TUKLAS RESEARCH PLAN FORMAT

Research Title: Phytochemical Screening of Secondary Metabolites and Comparison Between Cytotoxicity Level of Unripe And Ripe Saba (Musa acuminata × balbisiana) Ethanolic Peel Extract Using Brine Shrimp Lethality Assay

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A. RATIONALE:

Medicines are chemicals or compounds used to cure, halt, or prevent disease; ease symptoms; or help in the diagnosis of illnesses. According to WHO, an estimated 1 in 10 medical products in low- and middle-income countries is substandard or falsified . Some of these products are toxic in nature, with either fatal levels of the wrong active ingredient or other toxic chemicals that can affect our health and body. Natural products (NPs) have historically played an important role in medication discoveries, particularly for cancer and infectious disorders. It contains less heavy metals and higher molecular rigidity compared to synthetic compounds and combinatorial libraries. According to Hidayat Hussain et al., in April 2022, it was discovered that natural products such as peels have greater biological and pharmacological uses than other regions of the fruit in many cases. It also contains various nutrients, including fiber, vitamins, minerals, and antioxidants, and is said to be beneficial for the environment (Petre, 2022). Therefore, this study aims to contribute to the medicinal field by comparing the essential constituents of ripe and unripe saba peels through analyzing its cytotoxicity level by using brine shrimp lethality assay and investigating if those substances have safe therapeutic properties.

B. RESEARCH QUESTION OR PROBLEM BEING ADDRESSED

Countries have several problems in delivering good and efficient medical products. Some of those products contain harmful substances that can affect the human body. Due to these issues, the goal of this research is to identify the phytoconstituents present and their amount in both ripe and unripe saba peel extract.

Consequently, this study aims to answer the following questions:

1. What is the effect of Ripe saba (Musa acuminata balbisana) ethanolic extract on Brine Shrimp Lethality Assay in terms of:

a. Phytochemical content

    a.1 Flavonoid content

    a.2 Phenol content

    a.3 Tannins content

    a.4 Alkaloid content

    a.5 Glycosides content

b. Cytotoxicity level

2. What is the effect of Unripe saba (Musa acuminata balbisana) ethanolic extract on Brine Shrimp Lethality Assay in terms of:

a. Phytochemical content

    a.1 Flavonoid content

    a.2 Phenol content

    a.3 Tannins content

    a.4 Alkaloid content

    a.5 Glycosides content

b. Cytotoxicity level

C. GOALS/EXPECTED OUTCOMES/HYPOTHESES

HYPOTHESIS:

1. To determine what factors contribute to the cytotoxicity level of ripe and unripe saba peel extract.

    1. 1 Ripe saba (Musa acuminata balbisiana) ethanolic peel extract contain components that contribute to its cytotoxicity level.

    1.2 Unripe saba (Musa acuminata balbisiana) ethanolic peel extract does not contain components that contribute to its cytotoxicity level.

    1.3 Ripe saba (Musa acuminata balbisiana) ethanolic extract does not contain components that contributes to its cytotoxicity level.

    1.4 Unripe saba (Musa acuminata balbisiana) ethanolic peel extract contain components that contributes to its cytotoxicity level.

    1.5 Both ripe and unripe saba (Musa acuminata balbisiana) ethanolic peel extract contain components that contribute to its cytotoxicity.

    1.6 Both ripe and unripe saba (Musa acuminata balbisiana) ethanolic peel extract do not contain components that contribute to its cytotoxicity.

2. To determine the phytochemical constituents present in unripe and ripe saba peel extract.

    2.1 Ripe saba (Musa acuminata balbisiana) ethanolic peel extract does not contain phytoconstituents such as flavonoids, phenols, tannins, alkaloids, and glycosides.

2.2 Unripe saba (Musa acuminata balbisiana) ethanolic peel extract contains a considerable amount of phytoconstituents such as flavonoids, phenols, tannins, alkaloids, and glycosides.

2.3 Ripe saba (Musa acuminata balbisiana) ethanolic peel extract contains a considerable amount of phytoconstituents such as flavonoids, phenols, tannins, alkaloids, and glycosides.

2.4 Unipe saba (Musa acuminata balbisiana) ethanolic peel extract does not contain phytoconstituents such as flavonoids, phenols, tannins, alkaloids, and glycosides.

2.5 Both Unripe and ripe saba (Musa acuminata balbisiana) ethanolic peel extract contain a considerable amount of phytoconstituents such as flavonoids, phenols, tannins, alkaloids, and glycosides.

2.6 Both Unripe and ripe saba (Musa acuminata balbisiana) ethanolic peel extract does not contain phytoconstituents such as flavonoids, phenols, tannins, alkaloids, and glycosides.

D. PROCEDURES:

Research Design

To accomplish the objectives of the study, ripe and unripe saba peel will be collected and put through several laboratory procedures wherein the peels will be extracted with ethanol as the extractant. The extract will then undergo phytochemical screening to identify its components. To avoid biased results, the researchers will be using Completely Randomized Design (CRD) with three replicates in this study.

There will be five treatments with three replicates for Brine Shrimp Lethality Assay.

Trial				
Treatment	1	2	3	Average
1				
2				
3				
4				
5				

Table 1. Experimental Design Layout

Where:

Treatment 1: Dimethyl sulfoxide (DMSO) (Negative Control)

Treatment 2: Potassium dichromate (Positive Control)

Treatment 3: 100 µg/mL Ethanolic Peel Extract

Treatment 4: 50 µg/mL Ethanolic Peel Extract

Treatment 5: 25 µg/mL Ethanolic Peel Extract

Materials and Methods

Collection and Preparation of Saba Peel

The ripe and unripe saba will be purchased in Daet, Camarines Norte. The ripe and unripe saba will then be washed with tap water to eliminate any dirt and other particles that will not be needed in the study. The fruit inside will be removed once it has been cleaned. After that, the peel will be sun dried outside for 48 hours.

Ethanolic Extraction of the Peel

After the time for sun-drying of the peels, it will be crushed into coarse powder using a mechanical grinder. It will then be soaked in 2L of 95% ethanol for 72 hours and will be filtered and dried using a rotary evaporator at 65ºC under reduced pressure. The peel concentrate of the crude extract will be used in this study.

Phytochemical Screening of Ripe and Unripe Saba Peel

Qualitative phytochemical screening of ripe and unripe peel ethanolic extract

Test for Flavonoid (Shinoda's test)

Ten drops of dilute hydrochloric acid (HCL) and a piece of magnesium were added to 3 mL of extract, the resulting deep pink or red color indicating the presence of flavonoids.

Test for phenolic compounds and tannins (Ferric chloride test)

2 mL of 5% neutral ferric chloride solution were added to 3 mL of extract, the dark blue color indicates the presence of phenolic compounds and tannins.

Test for Alkaloid (Dragendorff 's test)

By adding 1 mL of Dragendorff"s reagent to 2 mL of extract, an orange red precipitate is formed, indicating the presence of alkaloids.

Test for Glycosides (Keller Killiani test)

A solution of 0.5 mL, containing glacial acetic acid and 2-3 drops of ferric chloride, was mixed with 2 mL of extract. Later, 1 mL of concentrated H2SO4 was added along the

walls of the test tube. The appearance of deep blue color at the junction of two liquids indicates the presence of cardiac glycosides.

		Presence of Phytoconstituents				
		Flavonoids	Phenols	Tannins	Alkaloids	Glycosides
Unripe Saba Ethanolic Peel	Replication 1					
	Replication 2					
	Replication 3					
Ripe Saba Ethanolic Peel	Replication 1					
	Replication 2					
	Replication 3					

Table 2. Presence of phytoconstituents on unripe and ripe saba ethanolic extract

Where:

+ (Positive) indicates the presence of phytoconstituents
- (Negative) indicates the absence of phytoconstituents

Cytotoxicity Test

To assess the difference in the cytotoxic property of ripe and unripe saba peel ethanolic extract, Brine Shrimp Lethality Assay will be conducted.

Brine shrimp lethality test

Maturation of Brine Shrimp

First, we need to make alternative seawater for the brine shrimp to live in. Using a measuring cylinder, measure 3 liters of distilled water and pour it into the rectangular jar. After that, add 27 grams of rock salt and mix the solution using a spatula. Then, place the tip of an airline from an air pump into the bottom of the jar to maintain proper aeration. Next, add about 15 g of brine shrimp eggs to the water inside the jar and mix it with water. Switch on a light (60-100 Watt bulb) and place it a few inches away from the jar. After 20-24 hours, the nauplii will hatch. Observe the eggs and nauplii then collect the nauplii after 24 hours. It is necessary to remove the hatched nauplii from the empty egg. It can be done by turning off the air and switching off the lamp. While the brine shrimp concentrate in the water column, the empty egg will float. Lastly, use a Pasteur pipette to transfer 10 nauplii to a test tube.

Treatment:

Dissolve 5 mg of the plant extract in 0.5 ml of dimethyl sulfoxide (DMSO) to produce a stock solution with a concentration of 1000 µg/mL and diluted it with 4.5 ml of artificial seawater. First, 2 ml will then be taken from the stock solution and will be diluted on 18 mL of artificial seawater to make the first concentration (100 µg/mL). To make the second concentration (50 µg/mL), 10 mL will be obtained from the first concentration and will be mixed with 10 ml of artificial water. Lastly, 10mL will be obtained from the second concentration and will be mixed with 10 mL of artificial seawater to create the third concentration (25 µg/mL).

To make the positive control, measure 3 mg of potassium dichromate using a digital weighing platform then mix it with 10 mL of artificial seawater

The negative control will be prepared by mixing 0.1 mL of DMSO and 9.9 mL of artificial seawater.

Following the placement of Treatments and Controls in each petri dish, the surviving nauplii will be counted after 24 hours of observation.

Methods of Data Collection

The researchers will be using the Completely Randomized Design (CRD) method in this study. The experimental units are randomly distributed to each treatment, ensuring that each experimental unit has an equal chance in receiving one of the treatments. CRD is suitable to use in laboratory experiments where the researchers can easily manipulate the environmental factors that may affect the accuracy of the results.

E. RISK AND SAFETY:

Mechanical grinder can cause potential hazards such as ill health due to dust inhaled. It is important to provide for good ventilation and use an approved dust mask to avoid inhaling excessive amounts of dust formed whilst grinding. Rotary evaporator is used for removal of solvents from samples by evaporation. However, it can have potential burn hazard from heating water bath (usually range from 25 – 95 °C) or cryogens used for cooling. Thus, an appropriate PPE must be worn when using the device. Substances and solutions that will be used in analyzing phyto-constituents present in ripe and unripe peel ethanolic extract can be toxic or harmful, a personal protective equipment (PPE) such as vapor respirators, rubber gloves, splash goggles and face shields should be used.

F. DATA ANALYSIS:

Phytochemical Screening of Ripe and Unripe Saba Peel

Quantitative Phytochemical Screening of ripe and unripe peel Ethanolic Extract

Total Flavonoid Content

50 mL of 80% aqeous methanol will be added to the 2.50 g sample in a 250 ml beaker, covered, and allowed to stand for 24 hours at room temperature. After discarding the supernatant, the residue will be re-extracted (three times) with the same volume of ethanol. Whatman filter paper number 42 (125 mm) will be used to filter the extract. The filtrate will later be transferred into the crucible and evaporated to dryness over a water bath. The content in the crucible will be cooled in a desiccator and weighed until constant weight was obtained.

Total Phenolic Content

To find the total phenolic content of saba peel ethanolic extract, Folin-Ciocalteu Assay will be used. One-hundred (100mg) of the extract will be mixed with 2.8 ml of deionized water and 2 mL of 50% Folin-Ciocalteu's phenol reagent and the resulting mixture will be incubated for 30 minutes at room temperature. Gallic acid solution in ethanol is going to be prepared as the standard.

The spectrophotometric absorbance of the reaction mixture will be measured at 765 nanometer (nm). The total phenolic content will be expressed as milligram galic acid equivalent per gram extract (mg GAE/g extract).

Total Alkaloid Content

200 mL of 10% acetic acid in ethanol is going to be added to 2.50 grams of fruit extract in a 250-mL beaker and allowed to stand for 4 hours. The extract will be concentrated on a water bath to one-fourth of the original volume followed by addition of 15 drops of concentrated ammonium hydroxide to the extract until the precipitation was complete immediately after filtration. After 3 hours of mixture sedimentation, the supernatant will be discarded and the precipitates will be washed with 20 mL of 0.1 m of ammonium hydroxide and then filtered using filter paper (12.5 cm). Using electronic weighing balance Model B-218 the residue will be dried in an oven.

Total Tannin Content

Folin-Denis will be used to quantitatively express the total tannin content of saba peel Ethanolic Extract. One hundred mg of sabba peel extract is going to be dissolved in 10 mL of distilled water. To the mixture, 5 mL of Folin-Denis reagent followed by 10 ml of Na_2CO_3 will be added. The mixture will be shaken well and kept for 30 minutes at room temperature. A set of reference standard solution of tannic acid will be prepared. The absorbance of the mixture will be read at 700 nm. The total tannin content will be expressed as milligram tannic acid equivalent per gram extract (mg TE/g extract).

Measuring the Cytotoxicity level of the ripe and unripe saba peel

The mortality endpoint of this bioassay is defined as the absence of controlled forward motion during 30 sec of observation. The percent of lethality of the nauplii for each concentration and control was calculated.

For each tube, count the number of dead and number of live nauplii, and determine the % death.

Plant Extract	Treatment/	Number of Surviving Nauplii		

	Concentration (µg/mL)	After 24 h			Total Number of Survivors	% Mortality
		T1*	T2	T3		
Unripe Saba	100					
	50					
	25					
	10 (DMSO)					
	10 (Potassium dichromate)					
Ripe Saba	100					
	50					
	25					
	10 (DMSO)					
	10 (Potassium dichromate)					

*T=Trial

Table 3. The cytotoxicity of ripe and unripe saba ethanolic peel extract using brine shrimp lethality assay

To get the %Death

%Death = Number of dead nauplii / Number of dead nauplii + Number of live nauplii x 100

To get the %Mortality

% Mortality = (no. of dead nauplii/initial no. of live nauplii) × 100.

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