Bioactivity-Guided Isolation and Partial Characterization of an Anti-inflammatory and Antibacterial Principles from *M. paradisiaca*

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Abstract

This study deals with the bioactivity-guided isolation and partial characterization of anti-inflammatory and antibacterial principles from M.paradisiaca.

The phytochemical screening shows the presence of leucoanthocyanins, flavonoids, saponins and tannins.

The Brine Shrimp Test shows that among the four extracts produced from serial extraction, methanolic extract has the highest mortality ranging from 63.33% – 90.00% at concentrations 10 - 1000 ppm.

The methanolic extract exhibited superior anti-inflammatory activity over the control both in the lower and higher concentrations in the casein solution. While in the albumin solution, methanolic extract only shows higher anti-inflammatory activity over the control at higher concentration.

Antimicrobial activity of the extract exhibits significant susceptibility to the following bacteria: S. aureus, B. subtilis, P. bulgaris, and E. coli. Data reveal that the extract exhibits greater bioactivity as the extract becomes purer.

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Spectroscopic analyses reveal that the bioactive fraction has alcohol functionality with bands at regions 3200 - 3600 cm^{-1} and 1050 cm^{-1}, for presence of aliphatic O-H stretch and aliphatic C-O stretch, with aromatic C - H stretch at 1050 cm^{-1} respectively. The UV spectrum reveals a peak at λ_{max} 290 nm, which denotes that the structure of the bioactive fraction may consist of several conjugated double bonds. With the IR and UV spectra, it can be hypothetically believed that the bioactive fraction may be a conjugated alcohol with benzene ring.

Keywords: Bioactivity Guided Isolation, Ant Inflammatory, Antibacterial, *M. Paradisiaca*

Introduction

For the past two centuries, the use of medicinal plants plays a great role in a locality, in a region, in a country, or even in the whole world. And today, there is an awakening interest among biologists, chemists, and research funding agencies and even within the pharmaceutical industry in a quest for bioactive natural products.

Most plants have folkloric use as medicine. On numerous occasions, the folklore records of many different cultures have provided leads to plants with useful medicinal properties. In the Philippines, a wealth of unexplored folk medicines is a unique valuable resource, which leads to chemical investigation and purification of extracts of plants and yield numerous purified compounds, which have been proven to be indispensable in the practice of modern medicine (Asis, 1987).

Ethnomedical information revealed that a number of our Philippine plants, indigenous or not, are used for the treatment of a variety of ailments. In fact, this is the practice by our herbalists who have been using plants in the cure of some common ailments particularly in places where modern medicine has yet to be fully accepted. The need to document scientifically the potentials of our plants must be strongly considered to validate the claims of these practices. Natural product scientists have joined efforts in the search for plants that may have potential as a new source of antibiotics. The bioactive compounds serve as lead structures for the development of analogues that can exhibit wider range of activities (Aguinaldo, et. al., 1995).

The banana plant, often erroneously referred to as a "tree", is a large herb, with succulent, very juicy stem (properly "pseudostem") reaching a height of 20 to 25 feet and arising from a fleshy rhizome or corm. Moreover, the banana has also folkloric backgrounds known to treat ulcers, bronchitis, dysentery, hemorrhoids and other digestive problems. Its sap is also considered a potent astringent (Asis, 1987).

The types we call "banana", are known by similar or very different names in banana-growing areas. Spanish-speaking people say *banana china, banana enano* (Costa Rica) *cambur or camburi* (Venezuela), *cachaco, colicero, cuatrofilos, carapi* (Colombia), *curro* (Panama), *zambo* (Honduras). For Portuguese they say *banana maca, banana de Sao Tome', banana da prata*. French islands or areas, the terms maybe *bananier nain, bananier de chine, figue, figue banana, figue naine*. For Germans, they say *echte banana, felge, or feigenhaum* (Encyclopedia Britannica, 1998).

In the quest for non-costly medicine, the practice of decoction, poultice and other methods using herbs or plants with medicinal value are still practiced today. Since plants in our surroundings have varying degrees of toxicity, it is imperative to employ methods in extraction and purification for subsequent applications to ensure safety and protection among the users (Quisumbing, 1978).

It has been studied that banana and its parts, specifically the leaves, flowers and roots have significant value in medicinal applications such as astringent, treatment for ulcers, bronchitis, dysentery, hemorrhoids, and other digestive disorders. Among its parts, only the fruit is considered for its nutritive value; thus, it really interests the researcher to investigate the fruit, specifically the peelings for its possible contribution to medicine. Consequently, the findings of this research study would possibly add to its list of contributions not only for record purposes, but also for its application in the pharmaceutical industry.

Banana is abundant everywhere. It grows favorably in the tropical region, especially in the Philippines. Since it is readily available and not costly, screening the phytochemicals present and possible application to the pharmaceutical world will be of great advantage.

Nevertheless, the peelings of the fruit of the banana are discarded. Its possible contribution to the medicine sphere must be established to appreciate the usefulness of this almost "futile" thing.

Materials And Methods

1 Sample Preparation

1.1 Collection of Sample

The fruits of the banana were left to ripen while attached to the plant, harvested, and individually peeled. It is then placed in a plastic bag and transported to the Chemistry Department of MSU-IIT, Iligan City. The sample was washed with distilled water, cut into small pieces and soaked in 80% ethyl alcohol for 48 hours. The collection of the sample was done during the first week of November 2003 in Buru-un, Iligan City.

1.2 Description of the Sample

The color of the peelings of the fully ripe banana is yellow. The peelings are thick and fleshy with some amount of soft pulp on the posterior part. It has a thickness of about 0.5 to 1.0 cm and a regular length of 3.5 to 12 inches.

1.3 Preparation of Ethanolic Extract for Bioassay-Guided Isolation

About 1.0 kg of homogenized banana peeling was soaked in 80% ethanol for 48 hours using a volume enough to cover the plant material. The mixture was filtered and the filtrate was concentrated in a rotary evaporator.

1.4 Preparation of the ethanolic extract for phytochemical screening

The crude EtOH extract was prepared by refluxing 300.0 g of the chopped fruit peeling material in a 1000-mL Erlenmeyer flask with 800 ml of 80% ethanol for one hour in a boiling water bath. The mixture was allowed to cool to room temperature and then

filtered. Sufficient amount of ethanol was added through the residue on the filter paper to make 500 ml of the extract.

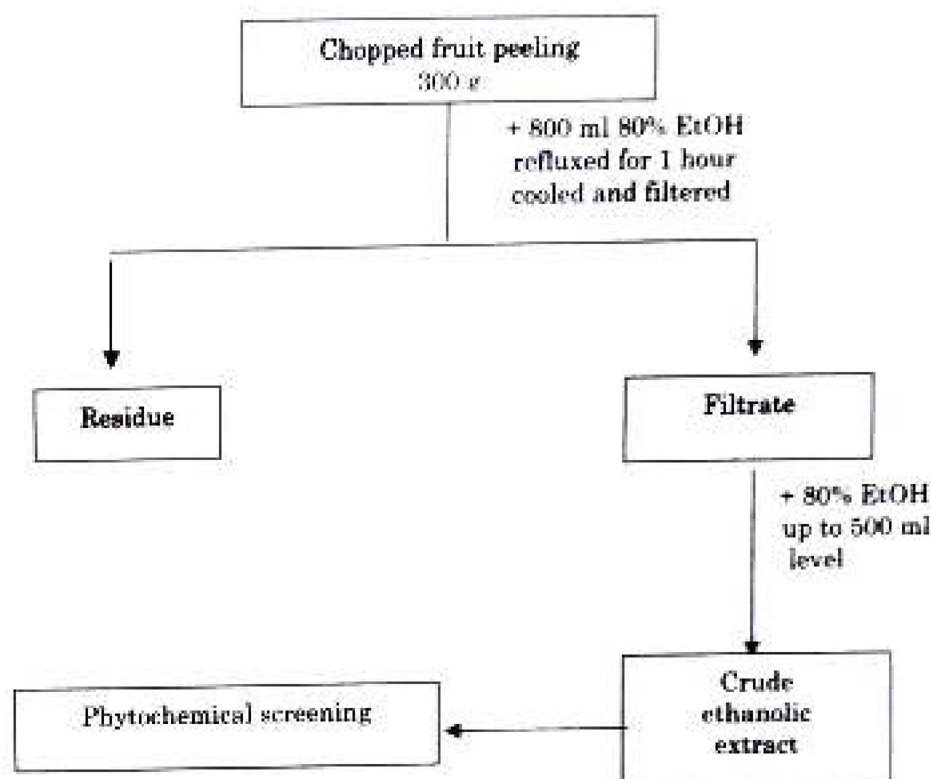

Figure 1. CRUDE Ethanolic Extract Preparation

2 Phytochemical Screening

The widely accepted phytochemical screening procedure outlined by Dr. Magdalena C. Cantoria was utilized in this study.

2.1 Screening for Alkaloids

A volume of 70 ml of the 80% ethanolic extract was evaporated to dryness on a steam bath. The residue was dissolved in 7 mL of 1% HCl aided by warming on the steam bath for 1 to 2 minutes. The solution was allowed to cool and filtered. The volume was adjusted to 7 mL by washing the residue on the filter paper with sufficient quantity of 1% HCl. A few grains of powdered NaCl was added to the filtrate and then stirred and filtered.

A sample of 1.0 mL of the filtrate was placed into each of the four small test tubes. To the first test tube, 3 drops of Mayer's reagent was added. To the second test tube, 3 drops of Valser's reagent was added. And to the third test tube, 3 drops of Wagner's reagent was added. The results were observed and recorded as follows:

(-)	for negative
(+)	for slightly turbid
(++)	for definite turbidity
(+++)	for heavy precipitation

2.2 Screening for Anthraquinones Heterosides

Borntrager's Test

A 5-mL portion of the crude ethanolic extract was evaporated to dryness on a steam bath and the residue was defatted with 5 to 10 mL of petroleum ether. To the defatted residue, 50 ml of distilled water was added. The solution was mixed well and filtered into a small separatory funnel. The contents in the funnel was added with 10.0 mL of benzene and mixed well, and the two phases were allowed to separate. The aqueous layer was then drained out and the benzene phase was transferred to a test tube. A 5-mL volume of ammonia solution was introduced into the tube and was shaken to mix the contents well. The benzene layer was then observed for color change. The presence of a red color indicates the presence of anthraquinones.

2.3 Screening for Unsaturated Sterols and Triterpenes

A volume of 30.0 mL of the 80% EtOH extract was evaporated to dryness on a steam bath. The residue was cooled to room temperature and defatted by mixing it with 15.0 mL of petroleum ether and filtered. An additional volume of petroleum ether was added and the procedure was repeated until the last volume of the filtrate was colorless. The ethereal filtrates were combined and the defatted residue was set aside for the screening of flavonoids and leucoanthocyanins.

The combined ethereal filtrates were evaporated to dryness and the residue was dissolved in 15 mL of $CHCl_3$. The chloroformic solution was dried over anhydrous Na_2SO_4, filtered, and the filtrate was divided equally into three dry test tubes with one of the test tubes as the control.

Liebermann-Bouchard Test

To the first test tube, 0.3 mL of acetic anhydride was added and mixed the solution gently. After which, a drop of concentrated H_2SO_4 was added and the immediate color change was noted. Further color change was observed within an hour. The appearance of red color indicates the presence of unsaturated sterols and triterpenes.

Salkowski Test

Five mL of the filtrate was transferred to a dry test tube and a ring test was performed with concentrated sulfuric acid. After 1-2 minutes, the tube was shaken and the change in the color was noted. A cherry-red color is indicative of the presence of unsaturated sterols. Similar tests with standard solutions were conducted.

2.4 Screening for Flavonoids and Leucoanthocyanins

The defatted residue from section C was dissolved in 30 mL of 50% ethanol. The resulting solution was filtered and then 1.0-2.0 mL of the filtrate was placed in each of the three test tubes.

To the first test tube, 0.5 mL concentrated HCl was added and warmed in a steam bath for five minutes. Color change was observed within one hour. The absence of a red-violet color indicates the absence of leucoanthocyanins.

To the second test tube, 0.5 mL concentrated HCl and 3-4 magnesium strips were added. Color change within 10 minutes was observed. The absence of any color change from orange to red, from crimson to magenta, and occasionally to green or blue indicates a negative result for flavonoids.

The last test tube served as the control. No reagents were added.

2.4.1 Screening for Saponins

A volume of 30 mL of the 80% ethanolic extract was placed in an evaporating dish and have it evaporated to dryness on a steam bath. To the residue, 30 mL of hot saline was added. The solution was stirred to mix the solution well while it was continuously heated on a steam bath. Then 2.0 g of MgO was added to the solution with continuous stirring for 5 minutes. The mixture was vacuum filtered and the volume of the filtrate was adjusted to 20 mL.

Hemolysis Test

About 2-3 drops of saline extract was placed on one cup of blood agar plate. To another cup, an equal volume of saline solution was added to serve as the control. The absence of a clear zone around the cups indicates that no hemolysis has taken place and that saponins are absent in the plant extract.

Froth Test

One-half of the saline extract (10 mL) was transferred to a 20-mL test tube, which was then stoppered and shaken vigorously. Using metric ruler, foam height was measured at the end of 3 minutes and 30 minutes after shaking. A foam height of 1 cm after 3 minutes and

the disappearance of the froth after 30 minutes indicates a negative result for saponins.

2.4.2 Screening for Tannins and Phenolic Compounds

From the 80% ethanolic extract, 100 mL was placed in an evaporating dish and dried on a steam bath. The residue was added with 25 mL of hot distilled water and stirred. The mixture was allowed to cool and centrifuged for several minutes. The supernatant was decanted from each tube used and added with 3-4 drops of 10% NaCl solution to precipitate non-tannin components. Any precipitate formed was filtered. Three clean small test tubes were prepared and placed 3 mL of the filtrate in each of the test tubes. To the first test tube, 2-3 drops of 1% gelatin solution was added; to the second test tube, same amount of gelatin-salt reagent was added; and to the last test tube several drops of $FeCl_3$ solution was added. Color changes in the three test tubes were observed. A negative result in the gelatin-salt block test associated with the production of a greenish black color after the addition of $FeCl_3$ solution indicated the absence of tannins and other phenolic constituents.

3. Bioactivity-Guided Isolation

3.1 Serial Extraction

The crude ethanolic extract concentrate was extracted sequentially with hexane, ethyl acetate, and ethanol using a separatory funnel. Washing the concentrate twice with the aforementioned solvents produced the desired volume of the crude extracts. When methanol extract was used, it was noted that the sap separated from the extract itself. Since, it was part of the whole sample; the sap was given consideration to be one of the test samples. It was dissolved in acetone and diluted to desired concentrations for subsequent test. Hence, there were a total of four extracts. Each crude extract was subjected to Brine Shrimp Lethality Test, and the extract that exhibited the most bioactivity was subjected to quick column chromatography and antimicrobial assay.

3.2 Gravity Column Chromatography

The crude extract with the most pronounced activity was subjected to first gravity column chromatography. Silica gel 60 Merck Cert. No. 7730 was packed into an acid burette. The packing material was packed to a height of 14 cm by gently tapping the glass funnel with a rubber-tipped rod. The dried sample with silica was placed uniformly on the surface of the column. It was eluted with solvents of increasing polarity as follows: pure hexane, hexane: ethyl acetate (3:1; 1:1; 1:3), pure ethyl acetate, ethyl acetate: methanol (3:1; 1:1; 1:3), and pure methanol, 2 x 20 mL of each solvent system was used. These isolates or fractions were subjected to antimicrobial assay for further isolation.

The fractions that did not exhibit bioactivity were discarded, while the fractions that exhibited bioactivity were pooled and subjected to second column chromatography. After the fractions with bioactivity were pooled, it was concentrated to dryness through the vacuo and placed it uniformly on the surface of the packed column. The same solvent systems used in the first column chromatography were also used in the second. There were 18 fractions collected that were subsequently subjected to antibacterial assay.

The third gravity column chromatography was done after pooling the fractions with bioactivity. Again, the same solvent systems were used. Consequently, nine (9) fractions were collected and subjected to antibacterial assay and Thin Layer Chromatography. Fractions with the same profile in the TLC were subjected to partial characterization using UV-VIS and Infrared spectroscopy.

3.3 Thin Layer Chromatography

This procedure utilized the silica- precoated plastic sheets. The fractions that were collected from the gravity column chromatography were concentrated in the vacuo. From the concentrate, a small amount was taken up using a capillary tube and was spotted on the TLC sheets. The solvent systems used were the corresponding solvent of the fractions (i.e. fraction eluted with pure methanol utilizes also methanol as its solvent). The solvent was then allowed to travel up to 10 cm to allow maximum separation of the possible components. The

R_f values were then computed. Fractions that exhibited similar profiles were subjected to spectroscopic methods.

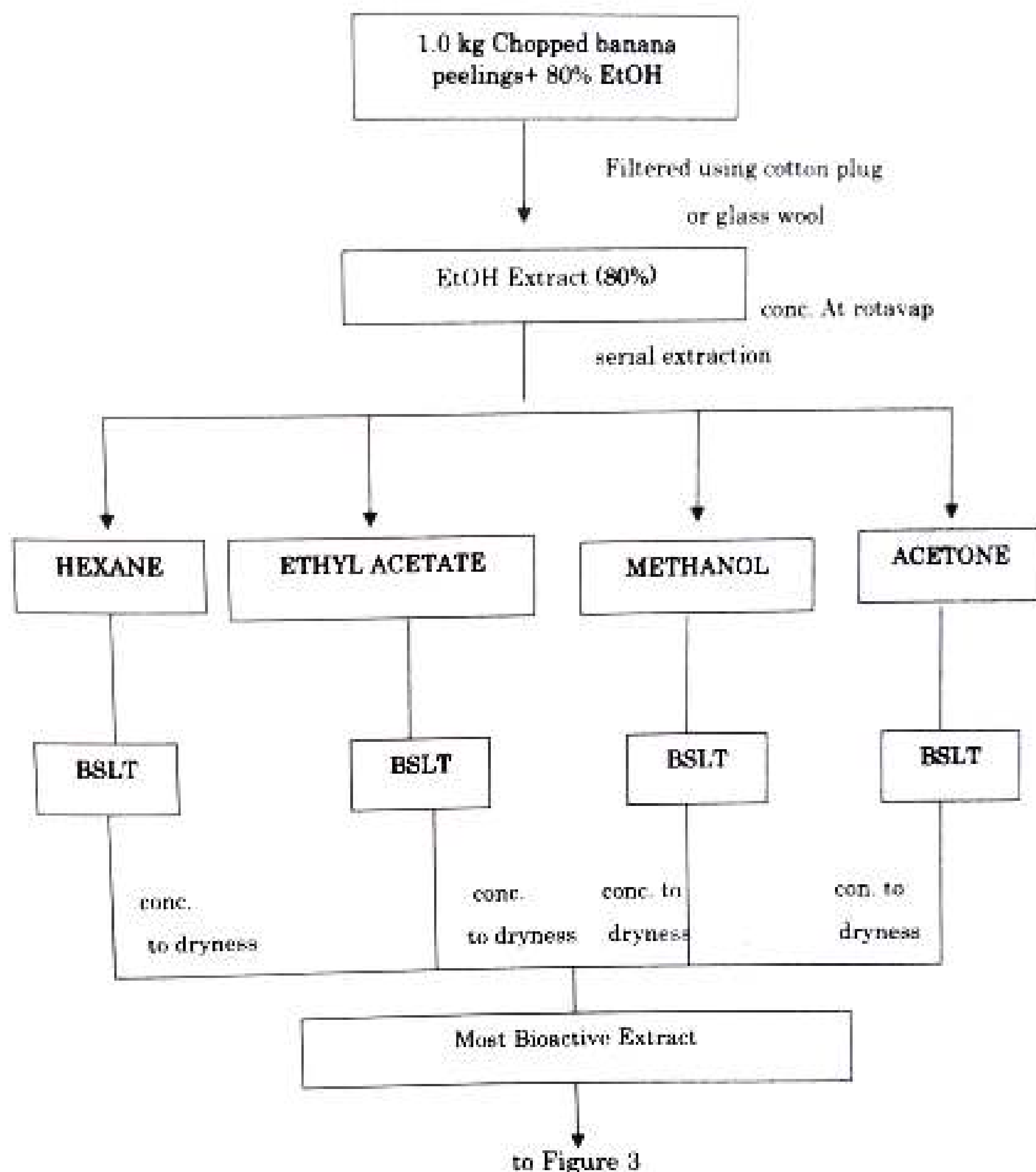

Figure 2. Serial Extraction Procedure

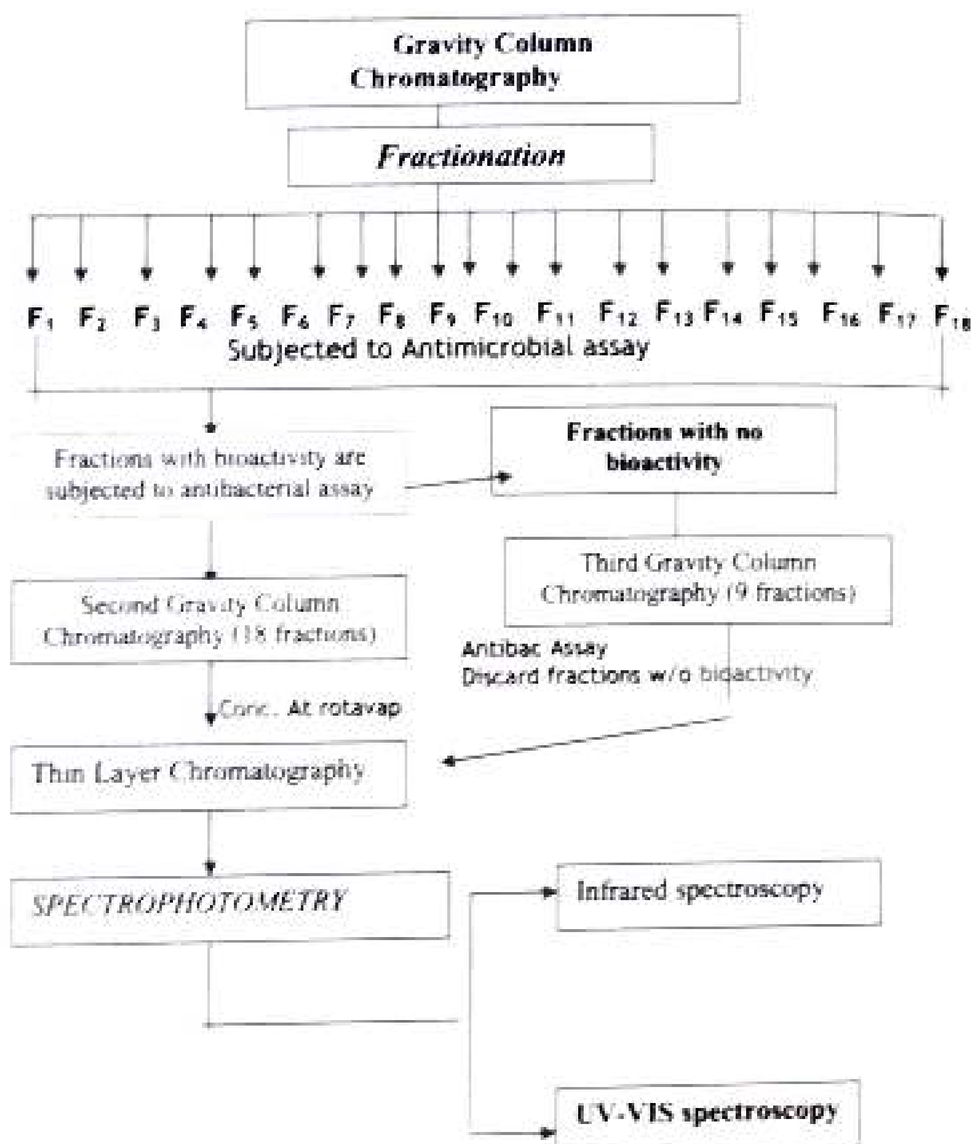

Figure 3. Isolation of the Bioactive Component From the Most Bioactive Fraction

4 Bioassay

4.1 Brine Shrimp Lethality Test (Meyer et. al., 1982)

- **Preparation of Test Samples**

A 1000-ppm solution was prepared by dissolving 5.0 mg of the dried extract from hexane, ethyl acetate, methanol, and acetone in 5.0-mL of their respective solvents. From these solutions, 1.0-mL aliquot was pipetted and diluted to 10 mL to make 100 ppm. Again, 1.0-mL aliquot was pipetted from each of the solutions and dilute to 10 mL to make 10 ppm. (see diagram below)

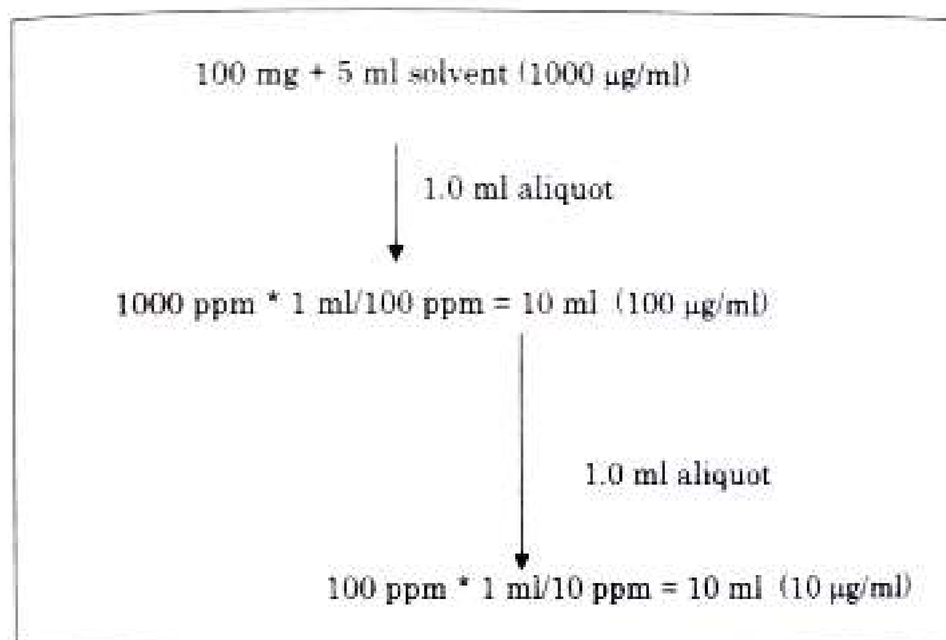

Figure 3.4. Preparation of Dilutions

- **Brine Shrimp Hatching**

Artificial seawater was prepared by dissolving 38 grams of ordinary table salt in one liter of water. Sufficient amount of the

prepared seawater was placed in a small tank with perforated divider. A pinch of brine shrimp eggs was placed in one compartment of the tank that was darkened. The other compartment was kept illuminated using a 10-watt bulb to attract the hatched shrimps (nauplii). A drop of yeast solution (3.0 mg yeast per 5.0 mL distilled water) was added to both sides of the tank as food for the shrimps. After 24 hours, the nauplii were transferred into the vials.

- **Actual Assay**

Approximately 0.5 mL of the test solutions was allowed to dry in a vial. Three replicates were prepared for each test solution. Ten active nauplii were placed in each vial and added with enough seawater 5 mL volume. A drop of yeast solution was added to each vial that serves as the food of the nauplii. After 24 hours, the number of dead shrimps in each vial was recorded.

4.2 Antibacterial assay of the *M. paradisiaca* Extract

- **Preparation of Nutrient Agar**

Soak 10.0 g of nutrient agar in distilled water and the solution was heated with constant stirring until nutrient agar was completely dissolved. The solution was placed in a volumetric flask and added with distilled water to reach the 1.0-L mark.

- **Preparation of Soft Nutrient Agar**

Dissolve 3.0 g of beef extract, 5.0 g of peptone and 5.0 g of pure agar in distilled water. The solution was then heated with constant stirring until ingredients were completely dissolved. The solution was added with enough water to reach the 1.0-L mark.

- **Preparation of Nutrient Broth**

Dissolve 3.0 g of beef extract and 5.0 g of peptone in distilled water. The solution was heated with constant stirring until ingredients were completely dissolved. Enough distilled water was added to 1.0-liter mark.

- **Preparation of Bacterial Suspension**

A loopful of bacteria was scooped and transferred to 20-mL culture tube containing 10.0 mL of nutrient broth. The culture tube was shaken for even distribution of the bacteria. The culture tube was then covered with a cotton plug and incubated at 37°C for 24 hours.

- **Actual Assay**

About 10-20 mL of nutrient agar was allowed to harden in sterile petri dishes. Into each petri dish, 0.1 mL of bacterial suspension was added. Approximately 5 mL of soft nutrient agar was also added to the petri dish. The overlay was allowed to set. Filter paper discs (6 mm diameter) were then dipped into the different extracts and isolates of rotavap-dried sample. Discs were also dipped into the positive control chloramphenicol, the solvent used (methanol), and the negative control (distilled water). After removing the excess liquid, the discs were arranged equidistantly on the hardened soft nutrient agar. The discs were wrapped and incubated in an upside down position at 37°C. After 24 hours, the zone of inhibition around the discs was measured. Three trials per microorganism were performed. The test microorganisms used were the *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, and *Proteus bulgaris*. Resting was done aseptically to minimize or avoid contamination.

Reading and Interpretation of Experimental Tests

After incubation, the presence or absence of circular zones was examined depending on the activity of the sample being tested.

Within limits, the diameter zones of inhibitions, which may or may not be knife-edged, were noted. Clear and well-defined inhibitory zones

around the disc were observed if the sample tested possesses antibacterial activity while failure of the test disc to exhibit zones of inhibition indicates absence of antibacterial effects.

The mean average diameter zones of inhibitions produced by each test sample in three replications were measured in terms of millimeters by means of a ruler. Control tests were likewise read.

5. **Anti-inflammatory Activity**

- **Preparation of Protein Solution**

Dissolve 4.0 g of casein or albumin in small amount of 0.01 M NaOH. The protein solution was then diluted to 400 mL of 0.9% saline solution, and the resulting 1% protein solution was stored in refrigerator.

- **Preparation of Saline Solution**

Dissolve 9.0 g of sodium chloride in distilled water. The solution was then added with enough distilled water to reach the 1-liter mark.

- **Actual Assay**

About 10.0 mL of 1% protein (casein/albumin) in saline solution was introduced into each of the eight test tubes. To each, 0.5 mL of the extracts with different concentration (10000 ppm, 1000 ppm, 100 ppm) was added leaving two of these test tubes blank; one for the casein, and the other for the albumin. The test tubes were warmed in a thermostat bath at a temperature of about 70°C for 20 minutes and were added with 0.3 mL of 1% HOAc. Thermal denaturation of proteins was noted by the turbidity of the solution and measured the absorbance levels using the UV-VIS spectrophotometer at 460 nm.

6. Spectroscopic Analysis

The spectroscopic methods include the Infrared and UV-VIS. The Infrared spectroscopy made use of the Thin Film Method, where two drops of the extract concentrate was allowed to dry on the surface of the KBr plate and have it analyzed using Infrared spectrophotometer (BUCK Scientific Spectrophotometer Model 500). The UV-VIS spectroscopy was analyzed using a double beam spectrophotometer (Shimadzu UV-160A).

Results and Discussion

Table1 Results of Phytochemical Screening

Possible Components	**Remarks**
Alkaloids	Absent
Anthraquinones	Absent
Unsaturated Sterols and Triterpenes	Absent
Flavonoids	Present
Saponins	Present
Leucoanthocyanins	Present
Tannins	Present

The table above shows the summary of phytochemical screening results. It is indicated by the presence or absence of some possible bioactive components in the sample *M. paradisiaca*.

Alkaloids were found to be absent. The minimal color change was due to the iodine content in the Wagner's reagent but no precipitation was noted. Anthraquinones, unsaturated sterols and triterpenes were also found to be negative, since there were no color changes or formation of precipitate when added with reagents.

However, leucoanthocyanins, flavonoids, saponins and tannins are found to be present in the sample. There were marked color changes and heavy precipitation noted. The presence of leucoanthocyanins, flavonoids, saponins and tannins in the sample *M. paradisiaca* implies their possible contribution to taste and flavors of food (flavonoids) and

their medicinal potential as antiviral, antifungal, anti-inflammatory, and astringent.

Table 2. Results of Brine Shrimp Toxicity Test (% Mortality)

TEST EXTRACT	Percent Mortality*			
	1000 ppm	100 ppm	10 ppm	Control
Hexane	66.67	56.67	33.33	0.00
Ethyl Acetate	73.33	63.33	36.67	0.00
Methanol	90.00	73.33	63.33	0.00
Acetone	43.33	26.67	10.00	0.00

*Average of three trials

The table above shows the Brine Shrimp Lethality Test (BSLT) of the extracts from serial extraction of the ethanolic extract using hexane, ethyl acetate, methanol, and acetone. The BSLT results are expressed in percent mortality at varying concentrations of 1000 ppm, 100 ppm, and 10 ppm, respectively. It is noted that among the four extracts, methanolic extract exhibits the greatest number of dead brine shrimps. This implies that the methanolic extract has the most bioactivity with an average percent mortality ranging from 63.33 to 90.00 at the given concentrations.

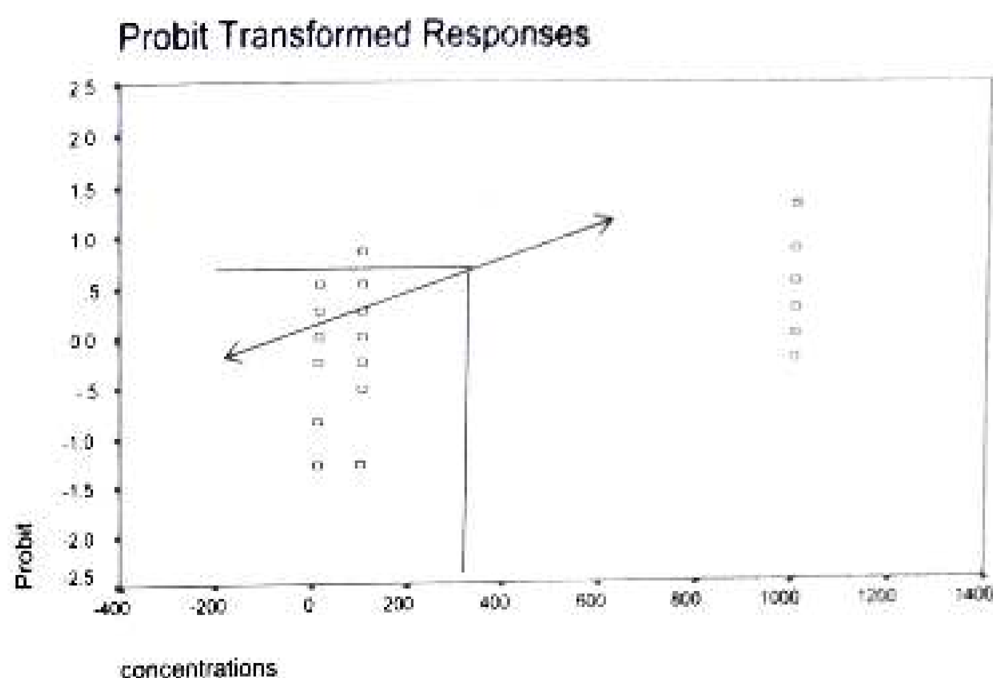

Fig. 4.1 Median Lethal Concentration of Brine Shrimp Lethality Test

The data obtained in brine shrimp lethality test was treated statistically to determine the median lethal concentration (LC_{50}), using Probit analysis at 95% level of confidence to allow comparison of data from the different tests. The analysis showed confidence limits for effective concentration, and it reveals that the concentration estimated to be lethal to the 50% of the total test population after 24 - hour exposure (to the different concentrations) is at 244.94 ppm.

This implies that one-half of the total number of brine shrimps used in the BSLT dies at 244.94 ppm.

Table 3. Anti-inflammatory Activity of *M. paradisiaca* Extract in 1% Protein

Concentration (ppm)		ALBUMIN		CASEIN	
		Absorbance	%Anti-inflammatory	Absorbance	%Anti-inflammatory
Methanolic Extract	0	0.542	60.87	0.138	32.85
	100	1.076	22.17	0.158	23.14
	1000	0.854	38.33	0.150	27.02
	10,000	0.511	63.13	0.092	55.50
Voltaren (control)	0	1.539	-8.94	0.158	30.70
	100	0.643	54.47	0.178	21.78
	1000	0.607	57.01	0.181	20.61
	10,000	0.603	57.30	0.139	39.03

Treated protein, casein 0.228 Untreated protein 0.129
Treated protein, albumin 1.413 Untreated protein 0.396

The table above shows the anti-inflammatory activity of the *M. paradisiaca* extract. It can be seen that the extract has lower absorbance levels compared to the control. This is because the extracts prevent the denaturation of proteins resulting to less turbid solutions that consequently display lower absorbance levels. These data further imply that the lower the absorbance levels the higher the anti-inflammatory activity. These data are supported with the following figures below.

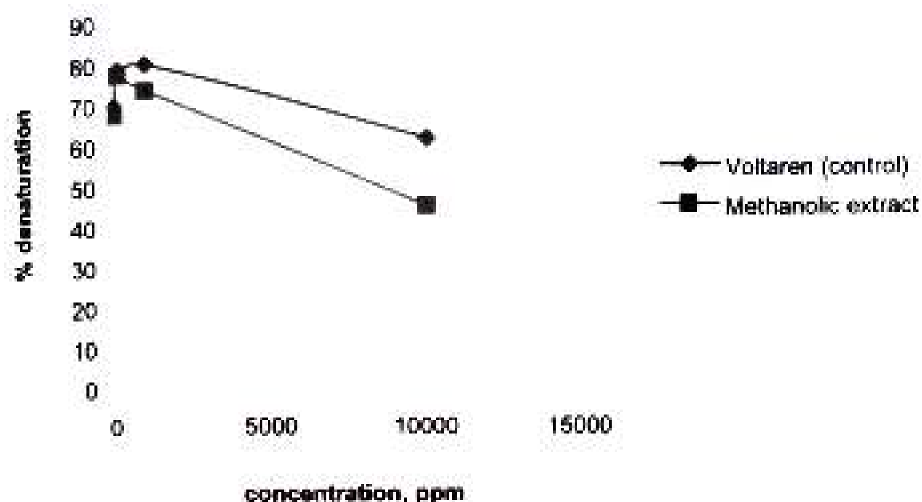

Figure 4.2. Percent Denaturation of Methanolic Extract and Voltaren in Casein

Protein structure is sensitive to environmental factors. Many physical and chemical agents can disrupt a protein native conformation. Depending upon the degree of denaturation, the molecule may partially or completely lose its biological activity. Figure 4.1 shows both the percent denaturation of the control (voltaren) and the methanolic extract in casein solution. It significantly shows that the methanolic extract has lower percent denaturation both in lower and higher concentration. It implies that there is a low protein disruption in the protein structure, thus, it can be inferred that anti-inflammatory activity of the methanolic extract is better compared to the standard both in the lower and higher concentrations. Hence, methanolic extract is a possible potent alternative to anti-inflammatory agent voltaren.

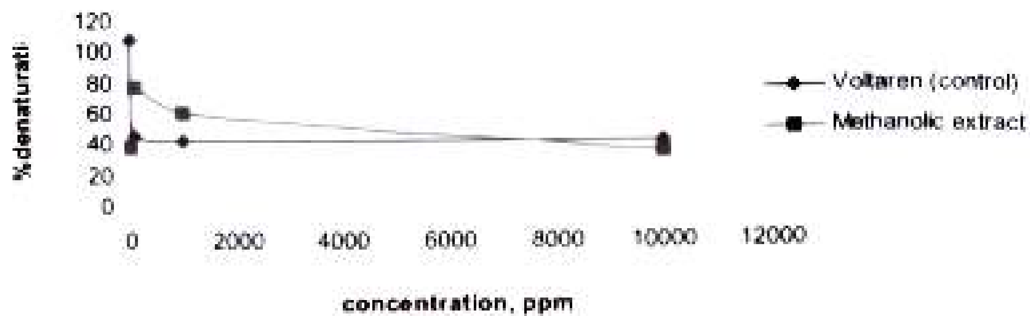

Figure 4.3. Percent Denaturation of Methanolic Extract and Voltaren in Albumin

The figure above shows that methanolic extract has lower percent denaturation over voltaren in higher concentration only. It is noted that the control has a better anti-inflammatory activity at lower concentrations evidenced by lower percent denaturation. Thus, it can be inferred that the extract is a potent alternative as an anti-inflammatory agent in albumin solution at higher concentration only (10000 and above).

Table 4.4. Zones of Inhibition (mm) in the Antibacterial Assay of the First Gravity Column Chromatography.

FRACTION	BACTERIAL SPECIES			
	S. aureus	*B. subtilis*	*P. bulgaris*	*E. coli*
1	0.0	0.0	0.0	0.0
2	0.0	0.0	0.0	0.0
3	7.0	7.0	0.0	0.0
4	0.0	0.0	0.0	7.0
5	8.0	9.5	8.0	9.0
6	8.5	8.0	8.0	8.5
7	0.0	7.0	7.0	0.0
8	0.0	0.0	0.0	0.0
9	8.5	8.0	8.0	8.0
10	8.0	8.0	8.0	9.0
11	9.0	8.5	9.5	8.0
12	8.0	9.0	9.0	8.5
13	8.5	9.5	8.0	8.0
14	8.0	8.0	8.0	9.0
15	8.5	8.5	9.5	8.0
16	9.0	9.0	8.0	8.5
17	8.0	8.5	8.5	9.5
18	8.5	8.0	8.0	9.0
Negative Control				
Water	0.0	0.0	0.0	0.0
Methanol	0.0	0.0	0.0	0.0
Positive Control				
Amoxicillin	36.5	14.5	27.0	15.0
Chloramphenicol	26.0	20.0	23.0	22.0

The antimicrobial assay of the first quick column chromatography shows that fractions 5, 6, 9, 10, 11, 12, 13, 14, 15, 16, 17, and 18 displayed significant antibacterial susceptibility to strains of *S. aureus*, *B. subtilis*, *P. bulgaris* and *E. coli* with zones of inhibition that ranges from 8.0 to 10.0 mm, respectively. These fractions with bioactivity were then pooled, concentrated and subjected to second column chromatography while the fractions with no bioactivity were discarded.

Table 4.5. Zones of Inhibition (mm) in the Antibacterial Assay of the Second Gravity Column Chromatography

FRACTION	BACTERIAL SPECIES			
	S. aureus	*B. subtilis*	*P. bulgaris*	*E. coli*
1	0.0	0.0	0.0	0.0
2	0.0	0.0	0.0	0.0
3	0.0	0.0	0.0	0.0
4	0.0	0.0	0.0	0.0
5	9.0	9.0	9.5	9.0
6	8.5	9.0	10.0	9.0
7	0.0	0.0	0.0	0.0
8	0.0	0.0	0.0	0.0
9	9.0	10.0	9.5	9.5
10	10.0	9.5	10.0	10.0
11	11.0	10.5	10.0	9.5
12	10.0	11.5	9.5	9.5
13	11.0	11.0	9.5	10.0
14	10.5	10.0	10.0	10.0
15	11.5	9.5	11.0	11.0
16	10.0	10.0	10.5	10.5
17	10.5	10.0	10.0	10.0
18	11.0	11.5	11.0	10.5
Negative Control				
Water	0.0	0.0	0.0	0.0
Methanol	0.0	0.0	0.0	0.0
Positive Control				
Amoxycillin	37.0	14.0	29.0	12.0
Chloramphenicol	27.0	26.0	22.0	23.0

The results in the second column chromatography were subjected to antibacterial assay and the results are found to be similar with the first antibacterial assay. Fractions 5, 6, 9, 10, 11, 12, 13, 14, 15, 16, 17 and 18 were the same fractions having pronounced bioactivity with zones of inhibition that ranges from 8.5 to 11.5 mm. Again, fractions with no bioactivity were discarded while the fractions with bioactivity were pooled, concentrated and subjected to third column chromatography.

Table 4.6. Zones of Inhibition (mm) in the Antibacterial Assay of the Third Gravity Column Chromatography

FRACTION	BACTERIAL SPECIES			
	S. aureus	*B. subtilis*	*P. bulgaris*	*E. coli*
4	12.0	11.5	12.0	12.5
8	12.5	12.0	10.5	14.0
9	14.0	13.0	11.0	13.5
Negative Control				
Water	0.0	0.0	0.0	0.0
Methanol	0.0	0.0	0.0	0.0
Positive Control				
Amoxycillin	37.0	14.0	29.0	12.0
Chloramphenicol	27.0	26.0	22.0	23.0

Nine (9) fractions were collected from the third column chromatography, which were subsequently subjected to Thin Layer Chromatography after each fraction has been concentrated. Results reveal that fractions 4, 8 and 9 have the same TLC profile characterize by a single spot with minimum tailing. These isolated fractions were subjected to antibacterial assay and results show that they exhibit very significant susceptibility to *S. aureus, B. subtilis, E. coli and P. bulgaris.* Comparing the first, second and third antibacterial assay, it is evident that the zones of inhibition increases. This simply shows that as the extract gets purer, its bioactivity increases.

Table 4.7. Thin layer Chromatography Profile

Isolated Fraction	Solvent System	Color	R_f Value
F_4	Pure EtOAc	Light yellow	0.428
F_8	EtOAc:MEtOH (1:3)	Yellow	0.434
F_9	Pure MEtOH	Dark Yellow	0.535

The most bioactive fractions obtained from the second chromatography and antimicrobial assay were subjected to Thin Layer Chromatography (TLC) to note their profiles and possible presence of other components.

The Retention Factor (R_f) of the isolates was then calculated using the equation below:

$$R_f = \frac{\text{Distance traveled by the isolate}}{\text{Distance traveled by the solvent}}$$

Note that the R_f values increases from pure ethyl acetate to methanol. This only shows that the eluting power of the solvents increases in the order of increasing polarities, i.e. pure ethyl acetate < EtOAc: MEtOH (1:3) < Pure Methanol. It further implies that the extract or isolate can be eluted best in a more polar solvent (which is the methanol) than in a less polar one.

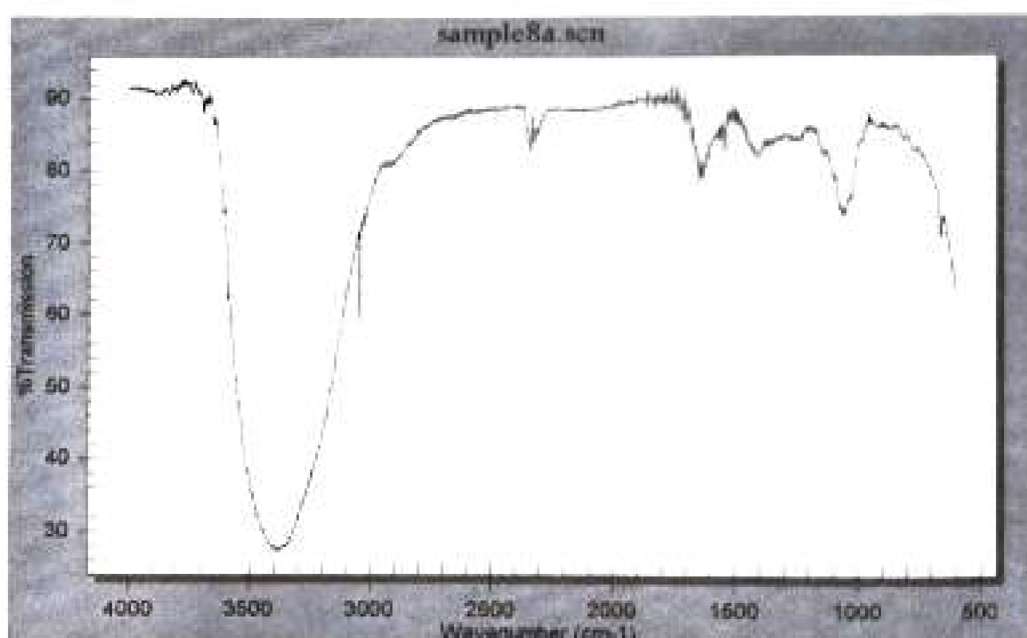

Figure 4.4. Infrared Spectrum of the Most Bioactive Fraction

The fraction with the most bioactivity was further analyzed using spectroscopic methods. The spectrum may show presence of impurities, but the characteristic absorption peaks of the most bioactive fraction can

still be figured out. As shown, strong, broad band from an O-H stretching absorption in the region 3200 – 3600 cm^{-1}. Also, spectrometric identification of organic compounds (Silverstein, 1981), the C-H stretching bands of aromatic ring absorb at 3050 cm^{-1}, C≡C stretching at 2330 cm^{-1} and C=C stretching at 1600 to 1650 cm^{-1} (feature of double-bond vibrations) that probably influences the possibility of a conjugated compound. Another broad band, due to aliphatic C-O stretching that appears in 1050 cm^{-1}. Thus, it is initially inferred that the bioactive fraction has a certain degree of unsaturation.

The most important implication is, despite the presence of impurities in the analyte as evidenced by the corrugations in the spectrum, it can still be inferred that an aliphatic –OH is positively present, this is confirmed by the appearance of aliphatic C-O stretch at 1050 cm^{-1}, which led to hypothesize that the sample is a conjugated alcohol with a benzene ring.

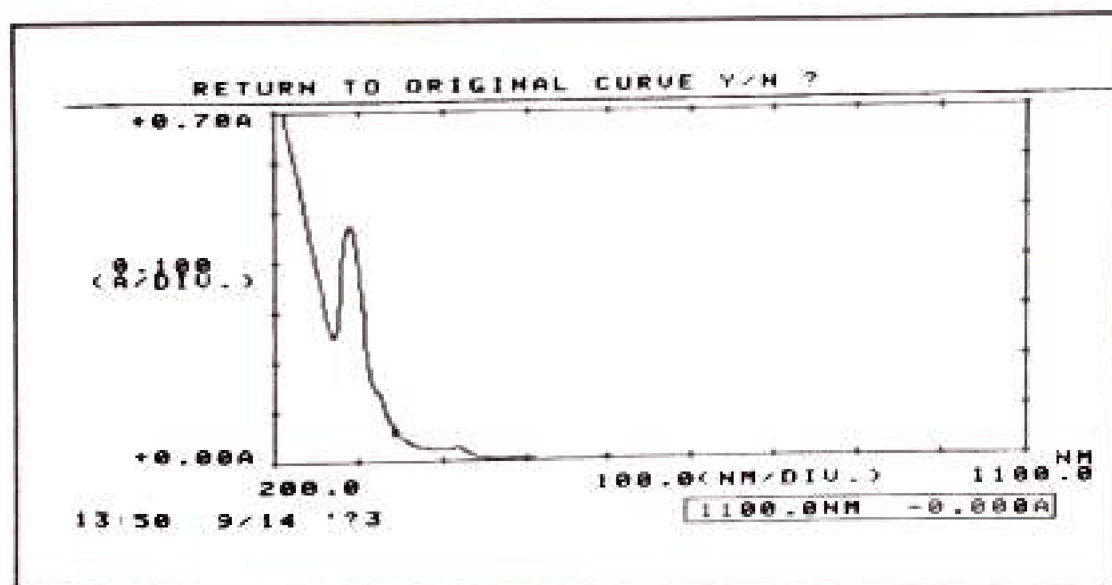

Figure 4.5. UV Spectrum of the Most Bioactive Fraction

A typical molecule has a very large number of different energy states and, is capable of absorbing many different wavelengths of radiation. The UV-VIS spectrum (as shown above) shows a peak at λ_{max} 290 nm indicating that the structure of the bioactive fraction may consist of several conjugated double bonds.

Summary and Conclusions

The crude ethanolic extract of M. paradisiaca that was used in the phytochemical screening contains leucoanthocyanins, saponins, flavonoids and tannins. Their presence would open the possibilities of their application in food flavors and colors, as well as in the medicine sphere being an antibacterial.

The methanolic extract was found to be the most bioactive fraction far better than hexane, ethyl acetate and acetone extracts. It showed the highest percent mortality that ranges from 63.33 to 90.00 at concentrations that varies from 10 ppm to 1000 ppm in the Brine Shrimp lethality Test.

The anti-inflammatory activity of the extract was determined through percent denaturation employing the Protein Stabilization Method. The methanolic extract exhibited lower percent denaturation of protein over voltaren (control) both in lower and higher concentrations in the casein solution. This implies that methanolic extract exhibited higher anti-inflammatory activity in casein solution in varying concentrations. On the other hand, methanolic extract exhibited lower percent denaturation over voltaren only in higher concentration in the albumin solution. This implies that the methanolic extract is found to be effective anti-inflammatory agent in albumin solution only in higher concentrations.

The sample from M. paradisiaca used in the Antibacterial Assay displayed toxicity evidenced by its susceptibility to strains of Staphylococcus aureus, Bacillus subtilis, Escherichia. Coli and Proteus bulgaris. Antibacterial assay proved its possible medical efficacy in the treatment of diseases like bronchitis, dysentery, inflammatory diseases, and etc.

The spectroscopic analyses showed that the presence of the impurities present in the sample have modified or influenced the peaks in the spectra, hence it is not conclusive. However, it is for certain that the bioactive fraction contains an aliphatic O-H stretch, C – H stretch for aromatic ring and C-O stretch evidenced by the absorption peaks in regions 3200 – 3600cm^{-1}, 3050 cm^{-1} and 1050 cm^{-1}, respectively. The UV-VIS showed a peak at λ_{max} 290, which led to infer that the structure of the bioactive fraction may consist of several conjugated double bonds. With

the IR and UV-VIS spectra, it can be hypothetically believed that the bioactive fraction may be conjugated alcohol with a benzene ring.

Recommendations

1. Similar study be conducted using the same plant part but of another species (maybe, a less sappy fruit) to verify its result.
2. Further isolation and purification be done to get the desirable IR spectrum for better characterization, and that, identification of functional groups would be conclusive.
3. Determine other physical properties of the extract like the pH, boiling point, specific gravity, etc.
4. The quantity of the sample should be measured before and after performing the procedures to determine the percent recovery.
5. Screening for toxicity, antibacterial and antifungal properties of other parts of banana such as pseudostem (trunk), flowers, leaves and even roots to verify its folk-medicinal uses.
6. To conduct other bioassays such as immunomodulatory, antineoplastic, antiviral, genotoxic activity, etc.

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