Synergy of green-synthesized silver nanoparticles and *Vatica diospyroides* fruit extract in inhibiting Gram-positive bacteria by inducing membrane and intracellular disruption

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**ABSTRACT**

Silver nanoparticles (AgNPs) are used in biomedicine applications. Other drugs combined with the AgNPs can improve efficacy in the treatment of diseases, and most such studies have focused on antibiotics. We determined the synergistic effects of *Phyllanthus emblica*-derived AgNPs in combination with *Vatica diospyroides* cotyledon extracts (VCE) against bacteria using agar well diffusion, broth microdilution, and minimum inhibitory concentration (MIC). Synergy of AgNPs and VCE was confirmed with the fractional inhibitory concentration index (FICI). To evaluate patterns of bacterial death, flow cytometry and electron microscopy were used. We found that the effective incubation time of AgNPs against bacteria was highly variable. Increasing AgNPs in the combination influenced antibacterial activity against *Staphylococcus aureus* and *Bacillus subtilis*. The MIC values interpreted through FICI showed synergy against *S. aureus* and indifference against *B. subtilis*. Flow cytometric profiles confirmed that the fraction of *S. aureus* that respond to a combination of VCE with AgNPs increased in dose-dependent manner. The response patterns of bacteria proceeded simultaneously as the cells lost intracellular components and suffered membrane damage. Synergy of AgNPs with a plant extract has become a promising approach, as green AgNPs and plant extracts are biocompatible and cost-effective resources that can utilized for the treatment of bacterial infectious diseases.

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1. Introduction

The development of safe and effective bactericides is pursued in an effort to improve infectious disease treatments. Several studies have reported that some medicinal plant extracts can inhibit the growth and kill Gram-positive and Gram-negative bacteria by affecting the cell membranes of a wide range of pathogenic bacteria, such as food pathogens and poultry gastrointestinal tract pathogens [1,2]. In many reports, a few plant species have been tested for various antimicrobial properties specified by bacterial species, and typically only high concentrations of plant extracts can kill bacteria [3,4]. Interestingly, it is difficult for the bacteria to develop a resistance to the plant extracts because many involved targets are hit by several active compounds [5].

Nanoparticles (NPs) with smallest dimension in the range 1–100 nm have general antibacterial properties against a wide range of bacteria, via mechanisms such as oxidative stress induction, metal ion release, and non-oxidative mechanisms [6]. Unfortunately, some potential risks in the use of NPs have been monitored and reported. Asare et al. [7] reported that normal cell functions were inhibited and DNA damage in human cells increased with prolonged exposure to a high concentration of NPs. Moreover, it is possible that bacteria may develop resistance to any nanoparticle upon continuous exposure to the NPs alone [8]. Thus, efficacy at a low concentration and short time of exposure should be proven for candidate NPs.

Interestingly, biosynthesized NPs have been confirmed as biologically safer and more environment-friendly than NPs from chemical synthesis [9]. Among the NPs synthesized, AgNPs are considered promising antibacterial agents with emphasis on bactericides [10]. Recently, AgNPs have been synthesized using various plant species, which has become a promising alternative to the chemical method [11,12]. Phyllanthus emblica is commonly known as an important herbal drug widely used in Thai traditional medicine. AgNPs can be formed by adding silver nitrate to extracts of P. emblica fruit without using reducing agents or stabilizers [13]. Although there have been reports of antibacterial combinations of NPs and antibiotics [14–18], the efficacy of NPs in combination with a plant extract has never been described.

In our previous study, antibacterial activities of antibiotics increased when combined with fruit extract of Vatica diospyroides, an endemic woody species in Dipterocarpaceae, which is known for potent medicinal properties [19]. We thus recommended the use of V. diospyroides fruit extract to inhibit bacterial species by combining it with biosynthesized AgNPs. To enable an alternative approach in controlling infectious diseases, this study suggests the synergistic use of AgNPs with V. diospyroides fruit extract, to inhibit the growth of pathogenic bacteria.

2. Materials and methods

2.1. Plant materials, chemicals and bacterial strains

Vatica diospyroides Symington fruit were collected from Khian Sa district in Surat Thani province, whereas the Phyllanthus emblica L. fruit were collected from Prince of Songkla University, Surat Thani campus, in Thailand. Silver nitrate (AgNO₃, +99.9% POCH CAS no. 7761-88-8, POLAND) was used as precursor in the preparation of silver nanoparticles (AgNPs). Four strains of bacteria, namely Staphylococcus aureus ATCC 25923, Bacillus subtilis ATCC 6633, Escherichia coli ATCC 25922 and Psuedomonas aeruginosa ATCC 27853, were prepared and maintained at the Scientific Laboratory and Equipment Center (SLEC), Prince of Songkla University, Surat Thani campus, Thailand.
2.2. Preparation of *V. diospyroides* and *P. emblica* fruit extracts

*V. diospyroides* cotyledon extraction (VCE) followed the method described previously [20]. Briefly, the cotyledon was separated from entire fruit and sliced into small pieces prior to drying. After the drying, acetone ((CH$_3$)$_2$CO) was used as the solvent in maceration. The pieces of dried cotyledon (270 g) were macerated for 5 days at room temperature. After that, the crude extract was prepared by evaporating the solvent used. Dried residue was obtained and stored in refrigulator ($4^\circ$C). The method used to prepare *P. emblica* fruit extract followed Ramesh et al. [21]. The dried fruit materials (25 g) were extracted with boiling water (100 mL) for 10 min, and then were filtered twice using Whatman filter paper No.1 resulting in a 250 mg/mL final concentration. The aqueous extract was stored in dark at $4^\circ$C prior to use as stabilizer and reducer in AgNPs synthesis.

2.3. Synthesis of AgNPs using *P. emblica* fruit extracts

A 10 mL sample of the aqueous fruit extract of *P. emblica* was added to 100 mL AgNO$_3$ (169 μg/mL). The mixture was incubated for 0.5–24 h at room temperature, and thereafter AgNPs were evaluated using UV-visible spectroscopy. The confirmation of synthesis was performed on GENESYS 10S UV-Vis Spectrophotometer (Thermo Scientific, USA) over wavelengths from 300 to 700 nm at a resolution of 1 nm. The synthesized AgNPs were in the absorbance range of 425–436 nm [21].

2.4. Antibacterial effect dependence on incubation time and concentration of synthesized AgNPs, *P. emblica* fruit extract, AgNO$_3^-$ or a combination of VCE and AgNPs

The AgNPs synthesized with various times of incubation and the optimum AgNPs concentration against four strains bacteria *S. aureus*, *B. subtilis*, *E. coli* and *P. aeruginosa* were investigated using an agar well diffusion assay [22]. *P. emblica* fruit extract, AgNO$_3^-$ and synthesized AgNPs were each evaluated in terms of antibacterial function. The potential antibacterial properties were assessed from zones of inhibition. The optimal time of AgNPs incubation was chosen to achieve sufficient AgNPs concentration against all tested bacteria. Briefly, $10^8$ cfu/mL of each bacterial culture was spread on nutrient agar plates with a sterile swab. A 6 mm diameter cork borer was punched into the agar medium and the hole was filled with 100 μL of pure but diluted AgNPs. The potential antibacterial properties of AgNPs were evaluated after incubation. Effects of combining VCE and AgNPs were also tested as described above.

2.5. Determination of minimal inhibitory concentration (MIC)

Minimum Inhibitory Concentrations (MICs) of tested substances, namely AgNPs and VCE, and synergistic effects of both substances against the bacteria were tested by broth microdilution and by Resazurin-based 96-well microdilution [23]. The first well had 280 μL of mixed solution with Mueller Hinton Broth (MHB) and twofold amount of the tested substances. Wells 2–12 had 140 μL MHB. To make twofold serial microdilution, 140 μL aliquot from the first well was pipetted and added into the next well and mixed, repeating up to well 10. Wells 11 and 12 had negative (10% Dimethyl sulfoxide) and positive (30 μg/mL Ciprofloxacin) controls, respectively. 10 μL of 6.75 mg/mL Resazurin was added into each well prior to adding 50 μL of bacterial suspension. The inoculated plate was incubated at
37°C for 24 h. After incubation, on evaluation of color, MIC was defined as the lowest concentration such that the resazurin color did not change from blue to pink.

### 2.6. Fractional inhibitory concentration index (FICI)

The synergy between VCE and AgNPs was assessed from the fractional inhibitory concentration index (FICI). The FIC was calculated as the MIC of VCE and AgNPs in combination, divided by the MIC of VCE or of AgNPs singly. FIC index indicates the interactions as follows: FICI < 0.5 indicates synergy, 0.5 < FICI < 1 indicates partial synergy, FICI = 1 indicates additivity, 1 < FICI < 4 indicates indifferent, and FICI > 4 indicates antagonistic [24].

\[
\text{FICI} = \frac{\text{FIC of VCE} + \text{FIC of AgNPs}}{1}
\]

\[
\text{FIC of ACE} = \frac{\text{MIC of ACE in combination}}{\text{MIC of ACE alone}}
\]

\[
\text{FIC of AgNPs} = \frac{\text{MIC of AgNPs in combination}}{\text{MIC of AgNPs alone}}
\]

### 2.7. Flow cytometric (FCM) analysis and electron microscopy (EM)

The final concentrations that bacteria respond to, namely 0.5 × MIC (1.48 μg/mL of AgNPs: 250 μg/mL of VCE), MIC (2.96 μg/mL of AgNPs: 500 μg/mL of VCE), and 2 × MIC (5.92 μg/mL of AgNPs: 1,000 μg/mL of VCE) for S. aureus and 0.5 × MIC (0.37 μg/mL of AgNPs: 62.5 μg/mL of VCE), MIC (0.74 μg/mL of AgNPs: 125 μg/mL of VCE), and 2 × MIC (1.48 μg/mL of AgNPs: 250 μg/mL of VCE) for B. subtilis were added to wells containing 5 × 10⁶ CFU/mL and incubated at 37°C for 0–24 h. The membrane integrity and the complete granularity of bacteria were measured by using flow cytometry and electron microscopy. Briefly, after incubation with the synergistic combination, the cells were washed and re-suspended in 950 μL of phosphate-buffered saline (PBS). For FCM analysis, propidium iodide (PI) staining was performed prior to analyzing bacterial response profiles (fluorescence intensity; FL2 area and side scatter; SSC) on a BD FACSCalibur flow cytometer (Becton Dickinson Biosciences, San Jose, CA, USA). The populations of viable cells, membrane-damaged cells, injured cells and dead cells in each sample were analyzed using WinMDI version 2.9 software (Scripps Institute, La Jolla, CA) [20]. For scanning and transmission EM, after incubation with the same combination as in the FCM test, 2.5% glutaraldehyde was added and mixed and then incubated at 4°C for 1 h. The mixtures were centrifuged at 8000 rpm for 10 min, and then washed twice with PBS followed by resuspension in 950 μL of PBS. Characteristics of membrane integrity and complete granularity of bacteria were observed by Transmission EM (TEM) and Scanning EM (SEM), respectively. The TEM and SEM were JEOL JEM-2010 (JAPAN) and FEI Quanta 400 (The Czech Republic), respectively.

### 3. Results

#### 3.1. Synthesis of silver nanoparticles (AgNPs) from P. emblica fruit extracts

Figure 1(a–c) shows the AgNO₃⁻ solution after adding the fruit extract of P. emblica, during 0.5–24 h of incubation. The color and turbidity of sample containing AgNO₃ changed
increasingly from light brownish to brown with high turbidity. The sample had an observable initial turbidity after 6 h of incubation. The UV–visible absorption spectra of the AgNPs at different incubation times were recorded. This showed the successful synthesis of AgNPs by *P. emblica* fruit extract, and the synthesis was confirmed by UV-visible spectrophotometer. Formation of AgNPs was initiated at 0.5 h of incubation, which was also the beginning of observing a brown color. The maximum absorption peak was located at 430–434 nm which is in the proper theoretical range of wavelengths (200–800 nm) for AgNPs synthesis [25].

**3.2. Antibacterial effect dependence on incubation time and concentration of synthesized AgNPs or combination of V. diospyroides cotyledon extract (VCE) and AgNPs**

To examine the antibacterial activity of the synthesized AgNPs, alone and in combination with VCE, antibacterial susceptibility tests were performed against gram positive and gram negative bacteria. As shown in Tables 1 and 2, AgNPs alone and proportions among *P. emblica* fruit extract, AgNO$_3$ and AgNPs with DMSO showed highest antibacterial activity against the tested bacteria with clear zone sizes of 13.0, 10.0, 12.0 and 12.56 mm,

*Figure 1. UV-vis spectra of synthesized AgNPs at different times during incubation; 0.5 (a), 3 (b), 6–24 h (c), during synthesis in *Phyllanthus emblica* fruit extract.*
respectively (Figure 2). All strains of bacteria responded to AgNPs in dose-dependent manner (Table 1). When the incubation time with AgNPs increased, the treatment had decreasing antibacterial activity against all tested strains. For *S. aureus* and *P. aeruginosa*, antibacterial activity significantly decreased (*p* = 0.05). As the concentration of AgNPs was diluted, the size of inhibition zone decreased with all bacterial strains. As shown in Table 3, VCE plus AgNPs showed remarkable antibacterial activity against *S. aureus* and *B. subtilis* with larger clear zone sizes than that for AgNPs singly (Figure 2). Effects of incubation time and concentration of AgNPs in the combination resembled the results obtained for AgNPs alone, but with larger inhibition zones (reaching 18 mm). On increasing AgNPs and decreasing VCE concentration, the combination gave reduced size inhibition zones for both *S. aureus* and *B. subtilis*. No activity was observed against the growth of *E.*

### Table 1. Effect of synthesis incubation time and concentration of synthesized AgNPs on antibacterial activity.

| Incubation time (h) | Concentration of AgNPs (%v/v) | Zone of inhibition (mm ± SD) |
|---------------------|-------------------------------|-----------------------------|
|                     |                               | *S. aureus* | *B. subtilis* | *E. coli* | *P. aeruginosa* |
| 0.5                 | 100                           | 13.33 ± 0.57 | 11.00 ± 1.00 | 9.00 ± 1.00 | 9.33 ± 0.57 |
|                     | 60                            | 12.67 ± 0.57 | 11.00 ± 1.73 | 9.00 ± 1.00 | 8.67 ± 0.57 |
|                     | 50                            | 12.00 ± 1.00 | 10.33 ± 1.15 | 8.33 ± 0.57 | 8.67 ± 0.57 |
|                     | 40                            | 11.33 ± 1.15 | 9.67 ± 1.15  | 8.00 ± 0.00 | 8.00 ± 0.00 |
|                     | 0                             | 0            | 0             | 0          | 0             |
|                     | 10% DMSO                      | 0            | 0             | 0          | 0             |
|                     | Ciprofloxacin                 | 33.00 ± 1.00 | 39.67 ± 0.57  | 34.67 ± 1.15 | 36.00 ± 1.00 |
| 3                   | 100                           | 13.00 ± 1.73 | 10.67 ± 0.57  | 9.33 ± 1.52 | 10.33 ± 1.15 |
|                     | 60                            | 11.67 ± 1.52 | 10.00 ± 1.00  | 9.67 ± 1.52 | 9.33 ± 1.15  |
|                     | 50                            | 11.67 ± 0.57 | 10.00 ± 1.00  | 9.00 ± 1.00 | 8.33 ± 0.57  |
|                     | 40                            | 11.67 ± 0.57 | 9.33 ± 0.57   | 8.33 ± 0.57 | 8.00 ± 0.00  |
|                     | 0                             | 0            | 0             | 0          | 0             |
|                     | 10% DMSO                      | 0            | 0             | 0          | 0             |
|                     | Ciprofloxacin                 | 29.67 ± 0.57 | 39.33 ± 0.57  | 34.67 ± 0.57 | 39.67 ± 0.57 |
| 6                   | 100                           | 13.00 ± 0.00 | 10.33 ± 1.15  | 9.33 ± 0.57 | 9.67 ± 1.15  |
|                     | 60                            | 12.67 ± 0.57 | 10.00 ± 1.00  | 9.33 ± 0.57 | 8.67 ± 0.57  |
|                     | 50                            | 12.00 ± 0.00 | 9.67 ± 1.15   | 8.67 ± 0.57 | 8.00 ± 0.00  |
|                     | 40                            | 12.00 ± 0.00 | 9.67 ± 0.57   | 8.33 ± 0.57 | 8.00 ± 0.00  |
|                     | 0                             | 0            | 0             | 0          | 0             |
|                     | 10% DMSO                      | 0            | 0             | 0          | 0             |
|                     | Ciprofloxacin                 | 33.33 ± 0.57 | 39.33 ± 0.57  | 35.67 ± 0.57 | 35.33 ± 0.57 |
| 9                   | 100                           | 12.00 ± 1.00 | 10.33 ± 0.57  | 9.00 ± 1.00 | 9.33 ± 1.15  |
|                     | 60                            | 12.00 ± 1.00 | 10.00 ± 0.00  | 9.00 ± 1.00 | 8.67 ± 0.57  |
|                     | 50                            | 11.67 ± 1.15 | 9.67 ± 0.57   | 9.00 ± 0.00 | 8.67 ± 1.15  |
|                     | 40                            | 10.67 ± 1.15 | 9.00 ± 0.00   | 8.00 ± 0.00 | 8.00 ± 0.00  |
|                     | 0                             | 0            | 0             | 0          | 0             |
|                     | 10% DMSO                      | 0            | 0             | 0          | 0             |
|                     | Ciprofloxacin                 | 32.33 ± 2.08 | 40.00 ± 1.00  | 35.67 ± 1.52 | 34.33 ± 0.57 |
| 12                  | 100                           | 12.67 ± 1.52 | 10.67 ± 1.52  | 10.33 ± 0.57 | 9.00 ± 0.00  |
|                     | 60                            | 11.33 ± 2.08 | 10.33 ± 0.57  | 9.67 ± 0.57 | 9.33 ± 0.57  |
|                     | 50                            | 11.33 ± 0.57 | 10.00 ± 1.00  | 9.00 ± 0.00 | 8.67 ± 1.15  |
|                     | 40                            | 11.00 ± 2.00 | 9.33 ± 0.57   | 8.00 ± 0.00 | 8.00 ± 0.00  |
|                     | 0                             | 0            | 0             | 0          | 0             |
|                     | 10% DMSO                      | 0            | 0             | 0          | 0             |
|                     | Ciprofloxacin                 | 34.00 ± 1.00 | 41.00 ± 1.00  | 36.67 ± 1.52 | 11.94 ± 11.85 |
| 24                  | 100                           | 11.33 ± 0.57 | 10.33 ± 0.57  | 8.33 ± 0.57 | 9.67 ± 1.52  |
|                     | 60                            | 10.33 ± 1.52 | 10.33 ± 0.57  | 8.00 ± 0.00 | 9.33 ± 1.15  |
|                     | 50                            | 11.00 ± 0.00 | 10.67 ± 0.57  | 8.00 ± 0.00 | 9.00 ± 1.73  |
|                     | 40                            | 9.67 ± 0.57  | 9.33 ± 0.57   | 8.00 ± 0.00 | 8.33 ± 0.57  |
|                     | 0                             | 0            | 0             | 0          | 0             |
|                     | 10% DMSO                      | 0            | 0             | 0          | 0             |
|                     | Ciprofloxacin                 | 30.67 ± 1.15 | 40.67 ± 1.15  | 35.67 ± 1.52 | 36.00 ± 0.00 |

*p* value higher than 0.05 (>0.05) is not statistically significant.
coli and *P. aeruginosa* (data not shown). Therefore, VCE and AgNPs were more effective against gram-positive bacteria than against gram-negative bacteria.

### 3.3. Minimum inhibitory concentrations (MICs) and fractional inhibitory concentration index (FICI)

AgNPs alone showed antibacterial ability against *S. aureus* and *B. subtilis* with MIC values of 11.83 and 0.74 μg/mL, respectively (*Figure 3* and *Table 4*). The MIC (2000 and 500 μg/mL) of VCE indicated growth inhibition of *S. aureus* and *B. subtilis*, respectively. Interestingly, in combination the AgNPs and VCE had MIC for *S. aureus* and *B. subtilis* that revealed a significant decrease (fourfold) (from 2000 to 500, 500 to 125, and 11.83 to 2.96 μg/mL) except for the MIC of AgNPs against *B. subtilis* being similar when used alone (MIC = 0.74 μg/mL) (*Tables 4 and 5*). The synergistic effects of AgNPs and VCE were assessed from the FICI shown in *Table 5*. Synergy existed for the AgNPs and VCE combinations against *S. aureus*, with FICI = 0.5, but not for *B. subtilis* (FICI = 1.25 indicates indifference).

### 3.4. AgNPs combined with VCE affected bacterial membrane and caused intracellular disruptions

Representative results of flow cytometric analysis for the synergistic antibacterial effects of AgNPs and VCE on *S. aureus* and *B. subtilis* strains are shown in *Figure 4*. The responses of bacterial membrane and intracellular status to the synergy are shown separately for

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**Table 2. Effects of proportions of *P. emblica* fruit extract and DMSO, AgNO₃ and DMSO, and AgNPs and DMSO, on inhibition of four bacteria.**

| Proportions *P. emblica* fruit extract with DMSO | Zone of inhibition (mm ± SD ) |
|-----------------------------------------------|---------------------------|
|                                               | *S. aureus* | *B. subtilis* | *E. coli* | *P. aeruginosa* |
| 100:0                                         | 10.00b ± 0.00 | 9.67b ± 1.00 | 8.67b ± 0.57 | 9.00b ± 0.00 |
| 60:40                                         | 8.67c ± 0.57 | 9.00bc ± 0.57 | 8.00c ± 0.00 | 8.00c ± 0.00 |
| 50:50                                         | 8.00d ± 0.00 | 8.33c ± 0.57 | –         | –             |
| 40:60                                         | –           | –             | –         | –             |
| 0:100                                         | –           | –             | –         | –             |
| 10% DMSO                                      | –           | –             | –         | –             |
| Ciprofloxacin                                 | 32.00a ± 0.00 | 39.33a ± 1.00 | 33.00a ± 1.15 | 31.33a ± 1.52 |
| P-value                                       | 0.00        | 0.00          | 0.00      | 0.00          |
| AgNO₃ with DMSO                               | 12.00b ± 1.00 | 12.00b ± 1.00 | 9.00b ± 1.00 | 9.33b ± 1.52 |
| 60:40                                         | 11.33b ± 0.57 | 11.67b ± 0.57 | 8.67b ± 0.57 | 9.67b ± 0.57 |
| 50:50                                         | 11.00bc ± 1.00 | 10.67b ± 0.57 | 8.33b ± 0.57 | 8.67b ± 0.57 |
| 40:60                                         | 9.67c ± 0.57 | 10.67b ± 1.15 | 8.00b ± 0.00 | 8.00b ± 0.00 |
| 0:100                                         | –           | –             | –         | –             |
| 10% DMSO                                      | –           | –             | –         | –             |
| Ciprofloxacin                                 | 40.33a ± 1.15 | 32.00a ± 1.00 | 34.33a ± 0.57 | 33.00a ± 9.86 |
| P-value                                       | 0.00        | 0.00          | 0.00      | 0.00          |
| AgNPs and DMSO                                | 12.56b ± 1.15 | 10.56b ± 0.85 | 9.22b ± 1.00 | 9.56b ± 0.98 |
| 60:40                                         | 11.78b ± 1.39 | 10.28b ± 0.89 | 9.11bc ± 0.96 | 9.11b ± 0.75 |
| 50:50                                         | 11.61b ± 0.69 | 10.06b ± 0.87 | 8.67b ± 0.59 | 8.56b ± 0.92 |
| 40:60                                         | 11.06d ± 1.21 | 9.39d ± 0.60 | 8.11d ± 0.32 | 8.06d ± 0.23 |
| 0:100                                         | –           | –             | –         | –             |
| 10% DMSO                                      | –           | –             | –         | –             |
| Ciprofloxacin                                 | 32.17a ± 1.85 | 40.00a ± 0.97 | 35.50a ± 1.24 | 36.33a ± 1.78 |
| p value                                       | 0.00        | 0.00          | 0.00      | 0.00          |

Statistically significant differences between the ratio are shown by different superscripts, based on DMRT (p < 0.05).
treated *S. aureus* and *B. subtilis* cells, exposed to various concentrations (0.5 × MIC, MIC and 2 × MIC). After 3–24 h treatments, the response patterns of tested bacteria were different. *S. aureus* incubated with 0.5 × MIC, MIC and 2 × MIC for 0, 3 and 12 h displayed continuously increased counts in dose-dependent manner relative to unincubated samples, increasing from 5.2% at 0 h in the 0.5 × MIC to 24.0 and 27.9% in the treated samples of MIC and 2 × MIC, respectively, and increasing by from 24.0 to 41.2 to 42.0%
at 3 h; and from 30.37 to 54.9 to 59.8% at 12 h, respectively. Incubation for 6 and 24 h had positive and negative effects on cell responses, increasing from 17.3 to 28.5%, and decreasing from 64.4 to 62.4%, respectively.

When *B. subtilis* was incubated for 3, 6 and 24 h, the highest fraction of responding cells was achieved by treating with the MIC concentration (20.6, 30.6, and 31.7%, respectively). Dose-dependent response (17.9, 31.1 and 33.5%, respectively) was observed when *B. subtilis* was treated and incubated for 12 h. Time-dependent response was achieved when treating *B. subtilis* with the MIC concentration (15.3, 20.6, 30.6, 31.1 and 31.7). This increase was not higher than those obtained when treating *S. aureus* with the same concentration. Synergistic efficacy appeared to be a consequence of *S. aureus* and *B. subtilis* membrane damage (high FL2) and intracellular disruption (high SSC), which was

| Incubation time (h) | Combination ratio of VCE and AgNPs (%v/v) | Zone of inhibition (mm ± SD ) |
|----------------------|-------------------------------------------|-----------------------------|
|                      |                                           | *S. aureus* | *B. subtilis* |
| 0.5                  | 100:0                                    | 17.67 ± 1.15 | 14.00 ± 1.00 |
|                      | 60:40                                    | 16.67 ± 0.57 | 13.33 ± 1.15 |
|                      | 50:50                                    | 16.33 ± 1.15 | 13.67 ± 1.52 |
|                      | 40:60                                    | 15.67 ± 0.57 | 13.00 ± 1.00 |
|                      | 0:100                                    | 14.33 ± 1.15 | 11.67 ± 1.15 |
|                      | 10% DMSO                                 | 0            | 0             |
|                      | Ciprofloxacin                            | 29.00 ± 0.00 | 36.33 ± 0.57 |
| 3                    | 100:0                                    | 16.33 ± 1.15 | 16.00 ± 1.00 |
|                      | 60:40                                    | 16.00 ± 1.00 | 15.33 ± 1.52 |
|                      | 50:50                                    | 16.00 ± 1.00 | 14.00 ± 1.00 |
|                      | 40:60                                    | 15.00 ± 1.00 | 13.33 ± 0.57 |
|                      | 0:100                                    | 13.67 ± 0.57 | 9.00 ± 1.73  |
|                      | 10% DMSO                                 | 0            | 0             |
|                      | Ciprofloxacin                            | 33.33 ± 1.52 | 41.33 ± 1.15 |
| 6                    | 100:0                                    | 17.33 ± 0.57 | 14.00 ± 1.00 |
|                      | 60:40                                    | 17.00 ± 1.00 | 14.67 ± 0.57 |
|                      | 50:50                                    | 15.67 ± 0.57 | 13.67 ± 0.57 |
|                      | 40:60                                    | 15.67 ± 1.15 | 13.33 ± 0.57 |
|                      | 0:100                                    | 13.67 ± 1.15 | 7.33 ± 6.42  |
|                      | 10% DMSO                                 | 0            | 0             |
|                      | Ciprofloxacin                            | 31.33 ± 1.15 | 35.33 ± 0.57 |
| 9                    | 100:0                                    | 17.33 ± 0.57 | 14.67 ± 0.57 |
|                      | 60:40                                    | 16.33 ± 1.15 | 13.33 ± 1.15 |
|                      | 50:50                                    | 16.00 ± 1.00 | 14.67 ± 0.57 |
|                      | 40:60                                    | 14.67 ± 1.52 | 14.00 ± 1.00 |
|                      | 0:100                                    | 13.00 ± 1.00 | 12.33 ± 0.57 |
|                      | 10% DMSO                                 | 0            | 0             |
|                      | Ciprofloxacin                            | 31.00 ± 0.00 | 37.33 ± 0.57 |
| 12                   | 100:0                                    | 18.33 ± 0.57 | 14.67 ± 0.57 |
|                      | 60:40                                    | 16.67 ± 1.52 | 13.00 ± 1.00 |
|                      | 50:50                                    | 14.67 ± 2.30 | 14.00 ± 0.00 |
|                      | 40:60                                    | 15.00 ± 1.00 | 13.33 ± 0.57 |
|                      | 0:100                                    | 14.00 ± 2.00 | 10.67 ± 1.15 |
|                      | 10% DMSO                                 | 0            | 0             |
|                      | Ciprofloxacin                            | 30.00 ± 1.00 | 36.33 ± 0.57 |
| 24                   | 100:0                                    | 18.33 ± 0.57 | 10.00 ± 8.66 |
|                      | 60:40                                    | 16.00 ± 1.00 | 12.00 ± 1.00 |
|                      | 50:50                                    | 16.00 ± 1.00 | 15.00 ± 1.00 |
|                      | 40:60                                    | 15.67 ± 0.57 | 9.67 ± 8.38  |
|                      | 0:100                                    | 13.00 ± 1.00 | 7.00 ± 6.08  |
|                      | 10% DMSO                                 | 0            | 0             |
|                      | Ciprofloxacin                            | 20.33 ± 17.61 | 25.00 ± 21.65 |

*p value* higher than 0.05 (>0.05) is not statistically significant.

Table 3. Effects of proportions of *V. diospyroides* cotyledon extract (VCE) and AgNPs incubated for 0.5–24 h on antibacterial activity against *S. aureus* and *B. subtilis*. 
associated with both AgNPs and VCE. These results were confirmed by SEM and TEM images. The SEM and TEM images of *S. aureus* and *B. subtilis* treated synergistically with AgNPs and VCE are shown in Figure 5(a–h), and 5(b,d,f,h), respectively, with clear evidence of pores formed in the outer membrane, showing rather wrinkled shapes and connected to 60 nm size of AgNPs (Figure 6), and thus confirming membrane damage and intracellular disruption with leakage, leading to cell death.

**Figure 3.** MIC determination of AgNPs (A and B), *V. diospyroides* cotyledon extract (C and D) and synergistic blend of both substances (E and F) against *S. aureus* (a) and *B. subtilis* (b) tested by broth microdilution and resazurin assay, with 2 replicate wells.
4. Discussion

In this study, we used P. emblica fruit extracts for synthesizing AgNPs with a general method, to avoid the risks of conventional synthesis using chemicals. We followed the Ramesh et al. [21] method using AgNO₃ to induce AgNP formation in P. emblica fruit extracts with incubation for 0.5–24 h. It is interesting to note that after 0.5 h of incubation, color of the solution appeared to be light brown and it continuously increased to dense brown with absorption at 432–434 nm wavelength. The color change might be due to phytochemicals in the plant [26] whereas the wavelength indicates the complete formation of maximum yield of AgNPs in the size of 60 nm diameter [25].

AgNPs can be synthesized by two types of methods, namely top down or bottom up. Antibacterial effects of AgNPs depend on various common factors such as the composition, size, shape, synthesis method, and material sources (plants or bacteria) [17,27–29]. Using plant extract or culture supernatant of bacteria to synthesize AgNPs are examples of the bottom-up approach [30] completed after 48–96 h of incubation [28,29]. Thus, we confirmed and reasoned that when the antibacterial activity was investigated, the widest clear zone of inhibition found in the treatment with 0.5 h incubation for all bacteria, indicated inefficient AgNPs that might have been complete early on during the incubation. From our results, P. emblica fruit extract is a most suitable resource for green AgNPs synthesis because it is eco-friendly, has high efficacy, and gives a rapid green AgNPs synthesis [21].

We found that combinations of VCE and AgNPs showed strong synergistic antibacterial activity against the tested bacteria, with synergy established by comparing to the activities of VCE or AgNPs used singly. Previous studies have reported that AgNPs are effective against both gram-positive and gram-negative bacteria [31–33]. On the other hand, our results showed that the AgNPs were more active against Gram-positive than Gram-negative bacteria. It can be seen that the concentration of AgNPs singly or in a combination affected antibacterial activity. Gram positive bacteria exhibited larger clear zones than the gram negative bacteria. AgNPs when used by themselves did not have any antibacterial activity against the gram negative bacteria, which indicates that the AgNPs in this study are especially appropriate for only Gram-positive bacteria. However, most previous studies have reported that Gram-negative bacteria are more susceptible to AgNPs [34]. Antibacterial activity of the AgNPs might be affected after attachment of AgNPs on the bacteria, and subsequently be affected by the type of bacterial wall which is thicker for Gram negative than for Gram-positive strains. As another explanation, the

| Bacteria  | MIC  | MBC  | MIC  | MBC  | MIC  | MBC  | MIC  | MBC  |
|----------|------|------|------|------|------|------|------|------|
| S. aureus | 11.83 | >11.83 | 2000 | >2000 | 500  | 2000 | 2.96 | 11.83 |
| B. subtilis | 0.74  | 2.95  | 500  | >2000 | 125  | 250  | 0.74 | 1.48  |

Table 4. Minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) of AgNPs and V. diospyroides cotyledon extract (VCE) when used singly or in combination against S. aureus and B. subtilis.

| Bacteria  | FIC  | FICI  |
|----------|------|-------|
| S. aureus | 0.25 | 0.50  | Synergy |
| B. subtilis | 0.25 | 1.25 | Indifferent |

Table 5. FIC values of AgNPs and VCE, and FICI for the two-substance combinations.
**Figure 4.** Flow cytometry dot plots for *S. aureus* and *B. subtilis* treated with 0.5xMIC, MIC and 2xMIC or combination of AgNPs and VCE incubated for 0–24 h. The regions divided by the lines were interpreted as: lower left for viable cells (FL2-negative and SSC-negative), lower right for membrane-damaged cells (FL2-positive and SSC-negative), upper left for injured cells (FL2-negative and SSC-positive), and upper right for dead cells (FL2-positive and SSC-positive). PI was used to evaluate bacterial membrane permeability. If loss or damage of membrane occurred, PI could penetrate to the cell and bind with nucleic acids (FL2-positive). In the injured cell with breakdown of intracellular components, loss of granularity of the cell or high complexity could increase the reflection of light (SSC-positive). Thus, the signals in the density plot are interpreted by each quarter.
AgNPs can penetrate into the cells and induce reactive oxygen species (ROS) and free radical generation [35,36] leading to damage to granularity, and consequently inhibited cell growth [37].
Figure 5. SEM and TEM images of *S. aureus* and *B. subtilis* treated with synergistic MIC of AgNPs and VCE: (a and c) SEM and TEM images of untreated *S. aureus*; (b and d) SEM and TEM images of treated *S. aureus*; (e and g) SEM and TEM images of untreated *B. subtilis*; and (f and h) SEM and TEM images of treated *B. subtilis* (scale bar is 1 μm).
In many studies on antibacterial activity, the efficacy results are not satisfactory, but when using advanced methods, the results are improved. Based on the synergistic model, both bacteria were killed in a shorter time at low concentrations of both VCE and AgNPs. Details of the interpretation of the broth microdilution plus Resazurin assay were based on the FICI as indicator. The FICI results showed that achieved bactericidal effect was synergistic against *S. aureus*, while an indifferent combination effect was found against *B. subtilis*. This is the first report that confirmed synergistic effect of AgNPs with a plant extract against a strain of gram positive bacteria. Antibacterial effects of VCE against *S. aureus* could be enhanced by AgNPs. In contrast, the combination of AgNPs and VCE led to decreased antibacterial effect of VCE against *B. subtilis*. The antibacterial activity of combined AgNPs and VCE might be due to phytochemicals in VCE (see Table 3). VCE has terpenoids and anthraquinones that possess antibacterial activity against only gram positive bacteria [20]. Unfortunately, AgNPs could effectively inhibit the activity of VCE since the activity of VCE was decreased. This issue may be due to the cell wall structure of gram-positive bacteria [19] and some possible mechanisms are outer membrane warping, free translocation on membrane, penetration to interior, and membrane embedding [38].

Cell envelope of gram positive bacteria includes peptidoglycan, which is a well-established target for antibiotics [39]. *B. subtilis* is non-pathogenic in healthy individuals and the bacteria have even been reported as potential therapeutic agent, whereas the pathogen

Figure 6. SEM images show the AgNPs effects after treating (a) *B. subtilis*, and (b) *S. aureus* cells and the red arrows point to locations of AgNPs adhesion with bacterial cells, whereas free AgNPs are indicated by yellow arrow. The calculated size of AgNPs was approximately 60 nm with spherical shape (scale bar is 1 μm).
S. aureus is a known cause of human diseases [40]. B. subtilis could inhibit the growth of S. aureus in natural competition. Therefore, our results could provide insights to deriving new bactericidal agents from medicinal plants that possesses safe and eco-friendly advantages with effects against only pathogenic gram positive S. aureus.

We have verified the synergy between AgNPs and VCE on the tested bacteria using flow cytometry and electron microscopy. We hypothesized that VCE could affect agent-induced membrane damage of bacteria. The bactericidal pathways of AgNPs and their challenges in the treatment of bacterial infections remain currently unclear [41]. Katva et al. [16] reported that the drug and AgNPs complexes release Ag⁺ at a higher rate. Therefore, the active compounds of VCE may result in conjugation of the compounds with Ag⁺. This will result in an effective concentration of the combination at a specific site and the loss of membrane integrity. As seen in Flow cytometric profiles and Electron microscopy images, the active compounds permeabilized bacterial membranes and might therefore provide the AgNPs access to internal target sites [42]. Flow cytometric profiles and images from Electron microscopy showed that combination of VCE and AgNPs affected cell membrane integrity and intracellular components in both bacteria. While other studies have confirmed treatments of gram-positive bacteria with AgNPs, without involving cell wall disruption [43], the actions of AgNPs plus plant extract have never before been explored. On the other hand, many recent studies have tried to explore the green synthesis of AgNPs using new effective plants or other organisms to reveal potential antimicrobial activity [44–46]. Those AgNPs may have high potential for applications in the treatment of bacterial infections when combined with plant extracts.

In conclusion, AgNPs and VCE act as antibacterial agents when used singly or in combination. The bacterial response patterns had synergy characterized by flow cytometric analysis and Electron microscopy images. This study confirmed the effects of green-synthesized AgNPs on increasing antibacterial activity of VCE. The mechanisms of action of the VCE and AgNPs combination need more investigation. The enhancement of the antibacterial activity due to added AgNPs could promote the use of medicinal plant extracts that are efficient and safe, providing new possibilities in healthcare and veterinary treatments.

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Declaration of interest statement

The authors have no conflicts of interest to declare.

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