Antibacterial Property of the Lipid Extract from Fresh African Nightcrawler (*Eudrilus Euginae*) Family Eudrilidae against *Staphylococcus Aureus* and *Escherichia Coli*

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Abstract: Antibiotics are drugs that are used to kill or slow down the growth of bacteria. The use of antibiotics is in demand because of the continuous occurrence of infectious diseases worldwide. Although there are many antibiotics available in the market, the rise of new infectious diseases as well as the emergence of drug resistance warrant the discovery of other antibiotic sources. The antibacterial activities of lipids have been known for over a century. Several publications reported research on the microbicidal effects of lipids. Earthworms have also been used in medicine for various remedies. They have been shown to have humoral and cellular immunity mechanisms which enable them to face pathogenic microorganisms in the environment. This research aims to determine if the lipid extract of *Eudrilus euginae* possesses antimicrobial property. Lipids were extracted from the earthworms by Folch method, yielding 0.343%. Cupric acetate test was then performed to confirm the presence of fatty acids. A two-fold serial dilution method was employed to prepare different concentrations of the extract which were then subjected to macrodilution method to determine the Minimum Inhibitory Concentration (MIC) against *Staphylococcus aureus* and *Escherichia coli*. Basing from the MIC against each test organism, three increasing concentrations of the extract (for *S. aureus* = 15.625, 31.25 and 62.5 mg/ml; for *E. coli* – 31.25, 62.5 and 125 mg/ml) were prepared and used to inhibit the growth of each test organism using disk diffusion method. The zones of inhibition were measured using a caliper. The mean zones of inhibition from the different concentrations were compared with each other and against the positive control, ciprofloxacin 5µg, using one-way ANOVA and Scheffe test. The common extract concentrations (31.25 and 62.5 mg/ml) were also compared using T-test to determine in which bacterium the extract is more effective. Based on the results, the extract showed inhibitory effect at a lower concentration against *S. aureus* (MIC = 15.625 mg/ml) compared to *E. coli* (MIC = 31.25 mg/ml). Against *S. aureus*, 62.5 mg/ml showed the greatest inhibitory effect. This concentration is also more effective compared to ciprofloxacin, while the 15.625 and 31.25 mg/ml were comparable to the positive control. Against *E. coli*, ciprofloxacin still has a greater inhibitory effect than the extract.

Keywords: lipid extraction; antibacterial activity of earthworm; coelomic fluid in earthworms; disk diffusion method

1. INTRODUCTION

1.1 Background

A disease is a disorder or incorrectly functioning organ, part, structure or system of the body resulting from the effect of genetic or developmental errors, infection, poison, nutritional deficiency or imbalance, toxicity or unfavorable factors, illness, sickness and ailment (Collins, 2014). Of the causes stated, infections are widely addressed through the administration of antibiotics. Through the years, the need for antibiotic drugs has become in demand because of the continuous spread of infection, not to mention the emergence of new infectious diseases and drug resistance worldwide.

Many antibiotic drugs have been discovered from plant sources. This study, however, is significant and unique because it explores an unlikely animal as a potential drug source. Other than being a fertilizing organism, this study offers another benefit from earthworms, and that is, being a cure. The species of earthworm under study is abundantly found in soil in different parts of the globe. They can easily reproduce and mature for a shorter period compared to plants. They can also be cultured. Thus, collection and use of the animal pose a very little risk, if any, to the ecosystem. Furthermore, multidrug antibiotic resistance is an increasing public health problem worldwide. Thus, exploring other antibiotic sources is very much warranted.

The earthworm was first documented in the Divine Farmer's Materia Medica Classic around 200 B.C. to 200 A.D. The 365 drugs listed in this book were classified into three groups: up, middle and low; low being the most toxic. The earthworm was classified under the "low" group. In later medical literature, however, the earthworm has been considered to be nontoxic and has been used in medicine for various remedies (Yu Shen, 2010).

Earthworms have been living with the aid of their defense systems since the early phase of evolution and have always faced the invasion of pathogenic microorganisms in their environments. The studies which have been continued for fifty years showed that earthworms have humoral and cellular immunity mechanisms (Arslan-Aydogyu, 2008). It has been found that the coelomic fluid of earthworms contains more than forty proteins and exhibits several biological activities: cytolytic, proteolytic, antimicrobial, hemolytic, hemagglutinating, tumorolytic and mitogenic activities. These investigations with earthworms have usually intensified with *Eisenia foetida, Lumbricus terrestris,* and *Dendrobaena venata*.

The antibacterial activities of lipids have been known for over a century. More than three decades ago, it was suggested that the clinical use of antimicrobial lipids could be advantageous. For the past four decades, there has been a growing interest in the effects of lipids on microbes, and numerous publications have reported research on the microbicidal effects of lipids (Halldor, 2011).

Since the effectiveness of the protein extract in other earthworms has been proven, the researchers want to determine if the lipid extract of the earthworm African Nightcrawler *(Eudrilus euginae*) also possess antibacterial properties that could inhibit the growth of common pathogens such as *Staphylococcus aureus* and *Escherichia coli* by measuring the zone of inhibition produced by the different concentrations of the lipid extract. This study also aims to determine the following: a) physical properties of the lipid extract; b) percentage yield; c) the Minimum Inhibitory Concentration (MIC) against *S. aureus* and *E. coli*; d) in which species of bacteria will the lipid extract produce greater antibacterial activity; and e) if there is a significant difference in the antibacterial activity between the different concentrations of the extract and ciprofloxacin 5µg.

This study involved the determination of the antibacterial property of the earthworm African Nightcrawler (*Eudriluseugeniae*) lipid extract with the aim of comparing its antibacterial property with the standard drug ciprofloxacin. The study also made use of several processes and procedures such as earthworm authentication, preparation of the earthworm lipid extract using Folch method, preparation of culture media, stock culture, 0.5 McFarland as the standard for turbidity, 0.05% Tween-80 as the solvent, and of different lipid extract concentrations. The minimum inhibitory concentration was determined using broth macrodilution method and the filter paper disk diffusion method was employed for observing the zones of inhibition created.

The researchers did not isolate, purify and determine the specific fatty acids in the lipids. Different methods of lipid extraction were not used other than the Folch method. The researchers made use of only three (3) concentrations of the extract against *Staphylococcus aureus* and *Escherichia coli*, all of which were based on the MIC results. The study also focused on the antibacterial activity of the lipid extract and not on other pharmacological activities. Lastly, the researchers did not formulate a dosage form.

2. METHODOLOGY

2.1 Earthworm Authentication and Collection

African Nightcrawler (*Eudrilus eugeniae*) earthworm was authenticated in the Bureau of Plant Industry (BPI) at 692 San Andres Street Malate, Manila.

Fully matured African Nightcrawlers weighing 900 grams were collected. The earthworms were washed with distilled water two times and immersed in boiling water for 10 minutes to inactivate lipolytic enzymes (Hansen RP, Czochanska Z, 1947). After immersion, the earthworms were placed on a filter paper to remove moisture.

2.2 Preparation of the Extract using Folch Method

The tissues were homogenized with chloroform/methanol (2/1) to a final volume that is twenty times the volume of the tissue sample (1 g in 20 ml of solvent mixture). After dispersion, the whole mixture was agitated for 15-20 min in an orbital shaker at room temperature. The homogenate was centrifuged to recover the liquid phase. The liquid phase was washed with 0.2 volume (4 ml of 20 ml) of 0.9% NaCl solution. After vortexing for some seconds, the mixture was centrifuged at low speed (2000 rpm) to separate the two phases. The upper phase was removed by siphoning. After centrifugation and siphoning from the upper phase, the lower chloroform phase containing lipids was evaporated under vacuum in a rotary evaporator. The percentage yield of the extract was computed after the extraction (Folch, 1957).

2.3 Preparation of Test Bacteria

Staphylococcus aureus (ATCC 25923) and *Escherichia coli* (ATCC 25922) were purchased from University of the Philippines, Microbiology Department – Manila. The bacteria were maintained in nutrient agar slants and stored at 4-8°C before use. Stock cultures of the test bacteria were prepared in Tryptic Soy Broth (TSB) placed in screw-capped tubes.

2.4 Preparation of 0.5 McFarland as the Standard for Turbidity

The procedure was carried out by the Clinical and Laboratory Standards Institute (2006).

A 0.5 ml of 1.175% Barium Chloride was added to 99.5 ml of 1% sulfuric acid with constant stirring to maintain the suspension. The correct density of the turbidity standard was verified using a spectrophotometer with the 1-cm light path and matched cuvette to determine the absorbance. The absorbance at 625 nm should be 0.08 to 0.10 for the McFarland Standard. The Barium Sulfate suspension was then transferred into screw-capped tubes of the same size as those used in growing or diluting the bacterial inoculum. These tubes were tightly sealed and stored in the dark at room temperature.

2.5 Preparation of Lipid Extract Concentration by 2-Fold Serial Dilution Method

The procedure was carried out by Benson's Microbiological Applications Laboratory Manual in General Microbiology (2001).

Two grams of the lipid extract was weighed and diluted with 2 ml of Tween 80 (0.05%). The dilution of the extract with Tween 80 leads to the concentration of 1000mg/ml.

Eleven test tubes were prepared. Into the first ten tubes, 0.5 ml of Tween 80 (0.05%) was transferred. One ml of the 1000 mg/ml concentration was transferred to the last tube. A 0.5ml aliquot of the transferred 1000mg/ml concentration was obtained and transferred to the second tube containing 0.5ml of Tween 80, making a 500 mg/ml

concentration in the second tube. A 0.5ml aliquot of the mixture in the second tube (500 mg/ml) was obtained and transferred into the third test tube containing 0.5ml of Tween 80, making a 250 mg/ml concentration. The dilutions were repeated until it reached the last tube (0.48 mg/ml). When the dilution reached the last tube, 0.5ml was discarded from it to have uniform volumes.

2.6 Determination of MIC using Broth Macrodilution Method

The procedure was carried out by the Clinical and Laboratory Standards Institute (2006).

For every bacterial isolate, 11 sterile screw-capped tubes were prepared with the respective lipid extract concentration to be tested. Two tubes were also labeled for growth control and sterility control. A 0.5 ml aliquot from each concentration of the lipid extract was added to the tubes (1-11). Tube 12 and Tube 13 were left without the lipid extract.

One ml of a bacterial suspension of each microorganism was transferred to sterile tubes. Their turbidity was adjusted using 0.5 McFarland Standard resulting in 1.5×10^8 CFU/ml. A 0.2 ml aliquot of the adjusted suspension was added to 19.8 ml of sterile broth, resulting in the final desired inoculum of 1.5×10^5 CFU/ml.

A 0.5 ml aliquot of the adjusted bacterial suspension was added to tubes 1 to 12. Sterile Tryptic Soy Broth was added to tube 13 for sterility control.

2.7 Determination of the Zones of Growth Inhibition using Disk Diffusion Method

The procedure was carried out by Benson's Microbiological Applications Laboratory Manual in General Microbiology (2001).

Twelve Muller Hinton Agar plates and 30 sterile filter paper disks 1/2" diameter were prepared. Each plate was properly labeled with the name of the test organism and the concentrations to be tested. A separate plate was used to test the positive and negative control. Triplicate of plates were prepared.

The agar plates were streaked with a sterile cotton swab that was subjected to the adjusted inoculum. Sterile disks dipped in the test concentrations were placed on the media at least 15mm from the edge of the plate. Ciprofloxacin 5ug disk and sterile disk dipped in Tween 80 were used as positive and negative controls, respectively. The plates were incubated at 37°C for 24-48 hours. After incubation, the zones of growth inhibition were determined by measuring the diameter of clear zones surrounding the disks as seen at the bottom of the plate using a caliper.

2.8 Statistical Analysis

The mean values were expressed as the Mean ± Standard Deviation (SD) and were analyzed using one-way ANOVA using the program SPSS 19.0 for Windows. Differences were considered significant at $p<0.05$.

ANOVA was used to determine significant differences in the mean zones of inhibition from the different concentrations and controls. Scheffe test was used after ANOVA for multiple comparisons.

The common extract concentrations used were also compared using T-test to determine in which bacterium the extract is more effective.

3. RESULTS AND DISCUSSION

3.1 The Lipid Extract

The lipid extract obtained from the earthworms appear as a semi solid brownish substance with a pungent odor.

Table 1. Percentage Yield Result

Weight of the Earthworms	Weight of the Lipid Extract	Percentage Yield
900 grams	3.09 grams	0.343%

As shown in Table 1, the percentage yield obtained in the extraction of lipids using the Folch method is low (0.343%). The great loss of the extract may have been due to filtration, evaporation and transferring of liquids. It also implies that there may be other methods of extraction other than the Folch method that is more suitable to achieve a higher yield.

3.2 Determination of Minimum Inhibitory Concentration (MIC)

Table 2 MIC Result against Staphylococcus aureus

Tube	Final Concentration of the Extract	Turbidity	Interpretation
1	500 mg/ml	(-)	No visible growth
2	250 mg/ml	(-)	No visible growth
3	125 mg/ml	(-)	No visible growth
4	62.5 mg/ml	(-)	No visible growth
5	31.25 mg/ml	(-)	No visible growth
6	15.625 mg/ml	(-)	No visible growth
7	7.812 mg/ml	(+)	With visible growth
8	3.906 mg/ml	(+)	With visible growth
9	1.953 mg/ml	(+)	With visible growth
10	0.976 mg/ml	(+)	With visible growth
11	0.488 mg/ml	(+)	With visible growth

Table 2 shows the MIC results against *Staphylococcus aureus*. Starting at 15.625 mg/ml concentration, no visible growth of the organism was seen as indicated by the negative turbidity. Hence, 15.625 mg/ml is the minimum concentration that may be used to inhibit the growth of *S. aureus*. Concentrations below 15.625 mg/ml were not effective in inhibiting the growth of bacteria as visible growth was seen.

Table 3. MIC Result against Escherichia coli

Tube	Final Concentration of the Extract	Turbidity	Interpretation
1	500 mg/ml	(-)	No visible growth
2	250 mg/ml	(-)	No visible growth
3	125 mg/ml	(-)	No visible growth
4	62.5 mg/ml	(-)	No visible growth
5	31.25 mg/ml	(-)	No visible growth
6	15.625 mg/ml	(+)	With visible growth
7	7.812 mg/ml	(+)	With visible growth
8	3.906 mg/ml	(+)	With visible growth
9	1.953 mg/ml	(+)	With visible growth
10	0.976 mg/ml	(+)	With visible growth
11	0.488 mg/ml	(+)	With visible growth

Table 3 shows the minimum inhibitory concentration results against *Escherichia coli*. Starting at 31.25 mg/ml, no visible growth of the organism was seen as indicated by the negative turbidity. This concentration is the minimum concentration that may be used to inhibit the growth of *E. coli.* Concentrations below 31.25 mg/ml were not effective in inhibiting the growth of bacteria.

Results for the MIC show that the lipid extract can inhibit the growth of both test microorganisms. The MIC of the extract against *S. aureus*, however, is lower than that of *E. coli.*, suggesting that a lower concentration of the extract is needed to inhibit the visual growth of the *S. aureus* compared to *E. coli*.

3.3 Antibacterial Activity

Table 4. Zone of Inhibition Results for Staphylococcus aureus

Group	Concentration	Average Zone of Inhibition (mm)
1	15.625 mg/ml extract	6.3333
2	31.25 mg/ml extract	7.3333
3	62.5 mg/ml extract	7.6667
4	Ciprofloxacin 5 ug	6.0000
5	0.05% Tween-80	0.0000

Table 5. Zone of Inhibition Results against Escherichia coli

Group	Concentration	Average Zone of Inhibition (mm)
1	31.25 mg/ml extract	5.3333
2	62.5 mg/ml extract	6.3333
3	125 mg/ml extract	7.0000
4	Ciprofloxacin 5 ug	10.3333
5	0.05% Tween-80	0.0000

All of the extract concentrations used and ciprofloxacin inhibit the growth of both *S. aureus* and *E. coli.* (Tables 4 and 5). Likewise, variable zones of inhibition were seen. Both microorganisms were consistently not inhibited by 0.05% Tween-80 as there were no zones of inhibition seen. This emphasizes the appropriateness in the selection of positive and negative controls. Tween-80 has no antibacterial property and hence, can serve as a basis for comparison if the extract indeed has antibacterial property. On the other hand, ciprofloxacin has both Gram-negative and Gram-positive coverage. Comparison with this standard can establish the effectiveness of the different extract concentrations against the test microorganisms.

Table 6. Percent Inhibition against Staphylococcus aureus and its Corresponding Zone of Inhibition Intensity

Group	Extract Concentration in mg/ml	Percent Inhibition	Zone of Inhibition Intensity
1	15.625	111.41%	Very Strong
2	31.25	149.38%	Very Strong
3	62.5	163.28%	Very Strong

Table 7. Percent Inhibition against Escherichia coli and its Corresponding Zone of Inhibition Intensity

Group	Extract Concentration in mg/ml	Percent Inhibition	Zone of Inhibition Intensity
1	31.25	26.63%	Weak
2	62.5	37.56 %	Weak
3	125	45.89%	Weak

Tables 6 and 7 show the computed percent inhibitions and corresponding inhibition intensity. The computed percent inhibitions for the three groups against *S. aureus* were classified as very strong, as the values are all greater than 100 %. Against *E. coli*, the computed percent inhibitions of the three groups were classified as weak as the values are all less than 50 %.

Meanwhile, Table 8 shows that there was a significant difference in the mean zones of inhibition among the different concentrations of the extract, positive and negative controls against *Staphylococcus aureus*. The mean values of each group were computed, the F computed resulting in a p-value of 0.000. The p-value of 0.000 which is less than the level of significance of 0.05 suggests that at least one of the five groups have a significantly different mean level of inhibition.

Table 8. ANOVA result for Staphylococcus aureus

Groups	Mean	F Computed	p-value	Decision	Conclusion
15.625mg/ml	6.3333	147.167	0.000	Reject Null	Significant
31.25mg/ml	7.3333				
62.5mg/ml	7.6667				
Positive (Ciprofloxacin 5µg)	6.0000				
Negative (0.05% Tween-80)	0.0000				

Table 9. Post Hoc Analysis Result for Staphylococcus aureus

(I) Test1		Mean Difference (I-J)	p - value	Decision	Conclusion
Negative	15.625mg/ml	-6.33333*	.000	Reject Null	Significant
	31.25mg/ml	-7.33333*	.000	Reject Null	Significant
	62.5mg/ml	-7.66667*	.000	Reject Null	Significant
	Positive	-6.00000*	.000	Reject Null	Significant
Positive	15.625mg/ml	-.33333	.928	Accept Null	Not Significant
	31.25mg/ml	-1.33333	.056	Accept Null	Not Significant
	62.5mg/ml	-1.66667*	.016	Reject Null	Significant
	Negative	6.00000*	.000	Reject Null	Significant
15.625mg/ml	31.25mg/ml	-1.00000	.191	Accept Null	Not Significant
	62.5mg/ml	-1.33333	.056	Accept Null	Not Significant
31.25mg/ml	62.5mg/ml	-.33333	.928	Accept Null	Not Significant

As shown in table 9, all of the extract concentrations showed significant zones of inhibition against *S. aureus* compared to the negative control. Comparing the different concentrations with the positive control, it shows that the 15.625 and 31.25 mg/ml extract concentrations produced comparable zones of inhibition with that of the positive control. However, 62.5 mg/ml extract concentration produced a greater zone of inhibition against *S.aureus* compared to ciprofloxacin as their mean difference was computed to be significant.

Table 10. ANOVA Result for Escherichia coli

Groups	Mean	F Computed	p-value	Decision	Conclusion
31.25mg/ml	5.3333	210.500	0.000	Reject Null	Significant
62.5mg/ml	6.3333				
125mg/ml	7.0000				
Positive (Ciprofloxacin 5µg)	10.3333				
Negative (0.05% Tween-80)	0.0000				

Table 10 shows that there was a significant difference in the mean zones of inhibition among the different concentrations of the extract, positive and negative controls. The mean values of each group were computed, the F computed resulting in a p-value of 0.000. The p-value of 0.000, which is less than the level of significance of 0.05, suggests that at least one of the five groups have a significantly different mean level of inhibition.

Table 11. Post Hoc Analysis for Escherichia coli

(I) Test1		Mean Difference (I-J)	p – value	Decision	Conclusion
Negative (0.05% Tween-80)	31.25mg/ml	-5.33333*	0.00	Reject Null	Significant
	62.5mg/ml	-6.33333*	0.00	Reject Null	Significant
	125mg/ml	-7.00000*	0.00	Reject Null	Significant
	Positive	-10.33333*	0.00	Reject Null	Significant
Positive (Ciprofloxacin 5µg)	31.25mg/ml	5.00000*	0.00	Reject Null	Significant
	62.5mg/ml	4.00000*	0.00	Reject Null	Significant
	125mg/ml	3.33333*	0.00	Reject Null	Significant
	Negative	10.33333*	0.00	Reject Null	Significant
31.25mg/ml	62.5mg/ml	-1.00000	0.19	Accept Null	Not Significant
	125mg/ml	-1.66667*	0.02	Reject Null	Significant
62.5mg/ml	125mg/ml	-0.66667	0.53	Accept Null	Not Significant

Table 12. T-test Result Comparing 31.25 mg/ml dose against S.aureus and E.coli

Bacteria	Groups	Mean	F computed	t-value	Decision	Conclusion
S.aureus	31.25 mg/ml	7.3333	4.8986	2.776	Reject Null	Significant
E.coli	31.25 mg/ml	5.3333				

Table 13. T-test Result Comparing 62.5 mg/ml dose against S.aureus and E.coli.

Bacteria	Groups	Mean	F computed	t-value	Decision	Conclusion
S. aureus	62.5 mg/ml	7.6667	4.8986	2.776	Reject Null	Significant
E.coli	62.5 mg/ml	6.3333				

The extract was able to significantly inhibit the growth of both organisms at 31. 25 mg/ml and 62.5 mg/ml concentrations. At 5% level of significance, however, both concentrations produced significantly higher mean zones of inhibition against *S.aureus* compared to *E. coli* as shown in tables 12 and 13. This finding corresponds to the greater percent inhibition created against *S. aureus* as shown previously in Table 3.

4. CONCLUSION

The lipid extract showed antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*. The extract is more effective in inhibiting the *S. aureus* since a lower concentration of the extract is needed to inhibit the growth of *S. aureus* (MIC = 15.625 mg/ml) compared to *E. coli* (MIC = 31.25 mg/ml). In higher concentrations, the extract also proved to be more effective in inhibiting the growth of *S. aureus*. A general conclusion on its effectiveness against Gram-positive bacteria, however, may not be made as the extract has yet to be tested against more Gram-positive and Gram-negative microorganisms.

Ciprofloxacin is still more effective against *E. coli* compared to the three concentrations used. Against *S. aureus*, 62.5 mg/ml showed the greatest inhibitory effect. This concentration is also more effective compared to ciprofloxacin, while the 15.625 and 31.25 mg/ml were comparable to the positive control.

There is a low percentage yield with using the Folch method for extraction of lipids in earthworms. Other methods of lipid extraction may be employed in future studies to obtain a higher percentage yield.

For further studies, isolation and identification of fatty acids present in lipid extract may be done. Increasing the number of test microorganisms,

including multi-drug resistant strains and fungi may also be conducted to determine the spectrum of activity. Also, further studies determining other pharmacological activities may also be conducted.

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