Atepa: In Vitro Anticoagulant of Catappa Leaves (*Terminalia catappa*)

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Abstract. Catappa leaves (*Terminalia catappa*), contain flavonoids, saponins, phenols, and tannins after being tested qualitatively. Flavonoids have strong antioxidant activity. Based on previous studies, this active compound can prevent platelet aggregation in the blood clotting process. Due to this activity, catappa leaf extract has anticoagulant activity. *Atepa* was made by diluting the extract into several concentration variations, namely 1%, 2%, 3%, 4%, 5%, and 6%. After testing with the Lee White Clotting Time method, it was found that in 1% concentration was able to inhibit the coagulation process. After observing the May-Grunwald Giemsa stain, it can be observed that the blood cells are still shaped according to their characteristics and have not changed. *Atepa* can prevent coagulation in vitro. The active compound of flavonoids, saponins, and tannins in catappa leaves is a substance that plays an important role in anticoagulation activity. *Atepa* still requires in vivo testing if it is to be used as an in vivo anticoagulant. *Atepa* can be redeveloped according to the intended implementation.

Keywords: Anticoagulant, catappa leaves extract, coagulations

1 Introduction

Blood tests are frequently performed to establish a diagnosis and provide concrete evidence of the severity of a disease. Various blood tests can be performed according to the disease and condition to be evaluated. For example, hemostasis examination is used to determine abnormalities in the blood clotting process, blood culture examination to determine bacterial infection or what is called bacteremia. Blood examination is not only done macroscopically, but can also be done microscopically.

Microscopic blood examination is carried out with the aim of observing abnormalities in the form of blood cells that are usually experienced by patients who experience inflammation, infection, and leukemia. This type of blood examination can also differentiate the type of anemia suffered by the patient. In addition, this examination is the gold standard for the diagnosis of malaria. Diseases caused by the Plasmodium parasite can be easily observed by microscopic blood examination because the parasite attacks the patient's red blood cells.

Microscopic examination can be performed using venous and capillary blood samples. In order for the blood to be transported from the collection site to the examination site, the blood is then added with an anticoagulant. Anticoagulant is an ingredient that is added with the aim of inhibiting coagulation activity or blood clotting. Anticoagulants have different mechanisms of action according to their type. Some of them are by activating antithrombin, interfering with the maturation of coagulation factor proteins, and binding calcium ions which results in an inhibited aggregation process (Weliyani, Nugroho, R., A., Syafrizal, 2015)

Anticoagulants that are often used in laboratory examinations are Calium Ethylene Diamin Tetra Acetate (K3EDTA), Sodium Citrate, Oxalate, Heparin, Acetic Acid Dextrose (ACD), and Sodium Polyanetol Sulfonate (SPS) (Kiswari, R, 2014). EDTA works by binding calcium ions that are needed in the blood clotting process, so the blood does not clot. Meanwhile, heparin can stop blood clotting by binding to antithrombin compounds and then inhibiting thrombin activation (Keohane, E.M et al, 2015). The use of anticoagulants greatly affects the results of the examination performed. An error in the selection of anticoagulants can certainly bring false results to the examination.

Microscopic examination is recommended using unanticoagulated blood with capillary blood samples. The addition of anticoagulants is done under certain circumstances. A common anticoagulant used in microscopic blood examination is EDTA (Ethylene Diamine Tetra Acetic Acid). But this is noteworthy because anticoagulants will not have an impact on the morphology of blood cells if observed immediately. According to research conducted by Narasimha, Kumar, and Prasad, one hour after EDTA anticoagulant was added, artifacts appeared on the blood smear. There were also some changes in the morphology of blood cells.

The quality of blood smear preparations used in microscopic examination is very important in the diagnosis and prognosis of disease. The presence of artifacts and changes in blood cell morphology can certainly affect the results obtained. Therefore, an anticoagulant that does not affect the results of the analysis is needed, which does not damage blood components, does not affect blood cell morphology, does not cause hemolysis, and can be used in blood tests, especially microscopic tests.

Atepa is a natural anticoagulant made from catappa (Terminalia catappa) leaves. Catappa is a plant that grows widely in Indonesia. This plant can usually be found in dry areas such as beaches and hills. Catappa is one of the potential tropical plants that can be utilized as a source of pharmaceutical primary ingredients, this plant is also widely found in Indonesia because it can live in tropical and subtropical areas (Pattola, P., 2020).

Based on phytochemical tests conducted by Pandya (Pandya, B.N, 2013), ethanol extracts of catappa leaves contain alkaloids, flavonoids, resins, saponins, steroids, and tannins with a total flavonoid content of 56.67 mg/g extract. Based on studies that have been conducted, flavonoid compounds show potential as platelet modulation. In addition, these compounds also have antiplatelet and antithrombotic activities (Shalebah et al, 2015).

Thus, flavonoids are compounds that have anticoagulant activity without processes that can cause changes in blood cell morphology. Flavonoids prevent blood clotting with antiplatelet activity that can stimulate a decrease in platelet count. Furthermore,

flavonoid compounds can also function as antithrombotics, where these compounds can prevent the activation of thrombin proteins that play a role in the blood clotting process. Therefore, Atepa can be an ideal anticoagulant in the process of microscopic blood examination.

2 Research Methods

2.1 Time and Place

This research was conducted at the Basic Chemistry Laboratory of the Polytechnic of the Ministry of Health of Denpasar in the process of making Atepa and phytochemical testing, and the Hematology Laboratory of the Polytechnic of the Ministry of Health of Denpasar for anticoagulant testing located at Sanitasi Street Number 1 Sidakarya, Denpasar in April - May 2023.

2.2 Tools

The tools used in the study were vacuum needle, heparin tube, EDTA tube, tourniquet, alcohol cotton, hypafix tape plaster, beaker glass, test tube, tube rack, measuring cup, stirring rod, gauze, filter paper, dropper pipette, micropipette, yellow tip, blue tip, tube rack, rotary evaporator, staining rack, glass object, glass cover, microscope, blender, analytical balance.

Material

Materials used in the study, namely catappa leaves (Terminalia catappa), 70% ethanol solvent, 10% NaOH phytochemical screening test, mayer reagent, wagner reagent, dragendorff reagent, distilled water, iron (III) chloride, anhydrous acetic acid, concentrated acetic acid, dimethyl sulfoxide (DMSO), Ethylenediaminetetraacetic acid (EDTA), May-Grunwald Giemsa (MGG) dye, and aluminum foil.

Sample Preparation

The collected catappa leaves were sorted according to the criteria, cleaned of dirt, then washed with running water until clean, drained, then dried using an oven at ± 37˚C for 48 hours. After the oven, the dried samples were crushed using a blender and then soaked using 70% ethanol in a ratio of 1:4 (250 grams: 1000 ml) for 1x24 hours.

Extraction

Sample extraction was carried out using the method of extracting natural materials, namely maceration extraction [7,8]. Catappa leaf sample powder was weighed as much as 250g, then soaked with 70% ethanol solvent in a ratio of 1: 4, stirred and allowed to stand at room temperature for 24 hours. The sample was filtered to produce a filtrate,

the filtrate was put into a rotary evaporator with a pressure of 57 rpm at a temperature of 60° C to produce a thick extract. This filtrate was then processed into Atepa, which was previously diluted with distilled water.

Phytochemical Screening of Secondary Metabolite Compounds of Catappa Leaf Extracts

Flavonoids Identification
The extract is dissolved with ethanol then put into a test tube and added 2 - 4 NaOH 10%, if it gives a yellow color then the reaction is positive.

Alkaloids Identification
The extract is dissolved with ethanol and then filtered to get the filtrate, then the filtrate is divided into 3 each 1 ml filtrate and put into a test tube and then added to the reagent. In mayer reagent positive reaction formed white or yellow precipitate, in wagner reagent positive reaction formed brown to black precipitate and in dragendorff reagent positive reaction formed orange precipitate. The extract is positive for alkaloids if two or three precipitates are formed in the test tube.

Phenols Identification
The extract was dissolved with ethanol and then added FeCl3 reagent 1% where the reaction results were positive if there was a purple-black or blue-black color change.

Tannins Identification
The extract is dissolved with ethanol and then FeCl3 reagent is added where the reaction results are positive if there is a change in purple purple-black or blue-black color.

Saponins Identification
The extract is added 5 ml of hot distilled water and dissolved while heated on a water bath and then shaken vigorously if foam is formed and after 10 minutes the foam does not disappear then HCl 2N is added the foam still does not disappear then the positive reaction is saponin.

Steroids and Triterpenoids Identification
The extract is dissolved with chloroform and then 0.5 ml of anhydrous acetic acid is added, then 1-2 ml of concentrated sulfuric acid is added through the tube wall. If the result obtained is a brownish or violet ring on the border of two solvents, the reaction is positive for triterpenoids, while if a bluish green color is formed, the reaction is positive for steroids.

Anticoagulant Testing of Catappa Leaf Extract on Blood Samples Lee White Method

The samples used in this study were whole blood taken from the median cubital vein using disposable syringes, namely vacuum needles and sterile heparin and EDTA tubes. The number of blood samples taken from each volunteer was 3 cc or 1 tube. Blood

samples for testing were obtained from 3 male volunteers aged 19-21 years. The volunteers were physically healthy and had no history of prolonged bleeding. It was assumed that the volunteers had no hemostasis abnormalities.

Before being added with blood, the extract was dissolved with distilled water. The concentrations used in the test were 1%, 2%, 3%, 4%, 5%, and 6%. The ratio of anticoagulant and blood was 1:1, i.e. 300µl of blood was added with 300µl of anticoagulant.

Clotting Time examination using the Lee White method was performed using 3 tubes, each containing 300µl of anticoagulant. Blood that has been taken is then pipetted as much as 300µl and then homogenized with the anticoagulant in the tube. Then the tubes were incubated at 37˚C. Every 30 seconds the tube was tilted so that the blood touched the wall of the tube and could be observed whether clotting occurred.

Blood Smear Method Anticoagulant Test

Blood was taken from the results of the anticoagulant test of the close system method, then a smear (SADT) was made. Then fixation with ethanol for 2 minutes, May-Grunwald dye was added for 2 minutes, then dripped with Buffer pH 6.4 solution for 2 minutes. Then, rinsing is done and Giemsa dye is added for 15 minutes and rinsed again with running water. Preparations that have been rinsed, dried and observed under a microscope magnification of 1000 times.

3 Results and Discussion

3.1 Phytochemical Test Results

Phytochemical tests were carried out to determine the content of secondary metabolites from catappa leaf extract. The results of the qualitative phytochemical test are presented in the following table.

Table 1. Catappa Leaves Extract Phytochemical Test Results

Secondary Metabolites	Result
Flavonoid	(+)
Alkaloid (Wagner)	(-)
Alkaloid (Dragendorff)	(-)
Alkaloid (Mayer)	(-)
Saponin	(+)
Steroid	(-)
Phenol	(+)
Tannin	(+)
Triterpenoid	(-)

From the phytochemical tests that have been carried out, it can be seen that the ethanol extract of catappa leaves contains flavonoids, saponins, phenols and tannins. The anticoagulant effect is shown by flavonoid chemical compounds. Epidemiological

studies show that compounds that function as platelet modulation are flavonoids (El Haouari, M., & A Rosado, J. 2011).

Saponins are amphipathic glycoside compounds that show characteristics that can cause foam. Structurally, saponins consist of lipophilic polycyclic aglycones which can be called sapogenins (He Y et al, 2019). Saponins can be useful as antioxidants and prevent cancer and cholesterol.

Saponins can act as antimicrobial agents (antibacterial and antiviral sources), strengthen the immune system, increase vitality, blood sugar levels, reduce blood clotting time, and saponins also affect collagen (the initial phase of tissue repair) by inhibition. Excessive scar tissue formation. Some studies have also reported saponins with pharmacological properties such as antithrombotic, antiplatelet, and anticoagulant (Kim K et al, 2019). The mechanism of saponins in inhibiting the coagulation system can use arachidonic acid as well as GPVI and enzymatic.

Some types of saponins have the ability to inhibit platelet aggregation induced by ADP. Therefore, these compounds are antagonists of ADP-activated transmembrane receptors such as P2Y1 and P2Y12. Both of these receptors interact with G proteins, but their downstream effectors are in different signaling pathways. Activation of the P2Y1 ADP receptor, which belongs to the Gq protein-coupled receptor, leads to activation of phospholipase C (PLC), whereas activation of the P2Y12 receptor coupled to Gi proteins triggers adenylate cyclase activity. It has been suggested that activation of both receptors is required for a complete platelet response to ADP (Storey, R.F., 2006).

In addition to saponins, there are also phenolic compounds which are compounds with one or more hydroxyl groups directly attached to the aromatic ring. Phenol (C6H5OH) or carbolic acid is the basic structure of all classes of compounds where the aromatic ring is called benzene. Phenolic compounds are secondary metabolites produced by plants that are involved in certain physiological functions such as growth, development and normal defense mechanisms.

Tannins are compounds that belong to the polyphenol class whose forms can be hydrolyzed and condensed (Kiss A. K., Piwowarski J. P., 2019). The anticoagulant activity of tannin compounds was tested by Xie et al. in 2017. The study used rats as the object of research. Tannins with bPGG type are proven to be able to extend APTT, PT, and TT values and reduce fibrinogen concentration. These results were obtained from plasma obtained from rats that had been injected with adrenaline to obtain blood with high coagulation status.

A study reported that hamamelitannin (ellagitannin) can inhibit factor VII. Where the active form of plasma hyaluronan-binding protein (PHBP) can convert factor VII to VIIa. PHPB automates itself from the pro-PHBP form, and this process is accelerated by polyamines such as spermidine or heparin. One role of heparin in the auto-activation of pro-PHBP is to determine the scaffold for the auto-activation reaction, whereas spermidine is mainly involved in intramolecular interactions in pro-PHBP. It has been shown that hamamelitannin (IC 500.19 μM) inhibits spermidine-enhanced autoactivation of human PHBP but does not affect heparin-enhanced autoactivation. This suggests a selective action of hamamelitannin and its ability to indirectly modify factor VII activity (Marcińczyk N et al, 2021).

Flavonoids belong to the group of polyphenolic compounds commonly found in plants. Flavonoids appear in plants as yellow and white colored compound pigments. Flavonoids are known for their benefits that can be used as anti-inflammatory, anti-allergic, antioxidant, and antithrombotic. Flavonoid compounds can dissolve in water and alcohol, but cannot be dissolved in organic solvents. Flavonoids function as anti-oxidants to eliminate free radicals and help growth and development, in some plants these compounds have a role in immune defense because they can function as antimicrobials (Panche A.N et al, 2016).

Flavonoids have an effect on platelet aggregation which is one part of hemostasis. Hemostasis is the process of blood clotting carried out by the body in response to bleeding. In a study conducted by Vaiyapuri et al. in 2015 (Vaiyapuri S et al, 2015), with citrus samples, it was found that flavonoid compounds can inhibit platelet aggregation that has been induced by collagen and CRP-XL. The activity of platelet aggregation inhibition in this study is highly dependent on concentration.

Flavonoids, such as EGCG and nobiletin, have been shown to inhibit calcium mobilization after being induced by collagen or CRP-XL in a study by Ok et al. in 2012 (Ok W.J et al, 2012). Calcium mobilization is a process needed in platelets to become active. This is because the entry of calcium ions into the cytoplasm from intracellular stores allows binding with protein kinases. Flavonoids with apigenin, genistein, quercetin, and catechin have also been shown to inhibit calcium mobilization by inducing low doses of thrombin, ADP and collagen in the study of Mosawy et al. conducted in 2013 (Mosawy S et al, 2013).

Lee White Method Anticoagulant Test Results

The anticoagulant test using the Clotting Time (CT) test with the Lee White method was carried out with an observation time of 2 days against variations in extract concentration can be presented in tabular form as follows.

Table 2. Anticoagulant Clotting Time Test Results Lee White Method

Treatments	Time					
	1 hours	2 hours	3 hours	4 hours	24 hours	48 hours
K (-)	(-)	(-)	(-)	(-)	(-)	(-)
Control (+)	(+)	(+)	(+)	(+)	(+)	(+)
1%	(+)	(+)	(+)	(+)	(+)	(+)
2%	(+)	(+)	(+)	(+)	(+)	(+)
3%	(+)	(+)	(+)	(+)	(+)	(+)
4%	(+)	(+)	(+)	(+)	(+)	(+)
5%	(+)	(+)	(+)	(+)	(+)	(+)
6%	(+)	(+)	(+)	(+)	(+)	(+)

Description: (-) coagulation occurs, (+) no coagulation occurs.

Clotting time (CT) is the length of time it takes for blood to clot. In this test, the results are a measure of the activity of blood clotting factors, especially factors that form thromboplastin and factors derived from platelets. The clotting time examination using whole blood is actually a crude examination but is expected to be able to represent the clotting process that occurs in the body in vitro, so among the examinations using whole blood. The normal value of the coagulation time examined by the Lee White method is 4-10 minutes (Nurhayati et al, 2021).

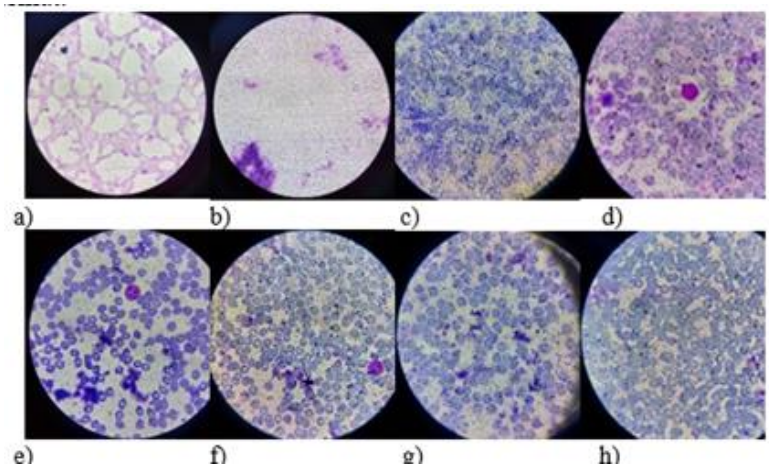
In the tests that have been carried out, the results show that all treatments with concentrations of 1% to 6% of Atepa anticoagulant, show test results that are far from normal numbers. It can be concluded that the active substances in the extract can prevent coagulation of blood. As for the negative control, the blood that was not given anticoagulant coagulated with a time of 4 minutes 30 seconds.

Clotting Time examination or what can be called clotting time is a test that can show the length of time it takes for blood to clot. The results of this examination can later become a measure of the activity of coagulation factors, especially factors that form thromboplastin and factors derived from platelets, and later become a benchmark of fibrinogen levels (Pearce, EC., 2011)

The elongated Clotting Time examination results in the tested samples may occur due to the active compounds contained in Atepa. Saponins have antithrombotic activity that can inhibit platelet aggregation. Antithrombotic activity is also possessed by flavonoids found in catappa leaf extract (Pasupuleti R et al, 2013). In addition to antithrombotic activity, flavonoids can also inhibit calcium mobility needed in the blood clotting process. The decrease in fibrinogen caused by tannins is also one of the factors causing the extension of the Clotting Time value (Xie P et al, 2017).

3.2 Anticoagulant Test Results by Smear

Blood smears were made from samples that had been tested with the Lee White method for 2 hours, the data obtained is presented in the following pictures.



Treatments: a) K(-), b) K(+), c) 1%, d) 2%, e) 3%, f) 4%, g) 5%, h) 6%

Fig. 1. Results of Anticoagulant Test of Blood Smear Method

In the examination that has been carried out, the results show that in the treatment with EDTA anticoagulant (positive control), after being observed, blood cells are still visible, but not in good shape. Blood cells that are not coagulated have characteristics that are round like coins. Non-clotting blood, blood cells do not clump and separate (Tangkery, R.A.B et al, 2013).

Samples with concentrations of 1% to 6% showed blood cells that did not clump and separate. As for the negative control (treatment without anticoagulant), the blood cells already appeared to have a characteristic change, namely the rupture of the red blood cell wall. Lysed red blood cells also began to appear at a concentration of 1%.

In laboratory examinations related to cell counts, the shape, color, and size of erythrocytes, leukocytes, and platelets will be seen. Red blood cells or erythrocytes have double concave (biconcave), disc-shaped cells without nuclei, red in color because they contain hemoglobin. Red blood cells are 7.5 microns in diameter and 2.0 microns thick. Their number in the body is a maximum of about 4.5-5 million/mm^3 and their shape is flexible, allowing them to change shape as they flow through the various types of blood vessels through which they pass (Nugraha, G., 2017). The lifespan of red blood cells is about 100-200 days. During blood circulation, red blood cells can be damaged when they bounce off the walls of blood vessels. Without a nucleus, red blood cells have no way to repair themselves. When the time comes, red blood cells must head to the spleen to be filtered (Jitowiyono, S., 2018).

Meanwhile, leukocytes or white blood cells have different characteristics or cellular properties. Usually, leukocytes are larger than erythrocytes, colorless, and able to move in the presence of pseudopods, and have a lifespan of 13 to 20 days. Leukocytes are the least abundant of the three types of blood cells in the body, around 4000-11000/mm^3. There are five types of leukocytes, namely neutrophils, eosinophils, basophils, monocytes and lymphocytes (Sahetapi, C.M and Aritonang, C.R.L., 2023).

In addition to erythrocytes and leukocytes, in the blood there are also platelets, also called blood pieces or blood pieces, are fragments or small pieces of megakaryocyte cytoplasm, the number of which in the adult body is 150,000-400,000 pieces/mm^3. Platelets are an important part of the hemostatic response, which is closely related to other hemostatic components. Platelets are very small, measuring about 2-4 microns, round or oval (White, J.G and Michelson A, 2007).

In making peripheral blood preparations, there are several obstacles in conducting research, namely delaying the examination after collection, resulting in damage to blood cells, dirty glass equipment that causes spots on the examination, and too much or too little. slow sliding drops and uneven coloring are not good (Arwie, D et al, 2018). For good results, the preparation should be removed within one hour after preparation. Examination with EDTA blood should be done immediately (Indra, S.A.Q et al, 2022). If there is a delay, then the limitation of storage time for each peripheral blood test to be examined for less than 2 hours must be considered. This has been discussed in the study of Narasimha et al. By delaying the examination for 2 hours with EDTA anticoagulant, it has an impact on cell morphology where the cytoplasmic granules along with

the cell membrane become irregularly shaped. There is also an increase in leukocyte size, crenation of red blood cells and abnormal color. Platelets are swollen and the platelet fraction is increased.

4 Conclusion

From the results of this study, it can be concluded that Atepa anticoagulant based on catappa leaf extract (Terminalia catappa) has anticoagulant activity in vitro against human blood samples, and can maintain the characteristics of blood cells after being observed microscopically.

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