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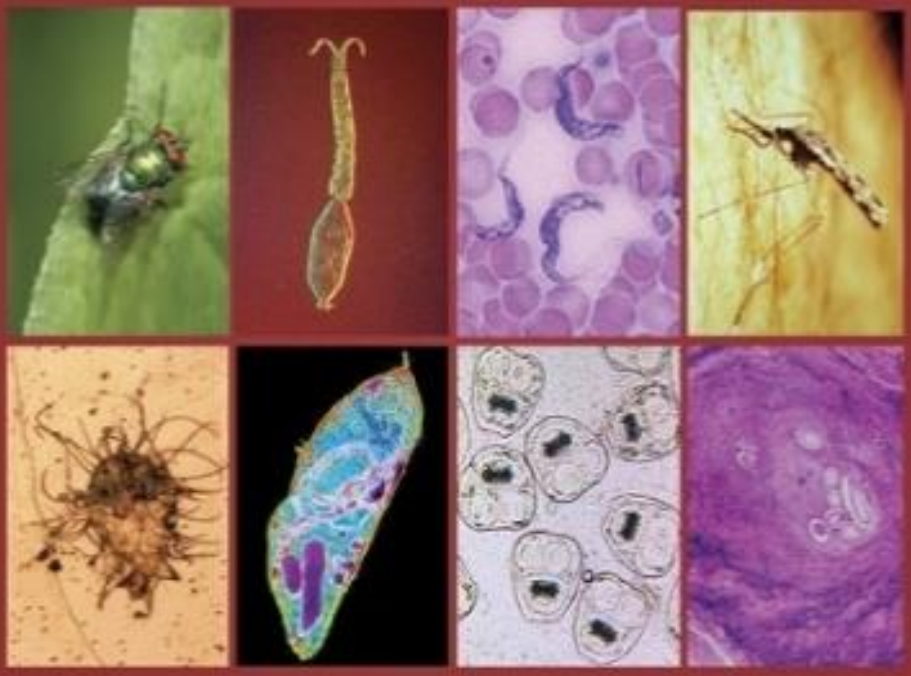

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A New Tool Against *Anopheles Gambiae* 4th Instar Larvae; *Ocimum basilicum* Mediated Silver Nanoparticles

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ABSTRACT

The excessive use or spillage of chemical insecticides is harmful to the environment and living organisms. Their replacement with plant-based insecticides is a cheap, and an excellent alternative to combat vector-borne diseases. *Ocimum basilicum*, commonly known as Sweet Basil, belongs to the genus Ocimum of the family Lamiaceae. Various species of *Ocimum* are known for their peculiar strong odours and culinary appreciation. This study presents the toxicity of silver nanoparticles obtained from an aqueous extract of *Ocimum basilicum* on the early 4th instar larvae of *Anopheles gambiae*, the main vector of malaria in Sub-Saharan Africa. The nanoparticles were characterised by ultraviolet-visible spectroscopy for their formation, infrared spectroscopy for the interface metabolites-silver and X-ray powder diffraction for their nature. The silver nanoparticles based on *Ocimum basilicum* are water dispersible, stable, pure and functionalized by the plant secondary metabolites and thus, they represent a new means for *Anopheles gambiae* vector control.

INTRODUCTION

A significant economic burden is associated with countries where vector-borne diseases are present and are responsible for important global public health plights (Ramalho-Ortigao & Gubler, 2020). Vector-borne diseases are responsible for a variety of infections including malaria, leishmaniasis, onchocerciasis, yellow fever, dengue, or chikungunya. They account for more than 17% of all infectious diseases, causing more than 700 000 deaths annually (WHO, 2020).

Over millennia, nature has perfected the art of biology at the nanoscale. Haemoglobin, the protein that carries oxygen through the body, is 5.5 nanometres in diameter and a DNA strand, one of the building blocks of human life, is only about 2 nanometres in diameter (Ullman, 2021). In the context of limited resources setting, plants present an interesting profile to combat vector-borne diseases. The reinforcement of the plant antivectorial capacity can be obtained using modern technologies.

Metal nanoparticles can exhibit unique properties dissimilar from the equivalent chemical compound in a larger dimension (Jeevanandam *et al*., 2018). Nanometals like platinum, palladium, titanium, or silver can be used to control instars of larvae and thus, larvae populations (Kojom Foko *et al*., 2020). Among these metals, biogenic silver from plants like *Psidium guajava* (Ntoumba *et al*., 2020) and *Moringa oleifera* (Idowu et al., 2021) present excellent IC_{50} data to control larval populations of *Anopheles gambiae*.

Ocimum basilicum L., commonly known as Sweet Basil, belongs to the genus Ocimum of the family Lamiaceae. Various species of Ocimum are known for their peculiar strong odours and culinary appreciated (Khair-ul-Bariyah *et al*., 2012). Studies indicate *Ocimum basilicum* to possess analgesic, anti-inflammatory, antimicrobial, antioxidant, anti-ulcerogenic, cardiac stimulant, chemomodulatory, CNS depressant, hepatoprotective, hypoglycemic, hypolipidemic, immunomodulatory and larvicidal activities (Bilal *et al*., 2012). Recent essential oils analysis by gas chromatography and gas chromatography coupled with mass spectrometry presented 65 compounds constituting 99.3% and 99.0% of the total oils. They have been obtained by solvent-free microwave extraction (SFME) and conventional hydro-distillation (HD) respectively. The main components of these oils after extraction were linalool (43.5% SFME; 48.4% HD), methyl chavicol (13.3% SFME; 14.3% HD) and 1,8-cineole (6.8% SFME; 7.3% HD) (Chenni *et al*., 2016).

The larvicidal properties of *Ocimum basilicum* essential oil have been evaluated against *Culex tritaeniorhynchus*, *Aedes albopictus* and *Anopheles subpictus* with an LC_{50} values of 14.01, 11.97 and 9.75 ppm and LC90 values of 23.44, 21.17 and 18.56 ppm, respectively (Govindarajan *et al*., 2013). Exposure of early fourth instars of *Aedes aegypti* with various concentrations of *Ocimum basilicum* essential oil for 24 h revealed a moderate LC50 and LC90 value of 141.95 ppm and 100.82 ppm respectively (Kumar *et al*., 2017). *Ocimum basilicum* showed remarkable potency against pre-adult stages and adult *Anopheles gambiae,* causing a 100% mortality rate at 0.4% concentration within 24h of treatment (Ileke & Adesina, 2019). The essential oil-induced 100% mortality of *Anopheles funestus* adults at 250 ppm (Akono Ntonga *et al.,* 2012). Also, *Ocimum basilicum* plant extract has been combined with neonicotinoid in the larvicidal management of *Anopheles stephensi* (Maurya *et al*., 2012) or on newly hatched larvae of *Spodoptera littoralis* (Mead, 2018). Anuradha and their co-worker described the first synthetic report of silver nanoparticles mediated *Ocimum basilicum* in 2014 (Anuradha *et al*., 2014). Further reports used *Ocimum basilicum* plants from India, Thailand, South Africa, Egypt and Iran.

This paper presents the larvicidal action of silver nanoparticles mediated *Ocimum basilicum* on 4th instar larvae of *Anopheles gambiae*,

MATERIALS AND METHODS

Plant Material:

Leaves of *Ocimum basilicum* were obtained from an experimental garden, Littoral region, Cameroon, in April 2018 and authenticated at the National Herbarium, Yaounde, in comparison with a voucher specimen previously deposited (6899/SRF Cam). The plant extract was prepared following the procedure of Eya'ane Meva and coworkers using 10g of plant material boiled at 80°C in distilled water (Eya'ane Meva *et al*., 2015).

Biological Synthesis of Silver Nanoparticles:

Silver nanoparticles were synthesized according to a well-known procedure with slight modifications (Eya'ane Meva *et al*., 2016a). Ten millilitres of freshly prepared aqueous extract were added to a 50 mL silver nitrate

aqueous solution (1 mM) for the bioreduction process. The mixture was incubated at room temperature in the dark to minimize the photoactivation of silver nitrate under static conditions until observation of a colour change (Figure 1). The mixture was then centrifuged at 7000 rpm for 20 minutes and washed twice with distilled water and once with ethanol 95%, and the pellets were stored for further analysis.

Fig.1 Change of colour during the synthesis: left silver nitrate, middle *Ocimum basilicum* leaf extract, right silver nanoparticles

Characterization Of Silver Nanoparticles:

Ultraviolet (UV)-visible spectroscopic measurement, Fourier transform infrared (FTIR) spectroscopy, Powder X-ray spectroscopy (PXRD) was carried out according to the literature (Belle Ebanda Kedi *et al*., 2018).

Evaluation of Larvicidal Activities:

Eggs of the susceptible *Anopheles gambiae* (Kisumu strain) were obtained from the 'Organisation de Coordination pour la lutte contre les Endémies en Afrique central', Yaounde, Cameroon. They were maintained and reared in the Insectarium of the University of Douala, Faculty of Medicine and Pharmaceutical Sciences to obtain 4th instar larvae. The larvicidal activity of the AgNPs produced from *Ocimum basilicum* extract was determined following the standard test procedures of the WHO with some slight modifications (Ntoumba *et al*., 2020). For the bioassay, twenty 4th instar larvae were placed in plastic bowls (6 cm diameter, 120 mL capacity) with distilled water in 4 replicates. The controls were set up with distilled water, *Ocimum basilicum* plant extract, $AgNO_3$ at ambient temperature, and $AgNO_3$ in the dark. Different concentrations of $AgNO_3$ in the range of 0 - 200 ppm were prepared through serial dilutions of 100 mL each. The experiments were carried out at $27^{\circ}C \pm 2^{\circ}C$, relative humidity of $75\% \pm 5\%$, and a photoperiod of 14 h/10h (light/dark). Larvae were considered dead if they did not respond to contact. The number of dead larvae was counted 24 h and 48 h after treatment and the percentage of mortality was determined.

Data were analyzed statistically using GraphPad Prism software version 9.1.1 for Windows (GraphPad Software, Inc., San Diego, CA, USA).

RESULTS AND DISCUSSION

Ocimum basilicum is known as a medicinal as well as a culinary aromatic plant. It is widely used by local people, who commonly grow it around their homes and in market gardening centres and then offer it in local markets. The translation from traditional pharmacological usage at the local level to industrial-scale poses the following constraints:

1) the huge mass of plants needed, an anthropic pressure that can lead to biodiversity extinction, 2) the hydrophobicity resolution of essential oils by the addition of ethanol or acetone and its environmental impact, 3) the flammable properties of essential oils and security plight to maintain huge amounts, 4) the high cost of essential oils.

To mitigate these problems, a formulation using nano-silver as a carrier is proposed in this study. The synthesis of the nanomaterial occurs by the reaction of an aqueous *Ocimum basilicum* extract with silver nitrate. The colour of the reaction medium changes from light brown (colour of the aqueous *Ocimum basilicum* solution) to dark brown. This observation is completed by an ultraviolet-visible spectroscopy analysis showing a characteristic plasmonic resonance at 426-428 nm which is in the range of 410-450 nm, similar to other synthesized silver nanoparticles mediated *Ocimum basilicum* plants (Wonsawat, 2014; Elumalai *et al*., 2019). At these plasmon resonances, the particles are considered spherical according to the Mie theory (Mie, 1908) and at the 20th minute, they tend to be anisotropic. This resonance corresponds to the collective motion of conduction electrons (plasmons) at the surface of the metal. The reaction proceeds rapidly and absorbance of 1AU is obtained at 5 minutes. Such rapidity was observed with the *Megaphrynium macrostarchium* (Eya'ane Meva *et al*., 2016b) and the carob leaf extract nanoparticles (Awwad *et al*., 2013). The solution was stable at the 96th hour without the formation of a precipitate. After centrifugation and drying, a powder pellet was obtained, ready for further characterization. Infrared analysis of silver nanoparticles showed a shift of the vibration at 1640 cm^{-1} corresponding to the functional groups of amides observed due to a bond formation with silver and indicating the presence of metabolites at the surface. The powder diffraction X-ray analysis of the nanopowder showed a structure corresponding to AgCl and Ag-type nanograins. Following instrument quantification, nanopowder issued from fresh plants compared to that of the dry plant was also examined. AgCl proportion was higher when the plant was used dry (95% AgCl; 5% Ag). The formation of AgCl has been discussed in our previous report (Belle Ebanda Kedi *et al*., 2020). Biological studies were carried out on nanoparticles obtained from fresh plants with composition (24% AgCl, 76% Ag) for environmental considerations. The Scherrer equation was used to determine nanoparticles' size yield for AgCl 41.36 nm and Ag 57.84 nm when all signals are taken into consideration (Eya'ane Meva *et al*., 2019). These nanograin sizes were in the range of 40 - 60 nm, similar to that obtained by Monica and Senthilkumar in 2020 using scanning electron microscopy (Monica & Senthilkumar, 2020). High levels of chloride in the root zone may compete with NO_3^- for the same channels and decrease the root-to-shoot translocation of NO_3^- (Rubinigg *et al*., 2003). The toxicity on the 4th instar of *Anopheles gambiae* larvae showed an IC_{50} of 1.540ppm after 24h and of 1.003ppm after 48h (Fig. 2). This suggests that the toxicity increases with time (Fig. 3). These values are lower than those obtained by *Psidium guajava* mediated silver nanoparticles (9ppm at 24h and 20ppm at 48h) (Ntoumba *et al*., 2020). Silver nanoparticles IC_{50} of 0.5 and 0.75ppm post-treatment of 24h and 48h have been obtained by Idowu and coworkers using *Moringa olifeira* or *Ficus exasperata* (Idowu *et al.,* 2021). The important toxicity of silver nanoparticles

obtained from *Ocimum basilicum* can be explained by the high surface toxicity to mosquitos' larvae. The synergy formed between nanometal and transported metabolites can explain the differences in toxicity. These nanoparticles disperse perfectly in aqueous reaction medium indicating their powerful ability if used as a bioinsecticide in water collections near houses and in plantations as sprays. Moreover, the preparation is rapid and simple thus, easily applicable in low resources settings. *Ocimum basilicum* plant doesn't cause larval death at all concentrations studied, a situation similar to *Psidium guajava* (Ntoumba *et al*., 2020).

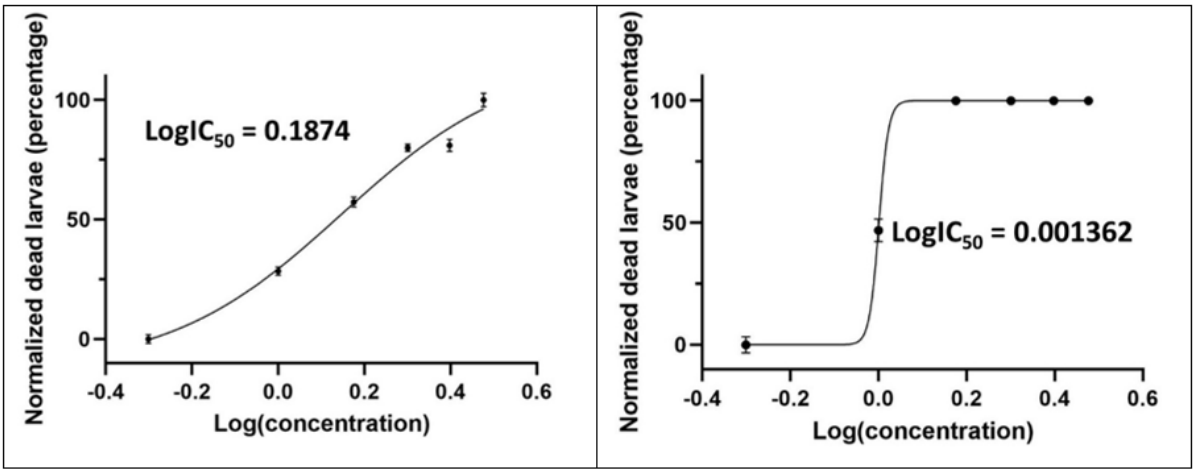

Fig. 2: Percentage of dead larvae as function of the logarithmic concentrations at 24 and 48h. Hillslope with R squared of 0.9610. (left) 24 h; (right) 48 h.

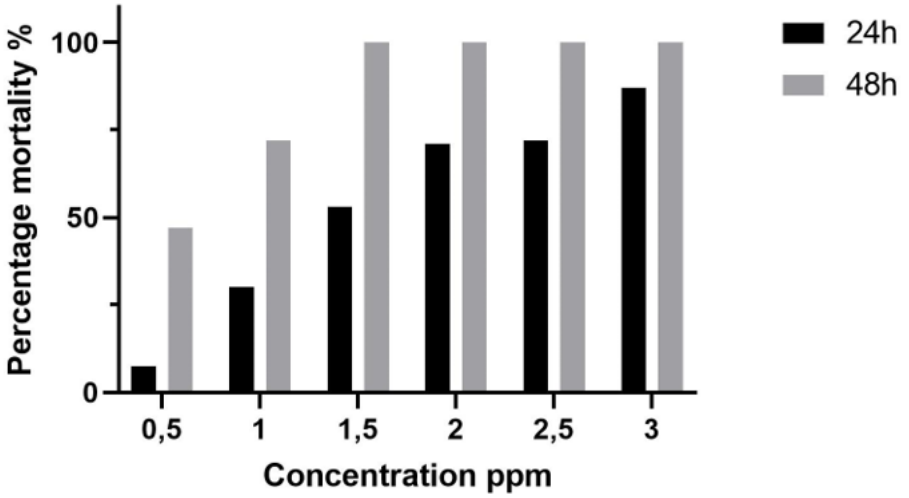

Fig. 3: Mortality rate of *Anopheles gambiae*

Conclusion

The transport of secondary metabolites by nanometals is a way to enhance the usability of plant secondary metabolites against vector-borne diseases. Pure silver nanoparticles functionalised with *Ocimum basilicum* plant were obtained. The nanoparticles were stable, functional and highly potent against the early 4th instar of *Anopheles gambiae*. The synthesized nanoparticles consisted of nanograins of silver chloride 41.36 nm and silver 57.84 nm. The formulation of plant extract and essential oils into nanoparticles can increase their activity and reduce anthropic pressure to plant extinction.

Contributions

AAN, FEM and LGL conceived and designed the study. AAN, SFJY, ATC, and MKNA screened the literature and performed data extraction. AAN and FEM analysed and interpreted the results. AAN, WEE, FEM and LGL drafted the manuscript, and all authors revised the manuscript. FEM and LGL supervised the work at all stages. All authors have read and approved the final manuscript.

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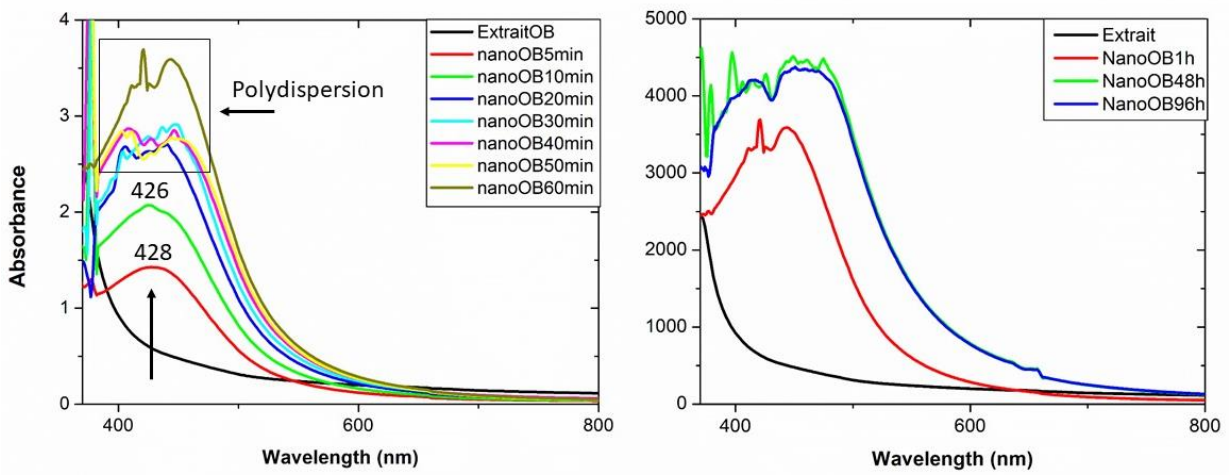
Supplemental Material

Characterization of silver nanoparticles.

Ultraviolet (UV)-visible spectroscopic measurement

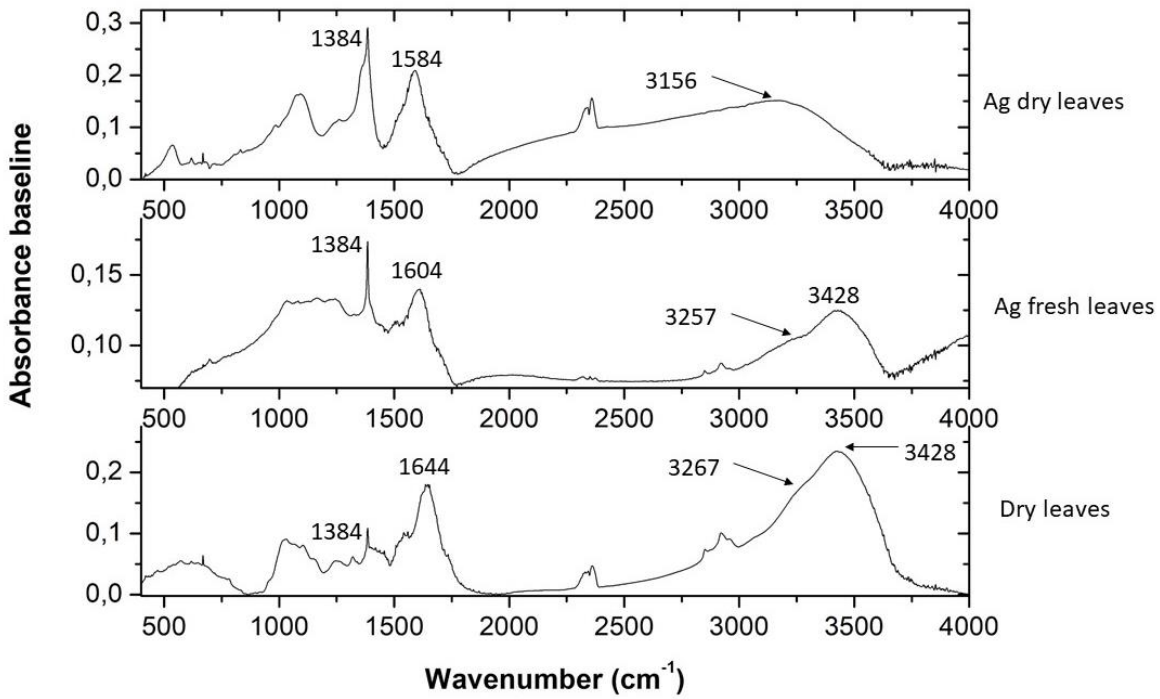
The reduction of silver ions was monitored by measuring UV-visible spectrum of the reaction mixture at 5, 10, 30, 40, 50, 60 minutes, 48 and 96 hours using the UV-visible spectrophotometer (UV-line 9,100 single beam, halogen light source, 1 nm resolution). Distilled water was used as a blank.



F1. Ultraviolet visible spectroscopic measurement (UV-Vis) of 2.5 mL samples of the reaction suspension at different time intervals. The spectrophotometer operated at 1 nm resolution and optical length of 10 mm. UV–visible analysis of the reaction mixture was observed for a period of 300 s.

Fourier transform infrared (FTIR) spectroscopy

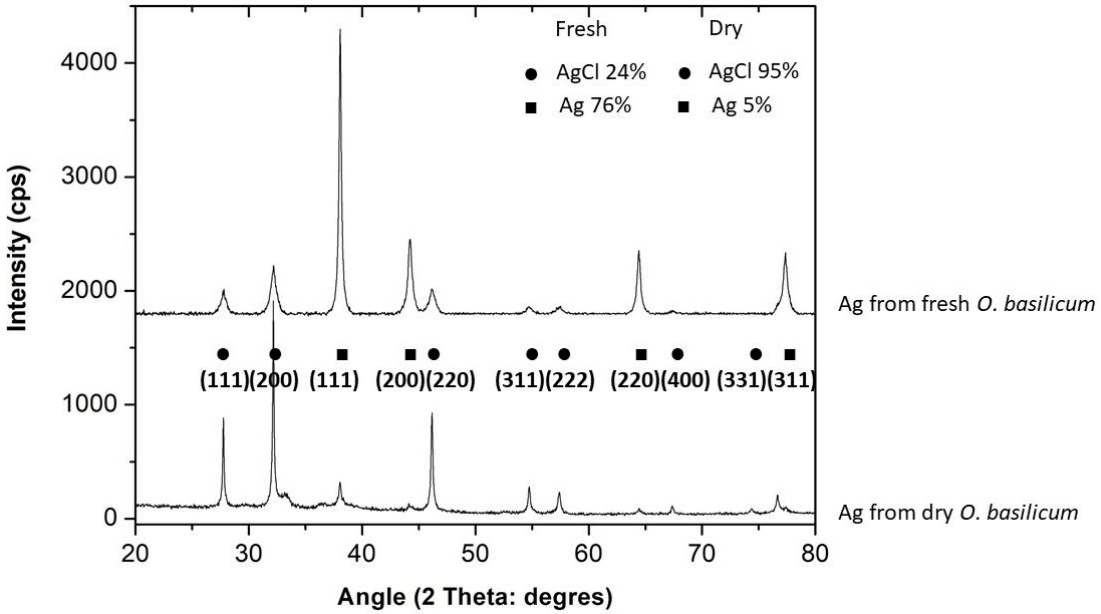
FTIR spectrum was recorded at room temperature through potassium bromide pellet method. Samples were grinded with KBr pellets and kept in infrared path, and the spectrum was measured using a Nicolet IS5 model of Thermo Scientific operating at a resolution of 0.4 cm^{-1}.



F2. Fourier-transform infrared spectroscopy (FTIR) FTIR spectrum was recorded at room temperature through potassium bromide pellet method. Samples were grinded with KBr pellets

Powder X-ray spectroscopy (PXRD)

The PXRD spectroscopy measurements of purified silver nanoparticles were carried out using a Panalytical Empyrean Serie 2 X-ray diffractometer (Cu K-Alpha1 [Å] 1.54060, KAlpha2 [Å] 1.54443, K-Beta [Å] 1.39225) by preparing a thin film on silicon substrate. PXRD pattern was compared to Joint Committee on Powder Diffraction Standards files (JCPDS 65-2871 and 31-1238).



F3. Powder X-ray spectroscopy (PXRD). The PXRD spectroscopy measurements of purified silver nanoparticles were carried out using a Panalytical Empyrean Serie 2 X-ray diffractometer (Cu K-Alpha1 [Å] 1.54060, KAlpha2 [Å] 1.54443, K-Beta [Å] 1.39225) by preparing a thin film on silicon substrate. Powder X-ray diffraction was used for the crystal structure.

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Characterization And Composition of The Nanoparticles.

Table 1: Full width at half maximum (FWHM) and size calculation of nanoparticles from fresh plant

No.	Pos. [°2Th.]	d-spacing [Å]	Height [cts]	FWHM Left [°2Th.]	Size nm
1●	27,813	3,20507	208,32	0,0936	91.36
2●	32,2029	2,77746	415,84	0,2808	30.77
3■	38,076	2,36147	2496,93	0,1872	46.91
4■	44,2538	2,04508	628,04	0,2496	35.90
5●	46,1791	1,9642	210,96	0,2184	41.32
6●	54,7192	1,67612	57,76	0,1872	49.93
7●	57,393	1,60422	47,24	0,624	15.17
8■	64,412	1,44531	552,24	0,2808	34.94
9●	67,4209	1,38794	22,24	0,4992	19.99
10■	77,36	1,23254	528,24	0,0936	113.62

Mean 41.356 nm, Ag 57.84 nm

Table 2: Full width at half maximum (FWHM) and size calculation of nanoparticles from dry plant

No.	Pos. [°2Th.]	d-spacing [Å]	Height [cts]	FWHM Left [°2Th.]	Size nm
1●	27,7555	3,21158	768,82	0,156	54.81
2●	32,1682	2,78038	1778,98	0,156	55.38
3■	38,0558	2,36267	197,85	0,2496	35.18
4●	46,1608	1,96493	836,35	0,156	57.84
5●	54,7477	1,67531	224,78	0,1872	49.94
6●	57,4173	1,6036	179,93	0,2496	37.92
7●	67,3768	1,38874	57,34	0,3744	26.65
8■	76,6817	1,24174	112,14	0,3744	28.27

Mean AgCl 47.09 nm, Ag 31.72 nm