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In-Vitro Determination of the Cytotoxicity and Antiproliferative Activity of the Semi-Purified Flavonoids from the Bauhinia Malabarica (Alimbangbang) Leaves Extract against Selected Cancer Cell Lines

Shiru F*, Jimenez JRM, Rodriguez AA

Department of Medical Oncology, University of Santo Tomas, Manila, Asia

***Corresponding author:** Shiru F, Department of Medical Oncology, University of Santo Tomas, Manila, Asia, E-mail: djiuen@163.com

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Abstract

The study aims to determine the cytotoxicity and anti-proliferative property of the semi-purified flavonoids of Bauhinia malabarica. In order to achieve this, the plant material undergone various experiment and tests. 80% Ethanol was used in the extraction process through maceration. The crude extract gave a brownish yellow liquid with characteristic odor. The semi-purification process was carried out giving a brownish black dried sample. Shinodas, Sodium hydroxide, Ethyl acetate, and lead acetate was used for confirmatory testing to determine the presence of flavonoids .Results showed a 0.24% of percent yield of flavonoids obtained from the 500-gram plant sample. FT-IR results showed absorbance with functional groups of alkane, acid, and aromatic bonding. HPLC results showed the active constituent percent in the extract is quercetin with 0.99%. Brine Shrimp Assay showed significant results against nautili eggs. Unfortunately, the MTT Assay in both cancer cell lines revealed inactive against MCF-7 and A549.

Keywords: Bauhinia Malabarica, Cytotoxic, Cancer Chemotherapy, Doxorubicin, Dimethylsulfoxide (DMSO), LC50, MTT Assay

Introduction

The Philippine Society of Medical Oncology stated that breast cancer is common in the Philippines that 1 in every 13 Filipinas is expected to develop it in the future. Moreover, the Philippine government has identified the highest incidence rate of breast cancer. Whereas, lung cancer is also the top cause of men's cancer-related deaths and cervical cancer is the third cause of women's cancer deaths in the Philippines. According to the related study, health experts stated that 10 Filipinos die of smoking-related diseases every hour. The data from World Health Organization show that there are 8518 lung cancer deaths in the Philippines.

Cancer is the most difficult and complicated medical problem in the world. The breast and lung cancer are the two major types of cancer leading the cancer death all around the world. Most of the time, cancer patients use chemotherapy to slower the growth of the cancer cells [1]. Up to now, there is no effective medicine to kill the cancer cells and cure the cancer. As for China, Chinese people choose different kinds of herbs to cure cancer diseases.

According to the data published by the World Health Organization, cancer is the second death related disease all around the world. There are 8.8 million cancer deaths. Studies showed that lung, liver cancer, stomach, prostate, colorectal, are the most common types of cancer in men. While the common types of cancer for woman is breast, lung, colorectal, cervix and stomach cancer.

With the above condition, the researchers are discerning to determine the cytotoxicity and anti-proliferative property of a plant which could be a good source of drug to solve the problem. The name of the plant is Bauhinia malabarica. It is also calling it Alimbangbang in the Philippines.

Bauhinia malabarica is a small deciduous tree. It is mainly distributed in the sub- Himalayan tracts. Bengal, Assam and south India are the most popular regions for Bauhinia malabarica. Bauhinia malabarica usually grows in the open region which has a long dry season. In the Philippines, Luzon is the ideal place for this plant. There is 1000 to 3000 rainfall in this region every year. The leaf of Bauhinia malabarica has many uses. It is consumed in Thailand, Philippines and other South Asia countries.

For medical uses, the leaves of Bauhinia malabarica could be used for curing headache, fever and wound healing. It is a good emmenagogue. For edible uses, it is a flavoring agent. The acrid leaves can eat together with the meat and fish. For the mineral content, the leaf of Bauhinia malabarica is the prominent source of iron and calcium. Aside from the leaves, in which young shoot can be eaten . It also has many medical uses. It is clearly know that it can treat the worm infection, wounds, scrofula, leprosy, wasting diseases and so on.

Materials and Methods

Plant Material Extraction and Isolation

The leaves of Bauhinia malabarica were collected in Antipolo City, Philippines. The leaves were air-dried at room temperature after collection for 48 hours. The dried sample was pulverized using a pulverizer. Dried and pulverized leaves sample was macerated in 80% ethanol for 72 hours and filtered by using Whatman filter paper. After filtration, crude extract was evaporated under the Rotary Evaporator and was obtained for phytochemical screening.

Computation of the percentage yield

The collected semi-purified flavonoids were weighed and the percentage yield was computed based on the formula.

$$\%yield = \frac{Weight\ of\ semi\text{-}purified\ flavonoids}{Weight\ of\ dried\ sample} * 100$$

Semi-purification of flavonoids

The crude extract of Bauhina malabarica was evaporated into incipient dryness using a water bath. The sample was dissolved in 2 M HCl and ethyl acetate until the extract became colorless. The ethyl acetate layer and aqueous layer were separated using a separatory funnel. The ethyl acetate layer was collected and evaporated into incipient dryness. The dried residue was the semi-purified flavonoids. The presence of flavonoids was confirmed by adding 80% ethanol.

Confirmatory test for flavonoids

Shinoda's test: Three pieces of Magnesium turnings and few drops of concentrated HCL were added to the semi-purified flavonoids [2]. The pink, orange, or red to purple coloration indicates the presence of flavonoids.

Sodium hydroxide test: Two ml of the 10% aqueous sodium hydroxide was added to produce a yellow coloration. Dilute hydrochloric acid was added. The color changed from yellow to colorless was an indication for the presence of flavonoids.

Ethyl acetate test: Ten mL ethyl acetate was added to the sample. Steamed bath for 3 minutes. Then added 4mL of the filtrate, 1mL diluted ammonia solution and stirred. Yellow coloration of the solution indicated the presence of flavonoids.

Lead acetate test: 1.2 mL of lead acetate was added and shacked. The formation of flesh-brown colored precipitate indicated the presence of flavonoids.

Physicochemical and instrumental analysis

The physical and chemical properties of the leaf extract of Bauhinia malabarica was evaluated through organoleptic analysis, solubility, and specific gravity. Instrumental Analyses was conducted through Fourier Transform Infrared Chromatography and High Performance Lipid Chromatography.

Organoleptic analysis: The crude extract and the semi- purified flavonoids of Bauhinia malabarica (Alimbangbang) underwent subjective evaluation of color, appearance, odor, and texture.

Solubility testing: The semi-purified flavonoids of Bauhinia malabarica was evaluated its solubility properties by using several common solvents such as water, alcohol, chloroform, ether, hexane, and acetone.

Fourier transfer infrared spectroscopy: Fourier Transform Infrared spectroscopy (FTIR) is a technique which is used to obtain an infrared spectrum of absorption or emission of a solid, liquid or gas. An FTIR spectrometer simultaneously collects high spectral resolution data over a wide spectral range. The semi-purified flavonoid of Bauhinia malabarica was subjected to Fourier Transform Infrared Chromatography to determine the presence of functional groups present in absorbance units. The procedure of the FTIR includes Sample Preparation. 1mg of powered sample with 50 mg of dry KBr was placed in the machine and sprinkled the grinded sample plus the KBr on top of the mirrored disk and ensured even coverage, gently inserted the other mirrored disk with its mirror down and inserts the metal piston rod. Gently push it into the shaft until it stops [3]. The constructed pellet maker was placed into the center of the press and hard-frighten the screw when the render of the graph was completed resulted.

High performance lipid chromatography: Analysis of extract and standard was performed by high profile lipid chromatography. The mobile phase was MeOH: H2O (75:25 v/v) at flow rate of 1.0 mL/min. The absorbance was monitored at λ =370 nm. It was needed to know the basis method to quantify the flavonoid. The High Performance Lipid Chromatography is the most useful method to identify the quantity of the flavonoids. The detector of the High Performance Lipid Chromatography was Shimadzu SPD-10AV. The pump is Shimadzu LC- 20AT. The Column was GL Sciences Inertsil ODS-3V RP, 4.6 × 150 mm, 5µm. The injection volume was 20 µL. The sample is 1.0 mg of Bauhinia malabarica in 10 mL H2O.

Bri ne shrimp lethality assay: Brine shrimp eggs were placed in a beaker filled with artificial seawater (38 grams of rock salt in distilled water) which served as a makeshift hatching chamber. Brine shrimp eggs were placed in the water and the set-up was exposed to constant light and aeration via an oxygen pump. Two days were allotted for the eggs to mature into Nauplius. Potassium dichromate in artificial seawater was prepared and used as the positive control. The negative control used was artificial seawater. Two (2) mL of the negative control, the positive control, and the different concentrations of the extract (100 ug/ml, 50 ug/ml, 25 ug/ml, 12.5 ug/ml, 6.25 ug/ml and 3.125 ug/ml) were placed in a corresponding well in a 24- well culture plate with each treatment having 6 replicates. Ten newly hatched brine shrimp nauplii were placed in each well. After 24 hours, the number of surviving and dead nauplii was tallied. A plot of the concentration versus the mortality rate was constructed. The LC50 was calculated from the plot previously generated for the crude test substance. This corresponded to the concentration in which mortality rate is at 50%. This was performed two more times, thus, three trials.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay: The MTT cytotoxicity assay performed in this study was adapted. In detail, cells were seeded at 4 or 6 × 104 cells/mL (depending on the cell culture used) in sterile 96-well microtiter plates. The plates were incubated overnight at 37°C and 5% CO2.

Eight two-fold dilutions of the sample were used as treatments starting from 100 µg/mL down to 0.78125 µg/mL. Doxorubicin served as positive control while Dimethyl Sulfoxide (DMSO) served as negative control. Following incubation, cells were treated with each extract dilution. The treated cells were again incubated for 72 hours at 37°C and 5% CO2.

After incubation, the media was removed and 3-(4,5-dimethylethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) dye at 5 mg/mL PBS was added. The cells were again incubated at 37°C and 5% CO2 for 4 hours. After which, DMSO is used to dissolve the formazan crystals formed by the reduction of the dye by the live cells. Absorbance was read at 570 nm. The Inhibition Concentration 50 (IC50) was computed using Graph Pad Prism 6. Graph Pad Prism 6 computes for the IC50 of the sample by employing non-linear regression curve fit on the computed percent inhibition per concentration of the sample.

Results and Discussion

Physicochemical and Instrumental Analysis

The results of the organoleptic analysis of Bauhinia malabarica (Alimbangbang) crude extract is shown on Table 1. It shows that the crude extract was brownish yellow liquid obtaining its characteristic odor [4]. In texture, the crude extract was non-viscous.

The semi-purified flavonoids extract is brown black in color, has a characteristic odor, and is sticky in appearance. The results of the extract is show in (Tables 1 and 2).

Properties	Results
Appearance	Brownish yellow liquid
Color	Brownish yellow
Odor	Characteristic odor
Texture	Non-viscous liquid

Table 1: Organoleptic Analysis of the crude extract of Bauhinia malabarica.

Properties	Results
Appearance	Brownish black
Color	Brownish black
Odor	Characteristic odor
Texture	Dryness

Table 2: Organoleptic Analysis of the semi-purified flavonoids of Bauhinia malabarica.

Confirmatory test results

The extract shows positive result in all of the confirmatory tests done indicating the presence of flavonoids in the sample (Tables 3 and 4).

Test	Actual Results
Shinoda's Test	Dark Red Violet
Sodium Hydroxide	Colorless Solution
Test	
Ethyl Acetate Test	Yellow Coloration
Lead Acetate Test	Flesh Precipitate

Table 3: Confirmatory test results for the presence of flavonoids.

Solubility testing

Solvents	Remarks
Water	Insoluble
Ethanol	Soluble
Chloroform	Partially soluble
Ether	Soluble
Hexane	Partially soluble
Acetone	Soluble

Table 4: Solubility Testing result of the Bauhinia malabarica (Alimbangbang).

Percentage yield

$$\%yield = \frac{1.2\ grams\ of\ semi-purified\ flavonoids}{500\ grams\ of\ plant\ sample} * 100$$

$$\%yield = 0.24\%$$

Fourier transform infrared chromatography

The semi-purified flavonoids of Bauhinia malabarica (Alimbangbang) were subjected to Fourier Transform Infrared Chromatography to determine the presence of functional groups present in absorbance units.

The result shows that the semi-purified flavonoids of Bauhinia malabarica (Alimbangbang) contain alkane bonding, acid and aromatic bonding, which indicated the presence of flavonoids (Tables 5-7).

Wavelength	Functional groups
2923.72	Alkane (C-H)
1709.68	Acid (RCOOH)
1605.48	Aromatic (C=C) bonding
1162.2	Alkane (C-H)

Table 5: FT-IR Chromatography of the semi-purified flavonoids of Bauhinia malabarica (Alimbangbang).

High performance lipid chromatography

Trial	Ret. Time	Area	Height
1	2.74	149714	9187.43
		4	
2	2.81	151461	9107.13
		6	
3	2.74	150150	9335.86
		3	
*Note: Bauhinia malabarica at 10.60ppm, D=2908.62,RSD=0.99%.			

Table 6: Result of the HPLC of 1.0mg of the Semi-purified flavonoids of Bauhinia malabarica (Alimbangbang) extract in10 ml water.

Concentrati on	Total number of dead naupilii eggs	% Mortality
100	24.7	0.855
50	21.9	0.719
25	15.7	0.608
12.5	8	0.391
6.25	1.7	0.065

3.125	0	0

Table 7: Brine Shrimp Assay using a number of Surviving Nauplii after 24 hours.

Statistical treatment

The statistical analysis of the MTT Assay of the semi- purified flavonoids of Bauhinia Malabarica (Alimbangbang) against A549 and MCF-7 cell lines are under One-way ANOVA and Tukey's Post Hoc Analysis. For trial 1, there is significant difference in the mean absorbance readings of three treatments ($F_{2,45}=28.176$, $p<0.0001$). For trial 2, there is significant difference in the mean absorbance readings of three treatments ($F_{2,45}=4.683$, $p<0.0001$). For trial 3, there is significant difference in the mean absorbance readings of three treatments ($F_{2,45}=31.980$, $p<0.0001$).

Tukey's post hoc analysis

Positive control is significantly different to negative control across three trials. Extract is significantly different to negative control in trial 2 and 3 with mean difference of 0.21 and 0.17, respectively. It is noticeable that in trial 1 extract is not significantly different to negative control with mean difference of 0.17. Extract is significantly different to positive control across three trials. Therefore, there is no significant difference in the antitproliferative activity of the positive control (Doxorubicin) and the semi-purified flavonoids of Bauhinia malabarica (Alimbangbang) leaves extract against human breast cancer (MCF-7) cell line and human lung cancer (A549) cell line.

One way Anova (MCF-7 CELLS)

For trial 1, there is significant difference in the mean absorbance readings of three treatments ($F_{2,45}=13.576$, $p<0.0001$). For trial 2, there is significant difference in the mean absorbance readings of three treatments ($F_{2,45}=40.467$, $p<0.0001$). For trial 3, there is significant difference in the mean absorbance readings of three treatments ($F_{2,45}=20.878$, $p<0.0001$).

Tukey's post hoc analysis

Positive control is significantly different to negative control across three trials. Extract is significantly different to negative control in trial 2 only with mean difference of 0.16. It is noticeable that in trial 1 and 3 extract is not significantly different to negative control with mean difference of 0.04 and 0.13, respectively. Extract is significantly different to positive control across three trials. Therefore, there is no significant difference in the anti-proliferative activity of the negative control (DMSO) and the semi-purified flavonoids of Bauhinia malabarica (Alimbangbang) against human breast cancer (MCF-7) cell line and human lung cancer (A549) cell line.

The statistical analysis of the Brine Shrimp Assay of the semi-purified flavonoids of Bauhinia Malabarica (Alimbangbang) under Probit regression. This procedure measures the relationship between the strength of a stimulus and the proportion of cases exhibiting a certain response to the stimulus [5]. It is useful for situations where you have a dichotomous output that is thought to be influenced or caused by levels of some independent variable(s) and is particularly well suited to experimental data.

The LC50=11.916ug/ml of the extract. The 90% confidence interval is 10.754ug/ml to 13.172ug/ml. The result of statistical analysis shows that at 11.92ug/ml of the extract, 50 % of the observations will respond to the treatment. It answers that the minimal concentration (LC50) of the semi-purified flavonoids of Bauhinia malabarica (Alimbangbang) exerts its cytotoxic activity against Artemia salina (Brine shrimp) using the Brine Shrimp Lethality Assay is 11.92 ug/ml. There is a significant difference in the cytotoxic property of the positive control (Potassium Dichromate) and the semi-purified flavonoids from Bauhinia malabarica (Alimbangbang) leaves extract against Artemia salina. There is no significant difference in the cytotoxic property of the negative control (Artificial Sea water) to the semi-purified flavonoids of Bauhinia malabarica (Alimbangbang) against Artemia salina.

Conclusion

The following conclusions were formulated after the study was concluded:

- Flavonoids is present in Bauhinia malabarica extract.
- Flavonoids is responsible for the anti-proliferative property of Bauhinia malabarica using Brine Shrimp Assay.
- The semi-purified flavonoids of Bauhinia malabarica was affirmative with various confirmatory testing such as Shinodas test, Sodium hydroxide test, Lead acetate test, Ethyl acetate test.
- Zero point twenty-four percent (0.24%) was the percentage yield of semi-purified flavonoids from the 500 grams of plant material.
- The Quercetin Standard of the semi-purified flavonoids of Bauhinia malabarica was 0.99%
- The semi-purified flavonoids of Bauhinia malabarica elicited an ant proliferative property using Brine Shrimp Assay with percent mortality of 85.5%, 71.9%, 60.8%,
- 39.1% and 6.50% at various concentrations.

Recommendation

In view of the findings and conclusions of the research, the following recommendations are hereby forwarded for consideration.

- Future researchers should formulate dosage forms that will use in the treatment and cure of lung and breast cancer.
- To apply in-vivo testing in either human or animal testing.
- Different parts of the Bauhinia malabarica can be used aside from its leaves.
- Different cancer cell line can be used aside from A549 and MCF7.
- Further researches maybe conducted to tests and validate the anti-proliferative property o Bauhinia malabarica (Alimbangbang).
- To conduct analysis of flavonoids as anti-proliferative agent.

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