BIOGENIC SYNTHESIS OF SILVER NANOPARTICLES USING NEEM LEAF EXTRACT AS REDUCING AGENT AND HYDROLYZED COLLAGEN AS STABILIZING AGENT

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Abstract. Silver nanoparticles (AgNPs) has been widely used as antimicrobial agent toward many bacterial strains and microorganisms. In this study, microwave-assisted green synthesis of silver nanoparticles (AgNPs) using *Melia dubia* (neem) leaf extract as reducing agent along with hydrolyzed fish collagen as stabilizing agent have been studied. Neem extract itself has the ability to inhibit bacteria. The reduction of silver nitrate (0.01M $AgNO_3$) solutions using neem extract was carried in microwave oven with and without collagen as stabilizing agent. The AgNPs produced were characterized by UV-Vis spectroscopy, scanning electron microscope (SEM) and x-ray diffraction analysis (XRD). The ability of AgNPs to inhibit two types of bacteria i.e *Escherichia coli (E. coli)* and *Staphylococcus aureus (S. aureus)* was conducted. The UV-visible spectra of AgNPs showed a shift to lower absorbance peak with the presence of collagen. Meanwhile, the spectrum of pure hydrolyzed fish collagen detected at the wavelength 215.5 nm. The microstructure analysis of AgNPs using SEM reveals that the synthesized silver nanoparticles particles produced around 63.4 nm for sample without collagen whereas around 39.5 nm for the sample with collagen. The XRD analysis proved the formation of AgNPs in both technique and demonstrated the crystalline nature of the AgNPs. The AgNPs produced with and without the presence of collagen are able to inhibit the growth of both bacteria. The AgNPs synthesized with collagen showed better antibacterial properties with the diameter of bacteria *S. aureus* and *E. coli* inhibition are 10.5 mm and 8.8 mm respectively. This study proved that collagen can be used as stabilizing agent to produce nano silver particles with smaller particles size and better antibacterial properties.

Keywords: silver nanoparticles, neem extract, collagen, microwave-assisted

Article Info

Received 14th January 2022
Accepted 11th April 2022
Published 20th April 2022

ISSN: 1823-7010, eISSN: 2600-7444

Introduction

Silver (Ag) is well-known material for its ability to kill bacteria and thus widely utilized in medical field as a topical bactericide [1]. Dental procedure, catheters and burn injuries are among the applications that utilized the function of Ag in order to control the bacterial growth [2]. In relation of this matter, the silver nanoparticles possess a wide surface to volume ratio, thus making it an ideal candidate for antimicrobial agent that able to attack on bacterial surface broadly [3]. In addition, comparing to microparticles, nanoparticles enhanced the physical as well as chemical characteristics due to its larger surface area. Nanoparticles enable work at the atomic, molecular and supramolecular level which ranging from 1 nm to 100 nm [4]. The potential of nanoparticles in the nanotechnology field has been recognized since ages and proven to be actively studied and developed by researchers [5]. Therefore, combining the positive traits possesses by Ag and nanoparticles, many previous researchers have developed silver nanoparticles (AgNPs) by different methods.

There are various methods in synthesizing the AgNPs include physical and chemical methods were reported [6]. Generally, the $AgNO_3$ solution was undergone redox reaction upon the addition of reducing agent in which the Ag ion reduced to Ag particle in nano size. The synthesis methods include physical and chemical methods. However, these types of typical redox syntheses demanded the utilization of particular energy input or hazardous chemicals which is harmful to the environment and will give rise to various side effects [7]. Therefore, it is only natural to opt for eco-friendly synthesis of AgNPs or also known as green synthesis. Previous study recommended that green synthesis is one of the best method due to its eco-friendly, biocompatibility and less toxic trait [8]. Green synthesis consists of synthesizing nanoparticles using microorganisms such as bacteria and fungus, plants and DNA [9]. Due to the ability of eliminating the elaborate process of cell culture, plant-synthesized was more preferable over microorganism-synthesized of AgNPs [3].

Neem is one of the mahogany (Meliaceae) family member distributed in tropical and subtropical regions. Two common species of neem are *Azadirachta indica* and *Melia dubia* which are actively studied as reducing agent for green synthesis of AgNPs. *Melia dubia* species most commonly found in India (with the exception of Jammu & Kashmir, Himachal Pradesh, Sikkim), the Malay Peninsula and tropical Asia [10]. *Melia dubia* species was chosen in this study as it can be easily found in Tanjong Malim, Perak Malaysia. *Melia dubia* leave extract act as an excellent reducing agent for its phytochemicals compounds which are terpenoids and flavanones [3]. Neem extract itself possesses antibacterial properties which is explained the wide use of it in various applications involving medical products [11]. Therefore, the green synthesis of AgNPs using neem extract developed in this study exhibit excellent antimicrobial activity.

Previous researcher conducted the green synthesis of AgNPs using *Melia dubia* (neem) leaves extract as reducing agent [12]. However, they were extracted neem leave at room temperature and it took about 24 hours to complete the extraction process They also used high molecular weight fish collagen as stabilizing agent and microwave irradiation to obtain spherical shape nano silver particles. This present study focusing on extraction of *Melia dubia* leave at high temperature (100 ^{o}C) with shorter extraction time (1 hours). The extracted neem was used as reducing agent and low molecular weight of collagen (below 3000 Da) for synthesis of AgNPs.

Room temperature synthesis of AgNPs using neem extract may take about 24 hours to complete the reactions. Therefore, microwave-assisted green synthesis of AgNPs is favourable as it offers less energy consumption and shorter reaction time [13]. In addition, the selection of stabilizing agent is highly recommended as it will prolong the lifespan of AgNPs [4]. Without stabilizing agent, the AgNPs particles have high tendency to become clustered and thus producing larger silver particles. Thus it is vital to embed a stabilizing or capping agent to maintain the nanoparticles at nanoscale [14].

Hence, in this study *Melia dubia* (neem) leave was extracted at high temperature and used as reducing agent for synthesizing of silver nanoparticles (AgNPs) by microwave irradiation and hydrolyzed fish collagen as the stabilizing agent.

Materials and Methods

The main materials are silver nitrate ($AgNO_3$) which was purchased from Sigma Aldrich in a form of white fine crystals, *Melia dubia* (neem) leaf was obtained locally from Tanjong Malim, Malaysia hydrolyzed fish collagen was purchased from Evachem Sdn Bhd, Selangor, Malaysia and Luria Bertani (LB) agar from Himedia.

Preparation of neem extract and silver nitrate solution. 100 g of fresh *Melia dubia* was washed with water to remove any impurities and boil in 500 ml of distilled water for 2 hours. The extract solution was filtered and keep in cold temperature until further used. About 0.1699 g of fine crystals of silver nitrate ($AgNO_3$) (molecular weight = 169.87 g/mol) was weighed and then dissolved in 100 ml distilled water to be made into 0.01 M of $AgNO_3$ solution.

Microwave-assisted green synthesis of AgNPs. The 0.01 M of 100 mL $AgNO_3$ solution was transferred into the 250 mL conical flask. Then, 0.1 g of fish collagen that act as stabilizing agent was added. Next, the mixture was being stirred using the magnetic stirrer for 1 hour until homogenized. After that, 10 mL of neem leaf extract was poured into the conical flask and the mixture was exposed to microwave irradiation at 510 W for 1 minute. Another set of $AgNO_3$ solution of the same concentrations were also prepared with the absence of fish collagen as stabilizing agent.

The characterizations of AgNPs. The characterization of synthesized AgNPs was conducted by using the Field Emission Scanning Electron Microscope (FESEM) in which Nova NanoSEM 450 was operated at 2.0 kV. Next, the UV-Vis Spectrophotometer (Cary 60 – Agilent Technologies) was utilized to scan the absorbance spectra ranging from 200 nm to 800 nm in wavelength. X-Ray Diffraction (XRD) analysis was also being carried out using Rigaku MiniFlex with the usage of voltage and current was 40 kV and 15 mA respectively, scanning from 3^o to 80^o with the rate of 3.0^o per minute.

Antimicrobial activity. The evaluation of antimicrobial activity was conducted by agar disk-diffusion method. The agar plates that act as the media were prepared by mixing 20 g of Luria Bertani (LB) agar into 500 ml of sterilized distilled water. Then, the solution was stirred with heat. After the solution was slightly cold, it was poured into numbers of petri dishes. This step was being done inside the laminar flow. Two set of agar plates were inoculated with two different types of bacteria which are *Escherichia coli (E. coli)* and *Staphylococcus aureus (S. aureus).* The bacteria were then left overnight to grow. Next, filter

paper discs (diameter = 5mm) that contained the AgNPs samples were placed on the agar surface. The agar plates were then incubated at 37 °C for approximately 16 to 18 hours. The bacteria inhibition then was observed.

Results and Discussion

Observation of color changes. The primary identification of the synthesized AgNPs was being done by merely visual observation specifically its color changes after the treatment of $AgNO_3$ solution with *Melia dubia* (neem) leaf extract as presented in Figure 1. Previous report stated that the production of AgNPs was proven by the color changes of the solutions [14]. Generally, the colorless solution of $AgNO_3$ changed into yellowish brown upon the addition of neem extract. Then, the solution turned into dark brown in color after the exposure to microwave irradiation. It is indicated that there is no silver salt left for further reaction when the solution becomes constant in color.

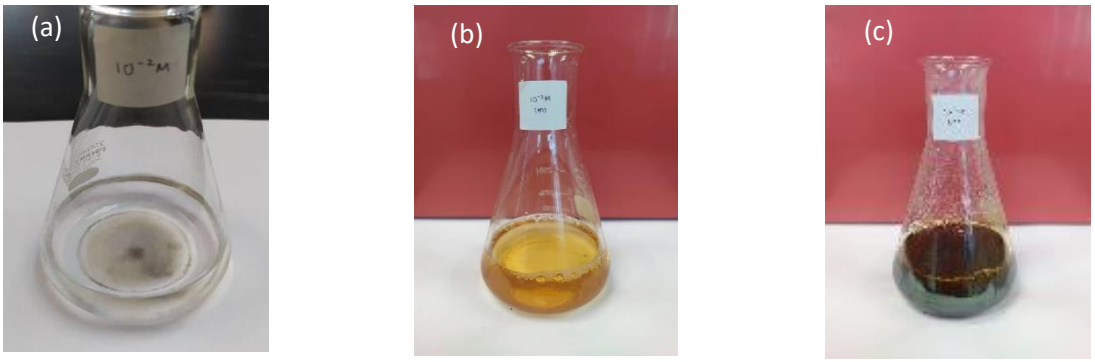

Figure 1. For the microwave-assisted green synthesis of AgNPs, (a) $AgNO_3$ solution was initially colourless, (b) the solution turned into yellowish brown after the addition of neem extract and (c) the solution turn into dark brown and becomes constant in color after exposing to microwave irradiation.

UV-Visible spectroscopy analysis. The synthesized of AgNPs, neem extract and collagen solutions were scanned from 200 nm to 800 nm using UV-vis spectroscopy and the spectrum is overlaid in Figure 2. It is observed that the formation of sharp peak at 440 nm and 443 nm were due to the present of AgNPs in the solution. Synthesized of AgNPs without and with the presence of collagen exhibited peak at 443 nm and 440 nm respectively. The results show that the shift of absorption peak to lower wavenumber could be due to the smaller size of AgNPs with the present of collagen as stabilizing agent. It was reported that Ag with smaller particles size (below 100 nm) exhibited stronger localize surface plasmon resonance effect in between 400 nm and 450 nm [5]. Previous research using other plant extract and silver ion reported the same observation of the absorbance peak formation at around 440 nm for the synthesized AgNPs [15]. Other researchers were reported that the smaller the particle size of the AgNPs, the lower absorbance peak [16]. Thus, the results from this study indicated that the presence of collagen in the reaction solution successfully stabilize the formation of nano Ag cluster to promote smaller particles size formation during green synthesis of AgNPs.

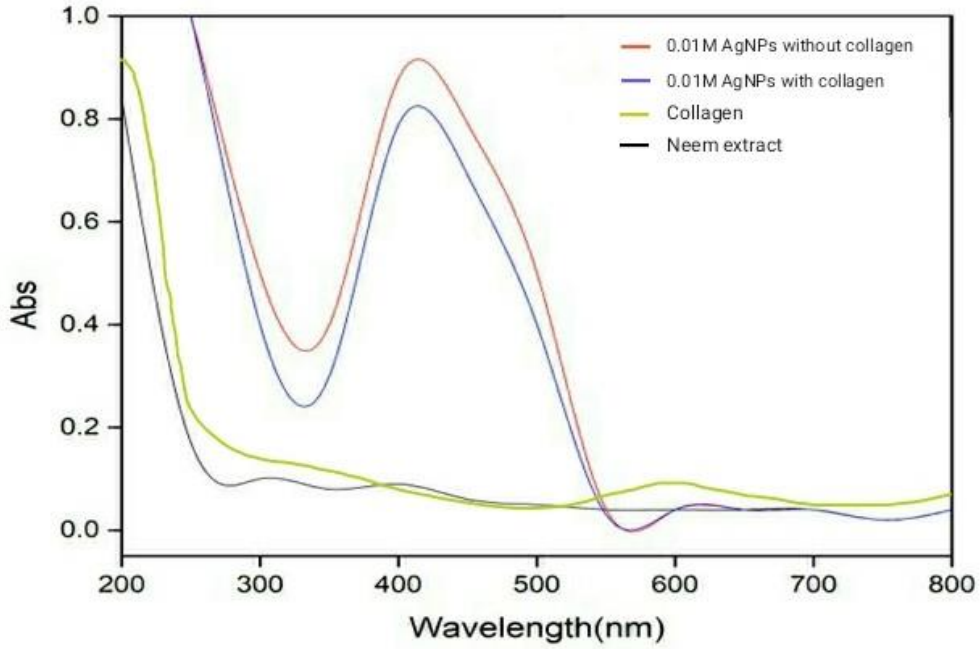

Figure 2. UV-Vis spectroscopy of the 0.01 M microwave-assisted green synthesized AgNPs (with and without the presence of collagen) and pure neem extract.

Field emission scanning electron microscopy analysis. Figure 3 represent the electron microscopy images of synthesized AgNPs from the $AgNO_3$ solution with the concentration of 0.01 M with and without collagen. The nanoparticles produced are more spherical in shape with the present of collagen and having a crystalline structure as shown in red circles. The particle size of the nanoparticles obtained from the ImageJ analysis for the sample without the presence of collagen is around 63.4 nm. On the other hand, the particle size for the sample with the presence of collagen is around 39.5 nm. The size of the synthesized AgNPs with the presence of collagen is finer thus proving the ability of collagen as the stabilizing or capping agent that maintained the nanoparticles at nanoscale [13]. The SEM analysis results seem to support the finding as shown in uv-vis spectroscopy analysis.

X-Ray Diffraction analysis. The XRD pattern of AgNPs samples is shown in Figure 4. The sharp peaks results proved the synthesis of AgNPs by the reduction of Ag salt using neem leaf extract is crystalline in nature. Based on the results, the diffraction peaks for synthesized AgNPs without collagen was observed at $2\theta = 12.8^o$, 19.6^o, 21.6^o, 29.6^o, 35.4^o and 39.0^o. Meanwhile, for the AgNPs that was synthesized with the presence of collagen showed peaks at $2\theta = 12.7^o$, 31.5^o, 32.2^o, 38.1^o, 44.3^o and 46.2^o. Both values of 2θ corresponding to the lattice planes of silver at (101), (111), (200) and (220). The average crystallite size of synthesized AgNPs was calculated using the Scherrer's equation [17]:

$$D = \frac{K\lambda}{\beta \cos \theta} \quad (1)$$

where,
D = crystallites size (nm)
K = 0.9 (Scherrer constant)

λ = 0.15406 nm (wavelength of the x-ray sources)
β = FWHM (in radians)
θ = peak position (in radians)

Based on Scherrer equation, the average crystallite size calculated for synthesized AgNPs sample without the presence is 55.5 nm whereas the synthesized AgNPs with the presence of collagen appeared to be finer in size which is 7.8 nm.

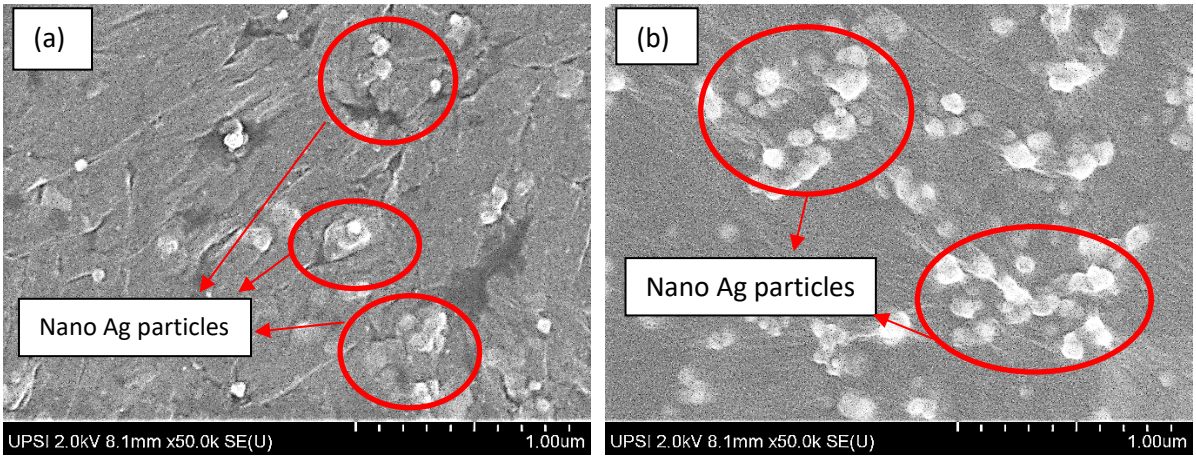

Figure 3. SEM micrographs of synthesized AgNPs (a) without collagen and (b) with collagen.

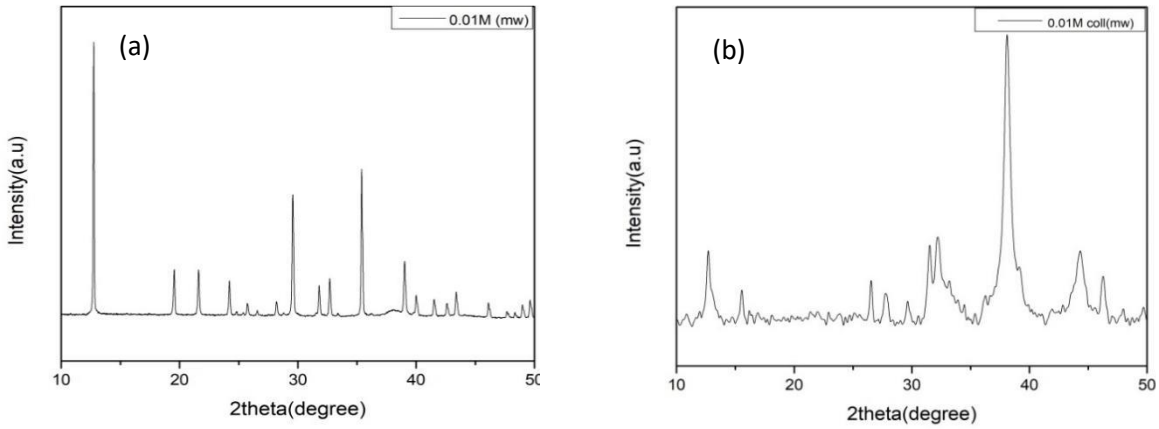

Figure 4. XRD patterns of AgNPs for microwave-assisted samples (a) without collagen and (b) with collagen.

Antimicrobial activity. The antimicrobial activity has been conducted using agar disk-diffusion method against two types of bacteria which are gram positive bacteria (*S. aureus)*, Figure 5 and gram negative bacteria (*E. coli*), Figure 6. This method is commonly used in determining the antimicrobial activity especially in the most institutions and hospitals [18]. *S. aureus* and *E. coli* were chosen to be tested against off as they are the most frequently isolated bacteria [19] as well as its availability in the laboratory. Among the gram positive bacteria, *S. aureus* included as the one of the very crucial pathogen in the medical field.

Furthermore, healthcare-acquired infections or nosocomial infections in adults are mainly caused by *S. aureus* and *E. coli* [20]. Therefore, antimicrobial activity against these bacteria is highly recommended.

Sterilized distilled water (dH_2O) and ethanol (etOH) acted as the negative and positive control respectively. The inhibition of antibacterial growth can be observed through the zone of bacteria inhibition. The diameter of zone of bacteria inhibition is presented in Table 1.

Table 1. The diameter of zone of inhibition for bacteria S. Aureus and E. Coli on microwave-assisted samples.

Samples		dH_2O	etOH	0.01M (mw)	0.01M coll (mw)
Zone of bacteria inhibition (mm)	S. Aureus	5.0	6.0	10.0	10.5
	E. Coli	5.0	6.0	8.0	8.8

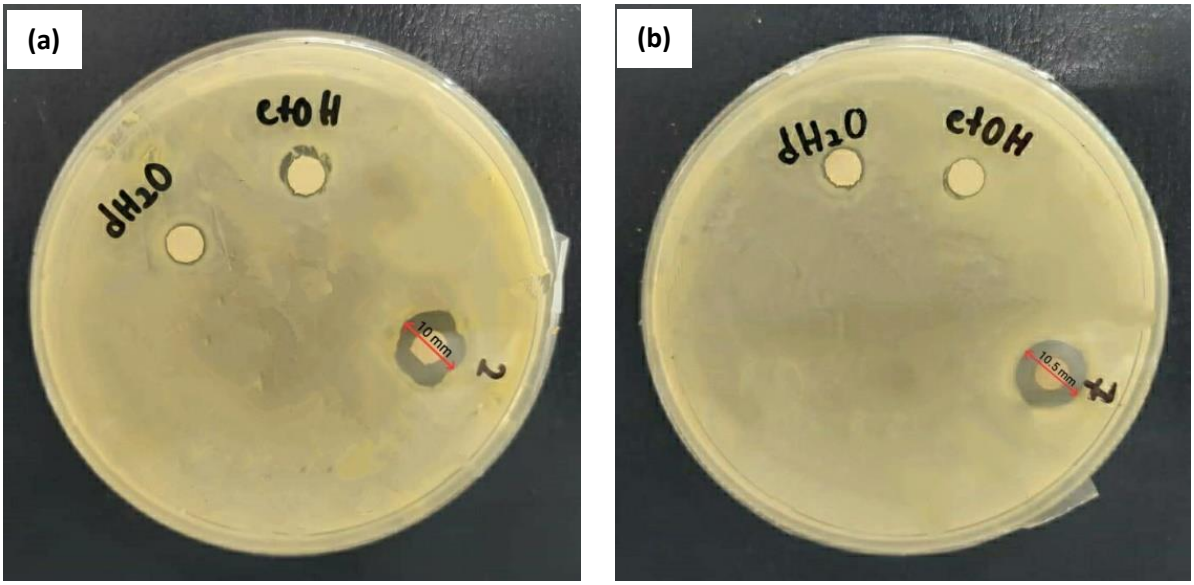

Figure 5. Inhibition zone of bacteria S. Aureus for microwave-assisted green synthesized AgNPs (a) without collagen (No. 2) and (b) with collagen (No. 7) samples. Sterilized distilled water (dH_2O) and ethanol (etOH) acted as the negative and positive control respectively.

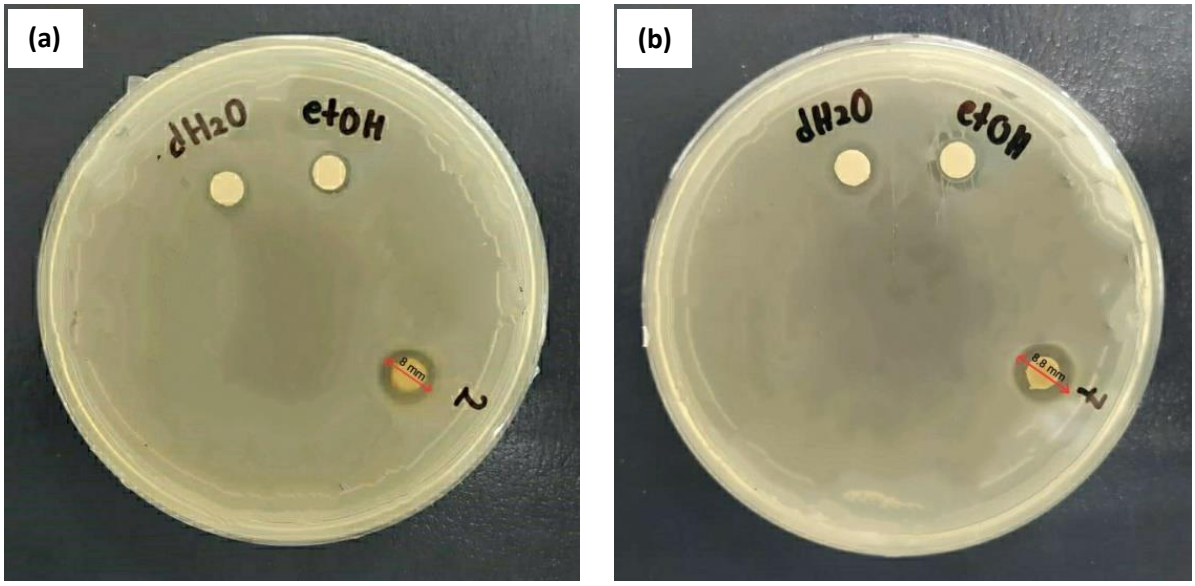

Figure 6. Inhibition zone of bacteria E. Coli microwave-assisted green synthesized AgNPs (a) without collagen (No. 2) and (b) with collagen (No. 7) samples. Sterilized distilled water (dH_2O) and ethanol (etOH) acted as the negative and positive control respectively.

The diameter of the filter paper disc that had been diffused with the sample tested was 5.0 mm. According to the results of antimicrobial activity on Table 1, the sterilized distilled water (dH_2O) as negative control showed no formation of inhibition zone proven that there was likely any contamination occurred. Meanwhile, ethanol (etOH) served as the positive control showcasing a poor resistance against both bacteria at 6.0 mm. The microwave-assisted green synthesized AgNPs from $AgNO_3$ solution with the concentration of 0.01 M and the presence of collagen (No. 7) showed a remarkable antibacterial property by inhibiting a large bacterial zone against *S. Aureus* and *E. Coli* bacteria with the diameter of 10.5 mm and 8.8 mm respectively. The sample of the same concentration (0.01 M) but without the presence of collagen (No. 2) shown that the diameter of inhibition zone against *S. Aureus* and *E. Coli* was slightly smaller than the sample with the presence of collagen at 10.0 mm and 8.0 mm respectively.

The antimicrobial study indicated the AgNPs sample with smaller particle size exhibited stronger antimicrobial properties. This result is in agreement with the finding by [21]. On the other hand, gram negative bacteria *E. Coli* showing a higher resistance of the AgNPs in compared with gram positive bacteria *S. Aureus.* This finding align with the research conducted by Bantawa et al [22] in which *E. Coli* exhibited the highest resistance to antibiotics as opposed to the other pathogens in the study which are *S. Aureus, Salmonella, Shigella* and *Vibrio*. As stated above, the results indirectly indicating that the microwave-assisted synthesized AgNPs with the presence of collagen has exhibited the most excellent antimicrobial property.

Conclusions

The microwave assisted-green synthesis of AgNPs using neem extract as reducing agent and collagen stabilizing agent successfully produced AgNPs with difference particle size. The exposure of silver pre-cursor to microwave irradiation at 510 W for 1 minute was economical procedure and potential to be used in commercial process for AgNPS production. The average particle size obtained from AgNPs which synthesized with collagen is about 39.5 nm and 63.4 nm for AgNPs sample without collagen. The UV spectra shown the absorption peak at 440 nm for AgNPs synthesized with collagen whereas 443 nm for AgNPs without collagen. Overall results indicated that the AgNPs samples synthesized with the presence of collagen produced smaller and finer particles. This proved the function of collagen as the stabilizing agent in maintaining the Ag particles at nanoscale. The antimicrobial activity against bacteria *S. Aureus* and *E. Coli* was tested and the AgNPs produced in this study exhibited an excellent antimicrobial property.

Acknowledgements

The financial support for this research was provided by Ministry of Higher Education Malaysia under FRGS Grant no. 2019-0147-103-02.

Author Contributions

All authors contributed toward data analysis, drafting and critically revising the paper and agree to be accountable for all aspects of the work.

Disclosure of Conflict of Interest

The authors have no disclosures to declare.

Compliance with Ethical Standards

The work is compliant with ethical standards.

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