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Antibacterial efficacy of *Senna alata* (L.) Roxb. (Fabaceae) against *Staphylococcus aureus*

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Key words: Antibacterial efficacy, *Shenna alata, Staphylococcus aureus,* Ethanol, Aqueous, Pure extract

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Abstract

The medicinal plant, *Sheena alata*, from Cebu elucidates an antibacterial potential. The study determined the antibacterial efficacy of *S. alata* against *Staphylococcus aureus*. It aimed to evaluate the bioactive compounds of *S. alata*, determine the antibacterial activity using ethanol, aqueous, and crude extract, and determine the minimum inhibiting concentration. As a result, *Shenna alata* has the presence of alkaloids, anthraquinone, cardiac glycosides, flavonoids, saponins, steroids, and tannins. The study used a Completely Randomized Design with subsampling to evaluate the specific extract concentration with the most efficient antibacterial activity using 70%, 65%,60%, 55%, and 50% concentrations. *S. alata* leaves have antibacterial properties against *Staphylococcus aureus*, as shown in the mean zone of inhibition with 9.02mm (aqueous), 15.18mm (ethanol), and 9.02mm (pure extracts). Therefore, it is implied that *S. alata* leaves have antibacterial properties with minimum inhibitory concentration at 55% of the ethanolic extract, which can be used in the pharmaceutical industry.

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Introduction

Medicinal plants play an essential part in humanity's various microbial diseases (Falodun *et al.*, 2006). People are so concerned with different illnesses caused by either bacteria or fungi where many of these pathogens (e.g., bacteria) have developed resistance to several antibiotics (Wei *et al.*, 2008). The increasing reports on antibiotic resistance have continued to challenge our pharmacological industries in search of better antibiotics. Thus, grassroots ethnomedicinal investigations of local plants' antimicrobial potential are being done and evaluated to accomplish this goal.

Senna alata is very useful to Filipinos and has proven to be of great use in medicine (Rai, 1987). They treat several diseases using different parts and preparations (Reezal *et al.*, 2002, Alalor, C. *et al.*, 2012). Previous studies have shown bioactive compounds in *S. alata*, which are essential in pharmacological areas . However, it is observed that there are differences in the presence of bioactive compounds in *S. alata,* as shown in some published papers collected in random localities globally. Future studies with higher extract concentrations are helpful to evaluate the actual antibacterial properties *of S. alata* leaves extracts (Somchit *et al.*, 2002). Thus, Shenna *alata* is of great use in the pharmaceutical industry, but extensive bioassay studies are needed (Fernand *et al.*, 2008).

In the Philippines, at least two conflicting results of previous studies presented the antibacterial effect of Shenna *alata*. In the study of Valle *et al.* (2015), twelve medicinal plants were investigated for their antibacterial activities against *Staphylococcus aureus, Enterococcus, Pseudomonas aeruginosa, and Acinobacter baumannii* and one of the plants was the Shenna *alata* which was taken from Angono, Rizal. The results showed that among the twelve plants, the leaf extracts of *Piper betel, Psidium guajava, Phyllanthus niruri, and Ehretia microphylla* showed a significant zone of inhibition. In contrast, Shenna *alata* and the rest of the leaf extracts showed low zones of inhibition. Meanwhile, the study of Paderes *et al.* (2016) on the antibacterial activity of *Shenna alata* in the Province of Abra showed a good significant result against *S. aureus* and *Escherichia coli*. These contrasting results may suggest that bioactive compounds with potential antibacterial activity may not be consistently present among *Shenna alata* cultivars nationwide.

Although this research did not delve into the ecotypes or influence of environmental factors on the variability of bioactive compounds in *S. alata* against bacteria, *S. alata* from Cebu may elucidate an antibacterial potential. Hence the study determined the antibacterial efficacy of *S. alata* against *Staphylococcus aureus*. Specifically, it aimed to (1) qualitatively evaluate the bioactive compounds of *S. alata* in Cebu, (2) determine the antibacterial activity of *S. alata* extract against *S. aureus* in terms of the type of solvent used such as (a) ethanol, (b) aqueous, (c) crude extract, and (3) to determine the minimum inhibiting concentration of plant extract against *S. aureus*. With the hypothesis, there is no significant difference in the antibacterial activity of *S. alata* using the different solvents.

Materials and methods

Research Design and Data Gathering Procedures

Preparation of Plant Sample

Young leaves of *S. alata* were initially washed with tap water and rinsed with distilled water. Next, the plant samples were air-dried for 7-14 days at room temperature. Dried leaves were cut into pieces, midrib removed, and powdered using an electric osterizer and were kept in a container at room temperature until needed to be processed (El-Mahmood *et al.*, 2008).

*Shenna alata l*eaves were submitted for identification at the University of San Carlos. Every phytochemical analysis has different methods "The extracts of the *S. alata* leaves were evaluated for the qualitative determination of phytochemical constituents such as alkaloids, flavonoids, tannins, saponins, steroids, anthraquinone, and cyanogenic glycosides as described and adapted from Veerachari and Bopaiah (2012)."

Extraction of Plants

The dried powdered leaves were extracted in different solvents: ethanol and aqueous. The crude extract (without solvent) was also used. Fifty grams (50g.) of dried powdered leaves were soaked in five hundred milliliters (500ml.) of ethanol and was left to stand for 72 hours (Ogunjobia and Abiala 2013) and was filtered with Whatman No.1 filter paper, after which the extract was concentrated through the rotary evaporator. For aqueous extract, fifty grams (50g.) of dried powdered leaves were boiled in five hundred milliliters (500ml.) of triple distilled water for two (2) hours (Chavan and Ghadage 2018) then it was filtered with Whatman No.1 filter paper. For pure extract (without solvent) the leaves were pounded to ease extraction. The pounded leaves were placed in cheesecloth and were squeezed to obtain the extracts.

Dilution Process

The extract produced after rotary evaporation was used for the dilution process with triple distilled water. There were five concentrations being used: 90%, 70%, 50%, 30% and 10% respectively. To make the different concentrations certain mixtures were prepared and the procedure was adapted from Petrucci *et al.* (2002).

Ninety percent mixture (9ml. of stock solution to 1ml. triple distilled water), 70% mixture (7ml. of stock solution to 3ml. triple distilled water), 50% mixture (5ml. of stock solution to 5ml. triple distilled water), 30% mixture (3ml. of stock solution to 7ml. triple distilled water) and 10% mixture (1ml. of stock solution to 9ml. triple distilled water). With the use of a micropipette, 0.5mL. per concentration was dispensed to every disc before it was impregnated into the petri dish with inoculated bacteria, and a zone of inhibition was observed.

Test Microorganism

Bacterial culture of *Staphylococcus aureus* served as the test microorganism receiving the treatments was used in this study. *S. aureus* was identified at the Department of Agriculture. Bacterial culture was sub-cultured in Mueller-Hinton broth medium, and the inoculum was used in the antibacterial assay.

Antibacterial Property Testing

The entire protocol for antibacterial screening was adopted from Habermeier (1978) and Barry *et al.* (1979).

Experimental Design

This experiment employed a Completely Randomized Design (CRD) with subsampling. Two different set-ups were prepared; one was for testing antibacterial activity using different solvents/treatments. Another set-up was laid to evaluate the specific concentration of extract that has the most efficient antibacterial activity. Pre-testing and optimization trials and final testing of the optimized protocol were also conducted.

Determination of Minimum Inhibitory Concentrations (MIC)

A plot of the square of the radius diameter of the zones of inhibition against the log concentration of the dilutions was done, and a suitable curve was drawn from the plots for each extract. Extrapolation of the curve was done to determine the log of MIC. From this log, the MIC was calculated as the antilog (Otto *et al.*, 2014).

Measurement of Zone of Inhibition (ZOI)

The zone of inhibition was measured after 16 to 18 hours or overnight incubation. The test plates were placed in an incubator while keeping the lid plate in place. The vernier caliper was used in measuring the zones of inhibition against the back of the petri dish. Measuring the zones of inhibition were done up to the nearest millimeter. If there were no inhibition, 6mm would be recorded. Colonies within the zone of inhibition would mean contamination (Barry *et al.* 1979).

Research Environment

The testing of the antibacterial activity of *S. aureus* was conducted at the Department of Agriculture (DA), Cebu City, and at the Cebu Normal University Biology Laboratory.

Data Analysis

The data gathered (zone of inhibition,mm $\pm$ S.D.) for each treatment were evaluated in MS Excel 2010. Mean scores of extracts were compared using a two-way Analysis of Variance (ANOVA) with sub-sampling.

Significant ANOVA results were further subjected to post-hoc analysis/Pairwise Comparison (Tukey's HSD test).

This post-hoc analysis was evaluated which among the treatments are significantly different from each other (zone of inhibition), statistically.

Results and discussion

Phytochemicals are naturally present in plants and show biological significance by showing antibacterial activity by inhibition mechanism (Tariq *et al.*, 2013).

In addition, phytochemical analysis conducted on *Senna alata* revealed constituents known to exhibit therapeutic and physiological activities.

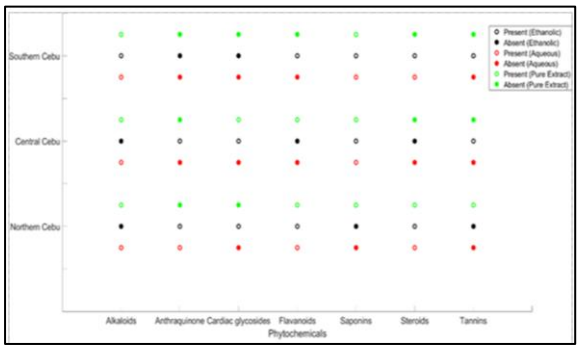


Fig. 1. Qualitative Phytochemical Parameters Present in the Ethanolic, Aqueous, and Pure Extract of *Shenna alata* leaves from Northern, Central, and Southern Cebu.

Phytochemical screening of *Shenna alata* leaves in Cebu, as shown in Fig. 1, revealed that for ethanolic extracts, alkaloids are found in Southern Cebu, anthraquinone, cardiac glycosides, and steroids are found in Northern and Southern Cebu, whereas, flavonoids are in Northern Cebu and saponins and tannins in Central and Southern Cebu.

For aqueous extracts, alkaloids are found in all extracts while cardiac glycosides and tannins are absent. In northern Cebu, it also contains flavonoids and steroids, while in Central Cebu, it contained alkaloids and saponins, and in Southern Cebu, it revealed alkaloids, saponins, and steroids.

On the other hand, for pure extract alkaloids, flavonoids, saponins, steroids, and tannins are present in Northern Cebu, whereas alkaloids, cardiac glycosides, flavonoids, and saponins in Central Cebu.

However, only alkaloids and saponins are present in Southern Cebu, and anthraquinone is absent in all extracts.

Phytochemicals of the plants serve as massive storage of compounds that have biological action (Gutierrez *et al.*, 2014). It is observed that Northern Cebu *S. alata* possesses several phytochemical properties than Central Cebu and Southern Cebu, respectively.

The results also revealed that of the different bioactive compounds present in plant alkaloids, flavonoids and saponins are primarily present in *S. alata,* which corresponds and agrees to the study of Gutierrez *et al.* (2014) and Ehiowemwenguan *et al.* (2014). They are known to have medicinal and antimicrobial properties.

Antibacterial Activity of Senna alata

Set – Up A

A two-way Analysis of Variance (ANOVA) was used in testing the null hypothesis of equal variance across all groups. Table 1 rejects the null hypothesis that variances are homogeneous across groups or that the variances are not equal across groups.

Test of between-subject effects – as seen in Table 1, there is a statistically significant difference between the dependent variable zone of inhibition and the concentration, sampling site, and interaction between concentration and sampling site, all with 0.000 significance level.

From the previous table, there is a statistically significant difference between the ZOI and concentration. But we don't know where those differences are. Therefore, performing the pairwise comparison was needed since we have six levels for comparison for concentration.

Table 1. Tests of Between-Subjects Effects with Zone of Inhibition as the dependent variable.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	232252.426[a]	35	6635.784	932.846	.000	.954
Intercept	196251.068	1	196251.068	27588.608	.000	.946
Conc	173082.159	5	34616.432	4866.313	.000	.939
Sampling Site	9801.544	5	1960.309	275.577	.000	.465
Conc * Sampling site	49368.724	25	1974.749	277.607	.000	.814
Error	11267.756	1584	7.113			
Total	439771.250	1620				
Corrected Total	243520.182	1619				

a. R Squared = .954 (Adjusted R Squared = .953)

Table 2. Ryan-Einot-Gabriel-Welsch Range[a,b] Range results between the independent variables with ZOI as the dependent variable.

Concentration	N	Subset		
		1	2	3
Aqueous	270	6.0000		
Pure extract	270	6.0000		
Control Aqueous	270	6.0000		
Control Ethanol	270	6.0000		
Ethanol	270		7.9759	
Control Antibiotic	270			34.0630
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed. Based on observed means. The error term is Mean Square (Error) = 7.113.

a. Critical values are not monotonic for these data. Substitutions have been made to ensure monotonicity. Type I error is therefore smaller.

b. Alpha = 0.95

In table 2, we have identified and grouped homogenous subsets of means that are not different from each other. For example, aqueous, Pure extract, and Control Aqueous, and control ethanol are contained in one subset since their means don't differ significantly from each other, having an average mean of 6.0. Thus, there is no statistically significant difference in their means (ZOI) between these samples.

However, we do have a statistically significant difference across groups or subsets. Ethanol and the subgroup, aqueous, pure extract, and control aqueous, have a statistically significant difference between their mean ZOI.

Also control antibiotic and the subgroup, aqueous, pure extract, and control aqueous, have a statistically significant difference between their mean ZOI. Moreover, Ethanol and control antibiotic are grouped in different subsets since their mean ZOI differs significantly from each other, with an average mean of 7.97 and 34.06, respectively.

Sampling Site

Again, from the previous table, we already concluded that there is a statistically significant difference between ZOI, the dependent variable, and sampling site, the independent variable. Therefore, a further test, Bonferroni for this case, would reveal which among the independent variable levels causes the significant difference on ZOI.

Table 3. Ryan-Einot-Gabriel-Welsch Range[a,b] results between the independent variable sampling site.

Sampling Site	N	Subset			
		1	2	3	4
Sirao	270	7.6130			
Babag	270	7.6259			
Danao	270		11.5667		
Carmen	270			12.7667	
Dalaguete	270			13.1148	13.1148
Argao	270				13.3519
Sig.		1.000	1.000	.340	.660

Means for groups in homogeneous subsets are displayed. Based on observed means. The error term is Mean Square (Error) = 7.113.

a. Critical values are not monotonic for these data. Substitutions have been made to ensure monotonicity. Type I error is therefore smaller.

b. Alpha = 0.95

Table 3 shows no statistically significant difference in the dependent variable ZOI between Sirao and Babag, Dalaguete and Carmen, Dalaguete and Argao, Carmen and Argao. However, we do have a statistically significant difference on the ZOI between Sirao and

Danao, Sirao and Carmen, Sirao and Argao, Sirao and Dalaguete, Babag and Danao, Babag and Carmen, Babag and Argao, Babag and Dalaguete, Danao and Carmen, Danao and Argao and Danao and Dalaguete.

Set – Up B

A two-way Analysis of Variance (ANOVA) in testing the null hypothesis of equal variance across all groups was performed.

Table 4. Tests of Between-Subjects Effects.

Dependent Variable: Zone of Inhibition						
Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	246977.218[a]	107	2308.198	908.286	.000	.953
Intercept	295877.432	1	295877.432	116429.109	.000	.961
Conc	160388.567	17	9434.622	3712.566	.000	.930
Sampling Site	9889.613	5	1977.923	778.321	.000	.450
Conc * Sampling Site	76699.038	85	902.342	355.076	.000	.864
Error	12076.100	4752	2.541			
Total	554930.750	4860				
Corrected Total	259053.318	4859				

a. R Squared = .953 (Adjusted R Squared = .952)

Test of between-subject effects – as seen in table 4, there is a statistically significant difference between the dependent variable zone of inhibition and the concentration, sampling site, and interaction between concentration and sampling site, all with 0.000 significance level.

We know from the previous table that there is a statistically significant difference between the ZOI and concentration. But we don't see where those differences are. Performing the pairwise comparison will be needed since we have 17 levels for comparison for concentration.

Table 5. Ryan-Einot-Gabriel-Welsch[a,b] Range results between the independent variable (concentration).

Concentration	N	Subset				
		1	2	3	4	5
Aqueous 70%	270	6.0074				
Pure Extract 70%	270	6.0074				
Pure Extract 65%	270	6.0074				
Pure Extract 60%	270	6.0074				
Pure Extract 55%	270	6.0074				
Pure Extract 50%	270	6.0074				
Control Aqueous	270	6.0074				
Control Ethanol	270	6.0074				
Aqueous 65%	270	6.0074				
Aqueous 60%	270	6.0074				
Aqueous 55%	270	6.0074				
Aqueous 50%	270	6.0074				
Ethanol 55%	270		7.0259			
Ethanol 50%	270		7.1759	7.1759		
Ethanol 65%	270			7.5296	7.5296	
Ethanol 70%	270			7.5463	7.5463	
Ethanol 60%	270				7.7352	
Control Antibiotic	270					31.3444
Sig.		1.000	.944	.109	.874	1.000

Means for groups in homogeneous subsets are displayed. Based on observed means.The error term is Mean Square (Error) = 2.541.

a. Critical values are not monotonic for these data. Substitutions have been made to ensure monotonicity. Type I error is therefore smaller.

b. Alpha = 0.95

In table 5, we have identified and grouped homogenous subsets of means that are not different from each other. For example, all levels of Aqueous (50%, 55%, 60%, 65%, and 70%), All levels of Pure extract (50%, 55%, 60%, 65%, and 70%), control ethanol, and control Aqueous are contained in one subset since their means don't differ significantly with each other. Also, Ethanol (55%, 50%, and 70%), and Ethanol (65%, 70%, and 60%) is contained in one subset, respectively. Thus, there is no statistically significant difference in their means (ZOI) between these samples. However, we do have a statistically significant difference across groups or subsets. That is, all levels of Ethanol (50%, 55%, 60%, 65%, and 70%) have statistically significant differences on the following: All levels of Aqueous (50%, 55%, 60%, 65%, and 70%), all levels of Pure extract (50%, 55%, 60%, 65%, and 70%), control aqueous, control ethanol and ethanol 55% have a statistically significant difference in their means ZOI on Ethanol 60%, Ethanol 65%, and Ethanol 70%. Lastly, the mean ZOI for Ethanol 50% is significantly different from that of Ethanol 60%. While control antibiotic is grouped in different subsets, its mean ZOI are known to differ significantly. However, the result did not conform to the study of Ocampo, A. *et al.* (2018) because in the study, at least 50% of the aqueous solution exhibited an inhibitory effect, while, in the result of this paper, the aqueous solution did not show an inhibitory effect, instead it showed in ethanolic solution.

Sampling Site

From the table below, we can conclude that there is a statistically significant difference between ZOI, the dependent variable, and sampling site, the independent variable. A further test, Bonferroni for this case, would reveal which among the independent variable levels causes the significant difference on ZOI. The table shows that there is no statistically significant difference on the dependent variable ZOI among Argao (Southern Cebu), Babag (Central Cebu), and Sirao (Central Cebu) only.

Table 6. Mean and Standard deviation results for the Zone of Inhibition (ZOI) against the geographical location (sampling site).

Location	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
Sirao	6.475	.056	6.365	6.585
Babag	6.454	.056	6.345	6.564
Danao	8.833	.056	8.723	8.943
Carmen	10.122	.056	10.012	10.232
Argao	8.456	.056	8.346	8.565
Dalaguete	6.475	.056	6.365	6.585

Table 7. Ryan-Einot-Gabriel-Welsch Range[a,b] results between the independent variable sampling site.

Location	N	Subset			
		1	2	3	4
Babag	810	6.4543			
Sirao	810	6.4753			
Dalaguete	810	6.4753			
Argao	810		8.4556		
Danao	810			8.8327	
Carmen	810				10.1222
Sig.		.999	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed. Based on observed means. The error term is Mean Square(Error) = 2.541.

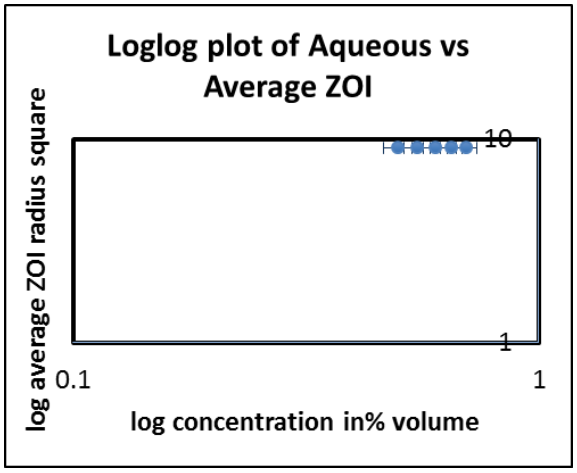
a. Critical values are not monotonic for these data. Substitutions have been made to ensure monotonicity. Type I error is therefore smaller.

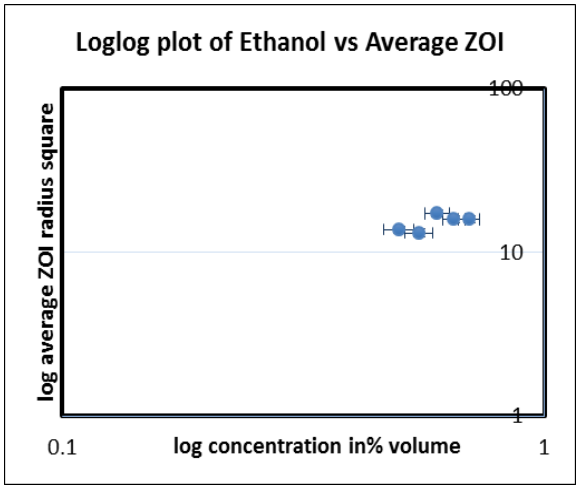
b. Alpha = 0.95

In table 7, we have identified and grouped homogenous subsets of means that are not different from each other. For example, table 7 shows that Babag, Sirao, and Dalaguete are all contained in one subset since their means don't differ significantly from each other. Thus, there is no statistically significant difference in their means (ZOI) among the samples taken from these various sampling sites.

However, there is a statistically significant difference in the mean ZOI between Argao and the following sites, namely Babag, Sirao, and Dalaguete. There is also a significant difference in the mean ZOI between Danao and the sites mentioned above, namely Babag, Sirao, and Dalaguete. The same three sampling sites mentioned in the previous findings where its mean ZOI is significantly different from that of Carmen. Also, between Argao, Danao, and Carmen, their mean ZOI is remarkably different from each other, with a value of p = 0.000.

Minimum Inhibition Concentration Determination





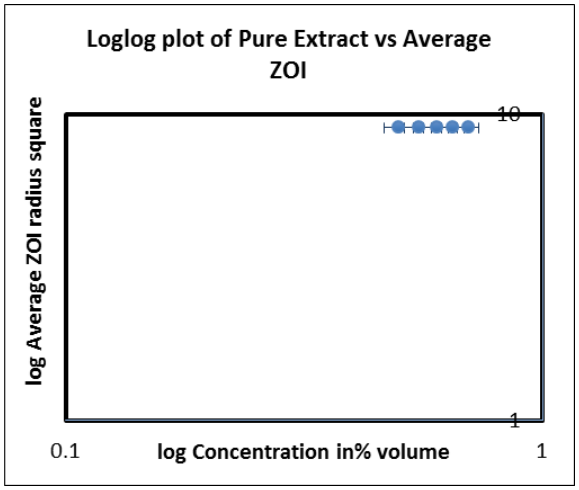


Fig. 2. Graphical Representations of Loglog Plot of Extracts vs Average Zone of Inhibition for (a) Aqueous, (b) Ethanol, and (c) Pure Extracts.

Fig.s 2a-2c Graphical Representations of Loglog Plot of Extracts vs. Average Zone of Inhibition for a) Aqueous, (b) Ethanol, and (c) Pure Extracts showed the minimum inhibitory concentration using a plot of the square of the radius diameter of the zones of inhibition against log concentration of the dilutions. These Fig.s reflect that all concentrations, both the aqueous and pure extracts, showed the same average results of radius squared (9.024074074mm^2), which suggests that there is no antibacterial activity. However, there is an observed antibacterial activity for ethanol solution with an average ZOI radius square of 13.74930556, 13.10185185, 17.26180556, 15.88240741 15.94699074 for concentrations 50%, 55%, 60%, 65%, and 70%, respectively.

Thus, it can be inferred from the results that the minimum inhibitory concentration is at 55% of the ethanolic extract. However, the result did not conform to the study of Ocampo, A. *et al.* (2018) because in the study, at least 50% of the aqueous solution exhibited an inhibitory effect, while, in the result of this paper, the aqueous solution did not show an inhibitory effect, instead it showed in ethanolic solution. The percentage is being used because the method used volume/volume while other studies usedmg/ug ormg/ml, but still, it possesses antibacterial activity, and the result of minimum inhibitory concentration is at 55% of the ethanolic extract.

Conclusions

Shenna alata leaves have antibacterial properties against *Staphylococcus aureus*. It has the presence of alkaloids, anthraquinone, cardiac glycosides, flavonoids, saponins, steroids, and tannins based from the result in the phytochemical screening and are known to have medicinal and antimicrobial properties. *S. alata* leaves have antibacterial properties against *Staphylococcus aureus*, as shown in the mean zone of inhibition with 9.02mm (aqueous), 15.18mm (ethanol), and 9.02mm (pure extracts). Therefore, it is implied that *S. alata* leaves have antibacterial properties with minimum inhibitory concentration at 55% of the ethanolic extract, which can be used in the pharmaceutical industry.

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