ANTI-LETHAL TOXICITY AND ANTI-PHOSPHOLIPASE A2 ENZYME ACTIVITY OF MAKAHIYA (MIMOSA PUDICA) ROOT EXTRACT AGAINST PHILIPPINE COBRA (NAJA PHILIPPINENSIS)

MELISA T. REBOLDERA, RPH, MPH, MSTChem

ABSTRACT

The study was conducted to evaluate the antivenom property of makahiya ethanolic root extract using its different concentrations against the median lethal dose of the Philippine cobra. The makahiya roots were extracted with 80% ethanol using a soxhlet apparatus and were concentrated until twenty milliliters were obtained with a rotatory evaporator. In the biological assay for the anti-lethal toxicity, a completely randomized design was used on twenty mice weighing about 16-20 grams, assigned for control and the three concentrations of makahiya ethanolic root extract with five (5) replications, using the Intravenous-tail vein route. Spectrophotometry-Turbidity method was used in the Anti-PLA2 enzyme activity assay. Egg yolk suspension was prepared with a 0.9% NaCl as the buffer. The egg yolk suspension was further diluted until the absorbance was 1.2. The different concentrations of makahiya previously mixed with venom was incubated with the egg yolk suspension. Absorbance and transmittance were measured for the five replications in each group. Based on the statistical analysis, there is no significant difference in the anti-lethal activity of the different concentration of makahiya ethanolic root extracts and the anti-PLA2 enzyme activity of Philippine cobra's venom. The biological assay results indicate that makahiya ethanolic root extract increases the survival rate and protection fold against the Philippine cobra venom. Therefore, the makahiya (Mimosa pudica) root extracts have a potential neutralizing effect on the venom of the Philippine cobra.

Keywords: *Antivenom, makahiya, phospholipase A2 enzyme*

INTRODUCTION

From antiquity, snakebite has been a major health problem especially in tropical countries like the Philippines. The estimate of the mortality rate of snakebite in the Philippines is about 200-300 each year (WHO South-east Asia, 2008). The death rate from Philippine cobra bite was estimated to be as high as 107.1 deaths per 100,000 population per year at one site. Most victims were young (median age 17) and 98% were males. Only 8% of the victims studied reached a hospital, and most of them never seek proper medical treatment. The confirmed death rate averaged 53.8/100,000 for the three populations noted in the study (U.S. Naval Medical Research, 2011).

In the treatment of snakebite, antivenom immunotherapy is available in the market. The commercial antivenom in the Philippines is manufactured only by RITM (Research Institute for Tropical Medicines). The preparation of the antivenom immune sera is very tedious and very costly because it requires the production of immune bodies in pure breed horses which are imported from other countries. This makes the commercial antivenom expensive and less accessible. Furthermore, Antivenom immune sera need storage at stable, low temperatures and need cold chain method (2-8 ^{o}C), but many villages do not have electricity or have an unstable supply of electricity. These hinder many commoners from accessing the medicines they needed. As a result, they often resort to indigenous healing practice like Tandok for treating snakebite. Tandok, however, is not recommended because it is highly invasive and may result in tetanus, hepatitis and other skin infections (Sun Star, 2013 issue).

The Family income and expenditures Survey (FIES) 2007 report showed that on average, two-thirds (67%) of all health care expenses by Filipinos were spent on buying medicines due to a high price of commercial medicines available in the market. Also, prices in the Philippines for the chosen drugs were a lot higher compared to other countries in Asia. (President of Philippine Government Corporation engaged in parallel importation of selected medicines, 2011 figures).

Retail prices of medicine in Indonesia, Malaysia, and Thailand, are 40 percent to 70 percent lower than in the Philippines (The Manila Times,

2012). As a result, low-income families cannot afford the medicines they needed for a quality healthcare. Most of the people in rural areas prefer traditional healing practices instead of professional medical treatment. A classic example is the case of snakebites.

The answer to this problem is the production of alternative medicine against snakebite from plants that are indigenous in the community. This plant which is readily available in the community will help common people that are financially incapable of buying the commercial antidote. A good example is the makahiya plant. Makahiya (Mimosa pudica) root extract shows a promising antivenom property (Pharmacognosy magazine, 2012).

Objectives of the Study

This study sought to evaluate the effectiveness of makahiya (Mimosa pudica) ethanolic root extract against the venom of Philippine cobra (Naja philippinensis).

Specifically, it aimed to determine which of the three (3) concentrations of makahiya ethanolic root extract is the most effective dose against the toxic effects of Philippine cobra venom; and to identify if there is a significant difference in the activity of the three concentrations of makahiya ethanolic root extract against the toxic effects of Philippine cobra venom.

Hypothesis

There is no significant difference in the activity of the three concentrations of makahiya ethanolic root extract against Philippine cobra venom.

METHODOLOGY

This chapter discusses the methods, preparations, and procedures that were utilized in the evaluation of the property of Mimosa pudica ethanolic root extracts against the venom of a Philippine cobra.

The complete randomized design-experimental method was employed in the study.

A. Collection of Plant Sample

The makahiya roots Http from Ballesteros, Cagayan. The gathered makahiya roots were washed with clean water and subjected to air drying. The air-dried roots were stored in a clean container ready for the extraction process.

B. Ethanolic Extraction

The extraction process and biological assay were conducted in St. Paul University Philippines – Science Laboratory, SP Building, Tuguegarao City, Philippines.

The air dried makahiya roots were cut into small pieces and washed with distilled water. Twenty (20) grams of the makahiya roots were macerated with 200 mL of 80% ethanol overnight and extracted with Soxhlet apparatus for 6 hrs refluxing at 60-80 °C. The extraction process yielded an extract which was dark-brown in color. The makahiya root residues were discarded properly. The makahiya extract was concentrated using a rotary evaporator until an approximate of twenty milliliters was obtained. The final extract was placed in a clean tightly stoppered amber glass container and stored in 4 °C after proper filtration process. The procedure was repeated until enough amount of makahiya ethanolic root extract was obtained.

100% makahiya ethanolic root extract concentration was the pure extract obtained after the extraction process using soxhlet apparatus and concentration procedure using the rotary evaporator. The pure extract was diluted with distilled water to come up with the 75% and 50% makahiya ethanolic root extract concentrations.

1. 100% concentration = pure makahiya ethanolic root extract after extraction and concentration processes
2. 5% concentration = 75mL of the pure makahiya ethanolic root extract diluted with 25 mL distilled water to make 100 mL, 3.50%

concentration = 50mL of the pure makahiya ethanolic root extract diluted with 50mL distilled water to make 100 mL.

C. Biological assay

The lyophilized powder of Philippine cobra venom was purchased from RITM (Research Institute of Tropical Medicine) in Alabang, Muntinlupa City. The venom has 0.56 mg/kg LD50.

The lyophilized powder of Philippine cobra's venom was diluted to the normal saline solution (1g lyophilized powder diluted to 100 mL normal saline solution – 1:100). The solution of Philippine cobra venom was used in the anti-Lethal toxicity and anti-PLA2 enzyme activity biological assay.

C.1. Anti-Lethal toxicity Activity

The graded dose of the venom was computed based on the weight of the mice using the 0.56 mg/kg LD50. The venom solution was mixed with equal amount of the three different concentrations of makahiya ethanolic root extract (100%, 75%, and 50%). Thirty minutes incubation period was allotted for the neutralization process to take place in the mixtures.

Control. Injection of the computed volume of the 0.56 mg/kg LD50 of Philippine cobra venom.

Treatment 1. Injection of the computed volume of the 0.56 mg/kg LD50 of Philippine cobra venom mixed with the 100% concentration of makahiya ethanolic root extract.

Treatment 2. Injection of the computed volume of the 0.56 mg/kg LD50 of Philippine cobra venom mixed with the 75% concentration of makahiya ethanolic root extract.

Treatment 3. Injection of the computed volume of the 0.56 mg/kg LD50 of Philippine cobra venom mixed with the 50% concentration of makahiya ethanolic root extract.

After the 30 min incubation period of the prepared three treatments,

they were filtered using a micro-filter. The filtered product was used for the biological assay for anti-Lethal toxicity effect of makahiya.

The test animals - albino mice both male and female weighing 16-20 g were individually identified and allowed to acclimatize to laboratory condition for seven days before the biological assay in standard cages. They were kept in a 12/12 hour light-dark cycle. Food pellets and water were available ad libitum. Each experimental group was matched with the parallel control group.The three treatments were injected into the tail vein of mice. Each treatment had five replications. Death or survival of the test animals with the time of survival was noted within the 24-hour observation.

Mouse Tail Vein Injection

The mouse was removed from the cage by its tail. Then, one of the two lateral veins of the tail was located. The needle was lined up straight to one of the lateral veins. The material was injected into the vein. The syringe was discarded properly. Tuberculin syringes were used for the administration of the mixture in the test animals.

The test animals were marked with a pen to identify their group. The time of death and survival of the test animals were recorded. After the observation, the dead albino mice were properly buried, and all the materials used in the experiments were properly washed or discarded as based on the standard operating procedures for laboratory practice.

C.2. Neutralization of Phospholipase A2 activity

A fresh egg yolk suspension was prepared in 0.9% NaCl. The egg yolk suspension was diluted further with 0.9% NaCl until the absorbance at 925 nm was 1.2. The three treatments mixed with venom were prepared using the same procedure used in the anti-Lethal toxicity assay.

Control. Solution of the 1:100 dilutions of Philippine venom plus the egg yolk suspension.

Treatment 1. Mixture of equal amount of 100% concentration of

makahiya ethanolic root extract and 1:100 dilution of Philippine cobra venom plus egg yolk suspension.

Treatment 2. Mixture of equal amount of 75% concentration of makahiya ethanolic root extract and 1:100 dilution of Philippine cobra venom plus egg yolk suspension.

Treatment 3. Mixture of equal amount of 50% concentration of makahiya ethanolic root extract and 1:100 dilution of Philippine cobra venom plus egg yolk suspension.

The three treatments and the control group were incubated at 37 ^{o}C for 30 min. After the incubation, the mixture was centrifuged for 10 min and the supernatant liquid was tested for the PLA2 enzyme activity using the spectrophotometry–turbidimetric analysis. Absorbance and Transmittance of each treatment and the control group were measured. Each of the treatments and the control group has five replications.

RESULTS AND DISCUSSION

This chapter discusses the result of the biological assay performed to evaluate the effectivity of makahiya ethanolic root extract against Philippine cobra venom. One way–ANOVA was used in the statistical analysis of data.

Table 1 presents the survival and death with the corresponding survival time of the test animals-albino mice in the biological assay of the Anti-Lethal toxicity of makahiya ethanolic root extract.

Legend: S- Survived D- Died

	Test animal (mice)	Survival Time	Dead/Survived
Control Group (Venom)	1	12 min	D
	2	4 min	D
	3	9 min	D
	4	8 min	D
	5	5 min	D

	Test animal (mice)	Survival Time	Dead/Survived
Treatment 1 (100% makahiya extract + Venom)	1 2 3 4 5	24 hrs 17 hrs & 45 min 24 hrs 24 hrs 24 hrs	S D S S S
Treatment 2 (75% makahiya extract + Venom)	1 2 3 4 5	14 hrs & 5 min 13 hrs & 32 min 24 hrs 24 hrs 16 hrs & 50 min	D D S S D
Treatment 3 (50% makahiya extract + Venom)	1 2 3 4 5	12 hrs & 10 min 8 hrs & 7 min 11 hrs & 20 min 24 hrs 24 hrs	D D D S S

Twenty albino mice (male and female) weighing 16-20 grams were randomly picked for the evaluation of the Anti-Lethal Toxicity activity of makahiya ethanolic root extract. In control, all of the 5 test animals died with a survival period of 4-12 min after the administration of the venom IV-tail vein as based on the 0.56 mg/kg LD50.

Among the treatments, treatment 1 (100 % concentration of makahiya ethanolic root extract) showed the most promising result with only one mortality and four of the test animals survived. The 75% and 50% concentrations showed the same result, with two survived mice and three mortality in each group.

Even if some of the animals died during the 24 hrs observation, still the makahiya extract significantly increased the survival rate of the test animals ranging from 8 hrs and 7 min to 17 hrs and 24 min. This indicates that makahiya ethanolic root extract increased the protection fold against the Philippine cobra venom.

Table 2. Contingency Table for the Significant Difference among the three concentrations of Makahiya Ethanolic Root Extract in Anti-Lethal Toxicity Assay

Dependent Variable	(I) treatment	(J) treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Survival time	control	treatment 1	-1357.40000*	181.32096	.000	-1922.6043	-792.1957
		treatment 2	-1101.80000*	181.32096	.000	-1667.0043	-536.5957
		treatment 3	-947.80000*	181.32096	.001	-1513.0043	-382.5957
	treatment 1	control	1357.40000*	181.32096	.000	792.1957	1922.6043
		treatment 2	255.60000	181.32096	.587	-309.6043	820.8043
		treatment 3	409.60000	181.32096	.207	-155.6043	974.8043
		control	1101.80000*	181.32096	.000	536.5957	1667.0043
	treatment 2	treatment 1	-255.60000	181.32096	.587	-820.8043	309.6043
		treatment 3	154.00000	181.32096	.867	-411.2043	719.2043
		control	947.80000*	181.32096	.001	382.5957	1513.0043
	treatment 3	treatment 1	-409.60000	181.32096	.207	-974.8043	155.6043
		treatment 2	-154.00000	181.32096	.867	-719.2043	411.2043

*. The mean difference is significant at the 0.05 level.

In the statistical analysis of the data in the anti-Lethal toxicity assay, there is no significant difference among the concentration of makahiya ethanolic extract. This indicates that the three concentrations (100%, 75%, and 50%) were equally effective against the Philippine cobra venom. While the control showed a significant difference when compared to the three treatments.

Table 3 presents the measured absorbance and transmittance of the three treatments and the control group with the corresponding five replications.

Table 3. Anti-Phospholipase A2 Activity

	Replications	Absorbance	Transmittance
Control (venom + Egg suspension)	1	0.949	11.8
	2	0.948	11.8
	3	0.948	11.8
	4	0.947	11.9
	5	0.949	11.8
Treatment 1 (100% makahiya extract + Venom + Egg suspension)	1	0.976	10.4
	2	0.980	10.1
	3	0.979	10.1
	4	0.978	10.2
	5	0.979	10.1

	Replications	Absorbance	Transmittance
Treatment 2 (75% makahiya extract + Venom + Egg suspension)	1 2 3 4 5	0.964 0.965 0.964 0.963 0.963	10.8 10.9 10.8 10.8 10.8
Treatment 1 (50% makahiya extract + Venom + Egg suspension)	1 2 3 4 5	0.960 0.959 0.959 0.957 0.958	11.2 11.2 11.2 11.1 11.2

In the spectrophotometric analysis, treatment 1 (100% makahiya ethanolic root extract) showed the highest absorbance and with the corresponding lowest transmittance followed by the 75% and 50%.

In the control group which is a mixture of venom and egg suspension, the venom increased the coagulation of the egg yolk suspension as shown by the decrease in absorbance from the initial 1.2 to 0.94+1. The control showed the lowest absorbance and the highest transmittance.

The findings indicate that makahiya ethanolic root extract can neutralize the PLA2 enzyme, thus preventing the venom to increase the coagulation of the egg yolk suspension over time.

Table 4. Contingency Table for the Significant Difference among the three concentrations of Makahiya Ethanolic Root Extract in the Anti-PLA2 activity

Dependent Variable	(I) treatment	(J) treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
absorbance	control	treatment 1	-.03400*	.00082	.000	-.0366	-.0314
		treatment 2	-.01700*	.00082	.000	-.0196	-.0144
		treatment 3	-.01280*	.00082	.000	-.0154	-.0102
	treatment 1	control	.03400*	.00082	.000	.0314	.0366
		treatment 2	.01700*	.00082	.000	.0144	.0196
		treatment 3	.02120*	.00082	.000	.0186	.0238
		control	.01700*	.00082	.000	.0144	.0196
	treatment 2	treatment 1	-.01700*	.00082	.000	-.0196	-.0144
		treatment 3	.00420*	.00082	.001	.0016	.0068
		control	.01280*	.00082	.000	.0102	.0154
	treatment 3	treatment 1	-.02120*	.00082	.000	-.0238	-.0186
		treatment 2	-.00420*	.00082	.001	-.0068	-.0016

*. The mean difference is significant at the 0.05 level.

The statistical analysis showed that the three concentrations of makahiya ethanolic root extract are not equally effective against the PLA2 enzyme present in the Philippine cobra venom. Treatment 1 (100% concentration of makahiya ethanolic root extract) showed the most promising effect against the PLA2 enzyme.

CONCLUSION

From the observations and results gathered from the study, it can be concluded that makahiya (Mimosa pudica) root extract has a potential antivenom effect against Philippine cobra (Naja philippinensis) and better result can be seen in higher concentration.

While 100% protection has not been achieved with the makahiya ethanolic root extract, the fact that there were survivors in the experiment was significant, since in the corresponding control group all of the animals died and in the anti-PLA2 activity assay, it was found that makahiya can neutralize the PLA2 enzyme.

The chemical constituents of makahiya ethanolic root extract can be held responsible for the neutralizing effect of makahiya root extract against the action of snake venom. The active constituents that have been identified to be present in the makahiya root extract were alkaloids, tannins, D-mannitol, beta-sitosterol, and flavonoids, based on the selected phytochemical screening as published in Pharmacognosy and medical journals.

These different chemical constituents shown to occur in this plant (Mimosa pudica) are capable of interfering with the macromolecule target.

RECOMMENDATIONS

Based on the findings and conclusion of the study, the following recommendations are drawn:

Further purification of the extract and isolation of the active constituents responsible for the antivenom effect of makahiya root.

Consider other solvents for the extraction process.

Identification of the minimum therapeutic dose and the median lethal dose (LD50) of the makahiya ethanolic root extracts.

Extend the duration of time for observation.

Consider another venom from other species of snakes in the evaluation of the antivenom property of makahiya.

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