Synergistic Antibacterial Efficacy of Biogenic Silver Nanoparticles Prepared Using Methanolic Stem Extract of Tinospora Cordifolia and Hydrogen Peroxide

Abstract: The research is aimed to assess the joint antibacterial effects of hydrogen peroxide and methanolic stem extract (SNP-MSE) - prepared silver nanoparticles against Klebsiella pneumoniae. Utilizing MSE under sunlight, biogenic silver nanoparticles were produced, with their properties characterised using FTIR and UV-Visible spectroscopies. Turbidimetry determined the Minimum Inhibitory Concentration (MIC) of SNP-MSE, H2O2, and their varied combinations at different concentrations. Agar well diffusion assessed the zone of inhibition. The study comprised three groups: Group 1 assessed SNP-MSE, Group 2 H2O2, and Group 3 their combination. A sample size of 9 (3 per group) was determined with an 80% pretest G power. At 1 μg/ml SNP-MSE, no inhibition zone was observed; for 0.625 mM H2O2, it measured 7.81 ± 0.464 mm; and for the combined treatment, 14.01 ± 0.478 mm. The combined treatment significantly (p=<0.001, p<0.05) outperformed SNP-MSE and H2O2 alone in antibacterial efficacy. Additionally, the Fractional Inhibitory Concentration (FIC) index, below 1, signified synergy between SNP-MSE and H2O2. These findings suggest their collaborative action in eradicating K. pneumoniae. Their synergistic potency proposes a potential novel antibacterial agent, potentially reducing H2O2 side effects, expediting infection recovery, and enhancing overall health.

KEYWORDS: Methanol Stem extract, Silver nanoparticles, Tinospora cordifolia, novel antibacterial agent, Klebsiella pneumoniae, Health.

1 Introduction

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for its antimicrobial properties against bacteria and fungi, has been a subject of study. This herb holds significant importance in the Indian System of Medicine (ISM) and is part of the Menispermaceae family. The plant, known as Amrita or Guduchi, is prevalent in Sri Lanka, India, Bangladesh, Myanmar, and China. It has gained a reputation for its antibiotic and blood-purifying qualities, being a remedy for various illnesses over time. Tinospora cordifolia, as per studies, possesses a range of attributes including anti-allergenic, anti-arthritic, anti-diabetic, anti-inflammatory, anti-oxidant, anti-spasmodic, anti-periodic, and radioprotective properties. Additionally, it's been recognised for its insecticidal, anti-fungal, and antibacterial properties. Analysis of extracts from its roots, stems, and leaves revealed antimicrobial effectiveness against harmful microbes. This plant demonstrates notable photocatalytic abilities and its antioxidant features make its extracts a viable alternative to synthetic chemicals. Polyphenols, comprising flavonoids with medicinal and antioxidant properties, are the primary constituents of this plant. These components play a crucial role in limiting or preventing the harmful reactions of free radicals in biological systems, which are commonly found in aromatic plants. Utilising either a combination of plants or their individual parts enhances their therapeutic, dietary, and antioxidant potential. Klebsiella pneumoniae, a Gram-negative bacterium, poses significant challenges in the medical field due to its ability to colonise and invade various human body parts, its emergence in hypervirulent forms, evasion of the immune system, and increasing resistance to antibiotics. With the global issue of antibiotic resistance, effective alternative treatments are needed. Nanomedicine offers promise in addressing these challenges. In a study exploring the use of silver nanoparticles derived from the ethanolic stem extract (SNP-MSE) of Tinospora cordifolia in combination with hydrogen peroxide (H2O2) – a common antiseptic and disinfectant – a Fenton-like reaction occurs, generating OH free radicals with potent bactericidal activity. Combining silver nanoparticles with H2O2 presents advantages such as lower dosage, reduced toxicity, and minimized chances of resistance. This combination effectively clears infections by producing highly reactive anti-microbial oxygen species like OH. The research investigated the synergistic antibacterial impact of SNP-MSE and H2O2 against K. pneumoniae. In the past five years, more than 504 research papers linked to this current study have surfaced on Google Scholar, alongside 32 papers on PubMed, 43 papers on Science Direct, and 6 papers on MDPI.com. The surge in antibiotic resistance has triggered a quest for alternative remedies to tackle microbial resilience. Silver nanoparticles, owing to their distinct size, shape, electrical characteristics, and antimicrobial properties, have garnered significant attention as a fresh antibacterial solution [1]. In response to the challenge posed by multidrug-resistant (MDR) superbugs, a straightforward single-step procedure was employed for producing silver nanoparticles using an aqueous extract sourced from Sisymbrium irio. Furthermore, silver nanoparticles synthesised with Terminalia mantaly extracts displayed notable antibacterial activity against Streptococcus pneumoniae and Haemophilus influenzae. Remarkably, silver nanoparticles derived from Vaccinium arctostaphylos aqueous extract exhibited enhanced antibacterial effectiveness against gram-positive bacteria in contrast to gram-negative ones [2]. These silver nanoparticles exhibited a combined antibacterial effect with H2O2 against both gram-positive and gram-negative bacteria [2,3]. Tinospora cordifolia finds extensive use in various domains such as its potential as an anti-diabetic, anti-cancer, and anti-HIV agent, alongside its effects on osteoporosis, immune modulation, systemic infections, and Parkinson's disease. However, the combined antibacterial influence of SNP-MSE and H2O2 against K. pneumoniae hasn’t been previously documented. The researchers hold expertise in nanofabrication and microbiology. In this particular investigation, they explored the combined antibacterial effects of SNP-MSE and H2O2 against K. pneumoniae.
2 Materials and Methods

2.1 Preparation of methanolic stem extract

The Tinospora cordifolia stem powder sourced from Seenthil chooranam was precisely weighed, measuring 2.5g, utilizing a Shimadzu analytical weight balance. This measured powder was dissolved in 50 ml of ethanol and left to incubate at room temperature for a duration of 24 hours. Following the incubation period, the solution underwent filtration using No.1 Whatman filter paper, and the resulting filtrate was collected in falcon tubes. Subsequently, the filtrate was air-dried, leading to the concentration of the extract after the ethanol solution had evaporated. This concentrated extract was then stored at 4℃ until further application.

2.2 Silver nanoparticles Biosynthesis

We first prepared individual aqueous solutions of AgNO3 (2 mM) and MSE (2 mg/mL) as stock solutions. Next, we combined 1.5 mL of AgNO3 and 0.5 mL of MSE in a test tube. This mixture was gently swirled for 15 minutes while exposed to sunlight. Interestingly, the initially colorless solution underwent a noticeable change, transforming into a rusty brown hue during this process.

2.3 Characterization of SNP-ESE

To identify the surface plasmon resonance (SPR) peak in silver nanoparticles, we utilised a UV-Visible spectrophotometer to scan the MSE, AgNO3, and SNP-MSE. Moreover, FTIR spectroscopy was employed to analyse potential alterations in functional groups for both the MSE and SNP-MSE.

2.4 Minimum inhibitory concentration (MIC)

In diagnostic labs, this technique is employed to determine the lowest antibiotic concentration capable of halting the growth of a specific bacterial strain. This process is vital for assessing bacterial resistance and gauging the effectiveness of new antimicrobial agents. To assess the antibacterial properties of silver nanoparticles, we utilised the CLSI M07-A8 standard broth dilution method. This involved observing microbes in agar broth to monitor their growth rate. The Minimum Inhibitory Concentration (MIC) was calculated using serial two-fold dilutions of SNP-ESE (128-1 μg/ml) and H2O2 (5-0.39 mM). These were adjusted with a bacterial concentration of 10^8 CFU/ml, based on 0.5 McFarland's standard, within Luria broth. The control consisted solely of inoculated broth and was incubated for 24 hours at 37 ℃.
MIC endpoint was identified as the concentration where no visible growth was observed in the wells. To validate this value, we assessed the visual turbidity of the wells both before and after the incubation period.

2.5 Synergistic antibacterial activity of SNP-MSE and H2O2

The combined antibacterial impact of SNP-MSE and H2O2 was assessed by exposing bacteria to diluted concentrations of SNP-MSE and H2O2, each kept below their individual MIC thresholds. After this exposure, the samples were kept at 37°C for 24 hours and checked for cloudiness to establish the MIC value for these combinations. We determined the MIC values of SNP-MSE and H2O2 when used separately and in combination, then computed the Fractional Inhibitory Concentration (FIC) index based on these MIC values.

2.6 Agar well diffusion method

Turbidity measurements were employed to assess the combined effects of various drug combinations. The concentrations demonstrating the most effective synergy were utilised to compare the individual and combined drug zones of inhibition. To examine the impact of these drugs on the K. pneumoniae bacterial strain, a microbial inoculum was uniformly spread over the agar plate. Volumes ranging from 20 to 100 of SNP-MSE and H2O2 were carefully added to wells using a sterile cork borer or tip. The antibiotics dispersed within the agar medium and restrained the growth of the bacterial strain. Subsequently, following a 24-hour incubation period, the zones of inhibition were gauged.

2.7 Statistical analysis

The mean zone of inhibition for Tinospora cordifolia’s NC, PC, MSE, and SNP-MSE against K. pneumoniae was compared using IBM-SPSS (28.0.0). Given that the variables were not interdependent, a one-way ANOVA test was utilized to determine the statistical significance in comparing the mean zone of inhibition of the various test compounds, with the aid of the IBM SPSS software.

3 Results

![Graph showing absorbance vs. wavelength for MSE, AgNO3, and SNP-MSE](image_url)

Fig. 1. UV-visible spectra of MSE, AgNO3, and SNP-MSE.
The FTIR spectra of the synthesized SNP-MSE displayed comparable functional groups to those found in the MSE alone. Testing the minimum inhibitory concentration (MIC) revealed that SNP-MSE showed a MIC of 32 μg/ml within a range of 1μg/ml to 128 μg/ml. Likewise, the MIC for H2O2 was determined within a range of 0.039 mM to 5 mM, with the observed MIC at 1.25 mM. When combined, SNP-MSE and H2O2 exhibited a MIC of 4 μg/ml for SNP-MSE and 0.156 mM for H2O2. To assess the zone of inhibition, an agar well diffusion assay was conducted using the MIC combination. Table 1 outlines the MIC values for SNP-MSE, H2O2, and their combination against active K. pneumoniae.

Table 1. Antibacterial efficacy of SNP-MSE, H2O2, and their Combination against K. pneumoniae.

<table>
<thead>
<tr>
<th>SNo</th>
<th>MIC of SNP-MSE (μg/ml)</th>
<th>MIC of H2O2 (mM)</th>
<th>Combination</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>128</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>64</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>32</td>
<td>1.25</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>16</td>
<td>0.625</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>8</td>
<td>0.3123</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>4</td>
<td>0.156</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>2</td>
<td>0.078</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>1</td>
<td>0.3906</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Fractional inhibitory concentration.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>MIC of SNP-MSE Alone (μg/ml)</th>
<th>MIC of H2O2 Alone (mM)</th>
<th>FIC index</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
<td>0.2498</td>
<td>Synergistic</td>
</tr>
</tbody>
</table>

The statistical significance of the findings was assessed using the IBM SPSS software, conducting a one-way ANOVA. Both Table 1 and Table 2 were instrumental in categorising the samples for this analysis via IBM SPSS software. Notably, the results indicated a significantly greater zone of inhibition (p=<0.001, p<0.05) when employing the combination of SNP-MSE and H2O2 in comparison to their individual usage.
a. The Fractional Inhibitory Concentration (FIC) value was derived by dividing the MIC (Minimum Inhibitory Concentration) of an antimicrobial when used in combination by its MIC when used alone. The FIC was then calculated by summing the FIC values of the two antimicrobials utilised together.

b. Combinations exhibit different antibacterial effects categorized as synergistic (FIC<1), additive (FIC=1), or antagonistic (FIC>1).

Table 3. Mean Zone of inhibition of SNP MSE, H$_2$O$_2$ and combination against K. pneumoniae.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Test substance</th>
<th>Mean zone of inhibition (mm)</th>
<th>STD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>SNP-MSE</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>H$_2$O$_2$</td>
<td>7.81</td>
<td>0.464</td>
</tr>
<tr>
<td>3</td>
<td>Combination</td>
<td>14.01</td>
<td>0.478</td>
</tr>
</tbody>
</table>

Table 4. One-way ANOVA for the comparative mean zone of inhibition performed using IBM SPSS software.

<table>
<thead>
<tr>
<th>Sum of squares</th>
<th>df</th>
<th>Mean square</th>
<th>F</th>
<th>Sig</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between groups</td>
<td>2</td>
<td>148.116</td>
<td>757.411</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Within groups</td>
<td>6</td>
<td>.196</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>8</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig. 2. FTIR of MSE.

Fig. 3. FTIR of SNP-MSE.
Fig. 4. Study of the zone of Inhibition of SNP-MSE, H$_2$O$_2$ and their combination against K. pneumonia using agar well diffusion method. X axis: Treatment groups such as SNP-MSE, H$_2$O$_2$ and their combination; Y axis: Mean zone of Inhibition (ZI). SD +/-

4 Discussion

The Minimum Inhibitory Concentration (MIC) recorded was 32 μg/ml for SNP and 1.25 mM for H2O2. When combined, the MIC reduced to 4 μg/ml for SNP-MSE and 0.156 mM for H2O2, yielding a calculated FIC index of 0.25. This indicates a synergistic antibacterial effect against K. pneumoniae. Furthermore, the combined treatment displayed a significantly greater mean zone of inhibition (p=<0.001, p<0.05) compared to individual treatments. Klebsiella strains are notorious for causing diverse illnesses, including pneumonia, urinary tract infections, bloodstream infections, and sepsis [14,15]. However, the prevalence of multidrug-resistant strains has limited effective treatment options [16-19].

Nanomedicine, particularly silver nanoparticles, when reacting with H2O2, generates OH free radicals, exhibiting potent bactericidal properties. Combining therapies can reduce dosage, toxicity, and resistance issues, offering a promising strategy against antibiotic resistance. The synergy observed between SNP-MSE and H2O2 is indicative of a Fenton-like reaction producing OH radicals, significantly enhancing the inhibition of K. pneumoniae [20]. Additionally, T. cordifolia demonstrates balanced immunomodulatory activity, implying that the synergistic effect of SNP-MSE with H2O2 may not only directly target bacterial components but also activate the host immune system, aiding in K. pneumonia eradication [21-23]. It’s important to note that this study only explored the antibacterial activity against a single bacterial strain. To establish its overall effectiveness, further investigations should evaluate its efficacy against various bacterial strains with differing resistance levels. Future research should focus on assessing this substance’s potential against a broader spectrum of bacterial strains that pose threats to human health [24-25].

5 Conclusion

In this study, the synergistic antibacterial efficacy of silver nanoparticles prepared using the methanolic stem extract of Tinospora cordifolia and hydrogen peroxide has been successfully tested against K. pneumoniae. The results indicate the synergism between the SNP-MSE and H2O2 in killing K. pneumoniae. The synergistic efficacy of SNP-MSE and H2O2 suggests that they may act as an alternative novel antibacterial agent which can reduce the side effects of H2O2 when used in combination for providing faster recovery from infection and better health.
against K. pneumoniae. The findings demonstrate a synergy between SNP-MSE and H2O2 in eradicating K. pneumoniae. This combined effectiveness implies that SNP-MSE and H2O2 could serve as a new antibacterial solution, potentially mitigating the side effects of H2O2 when used together. This promising combination could offer expedited recovery from infections and promote improved health outcomes.

References


