In vitro testing of antibacterial potential of Phyllanthus acidus (Karamay) Fruit Juice against Escherichia coli

Piolo Xander Flores, Jane Irish Blanche, Trisha Mae Alipalo, Marie Veth Macam, Jose John Nuqui, and Shenna Grace Mendoza

School of Medical Laboratory Science, College of Allied Health Sciences, Saint Jude College, Manila, NCR, Philippines

Abstract:

In this study, Phyllanthus acidus juice extract was tested for possible antibacterial activity against E. coli at various concentrations using the Kirby-Bauer Disk Diffusion Assay. The four different extract concentrations used in the experiment were 25%, 50%, 75%, and 100%. Gentamicin was used as the positive control and distilled water as the negative control. There were three trials in the experiment, and each trial's mean absorbance was 6 mm. The positive control's mean absorbance was 19 mm. By comparing the experimental group to the positive control, the statistical analysis revealed a P-value of 0.0001, which denotes a highly significant effect. The efficiency of Phyllanthus acidus extract at various concentrations against E. coli however, did not differ noticeably. as the P-value of 0.00 was higher than the confidence interval of 0.05. The conclusion drawn from this is that there are no discernible differences in the antibacterial properties of Phyllanthus acidus juice extract against varying levels of E. coli.

Keywords: Kirby-Bauer Disk Diffusion Assay; Antibacterial Activity; Gentamicin; Juice extract Phyllanthus acidus; Escherichia coli.

1. Introduction

According to the Centers for Disease Control and Prevention (CDC), Escherichia coli is a large group of bacteria that can widely be found in the environment, foods, people and animal's gut. There are various strains of Escherichia coli that are pathologic and can cause diseases such as diarrhea, urinary tract infection, pneumonia and other respiratory diseases. Escherichia coli is considered as a marker for food and water hygiene, there are various types of Escherichia coli strains that are classified as enteroinvasive (EIEC), enteropathogenic (EPEC), enterotoxigenic (ETEC), and verocytotoxigenic (VTEC). (Percival & Williams, 2014)

Every single day there are 200 million estimated individuals that are affected by different diarrheal diseases, and Escherichia coli accounts for the majority of its cause. This growing number made it a burden to public health. To prevent illnesses caused by Escherichia coli strains, it is important to control the environmental contamination and promote good hygiene. (Wanke & Sears, 2008) Phyllanthus acidus or "Karamay" (Ilk., Tag.), belongs to a family of Euphorbiaceae. A small plant capable of growing to 4 to 9 cm in height. The fruit of Phyllanthus acidus is a greenish white in color, it is fleshy and acidic with 3 or 4 celled stones with a single seed in each. (Stuart, 2020). The phytochemical test of Phyllanthus species shows phenolic compounds alkaloids, glycosides, flavonoids, tannins, and saponins where tannins account as the major constituents of Phyllanthus plants. (Nisar et al., 2018)

This study aims to test the antibacterial potential of Phyllanthus acidus fruit juice against Escherichia coli through In vitro testing. The purpose of this study is to acknowledge organic alternatives that can be used in eliminating pathogenic bacteria that are widely found in the environment.

2. Methodology

2.1. Research Design

This study used an experimental research approach to evaluate the extract and efficiency of Phyllanthus acidus (karamay) as an antibacterial agent against Escherichia coli. One of the most accessible methods for assessing susceptibility is the antibacterial Susceptibility Testing (AST); another name for this is the Disk Diffusion Method approach for detecting antibacterial activity (F.C. Tenover, 2019). The Phyllanthus acidus (karamay) will be extracted using electric manual extraction and after 24 hours of incubation, the researcher will assess the zone of inhibition against E. coli. It is crucial to measure the zone of inhibition since it will indicate whether or not it can eradicate E. coli, or not. The measurement tool the researcher will use is a caliper.

The experiment's methodology protocol was based on the Kirby-Bauer Disk Diffusion Susceptibility Test Protocol (Hudzicki, J. 2009). For the processing and antibiotic choice used in this test, Gentamicin will be used as a positive control, the 32nd edition of CLSI's Performance Standards for antibacterial Susceptibility Testing serving as the basis. By using McFarland Standard standards, it is possible to visually compare bacterial density with a suspension of either latex or barium sulfate particles. The range of the zone of inhibition for the extract of Phyllanthus acidus (karamay) is compared with Distilled for the negative control and Gentamicin for the positive control. Then, using a simple dilution process, we test our fruit extract with NSS at decreasing concentrations.

2.2. Locale of the Study

This study focused on St. Jude College PHINMA, corner Dimasalang and Don Quijote Street, Sampaloc, Manila. The researcher chose this place since it had the laboratory and the supplies they needed for their experimental research.

2.3. Population of the Study

This study used a simple dilution or microbroth dilution to recognize organic alternatives that can be used in Phyllanthus acidus (karamay) fruit extract to kill harmful bacteria. As illustrated below, this will consist of 4 test tubes filled with various quantities of Phyllanthus Acidus (karamay) extract and saline solution. This will imitate the gentamicin used as a positive control to kill Escherichia coli, and it will be incubated for 24 hours to determine its efficacy at various concentrations.

Table 1. PAE and Saline different concentrations for Simple Dilution

	1st test tube	**2nd test tube**	**3rd test tube**	**4th test tube**
Label (%)	100%	75%	50%	25%
Phyllanthus acidus Extract (PAE) (mL/uL)	1 mL	750 uL	500 uL	205 uL
Saline (uL)	none	250 uL	500 uL	750 uL
Time (hr/s)	24 hrs			

2.4. Data Gathering and Procedures

The research advisor wrote and recorded a letter requesting permission to carry out the experimental study that aims to test the antibacterial potential of Phyllanthus acidus (karamay) against Escherichia coli. The College of Allied Health Sciences (CAHS) Dean will receive the letter subject for review and approval of the said experiment. Prior to conducting the experiment, the researchers will ask for a letter of authorization that includes the permission to use the laboratory facilities. Escherichia coli, the bacteria to be employed, will be cultivated in the nearby tertiary laboratory in Quezon City so that the researchers have enough to complete the experiment.

Prior to the experiment, the researchers will confirm the identification of the fruit Phyllanthus acidus (karamay) by providing a certificate from the Bureau of Plant Industry. The fruit was obtained from Tarlac by one of the researcher's family members who also helped to transport the fruits from Tarlac City to Sampaloc, Manila. The researcher's method of choice is manual extraction for Star gooseberry. After dispersing the E. coli, these are put to the MHA agar, accordingly and performing microbroth dilution or simple dilution. After 24 hours of incubation, effectiveness will be assessed, and the researcher will measure the zone of inhibition.

2.5. Data Analysis

The processing and selection of antibiotics used in this test were based on the 32nd edition of CLSI's Performance Standards for Antimicrobial Susceptibility Testing, which provided the data for this study. Normal Saline is used for negative and Gentamicin for positive control which is then compared with the Extract of Phyllanthus acidus in terms of range of zone of inhibition. If the substance falls within the categories of Resistant (R), Intermediate (I), or Susceptible (S), measuring the Zone of Inhibition encompasses all of these conditions. The researchers used Two-Way ANOVA (Analysis of Variance) in differentiating the results of

positive control, negative control, and concentration of Phyllanthus acidus extract against E. coli in a disk diffusion test. We can accurately determine if it has antibacterial activity against E. coli by doing this.

2.6. Materials and Methods

2.6.1. Sample Preparation.

The Phyllanthus acidus (karamay) will be picked from Tarlac City, Philippines. The Manual Extraction of Phyllanthus acidus was carried out at Phinma St. Jude College of Medical Laboratory Science in Manila, Philippines. We will require 5 mL of Phyllanthus acidus (karamay) juice extract for this experiment. Using an electric manual press juicer, the operator must insert the fruit and firmly depress a lever to smash it. The fruit is squeezed against a sieve by a plunger that is pushed down by the lever. Only the juice flows out the bottom because the fine mesh filter prevents the pulp and rind from coming through. Of all the possibilities, this kind of juicer is the simplest to operate. Citrus fruits that have been split in half have a shape that makes it very simple for it to stay in position on a manual press juicer; as a result, that is where it is most usually seen as a tool. (Munsell, 2022)

2.6.2. Mueller-Hinton Plate Preparation

1. A MH agar plate (one for each microbe to be tested) should first be let to warm up to room temperature. It's better to keep plates in the plastic sleeve while they warm up to prevent moisture.
2. If a liquid is discernible on the agar's surface, invert the plate to let the surplus liquid drain and evaporate it. After that, leave the agar on the cover. In a laminar flow cabinet, plates can be dried at room temperature or in an incubator set to 35°C usually 10 to 30 minutes.
3. Each MH agar plate should be labeled suitably for the organism being studied.

2.6.3. Disc Diffusion.

Antibiotic susceptibility disks. Antimicrobial disks are prepared in the Laboratory of School of Medical Laboratory Science in PHINMA St. Jude College. By using the agar diffusion method, these disks are utilized to semi-quantitatively assess the in vitro susceptibility to antimicrobial drugs of numerous challenging species and quickly growing bacteria.

2.6.4. McFarland Standard.

A suspension of either barium sulfate or latex particles can be compared visually to bacterial density using McFarland criteria. One of the most typical was a little card called a Wickerham card, which has parallel black lines. A bacterial suspension containing between 1 and 2 x 108 E is referred to as a 0.5 CFU/ml. The following steps can be used to produce a 0.5 McFarland standard throughout.

1. dd a 0.5-ml aliquot of a 0.048 mol/liter BaCl2 (1.175% wt/vol BaCl2 • 2H20) to 99.5 ml of 0.18 mol/liter H2SO4 (1% vol/vol) with constant stirring to maintain a suspension.
2. Using a spectrophotometer with a 1-cm light path and matching cuvette, measure the absorbance to confirm that the density of the turbidity standard is accurate. The absorbance at 625 nm should be 0.08 to 0.13 for the 0.5 McFarland standard.
3. Transferring the barium sulfate suspension into screw-cap tubes the same size as those used to standardize the bacterial inoculums involves doing so in 4- to 6-ml aliquots.
4. At room temperature, store in the dark with a tight closure. (Hudzicki, J. 2009).

2.6.5. Preparation of Inoculum

1. Use a sterile inoculating loop or needles to contact four or five isolated colonies of the organism to be investigated.
2. The organism is then suspended in 2 saline tubes should be vortexed to aid
3. Adjust this suspension's turbidity to meet a 0.5 McFarland standard. Sterile saline should be used if the suspension is too thick.
4. After 15 minutes of preparation, this suspension should be used. (Hudzicki, J. 2009).

2.6.6. Simple Dilution

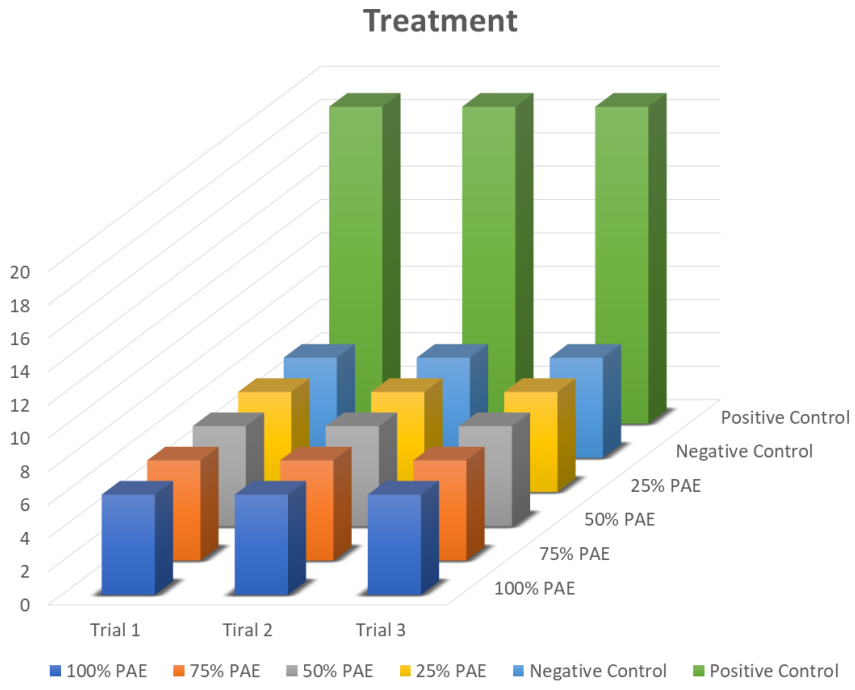
1. For the preparation star gooseberry fruit mix with saline in a clean new tube: 1st test tube (label 100%) - 1 mL star gooseberry fruit, ONLY, 2nd test tube (label 75%) – 750 uL star gooseberry fruit plus 250 uL saline, 3rd test tube (label 50%) – 500 uL star gooseberry fruit plus 500 uL saline and 4th test tube (label 25%) – 250 uL star gooseberry fruit plus 750 uL saline.
2. Assemble 14 pieces of 6 mm filter paper, then disinfect before using a puncher. Next, place three filter papers in each test tube and soak them in the solution.
3. Soak 1 6mm filter paper onto a test tube with saline.

Table 2. Number of Mueller-Hinton Agar to be used in this experiment

Label	Content	MHA Plates
Trial 1	Phyllantus acidus juice extract + NSS with 4 different concentration each screw capped	2 plates
Trial 2		2 plates
Trial 3		2 plates
Negative Control	Distilled Water	1 plate
Positive Control	Gentamicin	1 plate
Total		8 Plates

3. Results and Discussion

Table 3. The Total Antibacterial Activities of the Treatments in Different Trials



The Antibacterial Activities of Experimental Group which is the Phyllantus acidus juice extract in different concentrations of 25%, 50%, 75%, and 100%, Positive Control which is Gentamicin, and Negative Control which is distilled water. The experimentation had three (3) trials with mean absorbance of 6. The mean absorbance of positive control is 19. The P-value of less than <0.0001 which is lower than the confidence level of 0.05 making the effect considered extremely significant while within the different concentrations, the P-value of 0.00 is greater than the confidence level of 0.05 making the effect considered not significant.

Therefore, there is no significant difference in the effectiveness of antimicrobial components of extracted Phyllanthus acidus (karamay) juice against Escherichia coli by the zone of inhibition using different concentrations of saline.

4. Conclusion

The P-value is <0.0001 that shows that there is significant difference in the zone of inhibition of Escherichia coli against the different concentrations of Phyllanthus acidus. With these results, the researchers concluded that the different concentrations of Phyllanthus acidus juice extract is not effective in killing or inhibiting the growth of Escherichia coli than gentamicin, which is the positive control

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