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EVALUATION OF THE EFFECTIVENESS OF POLYCHAETA EXTRACT ANTICOAGULANT ON BLOOD COATING RATE

Evaluasi Efektivitas Antikoagulan Ekstrak Polychaeta Terhadap Laju Pembekuan Darah

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ABSTRACT

Atherothrombotic events such as myocardial infarction and ischemic stroke are the leading causes of morbidity and mortality from cardiovascular disease worldwide. The underlying mechanism of atherothrombotic events consists of plaque disruption and the formation of a blockage (thrombus). Thrombus occurs due to high blood flow velocity and increased activity of clotting factors and fibrin levels. Fibrinolytic enzymes are a group of serine proteases that are capable of destroying blood clots (fibrin) in various thrombotic diseases. It is known that Polychaeta organisms of the phylum Annelida contain substances with fibrinolytic and anticoagulative activities. This study was designed to analyze the effect of different doses of polychaeta extract on the response of blood clotting rate. The experimental design used was a completely randomized design with 3 treatments (P) of polychaeta extract, with treatment P1 = 20 µl, P2 = 40 µl, and P3 = 60 µl and 3 replications (U). The method used was the Lee White Method. Based on the ANOVA analysis, F count (6) > F table (5.14) which means that different doses of polychaeta extract have an effect on the blood clotting rate. Then, based on the DMRT analysis, it was concluded that treatments P3 and P2 have the same effect on the blood clotting rate, with the notation 'a', but treatment P1 has a different effect on the blood growth rate with the notation 'b'. However, the best treatment is P3 with an average blood clotting rate of 76.34 minutes. To determine the optimum dose value of polychaeta extract on the blood clotting rate, it was analyzed using polynomial regression with the equation Y = 56.32 + 14.19X − 2.51X 2 with an optimum concentration of 76.53 µl.

Keywords: Dosage, Extract, Freezing, Polychaeta

ABSTRAK

Kejadian aterotrombotik seperti infark miokard dan stroke iskemik merupakan penyebab utama morbiditas dan mortalitas akibat penyakit kardiovaskular di seluruh dunia. Mekanisme yang mendasari kejadian aterotrombotik terdiri dari gangguan plak dan pembentukan sumbatan

(trombus). Trombus terjadi akibat dari tingginya kecepatan aliran darah dan peningkatan aktivitas faktor pembekuan serta kadar fibrin. Enzim fibrinolitik merupakan kelompok protease serin yang mampu menghancurkan bekuan darah (fibrin) dalam berbagai penyakit thrombosis. Diketahui bahwa organisme Polychaeta filum Annelida mengandung zat dengan aktivitas fibrinolitik dan antikoagulatif. Penelitian ini dirancang untuk menganalisa pengaruh perbedaan dosis esktrak polychaeta terhadap respon laju pembekuan darah. Rancangan percobaan yang digunakan adalah rancangan acak lengkap dengan 3 perlakuan (P) ekstrak polychaeta, dengan perlakuan P1=20 µl, P2=40 µl, dan P3=60 µl serta 3 ulangan (U). Metode yang digunakan yaitu Metode Lee White. Berdasarkan analisis ANOVA menghasilkan F_{hitung} (6) > F_{tabel} (5,14) yang artinya bahwa dengan perlakuan dosis ekstrak polychaeta yang berbeda berpengaruh terhadap laju pembekuan darah. Kemudian berdasarkan analisis DMRT memberikan kesimpulan bahwa perlakuan P3 dan P2 memiliki pengaruh yang sama terhadap laju pembekuan darah, dengan notasi 'a', akan tetapi perlakuan P1 memiliki pengaruh yang berbeda terhadap laju pertumbuhan darah dengan notasi 'b'. Akan tetapi perlakuan yang terbaik adalah P3 dengan laju pembekuan darah rata-rata sebesar 76,34 menit. Untuk mengetahui nilai dosis optimum ekstrak polychaeta terhadap laju pembekuan darah, maka dianalisis dengan regresi polinomial dengan persamaan $Y=56,32+14,19X-2,51X^2$ dengan konsentrasi optimum sebesar 76,53 µl.

Kata kunci: Dosis, Ekstrak, Pembekuan, Polychaeta

INTRODUCTION

Atherothrombotic events, such as myocardial infarction and ischemic stroke, are the leading causes of morbidity and mortality from cardiovascular disease worldwide. The underlying mechanism of atherothrombotic events consists of plaque disruption and the formation of a blockage (thrombus). Thrombus formation results from high blood flow velocity and increased clotting factor activity and fibrin levels (Rohmah, 2020). The blood clotting process, or hemostasis, can stop and prevent bleeding. When a blood vessel is injured, vasoconstriction occurs, which can reduce blood flow. Furthermore, increased clotting factor activity and fibrin levels are caused by increased tissue factor activity, which enhances the coagulation cascade. Furthermore, fibrinolytic disorders can also be caused by impaired lysis of the thrombus (Asada et al., 2020).

Fibrinolytic enzymes are a group of serine proteases capable of destroying blood clots (fibrin) in various thrombotic diseases. Fibrinolytic enzymes have also been successfully obtained from various sources, including animals (lumbrokinase), fermented foods (natokinase and katsuwokinase), and microorganisms (streptokinase) (Sajuthi et al., 2011).

Controlling the coagulation process can reduce the potential for high thrombosis in atherothrombotic cases. Anticoagulant drugs, such as anticoagulants, antiplatelets, and thrombolytics, reduce blood clotting activity, prevent thrombus formation, and disrupt thrombus formation to prevent atherothrombosis. Some anticoagulant drugs have side effects. Aspirin and clopidogrel (antiplatelets) have side effects such as headache, abdominal cramps, vomiting, and gastric ulceration (Altman et al., 2012). Heparin (an anticoagulant) has side effects such as bleeding, osteoporosis, and thrombocytopenia (Oduah et al., 2016). Nattokinase (a thrombolytic) has side effects such as bleeding and triggers allergic reactions (Weng et al., 2017). Based on this background, research on anticoagulants, including natural products, is necessary. Efforts to find new sources of thrombolytic agents derived from marine biota are still ongoing. One potential source of thrombolytic agents is Polychaetes.

Polychaetes are a group of marine invertebrate species (Syomin and Zimina, 2020). One resource found in Indonesian waters contains chemical compounds that can be used as marine

bioproducts, including for marine pharmaceutical purposes. The pharmaceutical potential of this species can be used as a biota for testing thrombolytic activity in human blood.

Polychaetes, belonging to the Annelida phylum, are known to contain substances with fibrinolytic and anticoagulative activity. In recent years, fibrinolytic serine proteases have been explored in several marine polychaete worms, such as Arenicola cristata, Diopatra sugokai, Cirriformia tentaculata, Nereis virens, Neanthes japonica, and Marphysa sanguinea, which have been identified and purified from the coelomic fluid of Polychaetes. (Barzkar et al., 2020). Fibrinolytic activity has different names for each species, in the form of lyophilized powder from earthworms named lumbrokinase and N.V protease from Nereis virens, as well as fibrinolytic from N. japonica (Izuka), named NJF (EC 3.4.21.-) (Zhang et al., 2007). To date, research that has been conducted is only at the activity test stage, therefore, further research is needed regarding the effectiveness of polychaete extracts on rat blood.

RESEARCH METHODS

The method used in this study was a descriptive experimental method. This study tested the effect of different doses of polychaete extract on the blood clotting rate of mice. The method used was the Lee White method. The parameter observed was blood clotting time.

Extraction

Polychaete extraction was performed using the maceration method using ethanol as a solvent (Ebada et al. 2008). The cleaned samples were oven-dried at 500°C for 12 hours, then finely ground using a mortar for use in this study.

The maceration was carried out for 4 days. According to Ega et al. (2018), too short a maceration time can result in not all compounds being dissolved in the solvent used. Fauzana (2010) reported that curcuma rhizome extract yields with maceration times of less than 18 hours yielded low yields, below 12.60%. All samples treated with the ethanol solution were covered with aluminum foil. They were then stirred at 25 rpm for 24 hours using an orbital shaker. The extract solution was then filtered through filter paper to separate the filtrate from the residue, and the filtrate was collected. The filtrate was then evaporated using a rotary evaporator at a temperature of 50 oC for approximately 5 hours to produce a crude extract.

Research Stages

1) Preparation of blood test samples
   The research blood requirement was 9 ml taken from 3 male mice.
2) Blood Clotting Period Testing
   To determine the blood clotting time observed visually, the modified Lee-White method is used (Gandasoebrata, 1992). The working procedure of the modified Lee-White method is as follows:
   a) Clean test tubes labeled from number 1 to number 3. Observation of 3 test tubes with the addition of polychaeta extract with treatment doses P1 = 20 µl, P2 = 40 µl, and P3 = 60 µl.
   b) Treatment test tube P1 contains 1 ml of blood + 20 µl dose, treatment test tube P2 contains 1 ml of blood + 40 µl dose, and treatment test tube P3 contains 1 ml of blood + 60 µl dose.
   c) After 3 minutes using the stopwatch, the tube is lifted and tilted to see if blood clotting occurs.

Research Scheme

The research design used in this study was a non-factorial Completely Randomized Design (CRD) consisting of three treatments (P1, P2, and P3), each with three replications (U1, U2, and U3). The treatments used in this study were different doses of polychaete extract. The doses of handeuleum leaf extract used in this study were as follows:

P1 : 1 ml of mouse blood + 20 µl dose of polychaeta extract
P2 : 1 ml of mouse blood + 40 µl dose of polychaeta extract
P3 : 1 ml of mouse blood + 60 µl dose of polychaeta extract

The total experimental units consisted of 3 treatments and 3 replications, resulting in 9 experimental units. The treatment and replication containers were randomly assigned using Microsoft Excel. The randomization of the experimental units is shown in Figure 1.

P1U2	P1U3	P2U1
P3U3	P2U3	P1U1
P3U2	P3U1	P2U2

Figure 1. Randomization of Experimental Units

Data Analysis

To determine the effect of different polychaete extract doses on blood clotting rate, analysis was performed using Analysis of Variance (ANOVA) at a 95% confidence interval and a significance level of 0.05. If the calculated F value is greater than the F table, further testing was performed using the Duncan Multiple Rate Test (DMRT). The DMRT test aims to determine whether each different treatment has the same effect. This test is used because the Duncan test can be considered precise or has a high level of accuracy. The research data, according to RAL, are organized into a data analysis table, presented in Table 1.

Table 1. Analysis of Variance

Sources of Diversity	Degrees of Freedom (db)	Sum of Squares	Middle Square (KT)	F-Count	F table5% 1%
Treatment	t-1 = V_1	JKP	KTP = JKP/dbp	F = KTP/KTG	F (V_1,V_2)
Error	t (r-1) = V_2	JKG	KTG =JKG/dbg		
Total	rt-1	JKT			

Description:

Correction Factor (FK) $= \frac{\sum Y^2}{r \times t}$

Total Sum of Squares (JKT) $= \sum(Yij^2) - FK$

Sum of Squares of Treatment (JKP) $= \frac{\sum(Yi^2)}{r} - FK$

Sum of Squared Errors (JKG) = JKT - JKP

Degrees of freedom from treatment (dbp) = t - 1

Degrees of error freedom (dbg) = t (r - 1)

Total degrees of freedom (dbt) = tr – 1

If the calculated F value < Ftable (95% confidence interval; α = 0.05) or if the probability of error is less than 5%, then H0 is accepted while H1 is rejected and it can be stated that the treatment given has no effect on the observed response. However, if the calculated F value > Ftable (95% confidence interval; α = 0.05) or if the probability of error is less than 5%, then, H1 is accepted which means the treatment affects the response and further testing will be carried out. If the results of the ANOVA analysis produce calculated F> Ftable, then a further test analysis is carried out with the Dundan Multiple Rate Test (DMRT) which aims to whether

each different treatment has the same effect or not. The calculation formula for the Duncan Least Significant Distance Test (DMRT) is as follows:

$$DMRT = R_{(p;db;\alpha)}\sqrt{\frac{KT\ galat}{r}}$$

Description:

R	= Distance value
α	= Real level
db	= Degrees of error freedom
P	= Number of treatments
KT galat	= Mean square error
r	= Replication

Polynomial Regression

Polynomial regression is a multiple linear regression model formed by summing the influence of each predictor variable (X) raised to an increasing power up to the nth order. Polynomial regression is also known as regression with an independent variable as a factor with ordered powers. Polynomial regression is used to estimate the form of the relationship between the independent variable and the dependent variable and to determine the polynomial function that best fits a set of known data points, or is generally used to determine the optimum value of an existing treatment. In general, the polynomial regression model is written in the following equation:

$$Y = a + b_1X_1 + b_2X_2^2 + b_3X_3^3 + \ldots + b_nX_n^n + \varepsilon$$

Description:

Y	: Dependent variable
a	: Intercept
$b_1, b_2, \ldots, b_n$	: Regression coefficients
X	: Independent variable
ε	: Confounding factors that cannot be explained by the regression model

To determine the optimum X value, the following formula is used: $X_{opt} = -\frac{b}{2c}$

To determine the optimum Y value, the following formula is used:

$$Y_{opt} = a + bX_{opt} + cX_{opt}^2$$

RESULT AND DISCUSSION

Effectiveness of Polychaeta Extract Dose on Blood Clotting

The maceration soak for polychaete extracts in this study used 96% technical ethanol as a solvent. Sharon et al. (2013) stated that ethanol is a suitable solvent to replace methanol as an extractant. This is because ethanol is safer for pharmacology. The resulting ethanol extract, with a water content of <10%, was then formulated into a powder.

Based on the RAL analysis of different doses of polychaete extract on the blood clotting rate tested on rats, the blood clotting rate responses varied (in minutes), with blood clotting rates at P1, P2, and P3 being 68, 74.67, and 76.34 minutes, respectively. Based on the duration of blood clotting in rats, the P3 treatment, with a dose of 1 ml of rat blood + 60 µl of polychaete extract, had the longest blood clotting rate. Based on the average blood clotting rate results, it can be concluded that the higher the dose of polychaete extract, the greater the tendency for blood clotting to increase. However, this trend cannot be used to conclude that higher doses of polychatea extract also increase blood clotting rates. This requires further testing with a polynomial regression test to determine the pattern of blood clotting rates at optimal doses. The RAL results are shown in Table 2.

Table 2. RAL Results of the Blood Clotting Rate Test in Mice with Polychatea Extract Doses

Replication (U)	Treatment (P)		
	P1	P2	P3
1	69	76	77
2	68	75	77
3	67	73	75
Total	204	224	229
Average	68	74,66667	76,33333

Based on the ANOVA analysis of variance table used to test the hypothesis of whether the polychaete extract dosage treatment has an effect on blood clotting rate or not, the ANOVA analysis produced an F-value of 6 and an F-table of 5.14. Thus, it can be concluded that F-value (6) > F-table (5.14), which means that administering different doses of polychaete extract has an effect on blood clotting rate. The results of the ANOVA analysis are shown in Table 3.

Table 3. ANOVA Analysis of Variance Table

Source of Variation	df	SS	MS	F	P-value	F crit
Treatment	2	116,6667	58,33333	6	0,807518705	5,143253
Error	6	9,333333	9,722222			
Total	8	126				

The potential of fibrinolytic enzymes has been shown to increase endogenous plasminogen activator in animals and humans as an anticoagulant. Previous research has shown that fibrinolytic enzymes are found in polychaetes, such as in Zhang et al. (2007) who identified N.V. Protease from Nereis virens and N. japonica as NJF (EC 3.4.21.-). This study demonstrated fibrinolytic enzyme activity without testing the response of this activity in human blood.

The fastest blood clotting occurred with the P1 dose (20 µl) treatment (68 minutes), and the longest with the P3 dose (60 µl) treatment (76.33 minutes). Treatment 3, with a dose of 60 µl, had the longest blood clotting rate due to its greater fibrinolytic activity compared to the other treatments. The fibrinolytic enzymes in Polychaetes belong to the protease group of enzymes capable of degrading fibrin or fibrinogen (Ahmad et al., 2016). Substances with fibrinolytic and anticoagulation activity are found in the coelomic fluid of the polychaete Nereis (Neanthes) virens (Sars) (Tiemski, 2007). Proteases extracted from two polychaetes have demonstrated activity consistent with their function. These polychaetes produce their own specific protease, NV protease, with strong fibrinolytic properties (Zhang et al., 2007). Examples of the results of trials of different doses of polychaete extract on blood clotting rates are shown in Figure 2.

Figure 2. Results of trials of different doses of polycaeta extract on blood clotting rate, a) Control, b) P1, c) P2, d) P3

Fibrinolysis works by activating plasminogen into the proteolytic enzyme plasmin. Plasmin changes the shape of the thrombus and limits the development of thrombosis by proteolytically digesting fibrin (Aulia, 2018). The fibrinolytic enzyme then works by hydrolyzing fibrin, which causes the blood clot to form a soluble product that can be removed from the circulation, thus freeing the blood vessel from the clot and initiating the healing process of the blood vessel wall (Ajeng, 2015).

Results of the Effect of Inter-Treatment

The DMRT analysis aims to determine whether the applied treatments have the same or different effects. For simplicity, the conclusions of the DMRT test will produce specific notations (e.g., a, b, c, and so on). If the treatments have the same effect, the same notation will be given; however, if the treatments have different effects, different notations will be given. The results of the DMRT analysis are shown in Table 4.

Table 4. DMRT Analysis Results

Treatment	Average	Difference	DMRT	Notation
P3	76,34	1,67	6,455538	a
P2	74,67	6,67	6,228712	a
P1	68			b

Based on the DMRT analysis results, it can be concluded that treatments P3 and P2 have the same effect on blood clotting rate. However, treatment P1 has a different effect compared to treatments P3 and P2. However, considering the highest average (longest duration of blood clotting), treatment P3 can be considered the best dose for inhibiting blood clotting.

The best dose was treatment P3, with a formula of 1 ml of mouse blood + 60 µl of polychaete extract. However, although P3 with a dose of 60 µl of polychaete extract is considered the best dose, it cannot be considered the optimum dose. Therefore, to determine the optimal dose of polychaete extract for inhibiting blood clotting, a polynomial regression test was performed. The results of the polynomial regression analysis yielded the equation Y = 56.32 + 14.19X − 2.51X 2. Based on the model, the quadratic model with a downward curved direction (coefficient c=-2.51<0), then the optimum X value is 2.83, resulting in an optimum Y or optimum concentration of polychatea extract of 76.53 µl.

CONCLUSION AND SUGGESTION

Based on the ANOVA analysis, F count (6) > F table (5.14) which means that different doses of polychaete extract have an effect on the blood clotting rate. Then, based on the DMRT

analysis, it was concluded that treatments P3 and P2 had the same effect on the blood clotting rate, with the notation 'a', but treatment P1 had a different effect on the blood growth rate with the notation 'b'. However, the best treatment was P3 with an average blood clotting rate of 76.34 minutes. To determine the optimum dose of polychaete extract on the blood clotting rate, it was analyzed using polynomial regression with the equation Y = 56.32 + 14.19X − 2.51X 2 with an optimum concentration of 76.53 µl.

Based on the results of the discussion and conclusions, the researcher's suggestion is to conduct a species-specific determination test with different types of polychaetes.

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