Chapter 1.0 Introduction

Blood is an important tissue that plays a role in delivery oxygen and nutrients to all parts of our body. Therefore it is important to maintain a smooth blood flow in our body. Blood clotting is a normal and vital process that helps to prevent excessive bleeding when blood vessel is injured by forming clots at the damaged or injured vessels. Generally, platelets and proteins in our plasma work together to form a clot at injured sites. When an injury occurs, a clot usually forms within a few minutes. It is a complex process that needs to be carefully controlled as platelet hyperactivity or hyperaggregation will lead to internal clots which can be fatal.

Blood clots can occur in veins or arteries which are parts of the body circulatory system. The excessive clotting in the body can block the blood, oxygen and nutrient supply to a particular region of our body. These blood clots in arteries can cause atherothrombotic diseases such as myocardial infarction and cerebrovascular diseases if left untreated (Lee *et al*., 2009). These situations can be dangerous and require further accurate diagnosis and appropriate treatment. According to Smith *et al*., (2004), cardiovascular diseases are the most common cause of mortality in the developing countries. In western countries, more than 17 million people die from blood clotting related diseases. According to OECD/WHO (2012), the mortality rate of cardiovascular disease in Malaysia for 2008 was 263/100,000 populations. Thrombin, adenosine-5'-diphosphate (ADP), epinephrine and arachidonic acid are among the risk factors that contribute to platelet dysfunctions. Other risk factors causing the blood clotting related diseases are smoking, ageing, hypertension insulin resistance and hypercholesterolemia (Amrani and Harnafi, 2009).

Currently, the treatment available for arterial thrombosis includes the anticoagulants and the four classes of antiplatelet aggregation agents: aspirin, thienopyridines, phosphodiestherase inhibitors and glycoprotein IIb-IIIa antagonists. Although these currently available antithrombotic treatments show remarkable success in preventing arterial thrombosis, there are some limitations including drugs non-responsiveness and the determination of optimal dosage of drug that should be given to particular patients. A higher dose of drugs given to patient usually shows higher and better antithrombotic activity. However, this will lead to bleeding problem in a patient (Phillips *et al*., 2005). Therefore, there is a critical need for new anti-platelet aggregation drugs which do not increase the risk of bleeding.

According to Charles *et al*. (1997), common anti-platelet aggregation drugs can reduce the risk and protect from diseases such as strokes, myocardial infarction and cardiovascular death. However, these drugs like aspirin and clopidogrel have restrictions in their effectiveness and mode of action (Dominic, 2009; Fabre and Gurney, 2010). The consumption of anti-platelet aggregation drugs such as dipyridamole, clopidogrel, and ticlopidine can also cause bone marrow suppression, in particular leucopenia which decreases the number of white blood cells in the blood (Blann *et al*., 2002). Other than that, some blood thinners have carcinogenic side effect caused by the synthetic drugs (Sachez-Lamar *et a*l., 1999).

Many efforts have been done to search for better anti-platelet aggregation drugs. In bovine K-casein, an undecapeptide and the pentapeptide inhibit platelet aggregation in blood (Jolles and Caen, 1991). In soy protein, GE (Ser–Ser–Gly–Glu) and DEE (Asp–Glu–Glu) peptides inhibit rat platelet aggregation induced by adenosine-5'-diphosphate (ADP) (Lee and Kim, 2005). Other than that, medicinal plant such as

Rapanea melanophloeos (L.) Mez was known to be used by Zulu traditional healers to manage blood-clot related diseases. The extracts showed inhibition of rat platelet aggregation induced separately by thrombin, adenosine-5'-diphosphate (ADP) and epinephrine (Phiwamandla, 2011). Anti-platelet aggregation activities on rat platelets were also been observed in a medicinal plant namely *Bulbine natalensis* (Geraldine, 2011).

In recent years, the efforts to search for anticoagulant in variety of edible and medicinal mushrooms has started as recent studies showed that mushrooms have more medicinal benefits than what people previously realised. Based on Lindequist *et al*. (2005), the medicinal uses of mushroom have a very long tradition worldwide including Asia countries. According to Miles and Chang (1997), mushroom is a macrofungus which has distinctive fruiting bodies and is large enough to be seen by naked eye. Mushrooms were consumed since a long time ago due to its fine texture and flavor. Research done on mushrooms showed that they contained excellent nutritional values including essential and non-essential amino acids. Besides, these fungi provide large amount of carbohydrates, fiber, vitamins (B1, B2, B12, C and D) and mineral elements such as Mg, Na, Ca, K, P, Fe, Cu, Mn and Se (Guillamon *et al.*, 2010). In addition, mushrooms have low fat and cholesterol content and were excellent sources of protein (Ghorai *et al*., 2009). They also contained many physiologically active compounds.

Based on Chang (1996), extracts from mushrooms have been reported to exert antiviral, antitumorigenic, hypotensive and hepatoprotective effects. A number of antitumor substances have been identified from mushroom and polysaccharides are one of the substances that have antitumor together with immunomodulating properties (Wasser, 2002). Mushrooms are also important sources of thrombolytic agents. Their

fibrinolytic activity has been reported in many mushrooms such as *Flammulina velutipes* (Curtis) singer (Choi *et al*., 1999), *Pleurotus ostreatus* (Jacq.) P. Kumm. (Joh *et al*, 2004), *Schizophyllum commune* (Fr.) (Lu *et al*., 2010), *Armillaria mellea* (Vahl) P. Kumm. (Lee *et al*., 2005), *Grifola frondosa* (Dicks.) Gray (Nonaka *et al.*, 1997) and *Pleurotus ostreatus* (Jacq.) P. Kumm. (Shen *et al.*, 2007).

It is suggested that mushrooms can help in the prevention and in the treatment of thrombosis. Research have been done to search for platelet aggregation inhibitors. However, less potent platelet inhibitors without side effects have been developed especially from natural sources. Besides, few mushroom species are known to exert platelet aggregation inhibitory activity in their extract, the potent anticoagulant enzymes which contributed to the inhibitory activity have not been characterised. Anti-platelet aggregation activities have been reported in the extract of *Ganoderma lucidum* (Curtis) P. Karst**.** but the mechanism and the active principles remain to be further investigated (Tao and Feng, 1990). Besides that, it has been reported that *Auricularia polytricha* (Mont.) Sacc. contains platelet aggregating inhibitor in the crude dialysates of their aqueous extracts (Hammerschmidt, 1980; Hokama and Hokama, 1981).

It is believed that the anticoagulants present in mushrooms to be polysaccharide, protein or other compounds. For example, the polysaccharide in the extracts of *Auricularia auricula* (L.) Underw inhibits platelet aggregation and increases clotting times *in-vitro* and *in-vivo* (Yoon *et al.*, 2003). Other than that, lenthionine, a cyclic sulfur compound in *Lentinula edodes* (Berk.) Pegler was also known to inhibit platelet aggregation. From *Inonotus obliquus* (Ach. ex Pers.) Pilát, a tripeptide showed high platelet aggregation inhibitory activity in mice (Kwang *et al*., 2006). In this study, both medicinal and edible mushrooms were investigated. The protein in the mushrooms were

extracted and investigated for their anticoagulant properties. The medicinal mushrooms investigated were *G. lucidum*, *Cordyceps militarys* (L.) Link, and *Lignosus rhinocerotis* (Cooke) Ryvarden while the edible medicinal mushrooms investigated were *Pleurotus giganteus* (Berk.) S.C. Karunarathna & K.D. Hyde, *Pleurotus floridanus* (Singer) and *A. polytricha.*

Objectives

The objectives of this study were to:

i. investigate the potential antiplatelet aggregation activities in edible and medicinal mushrooms.
ii. identify and characterise the protein responsible for the antiplatelet aggregation activities.

Chapter 2.0 Literature Review

2.1 Blood clotting

Blood-clotting is a complex process that involves the activation of platelet and the coagulation cascades. The coagulation cascade is a stepwise process that involves various enzymes and coagulation factors.

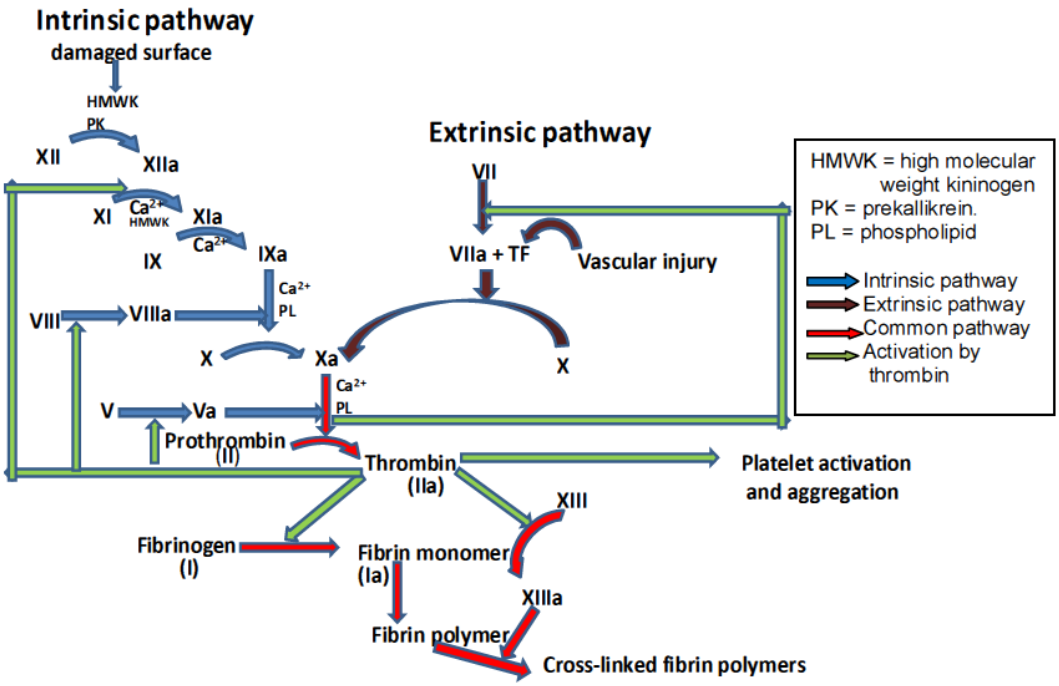

Figure 2.1: **The coagulation cascade.** The activation of the intrinsic and extrinsic pathways which converge at the final common pathway involving activation of factor X to Xa and generation of thrombin. Activated factors are designated with 'a' (King, 1996).

Based on King (1996) in Figure 2.1, there are intrinsic and extrinsic pathways. Intrinsic pathway is initiated when blood contacts with foreign surface. On the other hand, extrinsic pathway is initiated when there is vascular injury. The two pathways converge at a point that involves the activation of factor X to Xa. Factor Xa is known to convert prothrombin into thrombin. The thrombin is then converting the soluble fibrinogen into insoluble fibrin polymers. These fibrin polymers will form a meshwork together with the platelets to form blood clot. Other than that, thrombin can also

activate factors V, VIII, XI and XIII (Strukova, 2001). Factor XIIIa cross-links the fibrin polymers and stabilize the clot. Ca^{2+} also plays an important role in the activation of many proteins involved in the coagulation cascade.

2.2 Platelet activation and aggregation

Platelets are small irregularly-shaped anuclear cells. Resting platelets circulate in the blood in discoid shape. Upon blood vessel damage or injury, platelet activation begins. Generally, platelet aggregation involves several biochemical pathways. Platelet activation and aggregation is induced by binding of agonists on the various receptors found on the platelet surface. These agonists include thrombin, epinephrine, thromboxane A 2, collagen, platelet-activating factor and adenosine-5'-diphosphate (ADP). All of the agonists have their specific receptors on the platelet. The activated platelets rush to the site of injury and adhere to collagen after the exposure to the subendothelial matrix. Figure 2.2 shows the events that occur upon platelet activation followed by platelet aggregation. Platelet activation results in their morphological change. Granules such as adenosine-5'-diphosphate (ADP), thromboxane A 2, coagulation factors and inflammatory mediators are then released. Pro-aggregatory molecules such as thromboxane A2 and adenosine-5'-diphosphate (ADP) will amplify the platelet responses to collagens. Additional thromboxane A2 and adenosine-5'-diphosphate (ADP) will generate a number of second messengers, leading to platelet aggregation by the binding of fribinogen to platelets (Anderson *et al*., 1996). The binding of fibrinogen facilitates cross- linking of platelets resulting in platelet-rich thrombus formation. Then, the mature platelet-rich thrombus will be stabilised by fibrin polymers from the proteolytic activity of thrombin on the fibrinogen.

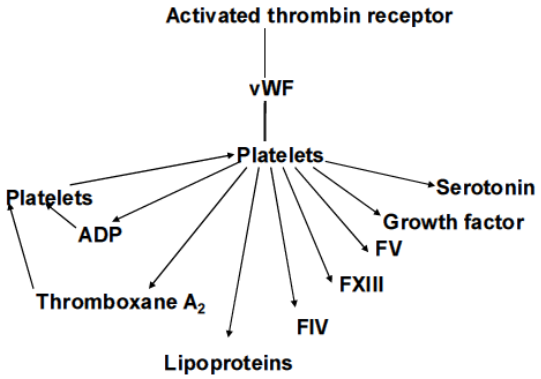

Figure 2.2: **Events that occur upon platelet activation followed by platelet aggregation.** Platelets undergo a morphological change and release granules (ADP, thromboxane A 2), some phospholipids and proteins that aid in coagulation (FIV, FV, FXIII) (Devlin,2006).

2.3 Platelet agonists

There are few platelet well-known agonists that bind to their specific receptors on the platelet surface and mediate the activation and aggregation of platelets. Platelet agonists include thrombin, adenosine-5'-diphosphate (ADP) and epinephrine.

2.3.1 Thrombin

Thrombin can mediate the activation and aggregation of platelets in two distinct pathways (Soslau *et. al*., 2001). The first pathway is the hydrolysis of protease-activated receptors-1 (PAR-1) on the membrane followed by glycoprotein llb/llla (GP llb/llla) dependent platelet aggregation. The second pathway does not include the hydrolysis of protease-activated receptors-1 (PAR-1). It involves the binding of thrombin to the platelet glycoprotein lb/lX/V complex. The binding site is known to be localized to the 45 000 Da N-terminal region of GP lb α-chain (De Candia *et al*.,1999). Fabre and Gurney (2010) suggested that thrombin acts through protease-activated receptors-1 (PAR-1) and protease-activated receptors-4 (PAR-4). Both of them are known to work together in the activation of platelets. The activation of membrane receptors following

by the shape changes, granular secretion, cytoskeletal remodeling and the aggregation of platelet is the characteristics of thrombin-induced platelet aggregation (Jardin *et. al*., 2007).

2.3.2 Adenosine-5'-diphosphate

Adenosine-5'-diphosphate (ADP) induced platelet activation is autocatalytic. After platelet activation induced by adenosine-5'-diphosphate (ADP), the platelet itself will release other adenosine-5'-diphosphate (ADP) molecules which in turn act on the platelet surrounding itself. Adenosine-5'-diphosphate (ADP) acts on G-protein with $P2Y_1$ and $P2Y_{12}$. According to Davi and Patrno (2007), $P2Y_1$ and $P2Y_{12}$ work together in the activation and the aggregation of platelet. The process is initiated by $P2Y_1$ and amplified by $P2Y_{12}$. The activation of platelet requires the present of Ca^{2+}. On the other hand, the inhibition of platelet activation will occur in the present of cyclic adenosine monophosphate (cAMP). Platelet activation by adenosine-5'-diphosphate (ADP) can be characterised by the morphological changes of platelet, the release of granules, the increase in Ca^{2+} but decreases in cyclic adenosine monophosphate (cAMP) concentration (Puri and Colman, 1997).

2.3.3 Epinephrine

Epinephrine is known as a weak platelet agonist. According to Lanza *et al*. (1988), epinephrine induces platelet aggregation by acting on α2-adrenergic receptors. It can also potentiate other aggregating agents such as fibrinogen biding and granular release on aggregation.

2.4 Antiplatelet aggregation agents

Platelet aggregation is one of the major contributors to thrombosis especially in arterial thrombosis. Currently, the treatment available for arterial thrombosis includes the anticoagulants and the four classes of antiplatelet aggregation drugs which are classified based on their action mode (Mahajan *et al*., 2012). The antiplatelet aggregation drugs are shown in Figure 2.4 and these antiplatelet aggregation drugs include:

i. Adenosine-5'-diphosphate (ADP) antagonists (thienopyridines)

ii. COX-1 inhibitors

iii. Phosphodiestherase inhibitors

iv. GPIIb/IIIa antagonists

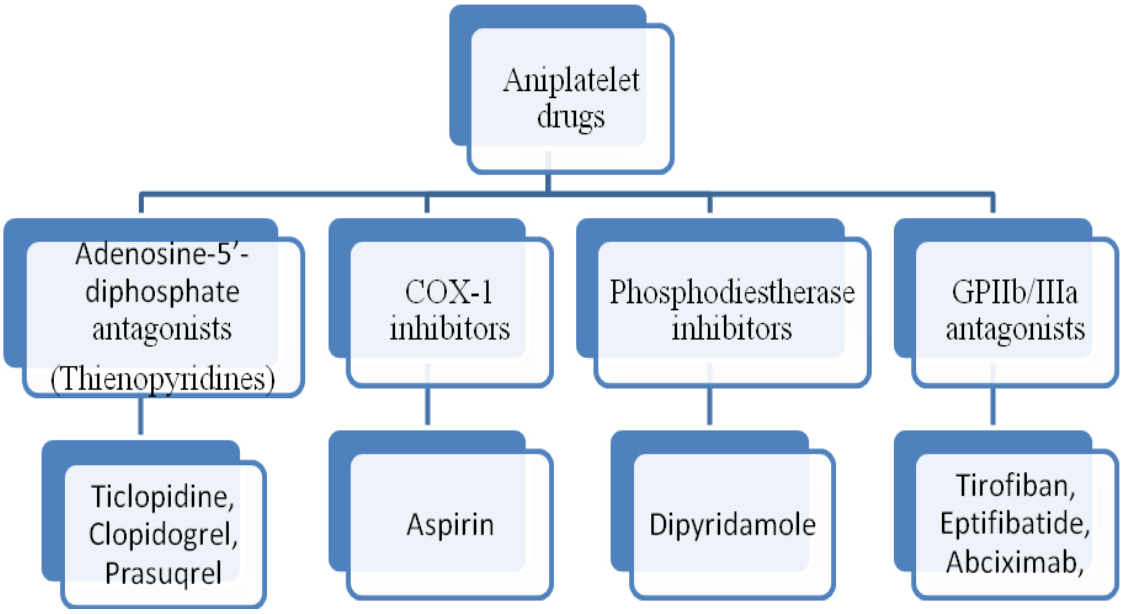

Figure 2.3: **Antiplatelet aggregation agents** (Mahajan *et al*., 2012).

2.4.1 Adenosine-5'-diphosphate (ADP) antagonists (Thienopyridines)

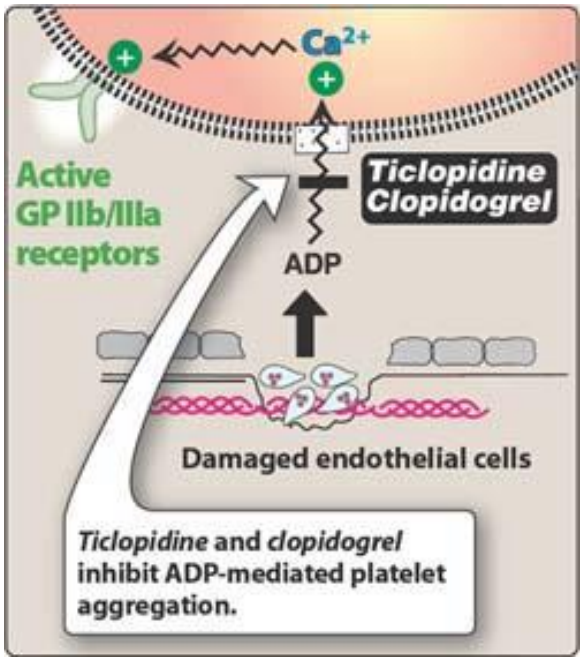

Figure 2.4: **Thienopyridines that block Adenosine-5'-diphosphate (ADP) receptors** (Mahajan *et al*., 2012).

Thienopyridines prevents platelet aggregation by inhibiting the adenosine-5'-diphosphate (ADP)-dependent pathways of platelet activation. The inhibition pathway is shown in Figure 2.4. The oldest drug in this class is the Ticlopidine. It can be used in patient intolerant to Aspirin. The side effects of this drug include neutropenia, thrombocytopenia and thrombotic thrombocytopenia purpura. Compared to Ticlopidine, Clopidogrel shows safer effects. It can be used to prevent atherosclerotic events following myocardial infarction or stroke. Prasugrel is a new antiplatelet aggregation product classified as Adenosine-5'-diphosphate (ADP)-antagonists.

2.4.2 COX-1 inhibitors

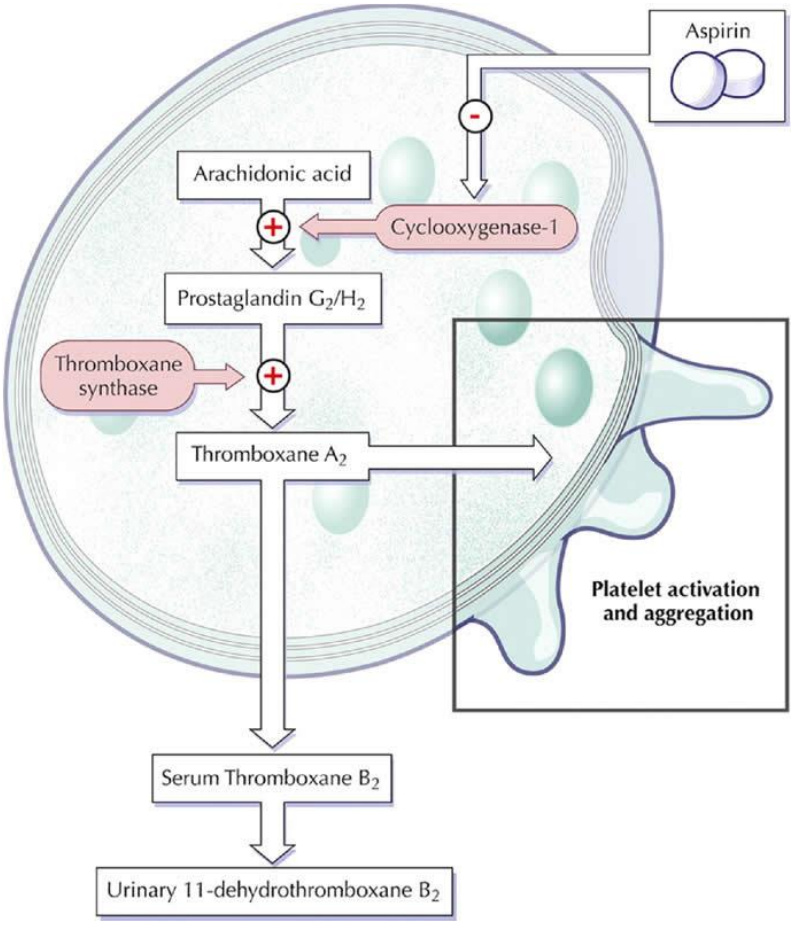

Figure 2.5: **Aspirin inhibition of COX-1** (Mahajan *et al*., 2012).

Aspirin is the only member in this class. Aspirin is also known as 2-acetyloxybenzoic acid. It is used to relieve minor aches and pains, to reduce fever, as anti-inflammatory medication besides acting as anti-platelet aggregating agent. As shown in Figure 2.5, Aspirin acts by inhibiting cyclooxygenase, which is an enzyme in thromboxane A2 (TXA2) generation. The recommended dose of this drug is in the range of 75 mg to 325 mg (Guzman, 2009). There are few side effects of this drug. The side effects include gastrointestinal bleeding and acute renal insufficiency.

2.4.3 Phophodiesterase inhibitors

Dipyridamole is an antiplatelet aggregation agent in this class. It prevents platelet aggregation by inhibiting the adenosine uptake and the cycic GMP phosphodiesterase activity. According to Mahajan *et al* (2012), the using of this drug together with other drugs such as Aspirin and Warfarin can increase the drug's effects.

2.4.4 Glycoprotein IIb/IIIa inhibitors

Glycoprotein IIb/IIIa antagonists act by preventing the cross-linking of platelets at the final stage of platelet aggregation. Abciximab is a monoclonal antibody directed against glycoprotein IIb/IIIa and has several side effects such as bleeding, intraverebral hemorrhage and thrombocytopenia.

2.5 Previous studies on platelet aggregation inhibition

Based on Gorden (1981), platelet adhesion and aggregation are known to contribute to the pathogenesis in thrombosis especially the arterial thrombosis. As variety endogenous substances can enhance the platelet aggregation activities, thrombus formation can be triggered. Further platelets aggregation can cause the development of thrombosis and cardiovascular disease. Therefore, platelet aggregation inhibition is one of the effective ways to prevent thrombosis and cardiovascular disease in human being (Kwang *et al*., 2006). There are many studies and efforts done to inhibit or prevent the platelet aggregation and thrombosis.

Based on studies by Fiat *et al*. (1993) and Rutherfurd and Gill (2000), peptides can inhibit platelet aggregation and thrombosis. The interaction of platelet membrane glycoprotein IIbIIIa with fribinogen and fribinectin can be inhibited by RGD-containing peptides and by peptides corresponding to a sequence unique to fibrinogen in the COOH-terminal domain of its γ chain (HLGGAKQAGDV). RGDS sequence at Aα 572- 575 and RGDF sequence at Aα 95-98 are the two tetrapeptide RGD sequences presented in fibrinogen Aα chain. In the study, polyclonal antibodies were developed from these two sequences to against the RGDF sequence and the γ COOH-terminal domain. Both of them reacted with fribinogen and immunoprecipitated the platelet aggregation factor (Andrieux *et al*., 1989).

Stimuli such as adenosine-5'-diphosphate (ADP) have been identified to enhance the platelet aggregation. In bovine K-casein, the undecapeptide Met-Ala-Ile-Pro-Pro-Lys-Lys-Asn-Gln-Asp-Lys (residues 106-116) has been shown to inhibit both the aggregation and binding of fribinogen to adenosine-5'-diphosphate (ADP) treated platelets. The pentapeptide of Lys-Asn-Gln-Asp-Lys (residues 112-116) has also been shown to inhibit platelet aggregation in blood (Jolles and Caen, 1991). Besides, SSGE (Ser–Ser–Gly–Glu) and DEE (Asp–Glu–Glu) peptides found in soy protein hydrolysate have also been shown to inhibit rat platelet aggregation induced by Adenosine-5'-diphosphate (ADP) (Lee and Kim, 2005).

2.6 Mushrooms

According to Miles and Chang (1997), mushroom is a macrofungus which has distinctive fruiting bodies and is large enough to be seen by naked eye. Mushroom is generally belongs to ascomycetes and basidiomycetes. Mushrooms are consumed since long time ago due to its fine texture and flavor. As mushroom is getting more attentions

nowadays, many research have been done on mushroom samples and it was shown that mushrooms contain excellent nutritional values. They contain essential and non-essential amino acids. Besides, mushrooms have low fat and cholesterol content but with excellent sources of protein (Ghorai *et al*., 2009). Due to these advantages, mushrooms are known to be capable to substituting meat in our daily meals.

Hundreds of edible mushroom species exist. However, only less than 20 species are consumed extensively and only about 10 species are cultivated regularly (Ghorai *et al*., 2009). Human beings usually consume the fruiting bodies of the mushroom. On dry weight basis, the fruiting bodies of the mushroom contain approximately 39.9% of carbohydrates, 17.5% protein, 2.9% of fats and other minerals (Demirbas, 2001). Throughout the history, some mushrooms have been treated as medicinal mushrooms due to their medicinal properties. These mushrooms contain bioactive substances with potent health enhancing properties.

As mushrooms are well-known for their nutrition and medicinal properties, many research has also been done in a variety of mushrooms to detect and characterise the potent anticoagulants present in the mushrooms. The anticoagulant effect of *G. lucidum* has been studied and it was suggested that this Chinese herbal medicinal *mushroom* can be an effective inhibitory agent of platelet aggregation. The result showed that the platelet aggregation was inhibited in the patient taking of 1 g *G. lucidum* three times per day for two weeks (Tao and Feng, 1990). Other than that, lenthionine, a cyclic sulfur compound in the Shiitake mushroom (*L. edodes*) was also known to inhibit platelet aggregation. From *I. obliquus*, a tripeptide (Trp-Gly-Cys) with molecular mass of 365 Da has been characterised and it showed high platelet aggregation inhibitory activity in mice (Kwang *et al*., 2006).

***Ganoderma lucidum* (Curtis) P. Karst.**

Ganoderma is a basidiomycete white rot fungus which is also known as Lingzhi, Munnertake, Sachitake, Reishi and Youngzhi. *Ganoderma* is popularly used for medicinal purposes mainly in China, Japan and Korea. Based on Chen *et al*. (2006), *Ganoderma* is widely used as immune-modulators in Asia. Its chemical constituents such as polysaccharides, triterpenes, sterols, lectins and proteins have been investigated extensively. *Ganoderma* can inhibit the proliferation of breast and prostate cancer cells; induces apoptosis of breast and prostate cancer cells; and suppresses cell migration of human prostate cancer cells. Besides, it can also induce apoptosis in colon cancer cells (Sliva, 2006). Anti-platelet aggregation activities have also been reported in the extract of *G. lucidum* but the mechanism and the active principles remain to be further investigated (Tao and Feng, 1990).

***Cordyceps militaris* (L.) Link**

According to Yu *et al*. (2004), *Cordyceps sinensis* is one of the precious mushroom species that are used in traditional Chinese medicine. It can benefit our body system such as in circulatory, immune, respiratory and glandular systems. However, the usage of this mushroom was limited due to the shortage of its supply. Following that, *C. militaris* has been found to be the alternative species to *C. sinensis* as they share same medicinal properties. Nowadays, *C. militaris* is popular due to its various physiological activities. Based on Seldin *et al*. (1997) and Zhou *et al*. (2002), cordycepin (3′-deoxyadenosine) is one of the major bioactive secondary metabolites which have shown antibacterial, antifungal, antitumor and antiviral activities. Fibrinolytic enzyme has been identified and characterised in this mushroom by Kim *et al*. (2006).

***Lignosus rhinocerotis* (Cooke) Ryvarden**

Lignosus rhicocerotis is a medicinal mushroom found in Malaysia. The local name of this mushroom is the 'cendawan susu rimau'. *Lignosus rhinocerotis* has been used for ages to treat asthma, fever, cough, cancer, food poisoning, to improve and maintain human health in local community (Tan *et al*., 2010).

***Pleurotus floridanus* (Singer)**

Pleurotus floridanus is one of the edible oyster mushrooms. It has high therapeutic value and is cultivated in large scale in many parts of the world. Studies have been done on this mushroom and it has been shown to contain large portion of protein, fat and ash on dry weight basis (Rout *et al*., 2006). According to Jose *et al*. (2004), *P. floridanus* has shown anti-inflammatory and platelet aggregation inhibiting activities in human.

***Auricularia polytricha* (Mont.) Sacc.**

Auricularia polytricha is also known as Jew's Ear, wood ear, red ear, black tree fungus or ear fungus. In Far East, this mushroom is popular as it is always consumed as food and well known as traditional medicine. The nutritional components of *A. polytricha* have been investigated and the constituents of this mushroom are known to have potential pharmacological applications (Sheu *et al*., 2004). *A. polytricha* has known to exert antioxidant activity, prevents lipid oxidation and has the ability to scavenge radicals (Mau *et al*., 2001). Other than that, it has been reported that *A. polytricha* contains platelet aggregating inhibitor in the crude dialysates of their aqueous extracts (Hammerschmidt, 1980 ; Hokama and Hokama, 1981).

***Pleurotus giganteus* (Berk.) S.C. Karunarathna & K.D. Hyde**

Pleurotus giganteus formerly known as *Lentinus giganteus* and *Panus giganteus* is an edible and medicinal mushroom belonging to Pleurotaceae family in Agaricales. *Pleurotus giganteus* has been reported to have hepatoprotective effects (Wong *et al*., 2012) and neurite outgrowth stimulating activity (Phan *et al*., 2012).

2.7 Review of Methods of recovery, purification and identification of proteins

Aqueous two phase system (ATPS)

Aqueous two phase system (ATPS) is a simple, easily scalable and mild separation technique for recovery of biomolecules from solutions (Albertsson, 1986). Basically, aqueous two phase system (ATPS) is formed when two mutually incompatible aqueous solutions, water soluble polymers, or one polymer and a high concentration salt are mixed together. The biomolecules are then separated into two different phases. According to Kula (1979), the most commonly used polymer/polymer system is the dextran and polyethylene glycol system. These polymers are chosen due to their non-toxicity properties and are environmental friendly. According to Li *et al*. (2001), PEG/salt system appears to be a good choice for bioseparation of protein as they have wide range of hydrophobicity between two different phases and are low in cost.

Aqueous two phase system (ATPS) is controlled by parameters such as the properties of the phase system, the substances and the interaction between substances (Albertsson, 1986). Based on Albertsson *et al*. (1990), the interactions such as hydrogen bond, van der waals' interactions, electrostatic bond and the conformational effects between the phase components and the substances can affect the partitioning of substances in aqueous two phase system (ATPS). Other factors such as the surface charges, existing of specific binding site and molecular weight of biomolecules can also

affect the portioning of substances in aqueous two phase system (ATPS) (Oliveira *et al*., 2003).

Aqueous two phase system (ATPS) allows the separation, concentration and the partial purification of target product to occur together; therefore it shortens the protein purification process. Besides, this method has attracted many attentions as it is able to hold high biomass load in large scale production as compared to other separation techniques. It is a straightforward method and requires only a simple equipment to operate. Other than that, the conditions needed for large scale production do not change much as compared to small scale production. Techniques such as chromatography, electrophoresis and precipitation are widely used but these methods are very costly. They also provide low yields and are not suitable to be used in large scale production (Ratanapongleka and Phetsom, 2011). Compared to conventional liquid-liquid extraction, aqueous two phase system (ATPS) has the advantage of preserving the targeted biomolecules as it provides mild environmental condition with high water content of both phases (70-90%) and low interfacial tension (Ramyaderi *et al.*, 2012).

Previous study in mushroom namely *Agaricus bisporus* (J.E. Lange) Imbach has shown that aqueous two phase system (ATPS) is an effective technique in recovery of laccase from the residual compost of mushroom (Mayolo-Deloisa *et al.*, 2009). The techniques used previously to recover laccase from residual compost of mushroom result in low yield and require higher cost for operation. These techniques include ultrafiltration, dialysis, one or more types of chromatography including exclusion, ion exchange and electrophoresis. In the study, various system parameters was tested to ensure the laccase was concentrates predominantly to the top PEG-rich phase. As result, PEG 1000- phosphate aqueous two phase system (ATPS) was selected and the overall yield of laccase in this one-single primary recovery phase process is as high as 95%.

The effectiveness of aqueous two phase system (ATPS) in the purification of lipase was also proven in the study of *Burkholderia pseudomallei* (Ooi *et al.*, 2009). In the study, aqueous two phase system (ATPS) was used to purify *Burkholderia pseudomallei* lipase from the fermentation broth. *Burkholderia pseudomallei* lipase is well known for its characterisations such as high temperature stability, organic solvent tolerance, and high catalytic activity for various substrates (Yang *et al.*, 2007). Due to its high versatile characteristic, the lipases has become great interest in industrial applications and is used in many fields such as in pharmaceutical industry (Jaeger *et al.*, 1999) and biodiesel fuel (Shimada *et al.*, 1999). The optimum conditions for the purification of lipase were obtained in polyethylene glycol (PEG) 6000-potassium phosphate system using tie-line length of 42.2% (w/w), with volume ratio of 2.70, and 1% (w/w) NaCl addition at pH 7 for 20% (w/w) crude load. The purification factor of lipase in the study was enhanced to 12.42 fold, with a high yield of 93% was obtained.

Recently, the aqueous two phase system (ATPS) has been used for the recovery of recombinant human protein expressed in plants green-tissue, alfalfa (*Medicago sativa*) (Ibarra-Herrera *et al.*, 2011). The aqueous two phase system (ATPS) system used was the PEG 8000/phosphate systems comprising of 16.1% (w/w) of polyethylene glycol (PEG), 10% phosphate, tie-line length of 35.7% (w/w), volume ratio equal to one and at pH of 7.0. As result, 88% of the rhG-CSF (granulocyte-colony stimulating factor) was recovered in the top phase. At the same time, 93% of alfalfa contaminant proteins were detected at the interface and the bottom phase.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS Page) and Native Page

The separation of macromolecules in an electric field is referred as electrophoresis. There are few forms of polyacrylamide gel electrophoresis (PAGE) which can be used to provide different information regarding proteins. For example, there are denaturing and reducing Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), nondenaturing PAGE which is also called native PAGE and two-dimensional (2D) PAGE (Thermo Fisher scientific, 2010).

a) Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is a very common method used to separate proteins by electrophoresis based to their molecular mass. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is chosen for routine separation and analysis of proteins due to its simplicity, reproducibility and low cost (Jay, 2007). This method can be used to estimate relative molecular mass, to determine the relative abundance of proteins in a sample and to determine the distribution of proteins. It uses a discontinuous polyacrylamide gel as a support medium and sodium dodecyl sulfate (SDS) to denature the proteins. In Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), the Sodium dodecyl sulfate (SDS) used is an anionic detergent. It poses a net negative charge when dissolved. When a polypeptide chain binds to the Sodium dodecyl sulfate (SDS), the negative charges on Sodium dodecyl sulfate (SDS) destroy most of the complex structure of proteins. When a current is applied, all Sodium dodecyl sulfate (SDS) bound proteins will migrate through the gel toward the positively charged electrode. Due to the sieving effect of the gel matrix, proteins with greater mass travel slower than the protein with less mass.

In sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), the protein samples can be heated with the Sodium dodecyl sulfate (SDS) before electrophoresis in order to denature the protein sample and to bind the Sodium dodecyl sulfate (SDS) tightly to the uncoiled protein sample. Dithiothreitol (DTT) can also be added to cleave protein disulfide bonds, thus ensure that no quaternary or tertiary protein structure remains in the sample (Thermo Fisher scientific, 2010). Protein samples may contain substances which can affect the migration of protein bands in the gel. Other than that, some substances including guanidine hydrochloride and ionic detergents can cause smearing effects in protein bands. To remove these interferences, dialysis, ultrafiltration, acetone precipitation of proteins and commercially available kits can be used to purify proteins before gel electrophoresis (Thermo Fisher scientific, 2010). To assess the relative molecular weights of proteins in a gel, protein molecular weight (MW) markers containing several proteins of known molecular mass is usually run alongside the test sample. After gel electrophoresis, the protein bands can be blotted to membrane for analysis by Western blotting or they can be visualized in the gel using various staining methods.

b) Non-denaturing PAGE (Native PAGE)

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is the most commonly used electrophoresis system to analyze proteins. However, it separates denatured proteins. In order to analyze a native; non-denatured proteins, to identify a protein in the gel by its biological activity such as the enzyme activity, receptor binding or antibody binding, a non-denaturing system or a native PAGE should be used. As no denaturants are used in this native PAGE, the protein structures can be retained and thus, it may be used for the preparation of purified active proteins. Based on John (2000), a band presented on gel would suggest a pure enzyme. However, contaminant might be

presented and we can only be convinced by carrying out biological test on the band observed on the gel.

In native PAGE, proteins are separated based on their net charges, size and shape of their native structure. The proteins carry a net negative charge in alkaline running buffer. The higher the charge density, the faster the protein travels. Other than that, due to the sieving effect of the gel matrix, proteins with greater size travel slower than the protein with less size. In order to maintain the protein structure during electrophoresis, the denaturation and proteolysis effects should be minimised. Extremes pH and temperatures should also be avoided as these might lead to protein denaturation (Thermo Fisher scientific, 2010).

Chapter 3.0 Materials and Methods

3.1 Mushroom sample preparation

Mushroom samples provided were cleaned and freeze-dried. The freeze-dried samples were then blended into powder form and kept in freezer for future use.

3.2 Tris-HCL buffer preparation

The buffer was prepared based on Kho (2008). A 2.423g of trizma was dissolved in 900 mL distilled water. Then, the pH was adjusted to pH 8 using HCl. Lastly, the solution was topped up to 1000 mL with distilled water.

3.3 Crude extracts preparation

Six samples of mushrooms (*G. lucidum*, *C. militaris*, *L. rhinocerotis*, *P. giganteus*, *P. floridanus* and *A. polytricha*) were screened for their anticoagulant activity. The crude extracts were prepared based on the standard protocol (Mushroom Research Centre, Universiti Malaya). Freeze dried sample of fresh mushroom was blended. Then, 10 g of the sample in powder form was suspended in 200 mL of 20 mM Tris-HCl buffer (pH 8). The sample was physically ground for 30 minutes in ice bath. After that, the sample was centrifuged at 10,000 g for 30 minutes at 4 $\pm 2^{o}$C. The supernatant was collected and freeze dried for future use. The freeze dried powder was designated as the crude extract in this study.

3.4 *In-vitro* preliminary anticoagulant activity screening assay

Microcentrifuge tubes (2 mL) were used to screen the anticoagulant activity in the mushroom supernatant samples. The test was done in triplicates. Whole fresh blood sample was collected from bovine slaughtering house. Different concentrations of mushroom extracts (1 mg/mL, 3 mg/mL, 6 mg/mL, 9 mg/mL) were added into each microcentrifuge tube and one mL of fresh blood was added into each tube and well mixed. The tubes were inverted to mix well the blood with the samples. Observation was done after 0 min, 5 min, 10 min, 15 min, 30 min and 90 minutes. The concentration of the mushroom samples that showed positive result was then increased and tested from 9 mg/mL to 12 mg/mL, 15 mg/mL, 18 mg/mL, 21 mg/mL and 24 mg/mL. Disprin (60 mg/mL) was used as positive control while one mL of whole fresh bovine blood was used as the negative control in the preliminary screening process.

3.5 *In-vitro* Anti-platelet activity assay

The *in-vitro* anti-platelet activity assay was performed based on the method of Lavanya *et al*. (2010) and Elumalai *et al*. (2012). Fresh human blood was drawn from volunteers. The human blood was centrifuged at 120 g for 15 minutes. The upper layer of platelet rich plasma was taken and haemocytometer was used to determine the platelet count. Then, the platelet rich plasma (1.3×10^8 platelets/mL) was used in each assay. The platelet aggregation activity was measured in percentage of transmittance values using spectrophotometer. Five concentrations (5 mg/mL, 10 mg/mL, 15 mg/mL, 20 mg/mL and 25 mg/mL) of mushroom extracts were tested and the assay was done in triplicates. Disprin 100 µg/mL was used as a positive control while ADP 125 µM was used as the negative control in this test. Adenosine-5'-diphosphate was used in all reactions to induce platelet aggregation.

Each extract of mushroom was dissolved in 20 mM Tris-HCL buffer (pH 8) to the desired concentration. Then, one mL of mushroom sample was added with one mL of platelet rich plasma in a 4 mL cuvette. The solution was mixed for 30 seconds before one mL of ADP was added. The solution was then well mixed for another 30 seconds before the spectrophotometer reading was taken. Transmittance changes during the reaction were determined at 660 nm. The cuvette was inverted continuously to ensure the interaction of platelet with the ADP and the sample. The percentage of transmittance value was calculated as follows:

Absorbance = -log (percent transmittance/100)

3.6 Dialysis and acetone precipitation of proteins

The crude sample was dialysed against distilled water overnight using dialysis tubing 12 kDa. The dialysed crude extract was precipitated using acetone precipitation of protein method (Wessel and Flugge, 1984). The acetone was first cooled to -20 ^{o}C. The protein sample was placed in acetone-compatible tube. Four times of the sample volume of cold (-20 ^{o}C) acetone was added. The mixture was mixed and vortex before incubation for 60 minutes at -20 ^{o}C. After one hour, the tube was centrifuged for 10 minutes at 13,000 - 15,000 x g. The supernatant was decanted and the acetone was allowed to be evaporated from the uncapped tube at room temperature. Appropriate amount of distilled water was added to dissolve the pellet. Vortex was used to fully dissolve the protein pellet in the distilled water. The *in-vitro* anti-platelet activity assay was performed (See section 3.5) again to test on the anti-platelet aggregation activity for the protein obtained after dialysis and acetone precipitation of proteins.

3.7 Aqueous two phase systems (ATPS)

The optimised aqueous two phase system in Ibarra-Herrera *et al*. (2011) was used in this study. Four grams of polyethylene glycol 50 % (PEG 8000) was added with 2.9 g of phosphate 40 %. Then, one g of mushroom sample was added. Following that, the system was topped up to 10 g by adding 2.1 g of distilled water. The mixture was centrifuged for 10 minutes. The separated top and bottom phase was then pipetted into two different tubes. The partially purified enzyme from ATPS was precipitated using acetone precipitation of protein method (Wessel and Flugge, 1984). Then, the *in-vitro* anti-platelet activity assay was performed (See section 3.5) again to test on the anti-platelet aggregation activity for protein samples obtained in the top and bottom phase in the ATPS.

3.8 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE)

The sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS) page was done based on the optimised protocol by Sivasangkary (2012). The casting frames were set. Two glass plates were clamped in the casting frames on the casting stands. Resolving gel (12%) was prepared by adding 4.4 mL of distilled water, 2.5 mL of 1.5 M Tris-HCL (pH 8.8), 0.1 mL of 10% SDS, 3 mL of 40% Bis-acrylamide, 50 µL of ammonium persulfate and 10 µL of TEMED. The solution was mixed gently before pipetted into the gap between the glass plates. To make the top of the resolving gel to be horizontal, the gap was filled with distilled water until overflow. Then, the resolving gel was left for 30 minutes for it to be solidified. After 30 minutes, the distilled water was discarded. The stacking gel prepared by the addition of 5.9 mL of distilled water, 2.5 mL of 0.5 M Tris-HCL (pH 6.8), 0.1 mL of 10% SDS, 1.5 mL of 40% Bis-acrylamide, 50 µL of ammonium persulfate and 15 µL of TEMED was pipetted onto the resolving gel.

Immediately, the well-forming comb was inserted without trapping any air under the teeth. The gel was left for another 30 minutes to solidify.

After 30 minutes, the comb was removed. The casting frames were taken out and were set in the running tank. Running buffer was poured into the inner chamber up to a required level. Mushroom sample was prepared by mixing 15 µL of the sample with 5 µL of sample buffer. The total 20 µL of sample was then loaded into the well. Protein marker was also loaded for references. The gel electrophoresis was performed at 110V at constant ampere for 120 minutes. The gel was then stained with brilliant blue R-250 for overnight before de-staining was done.

3.9 Native page (Nondenaturing polyacrylamide gel electrophoresis of protein)

The native page was done based on John (2002) to analyse the biological activity of the non-denatured protein. The preparation of the gel for native page is almost the same as in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS) page. However, few chemicals such as sodium dodecyl sulfate (SDS) and beta-mercaptoethanol were eliminated as these chemicals can denature the protein structure.

The resolving gel (12 %) was prepared by adding 4.4 mL of distilled water, 2.5 mL of 1.5 M Tris-HCL (pH 8.8), 3 mL of 40 % Bis-acrylamide, 50 µL of ammonium persulfate and 10 µL of TEMED. The solution was mixed gently before pipetted into the gap between the glass plates. To make the top of the resolving gel to be horizontal, the gap was filled with distilled water until overflow. Then, the resolving gel was left for 30 minutes for it to be solidified. After 30 minutes, the distilled water was discarded. The stacking gel prepared by the addition of 5.9 mL of distilled water, 2.5 mL of 0.5 M Tris-HCL (pH 6.8), 1.5 mL of 40% Bis-acrylamide, 50 µL of ammonium persulfate and 15 µL of TEMED was pipetted onto the resolving gel. Immediately, the well-forming

comb was inserted without trapping any air under the teeth. The gel was left for another 30 minutes to solidify.

After 30 minutes, the comb was removed. The casting frames were taken out and were set in the running tank. The running buffer without 10% sodium dodecyl sulfate (SDS) was poured into the inner chamber up to the required level. Mushroom sample was prepared by mixing 15 µL of the sample with 5 µL of sample buffer (without beta-mercaptoethanol). The total 20 µL of sample was then loaded into the well. The gel electrophoresis was performed at 110V voltages at constant ampere for 120 minutes. In order to excise the gel for biological test, the gel was stained for half an hour and de-stained for another half an hour before excision.

3.10 Protein elution from polyacrylamide gel

The protein was excised and eluted using modified protocol retrieved from Thermo Scientific (2009). The excised gel pieces were placed in a clean microcentrifuge tube. One mL of elution buffer (50 mM Tris-HCL, pH 8 and 150 mM NaCL) was added so that the gel pieces are completely immersed. The gel pieces were then crushed and were incubated in a rotary shaker at 30 ℃ overnight. Then, the protein in elution buffer was centrifuged at 5,000-10,000 × g for 10 minutes. The supernatant was carefully pipetted into a new microcentrifuge tube. The i*n-vitro* anti-platelet activity assay was performed (See section 3.5) again to test on the anti-platelet aggregation activity for protein eluted from the polyacrylamide gel.

Chapter 4.0 Results

4.1 Crude extracts preparation

The crude extract was freeze-dried and the freeze-dried powder was re-dissolved in 20 mM Tris-HCL buffer (pH 8.0).

4.2 *In-vitro* preliminary anticoagulant activity screening assay

Disprin was used as the positive control. Optimisation of the positive control was done and Figure 4.1(1) shows the result taken after 90 minutes of the screening test. The blood added with Disprin 6 mg/mL clotted within 5 minutes. No clotting was observed in blood added with Disprin 30 mg/mL and 60 mg/mL after 90 minutes.

Negative control was shown in Figure 4.1 (2). One mL whole fresh blood without any mushroom extract added was used as the negative control. The blood clotting time was determined. In both replicates of the negative controls, the one mL blood clotted within 5 minutes.

For the screening of anticoagulant activity in *A. polytricha* crude extract, one mL of whole fresh blood was added to different concentrations of *A. polytricha* crude extract (1 mg/mL, 3 mg/mL, 6 mg/mL and 9 mg/mL). Figure 4.1(3) shows the result taken after 90 minutes of the screening test. The blood added to *A. polytricha* crude extract (1 mg/mL, 3 mg/mL and 6 mg/mL) clotted up to 90 minutes. The turbidity of the blood increased and small clot was observed in the blood added with *A. polytricha* crude extract (9 mg/mL) after 90 minutes of the screening test. The result showed that *A. polytricha* may contain anticoagulant compound as 9 mg/mL can inhibit blood coagulation.

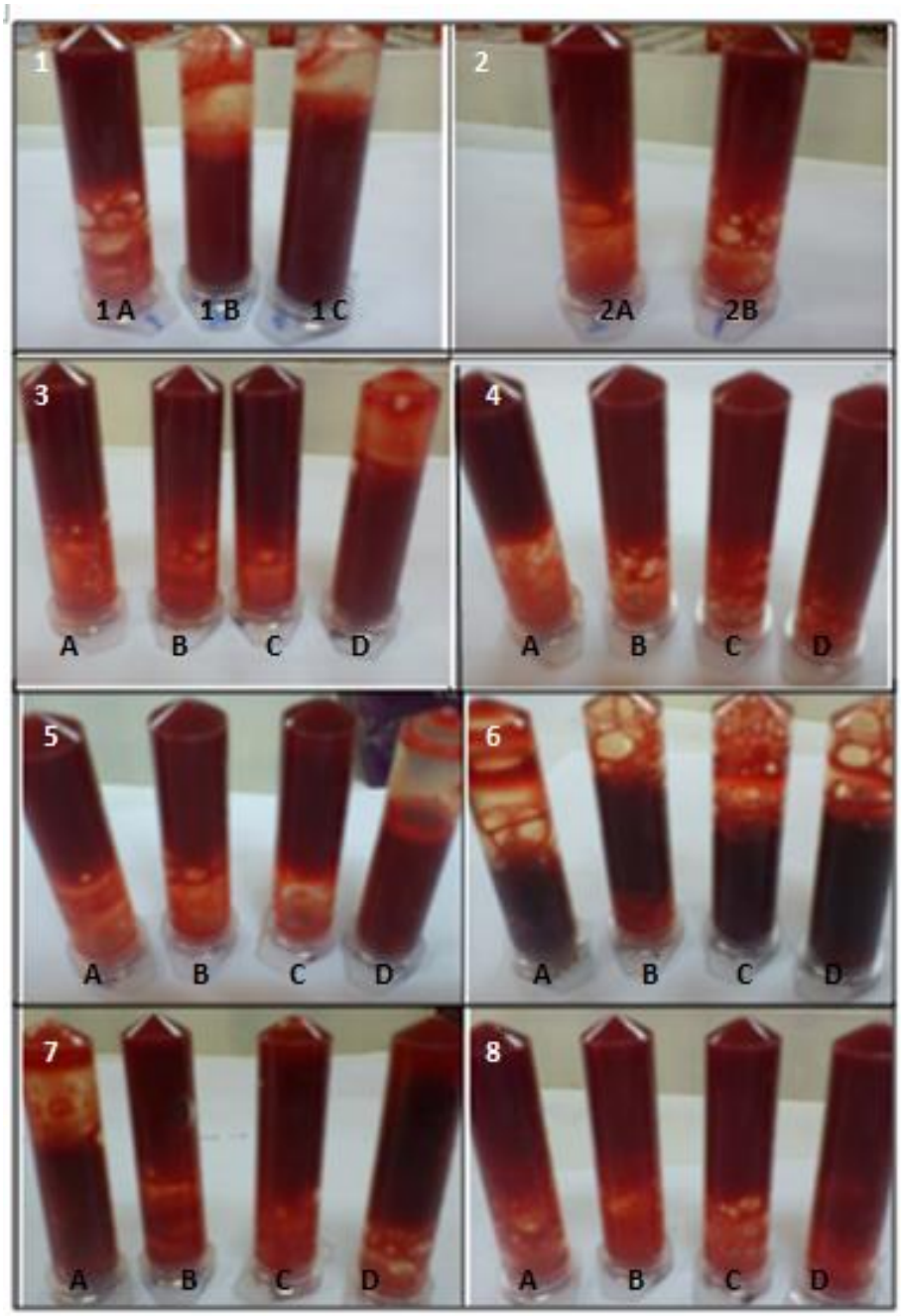

Figure 4.1: The anticoagulant activity in different concentrations of different mushrooms extract added with one mL whole fresh blood. (1): Dispirin. 1A:6 mg/mL. 1B: 30 mg/mL. 1C: 60 mg/mL. No clotting was observed in 1B and 1C. (2): One mL whole fresh blood as negative control. Blood clotted within 5 minutes. (3): *A. polytricha.* A: 1mg/mL. B: 3 mg/mL. C: 6 mg/mL. D: 9 mg/mL. (4): *C. militaris.* A*:* 1 mg/mL. B: 3 mg/mL. C: 6 mg/mL. D: 9 mg/mL. Blood clotted in all concentrations. (5): *L. rhinocerotis.* A*:* 1 mg/mL. B: 3 mg/mL. C: 6 mg/mL. D: 9 mg/mL. (6): *P. floridanus*. A*:* 1 mg/mL. B: 3 mg/mL. C: 6 mg/mL. D: 9 mg/mL. Blood clotted in all concentrations. (7): *G. lucidum* . A: 1 mg/mL. B: 3 mg/mL. C: 6 mg/mL. D: 9 mg/mL. Blood clotted in all concentrations. (8): *P. giganteus.* A: 1 mg/mL. B: 3 mg/mL. C: 6 mg/mL. D: 9 mg/mL. Blood clotted in all concentrations.

For the screening of anticoagulant activity in *C. militaris* crude extract, one mL whole fresh blood was added to different concentrations of *C. militaris* crude extract (1 mg/mL, 3 mg/mL, 6 mg/mL and 9 mg/mL). Figure 4.1(4) shows the result taken after 90 minutes of the screening test. All of the blood added to different concentrations of *C. militaris* crude extract (1 mg/mL, 3 mg/mL, 6 mg/mL and 9 mg/mL) clotted up to 90 minutes. The result showed that *C. militaris* in the concentrations tested did not inhibit blood coagulation.

For the screening of anticoagulant activity in *L. rhinocerotis* crude extract, one mL whole fresh blood was added to different concentrations of *L. rhinocerotis* crude extract (1 mg/mL, 3 mg/mL, 6 mg/mL and 9 mg/mL). Figure 4.1(5) shows the result taken after 90 minutes of the screening test. The blood mixed with *L. rhinocerotis* crude extract (1 mg/mL, 3 mg/mL and 6 mg/mL) clotted after incubation. The turbidity of the blood increased but no clot was observed in the blood added with *L. rhinocerotis* crude extract (9 mg/mL) after 90 minutes of the screening test. The result showed that *L. rhinocerotis* may contain anticoagulant compound as it can inhibit blood coagulation at 9 mg/mL.

For the screening of anticoagulant activity in *P. floridanus* crude extract, one mL whole fresh blood was added to different concentrations of *P. floridanus* crude extract (1 mg/mL, 3 mg/mL, 6 mg/mL and 9 mg/mL). Figure 4.1(6) shows the result taken after 90 minutes of the screening test. All of the blood added to different concentrations of *P. floridanus* crude extract (1 mg/mL, 3 mg/mL, 6 mg/mL and 9 mg/mL) clotted after incubation. The result showed that *P. floridanus* in the concentrations tested did not inhibit blood coagulation.

For the screening of anticoagulant activity in *G. lucidum* crude extract, one mL whole fresh blood was added to different concentrations of *G. lucidum* crude extract (1

mg/mL, 3 mg/mL, 6 mg/mL and 9 mg/mL). Figure 4.1(7) shows the result taken after 90 minutes of the screening test. All of the blood added to different concentrations of *G. lucidum* crude extract (1 mg/mL, 3 mg/mL, 6 mg/mL and 9 mg/mL) clotted after incubation. The result showed that *G. lucidum* in the concentrations tested did not inhibit blood coagulation.

For the screening of anticoagulant activity in *P. giganteus* crude extract, one mL mwhole fresh blood was added to different concentrations of *P. giganteus* crude extract (1 mg/mL, 3 mg/mL, 6 mg/mL and 9 mg/mL). Figure 4.1(8) shows the result taken after 90 minutes of the screening test. All of the blood added to different concentrations of *P. giganteus* crude extract (1 mg/mL, 3 mg/mL, 6 mg/mL and 9 mg/mL) clotted after incubation. The result showed that *P. giganteus* in the concentrations tested did not inhibit blood coagulation.

After the preliminary screening of anticoagulant activity in the 6 mushroom samples (*G. lucidum, C. militaris, L. rhinocerotis, P. giganteus, P. floridanus* and *A. polytricha*), only two samples, ie: *A. polytricha* and *L. rhinocerotis* were selected for further anticoagulant activity screening. The concentration of the mushroom samples was increased to 9 mg/mL, 12 mg/mL, 15 mg/mL, 18 mg/mL, 21 mg/mL and 24 mg/mL.

Figure 4.2 shows the result of different concentrations of *A. polytricha* crude extracts (9 mg/mL, 12 mg/mL, 15 mg/mL, 18 mg/mL, 21 mg/mL and 24 mg/mL) added to one mL whole fresh blood. The observation was made taken after 90 minutes of incubation. One mL whole fresh blood without any mushroom extract added was used as the negative control. The blood in the negative control clotted within 15 minutes. The one mL whole fresh blood added to Disprin 60 mg/mL was used as positive control. The blood added with Disprin 60 mg/mL did not clot after 90 minutes. Then, the one

mL whole fresh blood added to 9 mg/mL and 12 mg/mL of *A. polytricha* crude extract clotted after 20 minutes. The one mL whole fresh blood added to 15 mg/mL, 18 mg/mL, 21 mg/mL and 24 mg/mL of *A. polytricha* crude extract did not clot after 90 minutes. From the observation, the higher the concentration of the *A .polytricha* crude extract added to the blood, the better was the anticoagulation activity. A smooth flow of blood added to 24 mg/mL of *A. polytricha* crude extract is shown in Figure 4.3.

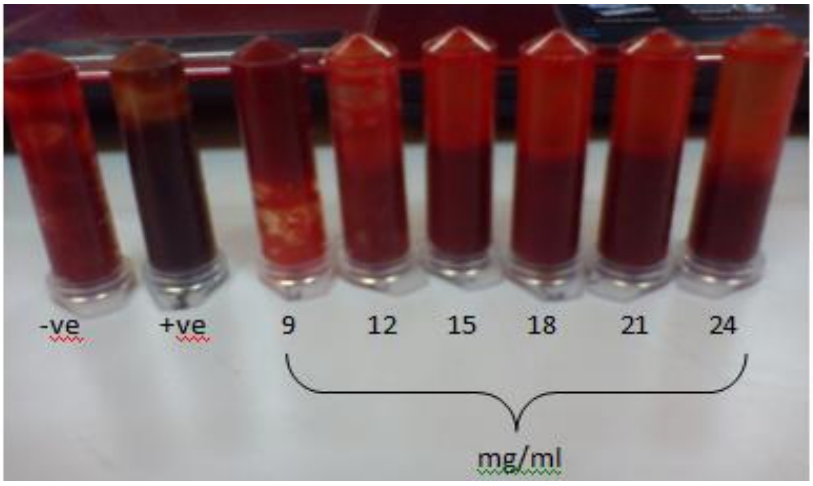

Figure 4.2: The anticoagulant activity in different concentrations of *Auricularia polytricha* crude extracts added with one mL of whole fresh blood. –ve: Negative control (whole fresh blood); + ve: Positive control (Disprin 60 mg/mL); 9: *A. polytricha* 9 mg/mL; 12: *A. polytricha* 12 mg/mL; 15: *A. polytricha* 15 mg/mL; 18: *A. polytricha* 18 mg/mL; 21: *A. polytricha* 21 mg/mL; 24: *A. polytricha* 24 mg/mL.

Figure 4.3: The blood mixed with *Auricularia polytricha* crude extract (24 mg/mL) did not clot up to 90 minutes.

Figure 4.4 shows the different concentrations of *L. rhinocerotis* crude extracts (9 mg/mL, 12 mg/mL, 15 mg/mL, 18 mg/mL, 21 mg/mL and 24 mg/mL) added to 1mL of whole fresh blood. The picture was taken after 90 minutes of the screening test. One mL whole fresh blood without any mushroom extract added was used as negative control. The blood in the negative control clotted within 5 minutes. The one mL whole fresh blood added to Disprin 60 mg/mL was used as positive control. The blood added with Disprin 60 mg/mL did not clot after 90 minutes. Then, the one mL whole fresh blood added to 9 mg/mL, 12 mg/mL and 15 mg/mL of *L. rhinocerotis* crude extract clotted after 15 minutes. The one mL whole fresh blood added to 18 mg/mL, 21mg/mL and 24 mg/mL of *L. rhinocerotis* crude extract did not clot after 90 minutes. From the observation, the higher the concentration of the *L. rhinocerotis* crude extract added to the blood, the better was the anticoagulation activity. A smooth flow of blood added to 24 mg/mL of *L. rhinocerotis* crude extract is shown in Figure 4.5.

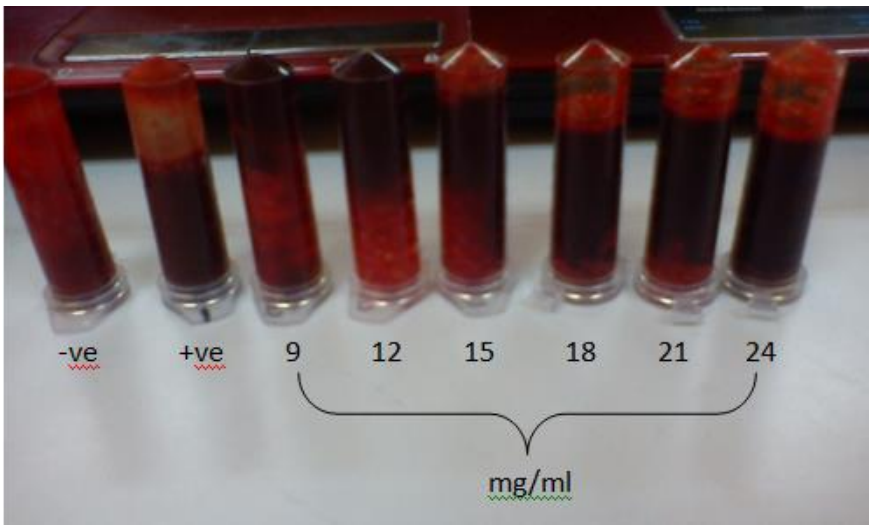

Figure 4.4: **The anticoagulant activity in different concentrations of *Lignosus rhinocerotis* crude extracts added with one mL of whole fresh blood.** –ve: Negative control (whole fresh blood); + ve: Positive control (Disprin 60 mg/mL); 9: *L. rhinocerotis* 9 mg/mL; 12: *L. rhinocerotis* 12 mg/mL; 15: *L. rhinocerotis* 15 mg/mL; 18: *L. rhinocerotis* 18 mg/mL; 21: *L. rhinocerotis* 21 mg/mL; 24: *L. rhinocerotis* 24 mg/mL.

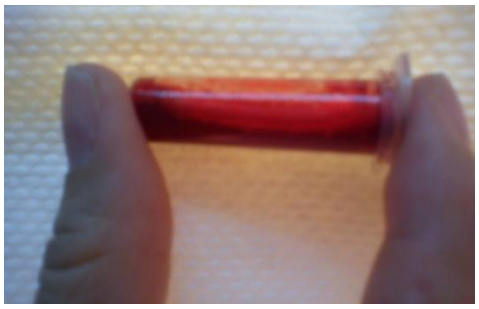

Figure 4.5: The blood mixed with *Lignosus rhinocerotis* crude extract (24 mg/mL) did not clot up to 90 minutes.

4.3 *In-vitro* Anti-platelet activity assay

The platelet aggregation activity was measured using spectrophotometer in percentage of transmittance values. Based on Zhou and Schaimer (2005), platelet aggregation occurs when adenosine-5'-diphosphate (ADP) is added to the platelet rich plasma. This aggregation event will result in the increase in light transmittance value. Therefore, the decreasing in percentage of transmittance value in the study refers to the disaggregation of platelets.

Optimisation for the positive control (Disprin) and the negative control Adenosine-5'-diphosphate (ADP) was done. As result, adenosine-5'-diphosphate (ADP) 125 µM and Disprin 100 µg/mL were chosen in the assay. Different concentrations of adenosine-5'-diphosphate (ADP) (5 µM, 10 µM, 75 µM, 100 µM and 125 µM) were tested in the study. Initially, adenosine-5'-diphosphate (ADP) 5 µM and 10 µM were tested based on Lavanya *et al*. (2010) and Elumalai *et al*. (2012). However, the concentration of the adenosine-5'-diphosphate (ADP) is not sufficiently strong, causing the platelets to disaggregate after few minutes (Zhou and Schemaier, 2005).

Following that, the concentration of the adenosine-5'-diphosphate (ADP) was increased based on Kwang *et al*. (2005). Three concentrations of adenosine-5'-diphosphate (ADP) 75 µM, 100 µM and 125 µM were tested and the result is shown in

Table 4.1. Adenosine-5'-diphosphate (ADP) 125 µM was lastly chosen as the negative control in this study as the percentage transmittance increased at the first minute to the seventh minute tested. This showed that platelet aggregation was induced from the first minute to the seventh minute tested.

The Disprin100 µg/mL was tested based on Lavanya *et al*. (2010) and Elumalai *et al*. (2012). The result is shown in Table 4.2. Platelet aggregation was decreased over time as the percentage of transmittance values obtained decreased from the first minute to the sixth minute.

Table 4.1: Percentage transmittance values for Adenosine-5'- Diphosphate on platelet activity

Concentration / Time	ADP 75 µM	ADP 100 µM	ADP 125 µM
1 min	20.559 ±0.002	24.927 ±0.002	29.603 ±0.007
2 min	22.387 ±0.016	25.922 ±0.019	30.549 ±0.014
3 min	23.281 ±0.022	28.162 ±0.006	31.420 ±0.015
4 min	22.944 ±0.024	27.883 ±0.009	32.459 ±0.007
5 min	22.336 ±0.028	27.458 ±0.008	33.291 ±0.005
6 min	21.677 ±0.023	27.416 ±0.112	33.755 ±0.005
7 min	21.478 ±0.018	26.833 ±0.006	34.409 ±0.09

Table 4.2: Percentage transmittance values for Disprin 100 µg/mL on platelet activity

Concentration / Time	Dispirin 100 µg/mL
1 min	62.86 ±0.020
2 min	60.81 ±0.020
3 min	59.84 ±0.020
4 min	58.84 ±0.020
5 min	57.81 ±0.030
6 min	57.59 ±0.02

***In-vitro* anti-platelet activity in *L. rhinocerotis* crude aqueous extract**

The percentage transmittance values obtained in the reaction of the platelet with different concentrations of *L. rhinocerotis* crude extract is shown in Table 4.3. From the result, all concentrations (5 mg/mL, 10 mg/mL, 15 mg/mL, 20 mg/mL and 25 mg/mL) of *L. rhinocerotis* crude extract showed anti-platelet aggregation activity as the percentage transmittance values decreases from the first minute to the sixth minute. However, *L. rhinocerotis* 15 mg/mL showed higher anti-platelet aggregation activity compared to other concentrations tested. The *L. rhinocerotis* showed dose dependent inhibition of ADP-induced human platelet aggregation.

Table 4.3: Percentage of transmittance values for *Lignosus rhinocerotis* mushroom extract

		Percentage Transmittance values					
Concentration		**1 min**	**2 min**	**3 min**	**4 min**	**5 min**	**6 min**
ADP 125µM		29.377 (±0.007)	30.374 (±0.014)	31.297 (±0.015)	32.248 (±0.004)	33.26**6** (±0.005)	33.574 (±0.009)
Disprin 100 µg/ml		62.86 (±0.02)	60.81 (±0.02)	59.84 (±0.02)	58.84 (±0.02)	57.81 (±0.03)	57.59 (±0.02)
***Lignosus rhinocerotis* mushroom extract**	**5 mg/mL**	40.179 (±0.013)	39.780 (±0.010)	39.385 (±0.011)	38.845 (±0.009)	37.786 (±0.016)	37.526 (±0.016)
	10 mg/mL	21.445 (±0.016)	21.054 (±0.010)	20.654 (±0.017)	19.922 (±0.022)	19.846 (±0.016)	19.350 (±0.015)
	15 mg/mL	22.257 (±0.022)	21.584 (±0.022)	20.827 (±0.026)	20.452 (±0.020)	19.907 (±0.027)	18.948 (±0.036)
	20mg/mL	16.921 (±0.010)	16.283 (±0.020)	15.931 (±0.016)	15.391 (±0.017)	15.115 (±0.020)	14.733 (±0.017)
	25mg/mL	14.240 (±0.013)	13.746 (±0.013)	13.267 (±0.011)	13.095 (±0.011)	13.012 (±0.004)	12.865 (±0.009)

In-vitro Anti-platelet activity assay in aquoues extract of *A. polytricha*

The percentage of transmittance values obtained in the reaction of the platelet with different concentrations of *A. polytricha* crude extract is shown in Table 4.4. All concentrations (5 mg/mL, 10 mg/mL, 15 mg/mL, 20 mg/mL and 25 mg/mL) of *A. polytricha* crude extract showed unstable result as the percentage transmittance values increased and decreased from the first minute to sixth minute.

Table 4.4: Percentage of transmittance values for *Auricularia polytricha* mushroom

Concentration		Percentage Transmittance values					
		1 min	**2 min**	**3 min**	**4 min**	**5 min**	**6 min**
ADP 125 µM		23.732 (±0.001)	26.202 (±0.002)	28.141 (±0.006)	30.246 (±0.004)	30.643 (±0.003)	31.502 (±0.002)
Disprin 100 µg/mL		64.417 (±0.006)	61.849 (±0.012)	60.767 (±0.011)	59.247 (±0.023)	58.300 (±0.023)	57.236 (±0.022)
***Auricularia polytricha* mushroom extract**	**5 mg/mL**	26.152 (±0.051)	26.638 (±0.054)	26.76 (±0.057)	26.73 (±0.060)	26.152 (±0.063)	25.586 (±0.064)
	10 mg/mL	22.777 (±0.071)	23.632 (±0.064)	24.21 (±0.057)	24.322 (±0.050)	23.851 (±0.047)	22.856 (±0.053)
	15 mg/mL	20.821 (±0.009)	21.677 (±0.012)	21.953 (±0.011)	21.602 (±0.007)	20.941 (±0.010)	21.989 (±0.017)
	20 mg/mL	20.449 (±0.006)	21.054 (±0.004)	21.660 (±0.007)	21.380 (±0.015)	20.973 (±0.016)	20.433 (±0.004)
	25 mg/mL	21.038 (±0.020)	22.267 (±0.030)	23.082 (±0.020)	22.834 (±0.020)	22.063 (±0.029)	21.627 (±0.017)

4.4 Dialysis and acetone precipitation of protein

As *L. rhinocerotis* crude extract showed anti-platelet aggregation activity in the *In-vitro* anti-platelet activity assay, the mushroom was further investigated. Dialysis and acetone precipitation of protein were done for the mushroom extract. The percentage transmittance values obtained in the reaction of the platelet with dialysed *L. rhinocerotis* mushroom extract (15 mg/mL) and with the dialysed *L. rhinocerotis* mushroom extract precipitated with acetone (15 mg/mL) is shown in Table 4.5. From the result, the dialysed *L. rhinocerotis* crude extract showed anti-platelet aggregation activity as the percentage transmittance values decreases from the first minute to the sixth minute. This showed that the anticoagulant compound in the mushroom is bigger than 12 kDa as the mushroom extract was dialysed against distilled water using the cellular membrane with molecular weight of 12 kDa cut off range.

After the test, the protein in the dialysed mushroom extract was precipitated using acetone. The *in-vitro* anti-platelet activity assay was performed again to test on the anti-platelet aggregation activity for the protein obtained after acetone precipitation. As result, the protein precipitated out from the dialysed *L. rhinocerotis* crude extract showed anti-platelet aggregation activity as the percentage transmittance values decreases from the first minute to the sixth minute.

Table 4.5: Percentage of transmittance values for dialysed and acetone precipitated *Lignosus rhinocerotis* mushroom extract

	Percentage Transmittance values				
Concentration	**1 min**	**2 min**	**3 min**	**4 min**	**5 min**
ADP 125 µM	34.319 (±0.001)	35.167 (±0.002)	36.243 (±0.006)	37.509 (±0.004)	38.152 (±0.003)
Disprin 100 µg/mL	64.445 (±0.006)	61.763 (±0.012)	60.500 (±0.011)	58.917 (±0.023)	58.300 (±0.023)
Dialysed mushroom extract (15 mg/mL)	31.141 (±0.001)	30.083 (±0.011)	29.107 (±0.012)	28.752 (±0.008)	28.205 (±0.006)
Acetone Precipitated of dialysed mushroom extract (15 mg/mL)	21.544 (±0.018)	20.797 (±0.014)	19.800 (±0.010)	19.573 (±0.008)	19.320 (±0.001)

4.5 Aqueous two phase systems (ATPS)

After the acetone precipitation of protein, aqueous two phase systems (ATPS) was performed to recover the anticoagulant enzyme from the solutions. Polyethylene glycol 50 % (PEG 8000) and phosphate 40 % was used to recover the anticoagulant enzyme and the *in-vitro* anti-platelet activity assay was performed again to test on the anti-platelet aggregation activity for the enzymes recovered in the top and the bottom phases of the aqueous two phase systems (ATPS). The enzymes that recovered from the top phase of the aqueous two phase systems (ATPS) showed anti-platelet aggregation activity as the percentage transmittance values decreases from the first minute to the

sixth minute. The enzymes that recovered from the bottom phase of the aqueous two phase systems (ATPS) did not show anti-platelet aggregation activity as the percentage transmittance values increases from the first minute to the sixth minute.

Table 4.6: Percentage of transmittance values for *Lignosus rhinocerotis* mushroom extract after aqueous two phase systems (ATPS)

		Percentage Transmittance values				
Concentration		**1 min**	**2 min**	**3 min**	**4 min**	**5 min**
ADP 125 µM		33.88 (±0.002)	34.28 (±0.003)	34.43 (±0.005)	35.40 (±0.008)	37.76 (±0.005)
Disprin 100 µg/mL		64.37 (±0.005)	61.56 (±0.013)	60.76 (±0.011)	59.24 (±0.017)	57.41 (±0.018)
***Lignosus rhinocerotis* mushroom extract**	**Blank for top phase**	17.061 (±0.029)	17.405 (±0.026)	18.058 (±0.025)	18.635 (±0.019)	19.172 (±0.015)
	ATPS for top phase	15.28 (±0.014)	14.62 (±0.018)	14.66 (±0.019)	14.45 (±0.018)	13.96 (±0.023)
	Blank for bottom phase	16.62 (±0.015)	17.02 (±0.016)	17.54 (±0.023)	19.63 (±0.011)	20.40 (±0.016)
	ATPS for bottom phase	14.00 (±0.025)	14.42 (±0.014)	14.62 (±0.010)	15.21 (±0.006)	15.50 (±0.009)

4.6 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE)

Following the aqueous two phase systems (ATPS), sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) page was performed to observe the enzymes that recovered from the top phase of the ATPS. The protein bands of the crude mushroom extract on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) are shown in Figure 4.6. Figure 4.7 shows the partially purified protein that recovered from the top phase of the aqueous two phase systems (ATPS) on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE). Two bands in the

range of approximately 45 kDa-60 kDa (~ 50 kDa, ~ 55 kDa) were observed on the gel for the ATPS product.

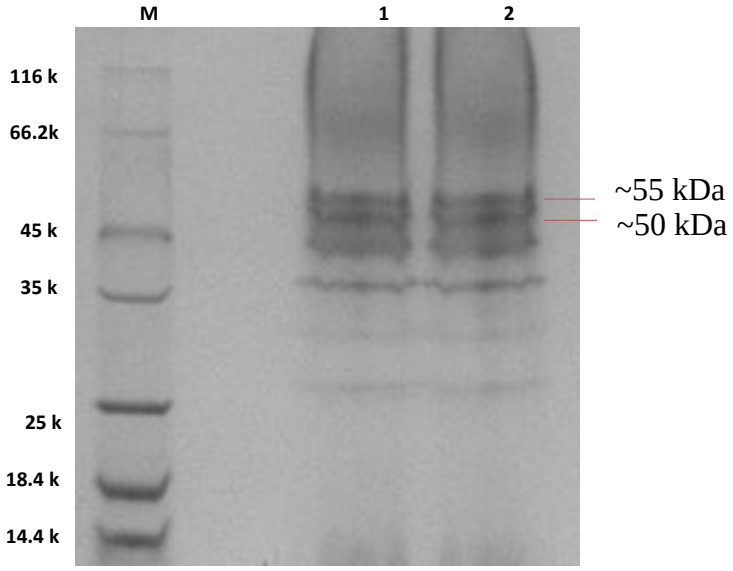

Figure 4.6: **Molecular mass determination of crude extract on SDS-PAGE**. Lane: M = Protein molecular weight standards; 1 & 2= Crude extract.

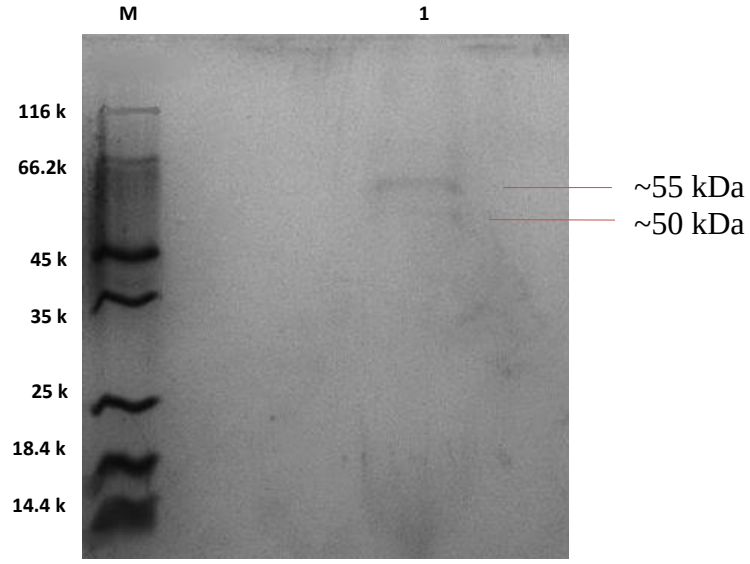

Figure 4.7: **Molecular mass determination of partially purified protease on SDS-PAGE**. Lane: M = Protein molecular weight standards; 1 = partially purified protease.

4.7 Native page and protein elution from gel

In order to confirm that the two bands observed on the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) are contributing to the anti-platelet aggregation activity, native page was performed and the bands were excised and tested again for the anti-platelet aggregation activity. The result is shown in Table 4.7. For both bands excised and tested, the percentage transmittance values decreases from the first minute to the sixth minute in the *in-vitro* anti-platelet activity assay. No anti-platelet aggregation activity was detected in the blank using elution buffer as control as the percentage transmittance values increases from the first minute to the sixth minute in the *in-vitro* anti-platelet activity assay.

Table 4.7: Percentage of transmittance values of protein eluted from gel for *Lignosus rhinocerotis* mushroom extract

		Percentage Transmittance values				
Concentration		**1 min**	**2 min**	**3 min**	**4 min**	**5 min**
ADP 125 µM		26.39 (±0.001)	28.59 (±0.031)	30.64 (±0.040)	32.64 (±0.040)	33.66 (±0.044)
Disprin 100 µg/mL		73.96 (±0.004)	71.67 (±0.008)	70.57 (±0.005)	69.50 (±0.002)	69.23 (±0.001)
Lignosus rhinocerotis	**Control (Elution buffer)**	11.22 (±0.083)	12.94 (±0.079)	13.29 (±0.083)	13.68 (±0.15)	15.28 (±0.15)
	Band excised (~50kDa)	26.26 (±0.014)	25.68 (±0.018)	25.09 (±0.019)	23.73 (±0.018)	22.54 (±0.023)
	Band excised (~55kDa)	17.28 (±0.006)	16.82 (±0.006)	15.87 (±0.001)	14.80 (±0.012)	13.68 (±0.009)

Chapter 5.0 Discussion

5.1 Crude extracts preparation

Based on Voet and Voet (2004), freeze-drying process can preserve enzyme in the frozen condition. As proven by Kho (2008), the fibrinolytic activity of the crude extract of freeze-dried fruit bodies of *Auricularia auricula-judae* was higher than the crude extract of fresh fruit bodies. Hence, freeze-dried mushroom was chosen in this study. The preparation of mushroom crude extract was done in ice bath as protein can be easily denatured at high temperature. A volume of 200 mL solvent buffer was used to re-dissolve the 10 g of crude extract powder in order to obtain relatively concentrated enzyme. In this study, Tris-HCL buffer (pH 8.0) was used in order to maintain the stability of protein extracted. Other than that, in order to minimise frothing of protein solution, the powder in the protein solution was grinded instead of blending and homogenizing (Voet and Voet, 2004).

5.2 *In-vitro* preliminary anticoagulant activity screening assay

In the *in-vitro* preliminary anticoagulant activity screening assay, bovine whole fresh blood sample was used instead of human blood as large amount of blood was needed. In the test, small glass tubes were firstly used. However, all blood clotted in a short time. Based on Stover and Stammers (1995), glass activates intrinsic blood coagulation pathway faster than plastic, causing the blood to clot in a very short time. Therefore, plastic microcentrifuge tubes were used to observe and screen for the anticoagulant activity of the mushroom extracts.

For the six mushroom samples investigated, only two mushroom samples, the *L. rhinocerotis* and *A. polytricha* showed anticoagulation activity in the bovine blood. As reported by Yoon *et al.* (2003), the extracts of the other *Auricularia* species known as *A.*

auricula had shown anticoagulation activity in blood. The polysaccharide extract in the *A. auricula* inhibits platelet aggregation and increases clotting time *in-vitro* and *in-vivo.* The anticoagulant activity was not observed in the mushroom extract of *G. lucidum, C. militaris, P. giganteus* and *P. florida*. This might be due to the absence of anticoagulant compounds in the mushroom or the concentration of crude extract used (1 mg/mL, 3 mg/mL, 6 mg/mL, 9 mg/mL) in the test might not strong enough to inhibit blood coagulation. For example, it was suggested that the Chinese herbal medicinal *G. lucidum* can be an effective inhibitory agent of platelet aggregation. However, the concentration, mechanism and the active principles of the inhibitory process remain to be further investigated (Tao and Feng, 1990).

After the preliminary screening of anticoagulant activity in the 6 mushroom samples, only 2 samples of *A. polytricha* and *L. rhinocerotis* were selected for further anticoagulant activity screening. The concentration of the mushroom samples was increased and from the observation, the higher the concentration of the *A. polytricha* and *L. rhinocerotis* extract added to the blood, the better was the anticoagulation activity result.

5.3 *In-vitro* Anti-platelet activity assay

Human blood was used instead of bovine blood due to the distance of bovine slaughtering house from Mushroom Research Centre, University of Malaya. As platelet function test should be performed immediately and completed within 3 hours after blood collection (Zhou and Schmaier, 2005), human blood was drawn and centrifuged immediately to obtain the human platelet rich plasma. The human platelet rich plasma freshly obtained in this study was turbid and it was mostly due to the platelets (Born and Cross, 1963).

The platelet aggregation activity was measured using spectrophotometer in the percentage of transmittance values. Based on Helem (1960), adenosine-5'-diphosphate (ADP) can induce platelet aggregation. Initially, adenosine-5'-diphosphate (ADP) 5 μM and 10 μM were tested. However, the concentration of the adenosine-5'-diphosphate (ADP) is not sufficiently strong, causing the platelets to disaggregation after a few minutes (Zhou and Schemaier, 2005). Based on Born and Cross (1963), when adenosine-5'-diphosphate (ADP) was added, the optical density or absorbance of the spectrophotometer increased again after its initial fall (percentage of transmittance decreased after its initial increment). This suggested that the aggregated platelets were able to disperse under some conditions as proven by Born and Cross (1963). The dispersion was always seen microscopically when low concentrations of adenosine-5'-diphosphate (ADP) was added in order to induce platelet aggregation (Born, 1962). This observation suggested that the dispersion of the aggregates might be due to the break-down of adenosine-5'-diphosphate (ADP) to adenosine monophosphate (AMP) and other substances which not only do not cause aggregation (Gaarder et al. 1961) but act as potent inhibitors to the aggregation brought by Adenosine-5'-diphosphate (ADP). In addition, human plasma contains enzymes which catalyse the break-down of adenosine-5'-diphosphate (ADP) to adenosine monophosphate (AMP) Jorgensen (1956).

Then, the concentration of the adenosine-5'-diphosphate (ADP) used in the study was increased to 75 μM, 100 μM and 125 μM. Adenosine-5'-diphosphate (ADP)125 μM was lastly chosen as the negative control in this study as the percentage transmittance increased from the first minute to the seventh minute tested. By using the same method, Mary *et al*. (2003) also showed that the addition of adenosine-5'-diphosphate (ADP) to platelet caused remarkable increase in light transmittance value at 600 nm due to the aggregation of platelets. For positive control, Disprin was used as it is a common anti-platelet agent. Same as Lavanya *et al*. (2010) and Elumalai *et al*.

(2012), Disprin100µg/ml decreased platelet aggregation over time as the percentage of transmittance values obtained decreased from the first minute to the sixth minute.

Different concentration of *L. rhinocerotis* crude extract (5 mg/mL, 10 mg/mL, 15 mg/mL, 20 mg/mL and 25 mg/mL) were tested for their anti-platelet aggregation activity in this study. Similar to the screening test for anti-platelet aggregation activity in *Couroupita guianensis* Aubl (Elumalai *et al*., 2012) and *Anisomeles malabarica* Linn (Lavanya *et al*., 2010), the result in this study showed that all concentrations of the *L. rhinocerotis* crude extract exhibited anti-platelet aggregation activity as the percentage transmittance values decreased over times. However, the mechanism and the active principles of the inhibitory process remain to be investigated.

For *A. polytricha* mushroom, all concentrations (5 mg/mL, 10 mg/mL, 15 mg/mL, 20 mg/mL and 25 mg/mL) of *A. polytricha* crude extract showed unstable results as the percentage transmittance values increased and decreased from the first minute to sixth minute. This might be due to the 'jelly-like nature' of *A. polytricha* crude extract used in the test. Thirty seconds of the reaction before spectrophotometer reading was taken might not be enough to ensure proper mixing between the mushroom sample, the platelet and the adenosine-5'-diphosphate (ADP). Zhou and Schmaier (2005) reported that platelet aggregation occurred only if there was an interaction between the adenosine-5'-diphosphate (ADP) and the platelet rich plasma in stirred condition. As the mushroom sample is too sticky to mix with the adenosine-5'-diphosphate (ADP) and the plasma with rich platelet, longer time might be needed to ensure proper interaction between the mushroom sample and the aggregated platelet induced by adenosine-5'-diphosphate (ADP).

Yoon *et al.* (2003) reported that the polysaccharide extract in the *A. auricula* inhibited platelet aggregation and increased clotting time *in-vitro* and *in-vivo*. Therefore,

the anti-platelet aggregation compound in the *A. polytricha* might also be polysaccharides. Other than that, the anticoagulant compound present in the mushroom might not inhibit platelet aggregation but inhibit other agents involved in blood clotting. Based on Jeffery *et al*. (2001), anticoagulants can inhibit thrombin generation or inhibit other coagulation factors such as factor Xa, factor IXa, and the factor VIIa/tissue factor complex which contributed to blood clotting.

5.4 Dialysis and acetone precipitation of protein

This study was mainly focused on the screening of the anticoagulant enzyme from the mushroom samples as protein can be an anticoagulant in mushrooms besides animals and plants. In mushroom namely *Inonotus. obliquus*, a tripeptide (Trp-Gly-Cys) showed high platelet aggregation inhibitory activity in mice (Kwang *et al*., 2006).

Based on Berg *et al*. (2002), bigger molecules such as proteins can be separated from other small molecules by dialysis using a semipermeable membrane. Anita *et al*. (2009) also used the same method to refine bioactive compounds in *G. Lucidum*. In this study, the dialysed *L. rhinocerotis* crude extract showed anti-platelet aggregation activity as the percentage transmittance values decreased from the first minute to the sixth minute. This showed that the anticoagulant compound in the mushroom is bigger than 12 kDa. In Choi *et al.* (2013), an anti-platelet aggregation enzyme 48.9 kDa was detected in *Codium fragile*. After overnight dialysis of the *L. rhinocerotis* crude extract, the molecules smaller than 12 kDa traverse the pores of the dialysis tubing and retained as dialysate outside the tube. The molecules that larger than the pore diameter were retained inside the dialysis tubing. The dialysed crude extract was then precipitated using acetone precipitation of protein method (Wessel & Flugge, 1984). The proteins precipitated out also exhibited anti-platelet aggregation activity.

5.5 Aqueous two phase systems (ATPS)

Based on Dixon and Webb (1979), enzymes are naturally found in complex mixture. In order to obtain specific enzymes from the complex mixture, purification step was performed. According to Voet and Voet (2004), protein precipitation using ammonium sulphate is commonly used due to its large solubility in water and with no harmful effect on most enzymes. It also can stabilize most enzymes and the precipitation process can bypass to be carried out at low temperature (Dixon and Webb, 1979). However, in previous study done by Kho (2008) in recovery fibrinolytic enzyme using ammonium sulphate precipitation, low purification yield of partially purified enzyme was obtained. Therefore, an aqueous two phase system (ATPS) was chosen in this study.

As stated in Li *et al*. (2001), PEG/salt system appears to be a good choice for bioseparation of protein as they have wide range of hydrophobicity between two different phases. Same as in Sharjahan *et al*. (2014), PEG 8000-phosphate aqueous two phases system (ATPS) was found to be a suitable system for recovery of the fibrinolytic enzyme (66 kDa) from the *A. polytricha* crude extract. After aqueous two phase systems (ATPS), the polyethylene glycol (PEG) was removed by acetone precipitation for subsequent *in-vitro* anti-platelet activity assay. Based on Dennison (2003), polyethylene glycol (PEG) is not easy to be separated from protein. However, the solubility of polyethylene glycol (PEG) in acetone enables the polyethylene glycol (PEG) to be separated from protein. The *in-vitro* anti-platelet activity assay performed to test on the anti-platelet aggregation activity for the enzymes recovered from the top phases of the aqueous two phase systems (ATPS) showed that the enzymes recovered at the top phase have the anti-platelet aggregation activity as the percentage transmittance values decreased from the first minute to the sixth minute. The enzymes recovered at the bottom phase of the aqueous two phase systems (ATPS) did not show anti-platelet aggregation activity. This showed that the anticoagulant enzymes from the *L.*

rhinocerotis extract exhibited top preference in the aqueous two phase systems (ATPS) where high molecular weight of PEG 8000 was used. In the study, a blank for the top phase and the bottom phase was prepared to ensure no false positive result given by the polyethylene glycol (PEG) or the phosphate salt used in the aqueous two phase systems (ATPS). No anti-platelet aggregation activity was observed for both blanks as the percentage transmittance values increases from the first minute to the sixth minute.

5.6 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE)

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) was used for protein separation and analysis in the study. To assess the relative molecular weight of proteins in a gel, protein molecular weight (MW) markers containing several proteins of known molecular mass was run alongside the test sample.

Six distinct protein bands of the crude mushroom extract were observed on the gel of the Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE). The bands were ranged from 25 kDa to 60 kDa. After aqueous two phase systems (ATPS), the partially purified protein recovered from the top phase was analyzed using Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE). Two bands within the range of approximately 45 kDa-60 kDa (first band ~50 kDa, second band~ 55kDa) were observed on the gel for the aqueous two phase systems (ATPS) product. As compared to the bands observed on the gel for the mushroom crude extract, the protein for these two bands were separated in the top phase of the aqueous two phase systems (ATPS) while the other 4 proteins for the other 4 bands might be separated in the bottom phase of the aqueous two phase systems (ATPS).

Based on Choi *et al.* (2013), the codiase enzyme purified from marine green alga, *Codium fragile* was estimated to be 48.9 kDa by Sodium dodecyl sulfate

polyacrylamide gel electrophoresis (SDS PAGE) and it was proven to have anticoagulant and anti-platelet aggregation activities. In order to confirm that the two bands (~ 50 kDa and ~ 55kDa) observed on the gel for the aqueous two phase systems (ATPS) product are contributing to the anti-platelet aggregation activity, native page was performed and the bands were excised and tested again for the anti-platelet aggregation activity.

5.7 Native PAGE and protein elution from gel

In order to analyze the two bands (~ 50 kDa and ~ 55kDa) observed on Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) in their native; non-denatured conditions, a non-denaturing system or a native PAGE was used. Anti-platelet aggregation activity was shown in the spectrophotometer reading for both bands tested. No anti-platelet aggregation activity was detected in the blank using elution buffer (control) as the percentage transmittance values increased from the first minute to the sixth minute in the *in-vitro* anti-platelet activity assay. This proved that the anti-platelet aggregation activity was solely from the bands excised from the native gel.

At the same time of screening for the anticoagulant activity from *L. rhinocerotis* extract, the fibrinolytic activity of the *L. rhinocerotis* extract was also investigated based on the study by Kho (2013). The fibrin plate method modified from Kim and Kim (1999) and fibrinolytic assay (Folin-spectrophotometery method) was used in her study. After the native page, the two bands with approximately 50 kDa and approximately 55 kDa were excised from the gel and fibrinolytic activity testing was done on fibrin plate. As result, lytic zone was observed for both of the bands on the fibrin plate. Based on Hassanein *et al*. (2011), a fibrinolytic enzyme could also be an effective antithrombolytic agent. In their study, they found that the *in vitro* application of K42

fibrinolytic enzyme extracted from the bacteria isolated from soybean flour on human blood serum prolonged the blood clotting time. Similarly, a new bi-functional fibrinolytic serine protease, the codiase enzyme, having thrombolytic, anticoagulant and antiplatelet aggregation activities was found in marine green alga, *Codium fragile* as reported by Choi *et al.* (2013). The purified enzyme was estimated to be 48.9 kDa by Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) and mass spectrometry. This enzyme was able to hydrolyze fibrin clot directly or by the activation of plasminogen on the fibrin plate assays. On the other hand, it also found to prolong the activated partial thromboplastin time (APTT), and prothrombin time (PT). Therefore, it is suggested that the crude extract and the partial purified crude enzyme of *L. rhinocerotis* possessed both anti-platelet aggregation capacity besides fibrinolytic capacity.

5.8 Recommendations for further studies

This is the first report of anti-platelet aggregation activity in *L. rhinocerotis*. As it has the potential to inhibit platelet aggregation in blood, further studies on the enzyme/s involved and the mechanism should be carried out in future. The enzyme/s can be further purified using suitable methods such as HPLC and sequencing of the pure anti-platelet aggregation enzyme/s can be done for further investigation. In addition, pure enzyme/s can be investigated for its *in vivo* anti-platelet aggregation activity in order to reduce the risk of cardiovascular disease. Thereby, *L. rhinocerotis* may become a new alternative for anti-platelet aggregation agent and potentially can be used to develop new therapeutic agent for thrombosis in future.

Besides, as polysaccharide extract in the *A. auricula* was reported to inhibit platelet aggregation, further study on the polysaccharide extract of *A. polytricha* in

inhibiting platelet aggregation can be done. For the other 4 species of *G. lucidum*, *C. militaris*, *P. giganteus* and *P. floridanus*, different concentrations of the crude extract can be tested for the screening of anticoagulant compounds in the mushrooms.

Chapter 6.0 Conclusions

Among the six mushroom samples (*G. lucidum*, *C. militaris*, *L. rhinocerotis*, *P. giganteus*, *P. floridanus* and *A. polytricha*) screened for their anticoagulant activity:

a) Two mushroom samples of *A. polytricha* and *L. rhinocerotis* contained the anticoagulant compound in the preliminary screening for the anticoagulant activity.

b) Anti-platelet aggregation activity was shown in *L. rhinocerotis* crude extract. The *in-vitro* anti-platelet activity assay performed to test on the enzymes recovered from the top phase of the aqueous two phase systems (ATPS) showed that the enzymes have the anti-platelet aggregation activity.

c) The partially purified protein recovered from the top phase of the aqueous two phase systems (ATPS) was analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE). Two bands of approximately 50 kDa and approximately 55 kDa were observed on the gel.

d) Both of the bands were excised and tested. Both of the bands showed anti-platelet aggregation activity.

e) *L. rhinocerotis* mushroom contains anti-platelet aggregation enzymes while the anti-platelet aggregation activity in other five species remains to be further investigated.

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Appendix

Appendix A

Preparation of reagents

7.1 20 mM Tris-HCL buffer (pH 8.0)

- Molecular weight of trizma = 121.14 g/moL
- To prepare 1000 mL,

$$\text{Mole} = \frac{20 \times 10^{-3}\,\text{M}\,(1000\text{ml})}{1000\,\text{ml}}$$

$$= 0.02\ \text{moL}$$

- $0.02\ \text{mole} = \frac{x}{121.14\,\text{g/mol}}$
- x = 2.4228 g

- To prepare 20 mM Tris-HCL buffer (pH 8.0), 2.4228 g of trizma was dissolved in 900 mL of distilled water.The pH was adjusted to pH 8.0 with HCL before the final volume was topped up to 1000 mL with distilled water

7.2 40% phosphate stock solution for ATPS

- To prepare 100 g,

21.7 g of K_2HPO_4 mixed with 18.3 g of KH_2PO_4 and 60 g of distilled water.

7.3 50% PEG stock solution for ATPS

- To prepare 100 g,

50 g of PEG 8000 was mixed with 50 g of distilled water.

Appendix B

7.4: Percentage transmittance values for Adenosine-5'- Diphosphate on platelet activity

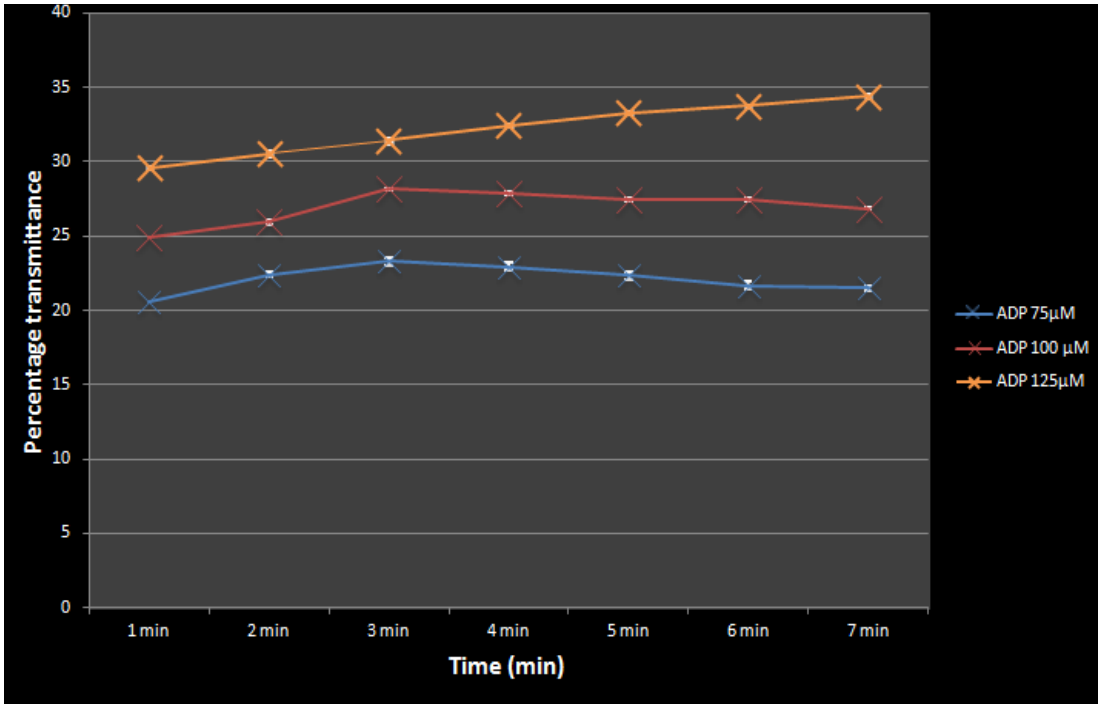

Figure 7.1: Effect of Adenosine-5'- diphosphate on platelet activity.

7.5: Percentage transmittance values for Disprin 100 µg/mL on platelet activity

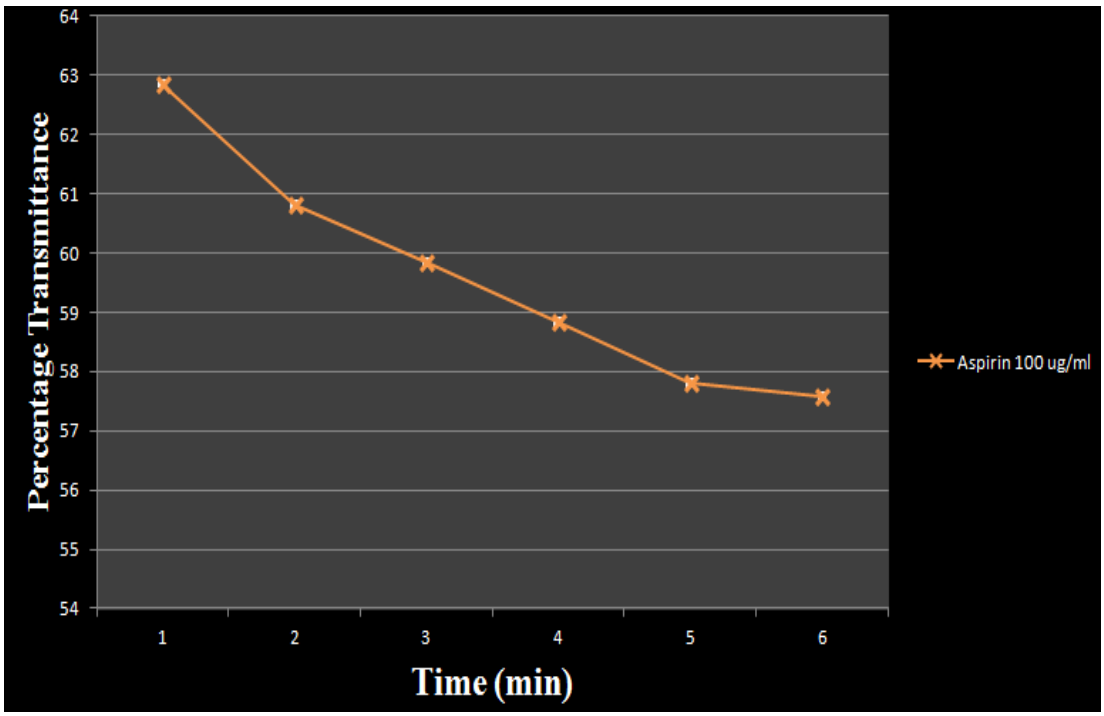

Figure 7.2: Effect of Disprin (100µg/mL) on platelet activity.

7.6: Percentage of transmittance values for *Lignosus rhinocerotis* mushroom extract

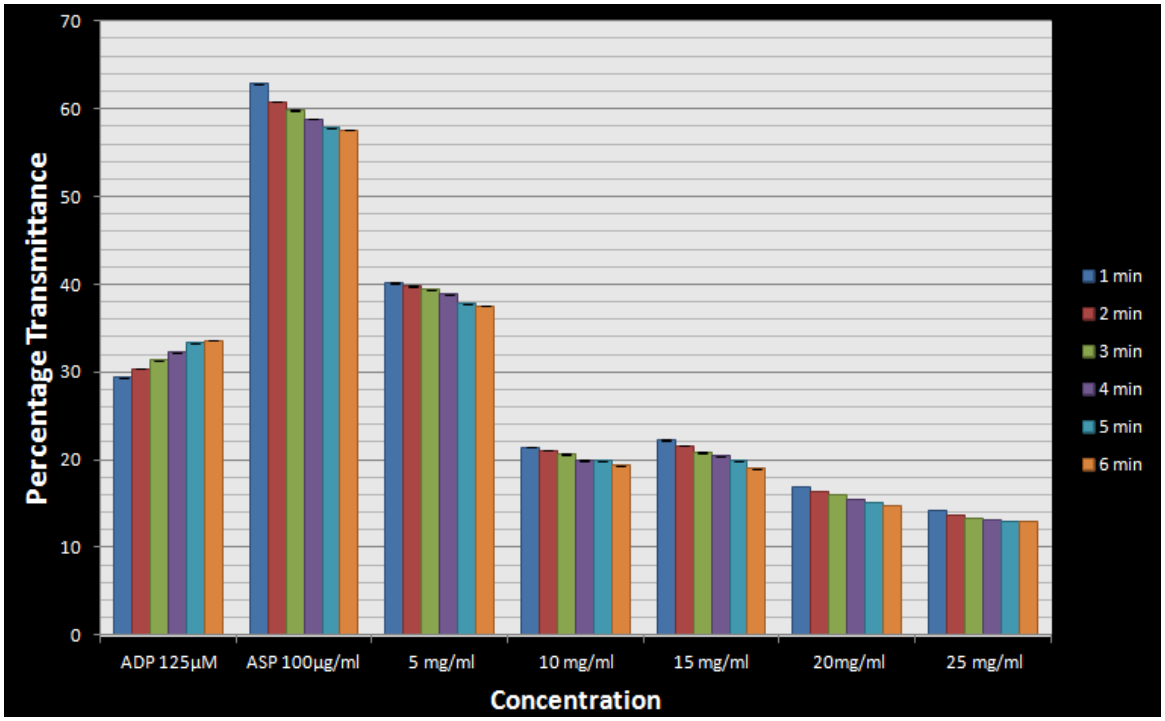

Figure 7.3: Effect of *Lignosus rhinocerotis* on anti-platelet aggregation activity.

7.7: Percentage of transmittance values for *Auricularia polytricha* mushroom

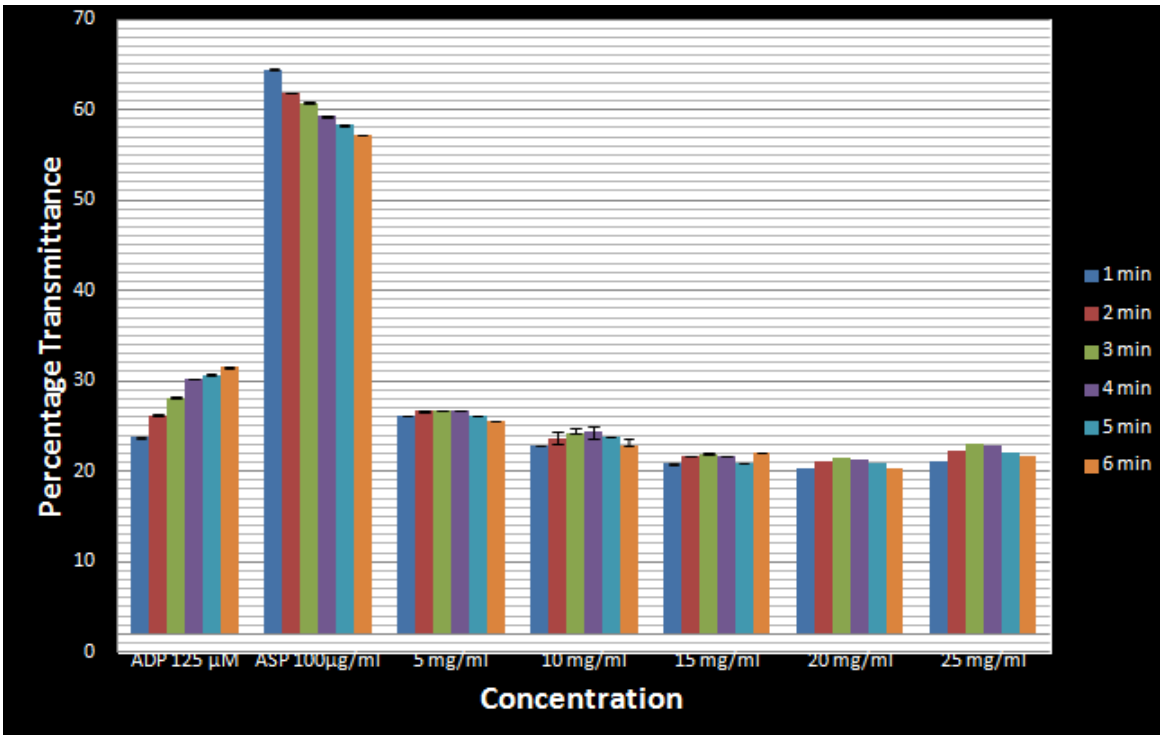

Figure 7.4: Effect of *Auricularia polytricha* on anti-platelet aggregation activity.

7.8: Percentage of transmittance values for dialysed and acetone precipitated *Lignosus rhinocerotis* mushroom extract

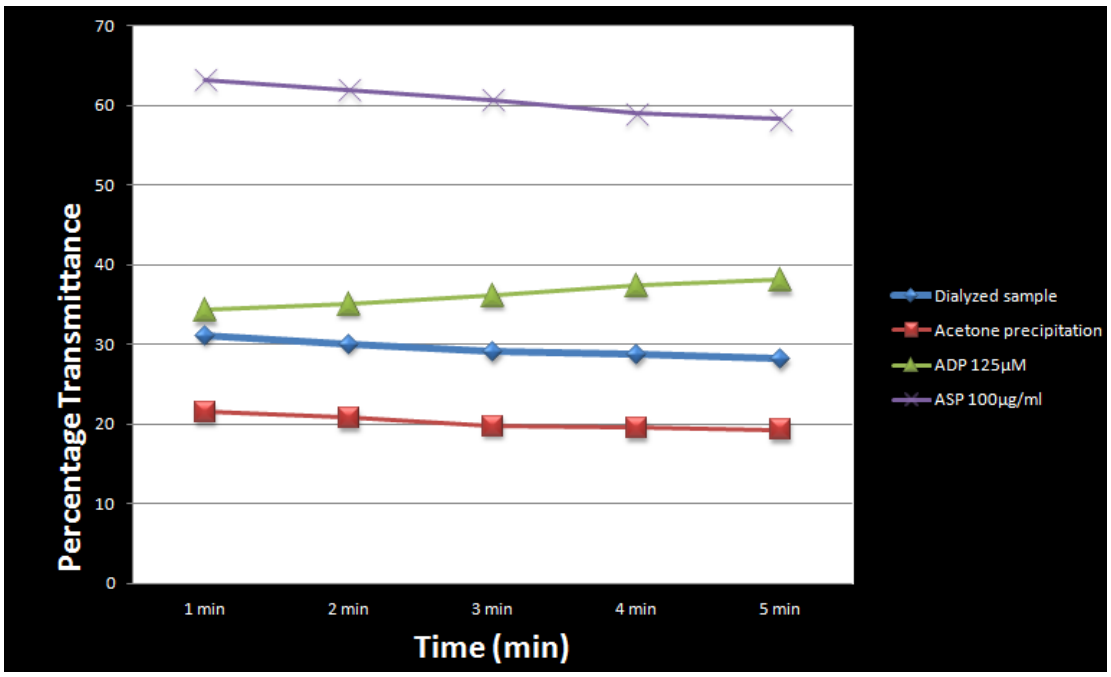

Figure 7.5: Effect of dialysed crude extract and acetone precipitated protein of *Lignosus rhinocerotis* extract on anti-platelet aggregation activity.

7.9: Percentage of transmittance values for *Lignosus rhinocerotis* mushroom extract after ATPS

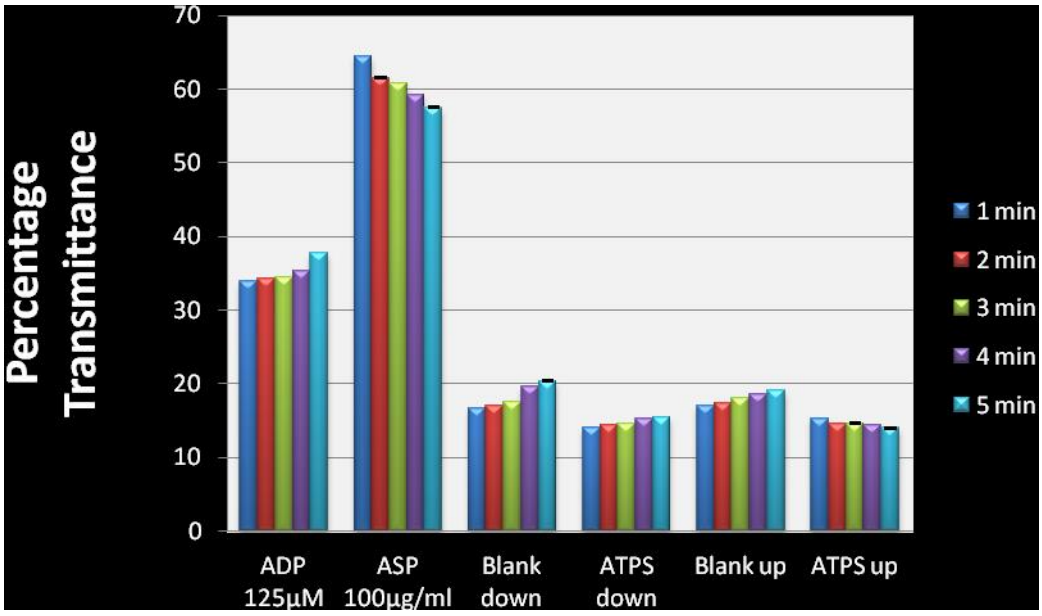

Figure 7.6: Effect of ATPS product of *Lignosus rhinocerotis* extract on anti-platelet aggregation activity.

7.10: Percentage of transmittance values of protein eluted from gel for *Lignosus rhinocerotis* mushroom extract

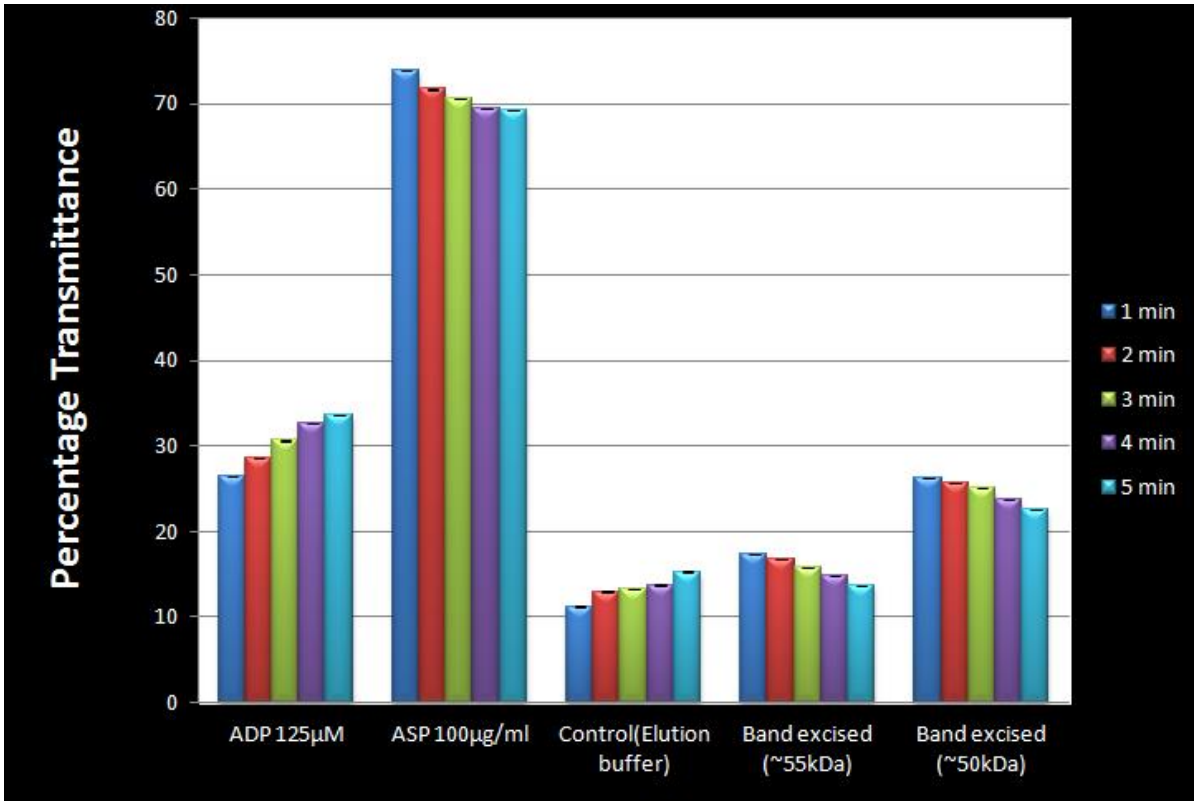

Figure 7.7: Effect of protein eluted from gel on anti-platelet aggregation activity.