Gaultherin Production From Gandapura (Gaultheria Fragantissima) By Enzymatic Inactivation Of Gaultherase

by Ari Yuniastuti

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Ari Yuniastuti, Mohammad Endy Yulianto, Indah Hartati

Abstract: Gaultherin is the active form of salicylate from plants Gandapura. Gaultherin has some characterictics which make it potential to become a natural aspirin, anti-cancer, antiinflamatory dan cardiopulmonary. Currently, aspirin (acetylsalicylic acid) is a medicine which is used by most of the people in this world because of its function as antipiretic, antiinflamatory, and analgesic. Approximately the need of pharmacy industry towards gaultherin will be increased in the following year. However, at the time being, there is still no any effective methods to produce gaultherin from gandapura. This difficulty in the process of taking gaultherin is based on the process of its extraction, where the tissue is broken, so, gaultherin will be hydrolyzed change to be its individual components, like methyl salicylate and disaccharides. The hydrolysis process is believed to be catalyzed by the enzyme gaultherase inside. This research is aimed to analyze the production of gaultherin form gandapura using the gaultherase enzyme inactivation process through extraction with alcoholic solvent and determine the correct condition to get the highest production of gaultherin. The result of the calculation shows that the bioextraction process variables of gaultherase enzyme inactivation which is mostly influential are pH and alcohol concentration. The more pH extraction, will increase the outcome of gaultherin active compounds The optimum condition of bioextraction enzyme inactivation is in pH 8 with 14,46% gaultherin active compounds and regression equation in $y = -4{,}8074x^2 + 78{,}301x - 305{,}28$. The bigger solvent concentration, the more gaultherin be extracted. The production of gaultherin will optimally reached in the 90% concentration of ethanol with the result of 13,10% active compounds

Keywords: Gaultherin, Gaultherase, Bioextraction

1 INTRODUCTION

Gandapura (Gaultheria fragantissima), which is known as Indian Wintergreen, also known as Gaultheria. punctuate, which is included in Ericeae family as one of essential oil producer. Gandapura is one of a plant which is included into commodity target list of Direktorat Jenderal Perkebunan based on Decree of Minister of Agriculture number 511/kpts/pd.310/9/2006. Gandapura can grow in highland specifically 1300 – 3300 meters above the sea [1]. Up to now, Gandapura has never being economically cultivated because there is no any technology to do that. Gandapura is harvested from plants which grow wildly on highlands like in Lawu Mountain, Tawangmangu, and Dieng Plateau, Wonosobo. One of gandapura oil producing industry which is located in Wonosobo is Tani Rukun Group which works in Sikunang village, Kejajar disticts, Wonosobo regency. Gandapura oil contains high methyl salycilate for about 93 – 98 %. Gandapura oil which is produced by farming group in Indonesia contains 82,23% of methyl salycilate [2].

- *Ari Yuniastuti, Department of Biology, Faculty of Mathematics and Natural Science, Universitas Negeri Semarang, Indonesia. 1st floor of D6 Building, Sekaran-Gunungpati, Semarang. 50229. Email: ari_yuniastuti@yahoo.co.id*
- *Mohammad Endy Yulianto, Faculty of Chemical Engineering, Diponegoro University, Indonesia.*
- *Indah Hartati, Faculty of Chemical Engineering, Wahid Hasyim University, Indonesia.*

Every months Indonesia still imports synthetic gandapura oil from China to fulfill the need of pharmacy industry [3]. In other words, the attempts to reveal the potency and development of gandapura industry in Indonesia needs to analyze the cultivation technology, the technology to improve the quality of gandapura oil, and the products diversification. Gandapura is a plant which contains very high concentrations of salicylate. The concentrations of salicylate of gandapura is 20 times higher than concentration of salicylate found in Filipendula and 100 times higher compared to concentrations of salicylate in Lemon Thyme. Most of salicylate in gandapura is actively formed as gaultherin, methyl salicylate conjugation by disaccharides. When the tissue of the plant is broken or ripped, gaultherin will be enzymatically hydrolyzed to be released as methyl salicylate. This process is known as the defense system of gandapura. Gaultherin has some characterictics which make it potential to become a natural aspirin, anti-cancer, antiinflamatory dan cardiopulmonary [4]. As a natural aspirin, gaultherin has a high healing power, yet, it has minimum negative effect than synthetic aspirin. Currently, aspirin (acetylsalicylic acid) is a medicine which is used by most of the people in this world because of its function as antipiretic, antiinflamatory, and analgesic. As what has been estimated, worldwide aspirin consumption is about 20 – 50 million every year [5]. Approximately the need of pharmacy industry towards gaultherin will be increased in the following year. However, at the time being, there is still no any effective methods to produce gaultherin from gandapura. This difficulty in the process of taking gaultherin is based on the process of its extraction, where the tissue is broken, so, gaultherin will be hydrolyzed change to be its individual components, like methyl salicylate and disaccharides. The hydrolysis process is believed to be catalyzed by the enzyme gaultherase inside. In order to handle this problem, it is important to find a method to extract gaultherin from the plants in the condition where gaultherase is minimum or disappear. In

the end, gaultherin's hydrolysis reaction which changes it to become methyl salicylate and disaccharides will not be happened. Taking gaultherin can be done under several methods. For instance; in 1928, gaultherin in Gaultheria procumbens could only be extracted with hot water and an addition of calcium carbonate. This process is followed by series of extractions using solvent. These series of extractions is using solvent, like acetic ester in the temperature of 100^{0}C. This process resulted 4g/kg gaultherin from fresh leaf. This small final result is happened because of gaultherin in Gaultheria procumbens has been hydrolyzed by gaultherase. Poulev et al. [6] stated that the activity of gaultherase can be obstructed with the addition of polar compounds. It is believed that alcohol can obstruct the activity of gaultherase. Some types of chemical compounds can result the same effect like alcohol, such as, methyl chloride, acetonitrile, or hot water. The alternative which can be offered in the process of production of gaultherin from gandapura is the production of gaultherin using gaultherase enzyme inactivation technology using alcoholic solvent (alcoholic solvent extraction). Polar solvent can create dual functions, inactivate the enzyme and extract the gaultherin. Polar solvent which is used is ethanol. The enzyme inactivation process using alcoholic solvent has an excellence in simplified three processes, inactivate the gaultherase enzyme, process the extraction, and process the osmosis dehydration [7][8]. This process can significantly increase the final result. The initial study of gaultherin production has ever been done for the process of extraction [8]. The result of the study showed, gaultherase enzyme inactivation using ethanol as a solvent is potential and prospective. This thing happens because gaultherin is not converted to be methyl salicylate and the usage of ethanol as the polar solvent is ingestible for nutraceutical products, so, gaultherin resulted can be used in the form of pills, tablets, and capsules. This research is aimed to analyze the production of gaultherin form gandapura using the gaultherase enzyme inactivation process through extraction with alcoholic solvent and determine the correct condition to get the highest production of gaultherin. The hypothesis of this study is by applying the enzyme inactivation technology through alcoholic solvent, gaultherase enzyme's activity can be obstructed or even deactivate it, so, the result earned will be very high.Gaultherin production using this technology can be an option for diversification product of gandapura. Hopefully, gaultherin production can improve the economic value of gandapura industry and positively impactful for the gandapura farmer groups.

2 MATERIAL AND METHODS

The study of gaultherin's productivity will be done in a month in Biochemical Laboratory of Semarang State University, Chemical Engineering Operating Laboratory in Diponegoro University Semarang, BPTP Laboratory Ungaran, and in the location of Rukun Tani group in Sikunang village, Wonosobo. The attempts to increase the productivity is adding drying agent (guillotine, sodium sulphate, dan calcium chloride) in the extraction of gaultherin. Generally, the level of productivity of gaultherin will be better with the addition of drying agent, like guillotine, sodium sulphate, and calcium chloride. The usage of drying agent will be advantageous to bond, decrease of even release the water in the solvents or the mixture of water, so, the hydrolysis reaction by gaultherase enzyme will not be happened. It is hoped that the addition of drying agent, will significantly improve the gaultherin production compare to not adding drying agent.

2.1 Procedure

The material used in this study is leaves and flowers of gandapura which are collected by Rukun Tani group in Sikunang village, Wonosobo. These materials is freezed using liquid nitrogen to obstruct the activity of gaultherase enzyme. The other materials used is chemical materials as the solvents, like buffer pH, drying agent, and material for analyzing the level of gaultherin, salicylate acid, and methyl salicylate. Gaultherin levels can be determined using LC-MS analysis methods and salicylate acid and methyl salicylate levels can be determined using Stable Isotope Dilution Analysis [9]. Identification and analysis of gaultherin can be done using spectrophotometer or using GC. This study used some standards, like sovent-feed ratio = 10:1; ethanol concentration = 90%; solvent pH = 4,8; mixer rotation speed = 75 rpm; chopper blade rotation speed = 125 rpm; extraction time = 60 minutes; and the product drying temperature = 80^{o}C. Somehow the free variable used is drying agent types of guillotine, calcium chloride, and sodium sulphate and drying agent concentration order of 3, 4, 5, 6 % (b/b). In this research, the leaves and the flower of gandapura is freezed by ice or liquid nitrogen. The extraction is executed in enzyme inactivation extractor with ethanol as the solvent. The extractor is equipped with chopper blade under it. The ratio of solvent-feed used in the experiment is 10:1. The ethanol added is ethanol with 90% concentration. Buffer pH is exploited for keeping the solvent in 4,8 pH. The rotation speed of the mixer and the chopper blade is 75 dan 125 rpm. Drying agent is added as the experiment variable. The extraction happens in 60 minutes. The solids is separated from the extract using filter or centrifuge. The extract which has been separated from the solids is added with chemical material or heated in order to release the solvent. To get a gaultherin solvent, the heated extract can be resuspended using buffer or water. The result of the extraction is analyzed to know the level of methyl salicylate and salicylate acid using stable isotope dilution method and the gaultherin level is revealed using GC. The composition of gaultherin is measured using chromatographic gas way. The conversion factor counted based on gaultherin formed from gandapura the leaves and flowers used. The salycilate acid is measured by stable isotope dilution method and gaultherin levels is measured using GC, the level of the water is measured using manual water levels determination, the level of methyl salicylate is measured using stable isotope dilution method, specific weight of the gaultherin is measured using picnometer, and bias index is measured using refractometer.

2.2 Data Analysis

The determination of dependent variable is executed in three months in Biochemical Laboratory of Semarang State University, Chemical Engineering Operating Laboratory of Diponegoro University Semarang, BPTP Laboratory Ungaran, and in the location of Rukun Tani group in Sikunang Village, Wonosobo. The planning of the experiment used is using 2^n factorial design. The

parameters researched here is the degree of acidity, inactivation time, the influence of gandapura solvent ratio, ethanol concentration, concentration of drying agent, and the rotation speed of mixer and chopper. The data analysis to determine the dependent variable is using normal probability plot, after the measurement of the main effect and the interactions or using statistics program, Matlab®. The modelling activity is initiated with arranging the mathematic equation regressively based on the data of optimation parameter process study. This modelling is executed in Computational Process Laboratory of Chemical Engineering of Engineering Faculty of Diponegoro University in a month. Regressive Model represents the process variable of enzymatic bioextraction inactivation in optimum condition and used for predicting condition and evaluate the work of bioextractor tools.

3 RESULT AND DISCUSSION

Gandapura extraction using ethanol as the polar compound has dual functions, which are inactivating the gaultherase enzyme and extract the active compound of gaultherin. Ethanol diffusion inside gandapura (Figure 1) is purposed make gaultherase enzyme in cytoplasm penetrated with the solvent, so, it can obstruct the activity of the enzyme. This statement is also explained by Poulev et al. (6) that the activity of gaultherase can be obstructed by polar compounds. The next mechanism is the ethanol solvent can infiltrate through the tonoplasm membrane wall and create a phase contact with the active compounds of gaultherin. That polar solvent will be diffused bringing gaultherin outside of the leaves cell, which is caused by the different solvency. Enzyme is a giant molecule with molecule weight varied between 5000 Da-5 million Da. Enzyme is included into macromolecule which is bigger than protein and consists of linear chain of specific amino acids. In its optimum condition, enzyme will encounter folding process (Figure 2). The process of forming folding arrangement in enzyme is a spontaneous process in seconds [10]. Therefore, if gaultherase enzyme is folding, and the tonoplas membrane is broken, it makes the enzyme catalyzes the hydrolysis reaction of gaultherin compounds to be methyl salicylate. This thing caused the gain of active compounds relatively low. The series of amino acid in the enzyme can form three certain dimensions which is specifically works through each enzyme (tertiary structure). The part of the tertiary structure if the enzyme which is responsible towards the catalytic activity of the enzyme is called active side. The total of active side of an enzyme can be around 10-20% of the total of enzyme volume [10]. The active side of the enzyme is usually a hydrophilic slit consists of amino acid chain which will bonds the substrates (Figure 3.a) or bonds a cofactor (Figure 3.b) and catalyze the reaction. The folding process in the enzyme is the process which involves hydrophobic amino acid chain to the central side of the enzyme and the releasing process of hydrophilic amino acid chain outside of three dimension of the enzyme. The study of gaultherin active compounds productivity is adding drying agent, like, guillotine, calcium chloride, and sodium sulphate. Figure 4 and 5 provides the graphic of the relation of adding drying agent towards every concentration of ethanol or osmosis dehydration concentration to the level of gaultherin. The increasing concentration of drying agent or ethanol causes the production of gaultherin higher, especially the addition of calcium chloride. Drying agent is functioned as the osmosis dryer, which is the process of taking water from a material which is executed with placing a material inside a high concentrated solvent where between both materials there is semipermeable membrane. The water inside of the liquid solvent will be diffused through the membrane to the solvent which has higher concentration continuously until the condition is balanced. As the character of semipermeable membrane can only be passed by water and small molecule weight compound, so, solute cannot diffuse the membrane conversely. Even if there is a diffused solute, the mass transfer will happen very slowly, so, the main mas transfer happens in this process is the mass transfer of water to the solvent which has higher concentration, As a result, the possibility of hydrolysis reaction which changes gaultherin to become methyl salicylate is low. The water mass transfer through semipermeable membrane can happen because there is a different chemical potential between two solvents, in this case, the chemical potential of water in liquid solvent is higher than in high concentration. This phenomenon is known as osmosis event. The chemical potencial of concentration function, temperature, and pressure. Chemical potential can only be influenced by concentration and pressure in isothermal condition. The increasing solute concentration will reduce the potential of solvent chemical potential. Table 1 and 2 provide gaultherin active compounds in every variable of extraction process. This study of experiment planning of is used to decide the most influential variable. The result of the measurement is known that from the value of the main effect and interaction, the most influential variable are pH and alcohol concentration (the biggest BD positive effect). Figure 5 shows that the higher the extraction of pH, will increase the active compounds of gaultherin. Somehow, the increasing pH makes the gaultherin production decreased. The optimum condition of bioextraction of enzyme inactivation is in pH 8 with 14,46% level of active compound of gaultherin and regression equation of:

$$y = -4{,}8074x^2 + 78{,}301x - 305{,}28.$$

This thing can be explained that gaultherase is a hydrolase enzyme, which has an optimum activity in low acid pH solvent. In consequence, the low base bioextraction condition make gaultherase enzyme unfolding, which will reduce the hydrolysis reaction of gaultherin to become methyl salicylate which is catalyzed by gaultherase enzyme. Figure 6 provides the graphic of relation of ethanol concentration towards the level of gaultherin. The more concentration of the solvent, the more extracted gaultherin will be. This thing happens because of enlarging the concentration of the solvent will enlarge the continuous phase, so, the liquid phase fraction volume will be dispersed to be smaller and the diameter of the particle will be smaller. With smaller diameter of the particle, it will widen the contact of the phases which causes the increasing solutes which is dragged into the solvent phase. Nevertheless, the increasing concentration of polar compounds will decrease the production of active compounds of gaultherin. It is possible when the condition of ethanol above 90%, cause some parts of diluent dragged

into the continuous phase because of there is an improvement of solvent. The optimum gaultherin production exists in 90% of ethanol concentration with the result of 13.10% active compounds.

4 CONCLUSION

The result of the calculation shows that the bioextraction process variables of gaultherase enzyme inactivation which is mostly influential are pH and alcohol concentration. The more pH extraction, will increase the outcome of gaultherin active compounds The optimum condition of bioextraction enzyme inactivation is in pH 8 with 14.46% gaultherin active compounds and regression equation in

$$y = -4{,}8074x^2 + 78{,}301x - 305{,}28.$$

The bigger solvent concentration, the more gaultherin be extracted. The production of gaultherin will optimally reached in the 90% concentration of ethanol with the result of 13,10% active compounds.

ACKNOWLEDMENT

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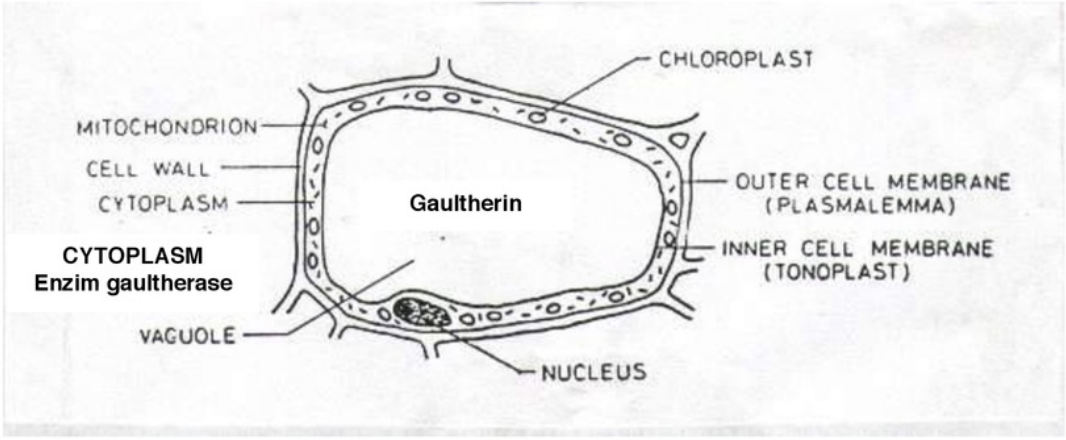

Fig. 1. *Gandapura leaf cell*

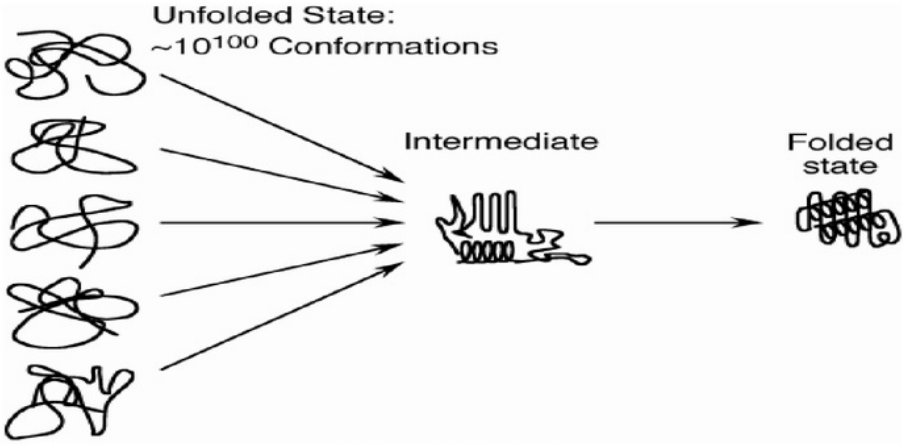

Fig. 2. *Folding process*

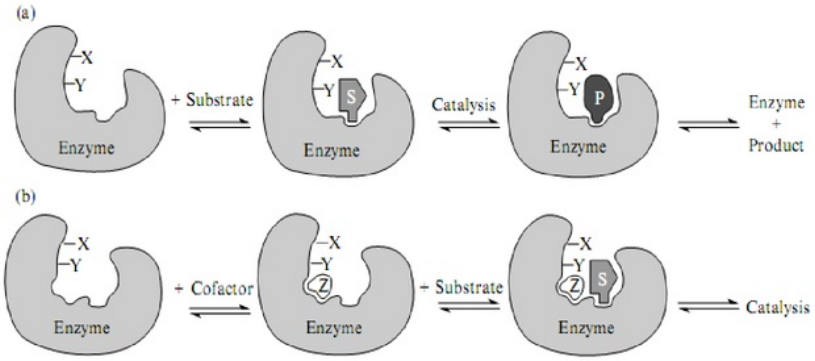

Fig. 3. *Enzyme's active side*

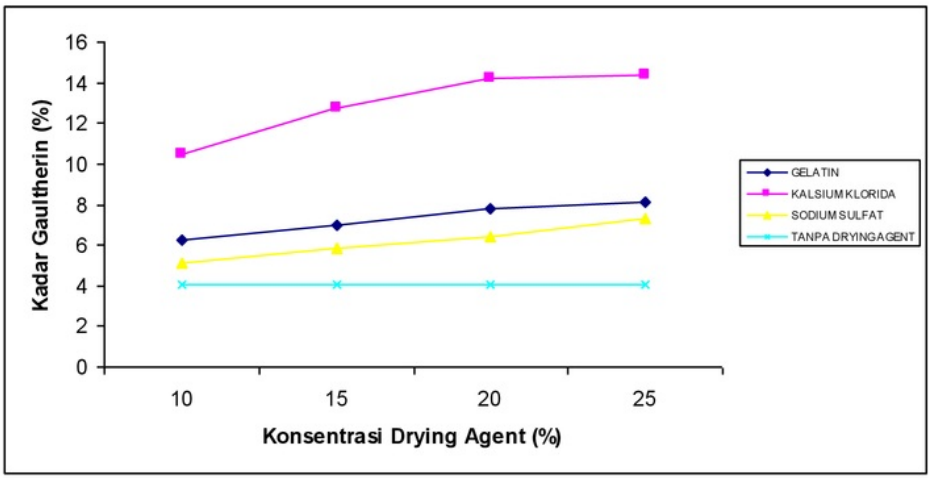

Fig. 4. *The graphic of relation between drying agent concentration to gaultherin level*

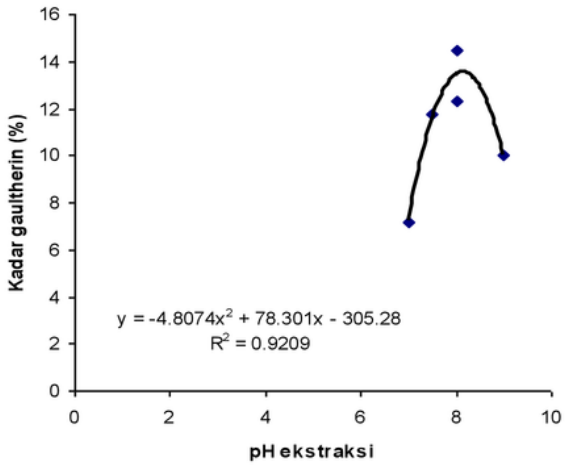

Fig. 5. *The graphic of relation between extraction pH to the level of gaultherin*

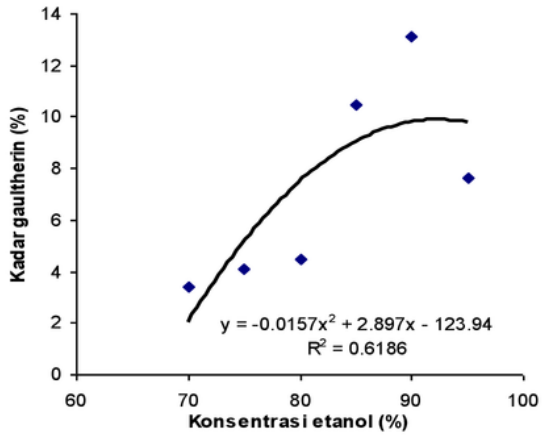

Fig. 6. *The graphic of relation between ethanol concentration to gaultherin level*

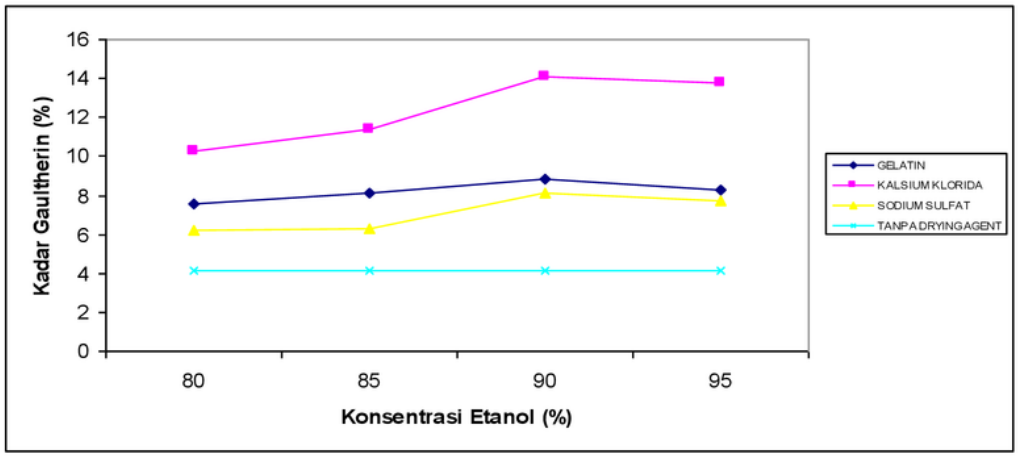

Fig. 7. *The graphic of relation between ethanol concentration to the gaultherin level resulted from many drying agent*

TABLE 1. *The value of the Main Effect and Interactional Effect in deciding the dependent variable*

Effect	Value of Effect	Effect	Value of Effect	Effect	Value of Effect	Effect	Value of Effect
A	0.7775	Ad	0.8175	ae	1.587	ade	0.06
B	-0.275	Bd	2.3087	be	-0.495	bde	1.73
Ab	-0.165	Abd	0.6675	abe	-0.007	abde	-0.427
C	1.3475	Cd	0.4475	ce	-0.19	cde	-0.9125
Ac	-0.925	Acd	0.395	ace	0.985	acde	0.8275
Bc	-1.1125	Bcd	0.205	bce	0.6725	bcde	-0.6475
abc	-0.985	Abcd	-0.6075	abce	0.6825	abcde	-0.08825
D	0.0425	E	1.2487	de	1.1875		

TABLE 2. *The data of the experiment result in deciding dependent variable*

Run in-	Variable					Levels of catechin
	A	B	C	D	E	
1	10%	80%	5:1	7	30	8.40
2	25%	80%	5:1	7	30	7.62
3	10%	95%	5:1	7	30	11.5
4	25%	95%	5:1	7	30	12.10
5	10%	80%	10:1	7	30	10.75
6	25%	80%	10:1	7	30	10.90
7	10%	95%	10:1	7	30	12.46
8	25%	95%	10:1	7	30	6.22
9	10%	80%	5:1	9	30	7.31
10	25%	80%	5:1	9	30	7.27
11	10%	95%	5:1	9	30	5.20
12	25%	95%	5:1	9	30	9.82
13	10%	80%	10:1	9	30	12.09
14	25%	80%	10:1	9	30	10.15
15	10%	95%	10:1	9	30	10.90
16	25%	95%	10:1	9	30	8.05
17	10%	80%	5:1	7	60	7.15
18	25%	80%	5:1	7	60	11.75
19	10%	95%	5:1	7	60	5.8
20	25%	95%	5:1	7	60	6.85
21	10%	80%	10:1	7	60	11.47
22	25%	80%	10:1	7	60	11.02
23	10%	95%	10:1	7	60	7.40
24	25%	95%	10:1	7	60	8.50
25	10%	80%	5:1	9	60	8.3
26	25%	80%	5:1	9	60	8.4
27	10%	95%	5:1	9	60	9.08
28	25%	95%	5:1	9	60	12.9
29	10%	80%	10:1	9	60	6.8
30	25%	80%	10:1	9	60	13.05
31	10%	95%	10:1	9	60	9.4
32	25%	95%	10:1	9	60	12.2

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