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In Vitro Anticoagulant Potential of *Caulerpa* sp. ("Lato"), *Eucheuma* sp. ("Guso"), *Ananas comosus* ("Pineapple") Peeling and *Psidium guajava* ("Guava") Leaf Extracts in ICR Mice

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ABSTRACT

Anticoagulants are valuable treatments for several circulatory diseases, thrombotic disorders and for hematologic analysis. In this study, the in vitro anticoagulant potential of *Caulerpa* sp. ("Lato"), *Eucheuma* sp. ("Guso"), *Ananas comosus* ("Pineapple") peelings and *Psidium guajava* ("Guava") leaf extracts were evaluated. Samples were washed with distilled water, air dried for 5-7 days, macerated and extracted for 2-3 days. In vitro anticoagulant potential of the obtained extracts was tested on 15 male ICR mice (10-12 weeks old), and were grouped into experimental, positive control (aspirin) and negative control (no treatment). Coagulation time was evaluated using the slide method. Two hundred ul of each of the extract was placed on separate slides (in triplicate). The mice were sedated and punctured at the facial vein. A drop of blood was placed on each slide on top of the prepared extract and was mixed gradually using a microhematocrit tube. Coagulation time was recorded when fibrin was seen to form. Results revealed that guso and pineapple extracts had longer average coagulation time (at least 12 minutes) than "lato" (7 minutes) and guava (5.2 minutes) extracts. One-way analysis of variance (ANOVA) revealed significant differences (p value = 0.022, F value = 3.314), which were accounted by guso and pineapple extracts (p value 0.033 each) in the post-hoc analysis. The present study showed the anticoagulant potential of the extracts from the whole plant of guso and pineapple peelings. Further studies must be explored in isolating specific components of the extracts for drug development.

KEYWORDS: *Ananas comosus, anticoagulant, Caulerpa sp., Eucheuma sp., Psidium guajava*

1 INTRODUCTION

Homeostasis includes maintenance of normal blood circulation. It is characterized by the continuous flow of blood to the different organ systems of the body. An anticoagulant substance (heparin) present in the blood prevents it from clotting to allow normal circulation (Hall et al., 2015). Anticoagulants are used in the treatment of several circulatory diseases and thrombotic disorders, including atrial fibrillation, pulmonary embolism, deep vein thrombosis, venous thromboembolism, congestive heart failure, stroke, myocardial infarction, and genetic or acquired hypercoagulability (Alquwaizani et al., 2013). It has also been used for treatment against snake venoms (Oliveira et al., 2005).

Anticoagulants are used in obtaining blood samples for routine hematological examination in human and animal disease diagnosis. Among the commonly used anticoagulants is the ethylenediaminetetraacetic acid (EDTA) (Oviedo and Rodríguez, 2003). EDTA is currently considered as an environmental pollutant (Thompson et al., 1983). It is usually the preferred chemical substance for blood samples for complete blood count. EDTA has been shown to cause hemolysis in common carp blood (Witeska and Wargocka, 2011).

The antithrombotic effect of aspirin (Gurbel et al., 2007) has been known to be medically significant, as it is a potent agent that could inhibit platelet aggregation (Weiss, 2003). In the past 30 years, aspirin as an anticoagulant has saved patients with cardiovascular diseases (Awtry and Loscalzo, 2000) and has reduced the formation of thrombus on the damaged surface of the arterial wall (Undas et al., 2007)

Anticoagulant therapy may be expensive, and alternative cheaper medicines from natural sources may be explored. The search for alternative sources of anticoagulants has risen as a result of the increasing demand for safer anticoagulant clinical therapy (das Neves Amorim et al., 2011). While several studies have demonstrated the anti-thrombin and anticoagulant properties of the marine algae *Caulerpa* sp. (Costa et al., 2012; Rodrigues et al., 2009; Rodrigues et al., 2011), only one study has specifically investigated *Caulerpa lentillifera* as a potential anticoagulant

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(Arenajo et al., 2017). Other natural sources, including *Eucheuma* sp. ("Guso"), *Ananas comosus* ("Pineapple") peeling and *Psidium guajava* ("Guava") also have reported anticoagulant properties. However, comparative in vitro evaluations of their potential has not yet been performed. Hence, this study was conducted.

2 MATERIALS AND METHODS

Research Design

The study is an experimental type. The potential anticoagulant properties of the selected extracts were evaluated in the ICR mice for two weeks (inclusive of one week for animal acclimatization). Mice were assigned into three groups: 1) negative control (no extract), 2) positive control (aspirin) and 3) experimental groups.

Research subjects and plant sources

Fifteen male ICR mice (10-12 weeks old; approximately 30g) were randomly allocated into three equal groups: experimental, positive control (aspirin) and negative control (no treatment). The mice were purchased from the University of San Carlos Laboratory Animal Facility, a BAI-registered and PALAS-accredited breeding facility. "Lato" and "guso" were obtained from Barangay Kalawisan, Cordova, Cebu, while pineapple peeling and guava samples were secured from commercial sources. Species identification was performed by a biologist from the Biology and Environmental Studies Program of the University of the Philippines Cebu. The study was conducted at University of the Visayas, Gullas College of Medicine, Cebu City.

Preparation of plant samples

Ten kilograms of fresh "guso" and "lato" and two kilograms of fresh guava leaves and pineapple peels were washed with distilled water to remove the foreign materials adhering to the plant samples. A 20 cm plastic strainer was used to hold the plant samples for one hour to let the excess water drip. Washed samples were transferred to a working table lined with filter paper to remove the excess water adhering to the plant samples. All plant materials were air dried for one to two weeks. After air-drying, samples were minced into small sizes (1.5 cm).

Preparation of the crude extract

The plant materials were macerated with 80% ethyl alcohol for two days. It was then subjected to reflux distillation for two hours to further exhaust the plant material. After distillation, it was evaporated to syrupy consistency to produce the extract. All extracts were placed in a tightly closed container and kept refrigerated at 2-80C until further use.

Animal marking and assignment to groups and cages

Upon arrival at the animal facility, the animals were randomly assigned to cages and marked at the base of the tail using permanent markers for identification. Cages were also randomly assigned to the different treatment groups. The health of animals was assessed by physical examination.

Animal acclimatization, monitoring, and maintenance

The mice were acclimatized for seven days. Commercial feeds and water were provided ad libitum. Lighting was on a 12 hour dark-light cycle, and ambient temperature was maintained between 24-260C. Beddings were changed every three days. Body score, appearance and behavior (Ullman-Culleré and Foltz, 1999; Bekkevold et al., 2013) were monitored.

Preparation and administration of aspirin and distilled water

The amount to be administered was computed based on the bodyweight (at 10 ml/kg). Aspirin and distilled water were administered orally at 24 and 12 hours prior to blood collection using a gauge 16 ball point gavage needle. Gavage needles were disinfected and flushed with distilled water for three times in between administrations. For the positive control (aspirin), one-fourth of a 300 mg-aspirin tablet was crushed and mixed in 75 ml distilled water and was orally administered at a dose of 5 mg/kg (Zhou et al., 2014).

Sedation, blood samples and evaluation of coagulation

The mice were sedated using a combination of tiletamine/zolazepam (Zoletil) administered intraperitoneally. After sedation, the facial vein was punctured using a hematocrit tube. One drop of blood was placed on each slide containing 0.2 ml of the experimental extracts (accomplished in triplicates) and was mixed gradually using a hematocrit tube. Coagulation time was recorded once fibrin was seen. Longest coagulation time was pegged at 12 minutes as it was observed that the slides would dry up after such time.

Data processing and analysis

Observations were manually recorded in a tally sheet. Gathered data were encoded in Microsoft Excel and imported into statistical software. Data was analyzed using one way analysis of variance (ANOVA) with post-hoc analysis by Tukeys method. The significance level was set at 5%.

Ethical consideration

The procedures performed in this study were guided by the principles of animal welfare, Animal Welfare Act of the Philippines (RA 8485) and AO 45 of the Bureau of the Animal Industry. The study was also approved by the Institutional Animal Care and Use Committee.

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Committee of Pet Science Laboratory Animal Facility and Veterinary Clinic, Cebu City, Philippines.

3 RESULTS AND DISCUSSION

The whole plant of guso and pineapple peeling extracts were found to have the longest average clotting time (at least 12 minutes), followed by the "lato" (seven minutes). Guava leaf extract was also found to prolong clotting time (5.2 minutes) compared to the negative control (0.9 minute), but almost the same result with that of the positive control (aspirin) (Table 1). Guso has been shown to have an anticoagulant effect because of its carrageenan component (Anderson et al., 1965; Rosa, 1972; McLellan and Jurd, 1992).

Pineapple contains a complex enzyme, bromelain, which may have an anticoagulant property. Studies have been conducted using stems (Milić et al., 2014) and fresh fruit juice extract (Evangelista et al., 2012), but it appears that the present study is the first to explore pineapple peels as a potential source of anticoagulant. On the other hand, Hsieh et al. (2007) also found an anticoagulant property of guava.

Statistical analysis revealed significant differences (p value 0.022, F value 3.314), which were accounted guso and pineapple extracts (p value = 0.033 each) in the post-hoc analysis (Table 2). The results indicate that guso and pineapple had longer clotting time, suggesting better anticoagulant properties than the other treatments.

Aspirin has properties that can reduce the ability of the blood to clot, and thus it is often used in the treatment of conditions associated with blood clots, including heart attacks (Guirguis-Blake et al., 2016). The present study showed that the studied extracts could have anticoagulant properties that can be equal to or much better than aspirin.

Table 1. Average clotting time (minutes) of male ICR blood mixed with Caulerpa sp., Eucheuma sp., Ananas comosus peeling and Psidium guajava extracts

Group	Mean	SD
Eucheuma sp.("*Guso"*)	12.0	0.0
Caulerpa sp. ("*Lato"*)	7.0	5.7
Ananas comosus (Pineapple) peeling	12.0	0.0
Psidium guajava (Guava) leaf	5.2	9.4
Positive Control (Aspirin)	6.2	10.0
Negative Control (Normal saline solution)	0.9	0.3

Table 2. Post-hoc analysis of the coagulation time between Eucheuma sp., Caulerpa sp., Ananas comosus, Psidium guajava and control groups

Group		Mean Difference	Std. Error	Sig.
Eucheuma sp.	*Caulerpa* sp.	4.977	3.070	.594
	Ananas comosus	0.000	3.070	1.000
	Psidium guajava	6.765	3.070	.275
	Aspirin	2.936	3.256	.942
	Negative control	10.763*	3.256	**.033**
Caulerpa sp.	*Eucheuma* sp.	-4.977	3.070	.594
	Ananas comosus	-4.977	3.070	.594
	Psidium guajava	1.788	3.070	.991
	Aspirin	-2.042	3.256	.988
	Negative control	5.785	3.256	.500
Ananas comosus	*Eucheuma* sp.	0.000	3.070	1.000
	Caulerpa sp.	4.977	3.070	.594
	Psidium guajava	6.765	3.070	.275
	Aspirin	2.936	3.256	.942
	Negative control	10.763*	3.256	**.033**
Psidium guajava	*Eucheuma* sp.	-6.765	3.070	.275
	Caulerpa sp.	-1.788	3.070	.991
	Ananas comosus	-6.765	3.070	.275
	Aspirin	-3.830	3.256	.843
	Negative control	3.997	3.256	.819
Aspirin	*Eucheuma* sp.	-2.936	3.256	.942
	Caulerpa sp.	2.042	3.256	.988
	Ananas comosus	-2.936	3.256	.942
	Psidium guajava	3.830	3.256	.843
	Negative control	7.827	3.432	.244
Negative control	*Eucheuma* sp.	-10.763*	3.256	**.033**
	Caulerpa sp.	-5.785	3.256	.500
	Ananas comosus	-10.763*	3.256	**.033**
	Psidium guajava	-3.997	3.256	.819
	Aspirin	-7.827	3.432	.244

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In a study by Rodrigues et al. (2011), the anticoagulant property of a related species Caulerpa cupressoides var. lycopodium was found to be dose-dependent. The aforementioned study also extracted sulfated polysaccharides (SP) from the selected seaweed.

SPs are structural components of the cell wall of marine algae, in which they are found in high concentrations (Painter et al., 1983; Pereira et al., 2005; Rodrigues et al., 2009; Rodrigues et al., 2010). The use of these molecules as alternative sources of anticoagulants is justified by the fact that algae are phylogenetically distant from mammals, significantly reducing contamination by viral particles (Leite et al., 1998).

Another study evaluated the in vitro anticoagulant activity of SP fractions from red alga Halymenia pseudofloresia using citrated rabbit plasma and observed marked changes in activated partial thromboplastin time (APTT) (Rodrigues et al., 2009). The fractions obtained in the first (464.20, 211.60, 103.50 and 101.70 IU mg-1) were more active compared to those from the third extraction (137.10, 96.50 and 89.20 IU mg-1). Its actions were considered superior to the existing heparin standard (100 IU mg-1) and to SPs from the same-genus species Halymenia sp. (Rodrigues et al., 2010).

4 CONCLUSION

The ethanolic extracts of guso and pineapple peelings apparently have in vitro anticoagulant potential. The potential of these extracts as an in vitro anticoagulant for diagnostic purposes and as a potential therapy for thrombotic disorders can be explored in vivo. Further studies in isolating specific components of the extracts for drug development are also recommended.

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