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RESEARCH ARTICLE

Evaluation of toxicity of *Vitex negundo L.* synthesized silver nanoparticles against *Aedes aegypti*

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Abstract

Dengue, chikungunya, and zika are some of the fatal diseases that are causing a high number of deaths. Therefore, this work is designed to provide an effective control measure against these species of mosquito. *Vitex negundo L.* leaves were used to synthesize silver nanoparticles (AgNPs), which were proven to have significant larvicidal and pupicidal activity when tested against the developmental stages of *Aedes aegypti*. The nanoparticles were synthesized using silver nitrate, and the synthesized nanoparticles were characterized using techniques such as UV-visible spectrometry, Fourier Transform Infrared Spectrometry, and X-ray diffraction to confirm the presence of nanoparticles. The conditions for the larval hatchability from the first instar to adult stages were optimized at different pH ranges with three water sources: reverse osmosis water, tap water, and stagnant water. The LC_{50} of the subjected stages was found to be 441.43, 308.74, and 490.66 µl/L for the third and fourth *instar* and pupal stages of *A. aegypti,* respectively. The plant secondary metabolites were utilized as ligand compounds to target mosquito juvenile hormone-binding protein. Our study attempted to identify a plant-based nanomaterial that showed promising results in controlling larval development.

Keywords

characterization; larvicidal activity; phytocompounds; silver nanoparticles; *Vitex negundo L.*

Introduction

Mosquitoes are the most abundant vector capable of spreading diseases like yellow fever, zika, malaria, hemorrhagic fever, dengue fever, lymphatic filariasis, Japanese encephalitis, and chikungunya (1-3). *Anopheles*, *Aedes,* and *Culex* are the three genera that comprise the majority of mosquito vectors and are responsible for causing higher incidences of mosquito-borne diseases (4). *Aedes aegypti* was found to be the most common species of *Aedes* in India, and illnesses continue to be the main source of mortality and morbidity (2, 5). For several decades, mosquitoes were controlled using synthetic chemicals and organophosphorus, and numerous health issues such as eye irritation, asthma, headache, sneezing, and breathing problems are reported. Concerns related to mosquitoes developing resistance to these repellents are a major limitation (2, 6). As effective vaccines are not available for this vector-borne disease, identifying a novel plant-based material becomes a crucial requirement. In contrast to

conventional insecticides, which are based on a single active component, plant-derived insecticides contain a mixture of safe and target-specific compounds that may work together synergistically to kill mosquitoes (2, 7, 8)

Plant-based nano synthesis is advantageous as it's simple, less energy-consuming, and can be executed with low chemical and biological contamination (9). The AgNPs from numerous plants' extracts, such as *Capsella bursa-pastoris, Brassica nigra, Azadirachta indica, Berberis vulgaris, Verbena officinalis, Origanum vulgare,* and *Lavandula angustifolia* were found to have a potent biological property (10-12). The AgNPs synthesized from the essential oil of *Curcuma zedoaria* pose potential activity against the larvae of *Culex quinquefasciatus* (7). The efficacy of biosynthesized AgNPs from leaf extract of *Leucas aspera* against the larval and pupal stages of *A. aegypti* and *Anopheles stephensi* was studied (13). The Ag-NPs showed a better mortality rate against the I-IV instar and pupae of *A. aegypti* and *A. stephensi* (13). Green-synthesized AgNPs obtained from the extract of *Ambrosia arborescens* was reported by Morejón *et al.* (3). The studies showed the potential activity of AgNPs against the third *instar* larvae of *A. aegypti*.

Vitex negundo L. is one of the key plants used in traditional medical practices throughout the world. Several parts of this plant are rich in phytocompounds, such as flavonoids, volatile oils, lignans, steroids, terpenes, etc., and exhibit several pharmacological properties (14). Secondary metabolites from *Vitex* are capable of acting as reducing agents in the synthesis of silver nanoparticles, which have proven to have larvicidal and ovicidal applications (15-17). Though the multiple properties such as antibacterial, antifungal, antibiotic, anticancer, antioxidant, anti-allergic, anti-inflammatory, hepatoprotective, and analgesic activity of *V. negundo* plant extracts have been explored, there are no reports that tested the efficacy of the extract against the mosquito developmental stages. The overall design of the study was to assess the *V. negundo* leaf extract-mediated AgNPs in controlling the developmental stages of *A. aegypti*.

Materials and Methods

Plant collection and extraction

Vitex negundo was collected from the Foundation for Revitalization of Local Health Tradition (FRLHT), Bangalore, India. The obtained plant sapling was further authenticated at The University of Transdisciplinary Health Sciences and Technology (TDU), Bangalore, India. The sample was washed with RO water, dried for seven days, and incubated in a hot air oven for 48 hours at 30°C. The dried leaves were ground into powder, and the Soxhlet extraction process was carried out at 60° C for 12 hrs using distilled water as the solvent material. The yield percentage of the extracted sample was calculated using the weight of the sample before and after the Soxhlet extraction (18, 19). The final yield percentage was calculated using the formula as follows:

Extraction Yield percentage = Iw-Dw/Iw * 100

Iw = initial dry weight, and **DW** = dry weight of the sample after Soxhlet extraction.

Phytochemical analysis of *Vitex negundo L.* leaf extract

The phytochemical analysis of leaf extract has been done to detect glycosides, alkaloids, flavonoids, steroids, phenols, tannins, saponins, and proteins. The alkaloids test was confirmed by three different tests, including Mayer's test, Dragendorff test, and Wagner test (20). The tannin content in the sample was confirmed by adding 2-3 mL of plant extract with 1% of lead acetate (21). The saponin test was done by vigorously mixing one gram of plant extract with 5 mL of distilled water (22). The carbohydrate test was carried out by adding 1.5 mL of Benedict's reagent to 3 mL of the sample (23). A biuret test was carried out for protein analysis (23, 24). The Salkowski test was performed to confirm the presence of sterols (24, 25). The flavonoid test was conducted by adding a few drops of diluted NaOH solution into the test tube containing 3 mL of plant extract (26). The Glycosides test was conducted by adding 1 mL of plant extract and 4 mL of diluted sulphuric acid into the test tube and was allowed to boil for 15 minutes. In the solution, potassium hydroxide (20%) was added after cooling. Equal Parts of Fehling's A and B solutions were added and heated for 5 minutes (27, 28). The phenols test was conducted using 3 mL of leaf extract and 1 mL of 2% ferric chloride (29).

Gas Chromatography-Mass spectrometric (GC-MS) analysis of *Vitex negundo* leaf extract

The aqueous leaf extract was subjected to GC-MS analysis. The phytocompounds were transferred to the volatile solution using methyl ester through solvent-solvent extraction to perform GC-MS. Later, the sample was analyzed in the GCMS-QP2010 SE-Shimadzu with helium as carrier gas. The compounds obtained from the GC-MS were identified and interpreted using the mass spectral library NIST (National Institute of Standards and Technology, USA) (30).

Synthesis and characterization of AgNPs from *Vitex negundo L.* leaf extract

About 25 mL of aqueous extract of *V. negundo* was slowly added to the equal volume of 1 mM of freshly prepared silver nitrate ($AgNO_3$) solution. The components were stirred at room temperature on a magnetic stirrer at 200 rpm for 24 hrs. The mixture was centrifuged at 10,000 rpm for 12 min to separate the nanoparticles. The collected nanoparticles were washed with deionized water and ethanol and were dried at 35-40°C for 14 hrs, and the obtained pellet was stored for further characterization (31, 32). The classification of biomolecules in the *V. negundo* in response to metal reduction and nanoparticle stability was characterized using Fourier Transform Infrared Spectrometry (FT-IR) (33). The XRD (X-ray diffraction) was used to characterize and confirm the crystalline nature and particle size of synthesized silver nanoparticles (34). UV–vis spectroscopy is the most crucial technique for analyzing the visual color changes, confirming the

presence of nanoparticles (35).

Hatchability optimization of *Aedes aegypti* stages

The *A. aegypti* eggs were procured from NCDC (National Center for Disease Control, Coimbatore, India). The eggs were immersed in trays containing reverse osmosis water and were maintained at room temperature. The eggs were hatched to develop into the first, second, third, and fourth instar stages. Later, the identification of different instar stages was based on characteristic features such as elongation in body size, antenna development, and increased eye coloration. The hatchability was analyzed using three different water sources: reverse osmosis water, tap water, and stagnated water. The study was carried out at 25°C and tested at three different pH (5, 7, 9). The test study was carried out with different larval stages at varying concentrations of AgNPs (36).

Larvicidal and pupicidal activity

Fifty larvae of the third and fourth instar stages were exposed to five different concentrations of AgNPs (200, 400, 600, 800, and 1000 µl/L) following the protocol of Poonguzali and Kalaivani, (16). The *A.aegypti* pupae were subjected to different test concentrations of silver nanoparticles. The percentage mortality of the larvae and pupae was calculated by exposing them to the AgNPs for 24 h, and the median lethal concentration (LC_{50}) was estimated using statistical tools (37).

In silico analysis

The compounds obtained from the *V. negundo* extract were analyzed by GC-MS, and the ligand molecules were determined. The ligands were selected by Lipinski's rule of 5 (http://www.scfbio-iitd.res.in/software/drugdesign/lipinski.jsp). The phytocompounds that were to act as the ligand material were downloaded from PubChem (https://pubchem.ncbi.nlm.nih.gov/). The selected targeted protein was 5V13 (a juvenile hormone-binding protein in *A. aegypti*). The structure was downloaded from RCSB (https://www.rcsb.org/structure/5V13). The protein was prepared with the addition of charges and hydrogen bonds using PyMol (http://www.pymol.org). The molecular docking of the prepared ligand compounds with the prepared protein was performed in PyRx using auto dock vina tools.

Statistical analysis

To analyze the lethal concentration of the larval and pupal mortality, the SPSS tool's probit analysis of regression was carried out. The Abbott formula was used to determine the mortality percentage.

Results and Discussion

The *V. negundo* is a medicinal shrub belonging to the *Lamiaceae* family. It is characteristically distinguished due to its aroma, with five lanceolate leaflets and a larger leaflet positioned at the center. *V. negundo* was selected for its medicinal properties and the presence of a higher quantity of secondary metabolites (14). The compound yield percentage was calculated to be 79%. The presence of sharp-edged peaks in the GCMS chromatogram indicated the presence of potential bio compounds. A list of active phytochemical compounds that can act as potential larvicidal compounds has been identified (Table 1). A similar study by Okoli *et al.* investigated the binding capability of *Vitex* essential oil against the mosquito odorant binding protein that causes the mortality of the

Table 1. Potential bio compounds present in the leaf extract of *V. negundo* identified using the NIST library

Peak number	Retention Time	Area	Area percentage	Name of the compound	Height	Height percent
1	7.159	41475	7.29	Undecane 4,7 -dimethyl-	19802	6.12
2	7.745	35980	6.32	Octane, 3-ethyl-2,7-dimethyl-	20280	6.26
3	8.845, 9.149, 10.140	38484, 31806, 51912	6.76, 5.59, 9.13	Hexadecane	28481, 24955, 29790	8.80, 7.52, 9.20
4	9.898	29441	5.18	3-Isopropoxy-1,1,1,7,7,7,-hexamethyl-3,5,5-t	24955	7.71
5	10.380	49992	8.79	Octadecane	29292	9.05
6	11.200	43458	7.64	Tetradecane,5-methyl-	26851	8.29
7	11.390	36310	6.38	Eicosane	24558	7.58
11	11.420	34275	6.02	1,1,1,5,7,7,7-Heptamethyl-3,3-bis (trimethylsiloxy	24651	1.39
12	12.019	39788	6.99	Octasiloxane,1,1,3,3,5,5,7,7,9,9,11,11,13,13	15307	4.73
13	13.215	36922	6.49	Heptacosane	9496	2.93
14	14.569, 15.547	33177, 36123	5.83, 6.35	Cyclononasiloxane,octadecamethyl-	19224, 18776	5.94, 5.80
15	15.976	29753	5.23	Z-11(13-Methyl)tetradecen-I-ol acetate	7972	2.46

mosquito (38).

Phytochemical analysis

Wagner's test, Drangondorff's test, and Mayers's test confirmed the presence of alkaloids with the respective change of colors. The saponins were confirmed by a formation foam layer of 2 cm. The formation of a reddish solution with the addition of Benedict's reagent confirmed the presence of carbohydrates. The formation of yellow precipitate with the addition of millions of reagents and the mixture turning into a light purple shade with the biuret test confirmed the presence of proteins. With the addition of chloroform, there was a distinguishable layer formation in the Salkowski test that confirmed the presence of sterols (24). The addition of sodium hydroxide solution into the leaf extract changed the color of the solution to deep yellow, which was further found to have disappeared with the addition of hydrochloric acid in its diluted form, which confirmed the presence of flavonoids. The presence of glycosides was tested using Fehling's solutions. Phenols tested using 2% ferric chloride turned the solution green, which confirmed the presence of phenols (29). A recent study revealed that the presence of a high amount of phytocompounds in the *V negundo* leaf extract contributes to the synthesis of silver nanoparticles (39).

Silver nanoparticle synthesis

The appearance of deep brown coloration during particle synthesis leads to visual confirmation of the silver nanoparticles formed at a yield rate of 05-08 grams. Studies conducted by Parthiban *et al.* reported the synthesis of AgNPs from *Annona reticulata* leaves was confirmed through the color transition from yellow to brown (40). Another experiment conducted by Pilaquinga *et al.* (41) synthesized the AgNPs from the fruit extracts of *Solanum mammosum* where the reduction of Ag^{+} to Ag^{0} was confirmed by observing the colour change from yellow to brown (41).

Characterization of silver nanoparticles

UV- visible spectroscopic analysis

UV visible spectral analysis of the synthesized nanomaterial exhibited a distinctive peak at 416 nm. According to Loganathan *et al.* (42), *Passiflora subpeltata* leaf extract was used to synthesize silver nanoparticles, which was confirmed by UV-visible spectrometry that indicated a peak at the range of 456 nm. The range of 200 to 450 nm confirmed the formation of AgNPs using the plant extracts. Similarly, other studies report that the synthesis of AgNPs from *Artemisia heba-alba* resulted in the formation of a UV-visible spectral peak at the range of 430 nm (42, 43).

Fourier transform infrared spectroscopy

The FT-IR method utilizes the infrared beam of light, which helps in the identification of the functional groups that are crucial in the reduction or capping of free silver ions that aid in the synthesis of AgNPs. The FTIR spectral stretches of AgNPs synthesized from cannonball leaves correspond to various bio compounds that aid in nano synthesis (44). Similarly, in our study, the band stretches at 3731.4 cm^{-1} corresponds to the O-H group, 3526.17 cm^{-1} corresponds to O-H stretching, 2931.2 cm^{-1} corresponds to the weak bond of the O-H group of alcohol, 1612.9 cm^{-1} corresponds to medium C=C stretching, 1321.14 cm^{-1} corresponds to

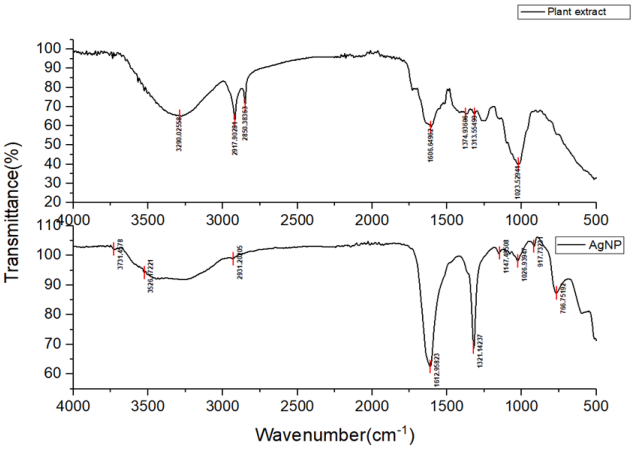

Fig 1. The spectral band stretching corresponds to the presence of bio compounds.

strong S=O sulfone group of compounds and 1147.48 corresponds to strong C- O ester stretching group (**Fig. 1**).

X-ray diffraction (XRD)

The XRD uses monochromatic X-rays in the analysis of the crystalline nature of the sample (**Fig. 2**). The *V. negundo* leaf extract indicated peaks at 27.6°, 32.1°,38.06°, 44.5° and 64.5° of two theta values. The analysis was further predicted using the JCPDS card no: 04-0783 to estimate its crystalline nature at plane angles to (111), (200), and (220). The XRD analysis confirmed the formation of AgNPs of a cubic crystalline nature. Similarly, the study from Santhoshkumar *et al.* (45) indicated the synthesis of AgNPs using *Nelumbo nucifera* (45) and found that the AgNPs formed were of fcc crystalline structure with the Bragg's reflection of (111), (200). (220) and (311). The studies also pointed to the two theta values of the X-ray diffraction

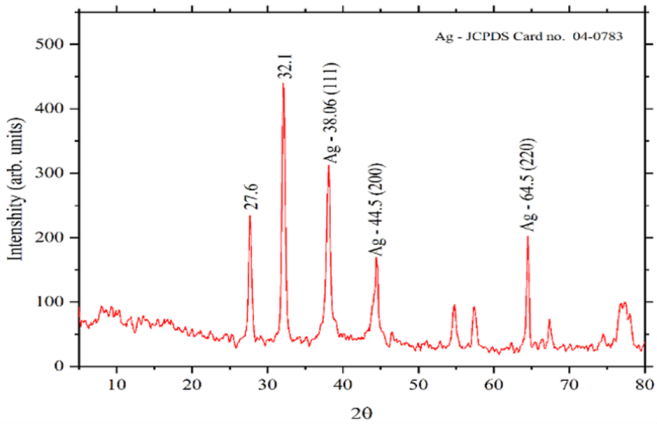

Fig. 2. The X-ray diffraction peaks of silver nanoparticles from the *V. negundo* leaf extract.

peaks at 38.8°, 53.62°, 65.67° and 76.67° that were found to be closely similar to the values that our study reported (45).

Hatchability optimization of *Aedes aegypti* stages

Compared to pH 7, the hatchability percentage was less in pH 9 and was at the lowest in pH 5 (**Fig. 3**). Stagnant water was studied as the best source for larval development compared to tap and reverse osmosis water. This trend is caused by the presence of a high concentration of minerals and other suitable particles in the stagnant water that could serve as a nutrient source for the uninterrupted growth of the larval stages. Studies have shown that the water stored or stagnated in containers and other human-made sources is most suitable for mosquito development (3,46). The study maintained throughout the experimentation was at a pH of 7, and the trays for larval hatching were maintained at room temperature, which has shown the highest hatchability.

The activity of silver nanoparticles against *Aedes aegypti* larvae and pupae

With the exposure of 50 larvae and pupae to varying concentrations of silver nanoparticles in triplicates, the study observed the mortality of larvae and pupae in 24 hours (**Fig. 4**). The highest mortality rate was found when the third instar larvae, fourth instar larvae, and pupae were exposed to 1000 µl/L of *V. negundo* synthesized AgNPs (71.66, 66.67, and 65.00%, respectively). The LC_{50} against the third instar was found to be 441.43 µl/L with 346.93 µl/L and 537.16 µg/L of upper and lower 95% confidence limits. The 50% of LC_{50} against the fourth instar larval stage of *A. aegypti* was found to be 308.74 µg/L with 154.27 µg/L and 422.45 µg/L of upper and lower 95% confidence limits for concentration. The LC_{50} against the pupal stage of *A. aegypti* was found to be 490.66 µg/L with 354.66 µg/L and 654.70 µg/L of upper and lower 95% confidence limit for concentration. The R^2 value of linear probit transformed responses was found to be 0.88, 0.94, and 0.90 for the third instar larvae, fourth instar larvae, and pupae, respectively. The study of Morejón *et al.* (3) synthesized AgNPs using *Ambrosia arborescens* and analyzed its larvicidal activity against the third instar larva of *A. aegypti*. The studies showed that the plant-synthesized AgNPs were highly toxic at lower concentrations (LC_{50} of 250 µg/L). The toxic moiety that causes larval mortality is due to the AgNPs synthesized from *V. negundo.* Synthesized nanoparticle penetrates the larval exoskeleton and causes damage to the surface epithelium and surface cuticle. Additionally, AgNPs bind to the sulfur particles in cello enzymes that disintegrate the organelles and enzyme network (35). Morphological observations like hair loss, necrosis, depigmentation, etc, are also reported due to the action of AgNPs.

In silico analysis

From the list of compounds that are present in the *V. negundo* leaf extract, six compounds were found to follow the rules of Lipinski's rule of five. The molecular mass of the selected compounds was screened for less than 500 Dalton, and the lipophilicity of the compounds was selected based on their LogP value, which was less than 5. The number of hydrogen bond donors was selected on a scale below 5; less than ten hydrogen bond acceptors and molar refractivity between 40-130 were selected as

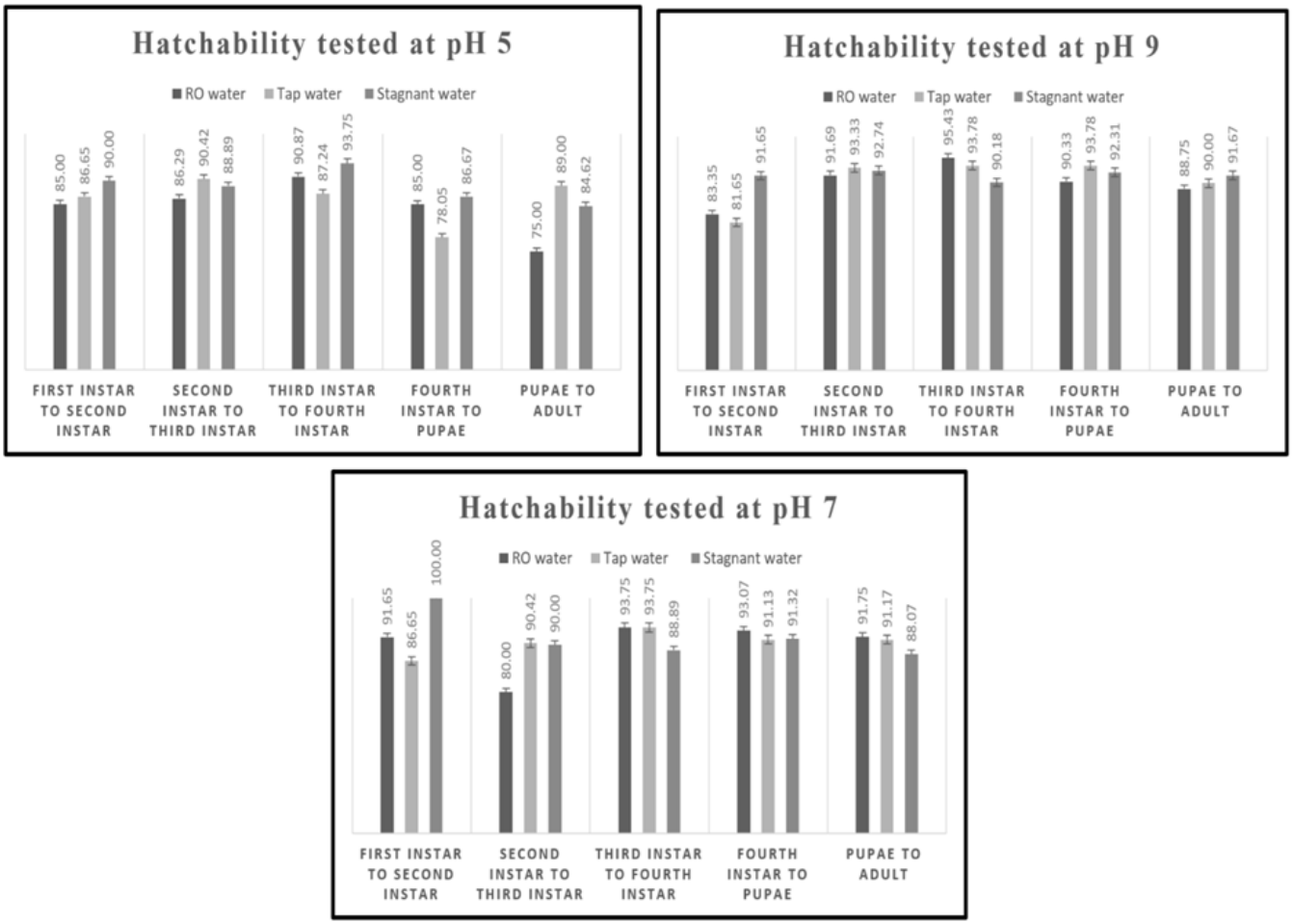

Fig. 3. The larval hatchability tested at three different pH like 5, 7, and 9.

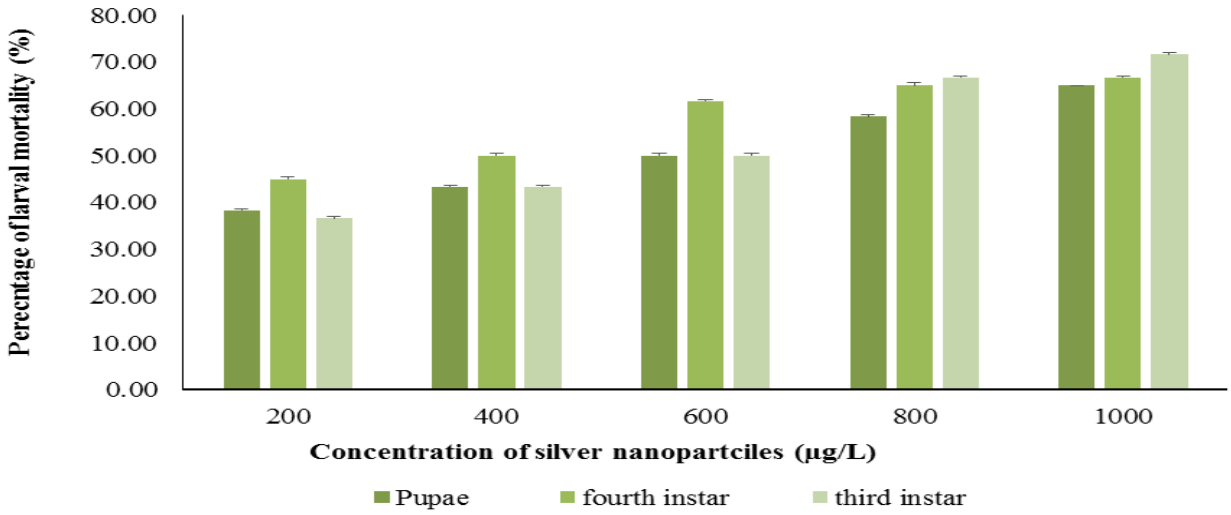

Fig. 4. The larval and pupal mortality percentage was estimated using Abbott's formula.

potential ligand molecules for the protein-ligand interaction studies (Table 2) (47). The bio compounds that were selected as ligand molecules were docked at the active site of mosquito juvenile hormone binding protein using the PyRX tool. Based on the binding affinity, it was found that Z-11(13-Methyl) tetradecane-I-ol acetate had the highest binding affinity with the energy of - 8.2 Kcal/mol, followed by Octadecane which possessed -8.1 Kcal/mol of binding affinity (Table 3). The binding affinity indicated the efficiency of the ligand molecule in inhibiting the JHBP (**Fig. 5**). The ligands are bound at the active site of the juvenile hormone-binding protein, which is assisted by hydrogen bonds. The main amino acids present at the region of the active site are Leu 37, Tyr 33, Phe 144, Val 34, Leu 30, Ala 281, Trp 278, Phe 269, Pro 55, Val 65, and Tyr 133, Ile140, Trp 50, Trp 53, Leu 72, Val 68, Tyr 64, Ser 69, Leu 74, Tyr 129, Val 51. Juvenile hormones play a significant role in the transformation of larvae into pupal stages. Other than their role in developmental roles, juvenile hormone also contributes to the regulation of reproductive and behavioral stimuli (47). Zero hatchability of the mosquito eggs is possible if the expression of Juvenile hormone is minimized (48). The mJHBP inhibition leads to the disruption of the mosquito developmental stages, thus marking the ability of the *V. negundo*-based compounds to act as potential mosquito control. Okoli *et al.* (38) reported that the plant metabolites from *V. negundo* can act as ligand molecules against the mosquito odorant binding proteins (OBP, OBP1, OBP4, and OBP7) (38).

Conclusion

There are widespread fatalities of diseases caused by mosquitoes. *A. aegypti,* being the most efficient in causing the spread of several types of viruses, is a primary target for abolishing fatal diseases. Multiple strategies for controlling these species have not endured long enough to control the diseases or the vectors. Therefore, alternate methods that could be sustained have to be implemented, which led us to design our study using a strong and sustainable source with minimal side effects on the environment. Thus, we used these plants to extract silver nanoparticles that can be more profound in acting against the developmental stages of *A. aegypti* Studies revealed that there is a high efficiency of the synthesized silver nanoparticles when analyzed for LC_{50} against *A. aegypti* larvae and pupae stages. The *in-silico* approach in analyzing the activity of the phytocompounds against the mosquito juvenile hormone binding protein revealed its binding at the active site of the protein that could be crucial in altering the characteristic features of the protein, which in turn can affect larval development. The larvicidal and pupicidal activities of the AgNPs against the *A. aegypti*

Table 2. Compounds selected by Lipinski's rule of 5

PubChem ID	Name of the compound	Molecular Mass (Daltons)	Hydrogen bond donors	Hydrogen bond acceptors	LogP	Molar refractivity
519389	Undecane 4,7 -dimethyl-	184.00	0	0	4.248	73.07
537329	Octane, 3-ethyl-2,7-dimethyl-	170.00	0	0	3.952	67.55
11006	Hexadecane	226.00	0	0	5.146	89.585
11635	Octadecane	254.00	0	0	5.759	100.519
98976	Tetradecane,5-methyl-	212.00	0	0	4.850	84.063
5367701	Z-11(13-Methyl) tetradecen-I-ol acetate	268.00	0	2	4.796	91.812

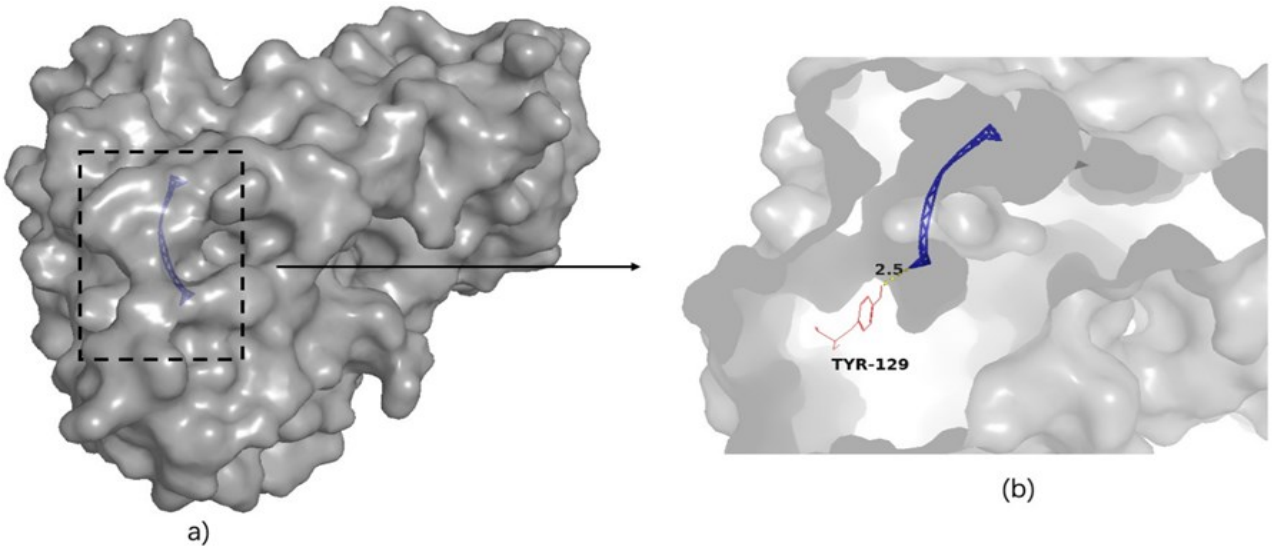

Fig. 5: a) Indicates the juvenile hormone binding protein highlighting its active site for ligand binding b) indicates the binding of Z-11(13-Methyl) tetradecen-I-ol acetate.

Table 3. The binding affinity of the mJHBP docking with compounds from *V. negundo* leaf extract.

Name of the compound	binding affinity (Kcal/mol)	RMSD (ub)	RMSD (lb)
Undecane 4,7 -dimethyl-	-7.5	0	0
Octane, 3-ethyl-2,7-dimethyl-	-7.4	0	0
Hexadecane	-7.7	0	0
Octadecane	-8.1	0	0
Tetradecane,5-methyl-	-7.7	0	0
Z-11(13-Methyl) tetradecen-I-ol acetate	-8.2	0	0

larval and pupal stages showed promising results that will allow it to be considered as a potential repellent candidate. Further investigation into this protein using the phytocompound of *V. negundo* can lead to enhanced drug development in the future.

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Authors' contributions

This research article was produced through collaboration between the authors. Conceptualization: K.A.P.; Writing original manuscript: J.A.G, J.Y.Y; Methodology, data curation, and formal analysis: J.Y.Y., J.A.G., B.B., M.P.; Review and editing, Interpretation: B.B., M.P., K.A.P; All authors have read and agreed to the published version of the manuscript.

Compliance with ethical standards

Conflict of interest: The authors hereby declare that they have no conflict of interest and have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability Statement: The data presented in this study are available on request from the corresponding authors.

Ethical issues: None.

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