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Philippine Wild Medicinal Mushroom, *Ganoderma lucidum* (Curtis: Fr.) P. Karst., Exhibits Anticoagulative Effect in Intrinsic Pathway

[1]Rich Milton R. Dulay, [2]Marwin L. De Guzman, [2]Roselito R. Magisa, [2]Jenny C. Mariano, [2]Ma. Cristina A. San Pedro, [2]Nancy Joy M. Mangansat, [2]Jo Neil T. Peria

[1]Department of Biological Sciences, College of Arts and Sciences, Central Luzon State University, Science City of Munoz, Nueva Ecija, Philippines
[2]College of Education, Nueva Ecija University of Science and Technology - Sumacab Campus, Cabanatuan City, Philippines

Address For Correspondence:
Rich Milton R. Dulay, Department of Biological Sciences, College of Arts and Science, Central Luzon State University, Science City of Munoz, Nueva Ecija 3120, Philippines.
E-mail: richmiltondulay@yahoo.com

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ABSTRACT

The significant contribution of blood coagulation in the growing number of incidences of cardiovascular diseases has prompted researchers worldwide to explore for treatments which will maintain it in its normal condition. In the present work, an attempt was made to investigate the anticoagulant activity of two Philippine medicinal mushrooms, *Ganoderma lucidum* and *Schizophyllum commune*. The active components of fruiting bodies were obtained through hot water extraction. *In vitro* anticoagulation assays such as activated Partial Thromboplastin Time (aPTT) and Prothrombin Time (PT) were performed on the citrated plasma of the five healthy volunteer blood donors. Coagulation times of the two mushroom extracts were compared to heparin, non-treated, and in-house control. Extract of *G. lucidum* significantly exhibited high anticoagulative activity in aPTT, but not in PT, indicating its apparent effect in intrinsic pathway of blood coagulation. In contrast, extract of *S. commune* has no effect on the blood clotting time in both aPTT and PT. Overall, hot water extracts of *G. lucidum* has inhibitory effect on blood coagulation specifically on the intrinsic pathway which may lead for its utilization as a novel organic anticoagulant in the future.

KEYWORDS: *Ganoderma lucidum*, anticoagulant, aPTT and PT, intrinsic pathway

INTRODUCTION

Blood coagulation (blood clotting) plays important roles in the incidences of cardiovascular troubles. Clotting may be due to the generalized activation of the cellular mechanisms resulting in clotting on the surface of monocytes and platelets in circulation, the activation of the coagulation and fibrinolytic systems, and the disruption of the vascular endothelium [1]. Thrombosis, the formation of an abnormal clot called a thrombus, can stop blood circulation in vessels (arteries or veins), and may cause thromboembolic disorders such as pulmonary emboli, deep vein thrombosis, strokes and heart attacks [2]. Tortora and Grabowski [3] explained that thrombin has a central role in haemostasis. It serves as the final enzyme in the coagulation cascade, converting fibrinogen to fibrin. It also activates factor XIII, accelerates the formation of factor V, which increases thrombin formation, and activates platelets, thus enhancing platelet aggregation and the release of phospholipids.

Activation both of blood coagulation and of platelets is important in the pathogenesis of thrombosis. Therefore, both anticoagulants and drugs that suppress platelet function are potentially effective in the prevention and treatment of thrombosis. Heparin is a natural anticoagulant that stimulates the activity of anti-

thrombin III and prevents the assembly of fibrinogen molecules into fibrin [4]. It also interacts with platelets and endothelial cells which may contribute to heparin-induced bleeding by a mechanism independent of its anticoagulant effect [5]. Dabigatran, rivaroxaban, and other agents currently in the pipeline of clinical development have the potential to replace warfarin in the two most frequent indications for anticoagulation, i.e. secondary prophylaxis of VTE and atrial fibrillation [4].

Mushrooms are one of the living components of the environment that contain certain bioactive compounds beneficial to human health. A wide variety of compounds isolated from many species of mushrooms, have been identified [6] and most of them were terpenoids, steroids, fatty acids, proteins, lectins, proteoglycans, and especially polysaccharides [7,8]. *Ganoderma lucidum* and *Schizophyllum commune* are wood-rotting basidiomycetes that commonly found growing in the Philippines. They are considered medicinal edible mushrooms since they exhibited various functional activities. For instance, the schizophyllan derived from *S. commune* and polysaccharides from *G. lucidum* have been approved in several countries as prescription drugs for the treatments of cancer [9,10].

Herein, we investigated the anticoagulative effects of the aqueous extracts of fruiting bodies of *S. commune* and *G. lucidum* in-vitro based on prothrombin time (PT) and activated partial thromboplastin time (aPTT).

MATERIALS AND METHODS

Mushroom Source:

Air-dried mature fruiting bodies of *G. lucidum* and *S. commune* were requested from the Center for Tropical Mushroom Research and Development, Central Luzon State University (CLSU), Science City of Muñoz, Nueva Ecija, Philippines.

Hot Water Extraction:

The air-dried fruiting bodies of mushrooms were pulverized using a food processor and extracted following the protocol of Eguchi et al. [11]. Five grams of each milled mushroom was extracted in 150 ml hot distilled water at 80-90°C in a water bath for 2 hours. Extracts were filtered using Double Rings Qualitative 102 filter papers (Vallery Enterprises). The extracts were used in anticoagulative effect evaluation.

Screening of Volunteer Blood Donors:

Five healthy volunteer donors, aged 18-25 years old, were sought for this study. The assistances of medical technologists and nurses from AC Medlinks Specialty Clinic and Diagnostic Center in screening for donors were requested. Preliminary measures including family history of cardiovascular diseases and other major coagulopathies were gathered and ensuring that the donor has not been injected with heparin nor taken aspirin or any blood-related drugs for at least two weeks was done. Blood samples were first compared with an in-house control (Spinreact) used by the clinic to ensure that the samples were normal and were appropriate for testing. Donors were informed of the reasons of conducting this research through a written letter and provided with written consent forms and signed. Data were treated with proper care and confidentiality. The procedures were carried out in compliance with the international ethical standards of research involving humans as subjects.

Blood Collection and Processing:

Blood samples were drawn through venipuncture at the *antecubital fossa* of forearms of the donors. Ten cubic centimeters (10 cc) of blood were extracted from each of the five donors. From this collected blood, 1.8 ml were deposited in a blue-top tube (Branden) containing 3.2% sodium citrate and were centrifuged at the speed of 4000 rotations per minute for 10 minutes. Addition of sodium citrate is needed to prevent the blood from clotting. Centrifugation was done to separate the components of the blood i.e. erythrocytes, leukocytes and thrombocytes from the plasma of the blood.

In-vitro Anti-coagulative Assay:

Two standard tests were used to examine the anticoagulative effects of the extracts of the two mushrooms. These were the activated Partial Thromboplastin Time (aPTT) test which measures the intrinsic pathway of blood coagulation and the Prothrombin Time (PT) test which monitors the tissue factor (extrinsic) pathway of clotting. In aPTT, each 100 microliters (100 µL) decalcified blood was mixed with 50 µL simplastin (Spinreact) and 50 µL extract and were incubated at 37 ° C for 3 minutes. Then, 50 µL calcium chloride (Spinreact) was added to initiate clotting. The tube was then swirled until the clot forms. The clotting time was defined as the time when the sample started to coagulate after the last substance was added on it. Prothrombin Time (PT) test was performed almost the same with APTT except that a tissue factor thromboplastin (TEClot) is added instead of simplastin. Heparin (Microcell Pharma Drug Mart), a proven commercial anti-coagulant, was used as the positive control. The untreated blood sample served as the negative control and the basis if the extracts can prolong clotting time.

Statistical Analysis:

The Statistical Package for Social Sciences (SPSS) 17 was used. Data were analyzed using one way Analysis of Variance (ANOVA) and treatment means were compared using Least Significant Difference (LSD) at 5% level of significance.

RESULTS AND DISCUSSION

Anticoagulative Effect in Intrinsic Pathway:

aPTT, a modified version of the activated Partial Thromboplastin Time test, is a test that measures the intrinsic pathway of coagulation. It is normally prescribed in patients with unexplained bleeding or clotting. The aPTT represents the time for clot formation after adding calcium, phospholipids, and kaolin to a sample of citrated blood. It is prolonged by heparin, direct thrombin inhibitors, a deficiency of or inhibitor of factors in the intrinsic and common pathways (e.g., factors II, V, VIII, IX, X, XI, and XII) as well as lupus anticoagulant, vitamin K deficiency, or severe liver disease [12]. In the present study, the anticoagulative effect of the two mushroom extracts in intrinsic pathway was evaluated using aPTT assay and the results are presented in Table 1. Among the two extracts, blood treated with *G. lucidum* extract significantly delayed the time of blood coagulation. A mean result of 74.8 seconds was remarkably greater than the 30 second-normal time. The blood samples actually clot 2.49 times longer than the normal, non-treated controls. This indicates that the hot water extracts of *G. lucidum* might have inhibited any of the following factors needed in the intrinsic pathway: fibrinogen, prothrombin, and factors V, VIII, IX, X, XI, and XII. Thus, bioactive chemical attributes of *G. lucidum* have apparent anticoagulative potential.

On the other hand, those blood samples treated with *S. commune* extract recorded the same time to non-treated and in-house control blood. No change in the clotting time was observed. It can be deduced that the extract of *S. commune* is not effective as source of anticoagulant via the intrinsic pathway. However, the blood with heparin did not coagulate even after the cut-off time of observing clotting time (300 seconds).

Table 1: Clotting time of blood treated with mushroom extracts in activated Partial Thromboplastin Time (aPTT) assay.

Treatment	Clotting Time (sec)					
	1	2	3	4	5	Mean
G. lucidum extract	54	75	60	47	138	74.8[b]
S. commune extract	30	30	30	30	30	30.0[c]
Heparin	>300	>300	>300	>300	>300	>300.0[a]
Non-treated	30	30	30	30	30	30.0[c]
In-house control	30	30	30	30	30	30.0[c]

In mean column, means with the same letter of superscript are not significantly different from each other at 5% level of significance in LSD.

Anticoagulative Effect in Extrinsic Pathway:

Simultaneously with platelet plug formation during a vessel injury, tissue factor and collagen were liberated upon vessel wall. The released tissue factor interacts with Factor VII. This binding activates factor X to its active form, Xa, which in turn, binds to the co-factor FV and is bound on membrane surfaces in the presence of calcium ions to generate the prothrombinase complex. The prothrombinase complex converts prothrombin to thrombin, which converts fibrinogen to fibrin to generate the fibrin clot. This coagulation initiated by tissue factor is the extrinsic pathway of coagulation [13]. During laboratory analysis of blood clotting, the extrinsic pathway of blood coagulation is evaluated using the prothombin time test [14]. The Prothrombin Time/International Normalized Ratio (PT/INR) is the time, in seconds, it takes for a blood sample to clot after the addition of a platelet activator inhibitor and a clotting factor i.e. a tissue factor. It is an assay designed to measure and screens for defects the activities of the extrinsic pathway of coagulation. The anticoagulative effect of the two mushroom extracts in extrinsic pathway was evaluated using PT assay was evaluated in this study. The results of clotting time of blood treated with mushroom extract in PT assay are presented in Table 2. Similar with the results in aPTT, heparin also inhibited the coagulation blood samples from clotting up to the 300 second cut-off time. Both mushroom extracts, however, did not influenced the clotting time of the blood samples added with tissue factor (extrinsic pathway). No change in the coagulation time of the blood samples despite the presence of the extracts. This only dictates that the two extracts have no effects on the enzymes involved in the extrinsic pathway of coagulation.

Numerous plant extracts were found to exhibit anti-coagulative activity. For instance, the aqueous extract of *T. capensis* (0.43 mg/ml) exhibited strong thrombin inhibition. The aqueous leaf extracts of *G. superba* (5.30 mg/ml), *L. leonurus* (9.69 mg/ml), *S. frutescens* (2.23 mg/ml) and *Z. aethiopica* (4.74 mg/ml) also showed moderate antithrombotic activity [15]. In addition, Kee et al., [15] also investigated the presence and absence of tannins in the anti-coagulative activity of plants. The plant extracts that showed activity in the presence of tannins were methanol leaf extracts of *G. superba*, *L. leonurus*, *S. frutescens* and *Z aethiopica*, stem extract of *L. leonurus*, rhizome extract of *T. capensis*, and aqueous leaf extracts of *A. ferox*, *A. spesiosa*, *G. superba*, *S.*

frutescens and *Z. aethiopica*, rhizome extract of *T. capensis*. After tannin removal, only the aqueous extracts of *G. superba* and *Z. aethiopica* retained their anticoagulant activity. Therefore, aside from tannins, there are other bioactive compounds present in these two plants that also exhibit anticoagulative activity.

Table 2: Clotting time of blood treated with mushroom extracts in Prothrombin Time (PT) assay.

Treatment	Clotting Time (sec)					
	1	2	3	4	5	Mean
G. lucidum extract	14	14	14	14	14	14.0[b]
S. commune extract	14	14	14	14	14	14.0[b]
Heparin	>300	>300	>300	>300	>300	>300.0[a]
Non-treated	14	14	14	14	14	14.0[b]
In-house control	14	14	14	14	14	14.0[b]

In mean column, means with the same letter of superscript are not significantly different from each other at 5% level of significance in LSD.

G. lucidum contains a wide variety of bioactive substances that shown many interesting biological activities, including anti-tumor, anti-inflammatory, anti-oxidant and antidiabetic effects [16-18]. The primary bioactive compounds are commonly considered to be polysaccharides and triterpenoids [19]. However, Choi and Sa [20] reported that a putative metalloprotease purified form *G. lucidum* mycelium showed anticoagulant activity in human plasma, which behaved as a competitive inhibitor of thrombin-catalyzed fibrin formation. Moreover, a crude polysaccharide fraction of *G. lucidum* inhibited the intrinsic pathway in blood coagulation and exhibited concentration dependent anticoagulation effects [21]. They also added that the carbohydrate moiety may be related to this activity. *G. lucidum* contains glucose, galactose, fucose, xylose, and arabinose. Aqueous extracts of other mushrooms also significantly showed moderate to high activity in aPTT. These include *Pleurotus ostreatus, Hericeum erinacium, Lentinus edodes, Auricularia auricula, Auricularia polytricha, Tremella fuciformis, Gyrophora esculenta, Tricholom matsutake, Agaricus bisporus, Grifola frondosa,* and *Flammulina velutipes* [21].

Conclusion:

Taken the data together, aqueous extract of *G. lucidum* exhibited anticoagulative effective in intrinsic but not in extrinsic pathway of blood coagulation while the aqueous extract of *S. commune* did not show anticoagulative activity in both pathways.

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