Use of Standard Inhibitory Zone Diameter to Compare Piper betel Leaf Extract's Efficacy Against Serratia Colony Over Kanamycin for Potential Antimicrobial Applications

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ABSTRACT

Aim: The purpose of this study was to assess the efficacy of *Piper betel* leaf extract against *Serratia* Colony versus Kanamycin by using standard inhibitory zone diameter. **Materials and Methods:** A total of 42 samples were computed by ClinCalc, including 21 samples in group 1 (Piper betel leaf extract) and 21 samples in group 2 (Kanamycin antibiotic isolate), using a power of 0.8, a confidence interval of 95 percent, and standard deviation of 1, the alpha-threshold was kept at 0.05. The findings of the mean results of the zone of inhibition and antibacterial susceptibility, as well as the standard deviation, standard error mean, and the significance of the study, were determined using SPSS version 26 software.The *Serratia* inoculum was made by inoculating pure *Serratia* colonies into 10ml of nutrient broth and incubating them for 24hrs. Standard disc diffusion techniques were used to assess antibiotic sensitivity with varied doses of Piper betel leaf extract and kanamycin discs. During MIC, a 96-well plate had been inoculated with 1000µl of nutrient broth, 10 liters of *Serratia*, and various concentrations of Piper betel leaf extract**.Results:**The zone of inhibition was estimated with a vernier caliper from the outer edge of the disc/well to the inhibitory zone's outer perimeter.The zone of inhibition was calculated to a P value of 0.036 alongside the P value of <0.001 for antibacterial susceptibility making it significant.Through the various OD values calculated, antimicrobial sensitivity was estimated. The data were analyzed in order to determine which antibiotic was the most successful. **Conclusion:** In terms of antimicrobial sensitivity, the antibiotic isolate kanamycin was proven to be highly active than the herbal extract *Piper betel* towards the *Serratia* colony.

Keywords: Novel extract, drug resistance, zone of inhibition, susceptibility, antibiotic isolate, antibacterial activity, betel leaf extract.

INTRODUCTION

The rising technology and advancements in the field of pathology ignores the importance of novel herbal extracts in antimicrobial, antifungal and antibacterial activity. The increasing problems of drug resistance in microbes urges the need to develop new compounds for bacterial sensitivity. Antibiotic resistance is a result of the microorganisms being subjected to selective pressure. Illiteracy, a lack of awareness, insufficient prescription procedures, unauthorized and uncontrolled drug sales, limited and poor diagnosis, ignorance of medications and their mechanisms of action, and finally, illegal pharmaceutical usage all contribute to these selection pressures.(Dey et al. 2021). The research states the importance and effectiveness of the antibacterial activity of *Piper betel* leaf extract towards *Serratia* through the measurement of the zone of inhibition. The inhibitory efficacy of an antibiotic may be visualized by measuring the width of the clear zone surrounding an antibiotic disc, known as the zone of inhibition (Barnard 2019).Microorganism resistance to conventional antibiotics requires immediate emphasis on the development of novel therapeutic compounds. Active components derived from plants have been utilized as remedies for a variety of illnesses and microbiological illnesses

since antiquity. A large range of historically utilized herbal remedies are still to be extensively explored against a range of microbiological infections, *Piper betel* being one among them.(Jain et al. 2015) The properties of antioxidation, detoxification and anti mutation suggests the ability of *Piper betel* to work against various pathogenic organisms and ailments which include fibrosis and carcinoma (Sarma et al. 2018).

A distinct method of prevention of antibiotic resistance of pathogens in pathology is the use of novel compounds, without the inculcation of existing synthetic antimicrobial agents (Bajpai, Kumar, and Kumar 2020).The decoction of such leaves is used in the process of wound healing. In addition to which, the leaves are useful in the treatment of cough, ozoena, bronchitis, fouls smell in the mouth, clears throat and lessens thirst (Bajpai, Kumar, and Kumar 2020; Chanda and Nair 2008).The focus on antimicrobial studies majorly concentrates on oral pathogens and aids in its treatment alongside the prevention of infections. The isolation of products obtained from betel leaves may be useful in reducing their toxicity and prevention of pathogenic growth (Nayaka et al. 2021). Literatures were scanned to identify similar articles across various softwares such as google scholar, elsevier, Pub med and Science direct. The highest antimicrobial activity was seen in *Piper betel* leaf extract amongst various other variants of ethanolic extracts against gram positive and negative (Prodhan and Yeasmin 2012). Antibacterial activity was found in ethanol extract of *Piper betel* leaves against a number of pathogens and spoilage microorganisms (Hoque et al. 2012). The methanolic and ethanolic extract's inhibitory zone diameter against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, was found to be higher than 20 mm. The zone of inhibition was less in *E.coli* with a value of approximately 17 mm (Tilak 2017). Inhibition of platelet aggregation occurred by the usage of *Piper betel* leaf and may be applied for the treatment and prevention of atherosclerosis and cardiovascular disease throughout the mechanism of antiplatelet and anti-inflammatory properties.(K and Leela 2020)*Serratia* isolates were far more responsive to sisomicin and gentamicin, while tobramycin and kanamycin were less effective (Waitz et al. 1972).

No studies were found in the existing literature to compare the action of antibiotics against *Serratia* alongside *Piper betel* leaf novel extract. Antibiosensitiviy and MIC identification were expertised prior to the study. The aim of the study is to compare and evaluate the better antibiotic among pure isolates and a herbal extract – kanamycin and *Piper betel* leaf extract respectively

MATERIALS AND METHODS

The study took place in Saveetha School Of Engineering in the microbiology laboratory. The paper consists of 2 groups with *Piper betel* leaf extract and kanamycin antibiotic as group 1 and group 2 respectively. Each group contains 21 samples, thus making 42 samples as the sample size in this research. The sample size was calculated through ClinCalc sample calculator with alpha value of 0.05 and a power of 0.8.The confidence interval was maintained at 95% with a standard deviation of 1 ((Waitz et al. 1972; Ajanaku et al. 2018). The instruments required for this study include the laminar air flow chamber, an autoclave for sterilization of

media, glasswares etc., a shaking incubator by REMI and a refrigerator with temperature to be maintained at - 4˚C for storage.

The first group consists of the drying of 250 grams betel leaves at 100˚C for 20 minutes in an oven followed by crushing and sieving the leaves to obtain a dry, fine powder. 5 grams of the leaf powder was dissolved in 50ml of ethyl acetate and allowed to incubate at room temperature overnight in a shaking incubator. The solution obtained was filtered and was allowed to concentrate in a hot air oven at 40˚C for 48 hours. The obtained product was scraped and resuspended in ethyl acetate. Different concentrations were formed with a minimum value of 0.05 µl/ml and a maximum of 1.00 µl/ml using nutrient broth dilution.Previously our team has a rich experience in working on various research projects across multiple disciplines(Balusamy et al. 2020; Arvind and Jain 2021; Zhao et al. 2020; Hani et al. 2020)

Kanamycin antibiotics, the second group was obtained in the form of discs and stored at -4˚C in sterile conditions. *Serratia* was inoculated in 10 ml nutrient broth using a heat sterilized inoculating loop from pure *Serratia* cultures. Turbidity is observed after incubation for 24 hrs, indicating the growth of the colony after which the inoculum is stored at -4˚C.

Muller hinton agar preparation was done by dissolving 38grams of agar in distilled water and the volume was made upto 1000 ml. The media was sterilized by autoclaving at 121˚C for 15 seconds and poured into 42 petri dishes with approximately 20 ml in each plate. After solidification, a sterile swab was dipped in the *Serratia* incolum and streaked on the media. Wells were cut and different concentrations of betel leaf extract was poured in the range of 0.05µl/ml - 1.00µl/ml. Similarly, kanamycin disks were placed using heat sterilized forceps. The plates were allowed to diffuse for 5 minutes and were incubated at room temperature. The zone of inhibition was observed and recorded the next day. On the other hand, nutrient broth of 1000 µl was prepared and proved with 10 µl of *Serratia* in 96 wells in a 96 well plate. The varying concentrations of betel leaf extract was added for analysis of minimum inhibitory concentration.

The test results were calculated by measuring the zone of inhibition from the edge of the well or disc to the end of the zone. The readings were compared to identify the better sensitive antibiotics. The OD values obtained from the 96-well plates were used to calculate the percentage of susceptibility using equation 1

Percentage of susceptibility = (Control OD - Sample OD)/ control OD X 100…….[1]

Statistical analysis

The statistical software used in the study consists of SPSS version 26 and ClinCalc sample calculator with optical density and zone of inhibition as independent variables, alongside the susceptibility percentage as a dependable variable. The SPSS software was used in the calculation of mean, standard deviation, standard error of mean and the significance of the results. Moreover, the graphs were plotted with a confidence interval of 95% and a standard deviation of 1. The final values were compared for analysis of a better antibiotic.

RESULTS

The zone of inhibition and the percentage of susceptibility was significant by 0.036 and extremely significant by a P value of <0.001 respectively. The samples were found to be analyzed better in the experiment of identifying the minimum inhibitory concentration than in the evaluation of the zone of inhibition. The zone of inhibition and the percentage of susceptibility was much higher in kanamycin than *Piper betel* leaf extract.

Figure 1 represents the inhibited growth of *Serratia* colony under the influence of kanamycin antibiotic and *Piper betel* leaf extract after incubation of 24 hours post streaking of *Serratia* and diffusion of the extract over the MHA media. The higher value of the zone of inhibition of kanamycin in the range of 6.5mm to 7.00mm is compared with the zone of inhibition of *Piper betle* leaf extract with zones of 2mm to 4mm in Fig. 2. This represents the increased sensitivity of kanamycin than that of the herbal extract. Fig. 3 indicated a value of 80% susceptibility for kanamycin making it a better efficient antibiotic with a difference of approximately 50% against betle leaf extract.

Table 1 indicated the raw data of the zone of inhibitions of the two groups, *Piper betel* leaf extract and kanamycin. The inhibitory zone diameter for *Piper betel* ranged from 0 mm to 4mm whereas that of kanamycin was within 5mm to 8mm. The increase in the diameter of the zone of inhibition indicated the better efficiency of the antibiotic isolate in comparison to a novel extract.

Table 2 represents the different values obtained for the antibacterial susceptibility from the OD obtained through the 96 well plate explains the efficiency of sensitivity against *Serratia* amongst the two groups.The values of susceptibility of *Piper betel* leaf extract contained a minimum value of approximately 11 % to 45%, however, kanamycin varied from 74% - 80% approximately.

Group statistics were depicted in Table 3. Wherein the mean, standard deviation and the standard error mean were calculated using the SPSS version 26 software. The mean zone of inhibition was found to be 2.4762 and 6.8095 for betel leaf extract and kanamycin respectively. Similarly, kanamycin's value of susceptibility was higher than betel leaf extracts by approximately 50%. Table 4. Indicates the independent sample tests wherein the zone of inhibition was found to be significant and the susceptibility with a P value of less than 0.001 was considered significant with a much higher margin.

DISCUSSION

The P value of the zone of inhibition was found to be significant (P value - 0.036), whereas the antibacterial susceptibility was found to be highly significant (P value - < 0.001).

Various studies were found similar to the results obtained, wherein an antibiotic isolate contained a higher desired value than a novel extract of herbal origin. The result is in accordance with a study by (Lancaster 1962) wherein, this bacterium seems to be resistant to all antibiotics besides kanamycin and neomycin; yet, despite popular belief, certain isolates are susceptible to various antibiotics. Among various microorganisms, the efficiency of antibacterial activity by betel leaf novel extract compared to that of a broad-spectrum antibiotic such as ceftriaxone indicates the potential of a much more

cost-effective and presumably harmless effective antibacterial (Tilak 2017). The findings suggest greater research into the separation, isolation, and characterization of active ingredients, as well as the toxicity of active ingredients, their adverse effects, and pharmacokinetic features in order to use them in in vivo experiments. (Antimicrobial Property of *Piper betel* Leaf against Clinical Isolates of Bacteria). The study did not find any opposing paper with respect to the results obtained in the existing literature ((Waitz et al. 1972; Ajanaku et al. 2018)).

Piper betel leaves were found to contain Vitamin A, Nicotinic acid, Vitamin C, Riboflavin and Thiamine. The leaf is rich in enzymes such as diastase, catalase, polyphenols and flavonoids (Kumar, Pauly, and G. 2020) This combination of compounds may lessen the effect of prioritized effect, thus limiting the study. The presence of an antibiotic isolate thus, presents an edge over the crude novel extract.

The isolation and characterisation of specified components in *Piper betel* may increase the efficiency, prevention and treatment of infections and diseases in the future.

CONCLUSION

Kanamycin was found to contain higher value of zone of inhibition and percentage of susceptibility ny 4 mm and approximately 50% against *Piper betel* leaf extract respectively, which could be due to the effect of an isolated pure antibiotic against a combination of components in the crude extract.

DECLARATIONS:

Conflict of interests

No conflict of interests in this manuscript

Authors Contributions

Author SA was involved in data collection, data analysis, manuscript writing. Author VAS was involved in conceptualization, data validation, and critical review of manuscript.

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REFERENCES

1. Ajanaku, C., J. Echeme, R. Mordi, T. Owoeye, O. Taiwo, O. Ejilude, and J. Ataboh. 2018. "Phytochemical Screening and Antimicrobial Studies of Stem and Root Extracts of Crateva Adansonii." *Annual Research & Review in Biology*. https://doi.org/10.9734/arrb/2018/39314.
2. Bajpai, Vikas, Nikhil Kumar, and Brijesh Kumar. 2020. *Phytochemistry of Piper Betle Landraces*. CRC Press.
3. Barnard, Ross T. 2019. "The Zone of Inhibition." *Clinical Chemistry* 65 (6): 819.
4. Chanda, Sumitra, and R. Nair. 2008. "Antimicrobial Activity of *Terminalia Catappa*, *Manilkara Zapota* and *Piper Betel* Leaf Extract." *Indian Journal of Pharmaceutical Sciences*.

https://doi.org/10.4103/0250-474x.43012.

5. Dey, Nibedita, C. Kamatchi, A. S. Vickram, K. Anbarasu, S. Thanigaivel, Jeyanthi Palanivelu, Arivalagan Pugazhendhi, and Vinoth Kumar Ponnusamy. 2021. "Role of Nanomaterials in Deactivating Multiple Drug Resistance Efflux Pumps - A Review." *Environmental Research* 204 (Pt A): 111968.
6. Hoque, M. Mahfuzul, M. Mahfuzul Hoque, Shemona Rattila, M. Asaduzzaman Shishir, M. L. Bari, Y. Inatsu, and S. Kawamoto. 2012. "Antibacterial Activity of Ethanol Extract of Betel Leaf (Piper Betle L.) Against Some Food Borne Pathogens." *Bangladesh Journal of Microbiology*. https://doi.org/10.3329/bjm.v28i2.11817.
7. Jain, Isha, Pankaj Jain, Dakshina Bisht, Alosha Sharma, Binita Srivastava, and Nidhi Gupta. 2015. "Comparative Evaluation of Antibacterial Efficacy of Six Indian Plant Extracts against Streptococcus Mutans." *Journal of Clinical and Diagnostic Research: JCDR* 9 (2): ZC50–53.
8. K, Leela, and K. Leela. 2020. "Phytochemical Screening and Antibacterial Activity of Three Medicinal Plants – Lawsonia Inermis, Mangifera Indica and Piper Betel." *Bioscience Biotechnology Research Communications*. https://doi.org/10.21786/bbrc/13.2/70.
9. Kumar, Sushma S., Sona V. Pauly, and Sowmyashree G. 2020. "PHYTOCHEMICAL SCREENING OF INFLORESCENCE OF PIPER BETLE." *International Journal of Current Pharmaceutical Research*. https://doi.org/10.22159/ijcpr.2020v12i6.40299.
10. Lancaster, L. J. 1962. "Role of Serratia Species in Urinary Tract Infections." *Archives of Internal Medicine* 109 (May): 536–39.
11. Nayaka, Ni Made Dwi Mara Widyani, Maria Malida Vernandes Sasadara, Dwi Arymbhi Sanjaya, Putu Era Sandhi Kusuma Yuda, Ni Luh Kade Arman Anita Dewi, Erna Cahyaningsih, and Rika Hartati. 2021. "(L): Recent Review of Antibacterial and Antifungal Properties, Safety Profiles, and Commercial Applications." *Molecules* 26 (8). https://doi.org/10.3390/molecules26082321.
12. Prodhan, Jobaer, and Sabina Yeasmin. 2012. "Antimicrobial Screening of Different Extracts of Mahagoni Seeds (Swietenia Mahagoni) and Betel Leaf (Piper Betle) against Selected Foodborne Pathogen and Spoilage Bacteria." *Journal of Food Science and Engineering*. https://doi.org/10.17265/2159-5828/2012.09.043.
13. Sarma, Chayanika, Prasad Rasane, Sawinder Kaur, Jyoti Singh, Joginder Singh, Yogesh Gat, Umar Garba, Damanpreet Kaur, and Kajal Dhawan. 2018. "Antioxidant and Antimicrobial Potential of Selected Varieties of Piper Betle L. (Betel Leaf)." *Anais Da Academia Brasileira de Ciencias* 90 (4): 3871–78.
14. Tilak, Kalpana. 2017. "STUDY OF ANTIMICROBIAL ACTIVITY OF BETEL LEAVES EXTRACTS AGAINST ESBL AND MBL PRODUCING PATHOGENES." *World Journal of Pharmaceutical Research*. https://doi.org/10.20959/wjpr20178-

9159.
15. Waitz, J. A., E. L. Moss Jr, C. G. Drube, and M. J. Weinstein. 1972. "Comparative Activity of Sisomicin, Gentamicin, Kanamycin, and Tobramycin." *Antimicrobial Agents and Chemotherapy* 2 (6): 431–37.

TABLES AND FIGURES

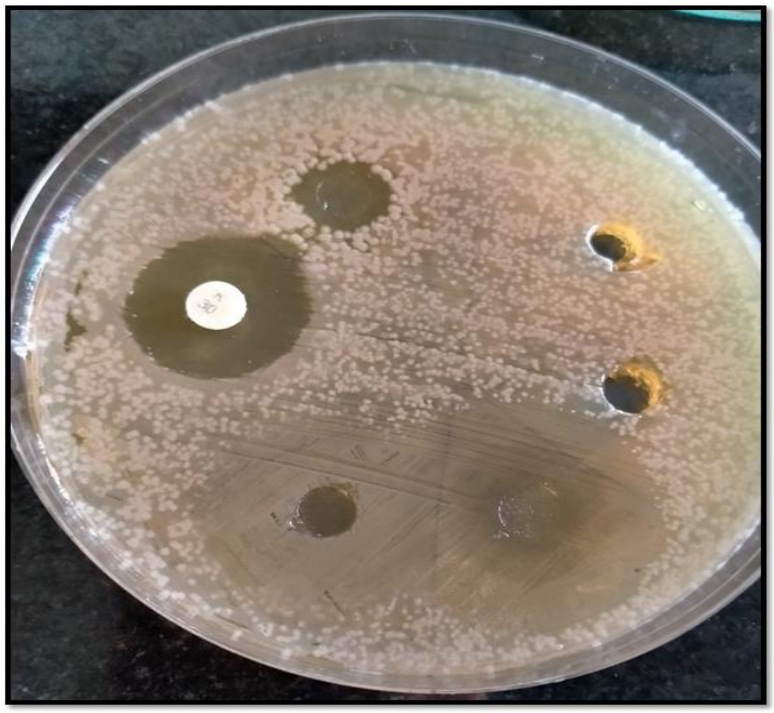

Fig. 1. Inhibitor diameter of the antibiotic kanamycin and the *Piper betel* leaf extract, against *Serratia* colony after incubation for 24 hours post streaking and diffusion.

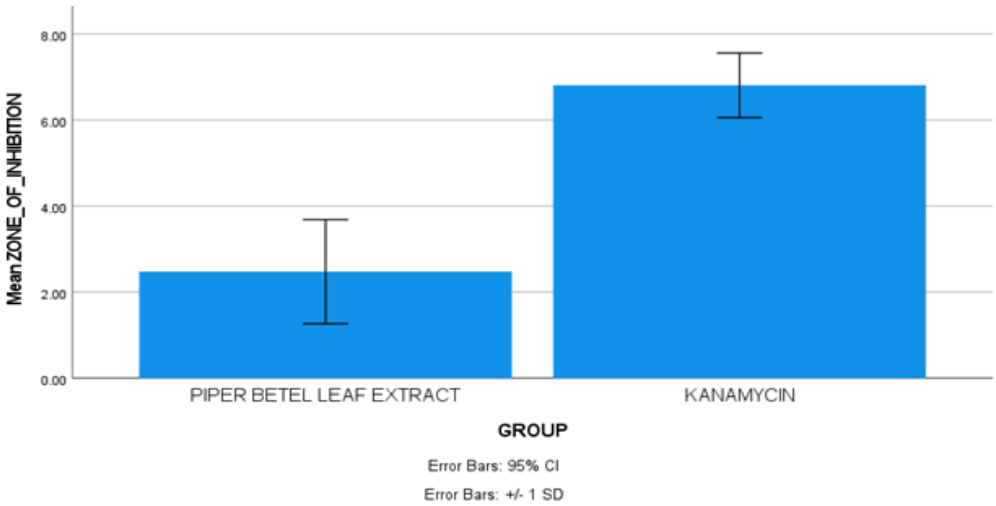

Fig. 2. Bar chart representing the mean zone of inhibition of the two groups, *Piper betel* leaf extract and kanamycin. The values of the inhibitory zone are seen to be of a higher value in kanamycin when compared to the betel leaf extract indicating a mean zone diameter difference of approximately 4 mm against *Serratia* colony. The result was found to be significant with a value of 0.036. The X-axis represents the two groups with the Y-axis indicating the zone of inhibitions in mm. SD+/- 1.

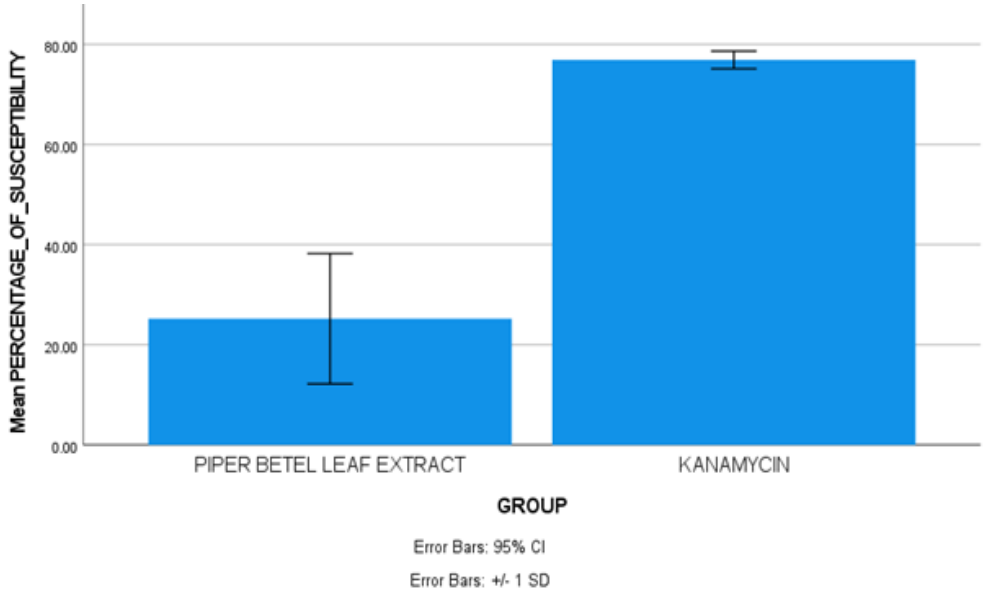

Fig. 3. Bar chart representing the percentage of susceptibility of the two groups, *Piper betel* leaf extract and kanamycin. The values of the antibacterial susceptibility are comparatively higher in kanamycin indicating a susceptibility of approximately 50 % higher than *Piper betel* extract against *Serratia* colony. The outcome was found to be significant with a value less than 0.001. The X-axis represents the two groups with the Y-axis indicating the susceptibility in percentage. SD+/- 1.

Table 1: Indicates the various inhibitory zone diameters of kanamycin and *Piper betel* leaf extract. The values of kanamycin are higher than betel leaf extract by a margin of 4 mm, making it more sensitive towards the *Serratia* colony.

S.No	CONCENTRATION	PIPER BETEL	KANAMYCIN
1	0.05	0	7
2	0.09	0	7
3	0.1	0	7
4	0.15	2	7
5	0.2	2	7
6	0.25	2	7
7	0.3	2	6
8	0.35	2	6
9	0.4	3	7
10	0.45	3	7

11	0.5	3	7
12	0.55	3	7
13	0.6	3	5
14	0.65	3	5
15	0.7	3	7
16	0.75	3	7
17	0.8	3	8
18	0.85	3	8
19	0.9	4	7
20	0.95	4	7
21	1	4	7

Table 2: The antibacterial susceptibility is represented in the table, highlighting a broader range for kanamycin with lesser values obtained in *Piper betel* leaf extract. The higher percentage of susceptibility indicated greater efficiency towards the *Serratia* bacterial colony.

S.No	CONCENTRATION	PIPER BETEL	KANAMYCIN
1	0.05	15.80357143	79.19642857
2	0.09	15.71428571	79.01785714
3	0.1	15.71428571	77.14285714
4	0.15	15.625	77.32142857
5	0.2	10.98214286	77.41071429
6	0.25	11.07142857	75.44642857
7	0.3	12.32142857	75.35714286
8	0.35	12.41071429	77.32142857
9	0.4	11.875	77.5
10	0.45	12.05357143	79.10714286
11	0.5	24.73214286	79.10714286

12	0.55	24.82142857	77.14285714
13	0.6	23.57142857	77.32142857
14	0.65	34.375	78.92857143
15	0.7	34.64285714	79.01785714
16	0.75	40.44642857	74.55357143
17	0.8	41.60714286	74.19642857
18	0.85	44.19642857	75.44642857
19	0.9	44.46428571	75.35714286
20	0.95	41.60714286	74.64285714
21	1	41.51785714	74.55357143

Table 3: Mean value, standard deviation and standard error mean of *Piper betel* leaf extract and kanamycin with respect to their corresponding zone of inhibition and the antibacterial susceptibility against *Serratia*. The mean values are equal amongst the two groups, indicating similar activity against the bacterial colony.

GROUP STATISTICS					
OUTCOMES	GROUP	N	MEAN	STD. DEVIATION	STD. ERROR MEAN
ZONE OF INHIBITION	PIPER BETEL LEAF EXTRACT	21	2.4762	1.20909	0.26385
	KANAMYCIN	21	6.8095	0.7496	0.16358
PERCENTAGE OF SUSCEPTIBILITY	PIPER BETEL LEAF EXTRACT	21	25.2168	13.01336	2.83975
	KANAMYCIN	21	76.909	1.75747	0.38351

Table 4: Depicts the significance of the outcomes of this study. The calculated P value of the zone of inhibition was significant with a p-value of 0.036 and the susceptibility was highly significant with a P value of less than 0.001, respectively.

INDEPENDENT SAMPLES TEST											
OUTCOMES		Levene's Test for Equality of Variances		t-test for Equality of Means							
		F	Sig.	t	df	Significance		Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
						One-Sided p	Two-Sided p			Lower	Upper
ZONE OF INHIBITION	Equal variances assumed	4.705	0.036	-13.959	40	<.001	<.001	-4.3333	0.31044	-4.96075	-3.70591
	Equal variances not assumed			-13.959	33.396	<.001	<.001	-4.3333	0.31044	-4.96464	-3.70203
PERCENTAGE OF SUSCEPTIBILITY	Equal variances assumed	69.697	<.001	-18.039	40	<.001	<.001	-51.692	2.86553	-57.4836	-45.9007
	Equal variances not assumed			-18.039	20.729	<.001	<.001	-51.692	2.86553	-57.6561	-45.7282