In Vitro Anti-inflammatory Assays on Hexane Extract of Sambong (*Blumea balsamifera*) Leaves

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***Abstract* — Anti-inflammatory drugs were developed to ease inflammation but side effects were soon developed, affecting the condition of patients. In this study, *Blumea balsamifera* (Sambong) leaves, with claimed therapeutic aids, was tested for anti-inflammatory potential through *in vitro* assays of albumin denaturation, HRBC membrane stabilization, and protease inhibition. Results showed that the percent inhibition and stabilization of hexane extracts were lower than 30%, owing to the nonpolarity of hexane and polarity of aqueous solutions, and washed polar phytochemicals during maceration. During statistical analysis, both 100 mg/mL and 75 mg/mL hexane extracts showed no significant difference at p>0.05 in protease inhibition and HRBC membrane stabilization when compared to all concentrations of aspirin. In conclusion, *Blumea balsamifera* leaves have anti-inflammatory potential in the assays, HRBC membrane stabilization and protease inhibition, contributed by non-volatile and volatile constituents possibly extracted which can minimize the side effects of anti-inflammatory drugs.**

***Keywords* — albumin denaturation, anti-inflammatory, *Blumea balsamifera*, HRBC membrane stabilization, protease inhibition**

I. Introduction

Inflammation is a biological process in which the body's white blood cells and chemicals produced defend the localized physical area of the body where the inflammation takes place but when it fails to carry on and preserve its process, symptoms of different diseases are triggered. Throughout the world, non-steroidal anti-inflammatory drugs (NSAIDs) and steroids are currently used to treat inflammation. They are widely used in the medicinal industry but are known to have severe side effects that affect the condition of a patient [5].

Medicinal plants, known to be therapeutically effective in treating different bioactivities, are culturally accepted by the common people, since most drugs and synthetic chemicals used in the medicinal industry have led to numerous side effects and undesirable hazards. *Blumea balsamifera*, commonly known as Sambong, belongs to the family Compositae, and is known to aid the treatment of different diseases. It has been traditionally used in the Asia-Pacific region, especially in Philippines, for medicinal purposes like anti-urolithiasis, anti-diarrhea, anti-spasm, dissolving kidney stones, and work as a diuretic [8]. *Blumea balsamifera* has been used over decades: however, some medicinal activities were not yet studied by researchers. Therefore, the present study was commenced to establish scientific evidence for the anti-inflammatory activity of *Blumea balsamifera* leaves.

The study aimed to analyze anti-inflammatory activity on hexane extract of *Blumea balsamifera* (Sambong) leaves. Specifically, it aimed to: (a) assess the three *in vitro* anti-inflammatory assays using different concentrations of the hexane extract; (b) use one-way ANOVA followed by Tukey post hoc test for statistical analysis between the hexane extracts and aspirin; and (c) evaluate interrelated discussions on the hexane extract results on albumin denaturation inhibition, HRBC membrane stabilization, and protease inhibition. With this, the study served as a baseline for possible incorporation of *Blumea balsamifera* leaf extract into cheap and safe remedy for inflammation and for practitioners in the same field of the present study. It contributed to the scientific industry through the gathered information and data which will help develop and improve the study.

II. METHODOLOGY

A. Research Design

This study is an experimental research which aimed to obtain the *in vitro* anti-inflammatory activity of *Blumea balsamifera* leaf extracts obtained from hexane at different concentrations (100, 75, 50, 25 mg/mL) in triplicates. The positive control was the Non-Steroidal Anti-Inflammatory Drug (NSAID), aspirin, while the negative control was the solvent, hexane. Fresh leaves of *Blumea balsamifera* were gathered in Dampas District, Tagbilaran City, Bohol. Maceration and evaporation of the extracts were done in the Chemistry Department of University of San Carlos – Talamban Campus, Cebu City. The assays were also done in the said institution. Alkaline Protease was purchased at University of the Philippines – Los Baños that would be utilized in the protease inhibition assay.

B. Plant Collection

The plant leaves were collected in Dampas District, Tagbilaran City, Bohol. They were identified and authenticated by the Department of Environment and Natural Resources-CENRO of the same place to secure the name of the plant. The leaves were washed and dried under shade and after five days, coarsely powdered using blender at 10,000 rpm.

C. Maceration

The powdered leaves, which weighed 200 grams, were soaked in hexane for 18 hours at room temperature and were

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then filtered with ordinary filter paper. Then, the solvent was separated from the extract by evaporation through a rotary evaporator for one hour. The solution was poured on an evaporating dish and was placed in a fume hood to allow the excess hexane to evaporate. When the excess hexane evaporated, the crude leaf extract was weighed for sample preparation.

D. Sample Preparation

The leaf extract samples were prepared by dissolving the crude extracts with hexane in concentrations 100, 75, 50, and 25 mg/mL. The positive control samples were prepared by knowing the weight of active ingredient, acetylsalicylic acid, in the commercial aspirin, Unilab LRi Aspilets. The commercial aspirin was 80 mg acetylsalicylic acid, of which the total weight of the commercial tablet was 122 mg. From this, the concentrations of 1.97, 1.48, 0.98, and 0.49 mg/mL were prepared.

E. In Vitro Anti-inflammation

Three assays were done for the study. Inhibition of albumin denaturation, HRBC membrane stabilization and protease inhibition were deliberated following the procedures of known and well-documented researches. All of the three assays were performed in triplicates for each of the hexane extracts, positive control, and negative control.

F. Albumin Denaturation Inhibition

Inhibition of albumin denaturation was assessed following the method of Murugan and Parimelazhagan (2014) with minor modifications [6]. Four mL of 1% bovine serum albumin was added to 2 mL of samples. Different adjustments of the pH to 6.3 were done using a small amount of 1 M HCl. The mixtures were allowed at room temperature for 20 minutes and then heated at 57°C for 20 minutes enough to destroy the secondary and tertiary structures of the albumin. The resulting solution was cooled down to room temperature and absorbance was recorded at 660 nm spectrophotometrically. The negative control used was hexane. Percent inhibition for albumin denaturation of the extracts and positive control were calculated using the equation in Sakat et al. (2010):

$$\% \text{ inhibition} = \frac{A_0 - A_1}{A_0} \times 100 \qquad (1)$$

where A_0 is the absorbance of the negative control and A_1 is the absorbance of the sample (hexane extract or positive control).

G. HRBC Membrane Stabilization in Heat-Induced Haemolysis

The method was carried out as per the method of Leelaprakash and Dass (2011) with slight modifications [4]. Human blood, about 20 mL, was collected by an authorized physician from a healthy human volunteer who has not taken any NSAIDs for the past two weeks prior to the study. The blood was transferred to heparinized centrifuged tubes. The tubes were centrifuged for 10 minutes at 3000 rpm and washed thrice using an equal volume of normal saline. The volume of the blood was measured to reconstitute as 10% v/v suspension with normal saline.

In heat-induced haemolysis, 4-mL reaction mixtures, each consisting of 2 mL of samples and 2 mL of 10% RBCs suspension, were prepared in separate test tubes, in triplicates. All the test tubes with the reaction mixture were incubated at 56°C for 30 minutes and subsequently, the test tubes were cooled under running tap water. The mixtures were read at 560 nm via spectrophotometer. Hexane served as the negative control. Percent membrane stabilization was calculated by the equation (1).

H. Protease Inhibition

Protease inhibition was determined according to the method of Gantait *et al.* (2013) with minor modifications [2]. Four mL reaction mixtures were prepared using 0.12 mg protease, 2 mL of 20 mM borate HCl buffer (pH 7.7) and 2 mL of the hexane extracts. The reaction mixtures were subjected to incubation at 37°C for 5 minutes. Two mL of 0.8% (w/v) casein was added and incubated again for 20 minutes. To terminate the reaction, 4 mL of 70% perchloric acid was added. The negative control used was hexane. Absorbance of the supernatant at 210 nm was measured against buffer as blank. Percentage protease inhibition was calculated by equation (1).

I. Sample Decontamination and Waste Disposal

In case of blood spills, household bleach with 5.25% concentration of sodium hypochlorite (NaOCl) was added until the solution of bleach and blood was one part of bleach and five parts blood (1:5) and the solution was left to stand for at least 30 minutes, making sure that no bubbles were observed [3]. For the wiping of surfaces, same procedure was followed: however, the solution of bleach and blood was one part bleach and one hundred parts blood (1:100). For the wiping of metal equipment in case of contact with blood, same ratio for the biohazard spills was incorporated. Then, the equipment was rinsed with water and alcohol after the appropriate contact time with the bleach [1]. The solution for the biohazard spills was poured down the drain with cold water [3].

For the waste disposal, aqueous solutions such as bovine serum albumin, trypsin, casein, and aspirin were disposed in the aqueous waste container. Biohazardous wastes, such as human blood, were disposed in the biohazardous waste container. All solutions with hexane were disposed in a special waste container for hazardous organic chemicals.

J. Statistical Analysis

The results were expressed in Mean ± Standard Deviation. One-way Analysis of Variance (ANOVA) was utilized for the determination of the significance between experimental groups of the hexane extracts and aspirin. Normality tests were done to support One-way Analysis of Variance (ANOVA) using the software SPSS Statistics. Means between the experimental groups were compared using Tukey Post Hoc Test with the software Minitab 17. The p-value of no significant difference between the groups were set at $p>0.05$.

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III. RESULTS AND DISCUSSION

The maceration method with hexane as the solvent was used to extract the *Blumea balsamifera* leaves. During the evaporation of the solvent hexane, a crude concentration weighing 3.12 grams was obtained. This concentration was dissolved in hexane to obtain the different concentrations of 100, 75, 50, and 25 mg/mL. Three replicates were prepared.

A. Albumin Denaturation Inhibition

The different hexane extracts and the controls were subjected to an acidic environment by addition of HCl. Only 1% fetal bovine serum albumin was utilized for denaturation. The results showed that the concentration of 100 mg/mL had the highest inhibition of 30.96 ± 3.58% for the hexane extracts while 97.47 ± 0.77% of the highest concentration of aspirin (1.97 mg/mL) as seen in Figure 1. An increasing concentration for both hexane extracts and aspirin showed that there was also an increasing % inhibition.

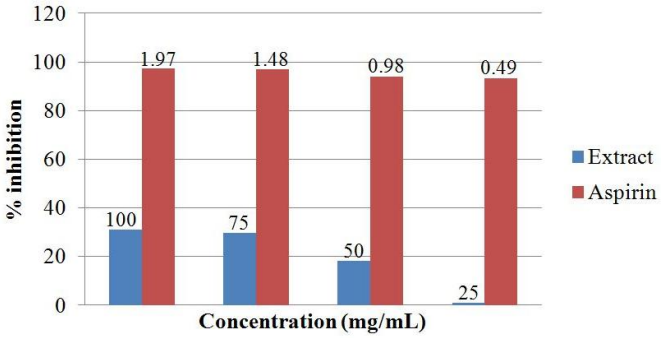

Fig. 1. Percent inhibition of Albumin Denaturation

Results of the Tukey post hoc test showed an adjusted p-value of 0.000, making it significantly different between each concentration of the hexane extracts and each concentration of aspirin where p>0.05. The relatively low % inhibition of the hexane extracts compared to aspirin could be due to the fetal bovine serum albumin which was immersed in an aqueous solution, making its interaction with the nonpolar hexane extract minimal.

B. HRBC Membrane Stabilization

Human blood was extracted by a medical technologist and placed in an EDTA tube to avoid coagulation. To separate the plasma and RBCs, centrifugation at 3,000 rpm for 10 minutes was done successfully. Figure 2 shows that the concentration of 100 mg/mL had the highest inhibition of 22.33 ± 0.87% for the hexane extracts while 54.20 ± 1.45% of the highest concentration of aspirin (1.97 mg/mL). Both aspirin and hexane extracts were also found to be concentration-dependent.

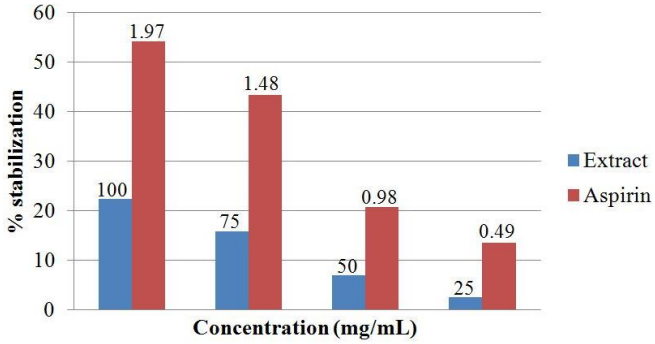

Fig. 2. Percent stabilization of HRBC Membrane

During Tukey post hoc test, each concentration of the hexane extracts were compared with each concentration of aspirin at p>0.05. Both 100 mg/mL and 75 mg/mL hexane extracts were able to stabilize the HRBC membrane with a comparable effect to 0.98 mg/mL concentration of aspirin at adjusted p=0.990 and p=0.234, respectively, which implies that there is no significant difference between the aforesaid concentrations. The 75 mg/mL hexane extract showed no significant difference at p=0.930 compared with the 0.49 mg/mL aspirin.

C. Protease Inhibition

As shown in Figure 3, the concentration of 100 mg/mL had the highest inhibition of 20.34 ± 2.17% for the hexane extracts while 22.09 ± 1.18% of the highest concentration of aspirin (1.97 mg/mL). In addition, both aspirin and hexane extracts were also found to be concentration-dependent.

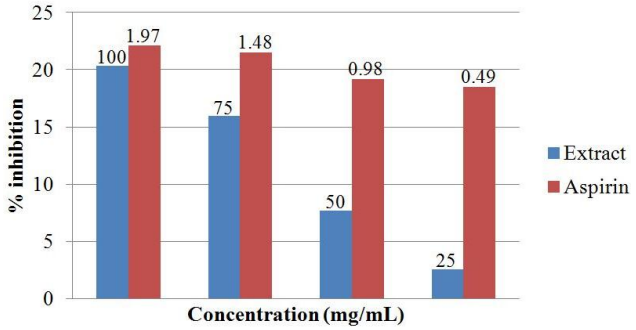

Fig. 3. Percent inhibition of protease

The Tukey post hoc test presented that the higher concentrations of the hexane extracts showed no significant difference when compared to most concentrations of aspirin. Specifically, the 100 mg/mL hexane extract was not significantly different when compared to the 1.97 mg/mL of aspirin with p=0.981. Also, the 100 mg/mL and 75 mg/mL hexane extracts showed no significant difference at p>0.05 in terms of aspirin with the concentrations: 1.48 mg/mL, 0.98 mg/mL, and 0.49 mg/mL at adjusted p-values seen in Table 1.

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TABLE I: ADJUSTED P-VALUES OF PROTEASE INHIBITION

Difference of Levels	P-Value
Aspirin (1.48 mg/mL) – Extract (100 mg/mL)	0.999
Aspirin (0.98 mg/mL) – Extract (100 mg/mL)	0.999
Aspirin (0.49 mg/mL) – Extract (100 mg/mL)	0.975
Aspirin (1.48 mg/mL) – Extract (75 mg/mL)	0.092
Aspirin (0.98 mg/mL) – Extract (75 mg/mL)	0.647
Aspirin (0. 49 mg/mL) – Extract (75 mg/mL)	0.856

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D. Anti-inflammatory Activity

In the albumin denaturation assay, denaturing the albumin through heating is a general result of inflammation and may possibly be caused by other physical and chemical agents inside the body. Further evaluation should be done in order to establish a potential for anti-inflammatory activity. In the results, the hexane extracts were not able to show a significant effect in albumin denaturation inhibition, owing to the different polarities of the solutions used.

The study by Steenkamp *et al.* (2013) showed that phytochemicals present in some plant materials are able to stabilize lysosomes, such as flavonoids, tannins, and saponins. Stabilization of lysosomal membrane could also mean the stabilization of HRBC membrane due to their analogous structure. *Blumea balsamifera* leaves have been known to be a rich source of flavonoids, which could be the cause for its stabilizing effect on the HRBC membrane as seen in the results [10]. The highest concentration of 100 mg/mL hexane extract was able to stabilize the HRBC membrane by 22.33 ± 0.87%. The percentage is lower than 30% which could be due to the low source of phytochemicals present in most hexane extracts of different plant materials. Some polar phytochemicals have possibly been washed away during maceration using the nonpolar hexane.

Among the three anti-inflammatory assays, the protease inhibition assay most likely showed the greatest significance when comparing the positive control (aspirin) and the hexane extracts. The 100 mg/mL hexane extract had the highest % inhibition of 20.34 ± 2.17% among other concentrations. The low % inhibition may still caused by the washed phytochemicals during the maceration of hexane extract that could substantially contribute to a higher % inhibition. Interestingly, aspirin had a low % inhibition which may be due to its mode of action. Aspirin specifically blocks the activity of the cyclooxygenase (COX) enzyme, and not on the protease enzyme.

These three anti-inflammatory assays were generally targeting the neutrophil activation during inflammation while aspirin and other non-steroidal anti-inflammatory Drugs (NSAIDs) are specific on the prostaglandin suppression by COX-enzyme to regulate inflammatory activity in the body. The hexane extract of the *Blumea balsamifera* leaves could have extracted non-volatile constituents of flavonoids and sterols which possibly contributed to its potential for anti-inflammatory activity in HRBC membrane stabilization. As for its potential anti-inflammatory activity in protease inhibition, monoterpenes, sesquiterpenes, fatty acids, and flavonoids are known to inhibit neutrophil constituents at some point in inflammation [7].

IV. CONCLUSION

This research successfully aimed to analyze the *in vitro* anti-inflammatory activity on hexane extract of *Blumea balsamifera* leaves through albumin denaturation inhibition, HRBC membrane stabilization, and protease inhibition assays. The results showed that in all anti-inflammatory assays, 100 mg/mL hexane extract had the highest % inhibition and % stabilization. Both hexane extracts and aspirin were also concentration-dependent.

Based on the results, the % inhibition and stabilization of the hexane extracts were below 30% which is attributed to the washed polar phytochemicals during maceration with nonpolar hexane. It has been concluded that the hexane extract of *Blumea balsamifera* leaves have a potential anti-inflammatory activity in the assays, HRBC membrane stabilization and protease inhibition, in comparison to the aspirin. *Blumea balsamifera* leaves are a potential candidate for anti-inflammatory agent, minimizing side effects from non-steroidal anti-inflammatory drugs (NSAIDs).

V. RECOMMENDATIONS

For aspiring studies related to this research, it is recommended to include other solvents such as polar (e.g. water) and semi-polar (e.g. ethanol) solvents to widen the scope and analysis of the study. It is also recommended to utilize other plant parts of *Blumea balsamifera* such as roots, flowers, trunk, fruits, stems, or bark for future studies regarding its medicinal properties. Characterization of volatile and non-volatile constituents of *Blumea balsamifera* leaves should be assessed using extraction techniques, such as steam distillation, CO_2 supercritical extraction, and column chromatography.

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