AgNPs as compared with the SA, whereas DNS did not promote mortality. Furthermore, the study of enzymes activity involved in the detoxification processes was significantly affected by the synthesized AgNPs, more than that of SA. Thus, the synthesized AgNPs may be employed as an eco-friendly insecticide and at low dosages to reduce mosquito vectors.

Chapter 4. Larvicidal and pupicidal evaluation of silver nanoparticles synthesized using *Aquilaria sinensis* and *Pogostemon cablin* essential oils against dengue and zika viruses vector *Aedes albopictus* mosquito and it's histopathological analysis.

Abstract

Mosquitoes pose a threat to humans and animals, causing millions of deaths every year. Vector control by effective eco-friendly pesticides of natural origin is a serious issue which requires urgent attention. The employment of green reducing extracts for nanoparticles biosynthesis in a rapid and single step process represents a promising strategy. In this study, silver nanoparticles (AgNPs) were biofabricated using an essential oil of *Aquilaria sinensis* (AsEO) and Pogostemonis herba essential oil of *Pogostemon cablin* (PcEO) in one step and cost effectively manner. UV–vis spectrophotometry, Fourier transform infrared spectroscopy, scanning electron microscopy, transmission electron microscopy, X-ray diffraction analysis and energy-dispersive X-ray spectroscopy were used to confirm for formation and biophysical characterization of Ag nanoparticles. The larvicidal and pupicidal toxicity of AsEO, PcEO, and biosynthesized AgNPs were evaluated against larvae and pupae of the dengue and zika virus vector *Aedes albopictus.* Compared to the tested essential oils the biofabricated AgNPs showed the highest toxicity against larvae and pupae of *Ae. albopictus.* In particular, the LC_{50} values of AsEO ranged from 44.23 (I) to 165.83 (pupae), LC_{50} values of PcEO ranged from 32.49 (I) to 90.05 (IV), LC_{50} values of AsEO-AgNPs from 0.81 (I) to 1.12 (IV) and LC_{50} values of PcEO-AgNPs from 0.85 (I) to 1.19 (IV). Furthermore, histological analysis of the midgut cells of the control and treated larvae exhibited that the epithelial cells and brush border were highly affected by the fabricated AgNPs compared to the essential oils (AsEO and PcEO).

Overall, the *A. sinensis* and *P. cablin* essential oils fabricated AgNPs have a potential to be used as a biopesticide for mosquito control through safer and cost effective approach.

Keywords: *Ae. albopictus*; essential oil; *Aquilaria sinensis; pogostemon cablin*; Zika virus; histological analysis.

4.1 Introduction

Mosquitoes (Diptera: Culicidae) are the primary vectors of several serious diseases that affect both animals and humans (Govindarajan et al. 2017). They play the important role in transmission of parasites and pathogens of high public health concerns including malaria, dengue, filariasis, yellow fever, Japanese encephalitis, West Nile, and Zika virus (Benelli 2016a, Benelli et al. 2016). *Aedes albopictus* mosquito is considered to be one of the world's fastest-spreading invasive animal species and it originates from the forests of tropical regions of south-east Asia (Cunze et al. 2016). This mosquito has recently invaded many countries, spreading rapidly to Europe, North and South America, the Caribbean, Africa, and the Middle East (Kumar et al. 2016). The *Ae. albopictus* mosquito is known to have transmitted a number of serious diseases including dengue fever, yellow fever, West Nile fever and Rift Valley fever, Japanese encephalitis (Buhagiar 2009) as well as Zika virus fever (Ngoagouni et al. 2017).

Mosquito control is a critical element in the prevention the outbreaks of mosquito-borne diseases. The application of synthetic insecticides such as organophosphates and pyrethroids and insect growth regulators such as diflubenzuron and methoprene are currently major tools for mosquito control (Govindarajan et al. 2016b). However, these options have created many health and environmental issues, such as expanding resistance, disturbance of the ecosystem's natural control mechanisms, and non-target

organism toxicity, and impact on aquatic species (Govindarajan et al. 2017). In order to deal with these challenges, effective and eco-friendly control methods for mosquito vectors are urgently required.

In the recent years, nanoparticles have emerged as potential pesticides and currently both plants and microbes are being used to fabricate metal nanoparticles (Kumar et al. 2016, Suresh et al. 2017). The synthesis of nanoparticles using plant extracts is rapid, low cost, eco-friendly, and a single step method for biosynthesis process (Govindarajan et al. 2016a). Essential oils (EOs) are the aromatic oily liquids present in the secretary cavities and glandular hair cells of the plant parts (Vilas et al. 2016b). These oily extracts of plants are becoming increasingly popular as natural products to be used for a variety of purposes including complementary medicine and natural therapeutics, insect repellents, antimicrobial agents and food preservation (Manju et al. 2016). The plant genus of Aquilaria (Thymelaeceae) is comprised of approximately 15 species distributed across the rain forests of Southeast Asia (Dahham et al. 2016). *Aquilaria sinensis*, the main plant resource in China for agarwood, is chiefly distributed in South China, and is widely cultivated in Hainan and Guangzhou provinces, with the planting area estimated to be covering more than 700 acres (Chen et al. 2011). The agarwood plays a role in Traditional Chinese Medicine (TCM) and a large amount of it is also being consumed by distillation to obtain an essential oil (Zhang et al. 2014b). On the other hand Pogostemonis herba is the aerial part of dried *Pogostemon cablin* (Blanco) Benth (Pogostemon, Lamiaceae) and it also plays a vital role in TCM for the treatment of various problems such as to remove dampness, relieve sunstroke, stop vomiting and increase appetite (Zhang et al. 2016). Patchouli oil is the essential oil of pogostemonis herba, and it has been widely used by traditional Chinese physicians to treat a wide array of medical conditions such as common cold, nausea, diarrhea, headache and fever since time memorial. Pogostone is the major chemical

constituent of Pogostemonis Herba and it has largely been responsible for the intensive aromatic odor of the essential oil of this herb (Chen et al. 2015).

In the present work, the larvicidal and pupicidal activity of agarwood essential oil of *Aquilaria sinensis* (AsEo) and Pogostemonis herba essential oil of *Pogostemon cablin* (PcEo) as well as silver nanopartilces (AgNPs) synthesized by both of the essential oils have been tested for acute toxicity against *Aedes albopictus* mosquito larvae. UV– Vis spectrophotometry, Fourier transform infrared (FTIR) spectroscopy, X-ray diffraction (XRD), scanning electron microscopy (SEM), transmission electron microscopy (TEM), and energy-dispersive X-ray (EDX) spectroscopy have confirmed the rapid and cheap biosynthesis of AgNP.

4.2 Material and methods

4.2.1 Essential oils and chemicals

The agarwood essential oil of *Aquilaria sinensis* (AsEo) and Pogostemonis herba essential oil of *Pogostemon cablin* (PcEo) were purchased from Jiangxi Medicinal Oil Refinery Factory, South China. The silver nitrate ($AgNO_3$) was purchased from Sigma Aldrich (USA). All other chemicals and reagents used were of the highest analytical grade purchased from local agencies.

4.2.2 Insects rearing

The eggs of *Ae. albopictus* obtained from mosquito colonies reared in the laboratory of Urban Entomology, Institute of Insect Sciences, Zhejiang University were allowed to hatch out under the controlled laboratory conditions at room temperature (RT: 26± 2C°, and 70–85% relative humidity RH) with a naturally prevailing photoperiod of 14:10 h (Light/Dark). The larvae were maintained in

dechlorinated tap water and were fed with finely ground rat food. The different developmental stages of mosquito larvae and pupae were used for the bioassays.

4.2.3 Synthesis of silver nanoparticles

In the present study, the silver nanoparticles (AgNPs) were synthesized using essential oil reduction method. The *Aquilaria sinensis* essential oil (AsEO) and *Pogostemon cablin* essential oil (PcEO) were utilized as both reducing and stabilizing agents. In a typical synthesis, the (0.01g) of each of AsEO and PcEO in 1ml of DMSO were separately diluted with 10ml of dechlorinated water. The pH value of the solutions was adjusted to 7 using 0.1 M of sodium hydroxide solution, and was dropped slowly into a boiling 100ml of 2 mM silver nitrate solution. The formation of AgNPs is indicated by the colour change from colourless to light yellow and further to the dark red.

4.2.4 Characterization of AgNPs

The synthesized nanoparticles were primarily characterized by UV–vis spectroscopy UV-2550 spectra (Shimadzu, Japan) at a resolution of 1 nm in the range of 200–800 nm. Furthermore, the reaction mixture was subjected to centrifugation at 15,000 rpm for 20 min, and the resulting pellet was dissolved in de-ionized water and filtered through a Millipore filter (0.45μm), and freeze-dried. An aliquot of this filtrate containing silver nanoparticles was used for scanning electron microscopy (SEM), transmission electron microscopy (TEM), energy-dispersive spectroscopy (EDS), X-ray diffraction (XRD) and Fourier transform infrared (FTIR). The FT-IR analysis was accomplished with the aliquot of reduced silver nanoparticles and recorded under identical conditions in the range of 500–4000 cm_1 region at a resolution of 4 cm_1 using Fourier transform infrared spectrometer (Vector 22, Bruker, Germany). The quality and formation of compounds were tested using Siemens X-ray diffractometer

(XRD) analysis. Where, XRD pattern was measured by drop coated film of dried powder of silver nanoparticles onto glass slides. The operation conditions were at a voltage of 45 keV and a current of 20 mA with Cu-Ka radiation as an X-ray source in the range of 20–80 at the 2h angle. Further, the morphology of the synthesized AgNPs was investigated by Scanning Electron Microscopic (SEM) using TM-1000 (Hitachi, Japan). Thin film of the sample was prepared on a carbon coated copper grid by simply dropping a very small amount of the sample on the grid. The instrument was equipped with an energy dispersive X-ray spectrum (EDX) to confirm the presence of silver metal.

The film on the SEM grid was then allowed to dry by putting the grid under a mercury lamp for 5 min. The structural characterization of AgNPs was carried out by Transmission Electron Microscopy (TEM) (JEM-1230, JEOL, Akishima, Japan). The extra sample was removed from carbon-coated copper grid using the cone of a blotting paper and sample was placed on the carbon-coated copper grid to make a thin film of the sample and then it was kept in a grid box sequentially.

4.2.5 Larvicidal and pupicidal bioassays

Mosquito larvicidal and pupicidal trials were carried out according to WHO standard procedures (WHO 1996), with slight modifications. The AsEO and PcEO were diluted in dimethyl sulfoxide (DMSO) in order to prepare a serial dilution of the test dosages. For experimental treatment, the desired dose of each essential oil in 1 ml DMSO was added to 100 ml of distilled water in a 250-ml beaker and mixed gently to produce a homogeneous test solution (Table 3). Twenty *Ae. albopictus* larvae (I, II, III, IV) instar and pupae were transferred in water into a bowl of the prepared test solution. For the experimental treatment of AgNPs, twenty *Ae. albopictus* larvae (I, II, III, IV) instar and pupae were separately introduced in a 250ml beaker containing 100ml of

dechlorinated tap water and added desired dosages of synthesized silver nanoparticles (Table 4). Four duplicate trials were carried out for every sample concentration, and for each trial, a negative control was included using distilled water containing the same amount of DMSO as the test sample. Mortality was determined after 24 h of exposure, during which no food was offered to the larvae.

4.2.6 Histological analysis

Histological analysis of the digestive system was performed using fourth instar larvae (treated and control). Twenty five fourth-instar larvae were exposed to a LC_{50} concentration for 24 h. The procedures were performed following the method of (Kjanijou et al. 2012) with a small modification. Briefly the larvae were fixed in 10% buffered formaldehyde for 24 h, dehydrated through a graded series of ethanol, and cleared with xylene solutions. They were embedded in a lock using melted paraffin at the embedding station. The paraffin blocks were sectioned in 5m thickness using a rotary microtome and stained with hematoxylin and eosin. The glass slides were examined for abnormalities using an Olympus BX61 light microscope and photographed by a Canon EOS 1100D digital camera.

4.2.7 Statistical analysis

The statistical analyses of data from larvicidal and pupicidal experiments were performed by probit analysis calculating LC_{50} and LC_{90} and following the method by Finney (Finney 1971) SPSS software package 16.0 version was used. The toxicity data were subjected to one-way ANOVA with two factors (i.e. the targeted mosquito instar and the tested dose). Means were separated using Tukey's HSD test. The acceptance level of statistical significance was $p \leq 0.05$ in all instances.

4.3 Results and discussion

4.3.1 Characterization of silver nanoparticles

The silver nanoparticles (AgNPs) were synthesized via reduction of silver nitrate using the AsEO and PcEO. This was a one-step silver nitrate reduction process in which the AsEO and PcEO acted as both the reducing and stabilizing agents. The formation of AgNPs was visually confirmed by the change of reaction mixture colors (Figure 11a). When the AsEO and PcEO were separately mixed with $AgNO_3$ solution under constant stirring at boiling degree, the color of reactant solutions changed from the colorless to light yellow color and further red color within one and half hours. Literature reports similar color changes due to the excitation of surface Plasmon vibrations of the synthesized AgNPs (Vilas et al. 2016a).

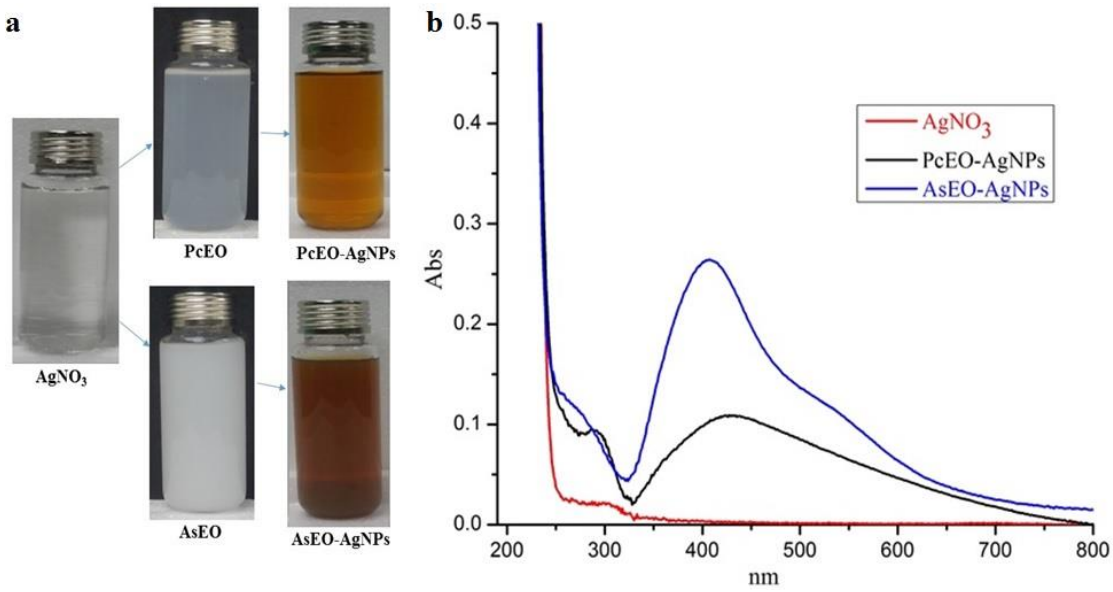

Figure. 11 Preparation process of AgNPs, and UV-vis spectrum. (a) Color of reactants before and after the reaction. (b) UV–Vis adsorption spectrum of AgNPs. The maximum absorption peaks of AsEO-AgNP and PcEO-AgNP were 408 and 430 nm, respectively after 90 min from the reaction.

The UV–vis spectroscopy has always been considered as one of the useful techniques for the preliminary characterization of metal NPs. UV–vis absorption spectra from the reaction solutions in the present investigation were monitored in the 200–800 nm range (Figure 11b). The maximum absorbance of AsEO-AgNPs was found at 408 nm due the excitation of the surface plasmon resonance to the fabricated AgNPs (Ramanibai and Velayutham 2016). Whereas the absorption peak of PcEO-AgNPs was broader and observed at 430 nm, indicating the formation of AgNPs (Suman et al. 2013b, Fouad et al. 2017a, Kalimuthu et al. 2017). Similar findings were recently reported by Shibani Basu and his colleagues (Basu et al. 2016).

Moreover, SEM analysis of our synthesized AgNPs was carried out in order to investigate the morphology and size distribution of AgNPs (Figure 12a, b). The AsEO-AgNPs and PcEO-AgNPs are mainly spherically shaped with the average range of particle size from 15 to 55nm, and 16 to 87nm, respectively And this is a good resemblance with shape of SPR band observed in the UV–Vis spectra (Manjamadha and Muthukumar 2016).

The EDX pattern of AsEO-AgNPs and PcEO-AgNPs were shown in (Figure 12c, d). The presence of strong peaks of silver element around 3 KeV were observed, which indicates that the Ag is the major element in AgNPs, thus confirming the successful biosynthesis of AgNPs (Rokade et al.). Similar peak (3 keV) has been reported by other researchers (Deepak et al. 2016)

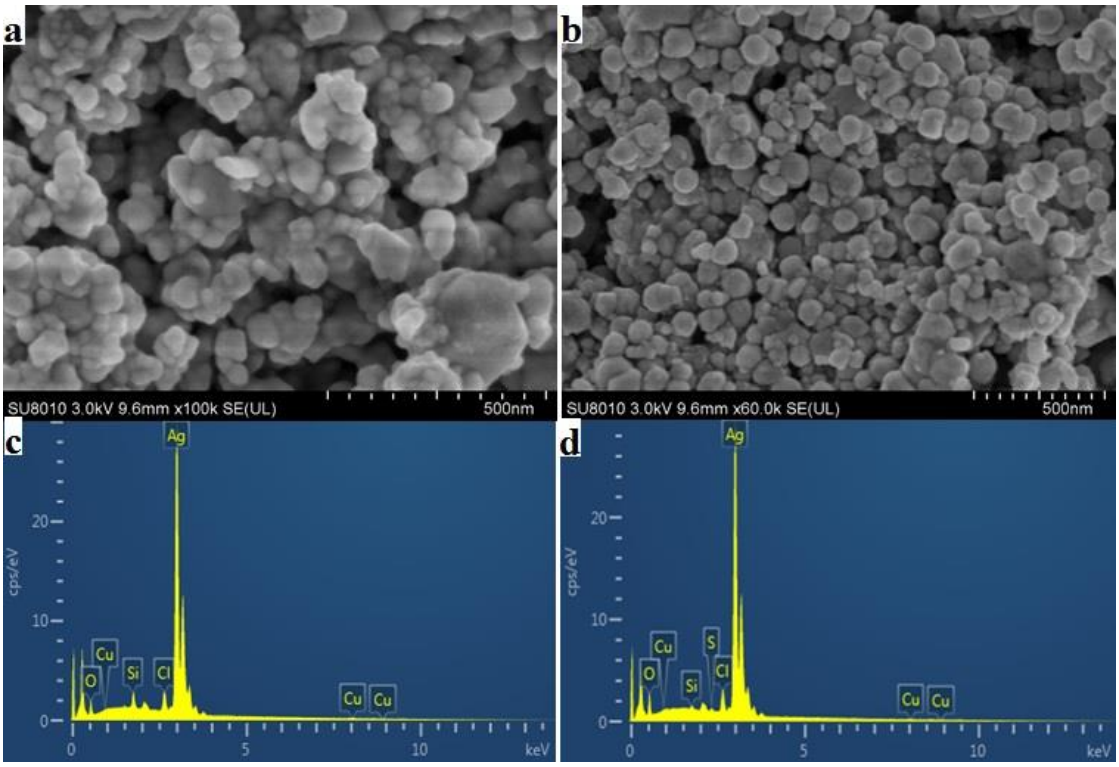

Figure. 12 (a) SEM image of synthesized AgNPs using *Aquilaria sinensis* essential oil (AsEO). (b) SEM image of synthesized AgNPs using *Pogostemon cablin* essential oil (PcEO). (C) EDX pattern of synthesized AsEO-AgNPs and (D) EDX pattern of synthesized PcEO-AgNPs.

Further investigations of the Transmission Electron Microscpoic (TEM) on the newly fabricated AgNPs using both AsEO and PcEO showed the polydispersity in nature. **(**Figure 13a, b) shows the TEM image of AgNPs and it indicates that the synthesized AgNPs are mainly uniform with spherical shape. The transparent organic layer coatings around the AgNPs were shown in the TEM image, It was also found that PcEO mediated AgNPs bound with clearly thin layer of biomolecule coating on their surface which was due to the phytochemicals that served as a capping agent, therefore, the particles were poly dispersed without direct contact and stable for long period of time to prevent agglomeration (Yuan et al. 2017).

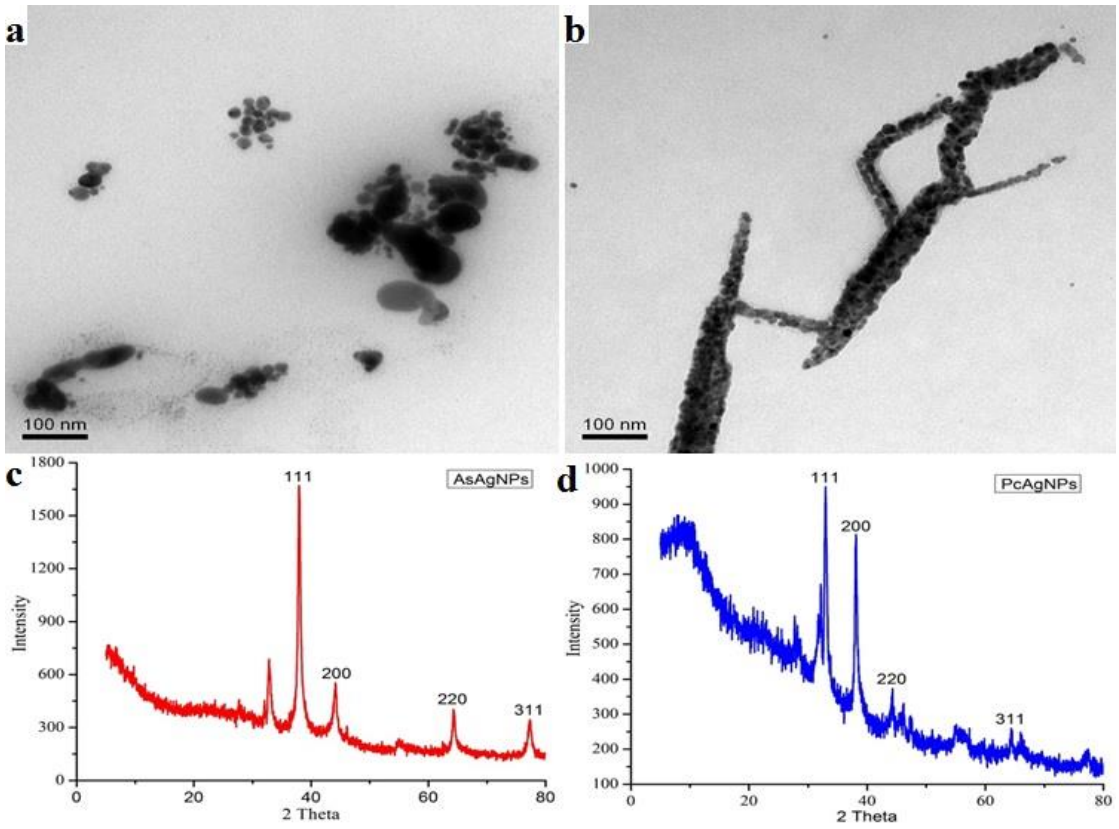

Figure. 13 (a) TEM image of synthesized AgNPs using *Aquilaria sinensis* essential oil (AsEO) (b) TEM image of synthesized AgNPs using *Pogostemon cablin* essential oil (PcEO). (C) XRD pattern of synthesized silver nanoparticles by AsEO and (D) XRD pattern of synthesized silver nanoparticles by PcEO.

XRD is a rapid analytical method to identify the crystalline structure, the analysis of XRD showed intense peaks at 2 theta. (Figure 13c, d) exhibits four well-defined characteristic peaks at scattering angles (2h) for each of AsEO-AgNPs and PcEO-AgNPs where the angles (2h) of 38, 44.28, 64.38, 77.36 corresponded to the lattice planes (111), (200), (220), and (311) in AsEO-AgNPs and the angles (2h) of 33.28, 38.38, 44.08, 64.44 corresponded to the lattice planes (111), (200), (220), and (311) in PcEO-AgNPs. The peak corresponding to the (111) is more intense than the other planes (Krishna et al. 2016). Thus, XRD highlighted that AgNPs formed by the

reduction of $AgNO_3$ with AsEO and PcEO were face-centered cubic (fcc) and crystalline in nature (Suresh et al. 2017).

FTIR analysis of the AsEO-AgNPs and PcEO-AgNPs was carried out to identify the molecules which may be responsible for reducing Ag ions and stabilizing AgNPs in the mixture. The FTIR spectrum of AgNPs prepared using AsEO were shown in (Figure 14).

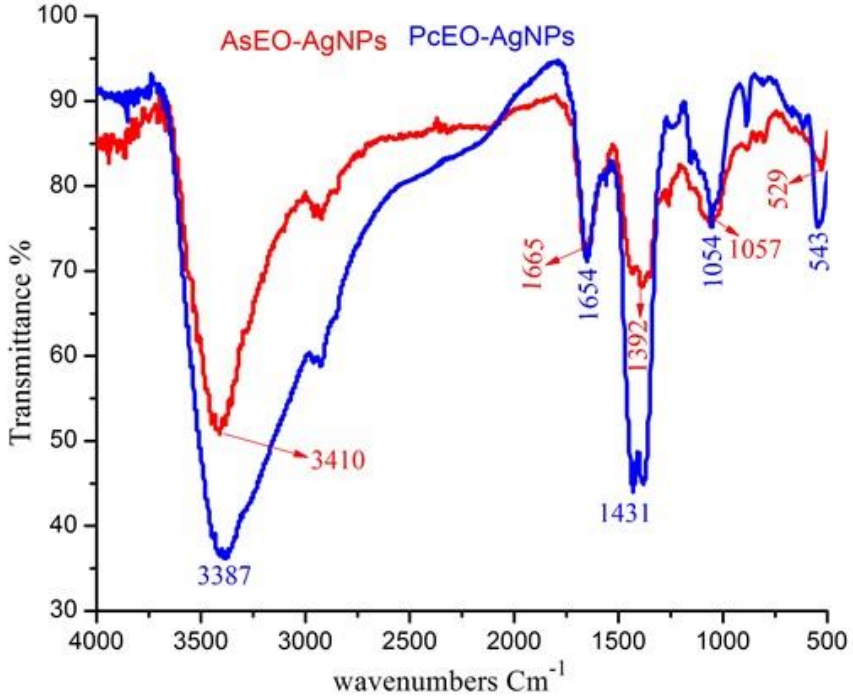

Figure. 14 Fourier transform infrared (FT-IR) spectrum of freeze-dried powder of silver nanoparticles synthesized using *Aquilaria sinensis* essential oil (AsEO) and *Pogostemon cablin* essential oil (PcEO).

The absorption bands due to vibration of chemical molecules at 3410, 1665, 1392, 1057, and 529 cm^{-1} were recorded in the spectrum of wave number between 4,000 and 500 cm^{-1}. The very strong broad absorption peaks at 3410, (O–H stretch and bending) ; strong and sharp peaks at 1665 cm^{-1} (carboxylic acid) and some variable stretching and bending peaks were observed for AsEO-AgNPs, which may be from $AgNO_3$ solution,

the metal precursor involved in the AgNP synthesis process (Gopinath et al. 2017). Also, IR spectrum of synthesized AgNPs using PcEO was shown in (Figure 4). The spectrum shows transmittance peaks at 3387, 1654, 1431, 1054, and 543 cm^{-1}. The strong broad absorption peak at 3387 represents the presence of hydroxyl group (stretch and bending) from polyphenols, proteins, enzymes and/or polysaccharides (Fouad et al. 2016b). The sharp absorption peak close to 1654 cm^{-1} might be linked with stretching vibration C=O of carbonyl group, amide I and nitro groups (Yuan et al. 2017). The peak at 1431 arises from the C-N stretching mode of the Aromatic amine group (Suman et al. 2013b). Overall, these different functional groups from biomolecules of both essential oils may responsible for reduction and capping of AgNPs (Rajaganesh et al. 2016).

4.3.2 Larvicidal and pupicidal activity of AsEO, PcEO and AgNPs against *Ae. albopictus*

In laboratory conditions, the larvicidal activity of aqueous essential oils of AsEO and PcEO, as well as the synthesized silver nanoparticles were evaluated against larval stages and pupae of *Ae. albopictus* mosquito. The 24h exposer of AsEO and PcEO were moderately toxic against larval instars (I–IV) and pupae of *Ae. albopictus* (Figure 15a). The LC_{50} values of AsEO and PcEO ranging from 44.23 to 166 ppm and from 32.49 to 90.05ppm respectively are shown in Table 3. Mortality was proportional to the tested concentrations and this was in agreement with a number of previously reported plant-borne extracts (Pavithra Bharathi et al. 2016, Elemike et al. 2017). Earlier Hye-Mi Park, and his colleagues studied the larvical activity of PcEO and showed significant effect against *Culex pipiens pallens* (Park and Park 2012). Also PcEO showed toxicity and repellency against urban ant's species (Albuquerque et al. 2013). Our present study reported that the PcEO did not cause any mortality against pupae of *Ae. albopictus* (Table 3). As mentioned above, the 24h exposer of AsEO was

moderately effective against dengue and zika vector mosquito. To the best of our knowledge, this is the first investigation of mosquitocidal activity of AsEO. Recently, the mosquitocidal potential of a number of plant essential oils have been reported. For example Zahran, H.E *et al* (Zahran et al. 2017) observed pronounced larvicidal activity of *A. monosperma, S. terebinthifolius* and *O. vulgare* oils after 24h of treatment against fourth instar larvae of *Cx. Pipiens*.

Table 3. Larval and pupal toxicity of essential oil *Aquilaria sinensis* (AsEO) and *Pogostemon cablin* (PcEO) against Zika vector *Ae. albopictus*

Treatment	Stage	$LC_{50}(LC_{90})$ ug/ml	95% confidence limit LC_{50} (LC_{90}) ug/ml		Regression equation	X2 (df=4)
			LCL	UCL		
AsEO	I	44.23 (397)	33.68 (220)	54.89 (1448)	Y= -2.83+1.71x	12.33
	II	54.94 (559)	43.18 (275)	70.95 (2884)	Y= -2.46+1.43x	11.96
	III	89.66 (652)	71.91 (329)	129.97 (2812)	Y= -3.23+1.71x	18.89
	IV	118.7 (809)	91.09 (385)	196.68 (4086)	Y= -3.43+1.71x	20.87
	Pupa	166 (2258)	108.5 (650)	513.82 (8666)	Y= -2.86+1.42x	12.70
PcEO	I	32.49 (287)	22.14 (172)	40.95 (863.9)	Y= -2.63+1.71x	11.02
	II	47.88 (591)	35.56 (275)	61.78 (3967)	Y= -2.46+1.43x	10.08
	III	60.03 (517)	48.45 (269)	77.34 (2165)	Y= -3.03+1.71x	14.61
	IV	90.05 (602)	72.79 (314)	128.33 (2351)	Y= -3.80+2.00x	19.16
	Pupa	n.m.	n.m.	n.m.		

LC_{50} = Lethal concentration that kills 50% of insects; LC_{90} = Lethal concentration that kills 90% of insects; LCL = lower confidence limit; UCL = upper confidence limit; $\chi 2$ = Chi-square value; *df* = degrees of freedom; n.m. = No mortality; Significant at $p < 0.05$ level.

Further Benelli, G. *et al*. (Benelli et al. 2017d) reported acute toxicity of montana essential oil and four other plant essential oils on *C. quinquefasciatus* larvae. Similarly, it has been reported that EO extracted from the leaves of *B. eriantha* showed high toxicity against 3rd instar larvae of Anopheles, Aedes and Culex species, with LC_{50} ranging from 41.61 to 61.33 µg/ml (Benelli et al. 2017c). The susceptibility of the Asian tiger mosquito larvae to the *Cannabis sativa* and *Humulus lupulus* essential oils with LC_{50} values of 302 and 331µg/l respectively were observed (Bedini et al. 2016).

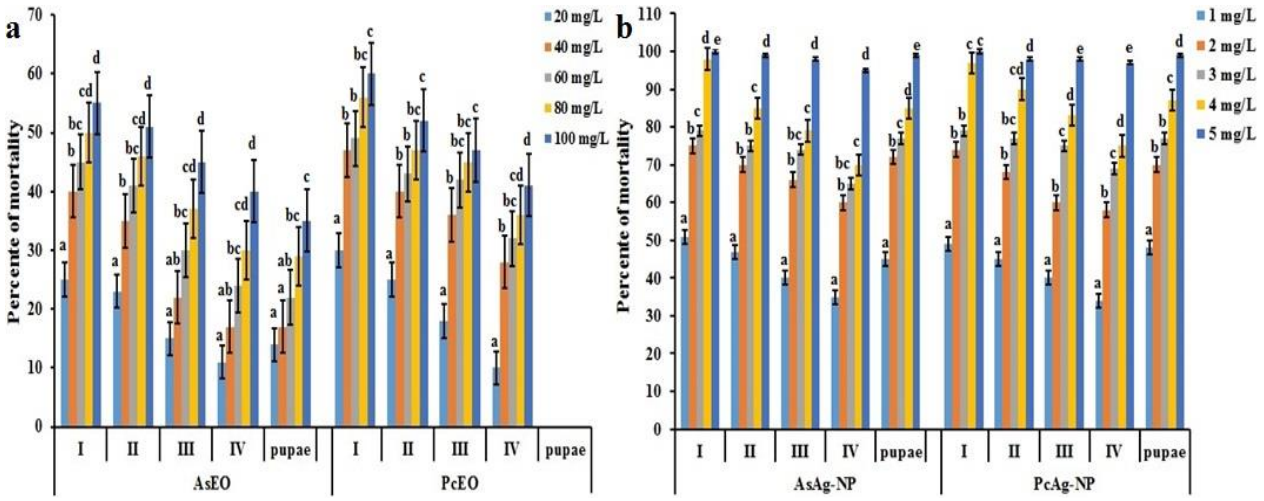

Figure. 15 Larvicidal and pupicidal efficacy of (a) *Aquilaria sinensis* essential oil (AsEO), *Pogostemon cablin* essential oil (PcEO) and (b) green synthesized silver nanoparticles using AsEO and PcEO against I-IV instar larvae and pupae of dengue and zika vector *Aedes albopictus*. Mortality was recorded after 24 h of exposure. No mortality was observed in the control. Different letters above each bar indicate significant differences among treatments (ANOVA, Tukey's HSD test, $P < 0.05$).

Notably the AgNPs synthesized using AsEO and PcEO were highly toxic against *Ae. albopictus* larvae and pupae (Figure 15b) with LC_{50} ranging from 0.81 to 1.12 ppm and from 0.90 to 1.19 ppm respectively (Table 4). In the recent years, the green-synthesized AgNPs showed comparable larvicidal and pupicidal toxicity against a number of mosquito vectors (Govindarajan et al. 2016a, Benelli et al. 2017d, Jinu et al. 2017).

For instance the *S. maritima*-synthesized AgNPs were highly toxic against *Ae. aegypti*. LC_{50} ranging from 8 to 17 ppm (Suresh et al. 2017). Another study by Kumar, P.M. *et al* (Kumar et al. 2016) reported that AgNPs synthesized from the leaf extract of *B. tinctoria* were highly effective against *Ae. albopictus* young instars, with LC_{50} ranging from 4.97ppm to 14.87ppm. In addition the *M. emarginata* leaf extract fabricated AgNPs revealed high toxicity against *A. stephensi, A. aegypti* and *C. quinquefasciatus* with LC_{50} values of 8.36, 9.20 and 10.02 µg/mL, respectively

(Azarudeen et al. 2017). In the present investigation, the larvicidal and pupicidal activity of both fabricated AgNPs showed highest toxicity and at low concentrations compared to AsEO and PcEO essential oils against *Ae. albopictus* mosquito. Vector control is one of the major tools due to the development of resistance against the usual insecticides. Therefore, the development of new alternative insecticides is highly needed and it is of great significance (Bhuvaneswari et al. 2016).

Table 4. Larval and pupal toxicity of *Aquilaria sinensis* and *Pogostemon cablin* essential oil synthesized silver nanoparticles (AsEO-AgNPs and PcEO-AgNPs) against Zika vector *Ae. albopictus.*

Treatment	stage	$LC_{50}(LC_{90})$ ug/ml	95% confidence limit LC_{50} (LC_{90}) ug/ml		Regression equation	X2 (df=4)
			LCL	UCL		
AsEO-AgNPs	I	0.81 (1.72)	0.61 (1.50)	0.97 (2.10)	Y= 0.0+5.0x	19.71
	II	0.83 (2.33)	0.56 (1.95)	1.02 (3.14)	Y= 0.2+2.8x	19.74
	III	1.02 (2.49)	0.81 (2.17)	1.19 (3.02)	Y= 0.1+3.6x	25.03
	IV	1.12 (4.36)	0.81 (4.36)	1.37 (6.48)	Y= -0.2+2.0x	28.46
	Pupa	0.90 (2.07)	0.68 (1.79)	1.06 (2.59)	Y= 0.1+4.0x	20.99
PcEO-AgNPs	I	0.85 (1.79)	0.65 (1.56)	1.00 (2.18)	Y= 0.2+5.0x	21.14
	II	0.91 (2.26)	0.68 (1.92)	1.08 (2.91)	Y= 0.2+4.0x	22.56
	III	1.04 (2.85)	0.79 (2.36)	1.23 (3.93)	Y= 0.1+4.0x	28.23
	IV	1.19 (3.43)	0.95 (2.89)	1.39 (4.42)	Y= -0.2+2.6x	32.09
	Papa	0.84 (2.15)	0.59 (1.82)	1.02 (2.78)	Y= 0.1+4.0x	19.30

LC_{50} = Lethal concentration that kills 50% of insects; LC_{90} = Lethal concentration that kills 90% of insects; LCL = lower confidence limit; UCL = upper confidence limit; $\chi 2$ = Chi-square value; *df* = degrees of freedom; Significant at $p< 0.05$ level.

4.3.3 Histological studies

The LC_{50} exposure at 24 h period and control 4th stage larvae of *Ae. albopictus* were used for histological analysis. Our investigations revealed histological structure alterations in the midgut epithelial cells from treated and control larvae (Figure 16).

Our observations are compatible with the findings of by Kalimuthu, K. *et al* (Kalimuthu et al. 2017) which noted that the midgut cells of 4th stage larvae of *Ae. aegypti* showed various degrees of apical swelling into the gut lumen, and reducing

intercellular contacts, in addition to degeneration of nuclei and brush border after treatment of rhizome-fabricated AgNPs. In the present study, our data showed that the midgut area including the epithelial cells and brush border were highly affected by the fabricated AgNPs compared to the essential oils (AsEO and PcEO). The midgut epithelium of control larvae exhibited normal cytoplasmic characteristics with regular microvilli lining (Figure 16a) whereas the essential oils treated larvae revealed moderate basophilia of the epithelial cells from the midgut (Figure 16b, c).

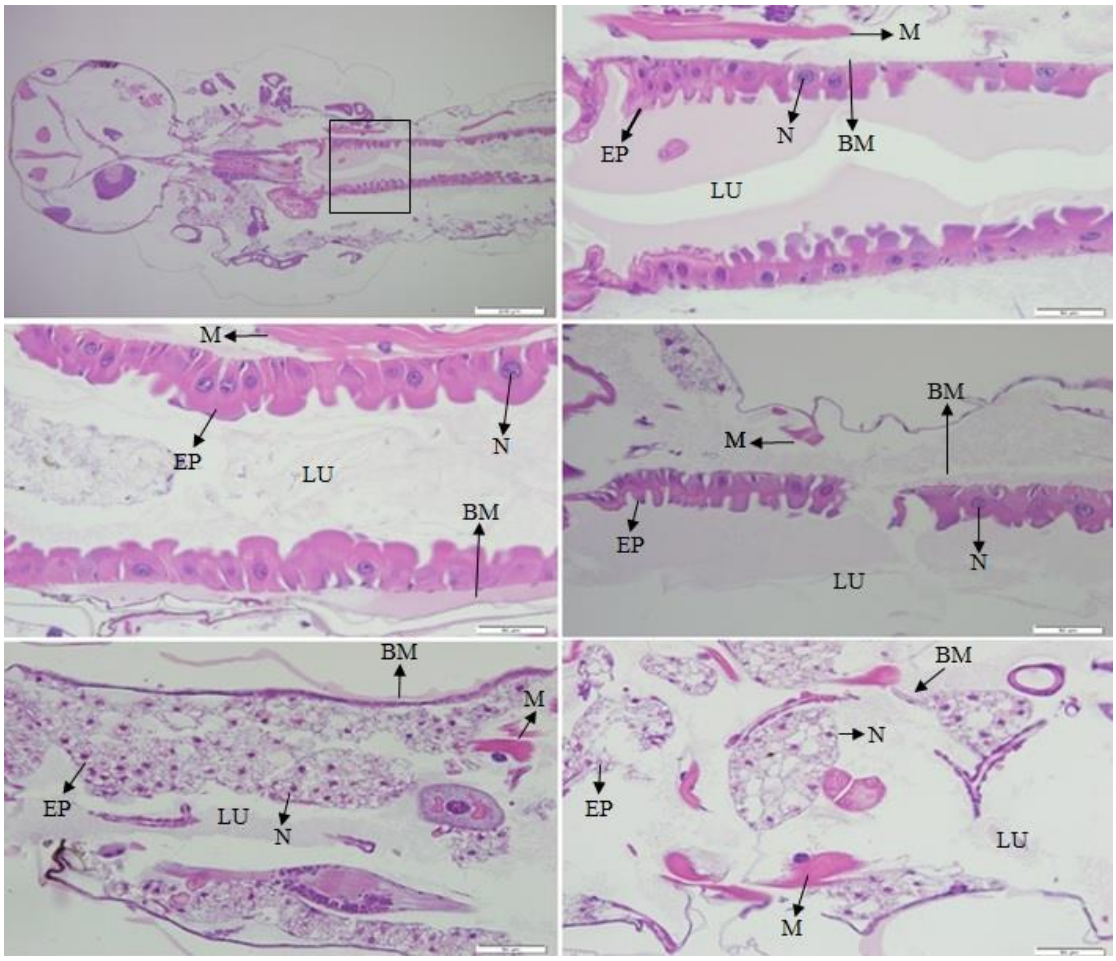

Figure. 16 Longitudinal sections of the midgut of an *Aedes albopictus* 4th instar larvae, magnification: 40×. (a, b) control, (c) a larva under treatment with *Aquilaria sinensis* essential oil, (d) a larva under treatment with *Pogostemon cablin* essential oil, (e) a larva under treatment with AsEO-AgNPs, (f) a larva under treatment with PcEO-

AgNPs. Epithelial cells = EP, basement membrane = BM, muscles = M, nucleus = N, gut lumen = LU,

Similar results were reported by Nunes, F.C. *et al* (Nunes et al. 2015). Also, morphological changes in the midgut cells of *C. quinquefasciatus* have been reported after exposure of essential oil of *Peumus boldus* Molina and its ascaridole-enriched fraction (de Castro et al. 2016). Notably the AgNPs treated larvae were highly affected, exhibiting severe damage in the midgut epithelial cells and showed cytopathological variations, such as the destruction of epithelial cells and degradation of nuclei (Figure 16d, e). Our result support earlier reports by Al-Mekhlafi, F.A (Al-Mekhlafi 2017). Furthermore the B. Sundararajan and his colleague observed histological alterations in the digestive tract and midgut of 3rd and 4th instar larvae of *Ae. aegypti* after exposure of gold nanoparticles (Sundararajan and Kumari 2017).

4.4 Conclusion

In this study, by adopting eco-friendly synthetic route for the fabrication of nanomaterials, we synthesized Ag nanoparticles using of *Aquilaria sinensis* essential oil (AsEO) and *Pogostemon cablin* essential oil (PcEO) as reducing and capping/stabilizing agents. Biosynthesized Ag nanoparticles were mostly spherical in shape, crystalline in nature with face-centred cubic geometry and their mean size ranged between 15–87 nm. The fabricated Ag nanoparticles showed significant larvicidal and pupicidal toxicity against *Ae.albopictus* mosquito even at low doses. Histological studies confirmed that Ag nanoparticles exert potential damage regarding the digestive tract and midgut cells of mosquito larvae. Further studies are needed to clarify the exact mechanism of action of Ag nanoparticles against mosquito vectors regarding skin impact as well as mineral balances and transportation within the cells of insect body.

Chapter 5. Mannosylerythritol lipids mediated biosynthesis of silver nanoparticles: An eco-friendly and operative approach against chikungunya vector *Aedes albopictus* mosquito.

Abstract

Mosquitoes are highly dangerous vectors of pathogens and parasites, which considered as serious health problems in the increasing world population of humans and animals. Current vector control strategies mainly rely on the use of synthetic insecticides which has resulted in resistance and adverse environmental impacts. Vector control by environmental friendly Insecticides of synthesized natural products have been a priority in this scope. In this study, larvicidal and pupicidal efficacy of fabricated silver nanoparticles (AgNPs) employing Mannosylerythritol lipids (MELs) biosurfactant produced from ustilaginomycetous yeast *Pseudozyma aphidis* was evaluated against larvae and pupae of dengue and Chikungunya viruses' vector *Aedes albopictus* mosquito. UV–vis spectrum was initially confirmed the synthesized AgNPs and further characterized by scanning electron microscopy (SEM), transmission electron microscopy (TEM), energy-dispersive X-ray (EDX), X-ray diffraction (XRD) and Fourier transform infrared (FTIR). Larvae and pupae were exposed to graduated concentrations of MELs biosurfactant and synthesized AgNPs for 24 h. The maximum toxicity was revealed in the synthesized AgNPs against larvae and pupae of *Ae. albopictus* with LC_{50} values ranging from 0.14μg/mL (I) to 0.38μg/mL (IV) compared to the MELs toxicity which have had LC_{50} values ranging from 45 μg/mL (I) to 100 μg/mL (pupae). Further investigations regarding to the systemic effects exerted by the tested materials against fourth instar larvae of *Ae. albopictus* showed an alterations in the levels of total protein, superoxide dismutase (SOD), peroxidases (POD) and catalase (CAT) enzymes activity. Moreover, the TEM images of the midgut cells of

the control and treated larvae explored AgNPs accumulation in the rough endoplasmic reticulum and other cell organelles such as nucleolus and mitochondria. Overall, this study suggest that AgNPs could be employed as innovative and operative tool in mosquito vector control strategies.

Keywords: *Ae. albopictus*, Mannosylerythritol lipids, glycolipids biosurfactant, MELs-AgNPs, cell organelles.

5.1 Introduction

Mosquitoes (Diptera: Culicidae) vectors represent one of the major public health problems in developing countries (Crocker et al. 2017). They are known to be efficient vectors for transmitting diseases to humans and animals, such as malaria, dengue, Chikungunya, Japanese encephalitis, lymphatic filariasis, yellow fever and recently Zika virus (Suman et al. 2016).

The Asian tiger mosquito *Aedes albopictus* (Skuse 1894) originates from tropical regions in Southeast Asia and has colonized almost over the world within last three decades (Oppold et al. 2015). The worldwide spread of *Ae. albopictus* has caused significant interest for researchers because these mosquitoes can transmit serious medically relevant pathogens, and therefore threaten to human and animal health (Kreß et al. 2017). The main movement modes of *Ae. albopictus* are the worldwide trade with used tyres and ornamental plants such as 'lucky bamboo' (*Dracaena* spec.) (Hofhuis et al. 2009). *Ae. albopictus* is one of the most invasive mosquitoes in the world and the reasons for the successful competition with native mosquito species are the high invasion potential, the vector capacity and its ecological plasticity (Fischer et al. 2011). The Asian tiger mosquito is socio-medical concern mosquito due to its aggressive daytime human-biting behavior and ability to transmit arboviruses, including dengue, chikungunya and Zika (Sherpa et al. 2017).

The control of mosquitoes is a potential tool in preventing the outbreaks of mosquito borne diseases (Ga'al et al. 2017a). Currently, the mosquito control practices depends primarily on continued applications of synthetic chemical insecticides such as organophosphates and insect growth regulators such as diflubenzuron and methoprene, in addition to bacterial larvicides such as Bacillus thuringiensis and Bacillus sphaericus (González et al. 2017). However, the continuous use of synthetic chemical insecticides has resulted many health and environmental problems, such as expanding resistance, disturbance of natural biological control systems, and impact on non-target organisms (Ga'al et al. 2017b). So as to overcome these challenges, there is a crucial need for alternative tools of effective and eco-friendly control methods for mosquito vectors (Benelli et al. 2017c).

Nature produces many biologically active products against mosquito vectors in the form of plant products, marine products, microbial products and other biological derivatives (Chellappandian et al. 2017).

Biosurfactants are biologically active compounds produced by various groups of microorganisms such as different bacterial strains, yeast and fungi (Gómez-Graña et al. 2017). They have been used in many fields, including food processing, cleaning purposes, oil recovery, bioremediation of oil-contaminated sites, and drug development (Parthipan et al. 2017).

On the other hand nanotechnology is an emerging sector of nanoscale particles that has gained spectacular growth in recent decades (Gómez-Graña et al. 2017). Nanomaterials provide solutions to health and environmental challenges including medicine, public health, cosmetics, water treatment, packaging and biotechnology (Płaza et al. 2014). The green synthesis of metal nanoparticles is evolving an important

aspect of nanotechnology as a simple, eco-friendly and cost-effective substitute for the synthesis of metal nanoparticles (Das et al. 2016).

In the present study the larvicidal and pupicidal toxicity of biosurfactant Mannosylerythritol lipids (MELs) against Asian tiger mosquito *Ae. albopictus*, and the possibility of synthesizing silver nanoparticles using MELs as reducing and stabilizing agent were studied. The production and properties of the fabricated AgNPs were verified by UV–vis spectrophotometry, transmission electron microscopy (TEM), scanning electron microscopy (SEM), Fourier transform infrared (FTIR) spectroscopy, X-ray diffraction (XRD) and energy-dispersive X-ray (EDX) spectroscopy techniques.

5.2 Materials and methods

5.2.1 Chemicals and reagents

The crude MELs produced from the ustilaginomycetous yeast *Pseudozyma aphidis* ZJUDM34 were kindly provided by the Institute of food science, Zhejiang University, China, (Fan et al. 2014). Silver nitrate (AgNO3), was purchased from sigma Aldrich Chemical Corporation, china. SOD, POD, CAT and Bradford protein assay kits were purchased from Nanjing Jiancheng Bioengineering Institute, china, and all other chemicals, and reagents used were of the highest analytical grade purchased from local companies, Milli-Q grade water was used in all the experiments.

5.2.2 Synthesis and characterization of silver nanoparticles

In this study, the silver nanoparticles (AgNPs) were synthesized using Mannosylerythritol lipids (MELs) reduction method. The MELs were used as both reducing and stabilizing agents. Briefly 0.01 g of MELs in 1 ml of acetone were diluted with 10 ml of dechlorinated water. The pH value of the solution was adjusted to 7 using 0.1 M of sodium hydroxide solution and was dropped slowly into a 100 ml of 2 mM silver nitrate solution under vigorous string at a room temperature. The bioreduction

of Ag^{+} to Ag^{0} was indicated by the colour change and initially characterized by UV–vis. The UV spectra were recorded on a Shimadzu UV-2550 spectrophotometer at a 1 cm optical path quartz cuvette, in the range of 200–800 nm. Furthermore, the morphology and nanoparticle size was investigated by scanning electron microscope (SEM) using a Hitachi TM-1000 operated at an accelerating voltage of 10 kV. The presence of silver metal was also confirmed by the energy dispersive spectroscopy (EDS) instrument equipped with the SEM. While the structure of synthesized AgNPs were observed by the TEM using a JEOL - 1230 transmission electron microscope operated at an accelerating voltage of 300 kV. TEM samples were prepared by dispersing the powder AgNPs in methanol and sonication the colloidal solution followed by placing a few drops onto a carbon coated copper grid to make a thin film of the sample and then it was kept in a grid box sequentially.

Powder-XRD was done using Siemens X-ray diffractometer (XRD) analysis. The dried AgNPs powder was coated on a glass substrate and the X-ray diffraction measurement were carried out using Siemens X-ray diffractometer (XRD) analysis, operating at 45 keV and 20 mA current with Cu-Ka radiation as an Xray source in the range of 20–80 at the 2 h angle.

The functional groups and the bond type present in the fabricated AgNPs which caused to stabilize were determined with a Fourier transform infrared (FTIR) spectrometer (Vector 22, Bruker, Germany) in a range of 4000–500 cm^{-1}.

5.2.3 Insects rearing

The eggs of *Ae. albopictus* obtained from mosquito colonies were reared as described by Hassan ga'al *et al* (Ga'al et al. 2017a) in laboratory conditions (RT: 26 ± 2 ℃; 70–85% RH and 14:10 h (light/dark)). Different developmental stages of *Ae. albopictus* larvae and pupae were used for the bioassays.

5.2.4 Larvicidal and pupicidal bioassays

Larvicidal and pupicidal assays were carried as per procedure by WHO with some modifications (WHO 1996). Briefly Twenty-five individuals of first, second, third, and fourth instar larvae and pupae were separately kept in a 250-ml glass beaker containing 100 ml of dechlorinated water and 1 ml of desired concentration (20, 30, 40, 50 and 60 ug/ml) of MELs dissolved in acetone as solvent or (1, 2, 3, 4 and 5 ug/ml) of MELAgNPs dissolved in water. The control was setup by adding 1 ml of acetone with 100 ml of dechlorinated water or corresponding concentration of $AgNO_3$. Each concentration was replicated four times against all instars and pupae. Acute toxicity was noted after 24 h of exposure to the tested doses.

5.2.5 Preparation of enzyme extraction

The enzyme extraction was prepared with the method described by Ga'al *et al* (Ga'al et al. 2017a) briefly, the exposed and control larvae were homogenized in phosphate buffer (0.1 M, pH 7.2) under ice-cold condition. Then, the homogenates were centrifuged at 9,000 rpm and 4℃ for 20 min and the resulting supernatant was collected and stored at -80 ℃ for further enzyme analysis.

5.2.5.1 Protein assay

Sample protein concentrations were estimated by using the method described by Bradford. Bovine serum albumin was used for the calibration curve. Measurements were performed at 595 nm using a microplate reader Thermo Scientific Varioskan Flash.

5.2.5.2 Enzyme activity assays

The antioxidant activity of superoxide dismutase (SOD), peroxidases (POD) and catalase (CAT) were determined using commercial assay kits (Nanjing Jiancheng, Nanjing, China) following to the manufacturer's instructions.

SOD activity was determined by using xanthine and xanthine oxidase systems at 550 nm and expressed as U/mg protein. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the xanthine and xanthine oxidase system reaction in 1 ml enzyme extraction of 1 mg protein

POD activity was determined by catalyzing the oxidation in the presence of H_2O_2 of a substrate at 420 nm and expressed as U/mg protein. One unit of POD activity was defined as the amount that catalyses 1 mg substrate per minute per mg protein.

CAT activity was determined by monitoring the absorbance values of H_2O_2 at 405 nm and enzyme activity was expressed as U/mg protein.

5.2.6 Determination of AgNPs content in *Ae. albopictus* larvae by TEM

The amount of AgNPs accumulation in the body larvae was evaluated by TEM as descried by (Kovendan et al. 2016). Briefly, fourth instar larvae of *Ae. albopictus* with LC_{30} AgNPs treated and control were collected, anaesthetized and fixed in 2.5% gluteraldehyde in 0.1 M phosphate buffer (pH-7.2) for 24 h at 4C and washed 3-4 times with PBS each 20–45 min, then post fixed in 2% aqueous Osmium tetroxide for 1-2 h. They were later washed with deionized distilled water for 4–6 times each 30– 45 min, dehydrated in series of alcohols infiltrated and embedded spur resin. Samples were incubated at 80C overnight for complete polymerization. Ultrathin (50–70 nm) sections were made with a glass knife on ultra-microtome (LEICA EM UC7 ultratome), and then mounted on copper grids. Microtome sections were stained by uranyl acetate and alkaline lead citrate for 5 to 10min respectively Samples were subsequently observed under TEM (Hitachi Model H-7650 TEM. Japan).

5.2.7 Data analysis

LC_{50} and LC_{90} values, as well as 95% CL of Larval and pupal toxicity were calculated by probit analysis (Finney 1971). Data were analyzed using the SPSS

Statistical Software Package version 16.0. A probability level of $P < 0.05$ was used for the significance of differences between values. One way Analysis of Variance (ANOVA) and the means were separated using Tukey HSD test.

5.3 Results and discussion

5.3.1 Characterization of AgNPs

The synthesis of AgNPs was indicated by the change of color intensity of MELs solution before and after adding with AgNO3 solution from pale yellow color to brownish red color, confirming the reduction of Ag+ to Ag0 (Figure17a). The brownish red color could be due to the excitation of surface plasmon resonance (SPR) in the AgNPs (Govindarajan et al. 2016a, Kovendan et al. 2016). In the current study, the UV-Vis absorption spectrum of the AgNPs solution was observed at 430 nm indicating the formation of AgNPs, metal NPs have an SPR in the UV–vis region (Figure17b). This result evidenced that the AgNPs can be fabricated using MELs glycolipid as both reducer and stabilizer agent (Farias et al. 2014). In addition to that, the absorption spectrum of the synthesized AgNPs showed a single SPR peak indicating spherical shape of AgNPs (Ga'al et al. 2017a).

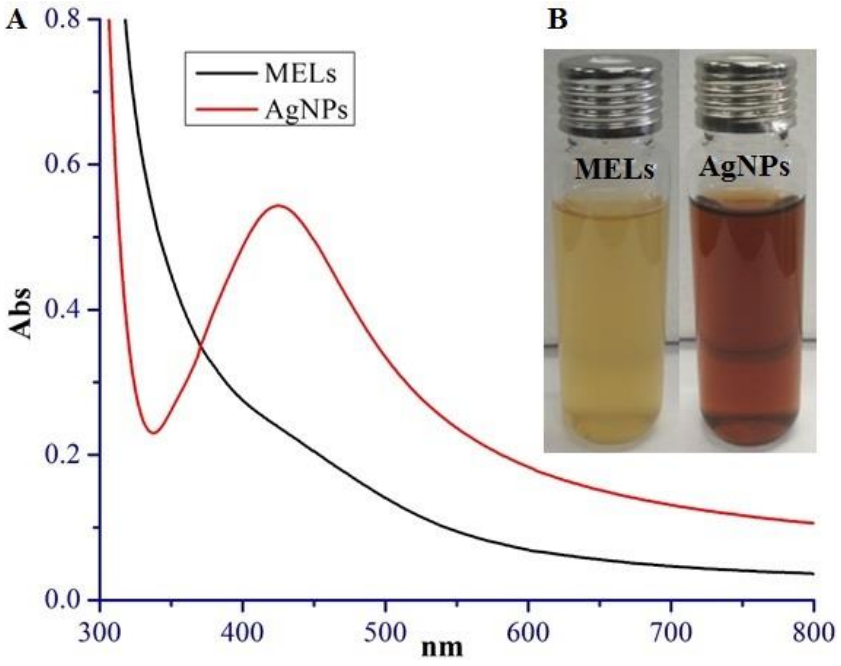

Figure. 17 (a) UV–Vis adsorption spectrum of AgNPs. The maximum absorption peak of MELs-AgNPs were 430 nm, after two hours from the reaction. (b) Color of the MELs solution and MELs-AgNPs solution.

Furthermore, the fabricated AgNPs was analyzed by the SEM in order to investigate the morphology and size distribution of AgNPs (Figure 18a). The SEM analysis displayed spherical nanoshapes with small sizes of AgNPs, similar results was reported by Giovanni Benelli *et al* (Benelli et al. 2017b). However, the EDS analysis in (Figure 18b) has confirmed the presence of a significant amount of silver element in our sample (Baláž et al. 2017).

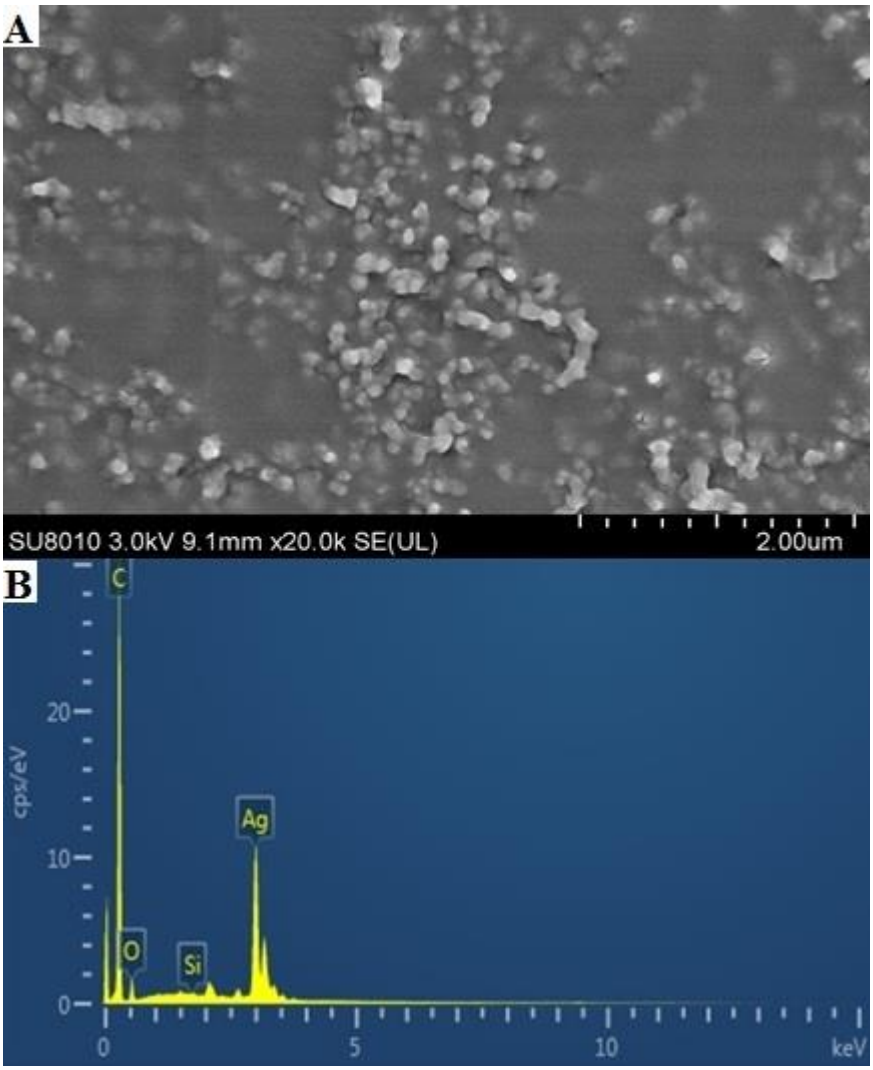

Figure. 18 (a) SEM images of synthesized AgNPs using Mannosylerythritol lipids (MELs) biosurfactant. (b) EDX patterns of synthesized AgNPs.

The TEM investigations of the synthesized AgNPs in (Figure 19a) also explored more details in the crystallinity shape and average sizes of the Ag NPs (Lee et al. 2018). XRD analysis of the MELs synthesized AgNPs in (Figure 19b) has demonstrated four characteristic peaks at 2θ region = 28.4°, 33.2°, 47.4°, and 56.3° which interpret to the corresponding lattice planes (111), (200), (220), and (311), respectively, these results affirms the crystalline and face centered cubic (fcc) structure of nanoparticles (Aarthi et al. 2017).

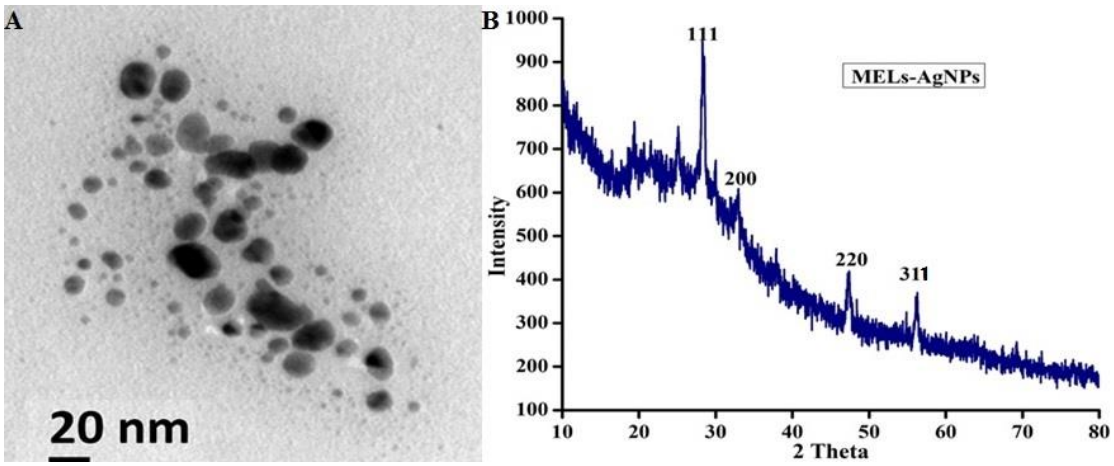

Figure. 19 (a) TEM images of synthesized AgNPs using Mannosylerythritol lipids (MELs) biosurfactant. (b) XRD patterns of synthesized silver nanoparticles by MELs.

The FTIR spectrum of the MELs-AgNPs is depicted in (Figure 20). and showed major peaks at 3337, 2923, 1742, 1562, 1344, 1093, 718 and 534 cm^{-1}. These bands confirmed the presence of different functional groups in the MELs Glycolipid which capped the AgNPs. The peak at 3337 cm^{-1} may be due to –OH stretching from polysaccharides (Kiran et al. 2010). The sharp band at 2923 cm^{-1} probably indicate to C–H stretching of Alkanes (Aarthi et al. 2017). Also the sharp and strong peak at 1562 cm^{-1} could be denoted by the carbonyl stretching vibration (Benelli et al. 2017a). The Peaks at 1466 and 1344 cm^{-1} can be assigned to (C–N) and (C–C) stretching vibration of aromatic and aliphatic amines, while the band at 1093 cm^{-1} could be assigned to (C–O) of an alkoxy groups (Ghramh et al. 2018). The peaks at 718 and 534 cm^{-1} for the CH_2 groups (Kiran et al. 2010).

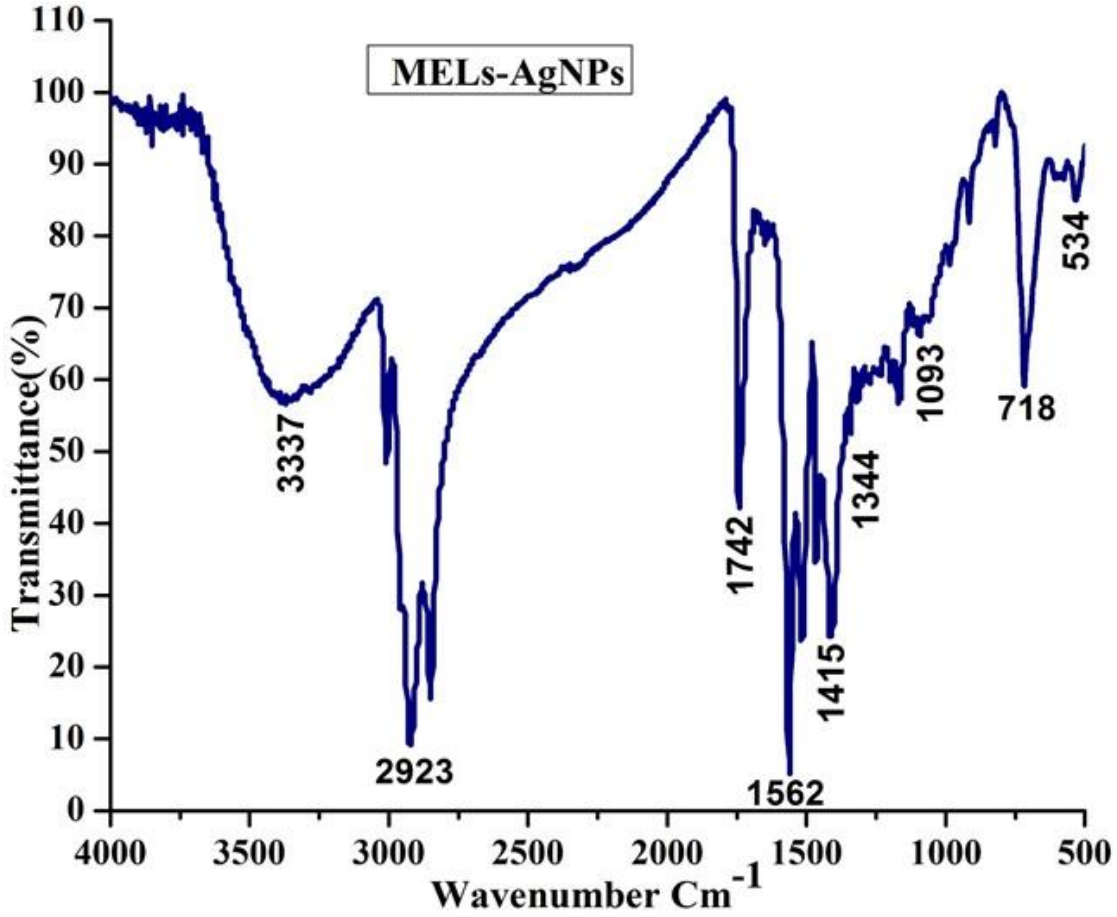

Figure. 20 Fourier transform infrared (FT-IR) spectrum of freeze-dried powder of silver nanoparticles synthesized using Mannosylerythritol lipids (MELs) biosurfactant.

5.3.2 Toxicity on *Ae. albopictus* young instars

The larvicidal and pupicidal activities of the MELs glycolipid biosurfactant and the synthesized AgNPs were showed in (Table 5). MELs glycolipid biosurfactant exhibited moderate toxicity on the larvae and pupae of *Ae. albopictus*. The LC_{50} values of MELs against 1st, 2nd, 3rd, 4th instar larvae and pupae of *Ae. albopictus* were 45 ug/ml, 55 ug/ml, 75 ug/ml, 90 ug/ml and 100 ug/ml, respectively. The results showed dose-dependent toxicity against larvae and pupae treated with MELs glycolipid. The mosquitocidal potential of MEL glycolipids biosurfactant has been scarcely investigated. However a few studies reported the use of other glycolipid biosurfactants

as biocontrol tools of mosquito invasion (Mnif and Ghribi 2016). The larvicidal efficacy of rhamnolipids biosurfactant produced by *P. aeruginosa* LBI 2A1 against *Aedes aegypti* larvae were reported by Silva *et al* (Silva et al. 2014). Also G. Prabakaran *et al* (Prabakaran et al. 2015), reported the pupicidal potency of di-rhamnolipids produced by *Pseudomonas fluorescens* Migula (VCRC B426) against *Culex quinquefasciatus*, *Anopheles stephensi* and *Aedes aegypti* mosquito vectors.

Table 5. Larval and pupal toxicity of Mannosylerythritol lipids and the synthesized silver nanoparticles (MELs-AgNPs) against chikungunya vector *Ae. albopictus.*

Tested compound	stage	LC_{50} µg/mL (95% LCL-UCL)	LC_{90} µg/mL (95% LCL-UCL)	Regression equation	$\chi 2$ (df = 3)	P-value
MELs	I	45(36.3-65.2)	524(202-13779)	y= -2.22+1.4x	1.62	0.65
	II	55(44.3-91.1)	552(217-10771)	y= -2.32+1.4x	0.93	0.82
	III	75(54.2-237)	1095(300-247423)	y= -2.16+1.2x	0.6	0.90
	IV	90(61.9-341.6)	1167(319-210395)	y= -2.62+1.4x	0.37	0.95
	pupae	100(67-434.9)	1231(333-213019)	y= -2.72+1.4x	0.06	0.99
MELs-AgNPs	I	0.14(0.04-0.37)	1.16(0.51-1.64)	y= 1.2+1.71x	0.52	0.91
	II	0.26(0,05-0.50)	1.69(1.21-2.20)	y= 1.0+2.00x	3.90	0.27
	III	0.27(0.05-0.53)	2.29(1.75-3.13)	y= 0.5+2.86x	7.67	0.05
	IV	0.38(0.12-0.64)	2.87(2.28-4.02)	y= 0.6+2.14x	5.85	0.12
	pupae	0.28(0.06-0.52)	1.89(1.41-2.46	y= 0.5+2.86x	1.95	0.58

LC_{50} = lethal concentration that kills 50% of insects. LC_{90} = lethal concentration that kills 90% of insects. LCL = lower confidence limit. UCL = upper confidence limit. $\chi 2$ = Chi-square value. df = degrees of freedom.

On the other hand, many researchers have been employed glycolipid biosurfactants for the biosynthesis of AgNPs and stabilization, bearing in mind the importance of developing eco-friendly and low-cost methods for the synthesis of AgNPs (Płaza et al. 2014).

The MELs-synthesized AgNPs were highly toxic even at lower concentrations against *Ae. albopictus* compare to MELs alone (Table 5). LC_{50} values were 0.14, 0.26, 0.27, 0.38, and 0.28 ug/ml for the 1^{st}, 2^{nd}, 3^{rd}, 4^{th} instar larvae and pupae of *Ae. albopictus*, respectively. Controls including testing Ag^{+} ions at the same concentrations

of the tested AgNPs recorded no mortality, in agreement with Marimuthu Govindarajan (Govindarajan et al. 2016a). Our results indicates that AgNPs increased significantly the bioactivity of MEL glycolipids biosurfactant against tested mosquito. Several previous researches have been demonstrated the larvicidal and pupicidal efficiency of green-synthesized AgNPs against various mosquito vectors (Pavunraj et al. 2017, Ghramh et al. 2018, Kovendan et al. 2018). For instance Palanisamy Mahesh Kumar *et al,* (Kumar et al. 2016) reported that AgNPs synthesized using the leaf extract of *Berberis tinctoria* are highly toxic against larvae and pupae of *Ae. albopictus*, with LC_{50} values ranging from 4.97 ppm (I instar) to 14.87 ppm (pupa). Also Hassan *et al* (Ga'al et al. 2017) highlighted the low doses of the AgNPs synthesized using *Aquilaria sinensis* and *Pogostemon cablin* essential oils were highly effective against *Ae. albopictus* larvae and pupae, with LC_{50} values ranging from 0.81 ppm (I instar) to 1.19 ppm (IV instar). We hypothesized that the considerable toxicity effect of MELs-synthesized AgNPs on *Ae. albopictus* larvae and pupae can be due to the small size of AgNPs, which allows penetration across the insect cuticle and incapacitated with physiological processes such as growth and molting (Kumar et al. 2016).

5.3.3 Effect of MELs glycolipid and MELs-AgNPs on the antioxidant enzymes of *Ae. albopictus* larvae

The effects of MELs glycolipid and MELAg-NPs on the total protein levels and antioxidant enzymes (CAT, SOD and POD) of forth instar larvae of *Ae. albopictus* were evaluated and the results are showed in (Figure 21). In normal conditions, there is a balanced regulation between the antioxidant enzyme system and the generation of reactive oxygen species (ROS) in the insect body (Li et al. 2014)**.** The total protein content of larval homogenate treated with MELs and MELs-AgNPs were investigated and compared to the untreated larval homogenate as shown in (Figure 21a). The total protein concentration of MELs-AgNPs exposed larvae was found to be significantly

decreased compared to the untreated larvae. Previously, several studies have investigated the effect of AgNPs on the total protein content in the mosquito vectors. For instance, Ga'al *et al* (Ga'al et al. 2017a) were found a decrease in total protein concentration in the fourth instar larvae of *Ae. albopictus* mosquito treated with AgNPs. Similar results were reported in the total protein contents of *Ae. aegypti* larvae which exposed to CNP, AgNP and CNP/AgNP nanocomposite (Solairaj and Rameshthangam 2017). In the present study, the depression of total protein levels in the MELs-AgNPs exposed larvae could be due to the toxic effect of reactive oxygen species (ROS) generated in the insect body upon exposure of MELs-AgNPs.

Our present report indicate that the treated samples with MELs and MELAg-NPs showed changes in the activity of CAT, SOD and POD in the larval body, these changes may be due to the presence of an efficient ROS scavenging system (Yasur and Rani 2015). As shown in (Figure 21c), CAT activity was significantly increased in MELs treated samples while it found to be significantly declined in MELAg-NPs treated samples compared to the control. CAT converts the toxic hydrogen peroxide to nontoxic molecules of molecular oxygen (O_2) and water (Parra Morales et al. 2017). Thus, the increase of activity in CAT upon treatment of MELs glycolipid could be happen in order to reduce the hydrogen peroxide toxicity among the larval body (Muthusamy and Rajakumar 2016). In contrast our study revealed that CAT activity was significantly decreased at MELAg-NPs treated samples as compared to control. The CAT activity decline of MELAg-NPs treated samples, may be due to the inhibition of CAT by the excess production and accumulation of ROS which may be caused by AgNPs (Zhang et al. 2017).

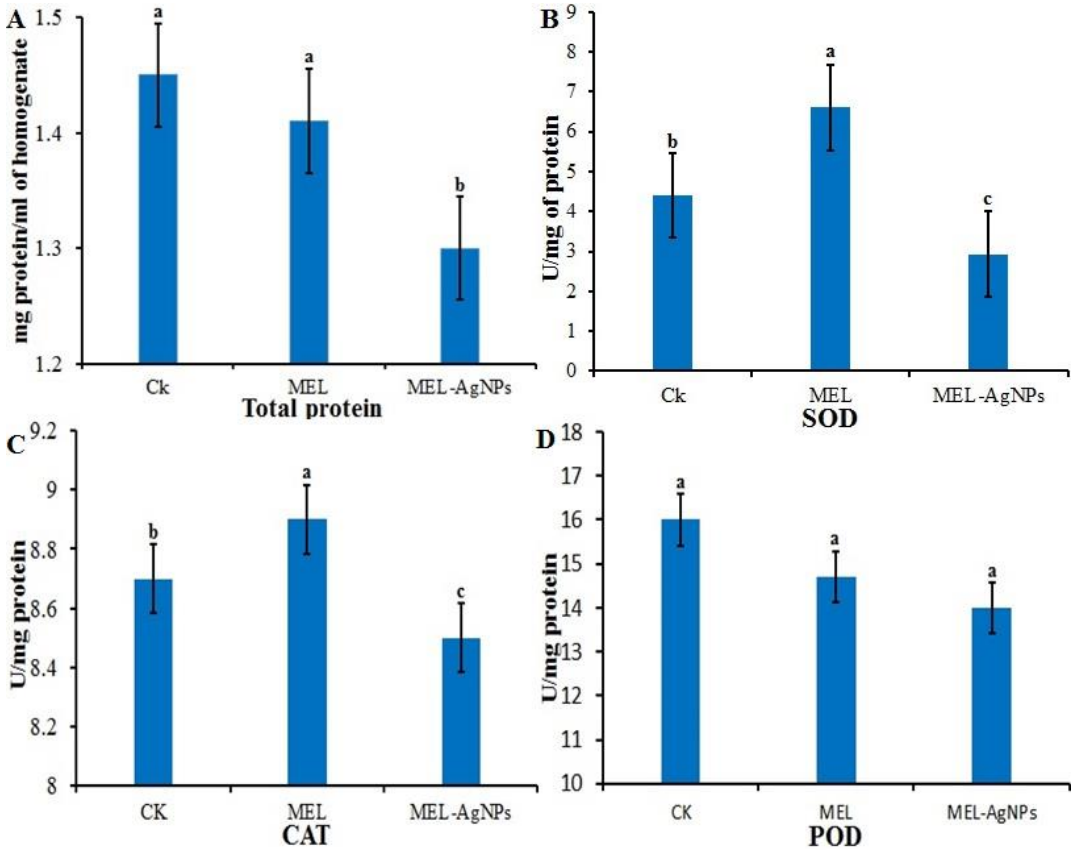

Figure. 21 effect of MELs and MELs-AgNPs on the antioxidant enzyme activities and total proteins. (a) Total protein concentration, (b) Superoxide dismutase (SOD), (c) catalase (CAT), (d) Peroxidases (POD). Each bar represents mean ± SE of four replicates using different preparations of larval homogenates.

The total SOD activity of the larval body which exposed to MELs and MELAg-NPs treatments had the same tendency as a CAT (Figure 21b), its activity significantly increased in MELs treated samples while it found to be significantly decreased in MELAg-NPs treated samples compared to the control. SOD enzyme is considered as the first enzyme of the detoxification process and catalyzes dismutation of superoxide anion (O_2^-) to form hydrogen peroxide (H_2O_2) which in turn is decomposed by the CAT. The noticed increase in SOD activity of this study may be due to the instant response of larvae to MEL glycolipids stress (Sezer and Ozalp 2015). The increase in SOD activity indicates high survival rate while low SOD activity affirms low survival rate

(Adamski et al. 2003). The current study also showed significantly decrease in the SOD activity of MELAg-NPs treated samples. The toxicity of the MELAg-NPs may be caused by the unbalance between the ROS and SOD enzyme activity, which might have resulted in inhibition of SOD activity (Wu et al. 2011).

In the present study, there were no significant differences in POD activity after exposure to MELs and MELAg-NPs treatments, compared with the control larvae (Figure 21d). However our results showed that POD activity was slightly decreased in MELAg-NPs treated larvae. The POD also catalyzes decomposition of H_2O_2 molecule into the nontoxic molecules by oxidation of co-substrates such as guaiacol or ascorbate as the electron donors (Liu et al. 2010, Zhang et al. 2017). Previously, it has been reported that the inhibition in the activity of the POD enzyme may be attributed to the presence of excess production of ROS (Yasur and Rani 2015). Also earlier reports showed that the susceptibility of antioxidant enzymes (SOD, CAT and POD) to different oxidative stress varies which resulting differences in antioxidant response of enzymes (Holovska et al. 1998).

5.3.4 AgNPs content in *Ae. albopictus* larvae by TEM

The LC_{30} exposure and control 4th-instar larvae of *Ae. albopictus* were used for TEM images to observe the AgNPs accumulation in the midgut cells of the larvae. Our investigations showed the microvilli structures in the midgut region (Figure 22a). Different ultrastructure of the cell organelles including mitochondria and other many vesicles can be seen in the micrograph. The ultrastructure of the cell organelles in the midgut of control and MEL glycolipid biosurfactant treated larvae exhibited normal cell characteristics with regular cell organelles content (Figure 22b, c). While the TEM images of the AgNP treated larvae explored accumulation of the Ag particles in the midgut cells as dark spots, pointed by red arrows (Figure 22d). The pictures of the

AgNPs treated larvae described the cytoplasm containing mitochondria, rough and smooth endoplasmic reticulum, and numerous of cell vesicles, it's distinctly reveals the localization and accumulation of the AgNPs in rough endoplasmic reticulum and other cell organelles such as nucleolus and mitochondria.

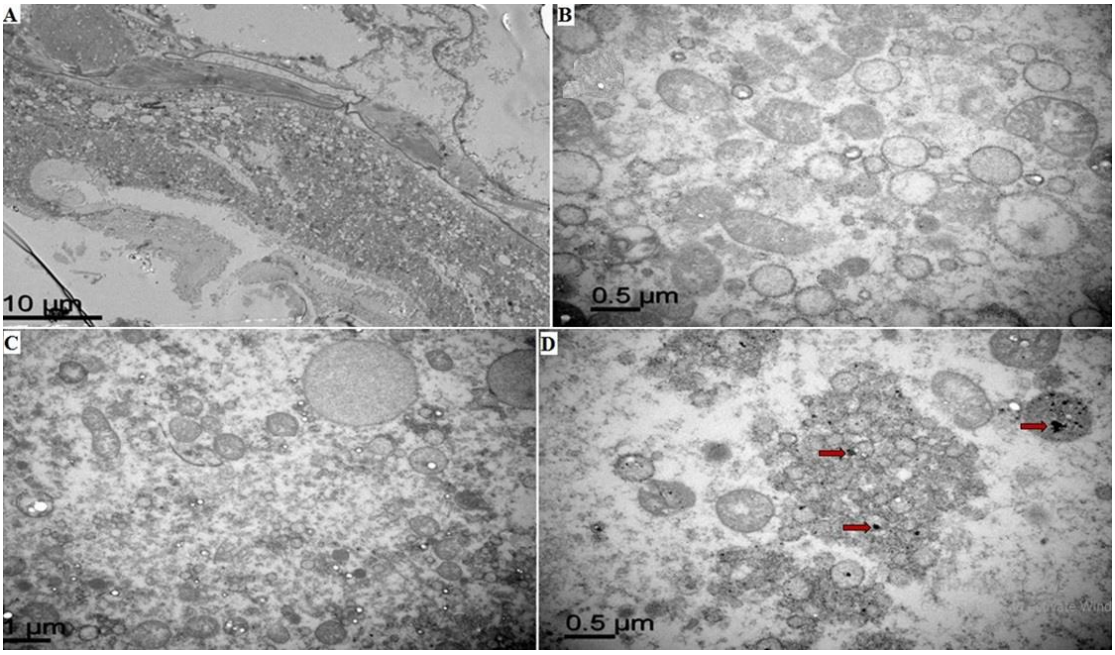

Figure. 22 TEM images of the MELs and MELs-AgNP treated larvae, beside untreated larvae. (a) Ultrastructure of the midgut in control larvae showing midgut microvilli. (b). Ultrastructure of the midgut in control larvae showing different cell organelles with normal characteristics. (c) Midgut of MELs treated larvae showing cell organelles including mitochondria, vacoules and vesicles. (d) Accumulation of AgNPs in the endoplasmic reticulum, nucleolus and other cell organelles of gut cells pointed by red arrows (dark spots).

Our results are in agreement with the findings of by Jyothsna Yasur and his colleague (Yasur and Rani 2015) which observed accumulation of the AgNPs in the in rough endoplasmic reticulum and some other cell organelles of the midgut cells of AgNP treated *A. janata* larvae. Similarly, earlier studies were reported gold nanoparticles accumulation in the rough endoplasmic reticulum and cell vesicles of treated *Drosophila melanogaster* (Pompa et al. 2011). Furthermore, Shu *et al* (Shu et

al. 2012) were observed alterations in the midgut cells and its ultrastructures in the cutworm *Spodoptera litura* larvae after feeding with Zn diets. From the TEM larval images, it can be deduced that amounts of absorbed AgNPs by the midgut cells are localized and accumulated in the cells organelles which could lead to detrimental effects including DNA damage, genotoxicity, mitochondrial dysfunction, altered cell morphology, and subsequently cell death by necrosis or apoptosis (Zhang et al. 2014a).

5.4 Conclusion

In the present study, we fabricated AgNPs by employing green mediated route as eco-friendly and cost-effective synthetic method. The synthesized AgNPs revealed larvicidal and pupicidal efficiency against *Ae. albopictus* mosquito even at low doses. TEM images described the localization of AgNPs and its accumulations in the midgut cell ultrastructures which may interfere the physiological activity of the midgut cells of mosquito larvae. Further studies are required to characterize the interaction pathway between AgNPs and the cell organelles including mitochondria, nucleus and cell vesicles.

Chapter 6. General conclusions

According to our laboratory study we can conclude the following outputs:

- The nanotechnology revolution in the recent years attended a significant interest as a source of eco-friendly insecticide formulation. The highly insecticidal efficiency of formulated nanoparticles and low amount of active ingredients are unique properties making them a promising strategy for pest management practices.
- The facile integration of AgNPs into different media and the flexibility of synthetic approaches as well as their unique chemical properties (i.e. size and shape) have gained remarkable interest for the researchers and encouraged to investigate the action mode aspects of insecticidal effects of AgNPs.
- The use of conventional pesticides in vector control strategy such as synthetic chemical insecticides have faced a number of challenges including resistance development at a wide range of mosquito species, an increasing environmental deterioration and soil disintegration. Thus the green approach mediated AgNPs formulations may overcome these challenges, and provide potential alternative for mosquito vector control without nature deterioration.
- Our study reported that *Aquilaria sinensis* and *Pogostemon cablin* essential oils are eco-friendly agents and convenient sources for green-mediated synthesis of AgNPs.
- This study suggested that fabricated AgNPs using *Aquilaria sinensis* and *Pogostemon cablin* essential oils showed high mosquitocidal efficiency. Thus they are considered as a promising instrument for vector management.
- The mode effect of AgNPs in the fourth instar larvae of *Ae. albopictus* was further examined by microscopic investigations of the larval midgut. These

microscopic analysis of AgNPs-treated larvae exhibited severe damage in the midgut epithelial cells, such as the destruction of epithelial cells and degradation of nuclei which may lead to the larval death.

- Data presented in this research have also suggested the employment of other natural resources including plant originated compound Salicylic acid and its derivative Dinitrosalycilic acid as an environmentally friend and potential reducing and stabilizing agents of AgNPs.
- The fabricated AgNPs by salicylic acid and its derivative dinitrosalicylic acid have revealed a strong mosquitocidal efficacy against Asian tiger mosquitoes. Further analysis on the activity of detoxifying enzymes including Esterases, Acetylcholine esterase, Phosphatase enzymes as well as total proteins in the fourth instar larvae of *Ae. albopictus* showed to be significantly affected by the synthesized AgNPs. Therefore, the employ of AgNPs as insecticide strategy may provide an efficient solution of insecticide resistance challenge in the vector mosquitoes.
- Our study also employed Mannosylerythritol lipids biosurfactant produced from the ustilaginomycetous yeast *Pseudozyma aphidis* as renewable source for the fabrication of AgNPs in one-step, rapid and cost-effective synthesis manner.
- The synthesized AgNPs by the glycolipid biosurfactant exhibited an extremely low LC50 values against treated larvae and pupae of *Ae. albopictus* mosquito. The investigation of the treated larval body by the TEM microscope was observed intracellular presence of the AgNPs, which may cause DNA replication damage, genotoxicity, mitochondrial dysfunction, and consequently cell death.

- In conclusion, the different conventional chemical and physical reduction methods for synthesis of metal NPs basically depend upon the employment of high toxic compounds which limit their applications.
- The biologically-mediated synthesis method of metal NPs is beneficial over physical and chemical routes, since it is cost-effective, single-step, rapid and does not require high temperature, pressure, energy, and also does not use high toxic chemicals.
- With the recent advance and the ongoing endeavor for improving NP synthesis efficiency and optimizing their pesticidal application, the implementation of the green synthesis approaches on a wide range and their production as a commercial tools in integrated pest management and mosquito vector control, will be highly beneficial to solve current challenges in this field including insecticide resistance and environmental deteriorations.

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