UNIVERSITI PUTRA MALAYSIA

IN VITRO STUDY OF ANTIBACTERIAL AND SYNERGISTIC EFFECT OF NEEM LEAF EXTRACT WITH CEPHALEXIN AGAINST *STAPHYLOCOCCUS PSEUDINTERMEDIUS*

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It is hereby certified that we have read this project paper entitled "*In Vitro* Study Of Antibacterial and Synergistic Effect of Neem Leaf Extract with Cephalexin Against *Staphylococcus Pseudintermedius",* by Varman a/l Sewasuppramaniam and in our opinion it is satisfactory in terms of scope, quality, and presentation as partial fulfilment of the requirement for the course VPD 4901 – Project.

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DEDICATION

This thesis is dedicated to my family, friends and the one that I love, who had stood by me in the entire journey of becoming a veterinarian.

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I wish to express profound gratitude to Associate Professor Arifah Abdul Kadir, my dedicated supervisor, for her unwavering guidance and support throughout the entirety of this project. Special appreciation extends to Dr. Sharina Omar, my co-supervisor, whose willingness to invest time and guidance in the bacteriology laboratory has been invaluable.

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LIST OF ABBREVIATIONS

℃	Degree Celcius
%	Percentage
G	Gram
mL	Mililiter
mm	Milimeter
rpm	Revolutions per minute
µL	Microliter
µg	Microgram

ABSTRAK

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4901 – Projek.

KAJIAN IN VITRO MENGENAI KESAN ANTIBAKTERIA DAN SINERGI EKSTRAK DAUN NEEM DENGAN CEPHALEXIN TERHADAP *STAPHYLOCOCCUS PSEUDINTERMEDIUS*

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Staphylococcus pseudintermedius adalah patogen oportunistik pada kucing dan anjing, menyebabkan penyakit kulit, telinga, dan dalaman. Untuk mengurangkan rintangan antimikrob, adalah penting untuk menyiasat rawatan alternatif dan mengkaji kesan gabungan mereka dengan antibiotik konvensional. Neem (Azadirachta indica) adalah tumbuhan ubat yang semakin mendapat perhatian disebabkan potensinya sebagai rawatan yang murah dan mudah didapati untuk pelbagai penyakit manusia. Kajian ini menilai sifat antimikrob ekstrak daun neem terhadap *S. pseudintermedius* dan kesan sinerginya dengan cephalexin. Dua isolat daripada telinga anjing dan dua lagi isolat dari jangkitan saluran kencing kucing telah diperolehi dari Makmal Bakteriologi Veterinari. Ujian resapan telaga (AWD) dijalankan untuk semua empat isolat sebanyak tiga kali, menggunakan 75%, 50%, dan 25% ekstrak daun neem, cephalexin; gabungan

75% ekstrak-cephalexine (nisbah 1:1), dan 95% etanol sebagai kawalan negatif. Kaedah pencairan kaldu digunakan untuk menentukan kepekatan perencatan minimum (MIC) menggunakan cephalexin, ekstrak 25% dan gabungan ekstrak-cephalexine 25% (nisbah 1:1). Kepekatan bakterisid minimum (MBC) ditentukan dengan menggunakan lima kepekatan lebih tinggi termasuk MIC. Analisis statistik telah melibatkan ujian Kruskal-Wallis H untuk AWD dan ujian Mann-Whitney U untuk data MIC. Dalam AWD, tidak terdapat perbezaan yang signifikan dalam zon perencatan antara cephalexin, tiga kepekatan ekstrak neem, dan gabungan. Walau bagaimanapun, semua kepekatan ekstrak dan gabungan menunjukkan aktiviti antibakteria yang signifikan ($p < 0,05$) terhadap semua empat isolat berbanding kawalan negatif. MIC dan MBC ekstrak daun neem adalah antara 0.20 - 1.56% dan 1.56 - 6.25%, masing-masing. Kewujudan sinergi adalah jelas disebabkan keperluan kepekatan antibiotik yang lebih rendah ($p < 0,05$) untuk mencapai MIC dalam gabungan berbanding dengan cephalexin sahaja. Kajian ini menunjukkan aktiviti antimikrob yang signifikan daripada ekstrak daun neem dan peningkatan keberkesanan cephalexin apabila digunakan bersama-sama.

Kata kunci: *Azadirachta indica*, antimikrob, *Staphylococcus pseudintermedius*, sinergi

ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine in partial fulfilment of the course VPD 4901 – Project.

IN VITRO* STUDY OF ANTIBACTERIAL AND SYNERGISTIC EFFECT OF NEEM LEAF EXTRACT WITH CEPHALEXIN AGAINST *STAPHYLOCOCCUS PSEUDINTERMEDIUS

by

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Staphylococcus pseudintermedius is an opportunistic pathogen in cats and dogs, causing skin, ear, and internal infections. It is essential to investigate alternative treatments and explore their combined effects with conventional antimicrobials to reduce antimicrobial resistance. Neem (*Azadirachta indica*) is a medicinal plant gaining increasing attention for its potential in developing affordable and accessible treatments for a range of human diseases. This study evaluates neem leaf extract's antimicrobial properties against *S. pseudintermedius* and its synergistic effect with cephalexin. Two isolates from canine ear infection and another two isolates from feline urinary tract infections were obtained from Veterinary Bacteriology Laboratory. An agar well diffusion assay (AWD) was conducted for all four isolates in triplicates using 75%, 50%, and 25% neem leaf extract, cephalexin; a 75% extract-cephalexin combination (1:1 ratio), and

95% ethanol as negative control. The modified broth dilution method determined the minimum inhibitory concentration (MIC) for cephalexin, 25% extract, and the 25% extract-cephalexin combination (1:1 ratio). The minimum bactericidal concentration (MBC) was determined by culturing five dilutions above the MIC. Statistical analyses involved Kruskal-Wallis H Test for AWD and Mann-Whitney U test for MIC data. In the antimicrobial susceptibility test, no significant difference was found in the zone of inhibition between cephalexin, three concentrations of neem extract, and the combination. However, all extract concentrations and the combination showed significant antibacterial activity ($p < 0.05$) against all four isolates compared to the negative control. The MIC and MBC of neem leaf extract is ranged from 0.20 - 1.56% and 1.56 - 6.25%, respectively. The synergy was evident with significantly lower ($p < 0.05$) antibiotic concentration needed to achieve the MIC in combination compared to cephalexin alone. This study demonstrates the significant antimicrobial activity of neem leaf extract and its enhancement of cephalexin's effectiveness, even at lower concentrations when used together.

Keywords: *Azadirachta indica*, antimicrobial, *Staphylococcus pseudintermedius*, synergy

1.0 INTRODUCTION

Staphylococci is a group of bacteria that can infect living organisms or colonise inanimate surfaces. It is also capable of withstanding unfavourable environmental conditions (França, 2021). *Staphylococcus pseudintermedius* is a commensal bacterium of skin and mucous membrane in more than 80% of some healthy dog populations (Rubin and Chirino-Trejo, 2011). However, it is also an opportunistic pathogen and one of the main causes of skin and ear, post-operative wound infection as well as infection of other body tissue cavities in cats and dogs (van Duijkeren, 2011). In addition, the worldwide emergence of methicillin-resistant *S. pseudintermedius* (MRSP) in the last two decades has caused the treatment of *S. pseudintermedius* a clinical challenge in veterinary medicine (Maali *et al*., 2018).

Cephalexin is a first-generation cephalosporin, one of beta-lactam antibiotics widely used in veterinary medicine used in cases of pyoderma, pneumonia, soft tissue and urinary tract infection against Gram-positive or Gram-negative bacteria (Aiensaard *et al*., 2021). However, a study carried out by Bardiau *et al*. (2013) showed that about 45% of *S. pseudintermedius* isolated from cats and dogs with dermatitis across south Japan were resistant to cephalexin. This prompted studies on alternative antibacterial agents are necessary to combat the issue of antibiotic resistance strains of *S. pseudintermedius.*

Azadirachta indica (neem) on the other hand is one of the most commonly used medicinal plant in India. The presence of active constituents such as alkaloids,

glycosides, saponins and flavonoids provides the leaves with antibacterial effect (Tyagi *et al*., 2013). The extract can be a potential alternative antibacterial agent used topically to treat bacterial skin infections in animals. It can also be a cheaper treatment option that will benefit shelters to reduce their financial burden. Moreover, there are limited studies that have been carried out on the synergism between cephalexin and neem leaf extract. Thus, the main objective of this study is to determine the antimicrobial activity of *neem* leaf extract and its synergistic effect in combination with cephalexin against *S. pseudintermedius*

1.1 The hypotheses of this study are:

1.1.1 Hypothesis 1

a) Null hypothesis: There is no significant antimicrobial activity of neem leaf extract against *Staphylococcus pseudintermedius.*

b) Alternative Hypothesis: There is a significant antimicrobial activity of neem leaf extract against *Staphylococcus pseudintermedius.*

1.1.2 Hypothesis 2

a) Null hypothesis: There is no significant difference between the antimicrobial activity of neem leaf extract when used in combination with cephalexin *in vitro*.

b) Alternative hypothesis: There is a significant difference between the antimicrobial activity of neem leaf extract when used in combination with cephalexin *in vitro*.

2.0 LITERATURE REVIEW

2.1 *Staphylococcus pseudintermedius* as common infective agent in small animals

About 84.7% of all *S. pseudintermedius* isolates is from canine skin, ear and urinary tract infections (Lynch and Helbig, 2021). A study by Bierowiew *et al*. (2021) proves that *S. pseudintermedius* is also isolated from respiratory tract infections, conjunctivitis, urogenital infections, dermatitis, otitis, and wounds in cats. Scherer *et al*. (2018) found multi resistant isolates of *S. pseudintermedius* from cases of otitis externa in dogs against commonly used antibiotics in veterinary medicine.

2.2 Cephalexin as common antibiotic in cats and dogs

Cephalexin, a first-generation cephalosporin, exhibits potent activity against Gram-positive cocci such as staphylococci and streptococci, including strains that produce beta-lactamase. It also demonstrates effectiveness against certain Gram-negative Enterobacteriaceae and anaerobes (Prescott, 2006). According to Foster, 2004, cephalexin is frequently used for treating abscesses, wounds, and various soft tissue infections in cats. In the United States and other countries, Cephalexin is approved for the treatment of skin infections in dogs caused by *S. pseudintermedius (Papich, 2016).*

2.3 Resistance of *Staphylococcus pseudintermedius* towards cephalexin

Cephalexin is advised for the treatment of diverse skin and soft tissue infections in dogs across all age groups because of its ability to kill common pathogens and its non-

toxic nature, particularly when gram-positive cocci, such as staphylococci, are responsible (Prodos *et al*., 2014). A study by Fungwithaya, 2017 shows that cephalexin treatment against *S.pseudintermedius* rapidly increases in MRSP due to selection pressure. About 45% of *S. pseudintermedius* isolated from cats and dogs with dermatitis in Japan were resistant to cephalexin (Bardiau *et al*., 2013)

2.4 Ethanolic neem leaf extract as an alternative medicine

Neem has been used in Ayurvedic medicine for over 4000 years due to its medicinal properties (Ahmed *et al*., 2023). *Azadirachta indica* or neem literally means “the free tree of India”, indicating its characteristics of being free from disease and insects (Subapriya & Nagini, 2005). Neem and its constituents have shown potential against cancer, diabetes, atherosclerosis and hypertension (Gupta *et al*., 2017). According to a study by Musa *et al*. (2019), soap made with neem extract is found to be an effective alternative against skin diseases in humans. An *in vitro* study by Ghadir *et al*. (2023) shows that neem can kill a sample of sporocyst entirely when used in high concentration. Even on oral administration, ethanolic neem extract did not cause any changes in liver and kidney parameters as well as their histopathology indicating no damage to liver and kidney in male rats (Seriana *et al*., 2021).

2.5 Synergistic antibacterial activity of plant extracts and antibiotics

Plant antimicrobials, when used in conjunction with conventional drugs, have been discovered to act as synergistic enhancers. While these plant compounds may not possess antimicrobial properties by itself, their combined administration with standard drugs amplifies the effectiveness of the drug (Chanda and Rakholiyam, 2011). De

Oliveira *et al*. (2011) explored the synergistic effects of norfloxacin, tetracycline, and erythromycin when combined with the ethanol extract of Mangifera indica L. peel against S. aureus strains. When used in combination with antibiotics, a four-fold reduction in the MIC values for tetracycline and erythromycin was observed. Combinations of plant extracts and antibiotics can lead to a decrease in the minimum dosage necessary for achieving effective antimicrobial effects. This has the potential to reduce both the risk of side effects and the overall costs associated with the treatment of infectious diseases (Silva *et al*., 2019).

3.0 MATERIALS AND METHODS

3.1 Preparation of test organism

Bacterial isolates of *Staphylococcus pseudintermedius* were obtained from Veterinary Bacteriology Laboratory, UPM. Two of the bacterial isolates are from clinical cases of Feline Lower Urinary Tract Disease (FLUTD) while the other two samples are from dog ear swabs. The isolates were revived into a nutrient agar and further sub-cultured before being used.

3.2 Collection and Preparation of Neem Leaves

Neem leaves were obtained from the residential area of Goodview Heights, Kajang, Selangor. Fresh leaves were collected from the plant in the evening by cutting the branches. The leaves were then plucked from the branch and those infected with parasites or damaged by insects are removed. All the selected leaves are washed three times with running tap water to remove dirt or soil on its surface. The leaves are then dried in the shade for 7 days as described by Mehta *et al*. (2022).

3.3 Preparation of Ethanolic Neem Leaf Extract

The neem leaf extract was prepared with a modified method described by Elaigwu *et al*. (2019). The dried leaves were ground into powder by a bench-top grinder (Panasonic, MX-AC400TSK). The powder was weighed and 60g of neem leaf powder was added to 600mL of 95% ethanol in a 1:10 ratio. The solution was macerated in a 1000mL conical flask for 3 days with 1 hour of mixing in a magnetic stirrer every day at noon. The solution is then filtered through Whatman filter paper No.1 resulting in 500mL

of filtrate. The ethanol solvent is then evaporated from the filtrate using a Heidolph rotary evaporator at 35℃ with rotation of 60 rpm. The crude extract obtained was centrifuged at 10,000 rpm for 15 minutes. The supernatant obtained was kept at -20℃ until further use.

3.4 Dilution Preparation of Ethanolic Neem Extract

According to the studies carried out by Sarmiento *et al*. (2011), the effects of neem leaf extract increase gradually as the concentration increases from 25%. In addition, the trial antimicrobial susceptibility test (AST) performed, showed that the bacteria is susceptible at 50% concentration of neem leaf extract. Hence, one concentration above and below 50% was chosen resulting in 75%, 50% and 25%. The neem extract was diluted with 95% ethanol and stored at -20℃ until further use.

3.5 Antibacterial Activity Test

3.5.1 Agar well diffusion assay for Antimicrobial Susceptibility Test (AST)

With slight modification of method described by Sivasamugham *et al*. (2021), 6 wells were made onto Mueller-Hinton agar using the base of sterile 1000 µL micropipette tips. Each bacterial subcultures are made into suspensions and standardised to 0.5 McFarland then lawned onto the agar with wells using sterile swabs. The wells were filled with:

I. 100 µL of 30 µg/mL cephalexin as positive control
II. 100 µL of 75% extract
III. 100 µL of 50% extract
IV. 100 µL of 25% extract

V. 100 µL of cephalexin and extract combination in 1:1 ratio

VI. 100 µL of 95% ethanol as negative control

The plates were prepared in triplicates for each bacterial isolate and incubated for 18-24 hours. The zone of inhibition is then measured using a calliper to evaluate the antimicrobial activity of antibiotic, extract and combination.

3.5.2 Determination of Minimum Inhibitory Concentration (MIC)

The MIC was determined through a modified broth dilution method described by Mehta *et al*. (2022). 150 µL of Mueller-Hinton broth was added to every well in the 96-well plate. Bacterial suspension equivalent to 0.5 McFarland standards was prepared and diluted to optical density (OD) 0.001 by adding normal saline to the ratio of 1:100 to obtain 10^6 cfu/mL. 150 µL of double the target concentration of antibiotic was added to the first well of 3 rows to obtain 30 µg/mL. Each test is performed in triplicates. The similar was done for 25% neem extract and extract-antibiotic combination in 1:1 ratio. Two-fold microdilution was performed from column 1 to 11 while column 12 of all wells functions as broth control without any extract or antibiotic. Then, 50 µL of bacterial suspension (OD 0.001) was added to all wells. The plates were incubated at 37℃ for 18-20 hrs and the lowest concentration of extract/ antibiotic/ extract-antibiotic that inhibits bacterial growth completely is identified as the MIC value.

3.5.3 Determination of Minimum Bactericidal Concentration (MBC)

MBC follows after determination of MIC, where 5 concentrations above and including MIC for 2 out of the 3 replicates in MIC test is cultured into respective Mueller-Hinton agar. The plates are recorded as 1xMIC, 2xMIC, 3xMIC, 4xMIC, 5xMIC and

6xMIC as the concentration increases. The plates are then incubated for 24 hours at 37℃. Standard plate count is done after incubation and the lowest concentration of cephalexin, extract and combination that inhibited any bacterial growth in the cultured plates is determined as the MBC.

3.5.4 Determination of MBC/MIC Ratio

The MIC/MBC ratio is calculated to determine if the neem leaf extract is bactericidal or bacteriostatic. If the ratio is less than 4, the extract is classified as bactericidal and if it is more than 4, it is determined as bacteriostatic (Mogana *et al*., 2020 and Jantorn *et al*., 2023).

3.5.5 Statistical analysis

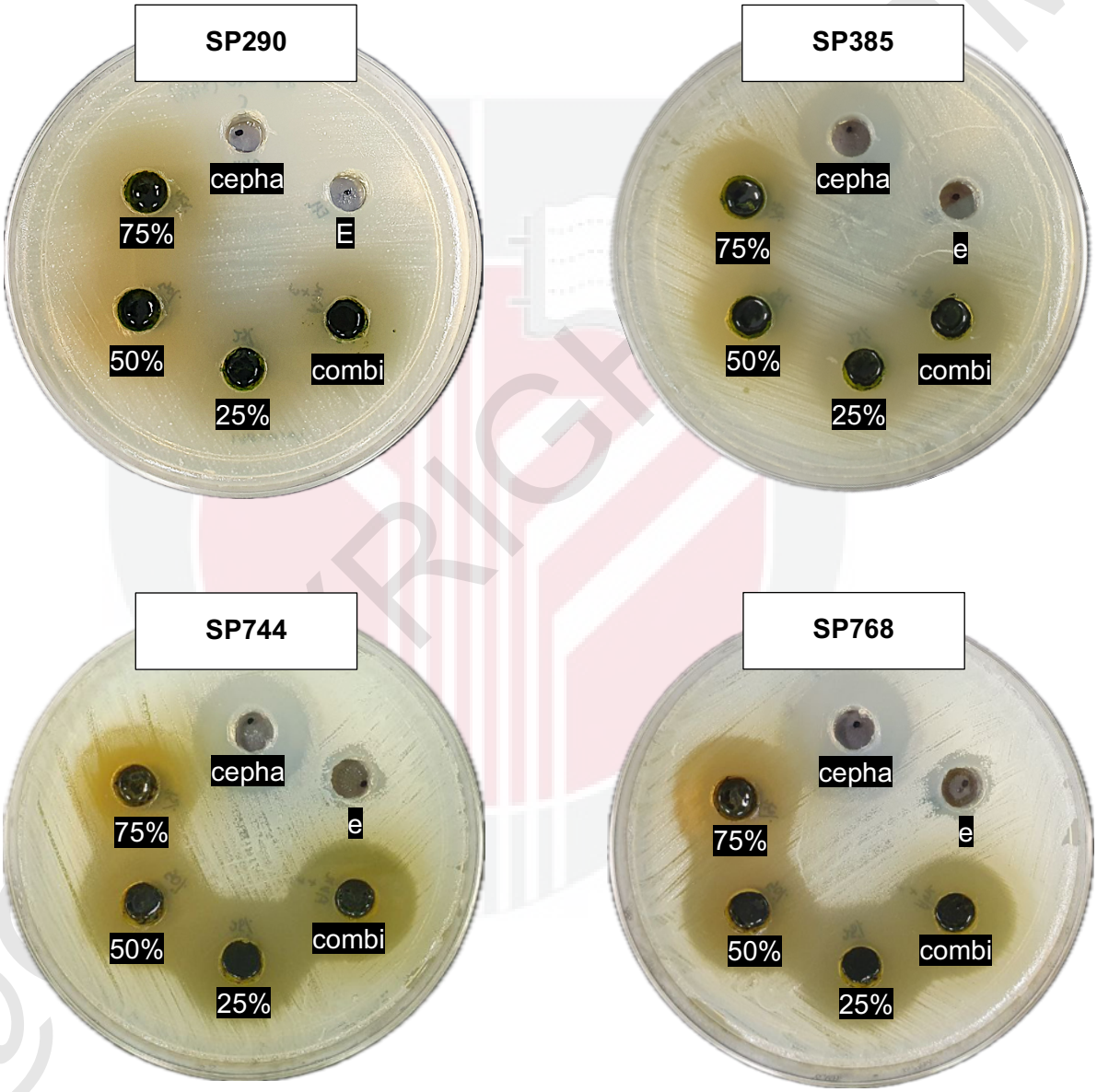
Test of normality was performed to determine if the data is normally distributed. Then, one-way ANOVA was done for the AST data to determine if there is a significant difference in zone of inhibition between cephalexin, neem leaf extract and combination. Then, T-test was performed to determine synergistic effect between neem leaf extract and cephalexin. Lastly, Mann- Whitney U Test was done for the result of MIC to prove the synergism between cephalexin and neem leaf extract.

4.0 RESULTS

Four clinical isolates of *S. pseudintermedius* were obtained from the Veterinary Bacteriology Laboratory, UPM. Two out of the four isolates are from dog ear swabs (50%) and the other two are from feline urinary tract infection (50%). Antimicrobial susceptibility test (AST) was done in triplicates for all 4 isolates using cephalexin, Neem extract at three different concentrations and combination. The mean zone of inhibition around the well is measured and expressed in mean ± standard error.

All three concentrations of neem leaf extract, combination and cephalexin shows antimicrobial activity against every clinical isolates of *S.pseudintermedius* (Figure 1*).* In this experiment where all of the zone of inhibition falls within the susceptible range of >18cm (Figure 2). The largest zone of inhibition is exhibited by 75% neem leaf extract against SP290 at 23.21 ± 0.56 mm compared to cephalexin that produced 22.44 ± 0.90 mm (Table 1). The zone of inhibition of 95% ethanol as negative control in every test is consistent at 8mm which is the diameter of the well itself. There is no significant difference in zone of inhibition between cephalexin, 3 concentrations of leaf extracts and combination for all bacterial isolates (Appendix 1). However, when their zone of inhibition is compared statistically to the negative control (95% ethanol), significant antimicrobial activity ($p < 0.05$) is evident (Appendix 2).

Figure 1: Zone of inhibitions produced by cephalexin, three concentrations of extract, Combination against SP768(left) and SP 744(right) isolates.



cepha= cephalexin, 75%= 75% extract, 50%= 50% Extract, 25%= 25% Extract, combi= Combination of cephalexin and extract, e= 95% ethanol

Figure 2: Mean diameter of zone of inhibition of cephalexin, various concentrations neem leaf extract and combination against *S.pseudintermedius*

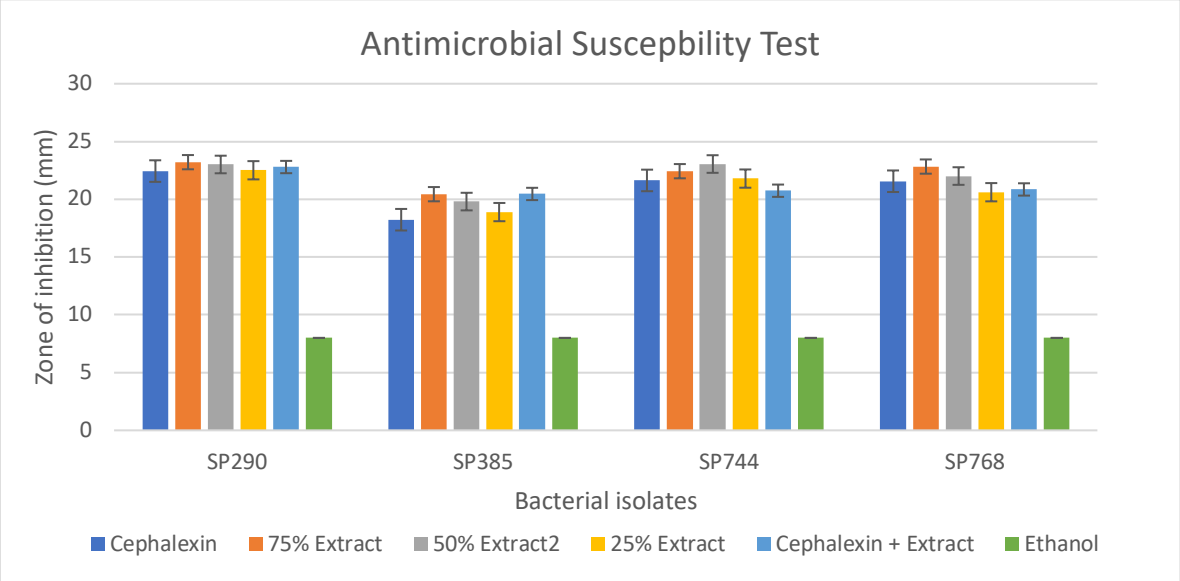


Table 1: Diameter of zone of inhibition of cephalexin, various concentrations of neem leaf extract and combination against *S.pseudintermedius*

Isolates	Cephalexin (mm)	75% Extract (mm)	50% Extract (mm)	25% Extract (mm)	Combination (mm)	Ethanol (mm)
SP290	22.44 ± 0.90	23.21 ± 0.56	23.01 ± 0.90	22.51 ± 1.08	22.79 ± 0.79	8.00 ± 0.00
SP385	18.23 ± 0.72	20.44 ± 0.70	19.80 ± 0.20	18.89 ± 0.14	20.46 ± 0.19	8.00 ± 0.00
SP744	21.63 ± 0.15	22.43 ± 2.08	23.05 ± 1.85	21.79 ± 1.30	20.74 ± 0.74	8.00 ± 0.00
SP768	21.56 ± 0.15	22.83 ± 0.19	22.01 ± 0.63	20.61 ± 0.56	20.85 ± 0.34	8.00 ± 0.00

*P < 0.05 when extracts compared to ethanol. Values are in mean ± standard error.

Based on the broth microdilution test, the minimum inhibitory concentration (MIC) of cephalexin ranges is 1.875 µg/mL for SP290 and SP385 while SP744 and for SP768 is at 3.750 µg/mL (Table 2). According to CLSI Third edition, cephalexin 50% of the bacterial isolates were susceptible to cephalexin (< 2 µg/mL) and the other 50% is intermediately susceptible (2-4 µg/mL). Only at 0.195% of concentration, neem leaf extract inhibited the growth of *S.pseudintermedius* isolates SP290 and SP385. The MIC for SP 744 and SP768 is higher at 0.391% and 1.563%, respectively. As for the MIC of combination, the concentration of cephalexin used in combination with Neem leaf extract is recorded. The MIC for three of the bacterial isolates (75%) is consistent at 0.117 µg/mL while 0.469 µg/mL only for SP768. The concentration of cephalexin alone compared to concentration of cephalexin in combination with neem leaf extract showed significant reduction ($p < 0.05$) in usage of cephalexin at MIC when used in combination (Appendix 3). The reduction in concentration of cephalexin at MIC when used in combination with neem leaf extract compared to cephalexin alone ranges from 87- 97% (Table 3).

Minimum inhibitory concentration (MBC) follows once MIC result is obtained. The MBC of cephalexin ranges from 15 to >30 µg/mL (Table 4). For SP744 and SP768, the MBC of Cephalexin could not be determined as bacterial growth is found even on the full concentration of 30 µg/mL. MBC of extract on the other hand ranges from 0.781% to 1.563%. While SP744 is killed even at concentration as low as 0.781% of neem leaf extract, SP768 managed to grow on culture plate with extract concentration lower than 6.25%. The concentration of cephalexin in combination with neem leaf extract is consistent as 0.469 µg/mL for three bacterial isolates except for SP768 at 1.875 µg/mL (Figure 3). When compared to concentration of cephalexin alone at MBC, significantly

lower ($p < 0.05$) concentration of cephalexin is needed at MBC when used in combination with neem leaf extract (Appendix 4).

MIC/MBC ratio was performed to determine if neem leaf extract is bactericidal or bacteriostatic (Table 5). Neem leaf extract against SP290 and SP385 has a higher ratio of 8, followed by SP768 at 4 and lastly SP744 where the MBC is only double the concentration of MIC.

Table 2: Minimum inhibitory Concentration (MIC) of cephalexin, Extract and Combination against *S. pseudintermedius*

Isolates	***Cephalexin, µg/mL***	***Extract, %***	***Combination, µg/mL***
SP290	1.875	0.195	0.117
SP385	1.875	0.195	0.117
SP744	3.750	0.391	0.117
SP768	3.750	1.563	0.469

*P < 0.05 when concentration of cephalexin is compared to combination at MIC

Table 3: Comparison of cephalexin concentration to cephalexin concentration in combination with neem leaf extract at MIC

Isolates	***Cephalexin(A), µg/mL***	***Combination(B), µg/mL***	***Percentage of Reduction, %***
SP290	1.875	0.117	94
SP385	1.875	0.117	94
SP744	3.750	0.117	97
SP768	3.750	0.469	87

Percentage of decrease = (A – B/ A) x 100, A= Concentration of cephalexin alone at MIC, B= Concentration of cephalexin at MIC when used in combination with extract

Table 4: Minimum Bactericidal Concentration (MBC) of four bacterial isolates

Isolates	*Cephalexin, µg/mL*	*Extract, %*	*Combination, µg/mL*
SP290	30	1.563	0.469
SP385	15	1.563	0.469
SP744	>30	0.781	0.469
SP768	>30	6.250	1.875

*P < 0.05 when concentration of cephalexin is compared to combination at MBC

Figure 3: Comparison of cephalexin concentration to cephalexin concentration in combination with extract at MBC

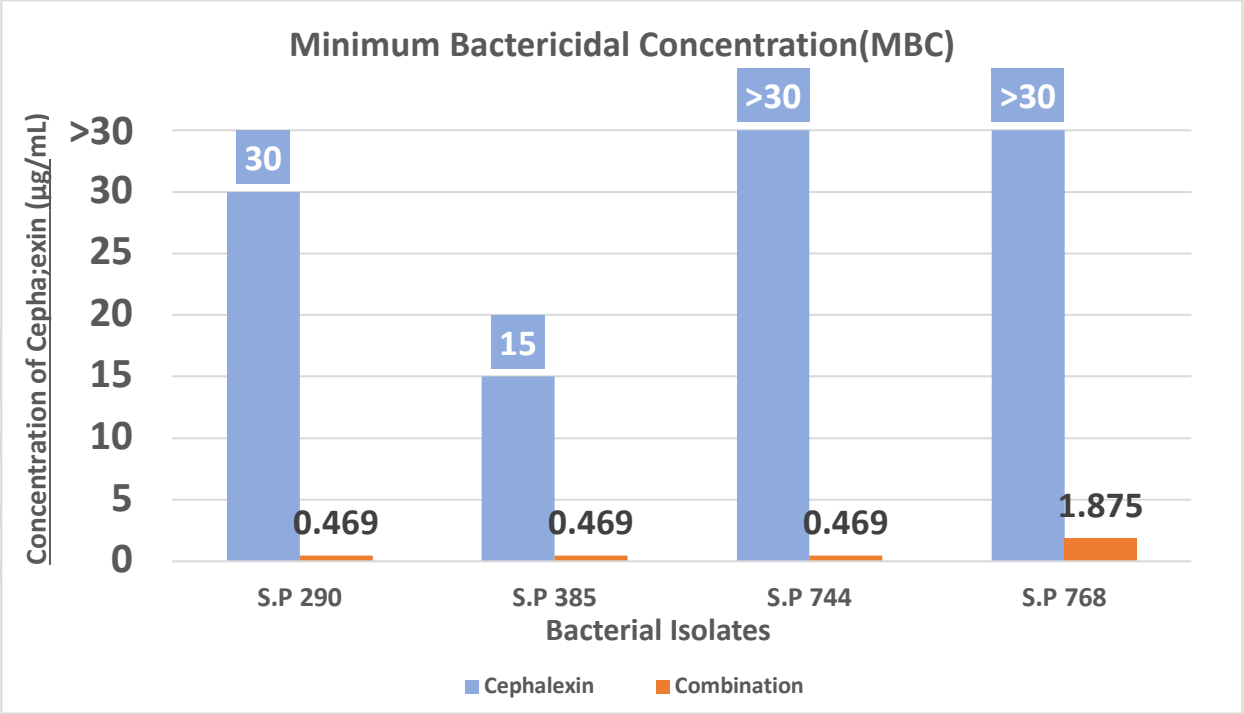


Table 5: MBC/MIC Ratio of four bacterial isolates

Isolates	*MBC (%)*	*MIC (%)*	*MBC/MIC Ratio*
SP290	1.563	0.195	8
SP385	1.563	0.195	8
SP744	0.781	0.391	2
SP768	6.250	1.563	4

5.0 DISCUSSION

The results of antimicrobial susceptibility test confirmed the excellent antibacterial activity by neem leaf extract. The absence of significant difference in antimicrobial activity between 75%, 50% and 25% can be due to the chemical properties of the ethanolic extracts. This is explained by a study by Eloff, 2019 where the researcher elaborated that leaf extracts usually have several antimicrobial components that are non-polar. When they are added to aqueous preparation of Mueller-Hinton agar, poor diffusion of non-polar components in the agar will lead to poor representation of real antimicrobial activity of the extracts.

The insignificant difference in zone of inhibition between cephalexin and cephalexin-neem combination on the other hand shows the synergism between neem leaf extract and cephalexin in an uncommon way. This is because only half of the concentration of cephalexin (15 µg/mL) is used when in combination with neem leaf extract to produce an effect similar to 30 µg/ml of cephalexin. This is supported by a study demonstrating maintained effectiveness of antibiotics of half their dosages when used in combination with neem leaf extract (Binge *et al*, 2023).

Based on the MIC results, both SP744 and SP768 is only intermediately susceptible (2- 4 µg/mL) to cephalexin. Both bacterial isolates are from dog ear wab samples while the other two bacterial isolates from cat urinary tracts infections are susceptible (< 2 µg/mL). This correlates the findings by Scherer *et al*. (2018) where 75% of bacterial isolates in cases of otitis externa in dogs were found to be resistant to cephalexin. Similar pattern is seen in tests of extract against bacterial isolates. The MIC

of extracts are 2 and 8 times higher for SP744 and SP768, respectively compared to isolates from feline urinary infection which is at 0.195%. The antimicrobial effectiveness of neem leaf may be compromised by genetic variations in bacteria. This is attributed to the expression of a specific gene in bacteria, which has the potential to modify or degrade the antimicrobial molecules present in the neem extract (Munita and Arias, 2016).

When the MIC result is analysed, it is evident that neem leaf extract produces a synergistic effect with cephalexin when used in combination. The concentration of cephalexin can be reduced as low as 97% to achieve MIC when used in combination with neem leaf extract compared to cephalexin alone. This again proves the synergism between neem leaf extract and cephalexin. Previously, a study by Aiemsaard *et al*. (2021) demonstrated the synergistic activity of lemongrass essential oil with cephalexin against *S.pseudintermedius*. The MBC of cephalexin, extract and combination is the highest against SP768. Even at full concentration of 30 µg/mL, cephalexin could not kill the bacterial isolates of *S.pseudintermedius* from dog ear swabs. However, in combination, MBC is achieved at much lower concentration of cephalexin.

The effectiveness of antibacterial properties of neem leaf extract is further analysed through MBC/ MIC ratio. According to Mogana *et al*. (2020) and Jantorn *et al*. (2023), plants extracts are bactericidal if MBC/MIC ratio is less than 4 and bacteriostatic otherwise. Hence, neem leaf extract is bactericidal to 2 bacterial isolates (50%) namely SP744 and SP768 where both are from dog ear swabs. This shows that despite cephalexin is ineffective against bacteria from dog ear swabs, neem leaf extract can kill them effectively. Towards other 2 bacterial isolates, neem leaf extract is bacteriostatic.

6.0 CONCLUSION

Azadirachta Indica (neem) leaf has good antibacterial properties against *Staphylococcus pseudintermedius*. It is effective against bacterial isolates from both urinary tract and ear infections. While being bacteriostatic to those from feline urinary tract infections, neem leaf extract is also bactericidal to *S. pseudintermedius* isolates from dog ear infections.

In addition to its own antimicrobial capabilities, it also shows evident synergism with cephalexin. When used in combination, it improves the effectiveness of cephalexin against *S. pseudintermedius*.

Thus, neem leaf extracts can be used a promising alternative treatment option in cats and dogs with *S. pseudintermedius* infections. The result of this study may act as a preliminary information for development of neem leaf extract as a nutraceutical by the pharmaceutical industry.

7.0 RECOMMENDATION

It has been demonstrated in this study that ethanolic neem leaf extract has antibacterial properties against *S.pseudintermedius*. In future studies, these results can be compared with aqueous neem leaf extracts as well as its practicality in real life application and preparation. Other solvents such as acetone can also be used for optimal extraction of phytochemical from the leaves. Bacterial isolates from other sources such as skin can be collected to be tested against neem leaf extract. This will help to broaden the possible usage of neem leaf extract as alternative treatment option.

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9.0 APPENDICES

9.1 APPENDIX 1

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	SP290	Statistic	df	Sig.	Statistic	df	Sig.
SP290_ZOI	SP290_Ce	.228	3	.	.982	3	.744
	SP290_75	.350	3	.	.829	3	.187
	SP290_50	.358	3	.	.814	3	.147
	SP290_25	.379	3	.	.764	3	.031
	SP290_Combi	.305	3	.	.906	3	.406
	SP290_NC	.	3	.	.	3	.

a. Lilliefors Significance Correction

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	SP385	Statistic	df	Sig.	Statistic	df	Sig.
SP385_ZOI	SP385_Ce	.202	3	.	.994	3	.854
	SP385_75	.330	3	.	.866	3	.284
	SP385_50	.189	3	.	.998	3	.906
	SP385_25	.323	3	.	.878	3	.320
	SP385_Combi	.287	3	.	.930	3	.487
	SP385_NC	.	3	.	.	3	.

a. Lilliefors Significance Correction

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	SP744	Statistic	df	Sig.	Statistic	df	Sig.
SP744_ZOI	SP744_Ce	.319	3	.	.885	3	.339
	SP744_75	.279	3	.	.939	3	.525
	SP744_50	.335	3	.	.858	3	.263
	SP744_25	.347	3	.	.836	3	.204
	SP744_Combi	.260	3	.	.958	3	.606
	SP744_NC	.	3	.	.	3	.

a. Lilliefors Significance Correction

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	SP768	Statistic	df	Sig.	Statistic	df	Sig.
SP768_ZOI	SP768_Ce	.295	3	.	.920	3	.453
	SP768_75	.200	3	.	.995	3	.862
	SP768_50	.216	3	.	.989	3	.797
	SP768_25	.344	3	.	.841	3	.217
	SP768_Combi	.321	3	.	.881	3	.328
	SP768_NC	.	3	.	.	3	.

a. Lilliefors Significance Correction

9.2 APPENDIX 2

Games-Howell	SP290_Ce	SP290_75	-.77333	1.05815	.965	-6.3377	4.7911
		SP290_50	-.56667	1.27033	.996	-6.5908	5.4575
		SP290_25	-.07000	1.40415	1.000	-6.8498	6.7098
		SP290_Combi	-.34667	1.19841	.999	-6.0743	5.3810
		SP290_NC	14.44000*	.89718	.014	6.9957	21.8843
	SP290_75	SP290_Ce	.77333	1.05815	.965	-4.7911	6.3377
		SP290_50	.20667	1.05998	1.000	-5.3725	5.7859
		SP290_25	.70333	1.21715	.986	-6.2038	7.6105
		SP290_Combi	.42667	.97262	.996	-4.4716	5.3249
		SP290_NC	15.21333*	.56102	.005	10.5583	19.8684
	SP290_50	SP290_Ce	.56667	1.27033	.996	-5.4575	6.5908
		SP290_75	-.20667	1.05998	1.000	-5.7859	5.3725
		SP290_25	.49667	1.40553	.999	-6.2867	7.2800
		SP290_Combi	.22000	1.20002	1.000	-5.5172	5.9572
		SP290_NC	15.00667*	.89934	.013	7.5445	22.4688
	SP290_25	SP290_Ce	.07000	1.40415	1.000	-6.7098	6.8498
		SP290_75	-.70333	1.21715	.986	-7.6105	6.2038
		SP290_50	-.49667	1.40553	.999	-7.2800	6.2867
		SP290_Combi	-.27667	1.34087	1.000	-6.9453	6.3920
		SP290_NC	14.51000*	1.08014	.020	5.5476	23.4724
	SP290_Combi	SP290_Ce	.34667	1.19841	.999	-5.3810	6.0743
		SP290_75	-.42667	.97262	.996	-5.3249	4.4716
		SP290_50	-.22000	1.20002	1.000	-5.9572	5.5172
		SP290_25	.27667	1.34087	1.000	-6.3920	6.9453
		SP290_NC	14.78667*	.79451	.010	8.1943	21.3790
	SP290_NC	SP290_Ce	-14.44000*	.89718	.014	-21.8843	-6.9957
		SP290_75	-15.21333*	.56102	.005	-19.8684	-10.5583
		SP290_50	-15.00667*	.89934	.013	-22.4688	-7.5445
		SP290_25	-14.51000*	1.08014	.020	-23.4724	-5.5476
		SP290_Combi	-14.78667*	.79451	.010	-21.3790	-8.1943

*. The mean difference is significant at the 0.05 level.

SP 290

Games-Howell	SP385_Ce	SP385_75	-2.21333	1.00559	.392	-6.9840	2.5573
		SP385_50	-1.57000	.74875	.485	-6.8842	3.7442
		SP385_25	-.65667	.73411	.920	-6.2809	4.9676
		SP385_Combi	-2.22667	.74671	.285	-7.5795	3.1262
		SP385_NC	10.23000*	.72090	.018	4.2484	16.2116
	SP385_75	SP385_Ce	2.21333	1.00559	.392	-2.5573	6.9840
		SP385_50	.64333	.72969	.925	-4.4969	5.7835
		SP385_25	1.55667	.71465	.469	-3.8955	7.0088
		SP385_Combi	-.01333	.72759	1.000	-5.1921	5.1655
		SP385_NC	12.44333*	.70108	.011	6.6262	18.2605
	SP385_50	SP385_Ce	1.57000	.74875	.485	-3.7442	6.8842
		SP385_75	-.64333	.72969	.925	-5.7835	4.4969
		SP385_25	.91333	.24524	.123	-.3342	2.1608
		SP385_Combi	-.65667	.28073	.348	-1.9890	.6757
		SP385_NC	11.80000*	.20232	.001	10.1213	13.4787
	SP385_25	SP385_Ce	.65667	.73411	.920	-4.9676	6.2809
		SP385_75	-1.55667	.71465	.469	-7.0088	3.8955
		SP385_50	-.91333	.24524	.123	-2.1608	.3342
		SP385_Combi	-1.57000*	.23893	.021	-2.7700	-.3700
		SP385_NC	10.88667*	.13860	<.001	9.7366	12.0367
	SP385_Combi	SP385_Ce	2.22667	.74671	.285	-3.1262	7.5795
		SP385_75	.01333	.72759	1.000	-5.1655	5.1921
		SP385_50	.65667	.28073	.348	-.6757	1.9890
		SP385_25	1.57000*	.23893	.021	.3700	2.7700
		SP385_NC	12.45667*	.19462	<.001	10.8418	14.0715
	SP385_NC	SP385_Ce	-10.23000*	.72090	.018	-16.2116	-4.2484
		SP385_75	-12.44333*	.70108	.011	-18.2605	-6.6262
		SP385_50	-11.80000*	.20232	.001	-13.4787	-10.1213
		SP385_25	-10.88667*	.13860	<.001	-12.0367	-9.7366
		SP385_Combi	-12.45667*	.19462	<.001	-14.0715	-10.8418

*. The mean difference is significant at the 0.05 level.

SP 385

Games-Howell	SP744_Ce	SP744_75	-.79667	2.09011	.997	-17.9405	16.3472
		SP744_50	-1.41667	1.85670	.952	-16.5996	13.7662
		SP744_25	-.15667	1.30905	1.000	-10.7068	10.3935
		SP744_Combi	.89333	.75479	.823	-4.8593	6.6460
		SP744_NC	13.63000*	.14731	<.001	12.4077	14.8523
	SP744_75	SP744_Ce	.79667	2.09011	.997	-16.3472	17.9405
		SP744_50	-.62000	2.78792	1.000	-13.9405	12.7005
		SP744_25	.64000	2.45739	1.000	-12.2939	13.5739
		SP744_Combi	1.69000	2.21243	.955	-12.9204	16.3004
		SP744_NC	14.42667	2.08491	.071	-.8727	31.7260
	SP744_50	SP744_Ce	1.41667	1.85670	.952	-13.7662	16.5996
		SP744_75	.62000	2.78792	1.000	-12.7005	13.9405
		SP744_25	1.26000	2.26220	.989	-10.1502	12.6702
		SP744_Combi	2.31000	1.99340	.833	-10.2893	14.9093
		SP744_NC	15.04667	1.85085	.052	-.3106	30.4039
	SP744_25	SP744_Ce	.15667	1.30905	1.000	-10.3935	10.7068
		SP744_75	-.64000	2.45739	1.000	-13.5739	12.2939
		SP744_50	-1.26000	2.26220	.989	-12.6702	10.1502
		SP744_Combi	1.05000	1.49664	.970	-7.1231	9.2231
		SP744_NC	13.78667*	1.30073	.031	2.9939	24.5794
	SP744_Combi	SP744_Ce	-.89333	.75479	.823	-6.6460	4.8593
		SP744_75	-1.69000	2.21243	.955	-16.3004	12.9204
		SP744_50	-2.31000	1.99340	.833	-14.9093	10.2893
		SP744_25	-1.05000	1.49664	.970	-9.2231	7.1231
		SP744_NC	12.73667*	.74028	.012	6.5943	18.8791
	SP744_NC	SP744_Ce	-13.63000*	.14731	<.001	-14.8523	-12.4077
		SP744_75	-14.42667	2.08491	.071	-31.7260	2.8727
		SP744_50	-15.04667	1.85085	.052	-30.4039	.3106
		SP744_25	-13.78667*	1.30073	.031	-24.5794	-2.9939
		SP744_Combi	-12.73667*	.74028	.012	-18.8791	-6.5943

*. The mean difference is significant at the 0.05 level.

SP 744

Games-Howell	SP768_Ce	SP768_75	-1.27667*	.23676	.036	-2.4301	-.1233
		SP768_50	-.45000	.64420	.966	-5.2149	4.3149
		SP768_25	.95000	.57936	.644	-3.2292	5.1292
		SP768_Combi	.70333	.36904	.532	-1.5508	2.9574
		SP768_NC	13.55667*	.14746	<.001	12.3331	14.7802
	SP768_75	SP768_Ce	1.27667*	.23676	.036	.1233	2.4301
		SP768_50	.82667	.65388	.791	-3.7498	5.4031
		SP768_25	2.22667	.59010	.176	-1.7648	6.2182
		SP768_Combi	1.98000	.38569	.062	-.1605	4.1205
		SP768_NC	14.83333*	.18523	<.001	13.2964	16.3703
	SP768_50	SP768_Ce	.45000	.64420	.966	-4.3149	5.2149
		SP768_75	-.82667	.65388	.791	-5.4031	3.7498
		SP768_25	1.40000	.84093	.608	-2.6147	5.4147
		SP768_Combi	1.15333	.71252	.638	-2.8258	5.1324
		SP768_NC	14.00667*	.62709	.007	8.8034	19.2099
	SP768_25	SP768_Ce	-.95000	.57936	.644	-5.1292	3.2292
		SP768_75	-2.22667	.59010	.176	-6.2182	1.7648
		SP768_50	-1.40000	.84093	.608	-5.4147	2.6147
		SP768_Combi	-.24667	.65449	.998	-3.7359	3.2426
		SP768_NC	12.60667*	.56028	.007	7.9578	17.2555
	SP768_Combi	SP768_Ce	-.70333	.36904	.532	-2.9574	1.5508
		SP768_75	-1.98000	.38569	.062	-4.1205	.1605
		SP768_50	-1.15333	.71252	.638	-5.1324	2.8258
		SP768_25	.24667	.65449	.998	-3.2426	3.7359
		SP768_NC	12.85333*	.33830	.002	10.0464	15.6603
	SP768_NC	SP768_Ce	-13.55667*	.14746	<.001	-14.7802	-12.3331
		SP768_75	-14.83333*	.18523	<.001	-16.3703	-13.2964
		SP768_50	-14.00667*	.62709	.007	-19.2099	-8.8034
		SP768_25	-12.60667*	.56028	.007	-17.2555	-7.9578
		SP768_Combi	-12.85333*	.33830	.002	-15.6603	-10.0464

*. The mean difference is significant at the 0.05 level.

SP 768

9.3 APPENDIX 3

Test Statistics[a]

	MIC
Mann-Whitney U	.000
Wilcoxon W	10.000
Z	-2.397
Asymp. Sig. (2-tailed)	.017
Exact Sig. [2*(1-tailed Sig.)]	.029[b]

a. Grouping Variable: Compound

b. Not corrected for ties.

Mann-Whitney U Test between Concentration of Cephalexin compared to Concentration of Cephalexin in Combination with extract at MIC

9.4 APPENDIX 4

Test Statistics[a]

	MIC
Mann-Whitney U	.000
Wilcoxon W	10.000
Z	-2.337
Asymp. Sig. (2-tailed)	.019
Exact Sig. [2*(1-tailed Sig.)]	.029[b]

a. Grouping Variable: Compound

b. Not corrected for ties.

Mann-Whitney U Test between Concentration of Cephalexin compared to Concentration of Cephalexin in Combination with extract at MBC