Adulticidal and Antimicrobial Potential of *Citrus aurantifolia* Fruit Peel-Derived Bioinsecticides against *Aedes aegypti* Linnaeus (Diptera: *Culicidae*) and Pathogenic Microbes

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Received: 12 September 2025; Accepted: 2 February 2026

ABSTRACT

Mosquito-borne diseases pose significant public health challenges, necessitating effective control strategies. Given the health risks associated with synthetic repellents, natural adulticides are crucial for mosquito management. This study investigated various form of *Citrus aurantifolia* Fruit Peel (CAFP)- derived bioinsecticides, including plant extract, essential oil, and silver nanoparticles (AgNPs) for its adulticidal against adult *Aedes aegypti*, as well as its antimicrobial property. WHO topical bioassay was used to test the efficacy of these adulticides, while histological analysis further assessed their toxic effects. The effect of LC_{90} of these adulticides were screened against *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans* using disc diffusion method. All bioinsecticides demonstrated adulticidal activity against *Ae. aegypti*. Overall, the essential oil from the peel of *Citrus aurantifolia* showed prominent effect with LC_{50} and LC_{90} value of 11.82 % and 23.41 %, respectively, followed by AgNPs- CAFP with 11.28 % and 30.99 %, and lastly was CAFP- plant extract at 14.51 % and 31.19 %. The order of potency was as follows: essential oil > silver nanoparticles > plant extract. Histological abnormalities observed in thoracic muscles of treated mosquitoes explains the toxicity and subsequent mortality caused by these insecticides. Essential oil showed promising inhibitory activity against the bacterial strains, whereas AgNPs were effective in inhibiting fungi. These findings pave the way for the development of novel herbal intervention, optimizing the most effective forms for controlling dengue vectors. Moreover, the demonstrated antimicrobial activity of these bioinsecticides further supports their other potential as multi-purpose agents in public health management.

Keywords— nanoparticles; essential oil; plant extract; mosquito; histology

1.0 INTRODUCTION

Mosquitoes are among the deadliest creatures on earth that threaten health hazards in humans, posing nuisance problems and mechanically transmitting pathogens causing diseases. One of the most prominent vectors among these is the *Aedes* spp., which is in charge of spreading deadly diseases including dengue, Zika, and chikungunya. These mosquitoes, particularly *Ae. aegypti* and *Ae. albopictus* are difficult to control as they can grow their populations quickly and flourish in urban areas. The diseases they transmit place an immense burden on healthcare systems worldwide, leading to millions of cases and thousands of deaths each year. At present, there is no vaccine effectively safeguard against mosquito-borne illnesses, with the exception of yellow fever and Japanese encephalitis [1]. Thus, mosquito vector control was considered the best strategy for preventing disease. There are several strategies for controlling and managing mosquito vectors, but chemical control is the strategy that has been used worldwide and extensively in everyday life.

Although chemical insecticides applications were intensified are considered as a successful vector control, yet, their frequent application has negatively impacted human health, non-target organisms, and the emergence of resistance. The extensive use of pyrethroids inevitably elicit levels of resistance in *Ae. aegypti* [2]. Early detection of resistance could assist authorities in deciding well-suited control strategies to minimize operational failures of *Ae. aegypti* control. Hence, there is a need to find alternative adulticides from plant-based options which are effective, safe, eco-friendly, biodegradable, non-toxic to humans and inexpensive as an alternative to chemical insecticides.

Plant extracts and essential oils have shown dominant activity against mosquitoes and other insect pests as claimed by most of the literatures. They are highly promising as safe alternatives to chemical insecticides. Plant extracts and essential oils are safe for human health and the environment, as they have been declared to be low-risk active substances by European Food Safety Authority (EFSA) [3]. They do not pollute the environment but rapidly degrade in soil and water. Moreover, it is difficult for mosquito vectors to develop resistance towards them [4].

Citrus aurantifolia (Christm.) commonly known as lime, is a small evergreen tree belonging to the Rutaceae family. Its fruit, known for its tart flavor, is widely used in culinary applications, beverages, and traditional medicine. The fruit peel, in particular, is rich in essential oils that contain bioactive compounds such as limonene, flavonoids, and terpenes, which have been found to possess various biological activities, including insecticidal, antimicrobial, and antioxidant properties [5,6,7]. These characteristics make *C. aurantifolia* an attractive candidate for use in pest control and other therapeutic applications.

Despite the growing body of literature highlighting the efficacy of various *Citrus* spp., particularly in the context of insecticidal properties, few of the research has primarily focused on the use of *Citrus aurantifolia* Fruit Peel (CAFP) essential oil as an adulticide. A critical gap remains in comparing the efficacy of *C. aurantifolia* Fruit Peel in various forms of adulticides against adult *Ae. aegypti* including the new rising adulticides in the form of silver nanoparticles (AgNPs) synthesized using this peel. Furthermore, the application of AgNPs synthesized using *C. aurantifolia* Fruit Peel against adult mosquitoes using standardized methods such as the WHO topical bioassay has not yet been explored in the existing literature.

In view of this, the present study seeks to address this knowledge gap by comparing the efficacy of various adulticidal forms derived from *C. aurantifolia* Fruit Peel (CAFP), including essential oil, plant extract, and silver

nanoparticle, using the WHO topical bioassay. To the best of our knowledge, this is the first study to evaluate the efficacy of AgNPs synthesized using plant based against adult mosquitoes, focusing using WHO topical bioassay method, as well as the histotoxic assessment. Additionally, this study is also the first study to directly compare these three forms of *C. aurantifolia* as adulticides, namely the plant extract, essential oil and silver nanoparticles, by providing novel insights into their relative effectiveness and potential applications in mosquitoes control. Thus, the study aims to conclude which adulticides are most effective in controlling *Ae. aegypti* adults, whether in the form of plant extract, essential oil or AgNPs. Additionally, the study evaluates their antimicrobial efficacy against pathogenic microbes. This comparison will not only clarify the most potent form but also provide valuable guidance for the development of sustainable pest control strategies, including broad-spectrum insecticides or also known as multipurpose agents, effective against both insect and microbes.

2.0 MATERIALS & METHODS

Extraction of aqueous fruit peel extract and essential oil

The fruit peels of *C. aurantifolia* was purchased from Chemical Engineering Pilot Plant (CEPP), Universiti Teknologi Malaysia, Malaysia, in the form of extract powder. Voucher specimens (KM 0115/24) were deposited in Institute of Bioscience, Universiti Putra Malaysia and authenticated by the Biodiversity Unit, Dr. Khairil Mahmud. 1 Gram of fruit peel powder was mixed with 100 mL of ultra-pure water and stirred. The mixture was boiled for 20 min at 60°C. The suspension thus obtained was cooled, filtered through Whatman filter paper No. 1 (25 µm). The extract was then freeze-dried using a Freeze Dryer (Labconco, USA) to obtain a powder form, for easier preparation of various concentrations to dilute with the solvent. It was stored under refrigeration for future use. The essential oil was extracted from the same batch of peel material by The Element Sdn. Bhd. (Kedah, Malaysia) using an automated cold-pressing machine.

Biosynthesis of silver nanoparticles

Silver nanoparticles used in this study were synthesized following the optimized protocol reported in our previous study [8]. The concentration of 4 mM aqueous solution of $AgNO_3$ was prepared using ultra-pure water and used for the synthesis of silver nanoparticles with the help of CAFP as a stabilizing and reducing agent. 10 mL of this extract was mixed with 30 mL of 4 mM of $AgNO_3$ solution, and kept in a water bath (70 °C; 4h). The formation of AgNPs then was confirmed as the colour changes into reddish brown colour. Then, AgNPs were centrifuged at 40,000 rpm for 15 min and washed to discard clear supernatant solution (to remove unwanted metabolites or so-called unbound plant extract residue). The pellet was then obtained and dried using a Freeze Dryer (Labconco, USA), forming a powder. The physicochemical characterization of the synthesized AgNPs, including UV–Vis spectroscopy, FTIR analysis, and FESEM imaging, has been previously reported in our earlier study [8]. In the present work, additional analyses including Dynamic Light Scattering (DLS) and High-Resolution Transmission Electron Microscopy (HR-TEM) were performed to further characterize the nanoparticles used in this biological evaluation.

High resolution transmission electron microscope (HR-TEM)

The sizes and morphology of the synthesized AgNPs were analyzed using a High-Resolution Transmission Electron Microscope (HR-TEM). The morphological analysis of the synthesized AgNPs was carried out following the methods described previously [43], with a few modifications. A dried powder of AgNPs was prepared by dispersing about 2

mg of the powder in acetone and ultrasonicating it for 5 min. The sample was placed on a copper grid coated with carbon film and dried at room temperature for 15 min. The HR-TEM measurements were performed on a F.E.I Technai G2 F20S-TWIN (Hillsboro, United States). The average diameter and shapes of AgNPs were determined from the HR-TEM micrographs, while the distribution of the nanoparticles was determined by counting the approximate number of nanoparticles on the micrograph. The photomicrograph obtained was analyzed using Image J (version 1.5 NIH) and Origin Lab software (Version 2022). The results of the average size and distribution of the nanoparticle were presented in a histogram.

Dynamic Light Scattering (DLS) and Particle Size

The average particle size, distribution, and zeta potential (ZP) of synthesized AgNPs were determined using a Zetasizer (Nano ZS-90 Malvern Instruments, Malvern, United Kingdom). The analysis was conducted following the method previously described [44], with some modifications. The synthesized AgNPs powder aliquot was dispersed in 15 mL of deionized water and sonicated for 15 min. Three millilitres of the solution were filtered through a 0.20 µm pore-sized syringe, and then the solution was analyzed. The average particle size distribution and ZP were determined by measuring the dynamic fluctuation of the light intensity of the colloidal particles. The measurement of the average hydrodynamic diameter of the particles, peak values in the hydrodynamic diameter distribution, and polydispersity index (PDI) were recorded and analyzed using Zeta Sizer software (version 7.11). All measurements were carried out in triplicate with a temperature equilibration time of 1 min at 25 °C. The results were presented in the graph showing the peaks and values.

Insecticides preparation

All form of *Citrus aurantifolia*-based adulticides such that the plant extracts, essential oil, and silver nanoparticles were formulated into the same concentrations viz: 0.5%, 5%, 10% and 18% for ease of the comparison. The CAFP- plant extract was prepared by combining with 0.1% acetone as a solvent or diluent to obtain the desired concentration. Similar to the preparation of CAFP- plant extract, the essential oil was mixed with 0.1% acetone, but with an additional one drop of Tween 80 as an emulsifying agent, preventing the oil from separation phase. Meanwhile for AgNPs-CAFP, this formulation was prepared by agitating the silver nanoparticle powder with 0.1% acetone using a sonicator (Clifton, United Kingdom).

Mosquitoes rearing

An established laboratory colony of *Ae. aegypti* (susceptible strain) which originated from Institute for Medical and Research (IMR) of Malaysia were reared under controlled conditions at the insectarium of Department of Medical Microbiology, Universiti Putra Malaysia. The conditions were maintained at 27 ± 1 °C, with 75 ± 5% humidity under light 12:12 h (day and night). Nulliparous 3-5 days old, non-blood-fed adult female Ae. aegypti mosquitoes were used for testing the effects of CAFP- plant extract, CAFP-essential oil and AgNPs- CAFP.

Topical application bioassay

The process of WHO Topical Application Bioassay was based on the recent study with slight modifications [9]. Female adult *Ae. aegypti* mosquitoes were aspirated out of the cage in groups of 25 and subsequently transferred into individual plastic cup designated for each of the concentration. These cups were then securely enclosed with netting and placed in a freezer (0 °C) for precisely 5 min 30 seconds to induce a state of stun through temperature shock. This duration

significantly influenced the mosquitoes' condition, as exposure for less than 6 min at 0 °C resulted in a brief recovery period, while exceeding 6 min led to thermal mortality. After the mosquitoes were sufficiently knocked down, each cup of mosquitoes was weighed to the nearest 0.1 g precision using an analytical scale. The mosquitoes were immediately transferred onto a Petri dish lined with filter paper and positioned on ice to ensure their immobilization. The entire procedure for transferring mosquitoes used an insecticide- free paintbrush, instead of forceps, aiming to minimize stress and physical damage to the insects which can lead to bias in result. Mosquitoes were thereafter dosed with 1 µl of control or adulticide solutions using a Multipette® Plus (Eppendorf, Germany). After dosing, the mosquitoes were poured back into their respective plastic cups, provided with 10% sucrose solution. Mortality was assessed 24 hr later. At the end of a 24 hr recovery period, the mosquitoes were considered dead if they showed no sign of movement such as lying on the bottom of the plastic cup and not responding to mechanical stimulation. Six replicates for each form of adulticides were conducted from different rearing batches. All treatments were treated in a similar manner in all forms of adulticides including the controls. Percent mortality was corrected by using Abbott's formula. Permethrin was used as a positive control, while for the negative control, 0.1 % acetone and 4 mM of (corresponding to the concentration used during nanoparticle synthesis) were employed. These dead mosquitoes from the treatments and controls were then collected for further histological and microscopy analysis.

The percentage mortality was calculated thus:

$$\text{Percentage mortality} = \frac{\text{Total number of dead mosquitoes}}{\text{Total number of mosquitoes}} \times 100$$

Histological analysis

The dead adult *Ae. aegypti* mosquito was preserved in 10% formaldehyde for histological analysis 24 hr post testing with the treatments. The samples were then processed using an overnight automatic tissue processor (Leica TP1020, German) with a succession of ethyl alcohol and washed with xylene. It was then embedded in paraffin wax using histoembedder tissue (Leica EG1160, German). They were trimmed and sectioned using a microtome (Leica RM2235 cwEU, German) at a thickness level of 20 um and 4 um respectively. All tissue blocks should be stored in the freezer before trimming and sectioning (Fig. 1). Trimming and sectioning should be performed immediately after removing the blocks from the freezer to ensure the tissue remains intact and does not become shredded. After sectioning, the paraffin ribbon was floated onto the surface of a warm water bath for fishing technique. The tissue then was air-dried and stained using standard technique of Hematoxylin and Eosin (H&E) staining with an automated slide stainer (Tissue-Tek Prisma®Plus Sakura, Japan). Following this, examination of tissue was conducted using a light microscope (Olympus CX33, Japan) at 600X magnification and photomicrographs were taken using a digital camera.

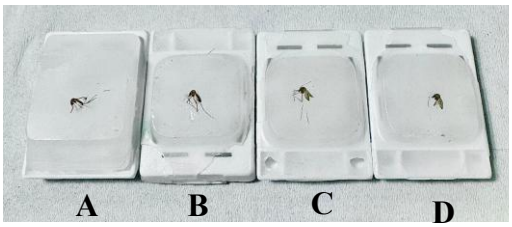

Fig. 1: Tissue blocks of adult *Ae. aegypti* after treatment with various form of adulticides after trimming process using microtome. (A) Negative control (B) Treatment with CAFP- plant extract (C) Treatment with CAFP- essential oil (D) Treatment with AgNPs- CAFP.

Antimicrobial disc diffusion assay: growth inhibition

A disc-diffusion assay was carried out according to the National Committee for Clinical Laboratory Standards Guidelines and previous study with small modifications [6,42]. he disk diffusion assay was conducted to assess the antimicrobial activity of various forms of CAFP, namely, plant extract, essential oil, and AgNPs against common microorganisms, including *Staphylococcus aureus* ATCC 27853 (a Gram-positive bacterium), *Escherichia coli* ATCC 25922 (a Gram-negative bacterium), and *Candida albicans* ATCC 60193 (a fungus). A representative microorganism from each category was selected to assess antimicrobial activity according to the LC_{90} value concentration for each treatment. The bacterial and fungal inoculum was prepared from an overnight culture on Mueller Hinton Agar (HiMedia, India) and Sabouraud Dextrose Agar (Oxoid®, United Kingdom) respectively. Colonies were directly suspended in 0.90% sterile saline solution to achieve a turbidity equivalent to the 0.5 McFarland standard (approximately 1.5x 10^8 CFU/ml), and this was compared with a commercialized McFarland standard. Aliquots (100 ul) of the suspensions were spread over the agar surface using a sterile cotton swab in order to get a uniform microbial growth. Under aseptic conditions, sterile paper discs (Whatman No. 1, 6 mm in diameter) were placed on the agar surface and immediately impregnated with 10 µL of LC_{90} concentration of the respective treatments. Blank paper discs (Liofilchem, Italy) were used as the negative control. As for reference control, Ciprofloxacin antibiotic disc (Oxoid®, United Kingdom) was used for the *S. aureus* and *E. coli*, whereas Fluconazole (Oxoid®, United Kingdom) antifungal disc was used for the *Candida albicans*. The plates were incubated at 37˚C for 24 hr for bacterial strains and 25 ˚C for 2 days for fungi, after which zones of inhibition were observed, measured and interpreted. After incubation, the inhibition zones were measured in mm. All experiments were carried out in six replicates and the mean ± SD values were presented. The scale of measurement was as follows: no inhibition zone < 12 mm, moderate inhibition zone 12- 20 mm and strong inhibition≥ 20 mm (disc diameter included) and. It is important to note that a fresh stock suspension was prepared each time, and all experimental conditions mentioned above were maintained constant under aseptic conditions.

Statistical analysis

Data were presented as means ± standard deviation (SD) of six replicates. Mean adult mortality and the effect of concentrations were calculated using the software IBM® statistical package for social sciences (SPSS) version 29, with a probit regression analysis for calculating LC_{50} and LC_{90} at a 95% confidence limit, and other statistics such as upper confidence limit (UCL) and lower confidence limit (LCL) also were obtained. To determine the most effective adulticides in vary concentrations, two-way ANOVA followed by a post hoc, Tukey test to determine the differences between each of the treatments.

3.0 RESULTS AND DISCUSSION

High-Resolution Transmission Electron Microscope (HR-TEM)

The High-Resolution Transmission Electron Microscope (HR-TEM) micrograph (Fig. 2) showed the size, shape, and distribution of biosynthesized AgNPs. The micrograph indicates nearly all the biosynthesized AgNPs were spherical in shape and the size of the particles ranges between 7.08- 28.15 nm, with an average mean particle size of 18.22 ± 5.43 nm. The HR-TEM micrograph (Fig. 3) showed spherical-shaped biosynthesized AgNPs at different magnifications of x25,000 and ×100,000. This is consistent with previous

findings that used plant extract in the synthesis of AgNPs [43, 45].

extract for AgNPs synthesis found that the AgNPs displayed spherical shape with size

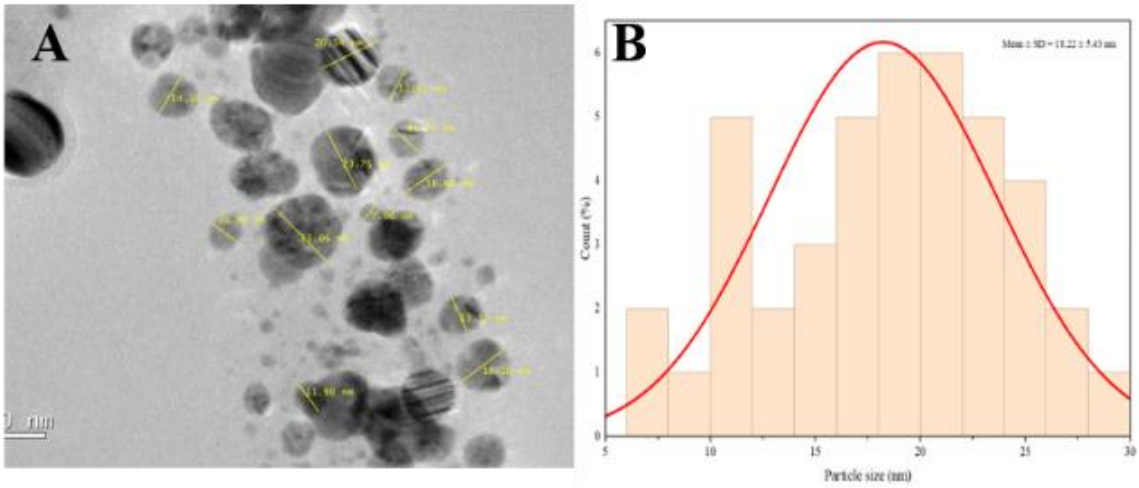

Fig. 2: (A) HR-TEM micrograph of AgNPs showing scattered, and spherical shape nanoparticles (B) HistoGram of average particle size of AgNPs (×100, 000 magnification).

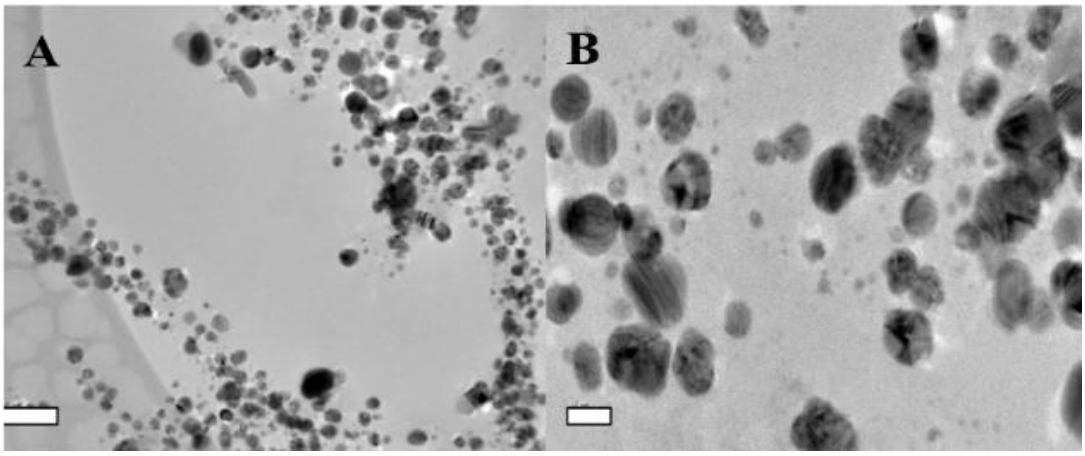

Fig. 3: HR-TEM micrograph of biosynthesized AgNPs showing spherical shaped nanoparticle under two different magnifications (A) ×x25,000 (B) × 100,000

Consistent spherical morphology has also been widely reported for AgNPs synthesized using *Citrus* peel extracts across different *Citrus* spp.. For instance, it is reported AgNPs synthesized using the peel extract of *Citrus tangerine* that is spherical, with average sizes of 10-70 nm [46].

Similarly, HR-TEM and FESEM from biosynthesis of AgNPs using *Citrus sinensis* peel extract revealed that the AgNPs are polydispersed and spherical in shape, with an average size of 10 ± 1 nm for the synthesis at 60 °C [47]. Likewise, a study employing *Citrus grandis* peel ranging from 20-30 nm [48]. Although AgNPs synthesized from different *Citrus* spp. consistently exhibit spherical morphology, variations in this particle size can be attributed to differences in synthesis parameters such as reaction temperature, plant extract concentration, $AgNO_3$ concentration, and pH [49]. These factors influence nucleation and growth processes during green synthesis, resulting in size variation when similar *Citrus* spp. peel extracts are employed.

Dynamic Light Scattering (DLS) Analysis

Dynamic light scattering (DLS) was employed to analyse the polydispersity index (PDI), hydrodynamic size, and zeta potential of AgNPs. The PDI serves as a key indicator of nanoparticle size distribution uniformity [50]. It provides insight into the degree of size variation within a sample, where values below 0.1 indicate a highly monodisperse system, while those above 0.4 reflect significant polydispersity. A PDI within the range of 0.1 to 0.4 represents a moderately polydisperse nanoparticle population [51].

In this study, the PDI values of AgNPs- CAFP was recorded as 0.665, demonstrating a highly polydisperse (Fig. 5).. This high PDI value signifies greater heterogeneity in particle size distribution. Thus, this finding is in line with the result obtained in the UV-Vis and HR-TEM micrograph, where the broad peak of surface plasmon resonance peak observed in the UV–Vis spectrum suggests polydispersity, and the HR-TEM micrographs reveal nanoparticles with slightly varied sizes.

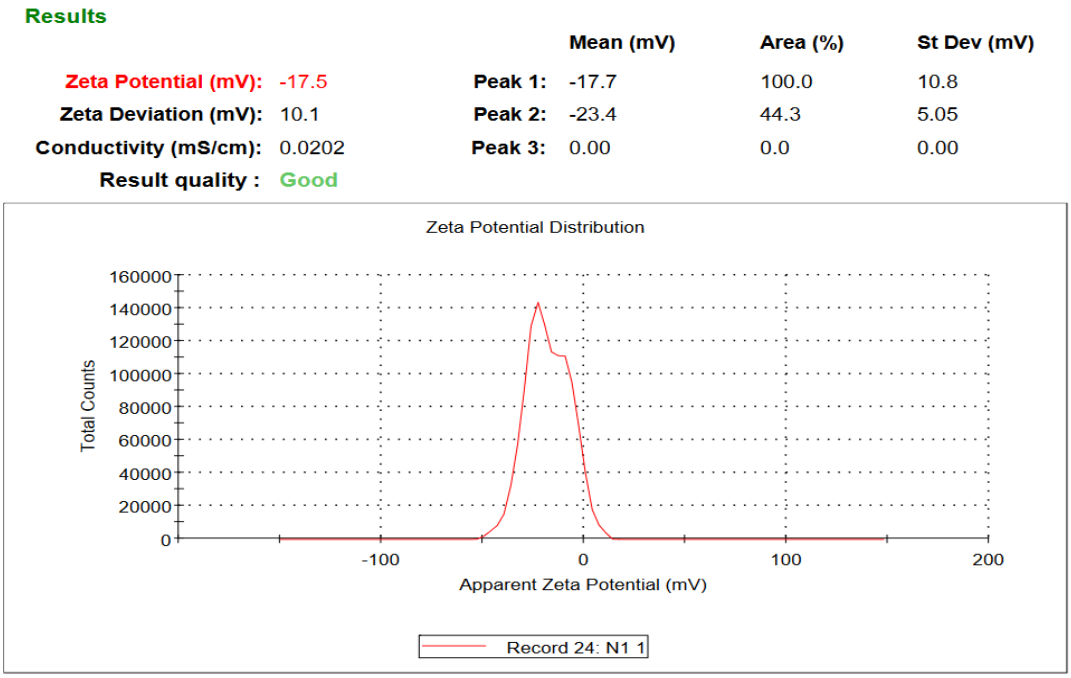

Fig. 4: Zeta Potential (ZP) of the AgNPs biosynthesized using CAFP extract

The possible reasons for the discrepencies observed in sizes of AgNPs could be method of synthesis used, variations in phytochemical compositions and uncontrolled nucleation, which can lead to the formation of AgNPs with diverse size distributions [52]. In fact, many studies point out that AgNPs produced using plant extracts often show a broad size distribution (polydisperse) due to the complex mixture of phytochemicals in the extract acting as reducing and capping agents [53, 55]. Hence, a PDI value below 0.7 is generally considered acceptable for plant- mediated AgNPs associated due to the complex plant extracts [56].

This finding is in line with several studies using *Citrus* peel in AgNPs synthesis. For instance, *Citrus unshiu* peel-mediated AgNPs reported a polydispersity (varied and broad size distribution), with a relatively high PDI value of 0.578 [57]. However, study using *Citrus microcarpa* peel extract which yielded a much low PDI value of 0.363, reflecting moderate polydisperse of AgNPs [58]. Similarly, *Citrus sinensis* peel–mediated synthesis of

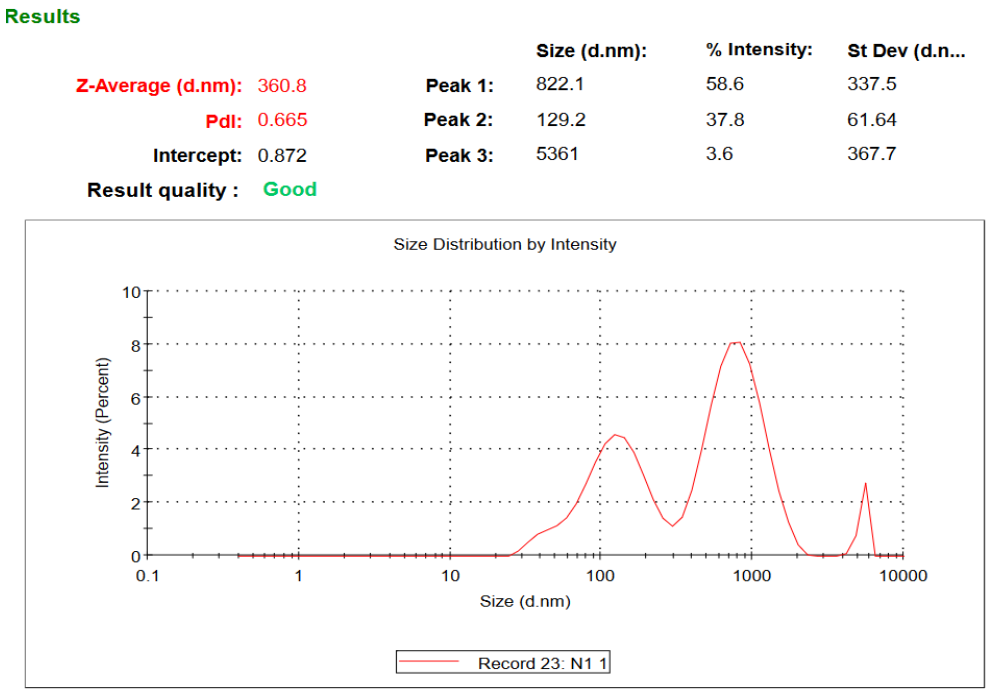

Fig. 5: Particle size distribution by intensity of AgNPs biosynthesized using CAFP extract

AgNPs yielded a PDI of 0.497 in DLS characterization, indicating moderate polydispersity [59]. Although these studies report different PDI values across *Citrus* spp., all values remain below 0.7, supporting the good quality of AgNPs which were commonly observed in plant-mediated AgNPs biosynthesis [56].

PDI remains a crucial parameter for evaluating nanoparticle uniformity and its implications for functional performance [60]. While monodisperse nanoparticles are often preferred for drug delivery systems and biomedical applications, polydisperse AgNPs on the other hand been reported to exhibit enhanced insecticidal activity. This enhanced efficacy is attributed to the presence of nanoparticles with varied sizes that can act through multiple mechanisms simultaneously, including cuticular penetration, varied surface reactivity, and prolonged interaction with insect tissues. Smaller nanoparticles may penetrate the cuticle more effectively, while larger particles may contribute to sustained toxicity through prolonged surface contact and gradual release of active nanoparticles [61].

The HR-TEM analysis confirms the PDI results, showing that AgNPs-CAFP has a significant polydispersed size distribution. Since PDI values above 0.4 indicates high polydispersity, the HR-TEM images support this finding by visually showing some variation in particle sizes. This suggests that factors like nanoparticle formation and interactions in the solution may influence their dispersion.

DLS measures the hydrodynamic size, including the particles, hydration shell, and surfactant coating. DLS often reports larger sizes than HR-TEM, which may be due to the influence of Brownian motion, which reflects actual particle size without the hydration layer [62]. Additionally, DLS measures particles in solution, where they may aggregate, while HR-TEM measures dried samples, providing more precise size estimates [63].

The hydrodynamic diameter of AgNPs- CAFP in this study is 360.8 nm. This finding is consistent with previous reports on fruit peel–mediated green synthesis of AgNPs, which demonstrate a

wide variation in hydrodynamic sizes. For instance, AgNPs synthesized using *Citrus sinensis* (orange) peel extract exhibited an average hydrodynamic diameter of 211.7 nm, while *Citrus microcarpa* peel–mediated AgNPs showed a size of approximately 235 nm [58, 59]. In contrast, pomegranate peel–mediated AgNPs displayed a much broader size distribution, with a mean hydrodynamic diameter of 624 nm [20]. The variation in hydrodynamic sizes could be due to the measured particles size which includes biomolecules that surrounds the surface of the silver particles and possible nanoparticle agglomeration (Brownian motion) in aqueous suspension.

Zeta potential (ZP) values provide an indication of nanoparticle colloidal stability. Nanoparticles with ZP values between 0 and ±10 mV are considered highly unstable, whereas values ranging from ±10 to ±20 mV indicate relative stability. ZP values between ±20 and ±30 mV suggest moderate stability, while values exceeding ±30 mV are generally associated with highly stable nanoparticle suspensions [19]. In this study, we obtained the ZP value of -17.5 mV, which indicates that the synthesized AgNPs are relative colloidal stability due to the presence of electrostatic repulsion between particles (Fig. 4). The negative zeta potential values are attributed to negatively charged compounds from *C. aurantifolia* peel extract acting as a capping agent [57, 13].

These findings align with previous studies reporting comparable zeta potential values for fruit peel–mediated AgNPs. For instance, *Citrus sinensis* peel–mediated AgNPs exhibited a zeta potential of −18.2 mV, corresponding to relative stability [59], while *Citrus unshiu* peel–mediated AgNPs showed a zeta potential of −24.6 mV, indicating moderate stability [54]**.** In addition to *Citrus* spp., AgNPs synthesized using pomegranate peel demonstrated a zeta potential of −20.1 mV, which also reflects moderate colloidal stability [20]**.** Hence, these results confirm that the zeta potential value obtained in the present study falls within the range commonly reported for fruit peel–mediated AgNPs which generally possess relative to moderate stability.

Adulticidal activity using WHO Topical Bioassay

The results of three forms of CAFP i.e., plant extract, essential oil and AgNPs against female *Ae. aegypti* adults after 24 hr of exposure were provided in Fig. 6 and Table I. Percentage of mortality were directly proportional to concentration. During the first hour of exposure to these treatments, it is visually observed that the mosquitoes initially showed episodes of excitement and then collapsed, displaying unusual behavior, erratic or unable to fly in coordinated manner in this recovery period. Plus, several of them (mainly at the highest concentration) remain static or moribund on filter paper of petri dish with symptoms of convulsion and paralysis prior to death.

The adulticidal activity of the CAFP was found to be form dependent. In general, all three forms exhibited effectiveness against female *Ae. aegypti* adults with results varying according to the concentration utilized. The adulticidal activity of CAFP against adults of *Ae. aegypti* 24 hr post exposure showed that essential oil was the most toxic form compared to the other forms. At the highest concentration of 18%, mortality percent of adult *Ae. aegypti* from CAFP- plant extract was 57.33%, 65.33% for AgNPs- CAFP and 70.67% mortality for CAFP- essential oil (Fig. 6), although no significant difference was observed between the adulticides at this concentration (p= 0.127).

Exposure to 4 mM $AgNO_3$ solution resulted in 0% mortality across all replicates, indicating no observable adulticidal activity under the tested conditions. The absence of mortality in the $AgNO_3$ treated group suggests that free Ag^+ ions alone did not contribute to adulticidal activity. Therefore, the enhanced bioactivity observed in the AgNPs- CAFP is likely attributable to nanoparticle

formation or synergistic interactions between silver and phytochemicals present in the plant extract.

The LC_{50} values of CAFP adulticides were recorded as 14.51%, 11.28% and 11.82% for CAFP- plant extract, AgNPs- CAFP and CAFP- essential oil respectively (Table 1), with a significant difference observed among adulticides (p= 0.042). Post hoc Tukey's test analysis further revealed a significant difference between the CAFP- plant extract and AgNPs- CAFP (p= 0.035), while no significant differences were observed between the CAFP- plant extract and CAFP- essential oil (p = 0.141), or between the CAFP- essential oil and AgNPs- CAFP (p= 0.736).

Meanwhile, the LC_{90} values were 31.19%, 30.99% and 23.41% for CAFP- plant extract, AgNPs- CAFP and CAFP- essential oil respectively, although no significant difference were observed among them (p= 0.080). These differences were also statistically significant (p = 0.014). Tukey's test analysis revealed a significant difference between the CAFP- plant extract and CAFP- essential oil (p = 0.012), whereas comparisons between the CAFP- plant extract and AgNPs-CAFP (p = 0.564), as well as between the CAFP- essential oil and AgNPs-CAFP (p = 0.089), did not yield significant differences.

Based on the adulticidal activity of CAFP- derived adulticides against adult *Ae. aegypti* mosquitoes, the order of effective percentage mortality can be arranged as follows, CAFP- essential oil > AgNPs-CAFP > CAFP- aqueous plant extract.

This present finding was in agreement with the previous report

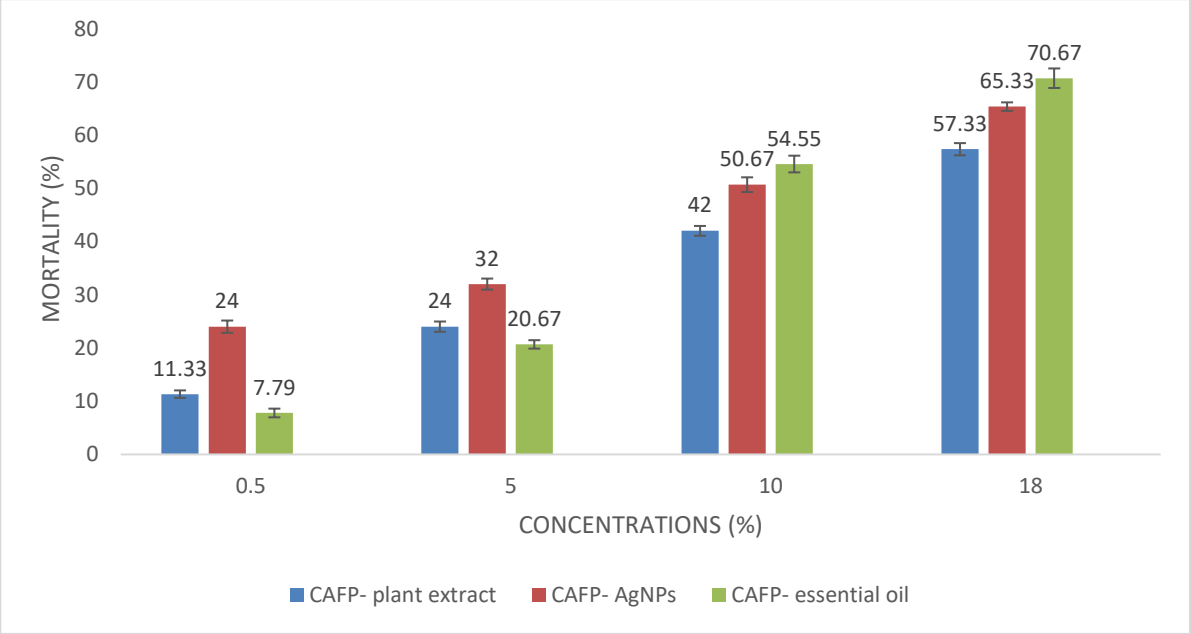

Fig. 6: Percent mortality of *Ae. aegypti* adults to four different concentrations of CAFP- plant extract, AgNPs-CAFP and CAFP- essential oil extracted from *C. aurantifolia* 24 hr after exposure. Data are presented as the mean ± standard error. All means obtained after 6 replicates. Vertical line denotes standard errors of the mean, while the numerical values displayed above each bar corresponds to the specific mortality percentages recorded.

TABLE I. Various forms of adulticides derived from *Citrus aurantifolia* Fruit Peel (CAFP) against female adults of *Ae. aegypti*, 24 hr post-exposure.

Adulticide	Conc. (%)	% Mortality (Mean ± S.D.)	LC_{50} (LCL-UCL)	LC_{90} (LCL-UCL)	Slope ± S.E.	$\chi 2$
CAFP-plant extract	0.5	11.33ab ± 1.72	14.51a (10.94 ± 21.80	31.19a (23.0 ± 57.65)	0.39 ± 0.94	1.316
	5	24a ± 2.37				
	10	42a ± 2.26				
	18	57.33a ± 2.80				
AgNPs-CAFP	0.5	24b ± 2.83	11.28ab (7.27 ± 18.50)	30.99a (21.93 ± 67.10)	0.34 ± 0.89	0.237
	5	32a ± 2.53				
	10	50.67a ± 3.39				
	18	65.33a ± 1.97				
CAFP-essential oil	0.5	7.79a ± 2.0	11.82b (9.33 ± 15.27)	23.41a (18.84 ± 33.41)	0.36 ± 1.51	1.527
	5	20.67a ± 1.94				
	10	54.55a ± 3.84				
	18	70.67a ± 4.50				
Positive control (Permethrin)		100	-	-	-	-
Negative control (acetone)		0	-	-	-	-
Negative control (4 mM $AgNO_3$)		0	-	-	-	-

Different superscript letters indicate significant differences ($p< 0.05$; One-way ANOVA with Tukey's post hoc test). For LC_{50} and LC_{90}, comparisons were made among adulticides; for mortality, comparisons were made among adulticides at the same concentration. Values with the same letter are not significantly different.

whereby claiming that CAFP-EO possesses potential adulticidal activity against adult *Ae. aegypti*, with 44% percent mortality at 0.1% concentration 24 hr after treatment using method of WHO tube susceptibility test [5]. A study using CDC bottle bioassay assessed the adulticidal activity of essential oils from *C. aurantifolia* peels against *Anopheles gambiae* mosquitoes proved that the EO exhibited concentration-dependent knockdown and adulticidal effects, with LC_{90} value of 1.84% [11].

However, the present study demonstrated that relatively higher concentrations (≥10%) of CAFP- EO are required to achieve more than 50% mortality at 24 hr post-exposure, as well as high LC_{90} value needed for CAFP-EO using the WHO topical application bioassay compared to previous studies. Nevertheless, this variation in results is probably due to differences in laboratory methodologies. Moreover, phytochemical composition, plant parts used, geographical origin and seasonal variation of the plant material, as well as differences in mosquito species tested also can influence the overall bioactivity [5, 12]. To date, there appear to be limited published studies where EOs and crude plant extracts have been directly compared for adult mosquito toxicity using WHO topical application bioassay in the literature, hence comparison with previous study is limited.

In comparing effectiveness of nanoparticles with aqueous plant extract, our finding demonstrated that AgNPs-CAFP presented better adulticides effect compared with the CAFP- plant extract. This current result is also comparable to earlier reports which conducted a comparison study of adulticidal effect between AgNPs and using plant extract only, on adult mosquitoes using WHO tube susceptibility test. For instance, maximum efficacy was observed with AgNPs synthesized using leaf aqueous extracts of *H. indicum* against the adult *Ae. aegypti* with $LD_{50} = 0.00296\%$ and $LD_{90} = 0.00543\%$, whereas for the treatment of *H. indicum* extract only, the value were much higher with $LD_{50} = 0.01235\%$ and $LD_{90} = 0.02192\%$. Hence, this signifies that AgNPs synthesized using plant extract was more effective in giving high mortality rate of adult *Ae. aegypti* compared to the plant extract only [14]. In other words, AgNPs required a significantly lower concentration to achieve high adulticidal efficacy against *Ae. aegypti*, indicating enhanced potency compared to the crude extract alone.

Other article also reported similarly whereby the AgNPs synthesized using *Chomelia asiatica* plant extract against adult *Ae. aegypti* was efficient in giving high mortality rate compared to the result of the plant extract only [15]. The maximum efficacy for AgNPs - *Chomelia asiatica* plant extract was observed at (LD_{50}= 0.00292%; LD_{90}= 0.00528%), whereas the plant extract only was at (LD_{50}= 0.01728%; LD_{90}= 0.03102%). Multiple studies confirmed that silver nanoparticles synthesized via plant extracts demonstrate significantly greater adulticidal activity than the crude extracts themselves, due to the attribution of their nanoscale size, greater penetration and ability to induce oxidative stress in mosquito cells causing cytotoxic effect.

The literature offers no publication on the toxicity of green synthesis of AgNPs against any adult mosquito spp., measured by topical application. While these studies highlight the potential of *Citrus* spp. in mosquito control, they may not have employed the WHO topical bioassay method. Thus, to our knowledge, this is the first report of AgNPs using plant extract overall against adult mosquitoes testing for WHO Topical Bioassay.

Collectively, these findings emphasize that the observed efficacy of plant-based adulticides are not solely dependent on the form of plant extract or the mosquito spp. tested, but is also significantly influenced by the methodological parameters employed in the assay. Factors such as the mode of exposure and bioassay type can markedly affect the LC values and mortality outcomes, thereby underscoring the importance of standardized protocols in evaluating botanical insecticides.

Variation in the activity of the three form of adulticides from the same plant against the adult stage of *Ae. aegypti* was another finding of the current study which may arise due to the difference in components in them. Chemically, essential

oils, are highly concentrated with only volatile aromatic compounds, whereas plant extract, contains a wider range of compounds including both volatile and non-volatile ones. In contrast, AgNPs are just particles of silver that range in size from 1 to 100 nanometers (nm). Different forms of adulticides from the same plant may act differently in regards to their mode of action. Essential oils and plant extract may act in a similar mode of actions with compounds playing roles in toxicity.

Overall, based on order or potency of CAFP- derived adulticides against adult *Ae. aegypti* mosquitoes are CAFP- essential oil > AgNPs- CAFP > CAFP- aqueous plant extract.

Penetration and Mechanism of action

CAFP- essential oil in the current study was the most effective form of adulticide compared to the CAFP- plant extract and AgNPs- CAFP, achieving the highest mortality number of adult *Ae. aegypti*. This is because essential oil is easily absorbed through the exoskeleton of adult mosquitoes due to its lipophilic nature. The exoskeleton of mosquitoes is composed largely of lipids, and many essential oil components are lipophilic (fat-soluble). This allows essential oils to dissolve and penetrate more readily into the cuticular layers, which are mainly composed of waxy substances. Lipophilic molecules are more likely to traverse this hydrophobic barrier such as those in essential oils traverse this hydrophobic barrier more effectively than hydrophilic agents like aqueous plant extracts or water-based nanoparticles.

However, little is known about the mode of adulticidal activity of plant extract and silver nanoparticles since this study is the first to report on the effect of AgNPs and plant extract on adult mosquitoes using WHO Topical Application Bioassay Test.

Given that the mosquito's water-resistant exoskeleton presents a significant barrier to penetration by water-based solvents, thus, the only way aqueous plant extract and AgNPs can enter the mosquito's body is through intersegmental membranes or cuticular sutures at the pronotum, which enables the toxins to be absorbed. It is hypothesized that only small amounts of AgNPs manage to enter the mosquito's body via these pathways causing histological changes as observed, while others accumulate on the surface of the exoskeleton [24], but still exerting histotoxic effects on the outer cuticular layer of mosquito. AgNPs has shown to be ineffective and hard in penetrating the human epidermal skin past beyond the stratum corneum [25].

In the present study, we hypothesize that a similar pattern of AgNPs penetration may apply to mosquito tissue, with difficulty of these nanoparticles to fully penetrate the exoskeleton as they are from water-based solvent. This limited penetration likely contributes to the reduced efficacy of AgNPs relative to essential oils as the total amount of AgNPs is not being fully absorbed compared to the essential oil. Prior studies have demonstrated that silver nanoparticles, particularly those exceeding 40 nm in diameter, struggle to pass through the stratum corneum and enter the skin. Meanwhile, for nanoparticles with a diameter less than 40 nm have been reported successfully penetrates the skin [23].

Therefore, in the present study, the synthesized AgNPs had an average diameter of 18.22 ± 5.43 nm as determined by HR-TEM analysis, with single spherical shape confirmed by FESEM [8]. Given their relatively small size, these nanoparticles may possess an enhanced ability to interact with and potentially penetrate the mosquito cuticle; however, direct evidence of transcuticular penetration was not established in this study. Further ultrastructural investigations scanning electron microscope (SEM) or transmission electron microscopy (TEM) are necessary to dig into the route of how AgNPs causing toxic to tissue.

The shape of nanoparticles also affects the tissue penetration capabilities. Spherical nanoparticles tend to penetrate deeper into the skin compared to ellipsoidal or rod-shaped particles. Studies have observed that spherical particles are located deeper in the epidermis and dermis, whereas ellipsoidal particles remain primarily in the stratum corneum and epidermal layers. Additionally, nanorods, despite their unique properties, have shown only penetrating a limited depth, at the outermost layers of the tissue [23]. Hence, size and shape significantly influences their ability to penetrate biological tissues.

Understanding the mechanisms by which these nanoparticles induce toxicity is crucial for developing effective mosquito control strategies. Metal nanoparticles exert toxic effects on adult mosquitoes through multiple mechanisms, including (1) oxidative stress, (2) cytotoxicity, and (3) genotoxicity. Certain metal nanoparticles (NPs) have been shown to generate DNA oxidation, mutations, decreased cell viability, distorted morphology, induced necrosis and apoptosis, and decreased proliferation [21]. In the present study, it was hypothesized similarly, whereby upon topical exposure with AgNPs, they interact with mosquito cells, leading to distorted morphology of the smooth muscle tissue, resulting in cellular dysfunction and increased mortality.

The nanoparticles has disrupt cell membranes, hindering essential cellular processes, and triggering apoptosis (programmed cell death). Silver and copper oxide NPs, have also been associated with DNA damage [21]. Additionally, a smaller size of nanoparticles means a greater surface area to volume ratio of nanoparticles, thus enhancing their chemical reactivity and biological activity, contributing to ROS production [22].

Unlike AgNPs which exert toxic effects through oxidative stress, cytotoxicity and genotoxicity, the biological activity of essential oil and plant are largely attributed to their major components. Although in some circumstances, major components may not always account for the total activity, the interaction of both major and minor components could somehow resulted in synergistic or antagonistic effects [16].

The present study demonstrated a tissue alteration in the thorax of the mosquitoes treated with essential oils and plant extract, as they were abundant with limonene (major compound), which belong to the monoterpenes group. This confirms with various studies reported that *d*-limonene as the predominant component, accounting for the major % area in *C. aurantifolia* fruit peel when analyzed using GC-MS in their respective studies designed for their own respective experiment [5, 6]. Using other insects, one research concluded that the application of *d*-limonene can led to significant mortality rate of bedbugs [17]. *d*-limonene effectively removes the waxy layer of an insect's respiratory system, leading to suffocation when applied directly [18]. In fact, *d*-limonene has been the most used botanical insecticide in California in the past decade (averaging >20,000 kg per year) [3].

Even though extracts from plants constitute a rich source of bioactive compounds that are biodegradable into non-toxic products, it is to note that essential oils are much more concentrated with compounds. Hence, this may explain the result from the present study, which shows that essential oils can cause a high mortality rate in adult *Ae. aegypti* compared to the plant extract, due to the concentrated compounds essential oils they contain, which are toxic or irritating to insects.

Effect of the adulticides on the histological changes.

Mosquito bodies are composed of three segments: the head, thorax, and abdomen. However, the histological studies in this research focused specifically on the thorax (pronotum) for several reasons: first, the treatment was applied directly to the pronotum area; second, the pronotum is a

crucial region for flight, as it contain flight muscles necessary for the mosquito to survive and obtain food; third, the head and abdomen part were rupture due to effect from the adulticide hence cannot be viewed.

Normal anatomy of untreated adult mosquito using H&E staining results were shown at 40X magnification in Fig. 7, comprising intact thoracic muscles, esophageal ganglions and intestinal tract. Nuclei and nucleoli stained blue-violet, while cytoplasm as well as extracellular matrix were colored in varying shades and intensities of pink in all specimens examined.

There were no signs of damage or disorientation in the smooth muscle at the thorax region of the control adult in Fig. 8(A). In contrast, *Ae. aegypti* adults treated with the three adulticides all showed similar alterations with the damage or deformation to most of the thoracic smooth muscle (or known as flight muscle), as shown in Fig. 8(B)- 8(D). The damage was observed solely in the thorax region of the adult mosquito.

An earlier study comparing the histological effects of gold nanoparticles and essential oil, both derived from *Artemisia vulgaris* leaf extract onto larvae stage of mosquito, demonstrated histological alterations in the digestive tract and midgut of third and fourth instar *Ae. aegypti* larvae following treatment [24]. Several histopathological alterations were observed in adult *Aedes* mosquitoes, including compound eyes degeneration, muscular damage with cellular infiltration, gut epithelial degeneration and necrosis, pyknotic nuclei in the malpighian epithelium, and ovarian cell degeneration were observed when treated with purple and white flower of *Ageratum conyzoides* Weed Extracts [16]. Another study also using a *Citrus* spp., namely, *Citrus sinensis* essential oil revealed obvious alterations in the cuticle, muscles, and midgut tissues including changes on the ultrastructural level of the third instar larvae of *Culex pipien* [26]. Although none of the studies mentioned above specifically attributed histotoxic effects to the treatment of AgNP, essential oils, or plant extracts derived from *Citrus* spp. specifically on the adult stage, the mechanisms observed are hypothetically similar to those in the current study, where histotoxicity was induced by various forms of adulticides derived from *C. aurantifolia* fruit peel.

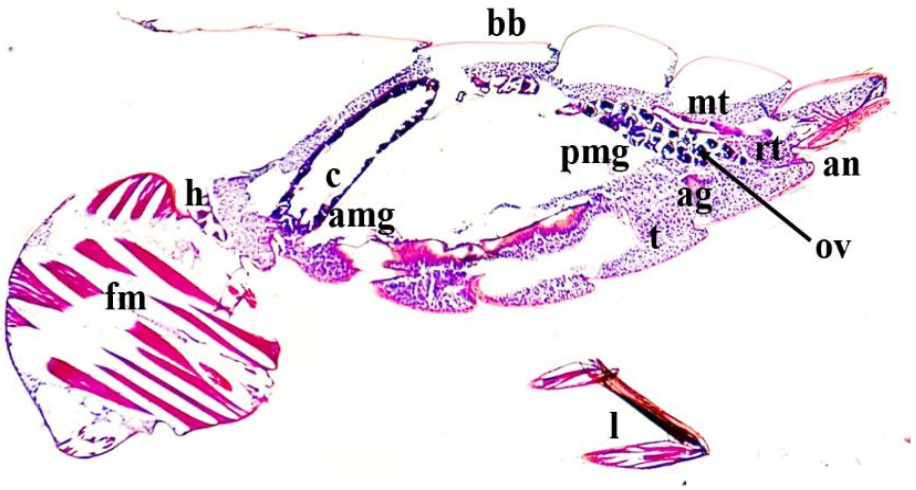

Fig. 7: Normal anatomy of untreated adult *Ae. aegypti*. Longitudinal section of a female. Hematoxylin and Eosin (H&E) staining of (an) anus; (ag) abdominal ganglia; (bb) bursh border; (c) cardia; (fm) flight muscle; (h) heart; (l) legs; (amg) anterior midgut; (pmg) posterior midgut; (mt) Malpighian tubules; (ov) ovary; (rt) rectum; (t) trophocytes, at 40X magnifications under light microscope.

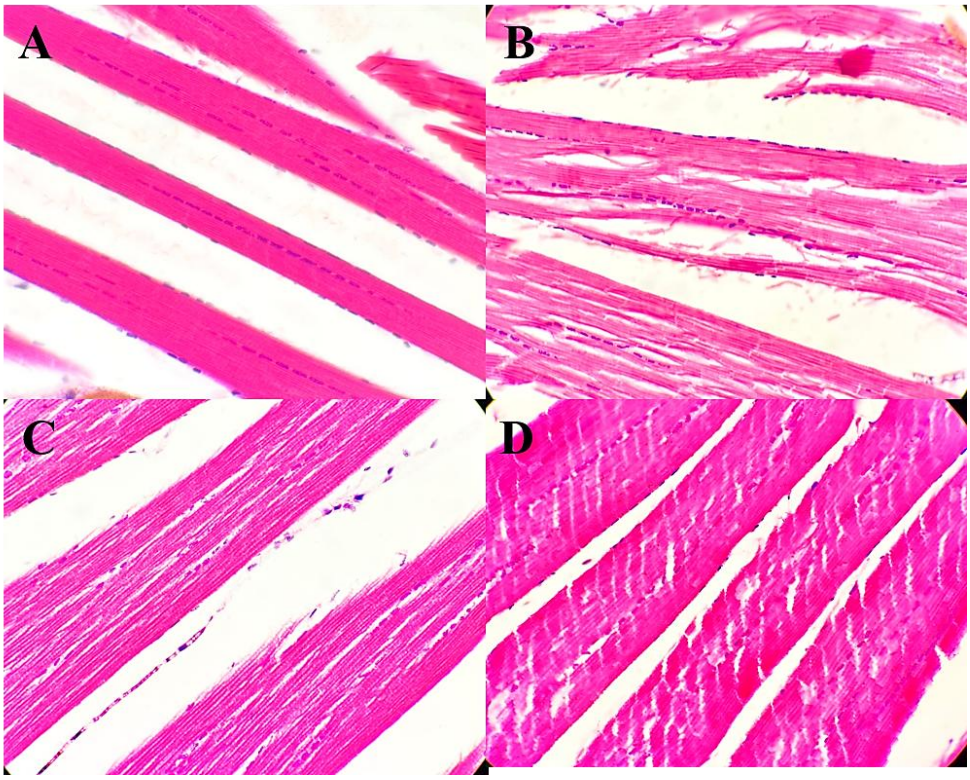

Fig. 8: Histological changes of the thorax of adult *Ae. aegypti* after treatment with various forms of adulticides derived from *Citrus aurantifolia* Fruit Peel (CAFP) at 600X magnification under light microscope. (A) Negative Control (B) Treatment with CAFP- plant extract (C) Treatment with AgNPs- CAFP (D) Treatment with CAFP- essential oil.

Despite the variation in the results using various forms of adulticides, they all still showed considerable mortality on *Ae. aegypti* mosquitoes, which is considered to be successful in managing the dengue vector. The thoracic smooth muscles are important organs that play a critical role in flight and the movement of the wings. It is essential for their ability to fly, survive and engage in reproductive activities. The thorax is made up of two primary parts: flight muscles and a thin-walled ellipsoidal or box-shaped exoskeletal cuticle where flight muscles attach [27]. As such, this cuticle is made up of chitin, water, and proteins, structured into different layers such as the endocuticle, exocuticle, and epicuticle [28]. Damage to these muscles could have created discomfort or difficulty in controlling the flight thereby leading to mortality [29].

Effect of LC_{90} value from different adulticides on the antimicrobial study

Disc diffusion assay, or known as the Kirby-Bauer test, is used to monitor antimicrobial resistance. Since the LC_{90} values that caused mortality in adult *Ae. aegypti* were obtained from each adulticide, thus, we intend to further investigate these LC_{90} values to evaluate their effects on common bacterial and fungal strains that are implicated in disease causation. In this study, AgNPs are effective in inhibiting the bacterial growth whereas CAFP- essential oil effective against fungal growth.

For comparison purpose, the antimicrobial activity of three different form of plant based adulticides against two bacterial and a fungal strain are summarized in Table II. The results represent the diameter of inhibition zone including diameter of paper disk (6 mm). A variation in antimicrobial properties of the analyzed adulticides were observed in the

study. Overall, CAFP- plant extract failed to inhibit all of the tested strains, both bacteria and fungi. However, LC_{90} concentration of AgNPs- CAFP showed consistently moderate antimicrobial activity (growth inhibition zone between 12- 20 mm) against both the bacterial spp. tested, whereas CAFP- essential oil showed an antifungal activity.

Previous studies have identified limonene as a major component of citrus oil, with evidence supporting its potent anti- *Candida* activity. This compound has been shown to inhibit *Candida albicans* growth by cell wall damage induced oxidative stress that leads to DNA damage. This cascade of events subsequently modulates the cell cycle and triggers apoptosis via nucleolar stress and a metacaspase- dependent pathway [30]. Although essential oil is hydrophobic and can penetrate the cell membrane of Gram-positive bacteria, thereby exerting their antibacterial effects through disruption of enzyme production, alterations in membrane functionality, and protein denaturation [31], this was not observed in our study, whereby it only susceptible to *C. albicans* but not to both of the tested bacterial strains, namely, *S. aureus* and *E. coli*.

Even though earlier study have reported that CAFP-EO (lime peel) exhibits stronger antimicrobial activity than grapefruit EO and orange peel EO against *S. aureus* (15 mm), *E. coli* (12 mm), and *C. albicans* (17 mm) [6], our study showed no antimicrobial activity when used CAFP-essential oil against the tested bacterial strains. This is perhaps due to several factors, including low concentrations, inadequate composition and bacterial resistance. The effectiveness of essential oils depends heavily on optimizing these variables. Studies have shown that at lower concentrations, essential oils may not exhibit significant inhibitory effects, and higher concentrations being required to inhibit microbial growth [32]. Essential oils consist of a complex mixture of volatile compounds, and the antimicrobial activity depends on the types and quantities of bioactive compounds like phenols, terpenes, aldehydes, and alcohols. Not all

TABLE II. Antimicrobial activity of three different forms of adulticides using *Citrus aurantifolia* Fruit Peel (CAFP) determined by disc diffusion assay.

Adulticides (LC_{90} Concentration)	*S. aureus*	*E. coli*	*C. albicans*
CAFP- plant extract (31.19%)	NI	NI	NI
CAFP- essential oil (23.41%)	NI	NI	12.0 ± 1.414
AgNPs- CAFP(30.99%)	11.83 ± 1.169	7.33 ± 0.516	NI
Permethrin (0.04%)	NI	NI	NI
Reference control[a]	32.0 ± 3.098	32.83 ± 0.408	20.50 ± 1.517
Blank disc[b]	NI	NI	NI

[a]Ciprofloxacin (5 µg) for bacterial and Fluconazole (25 µg) for fungal as a positive reference standard;
[b]Blank disc as a negative control;
NI- No zone of inhibition; Results were expressed as mean inhibition zone (mm) ± S.D of six replicates

essential oils contain sufficient quantities of compounds with antimicrobial activity, such that, phenolic compounds contribute significantly to antimicrobial action [33]. However, it is to take note that the evaporation of the essential oil and their constituents can affect the doses applied to paper discs and thus the result. Hence, additional studies using liquid culture assays to determine the minimum inhibitory concentrations (MIC) are necessary.

In present study, AgNPs- CAFP showed moderate antimicrobial activity (growth inhibition zone 12- 20 mm) against both the bacterial spp. tested, namely, *E. coli* and *S. aureus*. Other study also reported similarly whereby AgNPs synthesized from various extracts of *Citrus* fruit peels, including *C. aurantifolia*, exhibited notable antibacterial properties, with greater zone of inhibition in Gram-positive bacteria compared to Gram-negative. Specifically, inhibition zones measured in that study were 12.7 mm for *E. coli* and 13.3 mm for *S. aureus* [7]. These differences are largely attributed to variations in cell wall structure and composition, which influence how AgNPs may interact with the bacteria. The antibacterial effect for these particles might be due to the ability of the AgNPs to to disrupt cell membrane permeability, formation of free radicals, and interaction with thiol groups, prevent DNA replication, affect cellular signaling, and prevent biofilm formation [34].

The inhibitory activity of AgNPs against Gram-positive and Gram-negative bacteria is a controversial issue. A growing body of evidence reported that AgNPs are claimed to be more effective against Gram-negative bacteria [36, 37, 56]. This is because Gram-negative have a thinner peptidoglycan layer surrounded by an outer membrane containing lipopolysaccharides. The unique composition of this outer membrane can make them more susceptible to the disruptive effects of AgNPs [38]. However, interestingly, it seems that it has become a debate since few studies have claimed otherwise such that Gram-positive bacteria also is equally susceptible towards AgNPs [39]. Nevertheless, these variations in reported susceptibility between Gram-positive and Gram-negative bacteria are most likely attributable to differences in the physicochemical characteristics of the synthesized AgNPs.

The antimicrobial activity observed in the present study may be associated with the physicochemical characteristics of the synthesized AgNPs, including- particle size, shape and surface charge are also known to influence biological interactions and antimicrobial efficacy [38]. Smaller nanoparticles generally exhibit enhanced bioactivity due to their larger surface area, which facilitates greater silver ion release and direct interaction with microbial membranes [64]. A study demonstrated size-dependent biological effects in hepatic (HepG2) cells, showing that smaller AgNPs (10 nm) could enter the nucleus, whereas larger AgNPs (60 nm) remained aggregated in the cytoplasm [65]. In the present study, the AgNPs had an average size of 18.22 ± 5.43 nm, suggesting the same that AgNPs may penetrate bacterial cell walls and membranes, thereby contributing to their antimicrobial activity.

Nanoparticle shape morphology also influences antimicrobial activity. Synthesized AgNPs can be in various shapes, including spherical, triangular, cubic and rod-shaped [66]. A comparative study of synthesized AgNPs in three shapes showed that the highest bactericidal activity was observed in spherical forms, followed by disc-shaped and then triangular nanoparticles [67]. In the present study, FESEM analysis revealed that the synthesized AgNPs were predominantly spherical in shape, indicating that their morphology may have contributed to the enhanced antimicrobial activity observed.

Additionally, surface charge and colloidal stability also influence nanoparticle–bacteria interactions, such that a positively charged AgNPs exhibit

stronger interactions with bacterial cell membranes that contain lipopolysaccharides (LPS) and techoic acid, which are negatively charged [68]. Although the AgNPs synthesized in the present study with zeta potential (−17.5 mV) displayed a negative surface charge, the value was not highly negative. Thus, the observed antimicrobial activity may not be solely attributed to surface charge but rather to the combined effects of size, morphology, and surface characteristics.

In order to interpret the mechanism of AgNPs' antimicrobial activity, four different types of mechanisms have been proposed: (1) AgNPs' interaction with cell membranes, which alters membrane permeability and disrupts respiratory chain enzymes; (2) AgNPs' gradual diffusion into cells, which may negatively impact cellular enzyme activity and limit the transcription process by conjugation of silver particles to DNA; (3) Subcellular components leakage due to nanoparticles' interaction with the plasma membrane, which results in cell death; and (4) Free radical generation when the cell membrane is effected by silver ions [35].

Results of the current study also revealed that CAFP- plant extract did not display any inhibitory effect against the tested organisms, implying that any activity exhibited is attributed solely to AgNPs but not the plant extract which is used in the phytosynthesis. This is similar to the previous study whereby the *Citrus limon* peels extract alone does not inhibit bacterial growth when compared to the AgNPs-synthesized with *C. limon* [40]. This conclusion is drawn from a direct comparison of the antimicrobial effects of the AgNPs and the plant extract alone against these bacteria. In other words, the plant extract alone is ineffective in inhibiting bacterial growth unless it is in the form of AgNPs.

Moreover, in terms of CAFP- plant extract, one study claimed that both *Citrus sinensis* and *Citrus aurantifolia* peel extract, when prepared with ethanol, exhibited antibacterial property with moderate zone of inhibition against *S. aureus* and *E. coli* when used with much more higher concentration compared to the current study [20]. In addition, a study from Algeria also found that both leaf and peel extracts of *C. aurantifolia* extracted from ethanol and methanol exhibited significant antibacterial activity against multidrug-resistant *S. aureus* strains and *E. coli*, with inhibition zones ranging from 16.00 mm to 22.00 mm [41]. All of these previous study using *C. aurantifolia* peel aqueous extract that showed susceptibility against *S. aureus* and *E.coli* was due to the alcohol solvent used for the extraction. However, in the present study, water, a non-alcoholic solvent, was employed for the extraction of the plant material.

Different solvents target different compounds based on their polarity, thus, isolating a variety of compounds from plants. In this case, using water as a solvent for *C. aurantifolia* extraction in the formulation of CAFP- plant extract is not effective in inhibiting the growth of bacteria and fungi, as it fails to extract the targeted compounds that may act as antimicrobial agents. The antimicrobial potency of lime fruit is influenced by the solvent used, suggesting that certain active compounds in lime exhibit significant antimicrobial and antifungal effects, but are only released or extracted out with a specific solvent. Although water is the universal and most common solvent for preparing herbal extract. However, some bioactive compounds in plants are insoluble in water, meaning that methods such as preparing hot tea or boiling the plant in water may not effectively extract these components. Hence, the discrepancies may be attributed to factors such as differences in extraction methods, solvent types, plant cultivar variations, or experimental conditions.

4.0 CONCLUSION

In conclusion, the present study provides strong evidence supporting the

efficacy of lime peel extract of *Citrus aurantifolia* as an adulticidal agent against adult *Ae. aegypti* mosquitoes, with histological alterations further confirming its activity. Overall, all forms of adulticide demonstrated significant adulticidal effects, with the potency of *C. aurantifolia* fruit peel varying across different formulations. The order of potency was as follows: essential oil > silver nanoparticles > plant extract. The encouraging mosquito adulticidal activity of these three forms of adulticides might pave the way for the development of novel herbal mosquito formulations, optimizing the most effective form against *Ae. aegypti*. Moreover, the demonstrated antimicrobial properties of these bioinsecticides highlight their broader potential as multi-functional agents in integrated public health strategies. Overall, these findings suggest that lime peel could be effectively incorporated into vector control proGrams. However, to ensure more convenient outcomes, further field trials and a comprehensive analysis of the mode of action are recommended.

ACKNOWLEDGEMENTS

The authors acknowledged Universiti Putra Malaysia for awarding the IPM research grant (GP-IPM/2022/9736100) to support this research, as well as the Graduate Research Fellowship (GRF) program for aid in financial assistance and tuition fee waiver throughout the study. We wish to thank Universiti Putra Malaysia (UPM) for providing us with the facility to carry out the present study. The authors thank Mrs. Juita Chupri from Histology department UPM and Mrs. Wan Suhana Wan Mohd Shafie from Microbiology department UPM for their technical support and guidance, as well as providing the necessary facilities for histology and microbiology work.

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