GREEN SYNTHESIS OF SILVER NANOPARTICLES USING FRESH LEAF EXTRACTS OF *CRASSOCEPHALUM CREPIDIOIDES (BENTH.) S. MOORE*: EVALUATION OF ITS ANTIMICROBIAL PROPERTY AND PARTIAL CHARACTERIZATION

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ABSTRACT: *Green synthesis of silver nanostructures was successfully carried out using fresh leaves of Crassocephalum crepidioides (Benth) S. Moore. Fresh leaves were collected, weighed and extracted. The aqueous leaf extract was then allowed to react with 5.0 mM of $AgNO_3$ in a dark room at an ambient temperature. Five volume ratios in milliliters of plant extract to $AgNO_3$ were prepared and labelled as S_1, S_2, S_3, S_4, and S_5 that is 1:50, 5:50, 10:50, 20:50 and 30:50, respectively. Each ratio was allowed to react for three incubation time (i.e. 0, 24, and 48 hours). These ratios were tested against cultured bacteria (i.e., Staphylococcus aureus, Escherichia coli, and Bacillus subtilis) and fungi (i.e., Candida albicans and Aspergillus niger). The S_1 (1:50) possessed the highest antibacterial activity against E. coli, S. aureus, and B. subtilis and less for antifungal activity towards C. albicans. Scanning the λ_{max} of the five ratios were done using Ultraviolet-Visible (UV-Vis) spectrophotometer at 300 to 600nm. The 1:50 at 0 hour which exhibited the highest zones of inhibition for most pathogens was further characterized using Fourier Transform Infrared (FTIR) Spectroscopy. The presence of N=O with 1337cm^{-1} and 1373cm^{-1}, N-H with 1576cm^{-1} and 1633cm^{-1}, O-H stretch with 3429cm^{-1} and 3264cm^{-1} in the spectra indicates a nitro group, a primary amine, and an alcohol, respectively. Scanning Electron Microscope equipped with Energy Dispersive Spectrometer (SEM-EDS) analysis confirmed the reduction of silver ions to silver nanoparticle.*

Keywords: green synthesis, silver nanoparticles, antimicrobial, UV-VIS, SEM-EDS, FTIR

1. INTRODUCTION

Nanobiotechnology is a branch of nanotechnology which describes an application of biological systems for the production of new functional material such as nanoparticles [1]. Biosynthetic methods can be employed in either microorganism cells or plant extract for nanoparticles production [2]. Gold and silver nanoparticles are presently under intensive study for applications in optoelectronic devices, ultrasensitive chemical and biological sensors and as catalysts [3]. Historically, metallic silver had been used as hygienic and medicinal purpose though its mechanism is not fully known yet [4]. Various applications of silver disinfectants has emerged in consumer products ranging from home appliances to water treatment such as household water filters, antibacterial spray, cosmetics, detergents, and dietary supplements [5].

Nowadays, there is a growing need to develop eco-friendly processes of synthesizing silver nanoparticle which do not use toxic chemicals in the synthesis protocols. Most researchers used non-toxic solvent like water and a non-harmful bioreductant such as plants or microorganisms in silver synthesis. This method is known as *Green Synthesis*. Emerging studies regarding this method could lead to a safer and economical way of synthesis [6, 7]

Many antibiotics in the market had been known to fight against microbes, but due to their side effects and growing resistance of microorganisms to these conventional antibiotics, there is a need to develop new antimicrobial agent that will be synthesized in a non-toxic approach by the use of bio-inspired material like the plants. Many studies regarding the green synthesis of silver nanoparticle, its mechanism, growth, and toxicity were top on the list of research due to the interesting properties of silver nanoparticle, most especially as antimicrobial agents [8]. The study shows that silver nanoparticle in a reduced form from silver nitrate helps to fight pathogens by interacting bacterial membranes of sulfur-containing proteins as well as with phosphorus-containing compounds like DNA [9, 10].

Crassocephalum. crepidiodes (Benth) S. Moore is an invasive herb included in the Global Compendium of Weeds and classified as one of the most aggressive weeds occurring in tropical and subtropical regions [11]. In Africa and Benin, fleshy mucilaginous leaves and stems are eaten as vegetable [12]. In this study, the decoction extract from the fresh leaves of *C. crepidioides* was explored as potential reductant for the synthesis of silver nanoparticle from silver nitrate. Synthesized silver nanoparticles were evaluated for antimicrobial property against selected pathogens. UV-VIS Spectroscopy was used to determine the effect of reaction time towards the synthesis of silver nanoparticle. FTIR Spectroscopy was used to identify possible functional groups in the plant extract which has the potential to reduce silver ions to elemental silver. SEM-EDS analysis was used to determine the morphology of the synthesized silver nanoparticle.

2. EXPERIMENTAL DETAILS

Sample Collection and Preparation

Fresh leaves of *C. crepidioides* were collected from Marawi City, Philippines. The samples were washed three times with distilled water. Around 40.0g of finely cut *C. crepidioides* was soaked in 200 mL distilled water and heated to boiling for 20 minutes with constant stirring. The mixture was filtered using Whatman No.41 filter paper and the filtrate was immediately used for synthesis of silver nanoparticle.

Biosynthesis of silver nanoparticle

Five millimolar (5.00mM) of silver nitrate was prepared by dissolving 0.4247g $AgNO_3$ to 500mL distilled water. Five different v/v (volume/volume) ratio of plant extract to $AgNO_3$ were prepared and labelled as **S_1, S_2, S_3, S_4,** and **S_5**. For **S_1(1:50),** one mL of the aqueous extract was mixed to 50mL of 5.0mM $AgNO_3$ in an Erlenmeyer flask and covered with aluminum foil. Color change was monitored and time was recorded. The volume of the extract was varied while the volume of $AgNO_3$ was held constant to prepare the other mixtures **(i.e., S_2(5:50), S_3(10:50), S_4(20:50)** and **S_5(30:50)**. For each ratio, three replicates were prepared and were allowed to react at different reaction times (0, 24, and 48 hours). Synthesis was performed at room temperature and in the absence of light (dark room).

Antimicrobial screening

The antimicrobial screening was done using Paper Disc Diffusion Method [13]. Each ratio at different time was tested and determined for its antimicrobial potential. Biosynthesized silver nanoparticles were tested against selected pathogens. *Staphylococcus aureus, Escherichia coli, and Bacillus subtilis* were used for bacteria, while *Aspergillus niger* and *Candida albicans* were used for fungi. Fresh plant extract, 5.0mM $AgNO_3$, distilled water, Amoxicillin, and Nystatin were being tested as controls.

Ultraviolet-Visible (UV-Vis) Spectroscopy

The biosynthesized silver nanoparticle was characterized using *Lasany* Double Beam UV-Vis spectrophotometry LI-2800. The five ratios at different reaction time were analyzed to determine the absorbance of each ratio. Wavelength of maximum absorption (λ_{max}) was scanned within 300 – 600 nm range. The absorbance of the different sample ratio was determined at the λ_{max}. The analysis was conducted at the Chemistry Department, College of Science and Mathematics (CSM), MSU-Iligan Institute of Technology (MSU-IIT).

Fourier Transform Infrared (FTIR) Spectroscopy

The ratio that exhibited the highest zones of inhibition from the antimicrobial screening was further characterized using FTIR. A few drops of biosynthesized silver that exhibits the highest zones of inhibition and fresh *C. crepidioides* extract were placed in separate glass slides and were dried in an oven at 110°C for 5 minutes. Identification of the different functional groups present in the samples was determined using FTIR at the Physics Department, CSM, MSU-IIT.

Scanning Electron Microscope-Energy Dispersive Spectrometer (SEM-EDS)

The ratio that exhibited the highest zones of inhibition from the antimicrobial screening was subjected to morphological analysis using SEM-EDS to know the actual size and shape of the biosynthesized silver. A small drop of the sample was placed in a glass slide, dried in an oven for 110°C and analyzed. Silver nitrate solution was also taken for reference. The analysis was conducted at the Physics Department, CSM, MSU-IIT.

3. RESULTS AND DISCUSSION

Biosynthesis of silver nanoparticle

Table 1 shows the result of the biosynthesis of silver nanoparticle with its color change. A pale yellow solution turning to dark grey and with brownish orange color was observed at different reaction time. This qualitative test indicates the formation of silver nanoparticles. The collective oscillation of electrons in a nanoparticle upon interaction with light of suitable energy causes the nanoparticles to attain the color specific to a particular metal. Particle size, shape, and aggregation depend strongly on the frequency of oscillations [14].

Table 1. Color of silver nanoparticle at different incubation time

Ratio of plant extract to silver nitrate	Color of synthesized silver nanoparticle			
	Before 0	**0**	**24**	**48**
S_1(1:50)	Colorless	Pale yellow	Dark orange	Greyish orange
S_2(5:50)	Pale orange	Orange	Greyish orange	Greyish orange*
S_3(10:50)	Golden yellow	Slightly Dark yellow	Greyish dark orange	Greyish orange with**
S_4(20:50)	Brownish yellow	Brownish orange	Intense greyish orange*	Intense greyish orange**
S_5(30:50)	Golden yellow	Dark green	Intense greyish orange*	Intense greyish orange**

Note: *with less precipitate **with more precipitate

Antimicrobial screening

Tables 2 to 5 show the results of the antimicrobial assay of the synthesized silver nanoparticle. Means having the same uppercase letter are not significantly different at α=0.05 for each reaction time per treatment. Means having the same lowercase letter are not significantly different at α=0.05 for each treatment per reaction time.

Antibacterial Assay

Table 2 shows the results for antibacterial assay of synthesized silver nanoparticles using *E. coli* as test organism. Based on statistical analyses using One-Way ANOVA and Duncan Multiple Range Test (DMRT) for the effect of reaction time, it shows that at S_1 to S_5 have highest zones of inhibition at 0 hr. In addition, an abnormal decreasing trend of zones of inhibition conform as time of reaction for each treatment increases.

Table 3 shows the results for antibacterial assay of synthesized silver nanoparticles using *S. aureus* as test organism. Based on the statistical analyses using One-Way ANOVA and Duncan Multiple Range Test for 0hour, S_3 is significantly different from the rest and has the second highest zone of inhibition right after Amoxicillin. In addition, S_1 has the highest zone of inhibition at 48 hours. However, with respect to each time of reaction, no clear pattern for the zone of inhibition is observed.

Table 4 shows the results for antibacterial assay of synthesized silver nanoparticles using *B. subtilis* as test organism. Statistical analysis reveals that S_1 has the highest zones of inhibition at 0hr, 24hr, and 48hr. This only means

that S_1 shows the highest efficacy for suppressing the growth of *B. subitilis*.

Table 2 Mean diameter zones of inhibition for the antibacterial assay of synthesized silver nanoparticles using *E. coli* as test organism

Treatment	Mean diameter zones of inhibition (mm) against *E.coli*		
	0 hour	24 hour	48 hour
1:50 (S_1)	4.33^{Be}	2.67^{Ad}	2.33^{A}
5:50 (S_2)	4.67^{B}	2.00^{Ac}	1.67^{Ac}
10:50 (S_3)	5.33^{B}	2.00^{Ac}	1.67^{Ac}
20:50 (S_4)	2.33^{Ac}	2.00^{Ac}	1.33^{Ac}
30:50 (S_5)	2.67^{A}	2.00^{Ac}	1.67^{Ac}
Amoxicillin(A)	8.67^{Af}	12.67^{Be}	12.33^{Bd}
$AgNO_3$ (S_0)	3.00^{Bd}	1.00^{Bb}	0.67^{Ab}
Plant Extract (P)	1.67^{Bd}	0.00^{Aa}	0.67^{Ab}
Distilled water (DI)	0.00^{a}	0.00^{a}	0.00^{a}
Average mean	3.63	2.70	2.37

Table 3 Mean diameter zones of inhibition for the antibacterial assay of synthesized silver nanoparticles using *S. aureus* as test organism

Treatment	Mean diameter of zones of inhibition (mm) against *S. aureus*		
	0 hour	24 hour	48 hour
1:50 (S_1)	4.33^{Bcd}	2.33^{A}	4.00^{d}
5:50 (S_2)	4.33^{Bcd}	2.67^{A}	2.00^{Ab}
10:50 (S_3)	4.67^{Cd}	2.67^{B}	2.00^{Ab}
20:50 (S_4)	3.67^{c}	2.33	2.67^{bc}
30:50 (S_5)	4.00^{Ccd}	2.67^{B}	2.00^{Cb}
Amoxicillin(A)	5.33^{e}	5.33^{b}	5.33^{e}
$AgNO_3$ (S_0)	2.67^{b}	2.00	3.33^{c}
Plant Extract (P)	2.67^{Bb}	0.33^{Aa}	2.67^{Bbc}
Distilled water (DI)	0.00^{a}	0.00^{a}	0.00^{a}
Average mean	3.52	2.26	2.67

Table 4 Mean diameter zones of inhibition for the antibacterial assay of synthesized silver nanoparticles using *B. subtilis* as test organism

Treatment	Mean diameter of zones of inhibition (mm) against *B. subtilis*		
	0hour	24hour	48hour
1:50 (S_1)	3.00^{A}	2.67^{Ad}	2.00^{Ab}
5:50 (S_2)	1.67^{Ab}	1.67^{Ac}	0.67^{Aa}
10:50 (S_3)	1.67^{A}	1.67^{Ac}	1.00^{Aa}
20:50 (S_4)	2.00^{Bb}	1.00^{Bb}	0.67^{Aa}
30:50 (S_5)	2.00^{Bb}	2.00^{B}	0.67^{Aa}
Amoxicillin(A)	8.67^{Ac}	7.67^{Ae}	8.67^{Ac}
$AgNO_3$ (S_0)	0.67^{Aa}	0.67^{Aa}	0.67^{Aa}
Plant Extract (P)	0.67^{A}	0.00^{A}	0.00^{A}
Distilled water (DI)	0.00	0.00	0.00
Average mean	2.26	1.93	1.59

Antifungal assay

Table 5 shows the results for antifungal assay of synthesized silver nanoparticles using *C. albicans* as test organism. Based on the results, only Nystatin has shown positive results for antifungal activity at 0hr and 24hr. But in the 48hour, there is zone of inhibition from the sample with S_1 as the highest with 2.67mm. With respect to the antifungal assay of synthesized silver nanoparticles using *A. niger* as test organism, there were no observed zones of inhibition for S_1 to S_5.

Among the five different ratios, only S_1 exhibited the highest zone of inhibition for the three bacteria at the three reaction time and *C. albicans* at 48 hours. The S_1 (1:50) possessed the highest antibacterial activity against E. coli, S. aureus, and B. subtilis and less for antifungal activity towards *C. albicans*.

Table 5 Mean diameter zones of inhibition for the antibacterial assay of synthesized silver nanoparticles using *C. albicans* as test organism

Treatment	Mean diameter of zones of inhibition (mm) against *C. albicans*		
	0hour	24hour	48hour
1:50 (S_1)	0.0^{Aa}	0.0^{Aa}	2.67^{Ac}
5:50 (S_2)	0.0^{Aa}	0.0^{Aa}	2.00^{Ab}
10:50 (S_3)	0.0^{Aa}	0.0^{Aa}	2.00^{Ab}
20:50 (S_4)	0.0^{Aa}	0.0^{Aa}	0.33^{Aa}
30:50 (S_5)	0.0^{Aa}	0.0^{Aa}	0.33^{Aa}
Nystatin (N)	5.00^{Ab}	6.67^{Ab}	7.00^{Ad}
$AgNO_3$ (S_0)	0.0^{Aa}	0.0^{Aa}	0.00^{a}
Plant Extract (P)	0.0^{Aa}	0.0^{Aa}	0.00^{a}
Distilled water (DI)	0.0^{Aa}	0.0^{Aa}	0.00^{a}
Average mean	0.56	0.74	1.59

Characterization of silver nanoparticles

The formation of silver nanoparticle was confirmed using Ultraviolet-Visible (UV-VIS) spectrophotometer, Fourier transform infrared (FTIR) spectrometer, and scanning electron microscope equipped with energy dispersive spectrometer (SEM-EDS).

UV-Vis spectroscopy

Table 6 shows the wavelength of maximum absorption (λ_{max}) and the absorbance of each sample. From these data, S_1, S_2, S_3, and S_4 has the presence of silver nanoparticle because λ_{max} with 400-450nm will confirm that there is silver nanoparticle due to surface Plasmon resonance effect [14].

S_5, having no peak can be accounted to no silver nanoparticles has been synthesized because it is beyond the range and the instrument couldn't detect its wavelength and this may be due to increase in particle size and aggregation of colloidal particles forming precipitates which eventually could produce bigger particles not in the nanoscale. When nanoparticles are in solution, molecules associate with the nanoparticle surface to establish a double layer of charge that stabilizes the particles and prevents aggregation [15].

Only the S_1 and S_5 from the 24hour reaction time give the peak those are in the range for silver nanoparticles. The rest were above the range, since qualitatively the samples contained a precipitate that affects the absorbance from the UV-Vis region. These ratios were darker compared to S_1 and S_5. The S_1 having 442nm has synthesized silver nanoparticle with greyish orange solution while the other ratio exceed from 450nm and these account for the formation of bigger particles not in a nanoscale. The formation of silver nanoparticle can be detected at the color of yellow to orange from 400 to 450nm.

FTIR Spectroscopy

FTIR analysis was carried out to determine functional groups present in the extract which are possible reducing agents. The spectra of extracts were recorded before and after adding silver nitrate. S_1 at 0hour was selected for the spectroscopic analysis since this ratio exhibited the highest zones of inhibition for most pathogens. **Figure 2** and **Figure 3** show the spectra of the *C. crepidioides* extract without silver nitrate and *C. crepidioides* with silver nitrate sample, respectively.

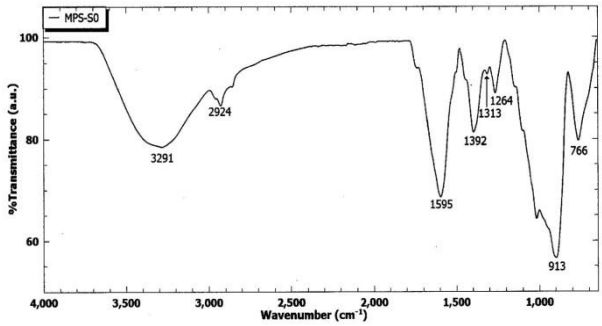

Figure 2. MPS-S0: *Crassocephalum crepidioides (Benth) S. Moore* extract without $AgNO_3$

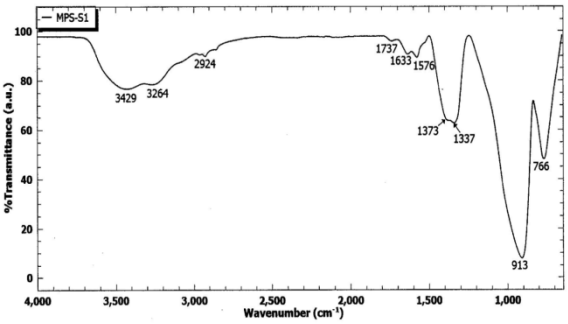

Figure 3. MPS-S1: 1:50 for 24 hours (*C. crepidioides* w/ $AgNO_3$)

From **Figure 2**, broad peak at 3291cm^{-1} and weak peaks at 2924cm^{-1} and 1264cm^{-1} indicate the presence of a phenol group with an O-H stretch. Phytochemical screening of the aqueous extract from *C. crepidioides* leaves revealed the presence of tannins which are polyphenols and this coincides with the spectral analysis [12]. This functional group reduces the silver ions to silver nanoparticle. The medium peak at 1595cm^{-1} is an N-H bend which corresponds to a primary amine functional group. The medium peak at 1392cm^{-1} is an N-H stretch which corresponds to an amide. The weak peak at 1313cm^{-1} is a C-N which corresponds to an aromatic primary amine. In relation to the secondary metabolites present in the plant, the presence of alkaloids was responsible for the reduction of the silver ions from the silver nitrate solution. Alkaloids contain organic nitrogen bases and are found primarily in flowering plants.

The broad peaks at 3429cm^{-1} and 3264cm^{-1} having an O-H stretch for alcohol in **Figure 3** changes in relation to **Figure 2** with 3291cm^{-1}. This change in the band peak is responsible for the formation of silver nanoparticle. Also, a weak band peak at 1737cm^{-1} corresponds to a C-H bend for an aromatic compound. Strong peaks at 1337cm^{-1} and 1373cm^{-1} is an N-O stretch for a nitro compound which increases its transmittance and this was due to silver nanoparticle formation. An N-H bend with a secondary amine was observed from 1576cm^{-1} and 1633cm^{-1}. This peak was also evident from plant extract. Thus, it can be accounted that this group was responsible for the reduction of silver nitrate to silver nanoparticle. From the antibacterial activity, S_1 exhibited the highest zone of inhibition and as with the spectral analysis it revealed the functional group responsible for the reduction of silver ions to elemental silver or silver nanoparticle and served as an effective antibacterial agent.

SEM-EDS

Analysis of S_1 using scanning electron microscope confirmed the presence of silver particles in colloidal mixture. **Figure 4** shows the results for silver particles viewed at 5000 magnifications. The exterior surfaces of small Ag-NPs become shiny in the spots' spherical shapes [16]. These results confirm that extract of *C. crepidioides* can control the shape and size of the Ag-NPs.

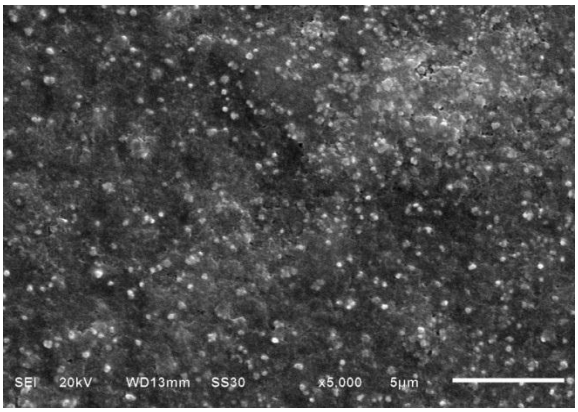

Figure 4. SEM result of the sample viewed at 5000 magnification

The optical absorption peak was observed at 2.983keV which is typical for silver nanocrystallite absorption due to surface plasmon resonance. In the recent study, individual spherical silver nanoparticles synthesized using alfalfa showed absorption peaks in the range of 2.5–4 keV [17]. The current profile for energy dispersive x-ray spectroscopy for silver nanoparticles of *C. crepidioides* indicate strong silver atoms signals at 3.0, 3.075, and 3.3keV, respectively.

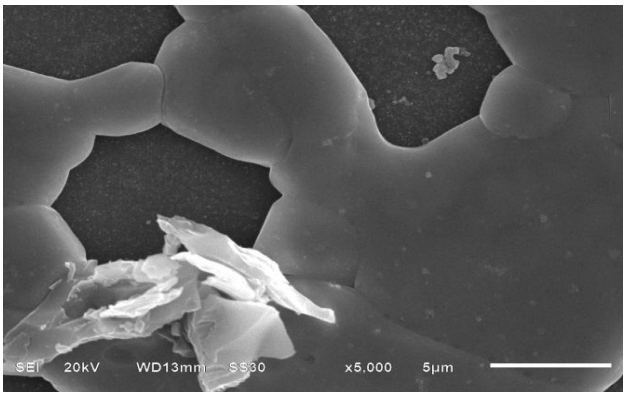

Figure 5. SEM result for $AgNO_3$ at 5000x magnification

Figure 5 shows the morphology of the blank sample ($AgNO_3$ only). Result of analysis shows difference in the size of the blank from that of the sample.

4. CONCLUSIONS

Silver nanoparticles were synthesized using fresh leaf extract of *Crassocephalum crepidioides (Benth) S. Moore*. The color change of each ratio were distinguishable with a color which ranges from yellow to orange and then to turbid orange. Among the five different ratios, only S_1 exhibited the highest zone of inhibition for the three bacteria at the three reaction time and *C. albicans* at 48 hours. Preliminary analysis using UV-Vis spectroscopy confirms the formation of silver nanoparticle. A broad peak was observed in the range of 400nm-450nm for all the ratios. Only the S_1 ratio gives 419nm, 446nm, and 442nm for the reaction time 0hour, 24hour, and 48hour, respectively. FTIR analysis revealed the presence of the functional groups such as phenols, aromatic primary amine, nitro group, and aromatic hydrocarbon. These results coincide with the preliminary tests and this would account that the biosynthesis of silver nanoparticle using the leaf extract of *C. crepidioides* was successfully done. Lastly, SEM-EDS analysis confirmed the reduction of silver ions to silver nanoparticle as shown by the morphological data.

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