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Larvicidal and Antibacterial Efficacy of Green Synthesized Silver Nanoparticles Using Aqueous Leaf Extract of *Cinnamomum Verum*

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KEYWORDS
Silver nanoparticles, *Cinnamomum verum*, antibacterial, larvicidal activity.

ABSTRACT:

Introduction: Green synthesized silver nanoparticles (Ag NPs) have wide applications in current scenario. The present study used aqueous extracts of *Cinnamomum verum* leaves for the green synthesis of silver nanoparticles.

Objectives: The study focused on the investigation of antibacterial and larvicidal potentials of green synthesized silver nanoparticles of *C. verum* aqueous leaf extracts against bacterial pathogens and *Aedes aegypti*'s (Diptera: Culicidae) fourth instar larvae respectively.

Methods: Fourth instar larvae of *A. aegypti* were exposed to various concentrations of Cinnamomum-Ag NPs (50, 100, 200, 400 and 800 ppm) and distilled water was maintained as control in two timespans namely 24 hours and 48 hours exposure. The antibacterial activity of green synthesized Ag NPs using *C.verum* leaf extract was carried out with disc diffusion method

Results: The results obtained from scanning electron microscopy, X-ray diffraction and Fourier transform infrared support the biosynthesis and characterization of silver nanoparticles. Transformation of colour from pale yellow to brick red of the leaf extract and $AgNO_3$ solution showed the formation of Cinnamomum-Ag NPs. Significant mortality occurred at all concentrations except 50 ppm during the 24 hour treatment and at all concentrations during the 48-hour treatment. The LD50 value was found to be 208.1 ppm (95% confidence limit between 175.34 and 249.67 ppm). Green synthesized Cinnamomum-Ag NPS showed antimicrobial effect against *Streptococcus mutans* (14mm), *Staphylococcus aureus* (8mm) and *Pseudomonas aeruginosa* (7mm).

Conclusions: The results suggests that Cinnamomum-Ag NPS is a potent larvicide against *A. aegypti* and an effective antibacterial agent.

1. Introduction

Mosquitoes are the major disease vectors, mainly in the tropics. They spread a wide range of diseases like Dengue fever, malaria and filariasis to both humans and other mammals. In order to reduce the incidence of disease, the mosquito population must be well controlled [1]. *Aedes aegypti*, commonly called the yellow fever mosquito, is a major vector for dengue fever, chikungunya, Zika fever and yellow fever virus, and other disease causing agents [2]. *A. aegypti* is one of the main vectors, affecting the lives of hundreds of millions of people every year [3]. The incidence of dengue fever has increased to 50 times in the last 30 years. The imperative reason for the surge of dengue fever are the increased breeding places for the *Aedes* mosquitoes, less effective control methods for mosquito, more urbanization as well as increased growth of population [4].

The main tools to control mosquitoes are larvicides. Organophosphates including temephos, methoprene, growth inhibitors as well as bacterial insecticides like *Bacillus thuringiensis israelensis* and *Bacillus sphaericus* are the most widely used larvicides [5-6]. Sometimes persistent use of chemical insecticides causes

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reduction in their efficiency [7] and ensues environmental pollution and ecological imbalance. To solve this problem and to reduce the incidence of mosquito borne disease biological products can be used as an alternative strategy. Biological control by natural products can be long lasting, economic, and harmless to living organisms and ecosystems.

As a possible effective replacement to chemical synthetic insecticides, silver nanoparticles (Ag NPs) can be used, as it is less likely to cause ecological damage. Silver exhibits strong toxicity to a varied array of microbes and are used in numerous antibacterial applications. Silver nanoparticles have been reported for its antifungal [8], anti-inflammatory [9] and anti-viral activities [10]. Depending on size and form silver nanoparticles have different properties and also stated that many plants can be used for the green synthesis of Ag NPs [11].

Pharmaceutical, industrial and biotechnological fields now have greater demand for nanoparticles. Owing to the significant application of nanoparticles in producing compounds displaying antimicrobial, anticancer and anti-oxidant activities nanoparticles and nano emulsion systems from the extracts of plants has gained great relevance in recent years [12-14]. The synthesis of nanoparticles expending plants are swift, cost-effective, environmental and harmless for human use [15]. Even low concentration of silver nanoparticles shows high toxicity against microbes including bacteria, viruses as well as fungi but on the other hand, low amounts of silver nanoparticles is known to be non-toxic to eukaryotic cells including humans [16].

Objectives

Objectives of the study included the preparation of silver nanopartciles using the aqueous extract of *Cinnamomum verum* leaves, to investigate the larvicidal activity of Cinnamomum-Ag NPs on the fourth instar larvae of *Aedes aegypti* and to study the antibacterial activity of the green synthesized Ag NPs of *C.verum*.

3. Methods

a. *Cinnamomum verum*-plant selected

Cinnamomum verum, true cinnamon or Sri Lankan cinnamon are small to intermediate sized trees, with oblong, ovate shaped, dark glossy green leaves. It has originated in the central hills of Sri Lanka. In India it is grown in Kerala and in the North eastern parts.

Cinnamon, the eternal tree of medical science, is aromatic and many of them are used as spices and herbal drugs by the people all over the world. Various compounds such as alkaloids, flavonoids, coumarins, tannins, terpenoids, saponins, glycosides and phenolics are found in the plant [17]. The compounds cinnamaldehyde, eugenol, caryophyllene, cinnamyl acetate and cinnamic aced were characterised from the leaf essential oil [18]. The cinnamaldehyde and trans-cinnamaldehyde in the essential oil are responsible for the fragrance and various biological activites. The essential oil extracted from the leaves of Cinnamon have antibacterial and antifungal properties [19].

b. Collection of larvae

Aedes aegypti larvae were collected from rain water filled pots located inside the Government College for Women, Thiruvananthapuram. The larvae were collected and transferred in to plastic bottles.

c. Preparation of *Cinnamomum verum* leaf extract

Cinnamomum verum plant leaves were collected from Paruthipara, Thiruvananthapuram district. The leaves were weighed and thoroughly washed with running tap water and followed by cleaning with distilled water for removing impurities. 50 g of fresh leaves were cut into tiny pieces and transferred in to a conical flask with 250 mL of distilled water and it is then left in a magnetic stirrer [20] at 70 degree Celsius for one hour. Then filtered with Whatman No. 1 filter paper and the filtrate was kept in the refrigerator in a container.

d. Preparation of $AgNO_3$ stock solution

$AgNO_3$ stock solution was made by dissolving 0.85 g of $AgNO_3$ in 500 mL distilled water.

e. Production of silver nanoparticles

Cinnamomum verum leaf extract and silver nitrate stock solution were mixed to the ratio of leaf extract: silver nitrate 1:10 v/v to obtain the final product [21]. This was performed by mixing 50 mL of *Cinnamomum verum* leaf extract with 500 mL of silver nitrate stock solution. The mixture was then incubated at room temperature for 24 h in a stirrer with continuous stirring, until the solution become brick red colour. After 24 h it was centrifuged at 5000 rpm for 20 minutes. The supernatant was discarded and 5mL of distilled water was added to the precipitate and centrifuged again at 5000 rpm for 20 minutes, this

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procedure was repeated once again to thoroughly wash the nanoparticles. The precipitate obtained was desiccated in a hot air oven for 24 h and the dried powder was used for further confirmation.

f. Larvicidal bioassay

The test included a control which is distilled water, and different concentrations of synthesized silver nanoparticles. Each bottle comprised 30ml tap water and different concentrations of silver nanoparticles such as 50ppm, 100ppm, 200ppm, 400ppm, 800ppm of green synthesized Ag NPs. 10 number of 4th instar larvae of *Aedes aegypti* were added to each bottle. The number of dead larvae were counted after 24h and 48h of exposure and the test is repeated for average value.

g. Statistical analysis

The data obtained from larval mortality were subjected to probit analysis for calculating LD50 and 95% confidence limits between upper and lower limits using the software SPSS 13 for Windows.

h. Antibacterial activity

The antibacterial activity of green synthesized Ag NPs using *C.verum* leaf extract was carried out with disc diffusion method against *Klebsiella pneumoniae* (ATCC 700603), *Pseudomonas aeruginosa* (ATCC 9027), *Staphylococcus aureus (*ATCC 33591), and *Streptococcus mutans* (ATCC 25175). The incubated bacterial culture poured in to respective Petri plates containing solidified agar. A sterile disc with distilled water was placed as control and a sterile disc dipped in Ag NPs was positioned in each plates and were subjected to overnight incubation. After incubation the clear zone appeared around the disc containing Ag NPs and it was measured. These clear zones are known as zone of inhibition. Around the control disc inhibition zone did not appeared.

i. Characterization of Ag NPs

The formation of silver nanoparticles were confirmed by X – Ray diffraction, FTIR and SEM analysis.

For studying the crystalline nature of the particle XRD was used. The well shaken mixture of silver nitrate solution and plant extracts were centrifuged at 5000 rpm for 20 minutes. Centrifugation was carried out 3 times. The resulting pellet was used for XRD, and FTIR analysis after converting in to powder form through drying. For XRD analysis Bruker D8 advance XRD instrument was used.

For FTIR analysis Shimadzu FTIR IR prestige 21 was used. FTIR is a procedure to detect organic, polymeric and inorganic materials. FTIR analysis can be used for qualitative as well as quantitative analysis. The potential reducing and capping biomolecules in the synthesized Ag NPs were found via FTIR analysis.

For identifying the morphology of synthesized Ag NPs SEM analysis was carried out using Carl Zeiss Evo 18 Research model instrument. Scanning electron microscopy was used for the characterization of surface morphology of Ag NPs. The sample was made by the centrifugation of Ag NPs solution at 5000 rpm for 20 minutes. 5mL distilled water was added to the resulting pellet and again centrifuged at 5000 rpm for 20 minutes. Repeated the procedure once again. The resulting pellet was dried in to powder form and used for the analysis.

4. Results

a. Characterization of Ag NPs

Ag NPs made from leaf extract of *C. verum* by the green synthesis method were characterized with different procedures such as colour change, X-ray diffraction, FTIR and SEM analysis.

i. Colour shift during the synthesis

Change in colour of the solution from pale yellow to brick red was observed.

ii. X-ray Diffraction analysis

The X-ray diffraction pattern of Ag NPs produced by leaf extract of *C. verum* is shown in Fig. 1. XRD analysis revealed four well resolved diffraction peaks at 2θ angle of approximately 568, 100, 208 and 223 which are associated with the crystallographic planes of the face cantered structure of silver nanoparticles 37.9°, 44.4°, 64.296° and 77.236°,respectively.

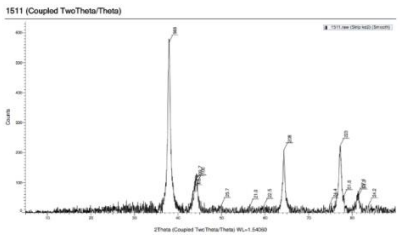

Fig. 1. XRD pattern of silver nanoparticles synthesized using leaf extracts of C. verum

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iii. FTIR –Fourier transform infrared spectroscopy

The spectra of Ag NPs showed various strong bands (Fig. 2). The strong band at 3209 cm^{-1} correspond to O-H stretch of alcohols or phenols [22].

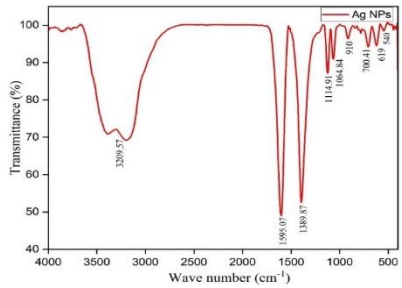

Fig.2. FTIR spectra of silver nanoparticles synthesized using leaf extracts of *C. verum*

The band at 1595 cm^{-1} correspond to C-C stretch of aromatic compounds. Band at 1389 cm^{-1} correspond to C-H bend of aldehyde groups. Band at 1114cm^{-1} and 1064 cm^{-1} correspond to C-N stretch of aliphatic amines. Band at 910 cm^{-1} correspond to N-H wag of primary, secondary amines. Band at 700 cm^{-1} correspond to alkenes. Band at 619 cm^{-1} correspond to alkyl halides. Peak at 540 cm^{-1} AgO or pure Ag NPs.

iv. SEM analysis

Carl Zeiss Evo 18 Research model Scanning Electron Microscope (SEM) was used for obtaining image to find out the shape of the particles developed. SEM analysis showed that the most of the nanoparticles were coarsely with spherical shape having smooth edges (Fig.3)

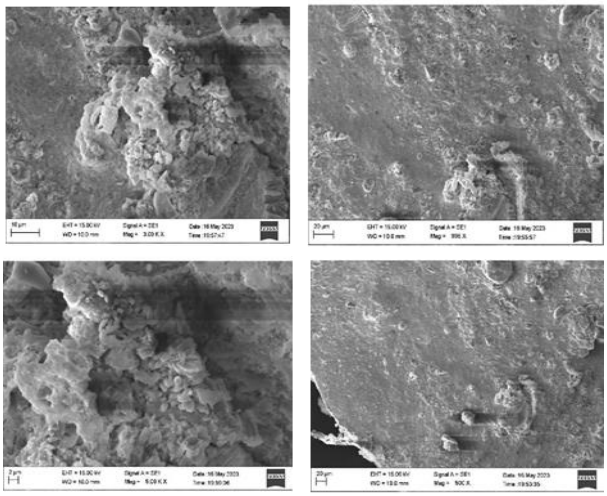

Fig 3. Scanning Electron Microscope images of silver nanoparticles synthesized using *C.verum* leaf extracts

b. Larvicidal activity of green synthesized Ag NPs from *Cinnamomum verum*

The green synthesized Ag NPs using fresh leaves of *C. verum* were subjected to larvicidal bioassay on 4th instar larvae of *Aedes aegypti* at varying concentrations (50, 100, 200, 400 and 800 ppm) and distilled water as control. Significant mortality occurred at all concentrations except 50 ppm during the 24 hour treatment and at all concentrations during the 48-hour treatment. (Table 1). The LD50 value was found to be 208.1 ppm (95% confidence limit between 175.34 and 249.67 ppm).

Table 1. Larvicidal activity of green synthesized Ag NPs on fourth instar larvae of *A. aegypti*

Concentration (ppm)	Mortality (%)	
	24hr	48hr
Control	--	_
50	0.33 ± 0.33	1.33 ± 0.33*
100	1.33 ± 0.33*	2.67 ± 0.33*
200	2.33 ± 0.33*	4.33 ± 0.33*
400	7.67 ± 0.33*	9.33 ± 0.33*
800	9.33 ± 0.33*	10.00 ± 0.33*

*values are mean ± SE of three replicates,
*significance at 0.01 level
– no mortality

c. Antimicrobial activity of green synthesized Ag NPs

The green synthesized Ag NPs showed antimicrobial effect against the Gram positive bacteria *Streptococcus mutans* (14mm), *Staphylococcus aureus* (8mm) and Gram negative bacteria *Pseudomonas aeruginosa* (7mm), and no antibacterial activity against *Klebsiella pneumonia* (Table 2, Fig. 4-7).

Table 2. Antibacterial activity of silver nanoparticles synthesized using the leaf extracts of *C. verum*

Sl. No.	Bacterial species	Zone size (mm)
1	*Streptococcus mutans*	14
2	*Staphylococcus aureus*	8

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3	*Pseudomonas aeruginosa*	7
4	*Klebsiella pneumoniae*	____

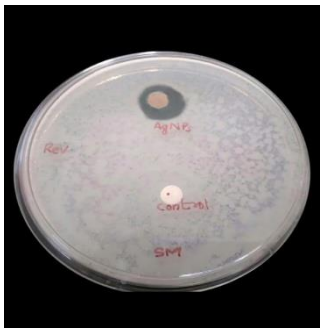

Fig .4 ***Streptococcus mutans***

Fig.5 ***Staphylococcus aureus***

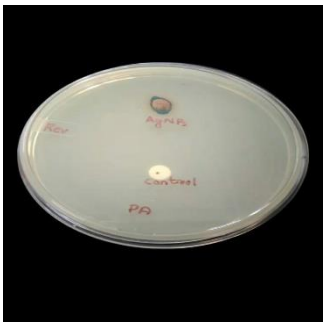

Fig .6 ***Pseudomonas aeruginosa***

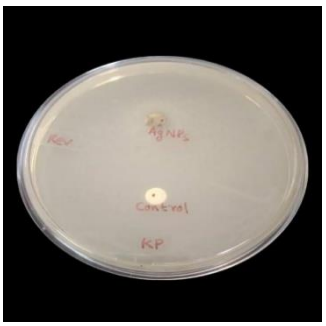

Fig.7 ***Klebsiella pneumonia***

Figures showing antimicrobial activity of silver nanoparticles synthesized using *C. verum* leaf extracts

5. Discussion

Formation of Ag NPs was confirmed by the colour change of the solution from pale yellow to brick red [23].

The synthesized silver nanoparticles from *Cinnamomum verum* were further confirmed by X-ray diffraction pattern analysis to study the crystalline nature of the Ag NPs. XRD results clearly showed that the Ag NPs formed by the reduction of Ag^+ ions by *C. verum* leaves extract are crystalline in nature.

Previous reports suggests that, various bands in the FTIR spectra are because of the presence of different functional groups like alcohols, phenols, aromatic compounds, aldehyde groups, amines, alkenes, and alkyl halides present in the plant extract [24].

The surface morphology of Cinnamomum-Ag NPs was investigated using SEM analysis. The spherical shape of the particles confirmed that they are nano particles.

Regarding the larvicidal activity, an increase in the mortality rate of the mosquito larvae occurred in a time and concentration dependent manner on treatment with green synthesized Ag NPs.

Ag NPs showed antibacterial activity against Gram positive bacteria and less or no anti-bacterial activity against Gram negative bacteria is assumed as a result of hard protective outer membrane of the Gram negative bacteria. Bacteria uses these outer membrane to repel antimicrobial agents. Silver nanoparticles can continuously release silver ions which may be considered as the mechanism of killing microbes [25].

As concluding remarks, this study produced Ag NPs in an economic, environment friendly manner using *C.verum* leaf extract in a less time. The Ag NPs revealed larvicidal and antibacterial activity against *Aedes aegypti* larvae and bacterial pathogens respectively. Introducing the eco-friendly, cost-effective green synthesized silver nanoparticles from *Cinnamomum verum* leaf extract could address health concerns caused by mosquito larvae and bacterial pathogens.

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