CYTOTOXICITY SCREENING AND ANTIBACTERIAL POTENTIAL OF BAKALI LEAF EXTRACT

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ABSTRACT

The study was conducted to screen the cytotoxic activity and to determine the antibacterial potential of the ethanol leaf extract of Bakali, a wild woody plant of the Bignoniaceae family and thrives on the slope of Mt. Arayat, Magalang, Pampanga. Four different concentrations (T1-10000 ppm, T2-1000 ppm, T3-100 ppm and T4-10 ppm), including T+ (ethanol) and T0 (saline solution) were prepared for cytotoxicity screening. Undiluted and diluted forms of the ethanol leaf extract, including T+ (chloramphenicol) and T- (distilled water) were utilized for antibacterial assay. Brine shrimps (*Artemia naupllii*) were used to determine the cytotoxic activity. The following percentages of deaths after nine hours of observation were recorded: T+ and T1 with 100%, T2 with 93.33%, and T3 with 3.33%. For T4 and T0, they had 0% deaths even after nine hours. The data also revealed that based on the Probit Method, the LC_{50}, LC_{70} and LC_{95} of Bakali leaf extract to the brine shrimps after nine hours are expected to be at 363.35 ppm, 520.66 ppm, and 1122.95 ppm, respectively. On the other hand, Gram-positive bacteria *Staphylococcus aureus* and Gram-negative bacteria *Escherichia coli* were used as test organisms for antibacterial assay using paper disc diffusion method. The results revealed that the ethanol leaf extract of Bakali had the potential to inhibit the growth of *S. aureus* and *E. coli*. Based on a 6-mm diameter paper disc, the extract is considered as "active" against *S. aureus* and "partially active" against *E. coli* when in the diluted form of aqueous solution.

Keywords: antibacterial; Bakali; Artemia naupllii; S. aureus; E. coli

1. INTRODUCTION

Herbal medicines are the synthesis of therapeutic experiences of practicing physicians of indigenous systems of medicine for generations, or for over hundreds of years. These are known to be the oldest health care products that have been used by mankind all over the world in the form of folklore medicines, traditional medicines, or ethnic medicines. The therapeutic use of herbal medicines is gaining considerable momentum in the world during the past decade (Kumari et al. 2011).

According to World Health Organization (WHO), more than 80% of the world's population relies on traditional medicine for their primary healthcare needs. Use of herbal medicines in Asia represents a long history of human interactions with the environment. Plants used for traditional medicine contain a wide range of substances that can be used to treat chronic as well as infectious diseases. A vast knowledge of how to use the plants against different illnesses may be expected to have accumulated in areas where the use of plants is still of great importance. The medicinal value of plants lies in some chemical substances that produce a definite physiological action on the human body. The most important of these bioactive compounds of plants are alkaloids, flavonoids, tannins and

phenolic compounds (Duraipandiyan et al. 2006). Therefore, such plants should be investigated to better understand their properties, safety and efficiency.

The screening of plant extracts and plant products for antimicrobial activity has shown that higher plants represent a potential source of novel antibiotic prototypes (Afolayan 2003). Since we are now living in a modern and modest world, proliferation of different drugs is being introduced that brought to mankind various conflict and difficulties in everyday living because of side effects. It affects the people of all ages and occurs equally among people of both sexes. Therefore, scenarios pushed individuals to return to natural healing method, in order to solve and alleviate these problems. Man need practical remedies for his common ailments and because of these, people must rediscover the healing elements of native plants (Lugtu 2007).

Bakali, a wild woody plant of the Bignoniaceae family, thrives on the slope of Mt. Arayat, a few kilometres from Barangay Ayala, Magalang, Pampanga, Philippines. Its leaves are opposite and compound. Plants of the Bignoniaceae have cork cambium and unilocular nodes. The bioactive components of this plant are primary alkaloid, steroid, flavonoid, saponin and tannin (San Andres 2013). The locals of Barangay Ayala use the leaf decoction as treatment for stomach ache. The plant parts (stems, leaves and roots) are claimed to treat kidney diseases. However, there is no scientific evidence to justify such claims of its effectiveness or safety, since it has not been studied so far.

With this information, the researchers thought of conducting this study which can provide scientific basis for knowing the cytotoxic and antibacterial potentials of Bakali leaf extracts. With the results of the study, the researchers hope that claims about its uses as alternative drug could be supported by scientific evidences.

2. OBJECTIVES OF THE STUDY

Generally, this study determined the cytotoxic potential of Bakali leaf extracts using the brine shrimp bioassay and its antibacterial property using paper disc diffusion assay.

Specifically, it aimed to answer the following questions:

1. What are the percent deaths of brine shrimps exposed to different treatments as affected by time intervals (3, 6 and 9 hours)?

2. At what concentration does the Bakali leaf extract exhibit LC_{50}, LC_{70} and LC_{95} at different time intervals (3, 6 and 9 hours)?

3. Is there a significant antibacterial potential of the Bakali leaf extract against Gram-positive *Staphylococcus aureus* and Gram-negative *Escherichia coli* as indicated by their zones of inhibition when compared with the positive control (chloramphenicol)?

3. METHODOLOGY

3.1 Plant Materials

Fresh Bakali leaves, not too young but not too old and free from damage and disease, were collected within the vicinity of Barangay Ayala, Magalang, Pampanga, Philippines. The collected leaves were washed using running tap water to remove the impurities adhering to the leaf. Then this was followed by thorough air-drying at room temperature for three days.

Figure 1: Bakali leaves

3.2 Preparation of the Plant Extract

Air-dried Bakali leaves were first ground, then weighed in an Erlenmeyer flask and treated with sufficient 80% ethanol to completely submerge the plant material. The flask was then stoppered and the plant material was kept soaked for 48 hours. The mixture was then carefully filtered and the filtrate was concentrated under rotary evaporator. Afterwards, the concentrated extract was transferred in an amber-colored container with proper label. The extract was stored tightly stoppered in a refrigerator at temperatures between 0°C to 5°C to prevent fungal growth, until it was needed for cytotoxicity and antibacterial studies.

3.3 Study 1. Cytotoxicity Screening

3.3.1 Preparation of the Plant Extract for Brine Shrimp Bioassay

About 0.5g of the concentrated plant extract was dissolved in 50ml of distilled water which served as the 10,000 ppm (T1). Then 1ml from the 10,000 ppm was obtained and mixed with 9ml distilled water to make a 1,000 ppm (T2). For 100 ppm (T3), 1 ml was obtained from 1,000 ppm and mixed with 9 ml distilled water. Lastly, 1 ml was obtained from 100 ppm and mixed with 9 ml distilled water to make a 10 ppm (T4).

3.3.2 Brine Shrimp Bioassay

The brine shrimp eggs were hatched in artificial sea water by dissolving 3.8 g of rock salt per 100 ml distilled water. After 48 hours, the hatched brownish orange nauplii (larvae) were pipetted.

Three Petri plates, which served as three replicates, were used per treatment. One (1) ml of artificial sea water was placed into each negative and positive control plates, followed by the addition of 4 ml of each of the negative control (saline solution) and the positive control (ethanol). For Treatments 1 to 4, four (4) ml of each of the previously prepared 10000 ppm, 1000 ppm, 100 ppm and 10 ppm was pipetted and each was diluted to 1 ml artificial sea water. With a Pasteur pipette, ten nauplii were transferred into each sample Petri plate labeled with the specified treatments.

The Petri plates with nauplii were kept under illumination. With the use of a 3x magnifying glass, the surviving shrimps were counted first after 3 hours, then after 6 hours and lastly after 9 hours. The percent deaths for each treatment were determined. The probability analysis method was employed to compute the LC_{50}, LC_{70} and LC_{95} of the extract to the brine shrimps.

3.4 Study 2. Antibacterial Screening

3.4.1 Sources of Bacterial Strains

Pure cultures of Gram-positive *Staphylococcus aureus* and Gram-negative *Escherichia coli* were obtained from the NMIS, City of San Fernando, Pampanga, Philippines. Bacterial slants were prepared from the given pure cultures, followed by the preparation of bacterial inoculums from the prepared slants**.**

3.4.2 Treatments Used

The following treatments were used in each antibacterial study against *Staphylococcus aureus* and *Escherichia coli*: T+ - chloramphenicol (positive control); T- – sterile distilled water (negative control); T1 – undiluted ethanol leaf extract of Bakali; and T2 – diluted ethanol leaf extract of Bakali.

3.4.3 Preparation of the Assay Plates

Twenty-four Petri dishes were used for the preparation of assay plates, twelve plates to test the treatments against *Staphylococcus aureus* and twelve plates to test the treatments against *Escherichia coli*.

3.4.4 The Assay Proper: Paper Disc Diffusion Method

A swabbed agar plate was laid on the paper circle pattern earlier prepared. Then the cover dish was removed. The sterile filter paper disc was dipped to the specific treatment, removing the excess liquid. It was then carefully placed onto the swabbed agar plate. The dish was covered immediately. Each Petri dish was labeled to identify the treatment and

replicate for assay to be delivered into the dish. The treatments to be assayed were delivered into their respective Petri dish. The plates were then incubated at 35°C. The results were observed after 24 hours.

3.4.5 Reading the Assay Plates and Documenting the Results

After the incubation period, a "halo" or "clearing", which is known as the zone of inhibition, was seen around each treated paper disc. The plates were inverted and the diameter of each inhibition zone was measured in millimeters with the use of a good ruler. The results were expressed as millimeter diameter zone of inhibition.

3.5 Analyzing the Results

No zone of inhibition should appear in negative control to make the other results valid. The mean values of the zones of inhibition of each of the three replicates per treatment were recorded. For a six millimeter (6 mm) diameter filter paper disc, the corresponding inferences were used for the diameter zones of inhibition observed (Guevarra *et. al*. 2005): <10 mm, may be expressed as inactive; 10-13 mm, partially active; 14-19 mm, active; and >19 mm, very active.

4. RESULTS AND DISCUSSION

4.1 Cytotoxicity Screening of Bakali Leaf Extract

Table 1 presents the percent deaths of brine shrimps exposed to different treatments as affected by time intervals. It reveals that T+ and T1 already had 100% deaths at 3 hours of exposure due to their toxic effect, while T2 had 86.67%, 90%, and 93.33% deaths after 3, 6, and 9 hours, respectively. On the other hand, T3 had percent deaths of 3.33 only after 6 hours of exposure, which could mean that this concentration of Bakali leaf extract will take some time for its toxicity to take effect. For T4, its percent death is 0 which is comparable to T-, which means that this concentration of Bakali leaf extract is not enough to kill brine shrimps.

Table 1: Percent deaths of brine shrimps exposed to different treatments as affected by time intervals

	Percent Deaths at Time Intervals		
Treatments	**3 Hours**	**6 Hours**	**9 Hours**
T+ (Ethanol)	100	100	100
T0 (Saline Solution)	0	0	0
T1 (10,000 ppm)	100	100	100
T2 (1,000 ppm)	86.67	90	93.33
T3 (100 ppm)	0	3.33	3.33
T4 (10 ppm)	0	0	0

Table 2 presents the different lethal concentrations of Bakali leaf extract at different time intervals. After 3 hours exposure of brine shrimps to concentrations of 10 ppm to 10,000 ppm Bakali leaf extract, the data revealed that based on the probability analysis, the

LC_{50} is at 686.67 ppm which means that at this concentration, 50% of the total population of brine shrimps is expected to die. While at 818.05 ppm (LC_{70}) and 1188.81 ppm (LC_{95}), it is expected that 70% and 95% of the total brine shrimps will die, respectively.

For 6 hour exposure of brine shrimps to different concentrations of Bakali leaf extract, the LC_{50}, LC_{70} and LC_{95} are as follows: 393.32 ppm, 576.17 ppm, and 1302.67 ppm, respectively. However, after 9 hours, the expected LC_{50}, LC_{70}, and LC_{95} are 363.35 ppm, 520.66 ppm, and 1122.95 ppm, respectively. The results suggest that for the Bakali leaf extract to function well as a cytotoxic agent, the concentration must be increased beyond its LC_{50}.

Table 2: LC_{50}, LC_{70}, and LC_{95} of Bakali leaf extract at different time intervals

	Percent Deaths at Four Levels of Bakali Leaf Concentration				**LC_{50}**	**LC_{70}**	**LC_{95}**
No. of Hours	**10,000 ppm**	**1,000 ppm**	**100 ppm**	**10 ppm**		**Ppm**	
3	100	86.67	0	0	686.67	818.05	1188.81
6	100	90	3.33	0	393.32	576.17	1302.67
9	100	93.33	3.33	0	363.35	520.66	1122.95

Antibacterial Potential of the Bakali Leaf Extract

Table 3 presents the data on the average zones of inhibition (in mm) of Bakali leaf extract against *S. aureus* after 24 hours. As shown in the table, T+ is considered as "very active" having a mean zone of 24.83 mm (>19 mm), while T2 is considered as "active" with a mean zone of 14.33 mm (14 to 19 mm). On the other hand, the mean zones of both the T0 (0 mm) and T1 (5.83 mm) are <10 mm, hence they are considered as "inactive." Based on statistical analysis, the data revealed that T2 is significantly different to T+, however, it is significantly higher than T1 and T0 which are comparable with each other (p-value at 0.05 = 1.33×10^{-5}).

The results imply that the Bakali leaf extract has an antibacterial potential against *S. aureus*. But this potential could work well in an aqueous solution, which could mean that the bioactive components of the extract as antibacterial may be activated upon this solution.

The same table also presents the data on the average zones of inhibition (in mm) produced by Bakali leaf extract against *E. coli* after 24 hours. As presented in the table, T+ with a mean zone of 35 mm (>19 mm) is considered as "very active," followed by T2 with a mean zone of 10.17 mm (10-13 mm) which is considered as "partially active." Both T0, with a mean zone of 0 mm, and T1, with a mean zone of 4.33 mm, are considered as "inactive" since their mean zones are <10 mm. Based on statistical analysis, the data revealed that T2 is significantly different to T+ but not to T1, while Treatments 0 and 1 are comparable with each other (p-value at 0.05 = 5.7×10^{-6}).

The results suggest that the Bakali leaf extract has the potential to act as an antibacterial agent against *E. coli*. However, its effectivity could perform well when it is in an aqueous solution which could mean that its bioactive components as antibacterial might be activated upon this solution. It has also been reported that the Bakali leaves are boiled with water by some of the residents in Brgy. Ayala, Magalang, Pampanga, Philippines and then the decoction is being used as treatment for stomach ache. However, it must be noted that

Bakali leaf extract was previously screened to have a cytotoxic activity at a certain concentration, hence proper dosage preparation should also be considered.

Table 3: Average zones of inhibition (in mm) produced by Bakali leaf extract against *S. aureus* and *E. coli* after 24 hours

	S. aureus		***E. coli***	
Treatment	**Mean***	**Analysis**	**Mean***	**Analysis**
T+(chloramphenicol)	24.83^{a}	Very Active	35^{a}	Very Active
T0(sterile dH_20)	0^{c}	Inactive	0^{c}	Inactive
T1-undiluted leaf extract	5.83^{c}	Inactive	4.33^{bc}	Inactive
T2-diluted leaf extract	14.33^{b}	Active	10.17^{b}	Partially Active

*Means of the same column having different letters are significant at 5% level of significance (Tukey HSD method).

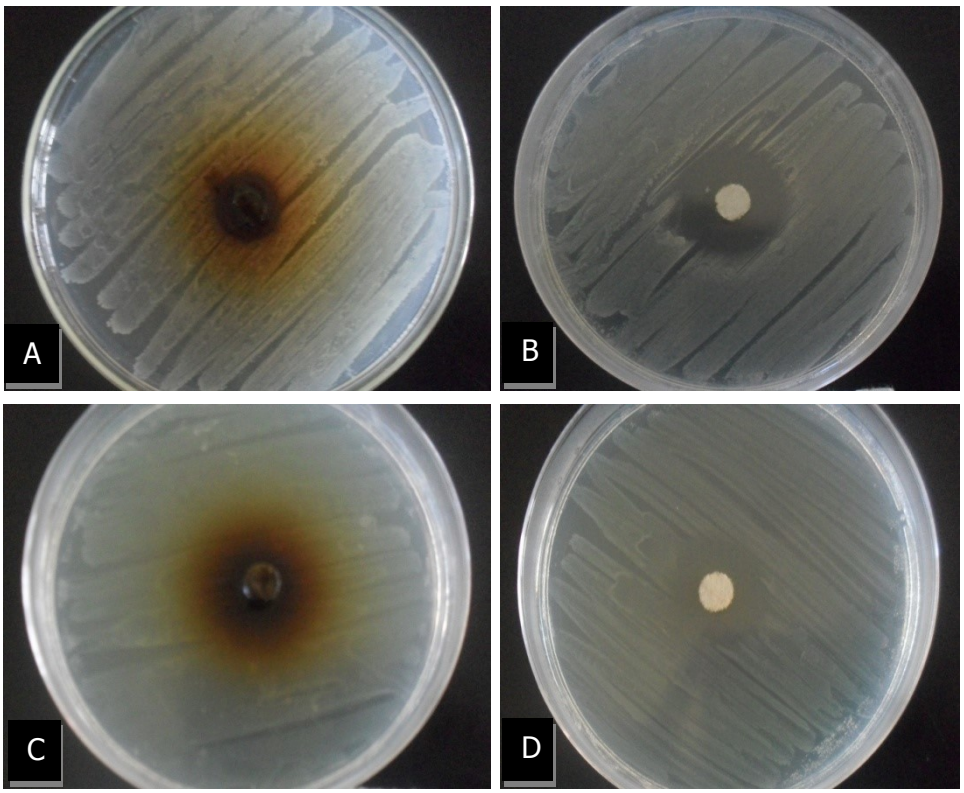

Figure 2: Zone of inhibition produced by Bakali leaf extract: A. undiluted leaf extract against *S. aureus*; B. diluted leaf extract against *S. aureus*; C. undiluted leaf extract against *E. coli*; and D. diluted leaf extract against *E. coli*

5. CONCLUSIONS

Based on the findings, the following conclusions were drawn: high percent deaths of brine shrimps exposed to higher concentration indicates the toxicity of the Bakali leaf extract which may be due to the presence of alkaloids, steroid, flavonoid, saponin and tannin, known to be poisonous at high concentration (San Andres 2013); the LC50, LC70 and LC95 of the Bakali leaf extract against the brine shrimps are 363.35 ppm, 520.66 ppm, and

1122.95 ppm, respectively. The Bakali leaf extract also has an antibacterial potential against *Staphylococcus aureus* and *Escherichia coli.*

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