Group B Strep in Pregnancy and *Allium sativum* L.

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Research Article

**Keywords:** Antepartum care, Pharmacology, Complementary and integrative therapies, Newborn care, Public health

**Posted Date:** April 8th, 2024

**DOI:** https://doi.org/10.21203/rs.3.rs-1149854/v5

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**Additional Declarations:** The authors declare no competing interests.
Abstract

Introduction: Maternal colonization with *Streptococcus agalactiae* or Group B *Streptococcus* (GBS) during pregnancy increases the risk of neonatal infection via vertical transmission from mother to fetus before or during labor.

Objective: This study aimed to evaluate the antimicrobial activity of the SP80 fraction derived from *Allium sativum* and, its synergistic potential with the antibiotics against GBS strains.

Methods: Antimicrobial activity and synergism were assessed using broth microdilution and disk diffusion assays. Fifty-five clinical isolates and one ATCC strain of GBS were tested using the disk diffusion method against the combination of the SP80 fraction with ampicillin and penicillin G, respectively and one ATCC strain was tested using broth microdilution assay.

Results: The Minimum Inhibitory Concentration (MIC) of SP80, ampicillin, and penicillin G against *Streptococcus agalactiae* (ATCC 12386) were found to be 5 µg/µL, 14 µM, and 3.75 µM, respectively. The combination of SP80 and antibiotics, assessed through the broth microdilution assay, demonstrated an additive effect. Statistical analysis revealed that the mean for ampicillin, when combined with the SP80 fraction, using the disk diffusion method, increased compared to ampicillin alone, although not significantly, and the mean for penicillin G, when associated with the SP80 fraction, remained unchanged. The inhibition halos obtained with isolated antibiotics indicated that the strains tested exhibited greater resistance to penicillin G compared to ampicillin.

Conclusions: The SP80 fraction displays antimicrobial activity against GBS. When combined with antibiotics, it exhibits an additive effect, suggesting a promising approach for combating GBS infections.

Introduction

*Streptococcus agalactiae* or Group B *Streptococcus* (GBS) are bacteria that inhabit the human gastrointestinal and vaginal tracts, and it can develop into invasive infections in newborns during the few first weeks of life [1].

Neonatal GBS disease can be classified according to the time of onset of infection. Early-Onset Disease (EOD) occurs 12 to 48 hours after birth or up to the first 7 days and is usually due to mother-to-child transmission during childbirth, and it can cause severe problems, such as meningitis, pneumonia, and sepsis. Late-Onset Disease (LOD) occurs between one week and several months after birth and it can cause meningitis, and the source of contamination may be in the infant’s home environment [2].

In 1996, prophylactic recommendations such as the use of antibiotics during labor were implemented to prevent Neonatal GBS infections were implemented by the American College of Obstetricians and Gynecologists (ACOG) [3] and Centers for Disease Control and Prevention (CDC) [4] and then in 1997 by the American Academy of Pediatrics (AAP) [5]. Following consensus reviews, the guidelines were revised
in 2002 and 2010, and universal recommendations were issued for the collection of vaginal secretions from all pregnant women between 35 and 37 weeks of gestation [6]. In 2018, the responsibility and leadership for updating the guidelines was transferred from the CDC to ACOG and AAP [7].

Currently, all pregnancies should undergo antepartum Group B *Streptococcus* screening between 36 and 38 weeks of gestation as part of routine antenatal care. Vagina and rectal cultures are obtained to optimize identification of those who should receive appropriate intrapartum antibiotic prophylaxis (IAP) [8,9].

The use of antibiotics during pregnancy has not been found to be beneficial, but at the beginning of labor, it is [10]. The use of 5,000,000 IU (intravenous) penicillin G is recommended, maintaining 2,500,000 IU every 4 hours, until the moment of delivery; or starting with 2 g (intravenous) ampicillin first and then administering an additional 1 g every 4 hours, until the moment of delivery [10]. In the case of allergic pregnant women, it is recommended 500 mg (IV) erythromycin every 6 hours, or 900 mg clindamycin every 8 hours is recommended; if the pregnant woman is GBS resistant, 1 g IV vancomycin every 12 hours is administered; however, this prophylaxis does not prevent LOD [2, 11,12].

Although prophylactic measures are already well established in conventional medicine, the use of medicinal plants for disease prevention is quite common in developing countries such as Brazil [13]. While researching the use of garlic for the treatment of GBS infection in traditional medicine, we were able to find some published articles describing positive results [14,15].

Garlic (*Allium sativum* L.), a member of the *Liliaceae* family, is one of the most studied medicinal plants in the world and has been used for centuries in cooking and traditional medicine [16]. In Brazil, this plant is included in a list of 71 plants of interest for the SUS (acronym for *Sistema Único de Saúde*, i.e. the Brazilian Universal Health System), issued by the National Program of Medicinal Plants and Herbal of Ministry of Health [13].

This plant species has been used as a remedy for several diseases, such as antioxidant [17], antitumoral [18], anti-inflammatory [19], immunomodulatory [20], antiviral [21], antimicrobial [22,23], and cardiovascular protective [24] actions, as well as promoting beneficial effects in diabetic [25] and obese patients [26].

Confirming the information on the antimicrobial activity of *Allium sativum*, the study carried out by Torres et al. [27] demonstrated the pharmacological efficacy of the SP80 fraction obtained from the bulbs of the plant against GBS.

The fact that the antimicrobial activity of *Allium sativum* has been identified opens the possibility of expanding the spectrum of therapeutic options in the prevention of GBS infections, as well as an option for the association with antibiotics already established in the treatment and prevention of GBS, allowing to increase the pharmacological efficacy or even reduce the adverse effects caused by high doses of conventional antibiotics. In this sense, this study aimed to evaluate the antimicrobial activity of the SP80
fraction obtained from *A. sativum* L. and its association with the antibiotic's ampicillin and, penicillin G against *S. agalactiae* strains.

**Material and Methods**

**Plant material**

Garlic bulbs from *A. sativum* L. (*Liliaceae*), a purple variety, were purchased in a local market in Campos Altos, State of Minas Gerais, Brazil; *Fazenda Tri "S"*, altitude of 1.132 m, in November 2017. For confirmation of the plant's botanical identity, samples of the bulbs were cultivated and the whole plant was subsequently provided to experts in Botany. A voucher specimen, (n° 502077), was deposited at the *Herbarium Maria Eneyda P. Kaufmann Fidalgo* where species identity was confirmed by Dr. Domingos Sávio Rodrigues. All study/experimental protocols involving plant materials were conducted by institutional, national, and international guidelines and legislation.

**RGE and SP80 fraction**

The SP80 fraction, obtained by purifying the raw garlic extract (RGE), on a Sep-Pak® C18 cartridge column, was carried out as previously described by Torres et al. [27]. The income obtained for SP80 fraction was 5.1%.

**Bioassays**

**Minimum Inhibitory Concentration (MIC)**

The SP80 fraction and antibiotics, reconstituted in ultrapure water, were subjected to a liquid growth inhibition test against the *S. agalactiae* (ATCC®12386™) strain, respectively. The assay was performed in 96-well microplates. For that, 20 µL of sample was applied to each well at a serial dilution of two-fold microtiter broth dilution and added to 80 µL of culture medium TSB containing the microorganism in the logarithmic growth phase for a final concentration of $10^3$ cells/mL. After 24 hours of incubation at 35 ± 2 °C under constant agitation, the absorbance at 595 nm was measured on a Victor 3 - 1420 (Perkin Elmer®) instrument. The MIC was considered the lowest concentration that completely inhibits the growth of the microorganism. The tests were performed in triplicate and the average of 3 readings was considered [28].
**Fractional Inhibitory Concentration Index (FICI)**

The checkerboard microtiter assay was applied for determining the interaction of combinations of SP80 with ampicillin and, penicillin G, separately. SP80 and antibiotics were dispensed into 96-well cell culture plates at final concentrations ranging from 1/4 to 4×MIC in each well. The samples were incubated in the dark at 35 °C for 18 h. The MIC of each antibiotic and SP80 was read and interpreted using previously described methods. For each antimicrobial combination, we calculated the FICI by computing the ratio of the MIC of the combination divided by the MIC of the antimicrobial alone for each agent and then adding those two ratios together (see Equation 1 and 2). The FICI data were interpreted using the following criteria: FICI is less than or equal to 0.5 is considered as synergy and more than 2.0 it is considered as antagonism. If FICI in between > 0.5 and ≤ 1.0 consider as an additive and FICI between > 1 and ≤ 2.0 is, considered as indifference [29].

\[
FICI_{[\text{PEN}]} = \frac{\text{MIC of SP80 in combination with PEN}}{\text{MIC of SP80 alone}} + \frac{\text{MIC of PEN in combination with SP80}}{\text{MIC of PEN alone}}
\]

\[
FICI_{[\text{AMP}]} = \frac{\text{MIC of SP80 in combination with AMP}}{\text{MIC of SP80 alone}} + \frac{\text{MIC of AMP in combination with SP80}}{\text{MIC of AMP alone}}
\]

**Synergism (disc diffusion assay)**

The method described by the CLSI [30] was followed. The test for the combined effects of SP80 fraction with antibiotics was carried out by disc diffusion assay with sterile filter paper discs containing 10 mcg of ampicillin and 10 UI of penicillin G, respectively.

For tests were used *S. agalactiae* strain (ATCC® 12386™) and fifty-five non-duplicate clinical isolates of *S. agalactiae* from positive vaginal and rectal cultures, kindly supplied by Salomão and Zoppi available in its library. These clinical isolates were confirmed by using the CAMP (*Christie, Atkins, Munch-Petersen*) test. For this purpose, ready-made Petri dishes were used with the Mueller-Hinton agar (MHA) with 5% sheep blood culture medium (150 mm), supplied by Probac, and *S. agalactiae* strains (previously prepared in saline), using alginate swabs, at a concentration of 1.5 x 10^8 CFU/mL, which corresponds to
0.5 in the MacFarland nephelometric scale. The strains of *S. agalactiae* were subjected to four treatments by inserting sterile absorbent filter paper discs immersed in the following solutions: (1) ampicillin; (2) penicillin G; (3) ampicillin with 15 µL SP80 fraction; and (4) penicillin G with 15 µL SP80 fraction. The plates, prepared as duplicates, were incubated at 35 ± 2 °C for 24 hours. The diameter of the clear zone around the disc was measured and expressed in millimeters [31-33].

**Statistical Analysis**

The statistical analysis was performed by specialized professionals from the Statistics Department of the Faculty of Medical Sciences of Santa Casa de Sao Paulo.

During planning, the sample size was (n) calculated based on the Analysis of Variance (ANOVA). A significance level of 5% (α) and test power of 80% (1-β) was adopted with a standard deviation of 5 units and a difference of 2 units, therefore finding n = 56 samples.

The results obtained were subjected to statistical analysis by using the IBM SPSS software, version 13.0, and were considered significant (*p* < 0.05). The non-parametric test used was Wilcoxon; whereas for paired data, *T*-test.

**Results**

*Minimum Inhibitory Concentration*

Bacterial suspensions (10^6 CFU/ml) were incubated with SP80 and antibiotics separately for 24 hours at 35 ± 2 °C with constant shaking. The concentration of SP80 and each antibiotic required to visibly change the suspensions from turbid to clear was considered the MIC. As shown in Table 1.

**Table 1.** Minimum Inhibitory Concentration (MIC) of SP80 and antibiotics against *Streptococcus agalactiae* (ATCC 12386).

<table>
<thead>
<tr>
<th></th>
<th>SP80</th>
<th>ampicillin</th>
<th>Penicillin G</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minimum Inhibitory Concentration (MIC)</td>
<td>5µg/µL</td>
<td>14µM</td>
<td>3.75µM</td>
</tr>
</tbody>
</table>

Values expressed as µg/mL and µM.

*Fractional Inhibitory Concentration Index*
The results for the effect of combining SP80 with ampicillin and SP80 with penicillin G are summarized in Table 2. The average FICI for *S. agalactiae* (ATCC 12386) for SP80 in combination with ampicillin was 0.65, indicating that an additive effect occurred with the combination, and the average FICI for *S. agalactiae* (ATCC 12386) for SP80 in combination with penicillin G was 0.65, again indicating an additive effect.

**Table 2.** Minimum inhibitory concentrations of SP80 and antibiotics alone and in combination.

<table>
<thead>
<tr>
<th></th>
<th>MIC</th>
<th>FICI</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC_A</td>
<td>MIC_A (with B)</td>
<td>MIC_B</td>
</tr>
<tr>
<td><em>S. agalactiae</em></td>
<td>5µg/µL</td>
<td>2.5µg/µL</td>
<td>14µM</td>
</tr>
</tbody>
</table>

|                | MIC\_A  | MIC\_A (with C) | MIC\_C | MIC\_C (with A) |
| *S. agalactiae*| 5µg/µL  | 0.625µg/µL | 3.75µM  | 1.875µM  | 0.625 | Additive effect |

*MIC*\_A, SP80; *MIC*\_B, ampicillin; *MIC*\_C, penicillin G; *MIC*\_A (with B), SP80+ampicillin; *MIC*\_B (with A), ampicillin+SP80; *MIC*\_A (with C), SP80+penicillin G; *MIC*\_C (with A), penicillin G+SP80.

The fractional inhibitory concentration index (FICI) data were interpreted using the following criteria: synergistic effect, FICI ≤0.5; additive effect, FICI 0.5-1.0; irrelevant effect, FICI 1.0-2.0; and antagonistic effect, FICI ≥2.0.

**Disk Diffusion Assay**

A total of 56 *S. agalactiae* strains, one standard strain (ATCC\textsuperscript{®} 12386\textsuperscript{TM}), and 55 clinical isolates were tested against the SP80 fraction in combination with antibiotics, respectively. The results are shown in Figure 1.

The mean results obtained were compared between the antibiotics (ampicillin and penicillin G) both isolated and associated with the SP80 fraction, respectively. Statistical analysis showed that the mean for ampicillin, when associated with the SP80 fraction, increased as compared to ampicillin alone, but it
was not significant and, the mean for penicillin G, when associated with the SP80 fraction did not change as shown in Table 3.

**Table 3.** Comparison of antibiotics, both isolated and in association with the SP80 fraction against *S. agalactiae* (median: minimum - maximum).

<table>
<thead>
<tr>
<th></th>
<th>Median</th>
<th>Minimum - Maximum</th>
<th><em>p</em> Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMP</td>
<td>28.46</td>
<td>23.00 - 40.00</td>
<td>*p = 0.05</td>
</tr>
<tr>
<td>SP80 + AMP</td>
<td>29.04</td>
<td>24.00 - 40.00</td>
<td></td>
</tr>
<tr>
<td>PEN</td>
<td>25.88</td>
<td>18.00 - 40.00</td>
<td>*p = 0.07</td>
</tr>
<tr>
<td>SP80 + PEN</td>
<td>25.53</td>
<td>16.00 - 39.50</td>
<td></td>
</tr>
</tbody>
</table>

*T-test. Significant (p < 0.05).*

The inhibition halos (mm) were compared between the antibiotic combinations (SP80 + ampicillin and SP80 + penicillin G), respectively, and the statistical analysis showed that the results were significant (*p < 0.001*). The association of the SP80 fraction + ampicillin showed a greater mean inhibition zone (mm) when compared to the mean value obtained for the association of the SP80 fraction + penicillin G for *S. agalactiae* (Table 4).

**Table 4.** Comparing antibiotics, both isolated and in association with the SP80 fraction, against *S. agalactiae* (mean: minimum - maximum).

<table>
<thead>
<tr>
<th></th>
<th>Median</th>
<th>Minimum - Maximum</th>
<th><em>p</em> Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP80 + AMP</td>
<td>29.04</td>
<td>24.00 - 40.00</td>
<td><em>p &lt; 0.001</em></td>
</tr>
<tr>
<td>SP80 + PEN</td>
<td>25.53</td>
<td>16.00 - 39.50</td>
<td></td>
</tr>
</tbody>
</table>

*Wilcoxon test. Significant (p < 0.05).*

According to the CLSI [30], when the inhibition halo formed by ampicillin and penicillin G, respectively is ≥ 24 mm, the strain is sensitive to the antibiotic in question. We compared the inhibition halos obtained with isolated antibiotics, and the result showed that the strains tested are more resistant to penicillin G as compared to ampicillin (Table 5).
Table 5. Comparison of the inhibition halo (mm) of the antibiotics ampicillin and penicillin G (isolated) against *S. agalactiae*.

<table>
<thead>
<tr>
<th></th>
<th>Inhibition halo ≥ 24 mm</th>
<th>Inhibition halo &lt; 24 mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>54 strains</td>
<td>2 strains *p &lt; 0.1</td>
</tr>
<tr>
<td>Penicillin G</td>
<td>31 strains</td>
<td>25 strains *p &lt; 0.05</td>
</tr>
</tbody>
</table>

Inhibition halo ≥ 24 mm: sensitive;  
Inhibition halo < 24 mm: resistant;  
*Wilcoxon test. Significant (p < 0.05).

**Discussion**

Garlic is one of the most studied medicinal plants in the world and has been used in traditional medicine for many centuries for the treatment of many diseases [34,35]. According to Majewski [16], the properties of garlic are due to the combination of several biologically active substances with antimicrobial properties, which are responsible for its curative effect, which aroused our interest in studying its antimicrobial activities. To this end, we evaluated its susceptibility and drug interaction when combined with the antibiotic ampicillin and penicillin G, both belonging to the class of β-lactams, against clinical isolates of *S. agalactiae*.

Ampicillin and penicillin G were used because they are first-line antibiotics for IAP in pregnant women [36]. In addition, no studies were found that evaluated the interaction of these antibiotics with garlic or the prospect of finding ways to enhance the action of these antibiotics, which could help in the prophylaxis and/or treatment of this infection. It is worth noting that our study is the first of its kind to be published in the literature to date.

The complete phytochemical analyses of the SP80 fraction obtained from the raw garlic extract (RGE) were performed by Torres et al [27] to isolate and identify the bioactive compounds present in garlic using reverse phase ultrafast liquid chromatography with UV spectroscopy (RP-UFLC-UV), mass spectrometry (LC-MS) and nuclear magnetic resonance (NMR). Some of the compounds identified in their study were γ-glutamyl-S-allylcysteine, γ-glutamyl-phenylalanine, and (E) and (Z)-ajoenes, and the yields of these compounds were 8.48%, 2.77%, and 0.31%, respectively. Therefore, the present study aimed to evaluate the synergistic activity of the association of the SP80 fraction with the antibiotic's ampicillin and penicillin G.

The SP80 fraction was used instead of the isolated fractions mentioned above because, according to Majewski [16], the properties of garlic result from the combination of several biologically active substances with antimicrobial properties, and since the yield of each isolated compound was very low, the ideal would be to use synthetic compounds purchased commercially, but this will be the objective of future work.
The methods used to evaluate antimicrobial and synergistic activity were disk diffusion and broth microdilution [37-41]. The disk diffusion method was used because it is the most used method in clinical analysis laboratories and hospitals, in Brazil, to verify antimicrobial susceptibility, and broth microdilution is used by the Microbiology Society.

The MIC of the SP80 fraction obtained by purification of the RGE and the antibiotics were determined by broth microdilution, in triplicate. The MICs used in our tests were the lowest that completely inhibited microbial growth and showed bacteriostatic activity [35]. As our work is pioneering, different extraction methods may lead to different biological responses.

When the MIC value was determined using the microdilution method, this garlic fraction was shown to have antimicrobial activity against *S. agalactiae*. This is an important finding that confirms all other studies described in the literature on the antimicrobial activity of this plant [37,42-45].

The association of the SP80 fraction with ampicillin was more efficient than the association of the SP80 fraction with penicillin G compared to the antibiotics alone. The combination of SP80 + ampicillin indicates an additive effect, but we cannot consider a synergistic effect.

According to CLSI [31], inhibition halos ≥ 24 mm formed by ampicillin and penicillin G indicate that the strains tested are sensitive to these antibiotics. We compared the antimicrobial effect of ampicillin and, penicillin G, respectively, isolated against *S. agalactiae* strains, and of the 56 strains tested, 25 strains were resistant to penicillin G and another 2 were resistant to ampicillin, thus demonstrating that the strains tested are more resistant to penicillin G than they are to ampicillin.

There is little information on the mechanism behind reduced penicillin G susceptibility in GBS. According to Hayes et al [46], β-lactams exert their antibacterial effect by blocking the action of enzymes involved in cell wall synthesis. Penicillin G inhibits bacterial peptidoglycan formation by binding the β-lactam ring to enzymes known as penicillin-binding proteins (PBPs) at DD-transpeptidase. Therefore, Chu et al [47] believe that modification of penicillin binding proteins is a possible explanation for the reduced penicillin G susceptibility observed in their GBS isolates.

According to Van der Linden et al [48], the acquisition of point mutations in the PBPs genes, leading to the production of proteins with low affinity for β-lactam binding, could explain why some *S. agalactiae* strains may be non-susceptible to penicillin. However, further molecular work is needed for confirmation.

Hayes et al [45] performed checkerboard and time-kill assays in vitro to determine the synergistic activity of erythromycin and nisin against clinical isolates of Group B *Streptococcus* against invasive and colonizing GBS strains. Their results suggest that erythromycin and nisin may act synergistically to inhibit the growth of GBS. This study differs from ours in the antibiotics used, and our results showed an additive effect of combining the SP80 fraction with antibiotics, respectively.

Garlic has a variety of bioactive compounds, including organosulfur compounds, saponins, phenolic compounds, and polysaccharides. The most important are organosulfur compounds, especially allicin.
According to Choo et al [50], the antibiotic activity of garlic is mainly due to allicin, which acts by destroying and inhibiting gram-positive and gram-negative bacteria.

In the work of Torres et al [27], the authors showed that other compounds present in garlic, in addition to allicin, have antimicrobial activity and can be used against strains of *S. agalactiae*, it is enough to know how they act to inhibit these bacteria.

From this research, the SP80 fraction from *A. sativum* L. has in vitro antimicrobial activity against the bacteria *S. agalactiae*, its association with the antibiotics showed an additive effect.

Based on our results, we believe that further studies should be conducted to pave the way for the development of translational research, as this drug-drug interaction may prove beneficial in the therapy of *S. agalactiae* infections in humans.

**Conclusion**

The current study demonstrates in vitro an additive effect between the SP80 fraction (a combination of several biologically active substances with antimicrobial properties) and ampicillin and penicillin G, respectively, against clinical GBS strains.

**Declarations**

**Acknowledgments**

We would like to thank the technical staff of the Faculty of Medical Sciences of Santa Casa of São Paulo (São Paulo/SP – Brazil) and the technical staff of the Laboratory for Applied Toxinology – Butantan Institute (São Paulo/SP – Brazil). We would also like to thank Salomão and Zoppi Clinical Laboratory (São Paulo - Brazil) for providing the strain *S. agalactiae* and also Dr. Domingos Sávio Rodrigues, for botanical identification.

**Competing Interests**

The authors declare no competing interests.

**Funding**

This research was funded by the Research Support Foundation of the State of São Paulo (FAPESP/CeTICS) (Grant No. 2013/07467-1), by the Brazilian National Council for Scientific and Technological Development (CNPq) (Grant No. 472744/2012-7), by the Coordenação de Aperfeiçoamento
Author Contributions

Lima SMRR: guided the work and final approval of the version to be submitted; Gamberini MT: statistical analysis; Silva-Jr PI: conception and design of the study; Rodrigues S: botanical identification of Allium sativum L.; Torres KAM: development of all experiments and writing of the manuscript.

Data Availability

All data generated or analyzed during this study are included in this published article.

Ethics declarations

The article contains no data concerning studies involving human subjects or the inclusion of identifiable human data or clinical trials; thus, no ethical approval was required.

References


**Figures**
Figure 1

SP80 fraction associated with ampicillin and penicillin G against *S. agalactiae*. (a) Picture of the experiment: Mueller-Hinton agar (MHA) with 5% sheep blood plate seeded with *S. agalactiae*, incubated at 35 ± 2 °C for 24 hours. The diameter of the clear zone around the disc was measured and expressed in millimeters (mm). (b) Result of inhibition halo the strains of *S. agalactiae* were subjected to four treatments by inserting sterile absorbent filter paper discs immersed in the following solutions: ampicillin; penicillin G; ampicillin with 15 µL SP80 fraction; and penicillin G with 15 µL SP80 fraction. The concentration for the SP80 fraction was 5µg/µL. The data represent mean ± SD (n = 56). AMP, ampicillin and PEN, penicillin G.