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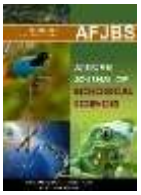

Leptogium marginelum (sw) mediated silver nanoparticles enhances radical scavenging ability and larvicidal activity against three mosquito species- *Anopheles stephensi, Culex quinquefasciatus* and *Aedes aegypti*.

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ABSTRACT

The present study was aimed to test the efficacy of *Leptogium marginellum* (*Lm*) extract and synthesized silver nanoparticles (AgNP's) against mosquito larvae. The synthesized *Lm*AgNP's was measured for its Surface Plasmon Resonance and the peak was observed at 428nm. FTIR results recorded a downward shift of absorbance band between 450-4000 cm^{-1} indicated the formation of *Lm* AgNP's which are spherically shaped with a size range from 150-200 nm confirmed using FESEM. Further, XRD analysis demonstrated *Lm*AgNP's are highly crystalline and exhibit a cubic, face centered lattice with characteristic (111), (200), (220) and (222) orientation. The bio-reduction of silver ions in solution was monitored by EDX. The zeta potential value was found to be -3.08 mV representing AgNP's were highly stable. Radical scavenging results revealed that the extracts and AgNP's demonstrated potent scavenging ability evidenced by DPPH, FRAP and H_2O_2 radical assay. *Lm* fractions and *Lm* AgNP's exhibited dose dependent larval mortality, highest mortality was observed in ethylacetate fraction against *Anopheles stephensi* ($IC_{c}50$=34.66; IC_{90}=158.647), followed by *Culex quinquefasciatus* ($IC_{c}50$=40.085; IC_{90}=213.777) and *Aedes aegypti* (IC_{50}=70.673; IC_{90}=248.836). Larvae treated with *Lm*AgNP's at different concentration represented potent mortality but at 500 ppm concentration demonstrated significant mortality with 88, 94.4, 98.4% mortality with highest activity against *Anopheles stephensi* ($IC_{c}50$=47.41) followed by *Aedes aegypti* and *Culex quinquefasciatus* (IC_{50}=71.66; IC_{50}=74.44). Based on the present findings it is suggested that the fractions and AgNP's of *Lm* is effective in inhibiting the growth of mosquito larvae.

Key Words: Lichen; *Leptogium marginellum;* silver nanoparticles; FESEM; Antioxidant; Larvicidal activity.

1. Introduction

Fundamental knowledge of atomic and subatomic physical, chemical, and biological properties is provided by nanoscience, which opens the door for new technologies in the creation of novel materials with superior and distinctive qualities [1]. Chemical reduction using polymer compounds [2,3], sodium hydroxide, sodium dodecyl sulfate, 2-mercaptobenzimidazole, or other organic reagents 3 [4-6] is used in a variety of ways to synthesize nanoparticles. The other is a physical approach that makes use of laser ablation method [5], thermal decomposition in organic solvents, gamma ray and solar irradiation, chemical deposition, UV photo-reduction, electrochemical method, and photochemical reduction [7-8]. Although the commercial methods for synthesizing AgNPs have been shown to be highly successful, the employment of hazardous chemicals and physical processes carries a number of warnings regarding potential negative effects on human health and the environment. Green synthesis is the result of the need for an alternate, environmentally acceptable way to synthesis nanoparticles. Because of the environmental issues around nanotechnology, the green and eco-friendly production of nanoparticles has gained popularity [9]. Because of their benign nature, manufactured nanoparticles are better suited for use in pharmaceutical and other biomedical applications [10].

Pt, Ag, Pd and Au are the common nanomaterials produced used for the synthesis of nanoparticles, nanowires and nanotubes. Among them, silver is most commonly used nanoparticle with wide range of application in the field of biomedical [11], medicines, agriculture [12], as a detection tool and has been proved to be a potent radical scavenger, anti-inflammatory and anticancer activities, larvicidal activity [13, 14], medical device coatings, drug delivery and personal healthcare products [15], antiviral, anti-bacterial activity and anti-fungal activity [16-18]. Synthesis of nanoparticles from plants or microorganism offers bio-compatibility in eliminating the problem. In this concern researchers are in search and are engaged using bacteria, algae, fungi, plants to develop nontoxic, efficient and low-cost metallic nanoparticles [19].

The primary mosquito-borne illnesses that affect the entire world include lymphatic filariasis, malaria, chikungunya, dengue fever, and Japanese encephalitis. According to reports, individuals residing in tropical and sub-tropical regions experience elevated rates of death and morbidity as a result of these illnesses [14, 20], which has an impact on labor output and commercial revenue. According to reports, these mosquitoes are hazardous insects sometimes referred to as "flying syringes" since they can spread disease and potentially kill millions of

people worldwide [20]. It has been documented that the *Aedes aegypti* vector can transmit chikungunya and yellow fever [20, 21], as well as lymphatic filariasis brought on by *Culex quinquefasciatus* [22]. *Anopheles stephensi*, a malaria-causing mosquito vector that accounts for 40–50% of malarial incidence annually, is the cause of millions of malaria cases that have been documented worldwide, with over 76% of those instances coming from India alone [23].

The need for an improved repellant and the distinction of lichens' symbiotic relationship with algae and fungi, which produces a variety of secondary compounds that are present in most plants [24]. Lichens are utilized as a food ingredient and traditional medicine worldwide. They have been shown to have a number of scientifically proven benefits, including anti-inflammatory, antioxidant, and anti-arthritic effects as well as strong larvicidal, antibacterial, and antipyretic effects [13, 14]. As a result, the current study builds on earlier research that used other lichen extracts and compares the outcomes of *Leptogium marginelum* (sw) extract with those of artificial nanoparticles, thereby examining the many uses of lichens.

2. Materials and Methods

2.1. Sample collection, identification authentication and extraction

Lichen *Leptogium marginellum* (*Lm*) was obtained in January 2018 from the Yercaud hills. The specimen, identified and authenticated at the National Botanical Research Institute (Govt. of India), located in Lucknow, Uttar Pradesh, India, was then preserved as voucher specimen number 16-030771. After being shade-dried to eliminate moisture content, the lichen samples were preserved for later use [25, 26]. Subsequently extraction process was followed.

2.2. *In-vitro* assays

2.2.1 DPPH assay

The DPPH (2,2-diphenyl-1-picrylhydrazyl) assay was used to assess *Leptogium marginelum's* capacity to scavenge free radicals by the use of produced AgNPs. A small adjustment was made in accordance with the protocol that employed [27]. 1.5 mL of a 0.85 mM/L DPPH solution and 1.0 mL of *Leptogium marginelum* of generated AgNPs at varied concentrations (20 to 100 µg/mL) were included in the reaction mixture. After that, the mixture was incubated with agitation for 30 minutes, and ascorbic acid was used as the control to measure the absorbance at 517 nm. The inhibition % was then calculated, which represents the ability to scavenge DPPH radicals.

$$\%\textbf{Decolourization} = \frac{\textbf{1} - \textbf{ABS sample}}{\textbf{ABS control}} \times \textbf{100}$$

2.2.2 FRAP assay

A slightly modified analysis of the ferric ions (Fe^{3+}) lowering antioxidant power assay of AgNPs was conducted by [28]. After diluting the reaction mixture with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide [K_3 Fe $(CN)_6$], which contained 20–100 µg/mL of AgNPs, it was incubated at 50º C for 20 minutes. A 10 min centrifugation at 3000 rpm was performed after adding 2.5 mL of 10% trichloroacetic acid. Lastly, 2.5 mL of deionized water, 0.5 mL of 0.1% $FeCl_3$, and 2.5 mL of supernatant were combined. Ascorbic acid served as the positive control for measuring the absorbance at 700 nm.

2.2.3 Hydroxyl radical scavenging assay

AgNPs' ability to scavenge hydroxyl radicals was assessed using a slightly modified version of the technique [29]. In distilled deionized water, prepare stock solutions of EDTA (1 mM), $FeCl_3$ (10 mM), ascorbic acid (1 mM), H_2O_2 (10 mM), and deoxyribose (10 mM). 0.1 mL of EDTA, 0.01 mL of $FeCl_3$, 0.1 mL of H_2O_2, 0.36 mL of deoxyribose, 1.0 mL of AgNPs extract (20–100 µg/mL), 0.33 mL of phosphate buffer (50 mM, pH 7.4), and 0.1 mL of ascorbic acid were added to the reaction mixture in that order. It was then incubated at 37 °C for 1hr. Lastly, 1.0 mL of the mixture was taken and individually combined with 1.0 mL of 10% TCA and 1.0 mL of 0.5% TBA. At 532 nm, the final mixture was measured.

2.3 Synthesis of silver Nano particle

Leptogium marginellum (*Lm*) lichen samples were used to synthesize AgNPs using the procedure outlined in [9]. The synthesized NPs, characterized using Fourier transform infrared spectroscopy, field emission scanning electron microscopy, energy dispersive X-ray analysis, X-ray diffraction, particle size analyzer, and zeta potential.

2.4 Collection of Mosquito larvae and Larvicidal bioassay

From the colonies of *Culex quinquefasciatus, Aedes aegypti*, and *Anopheles stephensi*, the three instar larvae were obtained from CRME, Madurai (Centre for Research in Medical Entomology). The feeding and care of the larvae followed the previous description [14]. First, one gram of (*Lm*) lichen extract and one mL of synthetic AgNPs were separately diluted in one hundred mL of water (stock solution). Water was used to prepare various concentrations from the stock solution, ranging from 100 to 500 ppm. The experiments took place at room temperature (28±2°C) for a duration of 24 hours. With minor adjustments, the WHO (1996) technique was used to evaluate the larvicidal activity [14, 30]. Following a 24hr exposure period, the percentage mortality of the larvae was determined by averaging the results of five repetitions.

2.5 Statistical analysis

Using the SPSS tool version 16.0, Probit analysis was conducted to determine the average larval mortality data, Specifically LC_{50} and LC_{90} (lethal concentrations). A probit regression model and chi-square values were then used to calculate the 95% fiducial limits including the upper confidence limit (UCL) and lower confidence limit (LCL).

3. Results

3.1 Characterization of Synthesized AgNPs

In order to determine the potential biomolecules in charge of the *Leptogium marginellum* extract-induced reduction of Ag_+ ions, samples of produced nanoparticles were subjected to FTIR analysis (Figure 1). The spectra revealed the sharp peak at 2919cm^{-1} responsible for presence of the carboxylic acids CH and OH stretches and followed that a broad band centering at 3425$cm^{-1}$is identified as the O-H stretching mode. The synthesised AgNPs were found to be spherical in shape and to be monodispersed, with a range of 150–200 nm in particle size (Figure 2). The existence of elemental silver is shown by the energy dispersive X-ray examination (EDX) of produced AgNPs employing *Leptogium marginellum* (Figure 3). The spectra show that the organic components in the lichen samples are responsible for the weak signals from Cl, K, and O and the strong signal from the silver atoms in the nanoparticles at 3keV. Four strong peaks (38.6°, 44.5°, 64.3°, 82.3°) were found in the entire spectrum of 2θ values at10 to 90°, according to the results of the XRD analysis pattern. These peaks correlate to the (111), (200), (220), and (222) crystallographic planes of FCC silver, respectively (Figure 4). Dynamic Light Scattering (DLS) investigations are used to determine the stability of the colloidal AgNPs, and the amplitude of the zeta potential indicates the potential stability of the colloid. Zeta potential (ζ) and hydrodynamic size of AgNPs synthesized by *Leptogium marginellum* are displayed in (Figure 5(a, b)). *Leptogium marginellum*-based AgNPs had an average hydrodynamic size of 168 nm and a negative surface charge of -3.08 mV.

Figure 1: FTIR analysis of synthesized AgNP's using *Leptogium marginellum* extract

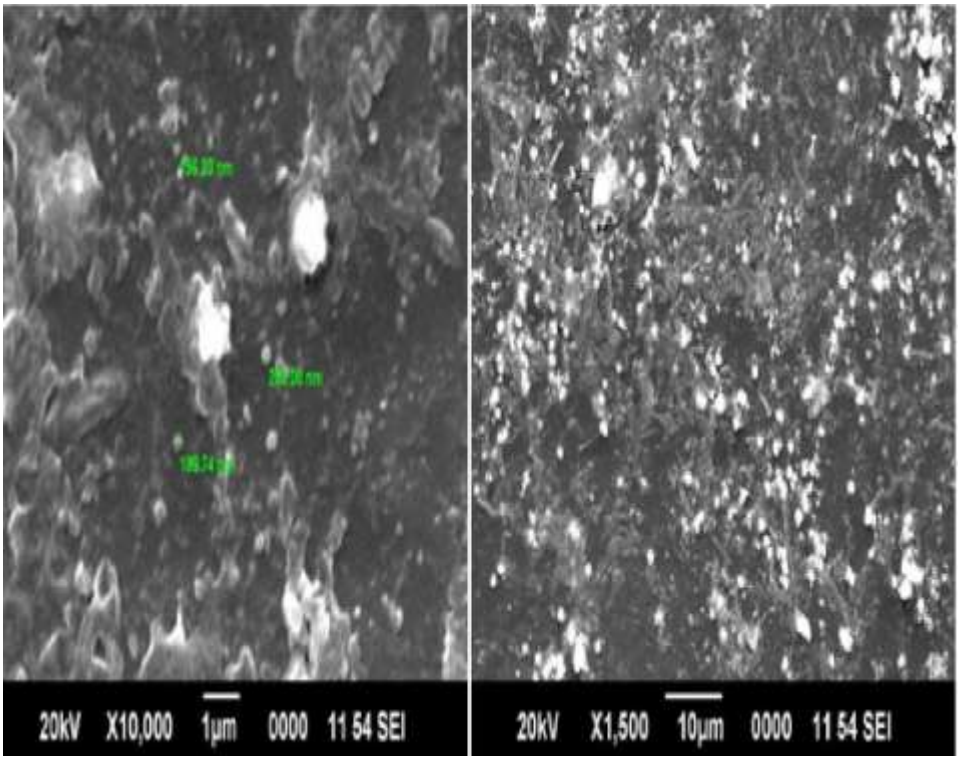

Figure 2: FESEM micrograph of synthesized AgNP's using *Leptogium marginellum* extract

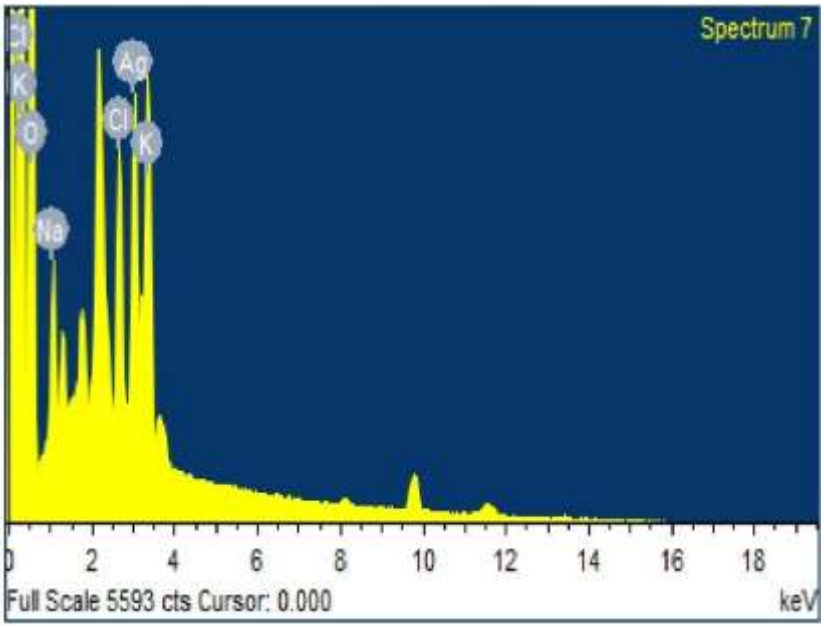

Figure 3: EDX spectroscopy unveiling the chemical components of the synthesized AgNP's using *Leptogium marginellum* extact

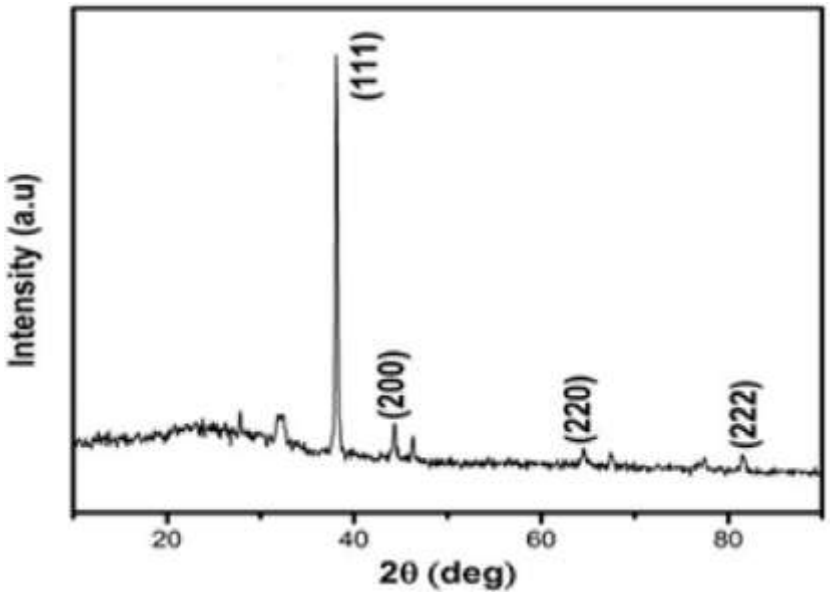

Figure 4: XRD pattern of synthesized AgNP's using *Leptogium marginellum* extract

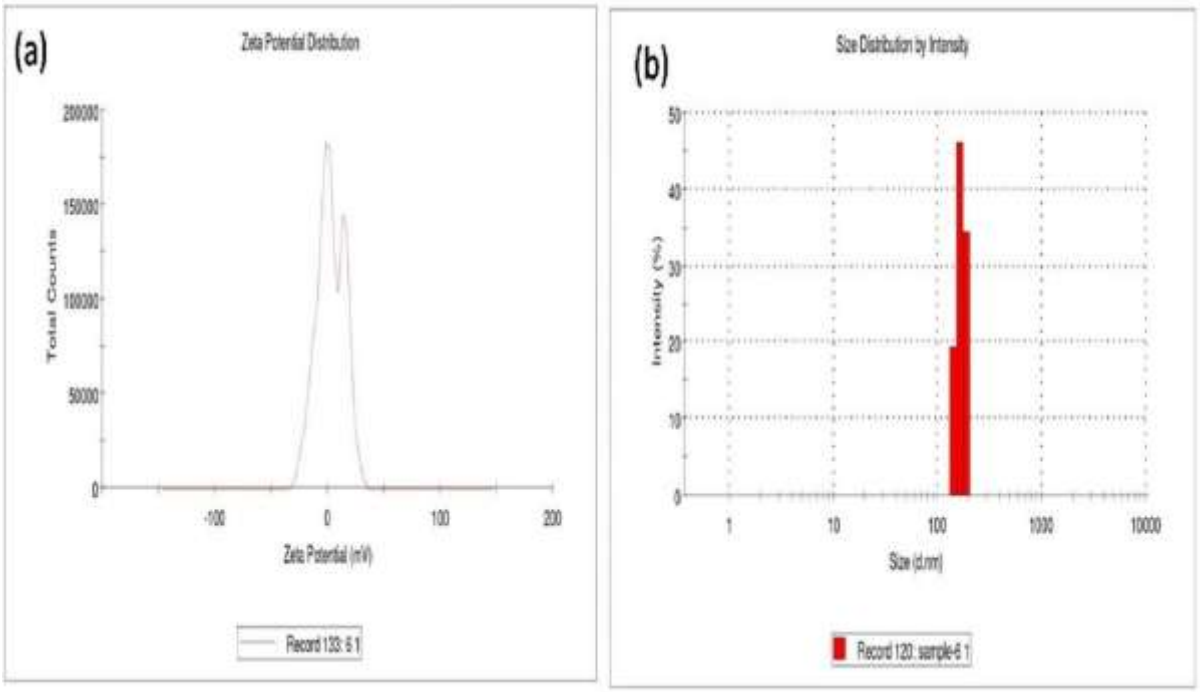

Figure 5 (a, b): Zeta potential and hydrodynamic diameter of AgNP's using *Leptogium marginellum* extract

3.2 Radical scavenging potential of synthesized AgNP's

The radical scavenging capacity of *Leptogium marginellum* and synthesized nanoparticles was evaluated using DPPH, FRAP, and H_2O_2 assays. According to the DPPH assay results, the extract exhibited 48-84% inhibition, while the AgNPs demonstrated 54-89% inhibition at concentrations of 20-100 µg/ml, comparable to conventional ascorbic acid (Figure 6). Similarly, the FRAP assay showed a dose-dependent response, with the *Lm* extract exhibiting 18.4-64.8% inhibition and the AgNPs showing 29.1-72.4% inhibition (Figure 7). Standard ascorbic acid exhibited the highest activity, reaching a maximum of 86.51% at a concentration of 100 µg/ml. In the hydrogen peroxide scavenging assay, AgNPs demonstrated dose-dependent scavenging activity, with 92.76% inhibition for ascorbic acid, while AgNPs and the extract exhibited 84.5% and 74.2% inhibition, respectively, at a concentration of 100 µg/ml (Figure 8).

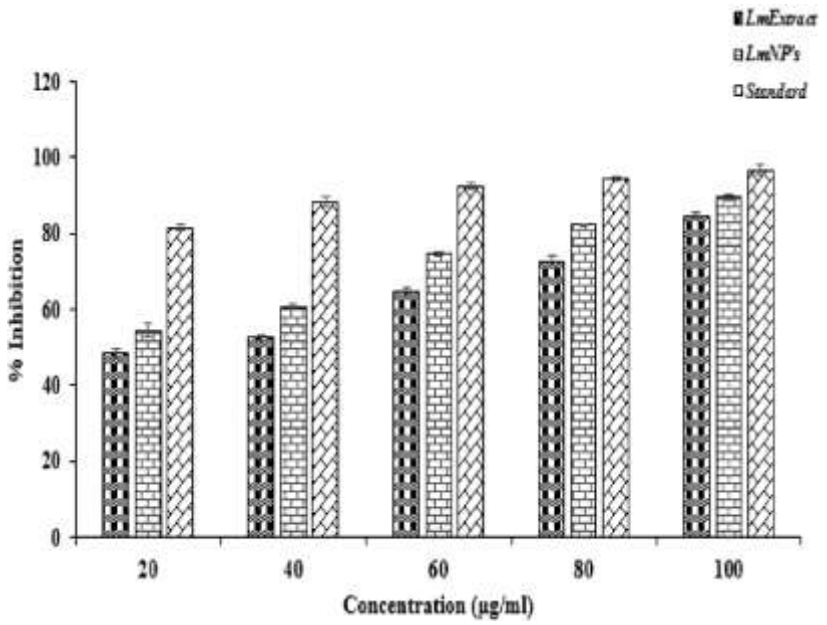

Figure 6: DPPH radical scavenging potential AgNP's synthesized using *Leptogium marginellum* extract

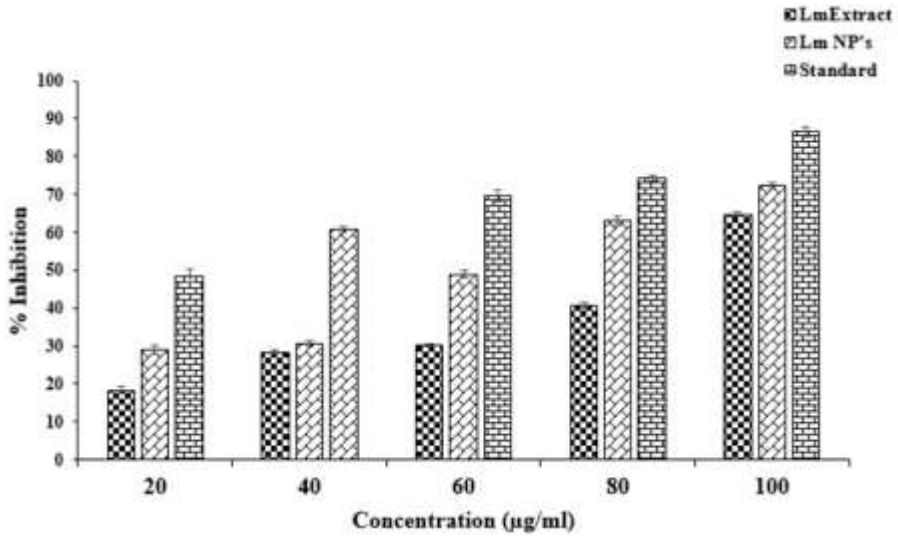

Figure 7: Ferric reducing-antioxidant power (FRAP) of AgNP's synthesized using *Leptogium marginellum* extract

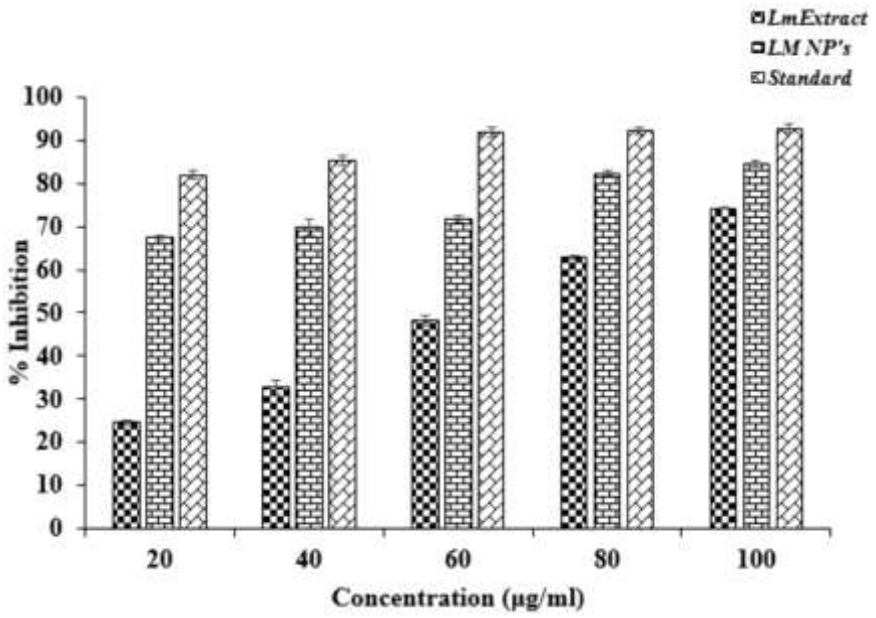

Figure 8: Evaluation of Hydrogen peroxide activity of AgNP's synthesized using *Leptogium marginellum* extract

3.3 Larvicidal potential of *Leptogium marginellum* fractions

The larvicidal activity of *Leptogium marginellum* against *Aedes aegypti, Culex quinquefasciatus,* and *Anopheles stephensi*, in ascending order of activity, is demonstrated by the following fractions: hexane fraction, ethanol extract, and ethyl acetate fraction (Table 1). The larvicidal values at different concentrations for *Aedes aegypti* (LC_{50} = 693.342, 161.435, and 70.673 ppm; LC_{90} = 955.035, 387.793, and 284.836 ppm), *Culex quinquefasciatus* (LC_{50} = 423.397, 131.088, and 40.085 ppm; LC_{90} = 836.400, 334.786, and 213.777 ppm), and *Anopheles stephensi* (LC_{50} = 368.607, 110.930, and 34.660 ppm; LC_{90} = 537.040, 317.693, and 158.647 ppm) were observed. Dose-dependent mortality was noted at 100, 250, and 500 ppm concentrations of *Lm* extract. At 500 ppm concentration, the observed mortality rate for the EA fraction against *Aedes aegypti, Culex quinquefasciatus,* and *Anopheles stephensi* was determined to be 90%, 94.4%, and 97.2%, respectively. The ethanol extract followed with 82%, 88%, and 92% mortality, while the hexane fraction showed the lowest percentages, with 40%, 41%, and 52% mortality, respectively (Figure 9).

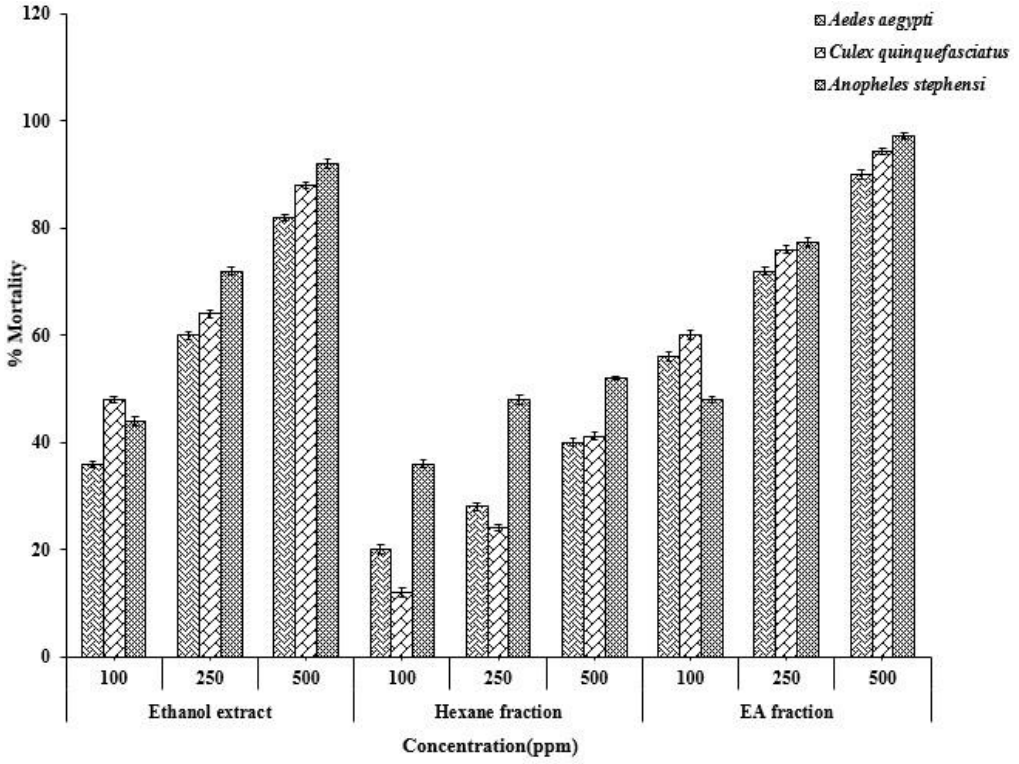

Figure 9: Graph representing Larvicidal activity (% mortality) of *Leptogium marginellum* extract and fractions

3.4 Larvicidal potential of synthesized AgNP's

The larvicidal potential of synthesized AgNPs against third instar larvae of *Aedes aegypti, Culex quinquefasciatus,* and *Anopheles stephensi* is presented in [Table 2]. The statistical data for *Lm* AgNPs against these mosquito species at different concentrations are as follows: for *Aedes aegypti*, LC_{50} = 71.66 ppm and LC_{90} = 935.78 ppm; for *Culex quinquefasciatus,* LC_{50} = 74.44 ppm and LC_{90} = 591.43 ppm; and for *Anopheles stephensi*, LC_{50} = 47.41 ppm and LC_{90} = 841 ppm. It was found that the observed mortality rate after AgNP treatment was dosage-dependent, with higher AgNP concentrations resulting in increased mortality rates. At a concentration of 500 ppm, the mortality rates for *Aedes aegypti, Culex quinquefasciatus,* and *Anopheles stephensi* were 88%, 94.4%, and 98.4%, respectively (Figure 10).

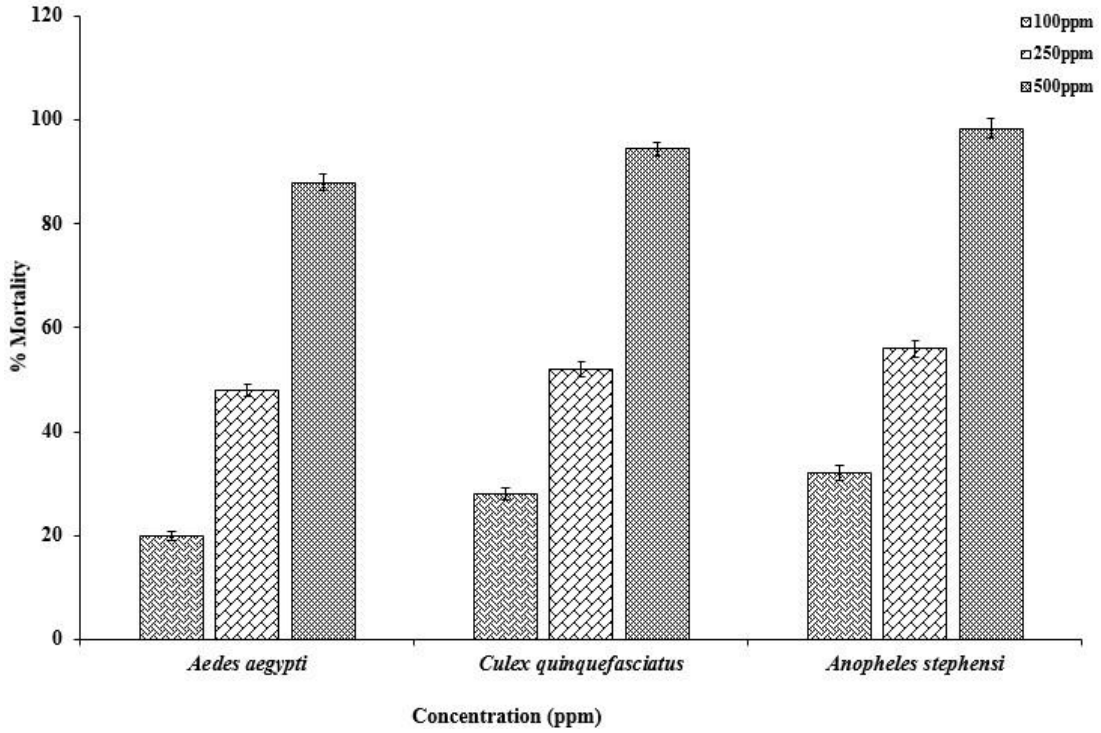

Figure 10: Graph representing Larvicidal activity (% mortality) of AgNP's synthesized using *Leptogium marginellum* extract

Table 1: Larvicidal potential of different fractions of *Leptogium marginellum* lichen against third instar larvae

Species	*Leptogium marginellum*	LC $_{50}$(UCL-LCL)/µg ml^{-1}	LC $_{90}$(UCL-LCL)/µg ml^{-1}	X^2 (df=7)
Aedes aegypti	Ethanol extract	161.435(119.830-221.843)	387.793(204.967-323.601)	0.993
	H- fraction	693.342(906.613-276.54)	955.035(839.806-489.66)	0.911
	EA- fraction	70.673(20.141-112.481)	284.836(461.739-301.372)	0.923
Culex quinquefasciatus	Ethanol extract	131.088(84.320-171.135)	334.786(538.578-2100.343)	0.970
	H- fraction	423.397(883.01-257.29)	836.40(809.641-602.106)	0.853
	EA- fraction	40.085(2.296-92.413)	213.777(493.417-204.381)	0.992
Anopheles stephensi	Ethanol extract	110.930(69.218-145.755)	317.693(429.358-1263.425)	0.850
	H- fraction	368.607(187.130-7.09110)	537.04(3517.738-1.049)	0.977
	EA- fraction	34.660(1.042-76.098)	158.647(370.329-6803.977)	0.992

Control - nil mortality. Significant at P<0.05 level LC_{50} lethal concentration that kills 50% of the exposed larvae, LC_{90} lethal concentration that kills 90% of the exposed larvae, UCL upper confidence limit, LCL lower confidence limit, $\chi 2$ chi-square; df degree of freedom.

Table 2. Larvicidal potential of synthesized AgNP's from *Leptogium marginellum*

Species	LC_{50}(UCL-LCL)/µg ml^{-1}	LC_{90}(UCL-LCL)/µg ml^{-1}	X^2 (df=7)
Aedes aegypti	71.66(17.993-116.144)	935.78(515.39-5789.81)	0.887
Culex quinquefasciatus	74.44(6.560-129.389)	591.43(334.68-7585.99)	0.128
Anopheles stephensi	47.41(3.630-91.632)	841.31(449.377-9392.502)	0.180

Control - nil mortality. Significant at P<0.05 level LC_{50} lethal concentration that kills 50% of the exposed larvae, LC_{90} lethal concentration that kills 90% of the exposed larvae, UCL upper confidence limit, LCL lower confidence limit, $\chi 2$ chi-square; df degree of freedom.

4. Discussion

In rural areas, lichens have long been used as insecticides and the current study compares the effectiveness of lichen *Leptogium marginellum*, which is known to have superior antioxidant qualities, with synthetic AgNPs to see how it affects mosquito larvae, which are the primary cause of many diseases. The use of FTIR spectra for characterization study reveals the distinctive fingerprint of organic compounds that stand out from the absorption patterns of all other compounds. These compounds exhibit rotational and vibrational modes of motion, indicating that they are optical isomers. While peaks at 1096cm^{-1} and 1047cm^{-1} indicate OH functional groups in polyphenols, the spectral band at 1634cm^{-1} is 1 indicates primary amines [31, 32]. Together with AgNPs, the presence of proteins, polyphenols, alcoholic groups, and carboxylic acids is explosively revealed by the stretching vibrations and bends seen in the spectrum. The findings revealed a diverse structure of nanoparticles, including rod-like, spherical, triangular, and platelet shapes. This could be because of absorption spectra of each AgNPs is correlated with its unique shape.

The quantitative examination indicates the presence of Ag with 29.05%, which was determined to be greater than other elements present. The findings of the EDX study showed by silver is the primary element that forms the nanostructures. X-ray diffraction analysis was used to determine the elemental composition and crystal structure of the synthesized

nanomaterials. The results showed that the face-centered cubic crystal structure of metallic silver is well matched with the synthesized AgNPs, and these findings are consistent with earlier reports [33, 34]. According to reports, particles should be regarded as stable if their zeta potential is within the range of +30 mV to -30 mV [35]. When two nanoparticles with either a positive or negative zeta potential come together, they usually repel one another and do not exhibit any changes in their properties. Nevertheless, because there is no repulsive force to stop this agglomeration in the case of low absolute zeta potential levels, these particles combine and flocculate.

Numerous studies have shown that flavonoids and phenolic compounds are the physiologically active substances that aid in transferring a hydrogen molecule to free radicals during the initial stages of lipid oxidation, hence upsetting the chain reaction. One possible explanation for the lichens strong capacity to scavenge free radicals is that one of their main secondary metabolites, phenolic hydroxyl groups, is present in them. The potential method of neutralizing DPPH, which is shown by a color shift from violet to yellow, by scavenging free radicals and giving e-from the compounds [36]. The FRAP assay is typically used to measure the antioxidant capacity of different foods, nutritional supplements, and beverages containing polyphenols. The results showed that *Lm* and AgNPs had a potent reducing ability, which may be because these foods contain various reductones that inhibit the formation of radical chains [36].

According to the World Health Organization, vectors are a major factor in agricultural crop damage, which results in lost revenue and the spread of disease [37]. Although using synthetic pesticides and indoor residual sprays has improved the control of mosquito larvae growth, there are still risks to human health and the environment [38, 14]. The scientific community is searching for eco-friendly control techniques that could lower the risk of these dangerous compounds [39]. According to a number of studies, when tested against several larval species, extracts and synthetic nanoparticles (*Heliotropium indicum, Euphorbia hirta, Mimosa pudica, and Nelumbo nucifera*) have the highest mortality rates at varied concentrations [14, 40–42]. Therefore, it is clear that *Leptogium marginellum's* existence of potent bioactive secondary metabolites affects its high mortality rates, a characteristic that has been documented in numerous studies. Numerous researchers have hypothesized that extracts or NPs affect DNA through a variety of mechanisms, including lowering membrane permeability, blocking or inhibiting transcription/translation of DNA, affecting neurosecretory cells and gut enzymes, and inhibiting respiratory chain reactions, all of which can inhibit larval growth and ultimately result in death [43–45].

5. Conclusion

Here, the lichen *Leptogium marginellum's* larvicidal potential and free radical scavenging activities are described in depth by the current work. A great way to create AgNPs that is safe, non-toxic, and least expensive while displaying the unique properties of the produced particles and providing a substantial in-vitro radical scavenging action. However, when tested against three distinct larval species, extracts and AgNPs demonstrated incredible larvicidal activity. These lichen extracts and NPs represent a potentially useful tool in the fight against viral and parasitic disorders, expressing safe and environmentally friendly medicinal agents.

6. Conflict of interest

No conflicts of interest.

7. Acknowledgement

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