Green synthesis of silver nanoparticles using *P. guajava L.* extract prepared by ultrasonic-assisted extraction, as reducing agent for wound dressing application: *In vitro* and *in vivo* evaluation

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Abstract

P. guajava L. leaf extract prepared by ultrasonic sound, is used as green reducing agent for silver nanoparticles (AgNPs) synthesis. Ultrasonic-assisted extraction provides not only a shortened process at low temperature but also a higher amount of reducing agents, compared to magnetic stirring method in same condition. A highly efficient radical scavenging activity of guava leaf extract, prepared by sonication (15 mins, 37 kHz, 150W), possesses the 50% inhibitory concentration (IC_{50}) of 43.29 µg/mL (1,1-diphenyl-2-picrylhydrazyl (DPPH•)). The synthesized AgNPs were confirmed by UV–vis spectrophotometry at 405 nm and X-ray diffraction. Field emission scanning electron microscopy and dynamic light scattering showed that the average sizes of the AgNPs were 20 to 40 nm and 27.3 nm respectively.

Guava leaf extract also acts as stabilizer for AgNPs. Zeta potential measurements (−56.5 mV) showed that the synthesized AgNPs had reasonably good stability. Antimicrobial activity assays of the AgNPs showed inhibition against selected bacteria E. coli. Healing effect on a second-degree burn of prepared AgNPs in an in vivo test is comparable to povidone. This study revealed the low-cost, fast and environmentally friendly synthesis of AgNPs, from P. guajava L. leaf extract, obtained by ultrasonic method and prepared AgNPs can be used as antimicrobial agent in nanobiotechnology field.

Keywords: Guava (*P. guajava L.*) leaf extract, silver nanoparticles synthesis, ultrasonic-assisted extraction, wound healing.

Introduction

Ultrasound-assisted extraction (UAE) has been extensively used for the plant's extraction due to increasing extraction rate and reduced processing time compared to heating magnetic stirring method[3,6,12,28]. In addition, UAE has some advantages such as low solvent volume, short ETs, few instrumental requirements and active-phytochemical retention. Energy from ultrasound destroys cell wall and cell membrane which supports the extraction of bioactive compounds from the plants[25]. Though UAE is a fast, effective extraction method for isolating plant material, this method depends on many parameters such as sonication intensity, extraction time, solvent to solid ratio which need to be further investigated.

Green synthesis of nanoparticles is an important subject due to many potential applications in environmental and biomedical fields[5,7,18]. Green synthesis aims at decreasing the usage of toxic chemicals and plant extracts are currently good candidates as green reducing reagents and stabilizers in the synthesis of metal nanoparticles[10,19]. To synthesize silver nano particles (AgNPs), many type of plants[1,4,14,24,26] and parts of plants like leaves, roots, flowers, fruits and rhizomes have been used and reported. Plant extracts have been obtained by using conventional techniques such as liquid-liquid extraction, solid-phase extraction and solid-phase micro-extraction, or modern techniques such as ultrasound-assisted extraction, pressurized liquid extraction, subcritical water extraction, supercritical fluid extraction, microwave-assisted extraction and instant controlled pressure drop extraction[29].

With different types of extracts from different plant sources, the synthesized AgNPs also have differences in morphology, physicochemical properties and long-term stability[21]. *P. guajava L.* is widely cultivated in Southeast Asia, especially Vietnam. Many studies indicate that *P. guajava L.* leaf extract contains a great number of bioactive compounds, such as polyphenol, polysaccharides, ascorbic acid, which may be considered as good reducing agent and stabilizer of nano particles[27]. According to review of the literature, *P. guajava L.* is primarily used to treat diarrhea and dysentery because of its antispasmodic and antimicrobial qualities.

It has also seen a lot of use as a hypoglycemic medication. In addition, other pharmacological properties of this plant are also recorded such as antioxidant, hepatoprotective, anti-allergy, antimicrobial, antigenotoxic, anti-plasmodial, cytotoxic, antispasmodic, cardioactive, anticough, antidiabetic, anti-inflammatory and antinociceptive qualities[8,15]. In this study, *P. guajava L.* leaf extract prepared by sonication was used as reductants and stabilizer, to reduce silver ions to rapidly form AgNPs at room temperature and at the same time to take advantage of the outstanding

biological activities of guava leaves for antimicrobic application.

Extraction time of sonication has been investigated to prepare *P. guajava L.* leaf extract, which has the highest reducing activity. Then, the obtained AgNPs were analysed using various techniques (UV–vis, FTIR, XRD, SEM, DLS, zeta potential) to determine the structure, size and stability in solution. Moreover, the biological activity of AgNPs - *P. guajava L.* combination was confirmed (antimicrobial activity, cytotoxicity study, histological study). This study offers to understand the properties of *P. guajava L.* leaf extracts prepared by sonication and the AgNPs prepared by this *P. guajava L.* leaf extract for further biomedical applications.

Material and Methods

Materials: Silver nitrate ($AgNO_3$) was purchased from Xilong Scientific Co. Ltd. (China, 96%) and sodium hydroxide (NaOH) was purchased from Samchun Chemical Co. Ltd. (Korea, 99,98%). 1,1-diphenyl-2-picrylhydrazyl (DPPH) was purchased from Tokyo Chemical Industry Co. Ltd. (Japan, 97%). The antibacterial test was carried out using *E. coli* (ATCC 25922). The L-929 cells were used to examine the *in vitro* cytotoxicity. The 8-weeks female white mice were used to examine the *in vivo* burn model. Deionized water (DI) was applied throughout the work.

Preparation of *P. guajava L.* leaf extracts: First, the fresh leaves collected from Tien Giang province (Vietnam) were cleaned with deionized water to remove any dirt and dust, dried for 24 h at 60 °C and ground into fine powder. Then, 10 g of *P. guajava L.* leaves were mixed with 150 mL deionized water and placed in ultrasonic bath (Elmasonic S60H, 37 kHz, 150W) for different times (5, 10, 15, 20, 25 mins). The pale brown extract was filtrated to remove contaminants. Finally, the obtained extraction was freeze-dried to remove H_2O and stored in a freezer at -20 ^oC for further experiments.

Preparation of AgNPs from $AgNO_3$ using *P. guajava L.* leaf extracts as reducing agent: *P. guajava L.* leaf extracts (50 mL) were added into solution of $AgNO_3$ 0.07 % (50 mL). This mixture was stirred for 2 hours, then dark brown solution was obtained, cooled and centrifuged in 10 mins to get AgNPs. AgNPs were dried in 50 ^oC to use for characterizations.

Characteristics of AgNPs: The collected *P. guajava L.* leaf extracts and formation of AgNPs were traced using UV-visible spectrophotometer (Agilent, Cary 60) at scan rate of 2400 $nm.min^{-1}$. The crystallographic structure of AgNPs was determined by X-ray diffractometer (XRD, Bruker, D8 Advance) using Cu Kα radiation (wavelength of ca. 0.154 nm). From XRD peak profile, full width at half maximum (FWHM), standing for the most intense plane, was analysed and then used for calculating crystalline size by the Scherrer equation. The microscopic structure was measured by Field emission scanning electron microscopy (FE-SEM) image (Hitachi, SU8000). The dispersion stability of AgNPs was confirmed using Zeta potential measurement (Horiba, SZ100).

The average particle size was recorded through dynamic light scattering (DLS) measurement (Horiba, LB550). The probable bioactive compounds, which were assumed responsible for the reduction of silver ions and capping of the reduced AgNPs, were identified using FT-IR analysis (Thermo, Nicolet 6700). 30 µg/mL of concentration of AgNPs was chosen for test on antibacterial study, *in vitro* and *in vivo* experiments.

Antioxidant activity: The antioxidant activities of *P. guajava L.* leaf extracts and AgNPs were evaluated by the 1,1-diphenyl-2-picrylhydrazyl (DPPH•) free radical scavenging activity[13]. The DPPH• radical scavenging activity (RSA) (%) is calculated based on the decreasing in absorbance at 515 nm of DPPH using the following equation:

$$RSA\ (\%) = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

where $A_{control}$ is the absorbance of DPPH• solution and A_{sample} is the absorbance of the mixture of DPPH• solution and the extract sample.

Antibacterial study: The antibacterial activity of AgNPs against Gram-negative bacteria (*E. coli*, ATCC 25922) was examined using the agar disk diffusion method.

***In vitro* cytotoxicity study:** Based on the method of ISO 10993-5, L-929 (mouse fibroblast cells) was used for cytotoxicity test by the clonogenic assay.

***In vivo* animal model study:** On the day of burn injury, anaesthesia was induced using ketamine. After adequate sedation was achieved, the fur was removed by close shaving with clippers. Burn injuries were induced by balancing the brass plate (1×1×0.1 cm), heated in fire in 15 seconds. After 24 hours, the scab was removed and treated with 50µL povidone (control) or AgNPs (30 µg/mL) per day. Mice were assessed for 14 days[16]. Skin from the region of burn injury was excised and fixed in ice-cold neutral-buffered formalin. The skin was dehydrated, embedded in paraffin and sectioned. Sections were dewaxed and stained with haematoxylin and eosin. Photographs were taken on an Olympus light microscope.

Results and Discussion

Preparation of *P. guajava L.* leaf aqueous extracts: The aqueous extracts of *P. guajava L.* leaves were obtained by sonication in different time (from 5 to 25 mins). Fig. 1 shows the UV–vis spectrum of the *P. guajava L.* extract in various sonication time (solid line). The results showed a strong absorption peak at 275 nm, specific to phenolic compounds[2],

with increase of intensity from 5 to 15 mins and slightly decrease from 15 to 25 mins. It means that the longer is the sonication time, the more time it exposes to crack the cell walls and reveal the cell contents.

However, prolonged extraction time may give a negative result due to increasing the chances of decomposition of bioactive compound in samples and motivating the reabsorption which lowers the yield[20]. In addition, fig. 1 also shows the UV–vis spectrum of the *P. guajava L.* extract from magnetic stirring at room temperature in 15 mins and 120 mins (dotted line). The results indicate that the amount of extraction from magnetic stirring method in same condition is much lower than that induced by ultrasound wave.

Synthesis of silver nanoparticles with *P. guajava L.* leaf aqueous extracts

The effect of extraction time to reducing ability of *P. guajava L.* leaf extracts: Silver nanoparticles were rapidly synthesized from Ag^+ ion using the prepared aqueous extracts of *P. guajava L.* leaves as reducing agent. The UV–vis spectrum in fig. 2 shows a strong absorption peak at 405 nm which is the characteristics for surface plasmon resonance band of AgNPs[11]. At 405 nm, the intensity of absorption peak increases when using the *P. guajava L.* leaf extracts with longer sonication time. The highest intensity when using the *P. guajava L.* leaf extracts with 15 mins sonication indicates the largest amount of reducing agent included. This result is consistent with the data shown in fig. 1.

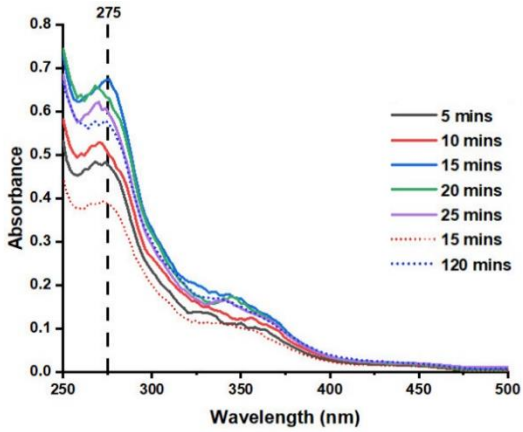

Fig. 1: UV–vis spectra of *P. guajava L.* leaf extracts prepared by ultrasound-assisted extraction from 5 to 25 mins (solid line) and heating magnetic stirring method from 15 mins to 120 mins (dotted line) in the range of 250–500 nm.

The effect of reaction time to reducing process: Fig. 3 showed UV–vis spectra of colloidal AgNPs in *P. guajava L.* leaf extracts in different reaction time from 15 to 240 mins in the range of 350–800 nm. As the reaction time increases from 15 to 240 minutes, the intensity of absorption peak at 405 nm gradually increases. It indicates that the longer is reduction time of *P. guajava L.* to $AgNO_3$, the more AgNPs were obtained. But there is no significant difference from 120 to 240 minutes. It means that the reaction almost finished after 120 minutes.

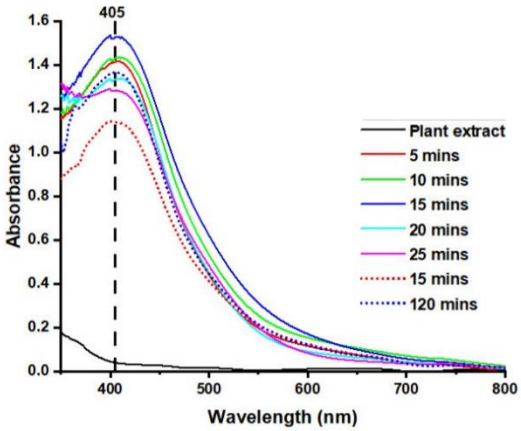

Fig. 2: UV–vis spectra of colloidal AgNPs in *P. guajava L.* leaf extracts prepared by ultrasound-assisted extraction from 5 to 25 mins (solid line) and magnetic stirring at room temperature from 15 mins to 120 mins (dotted line) and orgin *P. guajava L.* leaf extracts (black line) in the range of 350–800 nm.

Characteristics of AgNPs

X-ray diffraction (XRD): As shown by the XRD pattern of the AgNPs (Fig. 4), the analysed sample has strong diffraction peaks at 2θ = 38.24°; 44.22°; 64.52°; 77.52°; 81,67°. Those diffraction peaks are characteristic of the (111), (020), (022), (131) and (222) lattice surfaces. This is the face-centered cubic (FCC) structure of Ag metal. This allows us to confirm the formation of silver crystals in the solution. The AgNPs crystallite size, which is derived from Scherrer equation, is 13.9 nm.

Microscopic structure of AgNPs: The shape, morphology and dispersal characteristics of the nanoparticles were analysed by a FE-SEM analysis technique (Fig. 5). FE-SEM images of AgNPs show that each individual AgNPs is covered by a thin organic shell to stabilize them. Most AgNPs are spherical and have the range sizes of 20-40 nm.

Fourier transform infrared spectrometer (FTIR): The probable bioactive compounds responsible for the reduction of silver ions and capping of the reduced AgNPs produced using *P. guajava L.* leaf extracts were identified using FTIR analysis. Some strong signals corresponding to heterocyclic compounds such as polyphenols and alkaloid in extract which are responsible for reduction and efficient stabilization of the produced AgNPs could be seen in fig. 6: 3274 cm^{-1} (O–H and N–H stretching vibrations), 2927 cm^{-1} (C–H stretching vibration), 1600 cm^{-1} (C=C stretching vibration), 1405 and 1028 cm^{-1} (C–O stretching vibration), 1249 cm^{-1} (C–N bending vibration)[9]. The FTIR spectra obtained from AgNPs (blue line) also showed characteristic peaks indicating the presence of polyphenols and alkaloid at the surfaces of nanoparticles as stabilizer of AgNPs such as 3382, 2924, 1606, 1408, 1249 cm^{-1} for –O–H/N–H, C–H, –C=O, C–O stretching vibration respectively.

Comparison of the FTIR spectrum của *P. guajava L.* leaf extracts and AgNPs exhibits a band shift towards higher frequencies from 3274 to 3382 cm^{-1} (O–H stretching vibrations), 1600 to 1606 cm^{-1} (C=C stretching vibrations), 1405 to 1408 cm^{-1} (C–O stretching vibrations). Secondly the peak at 607 cm^{-1} were assigned to O-H bond between oxygen atom of Ag_2O and hydrogen atom of phenolic compound on the surface of the nanoparticle. Finally, a sharp decrease in signals at 617 cm^{-1} (N-H bending vibration) and 1249 cm^{-1} (C-N bending vibration) on FTIR spectrum of AgNPs demonstrates is the evidence that alkaloid acted as a silver reducing agent. The formation of AgNPs and the binding of the bioactive compounds in the extract to the surface of AgNPs was considered.

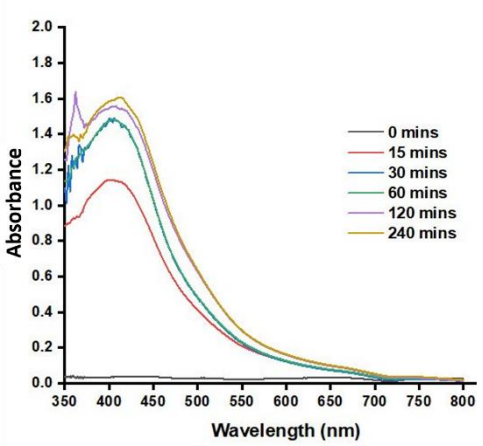

Fig. 3: UV–vis spectra of colloidal AgNPs in *P. guajava L.* leaf extracts prepared by ultrasound-assisted extraction in different reaction time from 15 to 240 mins in the range of 350–800 nm.

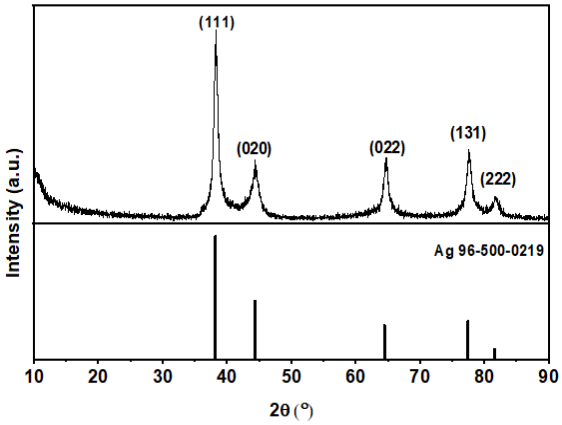

Fig. 4: XRD patterns of AgNPs.

Dispersion stability: The zeta potential of a sample is often used as an index of dispersion stability. Large zeta potential predicts more stable dispersion and vice versa. The zeta potential value for AgNPs prepared by *P. guajava L.* extract on the first day was -56.5 mV (Fig. 7a) which shows that the synthesized AgNPs are quite stable and well dispersed. The average particle size recorded through DLS results is 27.3 nm, this size is in agreement with the SEM results above. Moreover, the zeta potential and DLS synthesized AgNPs sample after 30 days are -22.7 mV and 55.3 nm respectively (Fig. 7b). This shows that AgNPs cluster maintains their nanostructure and relatively high stability.

It has been proven that *P. guajava L.* extract, in addition to being a reducing agent, also acts as a good stabilizer for AgNPs. This is consistent with the results of previous studies, when using guava leaf extract to synthesize AgNPs.

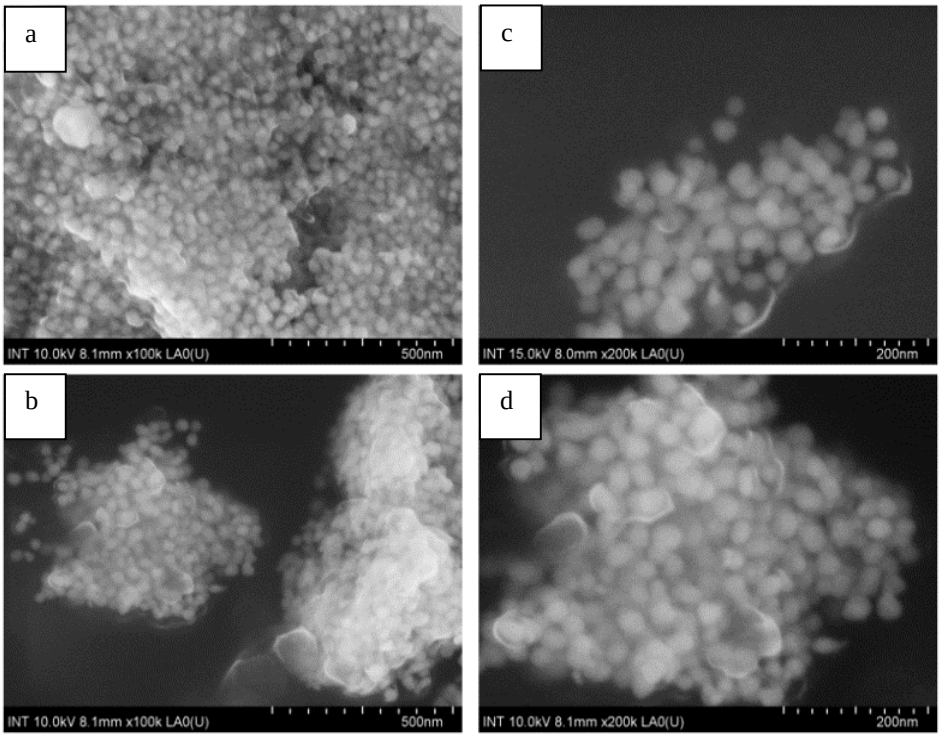

Fig. 5: FE-SEM image of AgNPs at (a and b) 100kx magnification and (c and d) 200kx magnification.

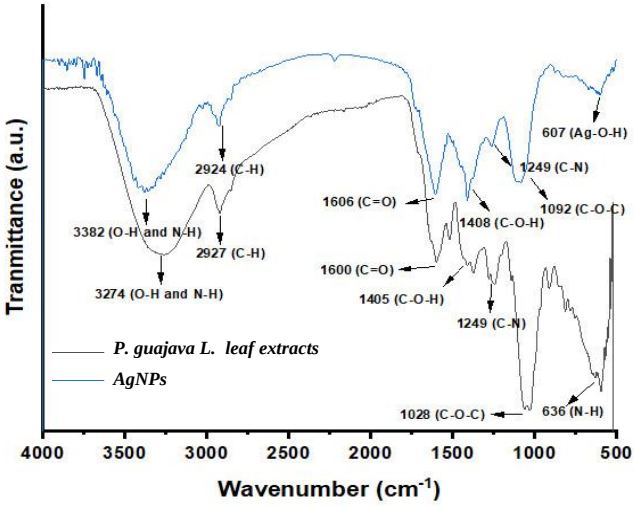

Fig. 6: FTIR absorption spectra of AgNPs and *P. guajava L.* leaf extracts.

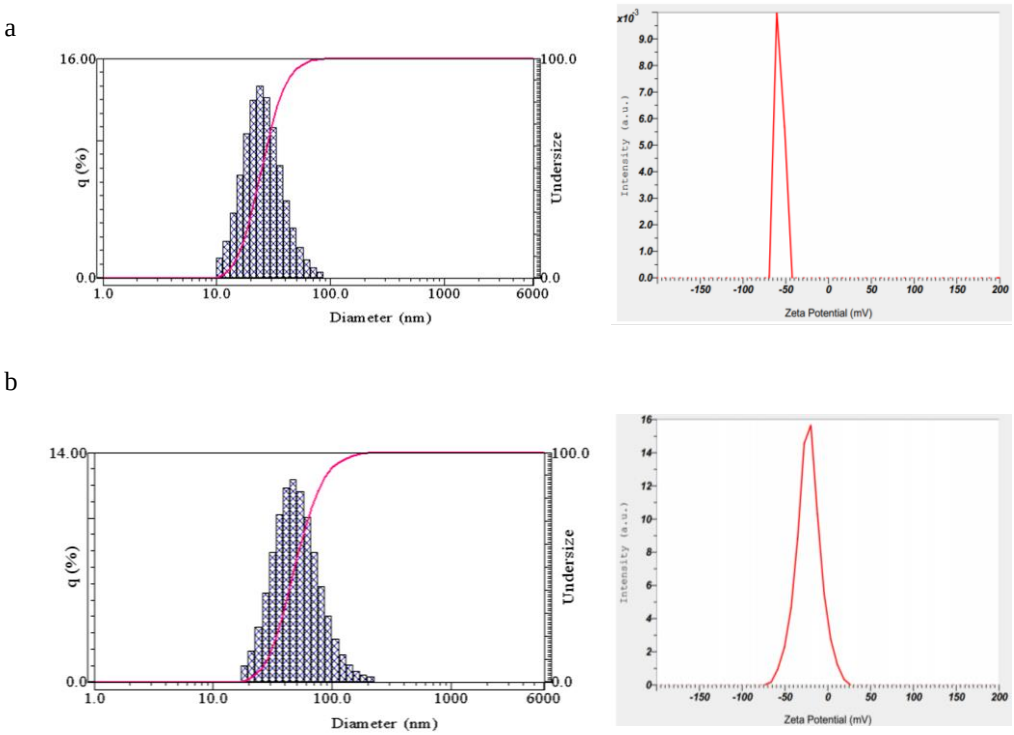

Fig. 7: DLS (left) and zeta potential (right) of AgNPs at (a) Day 1 and (b) Day 30.

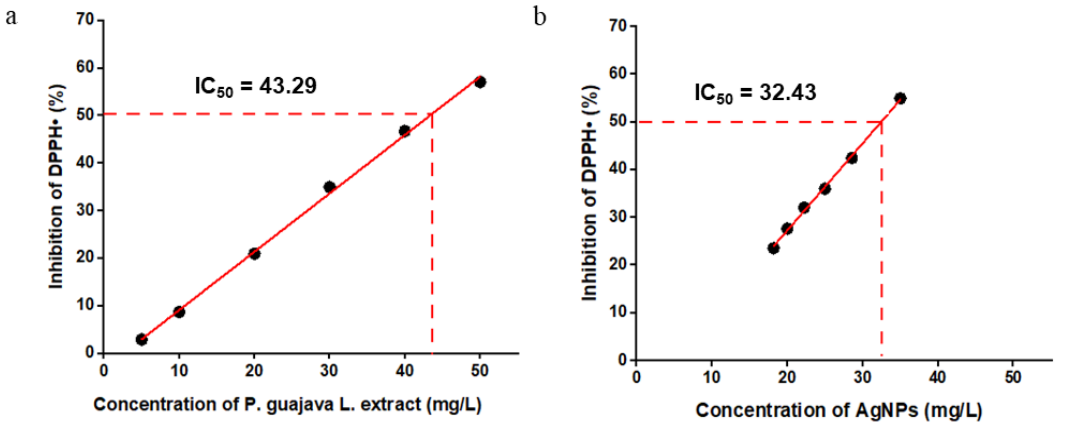

Fig. 8: DPPH• inhibitions of (a) *P. guajava L.* extract and (b) AgNPs respectively

From the previous reports which synthesize AgNPs from plants extract prepared by traditional heating method, AgNPs sizes were 62 nm[22], 14 – 35 nm[17] and 52.12 – 65.02 nm[23].

Antioxidant activity: Antioxidant activity was expressed through DPPH measurement. The results (Fig. 8) recorded that the IC_{50} index of *P. guajava L.* extract after 15 minutes of ultrasound was 43.29 µg/mL compared to the positive control ascorbic acid which was 25.69 µg/mL. This shows the antioxidant capacity of *P. guajava L.* extract. This extracted sample, after being reduced to Ag, still maintained its antioxidation ability. IC_{50} index was 32.43 µg/mL. This proves that AgNPs-reduced by *P. guajava L.* leaf aqueous extract was not only formed successfully but also gives advantage of the antioxidant properties of natural guava leaf extract.

Antimicrobial activity: The Gram-negative bacteria - *Escherichia coli (E. Coli*) was used to evaluate the antibacterial ability of the AgNPs sample. All AgNPs samples appear antibacterial circles (Fig. 9), with an average diameter of 13.6±0.7 nm. This shows the antibacterial ability of AgNPs against *E. coli.*

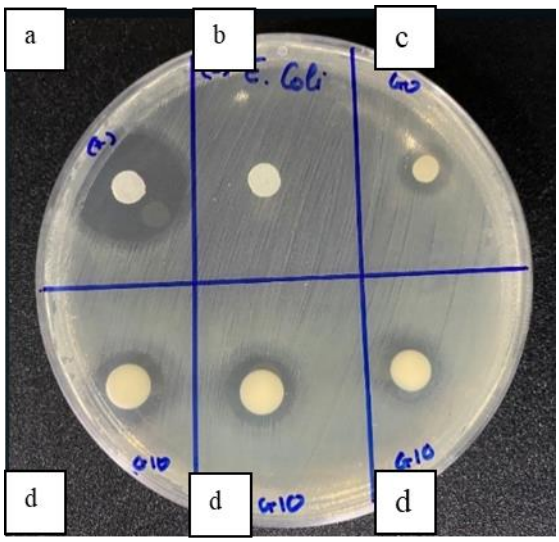

Fig. 9: Antimicrobial activity of AgNPs against *E. Coli* depicting zones of inihibition of (a) positive control-gentamicin, (b) negative control-water, (c) pure guava leaf extract, (d) AgNPs (30 µg/mL).

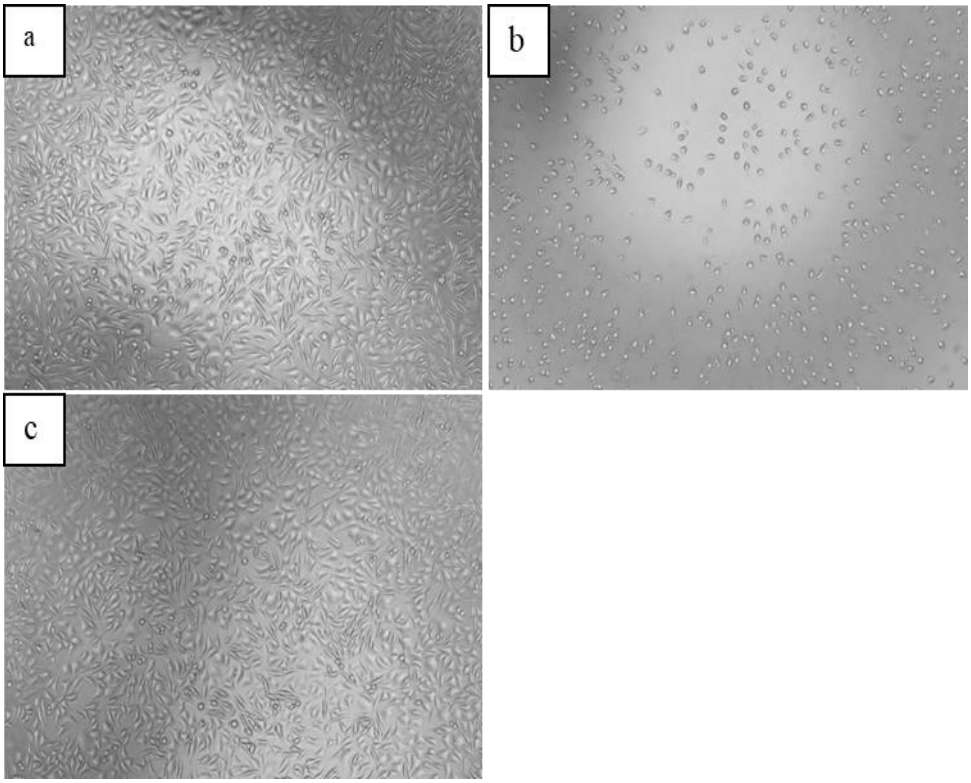

Fig. 10: Toxicity activity of (a) negative control-culture), (b) positive control-culture and 20% DMSO and (c) AgNPs (30 µg/mL on L-929 cell line.

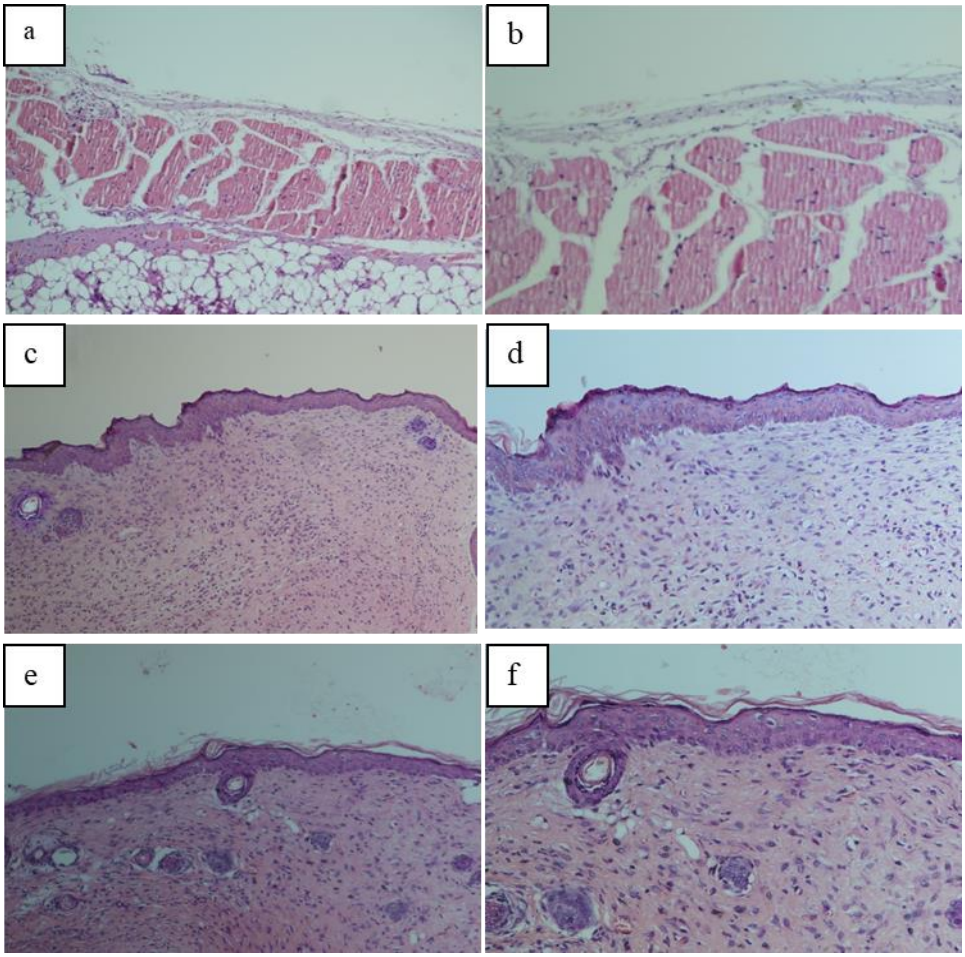

Fig. 11: Histological results of 2nd degree burn at (a) 10×, (b) 20×; treated with povidone after 14 days at (c) 10×, (d) 20×; treated with AgNPs after 14 days at (e) 10×, (f) 20×.

Toxicity: *In vitro* testing on the L-929 cell line showed the toxicity ability of the AgNPs sample. The concentration of AgNPs in this test was 30 µg/mL. The result (Fig. 10) showed that the percentage of living cell of the AgNPs sample was 91.5% compared to the negative control sample. The formed Ag is not toxic to L-929 cells according to ISO 10995-5 standards.

Histological results: The effectiveness of AgNPs on wound healing was evaluated through *in vivo* testing, with burn models on mouse skin and examined in 14 days. Based on the results in fig. 11a-b, the epidermis and dermis of the mouse skin were completely destroyed, showing that the second-degree (2^{nd}) burn model was successfully formed. The results (Fig. 11c-d and 11e-f) on both groups treated with povidone and AgNPs after 14 days, showed re-epithelialization, angiogenesis and fibroblast proliferation. The effectiveness of AgNPs in promoting wound healing was quite similar to that of povidone.

Conclusion

AgNPs were successfully green synthesized using *P. guajava L.* leaf aqueous extract prepared by sonication in short time (15 mins), which acts both as a reducing agent and a stabilizer. The AgNPs maintained their stability and nanostructure for up to 30 days (-22.7 mV – zeta potential). XRD spectra showed that AgNPs were formed with a single phase of Ag and had a nanostructure. From FE-SEM images, the particles have a uniform size, distributed from 20 - 40 nm and AgNPs are covered by a thin organic shell, which can give durability to the particles.

AgNPs exhibit antioxidant activity. IC_{50} was 32.43 µg/mL, good antibacterial ability against Gram-negative bacteria *E. coli* and did not cause toxicity to L-929 cells. *In vivo*, the healing effect of AgNPs on 2^{nd} degree burn is equivalent to povidone. Our study result demonstrated that ultrasound method has proven its advantages in supporting the extraction process and *P. guajava L.* leaf extracts are effective reducing agents in the synthesis of AgNPs. Moreover, synergistic effects of *P. guajava L.* leaf extracts and AgNPs were evidenced in their bioactivities.

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