Antimicrobial And Synergistic Activity of The Drug Association of *Allium Sativum* And Antibiotics Against Group B *Streptococcus*

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Abstract

Maternal colonization by Group B *Streptococcus* during pregnancy increases the risk of neonatal infection due to vertical transmission from mother to fetus before or during labor. The aims of this study were to evaluate the antimicrobial activity of SP80 (obtained from RGE) and its synergism associated with the antibiotic against strains of *Streptococcus agalactiae*. Biomonitoring of SP80 disclosed antimicrobial activity only in fractions F18, F19, F20 and F42. The broth microdilution was used to determine the antimicrobial activity of SP80 and fractions from SP80 and to establish the MIC of SP80 (2.40 mg/mL). By using the disk diffusion method, fifty-five clinical isolates of *S. agalactiae* and 1 ATCC were tested against the association of SP80 with antibiotic penicillin G and ampicillin, respectively, for synergistic assessment. The association of SP80 with penicillin G showed that the mean of the inhibition halos decreased, but it was not significant, with $p<0.07$. In contrast, the association of SP80 with ampicillin caused the mean inhibition halos to increase with a $p<0.001$, a significant result. SP80 has antimicrobial activity against *S. agalactiae* Gram-positive bacteria, and the association with the antibiotic ampicillin showed a synergistic effect, which did not occur when in association with penicillin G.

Introduction

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

GBS disease in newborns can be classified according to the time of onset of infection. Late-Onset Disease (LOD) occurs in the first week of life and is generally due to mother-to-child transmission during childbirth, and Early-Onset Disease (EOD) occurs between the first week and three months after the infant has been born, for which the contamination source 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 GBS infections in newborns 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].

According to the Consensus Reviews, universal recommendations were issued in 2002 for the collection of vaginal secretions from all pregnant women between 35 to 37 weeks of gestation to optimize the identification of those who should receive intrapartum prophylaxis [6].

The use of antibiotics during pregnancy was not seen to be beneficial, but at the beginning of labor it is [7]. 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 childbirth [7]. In case of allergic pregnant women, it is recommended 500 mg (intravenous) erythromycin every 6 hours, or 900 mg

clindamycin every 8 hours; if the pregnant woman is GBS resistant, 1 g vancomycin is administered intravenously every 12 hours [8, 9].

Although prophylactic measures are already well established by conventional medicine, the use of medicinal plants in disease prevention is quite common in developing countries like Brazil [10]. When researched the use of garlic for the treatment of GBS infection in traditional medicine, we were able to find some published articles describing positive results [11, 12].

Garlic (*Allium sativum* L.), part of the family Liliaceae, is one of the most researched medicinal plants in the world and has been used for centuries in cooking and traditional medicine [13]. In Brazil, this plant belongs to a list comprising 71 plants of interest to the *SUS* (acronym for *Sistema Único de Saúde*, i.e. Brazil's Universal Health System) issued by the Ministry of Health's National Program of Medicinal Plants and Herbal [10].

Organosulfur compounds are the main bioactive compounds present in garlic, especially allicin, which is responsible for the characteristic odor and antimicrobial properties of this plant [14].

Allicin was identified by Cavallito and Bailey [15] and has demonstrated antimicrobial activities against Gram-positive and Gram-negative bacteria [16]. It is converted from its precursor alliin by the enzyme alliinase when tissue damage occurs. It is quite unstable and quickly participate in a cascade of non-enzymatic reactions to produce compounds such as vinyldithiines, ajoenes and (poly)sulfides which have been reported to exhibit antimicrobial activity [16, 17].

This study aimed to assess the antimicrobial activity of *A. sativum* L. and its association with antibiotics penicillin G and ampicillin against *S. agalactiae* strains.

Results

SP80 by reverse-phase ultra-fast liquid chromatography (RP-UFLC) provided fractions according to retention time (Rt) and polarity. This preliminary phytochemical study confirms the presence of four fractions with antimicrobial activity (F18, F19, F20 and F42) against the *Streptococcus agalactiae* strain (ATCC® 12386™), showed in Figure 1.

The Minimum Inhibitory Concentration (MIC) of SP80 was determined, as triplicates, by employing the microdilution method. The mean and standard deviation of the results obtained was 2.40 ± 0.42 mg/mL. This concentration was used in the disk diffusion tests.

A total of 56 *S. agalactiae* strains, one standard strain (ATCC® 12386™), and 55 clinical isolated were tested against penicillin G, ampicillin, and SP80 in association with the respective antibiotics using the disk diffusion test (Figure 2). The results are shown as mean ± standard deviation as can be seen in Table 1.

Table 1. Inhibition halo diameter (mm) of the tested groups against *S. agalactiae* (mean ± standard deviation).

Ampicillin	**28.46 ± 3.41**
Ampicillin + SP80	**29.04 ± 3.50**
Penicillin G	**25.88 ± 5.29**
Penicillin G + SP80	**25.53 ± 5.75**

The mean results obtained were compared between the antibiotics (Ampicillin and Penicillin G) both isolated and associated with SP80, respectively. The statistical analysis showed that the mean for Ampicillin, when associated with SP80, increased as compared to Ampicillin alone (*p<0.001*), a significant increase. Otherwise, the mean for Penicillin G, when associated with SP80, decreased as compared to Penicillin G alone (*p<0.07*), a non-significant decrease (Table 2).

Table 2. Comparison of antibiotics, both isolated and in association with SP80 against *S. agalactiae* (median: minimum – maximum).

Ampicillin	**28.46 (23.00 - 40.00)**	***p<0.001**
Ampicillin + SP80	**29.04 (24.00 - 40.00)**	
Penicillin G	**25.88 (18.00 - 40.00)**	***p<0.07**
Penicillin G + SP80	**25.53 (16.00 - 39.50)**	

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

The inhibition halos (mm) were compared between the antibiotic combinations (Ampicillin + SP80, and Penicillin G + SP80), respectively, and the statistical analysis showed that the results were significant (*p<0.001*). The association of SP80 with Ampicillin showed a greater mean inhibition zone (mm) for *S. agalactiae* when compared to the mean value obtained for the association of SP80 with Penicillin G (Table 3).

Table 3. Comparing antibiotics, both isolated and in association with SP80, against *S. agalactiae* (mean: minimum – maximum).

Ampicillin + SP80	**29.04 (24.00 - 40.00)**	***p<0.001**
Penicillin G + SP80	**25.53 (16.00 - 39.50)**	

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

According to the CLSI (2015), when the inhibition halo formed by Penicillin G and Ampicillin is ≥ 24 mm, the strain is sensitive to the antibiotic involved. We compared the inhibition halos obtained with isolated

antibiotics, and the result showed us that the strains tested are more resistant to Penicillin G as compared to Ampicillin (Table 4).

Table 4: Comparison of the inhibition halo (mm) of the antibiotics Penicillin G and Ampicillin (isolated) against *S. agalactiae*.

	N	Inhibition halo ≥ 24 mm	Inhibition halo 24 mm	
Penicillin G	**56**	**31 strains**	**25 strains**	***p<0.05**
Ampicillin	**56**	**54 strains**	**2 strains**	***p<0.1**

Inhibition halo ≥ 24 mm: sensitive;

Inhibition halo 24 mm: resistant;

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

Discussion

Garlic is one of the most researched medicinal plants in the world and has been used in traditional medicine for many centuries for the treatment of numerous diseases [18]. According to Majewski [13], garlic's properties result from the combination of several biologically active substances with antimicrobial properties responsible for its healing effect. That aroused our interest in studying their antimicrobial activities. To that end, we evaluated their susceptibility and drug interaction when associated with the antibiotic penicillin G and ampicillin, both of which belong to the class of β-lactams, against clinical isolates of *S. agalactiae.*

Penicillin G and ampicillin were used as they are antibiotics of first choice used in the intrapartum prophylaxis of pregnant women [19]. 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 aid in the prophylaxis and/or treatment of this infection. It is worth noting that our study is the first one of its kind to be published in the literature to date.

The chromatography profile was performed using reverse-phase ultrafast liquid chromatography with UV (RP-UFLC-UV) system, and the fractions with antimicrobial activity found are F18, F19, F20 and F42. The complete phytochemical analyses will be carried out to identify the bioactive compounds presented in these fractions and will be presented in future works.

The SP80 was used instead of the isolated fractions as mentioned above, according to Majewski [13], garlic's properties result from the combination of several biologically active substances with antimicrobial properties.

The methods most used for assessing antimicrobial activity are disk diffusion and broth microdilution [20–24]. The disk diffusion method was used because it is a quantitative method that also enables a synergistic assessment between SP80, and the antibiotics used in our study.

The Minimum inhibitory concentration (MIC) of SP80 obtained by purifying the raw garlic extract (RGE) on a Sep-Pak® C18 cartridge column was determined by the broth microdilution, in triplicate. The MIC used in our tests was the lowest value that completely inhibited microbial growth and exhibited a bacteriostatic action [25]. As our work is pioneering, different extraction methods could lead to distinct biological responses. For this reason, in our study, the MIC established for SP80 was 2.40 mg/mL. This concentration was used in the disk diffusion tests.

When determining the MIC value by using the microdilution method, it was demonstrated that this garlic fraction has antimicrobial activity against *S. agalactiae*. This is an important finding that corroborates all other studies described in the literature regarding this plant's antimicrobial activity [20, 26–29].

According to the CLSI [30], inhibition halos ≥ 24 mm formed by penicillin G and ampicillin demonstrate that the strain tested against these antibiotics, respectively, is sensitive. We compared the antimicrobial effect of penicillin G and ampicillin, isolated, respectively, against *S. agalactiae* strains. 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 a dearth of information regarding the mechanism behind reduced penicillin g susceptibility in GBS. However, Chu *et al.* [31] believe that modification of penicillin-blinding proteins is a possible explanation for the reduced penicillin G susceptibility seen with their GBS isolates. Nevertheless, further molecular work is needed for confirmation.

Garlic has a variety of bioactive compounds, including organosulfur compounds, saponins, phenolic compounds, and polysaccharides. The main ones are organosulfur compounds, especially allicin [32, 33]. According to Choo *et al.* [34], garlic's antibiotic activity is mainly attributable to allicin, which acts on the destruction and inhibition of Gram-positive and negative bacteria.

Cuttler *et al*. [20] evaluated the *in vitro* antibacterial activity of an allicin aqueous extract and a gel formulation incorporating allicin against 76 *S. agalactiae* strains. By the results, the allicin in gel produced inhibition halos of 23.00 ± 6 mm, whereas allicin in water, 21.00 ± 6 mm. In our study, a combination of garlic compounds was used instead of an isolated substance as allicin.

Arzanlou [27] assessed the antimicrobial activity of allicin against Group A *Streptococcus* (GAS) by the microdilution method. The results showed that allicin strongly inhibits the maturation of SpeBz and proteolytic activity of SpeBm in a concentration-dependent manner. This study differs from ours in its methodology and in using the compounds allicin isolated from garlic, but both studies corroborate the antimicrobial action attributed to garlic.

In a North American research cited by Bontempo [26] the author presented the results of microbial growth inhibition for 14 bacterial species using a dilution of fresh garlic extract. Marchese *et al*. [28] carried out a review based on the antifungal and antibacterial activities of *A. sativum* L. and its main compound, allicin. Our findings also corroborate these studies, to confirm the antimicrobial activity of *A. sativum* L.

Hayes *et al*. [29] carried out checkerboard and time-kill assays *in vitro* for determining 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 can act synergistically to inhibit the growth of GBS. This study differs from ours in relation to the antibiotics used and the methodology.

From this research, the fraction SP80 from *A. sativum* L. has *in vitro* antimicrobial activity against the bacteria *S. agalactiae*, with a MIC of 2.40 mg/mL; its association with the antibiotic ampicillin showed a synergistic effect, which did not occur when in association with penicillin G.

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

Conclusions

The current study reveals that SP80 (a combination of several biologically active substances with antimicrobial properties) has antimicrobial activity against clinical isolates of *Streptococcus agalactiae* tested, and when associated with the antibiotic ampicillin, it has shown a synergistic effect resulting from this association.

Material And Methods

Plant material

Garlic bulbs from *A. sativum* L. (Liliaceae), purple variety, were purchased in local market at 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 subsequently provided to experts in Botany. A voucher specimen, (n ° 502077), was deposited at the *Herbarium Maria Eneyda P. Kaufmann Fidalgo*, São Paulo/Brazil where species identity was confirmed by Dr. Domingos Sávio Rodrigues. All study/experimental protocols involving plant materials was conducted in accordance with institutional, national and international guidelines and legislation.

Raw Garlic Extract (RGE) and Solid Phase Extraction (SPE)

Raw Garlic Extract (RGE): Two hundred grams of fresh *A. sativum* L. bulbs, undamaged and fungi-free, were carefully peeled, washed under running water and placed in a mini processor (Cadence®) for approximately 1 minute. The processed material was then filtered through a No. 20 mesh sieve and the

liquid obtained was lyophilized (Thermo Super Modulyo Pirani 501), stored in a freezer (-20 ºC), and named Raw Garlic Extract (RGE) [35].

Solid Phase Extraction (SPE): RGE (2 g) was solubilized with 5 mL of a 2M acetic acid solution under magnetic stirring in a refrigerated system (ice bath) for 30 minutes. Subsequently, the mixture was centrifuged (14680 x g) for 5 min. The supernatant (4.0 mL) was loaded onto the column Sep-Pak® C_{18} cartridges equilibrated in 0.05% trifluoroacetic acid (TFA) solution. The sample was eluted in acetonitrile (ACN) 80%, then lyophilized, to completely remove the solvent, and labeled as Sep-Pak 80% (SP80), and stored in a freezer (-20 ºC) before use in antimicrobial activity assays [36].

SP80 Chromatography Profile

The chromatography profile was performed using reverse-phase ultrafast liquid chromatography with UV (RP-UFLC-UV) system on a Prominence chromatograph (Shimadzu Corp., Kyoto, Japan) with preparative reverse phase column Shim-Pack prep ODS (H) kit (250 mm x 20 mm x 5 µm). The SP80 was reconstituted in 6 mL of acidified water (TFA 0.05%) and divided into three runs. The fractionation of the sample was carried out using a gradient of 0 to 80% ACN at a flow rate 2.0 mL/min for 120 minutes. All eluted peak were manually collected and were vacuum-dried to completely remove the solvent and were reconstituted in ultrapure water, before being used in antimicrobial activity assays [36].

Bioassays

Antimicrobial Activity: The antimicrobial assay of all peaks collected from SP80 were performed against *S. agalactiae* (ATCC®12386™) strain using TSB (Tryptic Soy Broth) medium. Antimicrobial activity was determined using broth microdilution assay in 96-well sterile plates. The dried fractions were dissolved in 250 µL of ultrapure water and then 20 µL were aliquoted into each well with 80 µL of microbial dilution at a final volume of 100 µL. Sterile water and TSB were used as negative control and penicillin G and ampicillin, the antibiotics of choice in the treatment of *S. agalactiae* infections, was used as positive control. After 24 h of incubation at 35 ± 2 ºC, the microbial growth was measured by monitoring the increase in optical density at 595 nm using a Victor 3 – 1420 (Perkin Elmer®) instrument [25].

Minimum Inhibitory Concentration (MIC): The SP80, reconstituted in ultrapure water, was subjected to liquid growth inhibition test against *S. agalactiae* (ATCC®12386™) strain. The assay was performed in 96-well microplates. For that, 20 µL of SP80 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 ºC 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 growth of the microorganism. The tests were performed in triplicate and the average of 3 readings was considered [25].

Disk Diffusion Assay: SP80 was carried out by disc diffusion test and the concentration used was established from the MIC assay results for *S. agalactiae* (ATCC® 12386™). For antimicrobial 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 [37].

For this purpose, ready-made Petri dishes were used with the Agar Miller-Hinton blood culture medium (150 mm), supplied by Probac, and *S. agalactiae* strains (previously prepared in saline), using alginate swabs, at concentration of 1.5 x 10^8 CFU/mL, which corresponds to 0.5 in the MacFarland nephelometric scale [38]. The strains of *S. agalactiae* were subjected to four treatments by inserting sterile absorbent filter paper discs immersed in the following solutions: (1) penicillin G (10 IU); (2) ampicillin (10 mcg); (3) penicillin G (10 IU) with 15 µL SP80; and (4) ampicillin (10 mcg) with 15 µL SP80. 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 [30, 39].

Statistical Analysis

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

Declarations

Competing Interests

The authors declare no competing interests.

Data Availability

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

Ethics declarations

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

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Author Contributions

K.A.M.T. was responsible for the conception and design of the work;

M.T.G. was responsible for statistical analysis;

D.S.R. was responsible for botanical identification;

S.M.R.R.L. and P.I.S.J. were responsible for funding acquisition;

D.S.R. and K.A.M.T. were responsible for data collection;

S.M.R.R.L., M.T.G., P.I.S.J. and K.A.M.T. were responsible for drafting the manuscript;

All authors were responsible for critical revision of the manuscript and analysis and interpretation of the data.

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Figures

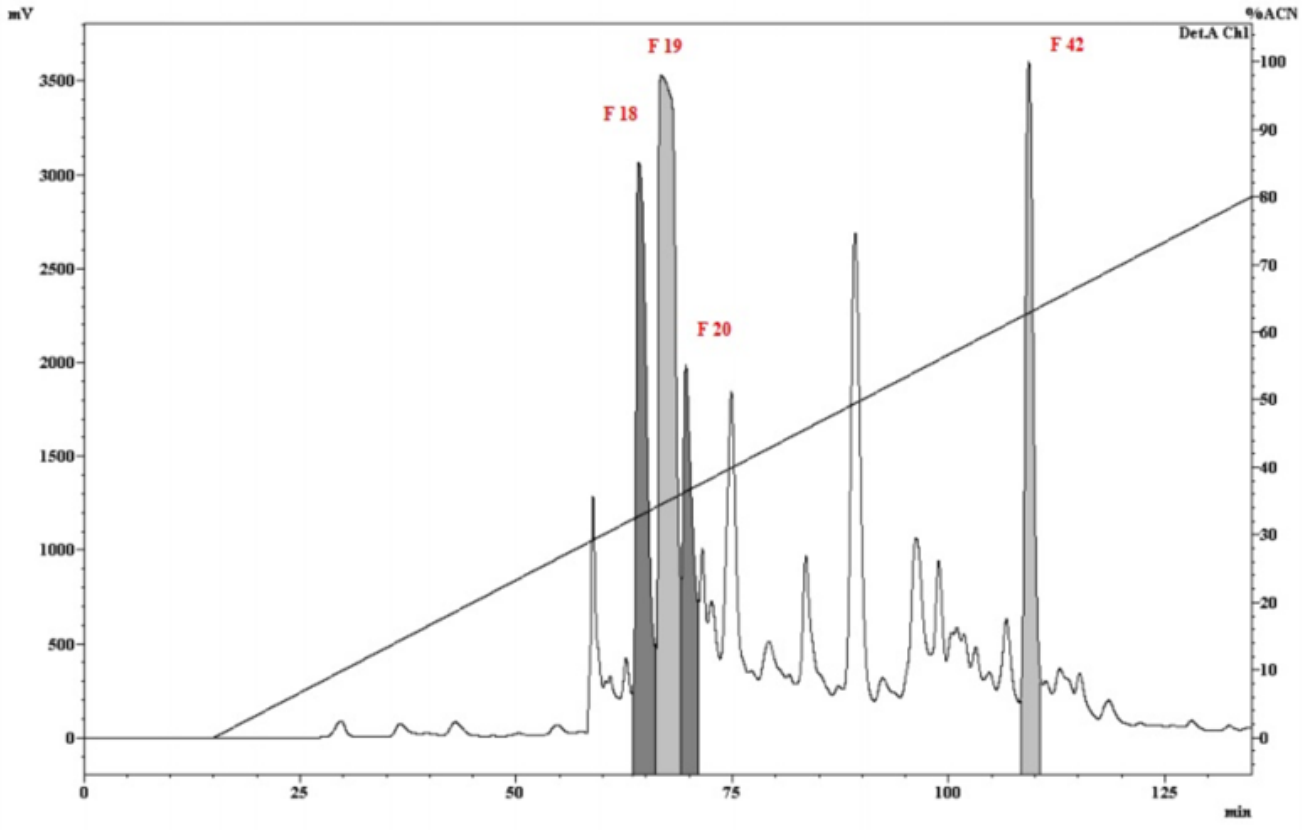

Figure 1

Chromatographic profile of SP80, obtained from Sep-Pak® C18 cartridges. Analysis was performed using a RP-UFLC system Shimadzu model Prominence device with preparative reverse phase (RF) column Shim-Pack prep ODS (H) kit (250 mm x 20 mm x 5 µm) and the absorbance was monitored at 225 nm. Note peaks F18, F19, F20 and F42 that correspond to fractions with antimicrobial activity against *S. agalactiae* (ATCC® 12386™).

Zone diameter (mm)	SP80 + penicillin	SP80 + ampicillin	penicillin	ampicillin
40		1	1	1
39.5	1			
39				
38				
37				
36.5		1		
36				1
35				
34.5		1		
34	1			1
33		1	1	
32	3	4	8	5
31.5	3	3	3	
31	2	4		6
30.5	5	7		
30	10	6	10	13
29.5		2		
29	3	1	3	3
28.5	1	1		
28		2	2	2
27.5		1		
27		1		5
26.5		1		
26	1	3	1	4
25.5	1	4		
25		7	1	10
24.5		1		
24		3	1	3
23		1	1	2
22.5	1			
22	4		5	
21	2		9	
20.5	2			
20	4		6	
19.5	3			
19	3			
18.5	1			
18	2		4	
17.5	1			
17	1			
16	1			

Figure 2

Result of inhibition halo the strains of *S. agalactiae* subjected to four treatments by inserting sterile absorbent filter paper discs immersed in the following solutions: penicillin G (10 IU); ampicillin (10 mcg); penicillin G (10 IU) with 15 µL SP80; and ampicillin (10 mcg) with 15 µL SP80. The MIC established for SP80 was 2.40 mg/mL. The zone diameter around the disc was measured and expressed in millimeters (mm).