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Synthesis of Green Engineered Nanoparticles From Three Himalayan Medicinal Plants And Their Effects on Mosquito Larvae

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Abstract

Mosquitoes pose a serious threat to the world population by serving as vectors for various dreadful diseases. For the control of their growth and propagation various conventional pesticides, microbial control agents, insect growth regulators, etc. have been employed. But, the use of these common conventional pesticides has resulted in various negative impacts on humans, wildlife and the environment. To address these concerns, eco-friendly control strategy of insect vectors is becoming increasingly important in our fight against various mosquito-borne diseases like dengue, chikungunya, West Nile, St. Louis encephalitis, Zika virus, Japanese encephalitis, etc. However, research efforts in this direction are experiencing the lack of effective and eco-friendly pesticides and limited success of most of the current biocontrol agents. Nanotechnology is emerging as a promising field of sciences particularly because of its role in the agricultural sector to improve crop yield and in crop protection. The synthesis of various green nanoinsecticides using different plant products in recent times are thought to be a promising field for the exploration of various pest populations. Therefore, there is a need to screen various plants for their utility in the synthesis of environmentally benign materials like silver nanoparticles (AgNPs) that offer various benefits like eco-friendliness and compatibility for larvicidal application. Indian greeneries are the source of a number of medicinal plants and other beneficial products. These plants have attained importance because of their unique properties, which render them very attractive in the field of research. In present study, three Himalayan medical plants, *Zanthoxylum* (leaves), *Melia* (seeds), *Vitex* (barks), were used for the biosynthesis of AgNPs and the larvicidal activity of these plants extract and their respective nanoparticles were tested against the mosquito larvae, *Culex quinquefasciatus*. Our results indicate that the corresponding AgNPs are more effective than their plants extracts and can, thus, be employed in various mosquito control programmes.

Keywords: Silver nanoparticles, *Zanthoxylum*, *Melia*, *Vitex*, Mosquito larvae.

1. Introduction

Mosquitoes are vectors of a number of pathogens that are threat for the human life. Mosquitoes cause diseases like dengue, West Nile, chikungunya, Zika virus, St. Louis encephalitis, Japanese encephalitis, *etc*. In the recent report of WHO, about 219 million of cases were recorded in 2017, out of which about 435,000 died due to malaria (1). In India, about 1.6 million cases of malaria occur each year out of which 1000-1500 persons die. Control of mosquitoes is very important and critical requirement in controlling the mosquito-borne diseases. Among several mosquito species, *Culex quinquefasciatus* (Diptera: Culicidae), having a worldwide distribution, is regarded as the major vector of *Wuchereria bancrofti*, the filarial parasite responsible for the transmission of lymphatic filariasis in humans [2].

ISSN: 0971-1260
Vol-22-Issue-17-September-2019

Various insecticides namely, DDT, dieldrin, organophosphrous, fenithothion, *etc*. have been widely used in India to control mosquito populations [3]. The indiscriminate use of these synthetic chemical products has resulted in a plethora of undesirable consequences like generation of resistant mosquito strains, elimination of biodiversity by affecting the non-target species, ecological degradation, *etc*. [4]. This has, therefore, prompted mankind to look for some safer alternatives. In last few decades, various attempts have been made to devise and formulate the safe and acceptable pest control agents to mitigate one of the major problems that the mankind has been facing since time immemorial. Nanotechnology has emerged as one of the most active research areas in present day material sciences [5]. Nanomaterial have been defined as the substances possessing at least one dimension in the size range of 100 nm [6] having various application in pharmaceutical, biotechnological and industrial fields [7]. Silver nanoparticles (AgNPs) have emerged as one of the materials of choice because of their unique physico-chemical and biological properties. They have also been reported to possess anti-bacterial, antiviral and anti-fungal properties [8-10]. Nanotechnology is also emerging as the principal branch of science where all medicinal plants as well as normal plant are used for the synthesis of different kind of nanoparticles [11]. These green engineered nanoparticles have the potential to solve this major life-threatening problem as several reports emerging from different parts of the world have confirmed the efficacy of these NPs to control various mosquito species. Naik *et al.,* [12] have reported to have synthesized green AgNPs from *Pongamia pinnata* showing larvicidal activity against the dengue vector. AgNPs were also synthesized from the *Leucas aspera* leaf extract and it indicated the larvicidal activity against *Aedes aegypti* [13].

Indian greeneries are the source of a number of medicinal plants and other beneficial products. These plants have attained importance because of their unique properties, which render them very attractive in the field of research. Therefore, there is a need to screen various plants for their utility in the synthesis of environmentally benign materials like AgNPs that offer various benefits including their eco-friendliness and larvicidal application compatibility. In this regard, we chose three Indian medicinal plants, *Zanthoxylum armatum (*leaves), *Vitex negundo* (bark) and *Melia azedarach* (seed), with an attempt to biosynthesize Ag NPs and investigate the larvicidal effects of these NPs and their corresponding phyto-extracts.

2. Methodology

2.1. Collection of plant samples

Three Himalayan medicinal plants, *Zanthoxylum armatum (*leaves), *Vitex negundo* (bark), *Melia azedarach* (seed*),* were collected from kangra, Himachal Pradesh (India) on the basis of medicinal properties, cost effectiveness, ease of availability, *etc*.

ISSN: 0971-1260
Vol-22-Issue-17-September-2019

Figure 1. *Zanthoxylum* (A), *Vitex* (B), *and Melia* (C) plant species

2.2. Collection of mosquito larvae

The mosquito larvae were collected from a pond near the campus of lovely Professional University, Punjab and brought to the laboratory. The larvae were fed dog biscuit and yeast (3:1).

2.3. Preparation of plants material extracts

Fresh, clean and healthy plant material (*i.e.*, leaves, seeds and barks) were collected and firstly washed with tap water followed by distilled water in order to remove the dust and unwanted

ISSN: 0971-1260
Vol-22-Issue-17-September-2019

material on them**.** The plant material were subsequently cut into small pieces and dried at room temperature separately. From each plant sample material, 10g of powder were weighed separately and then transferred into 250ml beakers containing 100ml of distilled water. The mixture was then boiled for 20 min. Thereafter, to remove the impurities on them, the extracts were filtered three times through Whatman No. 1 filter paper and the clear solution obtained was subsequently stored in refrigerator at 4 °C.

2.4. Preparation of 1mM $AgNO_3$ solution

1mM solution of silver nitrate was prepared by dissolving 0.017gms of silver nitrate in 100 mL of deionized water (DIW) and stored in colored bottle in cool and dry place for future used.

2.5. Synthesis of silver nanoparticles

In 250 ml flasks (A, B, C)**,** 22.5 mL of aqueous solution (1mM) of silver nitrate ($AgNO_3$) was taken in each flasks and 2.5 ml of *Zanthoxylum* leaf extract was added to flask A; 2.5ml of *Melia* seed extract to flask B and 2.5 ml of *Vitex* bark extract to flask C. Flasks containing mixtures of different extracts and silver solution were placed in incubator (BOD INCUBATOR) for 24 hours for the bio-reduction. After 24 hours, change in colour appears in all plants extract containing silver nitrate solution, indicating the formation of AgNPs. The plant extracts were centrifuged at 5000 rpm for 15 minutes, the supernatant was discarded and the resulting pellet was taken, which was then rinsed with deionized water. After that, 4ml of deionized water was added and then centrifuged at 10000 rpm for 8 minutes. Again, the supernatant was discarded and pellet was taken to which 2 ml deionized water was added, and the solution was stored in 4 °C for further use.

2.6. Characterization of AgNPs

2.6.1 UV-vis spectra analysis

To monitor the completion of bio reduction of Ag^+ in aqueous solution, 1ml of each sample was collected periodically followed by its dilution using 2ml of deionized water and then scanned under the UV –vis spectra between the wave lengths of 300 to 800 nm in a spectrophotometer

2.6.2. Fourier transform infrared spectrophotometer analysis

FTIR analysis of the AgNPs was used for the identification of the functional group in these NPs. In the Procedure of FTIR, firstly the pellet of the AgNPs dried. Then dried sample used for the analysis. IR Spectra done in the range of 450 to 4000 cm^{-1}.

2.7. Larvicidal bioassay

The larvicidal activity was estimated by the standard procedure elected by the World Health Organization with little modification [14]. Based on the high and low range of tests, 30, 60, 90, 120, and 150 μg/mL concentration of crude leaves, seeds, barks were taken and 30, 60, 90, 120,

ISSN: 0971-1260
Vol-22-Issue-17-September-2019

150 μg/mL concentration of respective AgNPs were taken. For our experiments, sixty mosquito larvae (third and fourth instar) were added into 100 ml of beakers containing 99 mL of double distilled water (DDW) and 1ml concentration of crude plant extracts or their respective nanoparticles were added. Three replicates of each concentration of crude plants extract and their AgNPs were run. Larval mortality was recorded after 0 hour, 6 hours, 12 hours, 24 hours period at room temperature. During the experimental period no food was offered to larvae and for each test one control was taken. The mortality was calculated using the following Abbott formula:

$$\textit{Mortality} = \frac{\textit{Number of mosquito dead after treatment}}{\textit{Total number of mosquitos used in treatment}} \times 100$$

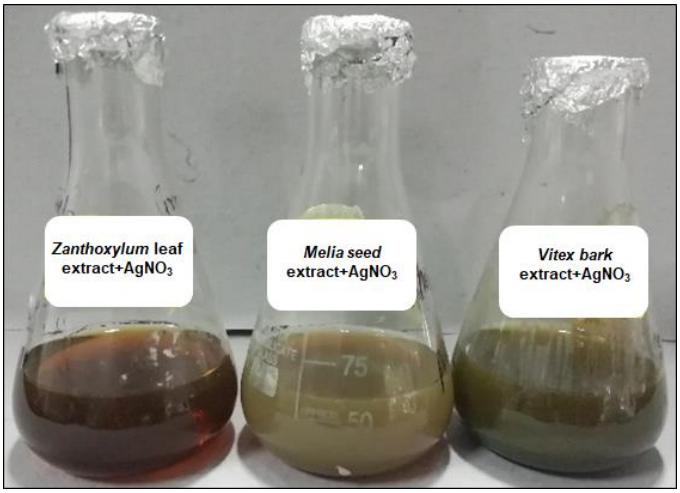
2.8. Statistical analysis

Mean mortality caused due to the exposure of different concentrations of plant extracts and their respective AgNPs after various time points were analyzed by using analysis of variance method. Two-way ANOVA was used to compare the results of various groups. In all the tests, 5% significance level was used and the statistical significance of mean differences was assessed by Dunnett's multiple comparison test. The analyses were made by using Prism GraphPad version 6.0.

3. Result

3.1. Colour confirmation

The primary confirmation of synthesis of AgNPs was done by the colour change of the solution (Fig. 1). The incubation of Leaf extract of *Zanthoxylum* and AgNO3 solution for 24 hours resulted in change in colour of solution. The colour of solution at the starting of experiment was wine red which turned into dark red after incubation indicating the formation of the AgNPs by *Zanthoxylum* leaf extract. In second plant, after the incubation of *Melia* seed extract and silver nitrate solution for 24h, the colour changed from the lemon colour to the greenish gray. In third plant, the colour of the mixture of *Vitex* bark extract and silver nitrate solution changed from the light brown to olive after 24 hr incubation. The change in the colour of the mixture of silver nitrate and different plants extract was due to surface plasmonic resonance of different types of AgNPs.



ISSN: 0971-1260
Vol-22-Issue-17-September-2019

Figure 2. Colour change confirming the formation of AgNPs by the *Zanthoxylum aramtum* leaf extract, *Melia azedarach* seed extract and *vitex negundo* bark extract.

3.2. Uitra violet visible spectroscopy

Silver nanoparticles synthesized from the mixture of *Zanthoxylum* seed extract and $AgNO_3$ solution showed maximum absorption in 480 nm (Fig. 2). This absorption peak confirms the synthesis and fabrication of AgNPs. AgNPs synthesized from the mixture of seed extract of *Melia* and $AgNO_3$ solution showed the maximum absorption in 470 nm (Fig. 3) indicating the synthesis of AgNPs. Similarly, AgNPs synthesized from the mixture of *Vitex* bark extract and $AgNO_3$ solution showed the maximum absorption in 400 nm (Fig. 4) indicating the bio reduction of silver ions into AgNPs.

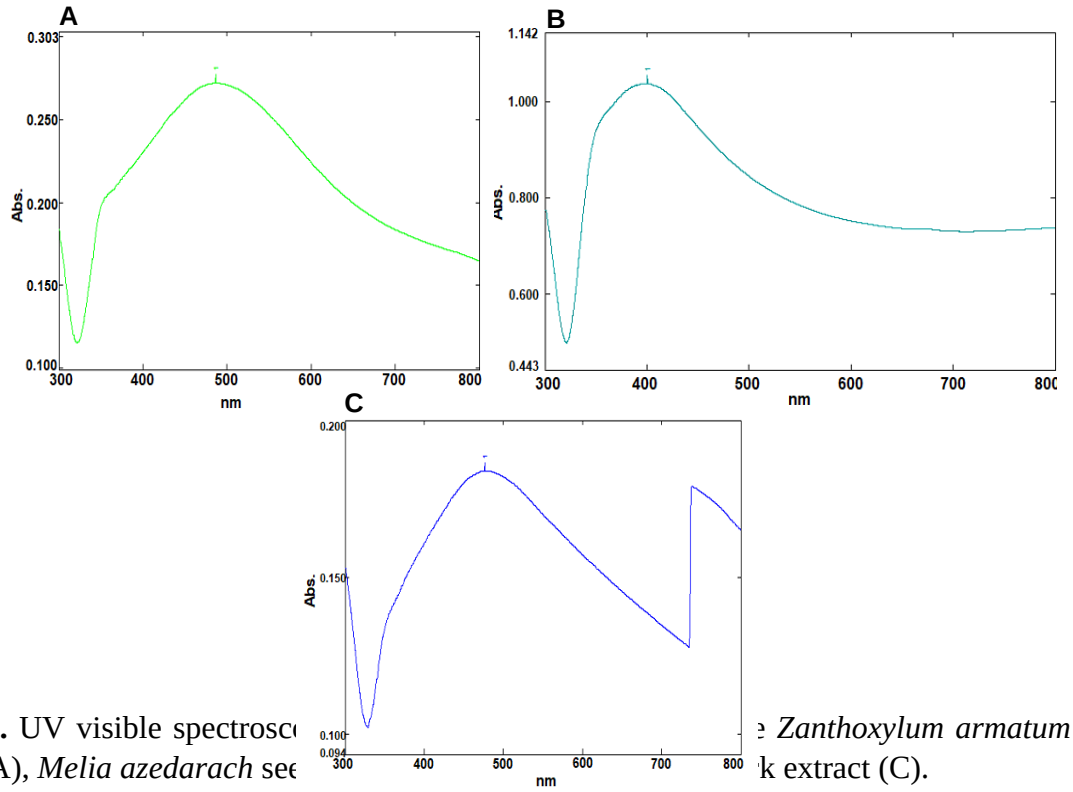


Figure 3. UV visible spectrosc e *Zanthoxylum armatum* leaf extract (A), *Melia azedarach* see k extract (C).

3.3. Fourier transform infrared spectroscopy analysis

The FTIR analysis of the *Zanathoxylum* leaf extract showed the major peaks (1066.74, 1572.04, 2339.73, 3724.67 cm^{-1}) in both finger print region as well as functional group region (Fig. 5A). The broad absorption peak at 1066.74 cm^{-1} may be due to the presence of the C-O streaching vibration of alcohal group, another broad peak at 1572.04 cm^{-1} because of the presence of C=C streaching of the cyclic alkene, and the strong peak at 2339.73 cm^{-1} due to the O=C=O steaching vibration of carbon dioxide. The last weak peak at 3724.67 cm^{-1} represents OH streaching of alcohol. The FTIR analysis of AgNPs synthesized from *Zanthoxylum* leaf extract showed the major peaks at 1064.74, 1585.54, 2355.16, 3257.88 cm^{-1} (Fig. 5B). The strong absorption peak at 1064.74 cm^{-1} also resprented C-O streaching vibration of alcohal group. The broad absorption

ISSN: 0971-1260
Vol-22-Issue-17-September-2019

peak at 1585.54 cm^{-1} assigned to C=C streaching vibration of cyclic alkene. The weak peak at 2355.88 cm^{-1} indicated O=C=O streaching vibration of carbon dioxide. The broad peak at 3257.88 $cm^{-1}$was due to OH streaching of alcohal group. The reduction in peaks from extract to the AgNPs formartion indicates the reduction of functinal group in C-O streaching , C=C streaching and O=C=O streaching, OH streaching as a capping and reducing agent.

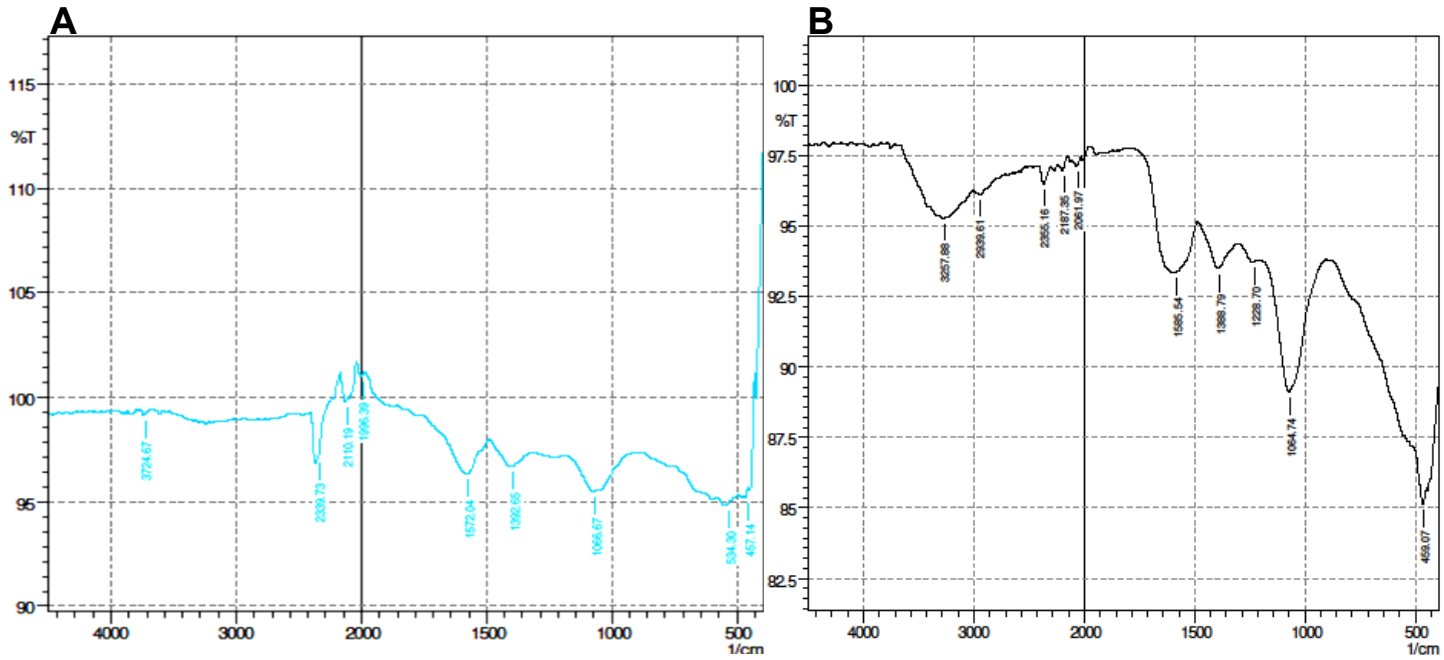


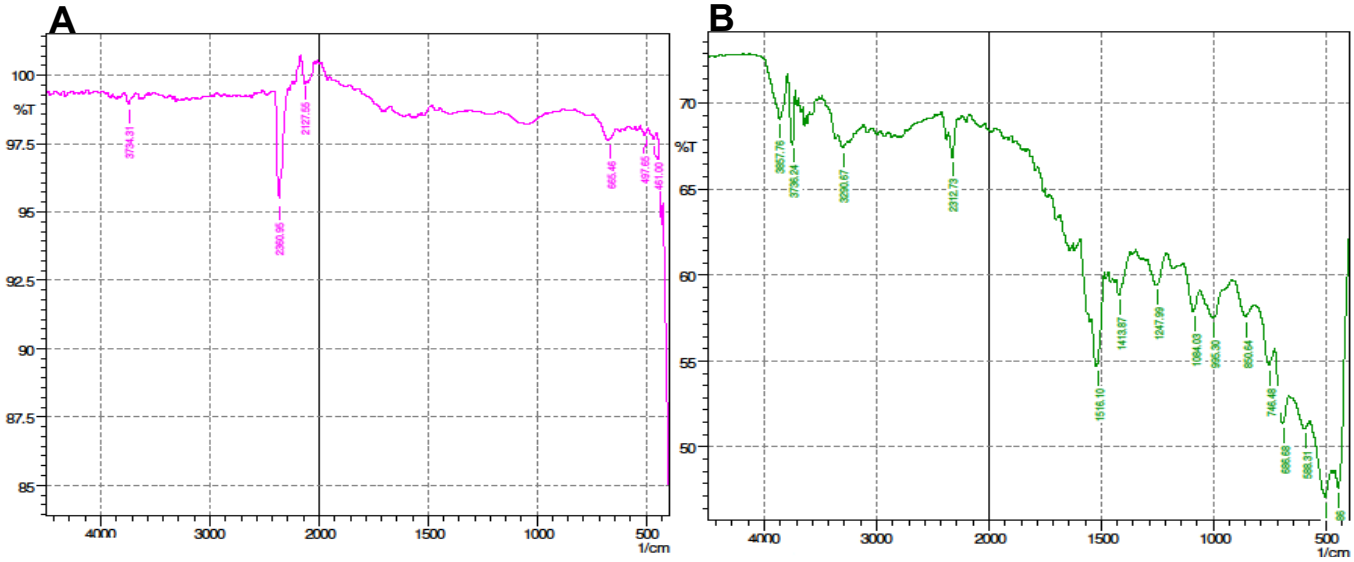
Figure 4: - FTIR analysis of *Zanthoxylum* leaf extract (A) and AgNPs synthesized from it (B).

The FTIR analysis of the *melia* seed extract showed the major peak in both finger print region as well as functional group region. Melia seed extract showed the major peak at 665.46, 2360.95, 3734.31 cm^{-1}. The weak absorption peak at 665.46 cm^{-1} might be becaue of C=C bending of alkene group. Very sharp and strong absortption peak at 2360.95 cm^{-1} signaled to O=C=O streaching of carbon dioxide. The little sharp and weak peak at 3734.37 cm^{-1} may indicate the presence of OH streaching of alcohol group. FTIR analysis of silver nanoparticles synthesized from the *Melia* seed extract showed a number of major peaks at 1024.24, 2362.88, 3267.52 cm^{-1} . The broad and strong absorption peak at 1024.24 cm^{-1} indicates C=C streaching of alkene group. The medium sharp and strong peak at 2362.88 cm^{-1} may be assigned to O=C=O stretching of carbon dioxide.The reduction in peaks from extract to the silver nanoparticles formartion indicate reduction of functinal group in C=C streaching , C=C streaching and O=C=O as a capping and reducing agent (Fig. 5).

ISSN: 0971-1260
Vol-22-Issue-17-September-2019

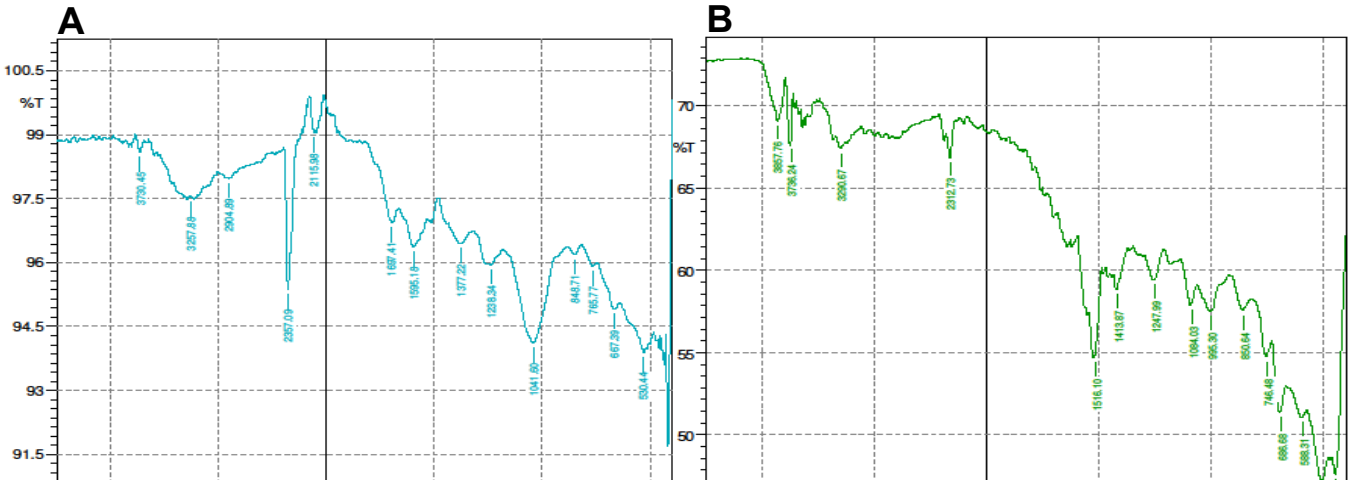
Figure 5. FTIR analysis of *Melia* seed extract (A) and AgNPs synthesized from it (B).

The FTIR analysis of bark extract of *Vitex* showed the major peak in the finger print region and functional region. The major absorption peaks at 1041.60, 1595.18, 2357.09 and 3257.58 cm^{-1}. The broad and strong peak at 1041.60 cm^{-1} was due to presence of the C-O streaching of alcohal. Broad and weak peak at 1585.18 indicated C=O streaching of nitro compounds group. the very strong and sharp peak at 2357.09 indicated the presnce of O=C=O streaching of carbon dioxide.



The broad and weak peak at 3257.58 in the presence of OH streaching of carboxylic acid. The FTIR analysis of AgNPs synthesized from *Vitex* bark extract showed major peak in the functional group region as well as functional group region. The major absorption peaks at 1084.03, 1516.10, 2312.73 and 3290.67.

The sharp and weak peak at 1084.03 cm^{-1} may indicate the presence of C-O steraching vibration of primary alcohal group. Thestrong and sharp peak at 1516.10 signaled to C=O streaching vibration of nitro compund. The sharp absorption peak at 2312.73 cm^{-1} O=C=O streaching of carbon dioxide. The broad peak at 3290.67 may indicate the presence of the OH streaching of carboxylic group. The reduction in peaks from extract to the AgNPs formation indicates the reduction of functinal group in C-O streaching , C=O streaching., O.=C=O steraching, OH streaching .as a capping and reducing agent.



ISSN: 0971-1260
Vol-22-Issue-17-September-2019

Figure 6. FTIR analysis of *Vitex* bark extract (A) and the AgNPs synthesized from it (B).

5.3 Effect of Plants extract and silver nanoaprticles on msoquito larvae

Three diffferent plants extract and their respected AgNPs were used to observe their effects on mosquito larave. Five different concentration 30µg/mL, 60µg/mL, 90µg/mL, 120µg/mL, 150µg/mL of each plant extract and their respective AgNPs were used in the present study to observe the time dependent and dose dependent responses.
The analysis of larvicidal activity of leaf extract of *Zanthoxylum* and the respective NPs synthesized from it against the *Culex* mosquito are shown in Fig. 7. Although the leaf extract alone showed significant larvicidal activity at higher concentrations, the resulting NPs showed significant larvicidal activities at every time point even at lower concentrations (Fig. 7 A & B). The larval mortality increased with increase in dose as well as larval exposure time reching the maximum mortality of 63% recorded in 150µg/mL after the 24 hours of treatment.

Fig. 8 indicates the comparison of mortality caused by different concentration of *Vitex* bark extract and the AgNPs synthesized from *Vitex* bark extract after different time intervals. In the case of bark extract, significant mortality was induced by the highest concentration (150µg/mL) after 12 hours of exposure. As the time of exposure increased, apart from the highest concentration (150µg/mL), even the lower concentrations (60µg/mL, 90µg/mL, and 120µg/mL) induced significant mortalities (Fig. 8A). Compared to this, the AgNPs syhthesized from the bark extract was observed to result in significant larvicidal activity after 6 hour exposure in the case of higher concentrations, *i.e.*, 90µg/mL, 120µg/mL and 150µg/mL), and after 12 and 24 hours for all concentrations used. The larval mortality increased in response to dose as well as exposure time (Fig. 8B).

The comparison of mortality caused by different concentration *Melia* seed extract and the AgNPs synthesized from this seed extract after different time intervals indicates that at lower concentrations the extract doesn't induce significant mortality for any of the concentrations used. Significant larvicidal activity was observed for the higher concentrations after 12 hour and subsequent exposure time (Fig. 9A). But, in the case of the respective NPs, significant mortality was induced for all the concentrations above 30µg/mL after 6 hours of exposure. For rest of the exposure times all the used concentrations of the NPs were observed to cause significant larval mortality. The highest mortality was recorded in the highest concentrations after every exposure time and all the concentrations showed maximum activity at their maximum exposure time thus indicating dose and time dependent activity of these NPs.

ISSN: 0971-1260
Vol-22-Issue-17-September-2019

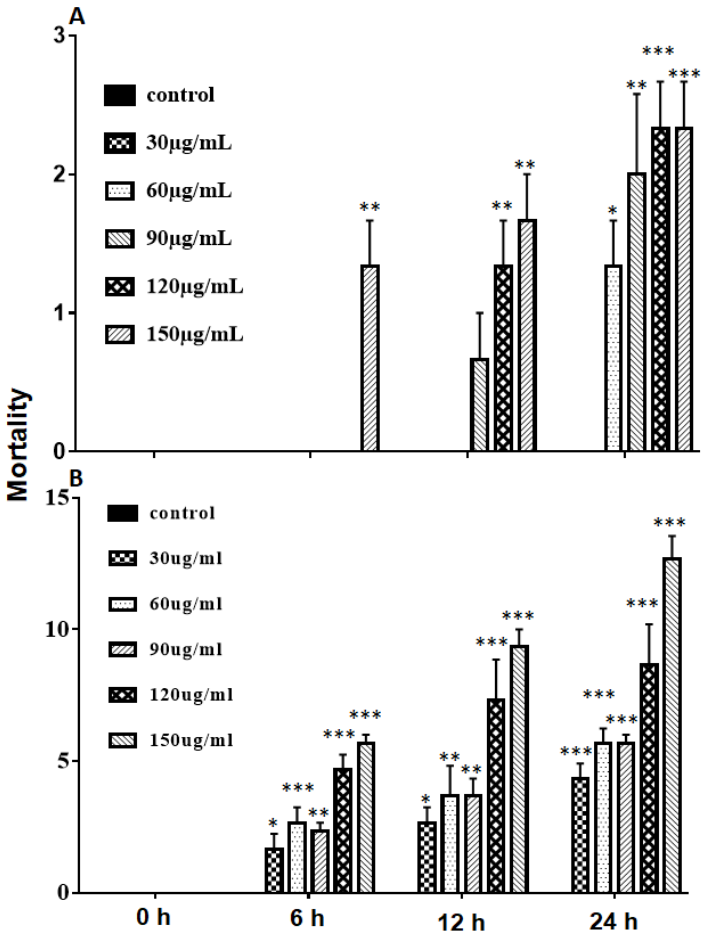


Figure 7. The larvicidal activity of *Zanthoxylum* leaf extract (A) and the AgNPs synthesized from it (B) against mosquito larvae after 0h, 6h 12h and 24h. The data represents the mean mortality (n=60) induced by the treatment, the statistical analysis of which was done by Two-way ANOVA test, followed by Dunnett's test for multiple comparison. *P≤ 0.05, **P≤0.01, ***P≤ 0.001 *vs* control.

ISSN: 0971-1260
Vol-22-Issue-17-September-2019

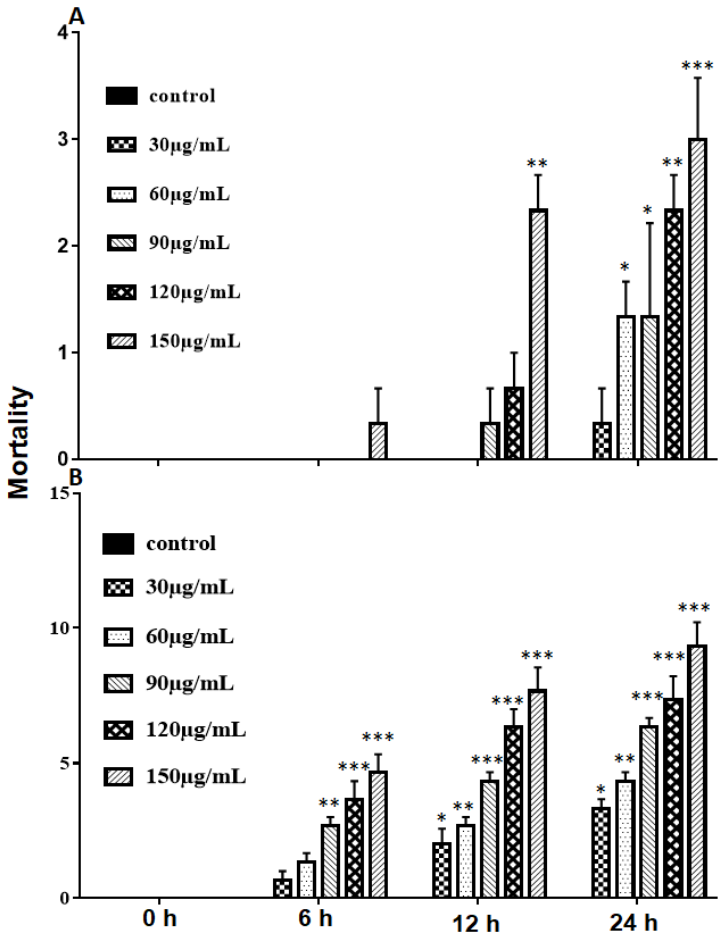


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Figure 8. The larvicidal activity of *Vitex* bark extract (A) and the AgNPs synthesized from it (B) against mosquito larvae after 0h, 6h 12h and 24h. The data represents the mean mortality (n=60) induced by the treatment, the statistical analysis of which was done by Two-way ANOVA test, followed by Dunnett's test for multiple comparison. *P≤ 0.05, **P≤0.01, ***P≤ 0.001 *vs* control.

ISSN: 0971-1260
Vol-22-Issue-17-September-2019

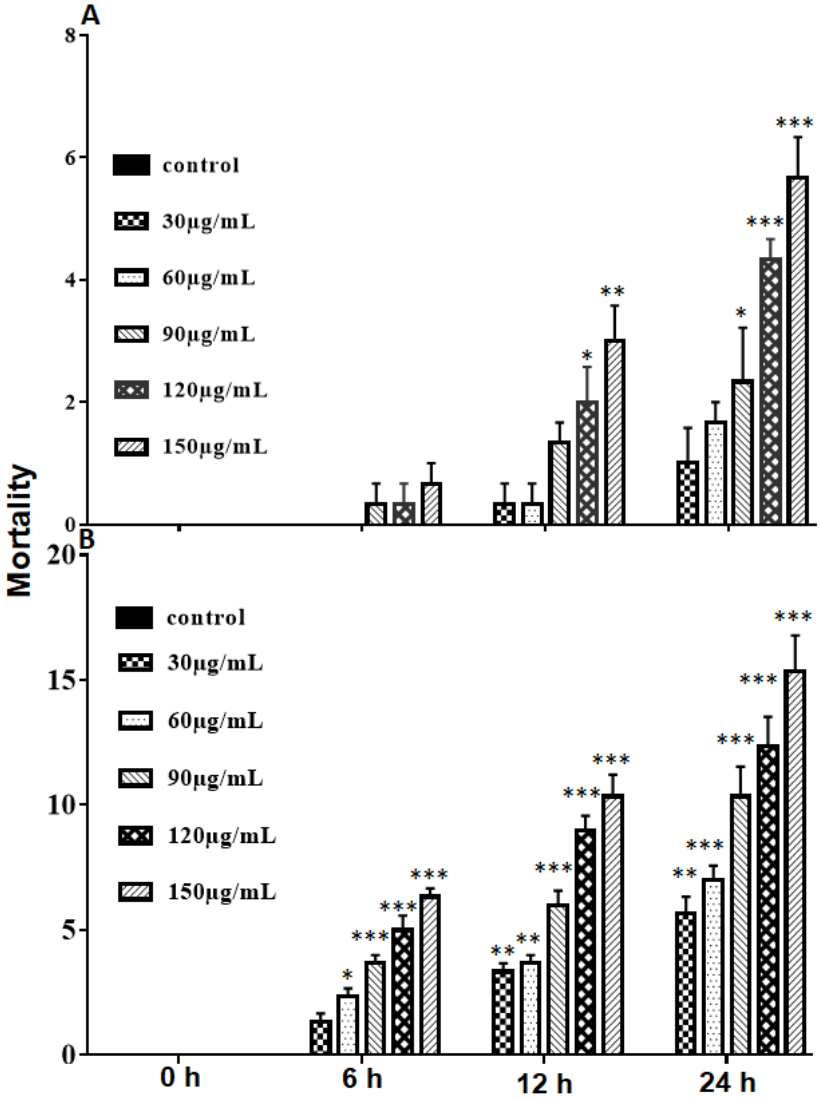

Figure 9. The larvicidal activity of *Melia* seed extract (A) and the AgNPs synthesized from it (B) against mosquito larvae after 0h, 6h 12h and 24h. The data represents the mean mortality (n=60) induced by the treatment, the statistical analysis of which was done by Two-way ANOVA test, followed by Dunnett's test for multiple comparison. *P≤ 0.05, **P≤0.01, ***P≤ 0.001 *vs* control.

Discussion

Nanotechnology is one of the fast developing technologies in the field of science in present world. In nanotechnology, different types of nanoparticles are synthesized. These nanoparticles are of different sizes as well as different shapes. In recent times, metallic nanoparticles like AgNPs, platinum nanoparticles, gold nanoparticles, etc. have been used to control the insect and other harmful organism. These nanoparticles also used in different pharmaceutical products [15].

The colour change is the key factor that determines the reduction of silver ions leading to the formation of corresponding NPs [16]; for instance, when leaf extract of *Parthenium* was mixed

ISSN: 0971-1260
Vol-22-Issue-17-September-2019

with aqueous silver ion solution, its water color started to change to yellowish brown, probably, due to extraction of surface plasmon vibrations indicating the formation of AgNPs. The plant extracts are emerging as potential agents for the control of various mosquito populations due to their cost effectiveness, easy-to-administer, ecofriendly properties, *etc*. [17, 18].

In this study, when *Zanthoxylum* leaf extract, *Melia* seed extract and *Vitex* bark extract were added in the silver nitrate solution resulting in colour changes due to the surface plasmon vibrations occurring due to the formation of AgNPs. Thereafter, the effects of these phytoextract and their respective silver nanoparticles on mosquito larvae were also analyzed. Our results indicate that the synthesized Ag NPs of the plant extracts are stable and have significant larvicidal properties against the mosquitoes. Furthermore, all three plants extract were comparatively less effective than their respective AgNPs, and the AgNPs synthesized from *Melia* seed extract were more effective than other two nanoparticles resulting in as high as 76% larval mortality. Previous studies have also reported that phyto-synthesized AgNPs are effective against the mosquito larvae and increase the mortality of mosquito larvae with increase in the concentration [19]. The present study, therefore, opens the possibility for future screening of other plant species for their repellent and larvicidal properties. Therefore, Nanopesticides provide novel class of plant protection products that promise a number of benefits to humans, wildlife and the environment. However, both benefits and potential risks to the environment are still poorly understood. Research in this area although active is still in its infancy and it is expected to provide answers on their future applicability, possible risks and economic acceptance.

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Vol-22-Issue-17-September-2019

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