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Determination of Total Flavonoid Levels and Cholesterol Reduction Activity Test of Saffron (*Crocus sativus*) Ethanol Extract Fraction : In Vitro Study

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Abstract: *Saffron is one of the herbal plants that has an important role in the medical. Saffron contains flavonoid compounds that have cholesterol reduction activity. This study aims to determine the highest concentration that provides the greatest reduction in cholesterol levels from the saffron ethanol extract fraction in vitro and to determine the differences in total flavonoid levels and cholesterol reduction activity of the saffron ethanol extract fraction in vitro. The extraction method used is remaceration using 50% ethanol as solvent. Fractionation by preparative thin layer chromatography method using mobile phase ethyl acetate : isopropyl alcohol : aquadest (65:25:10). Test of total flavonoid levels using AlCl3 colorimetric method and cholesterol lowering activity test using the Liebermann-Burchard method. The saffron ethanol extract fraction had a total flavonoid content of 3.8518 ± 0.43; 6.0802 ± 0.18; 6.2040 ± 0.17 and 7.2282 ± 0.07 mgEQ/g. Cholesterol reduction results showed a concentration of 700 ppm in fraction 5 of saffron ethanol extract with an average percent reduction in cholesterol of 58.01%. The statistical results of the saffron ethanol extract fraction had a significance value of <0.05, indicating that there was an effect of differences in total flavonoid levels and an effect on cholesterol reduction activity.*

Keywords: *Cholesterol, Liebermann-Burchard, Saffron (Crocus sativus), Flavonoid Levels, TLCP*

I. INTRODUCTION

In the era of digitalization with sophisticated technological developments, it not only has a positive impact on the country's development but also has a negative impact on society, especially in health problems. Generally, people have an unhealthy lifestyle by rarely exercising and often eating fast food. Eating too often fast food can affect the increase cholesterol in the body[13]. Cholesterol is a group of lipids (fats) synthesized in the liver and intestinal walls to make and maintain nerve cells and to synthesize hormones in the body. Normal cholesterol levels in the blood between 160-200 mg/dL[8]. Cholesterol that exceeds normal levels can cause various dangerous diseases, one of which is atherosclerosis. Atherosclerosis is the accumulation of plaque on the walls of blood vessels [17]. Patients with hypercholesterolemia generally require long-term therapy in consuming synthetic drugs to lower cholesterol levels, this is dangerous for the body, especially in the kidneys. Another alternative is to consume traditional plant medicines that are efficacious in lowering cholesterol levels. Treatment with plants is proven to be effective and the side effects are lower than synthetic drugs [16].

Saffron is one of the plants used in traditional medicine to treat various diseases such as cough, fever, sedative, expectorant, anti-asthma, anticancer, and antihyperlipidemic [1]. Saffron contains flavonoid compounds, anthocyanins, carotenoids, riboflavin, thiamine, protein, starch, amino acids and mineral ingredients [14]. Flavonoid compounds can reduce cholesterol levels by eroding cholesterol deposits on the walls of coronary blood vessels. The ethanolic extract of saffron contained the total flavonoid content of quercetin of 19.14 ± 0.9 mgQE/g. Saffron extract with a concentration of 400 ppm can reduce cholesterol levels by 13.22% in vitro [10]. In another study, saffron supplementation at a dose of 80 mg/kg for 3 weeks and a dose of 100 mg/day after 9 weeks was able to reduce total cholesterol, triglycerides, LDL, atherogenic index (TC/HDL) and increase HDL[14].

This study was to determine the total flavonoid levels and cholesterol reduction activity of the saffron ethanol extract fraction. The extraction method used is remaceration using ethanol solvent 50% resulted in a large yield value and ethanol solvent was chosen because it has polar properties that are suitable for attracting flavonoid compounds in saffron. Fractionation was carried out using the preparative thin layer

chromatography (TLCP) separation method. Test for determination of total flavonoid content using $AlCl_3$ colorimetric method with quercetin standard solution. Test of cholesterol reduction activity using the *Liebermann-Burchard* method.

Based on this description, the total flavonoid content and cholesterol level reduction activity of the saffron ethanol extract fraction in vitro using a UV-Vis spectrophotometer.

II. MATERIALS AND METHODS

2.1 Plant material Collection and Authentication

The pistil of saffron flower (*Crocus sativus*) were collected from dr.saffron.id, Islamic Republic of Afghanistan.in the month of September, 2020. The plant materials were tested by Eng Mohammed Sami, Manager and Specialist of Saffron Quality Control Lab and approved by The Agriculture Irrigation and Livestock of Herat Province.

2.2 Chemicals

The chemical agents such as ethanol 50%, silica powder, ethyl acetate, isopropyl alcohol, aquadest, quercetin, methanol, aluminium chloride ($AlCl_3$), sodium acetate anhydrous, cholesterol standard, chloroform, anhydrous acetic acid, sulfuric acid (H_2SO_4). All the chemicals used were of analytical grade.

2.3 Preparation of the extract and phytochemical analysis

10 g saffron flower pistil were extracted by 300 ml etanol solvent 50% using remaceration method until a clear filtrate was obtained and the solvent was changed every 24 hours. The obtained filtrate is concentrated with a rotary evaporator at a temperature of 60ºC, then when the amount of extract has reduced to a little, it can be concentrated with a waterbath until a thick extract is obtained. In this study, the yield value of saffron extract was 23.66%. Saffron etanol extract was tested in the phytochemical screening include phenolics, flavonoids, saponins, tannins, alkaloids and terpenoids.

2.4 Preparation of the fraction

40 grams of silica powder was dissolved with 80 mL aquadest (1:2) homogenized for ± 2 minutes. A silica plate measuring 20 x 20 cm was made. The silica plate was allowed to dry and baked for ± 30 minutes at 110°C. Longitudinal spotting of the extract was carried out using a capillary tube and then eluted in the chamber with saturated ethyl acetate: isopropyl alcohol: water (65:25:10) mobile phase. Observed the visible band under UV 254 nm for further scraping. The results of the tape scrapings were dissolved with chloroform: ethanol (1:1) and homogenized with a magnetic stirrer for 15 minutes, then filtered and the filtrate was obtained to be evaporated to obtain a dry fraction. Fractions were tested by TLC using ethyl acetate mobile phase: isopropyl alcohol: aquadest (65:25:10).

2.5 Total Flavonoid Content (TFC) Test

50.0 mg quercetin standard was dissolved with 50 ml ethanol. The concentration 1000 ppm was diluted to a concentration of 100 ppm then made series of concentrations of 30, 40, 50, 60, 70, 80 and 90 ppm. 50.0 mg of saffron ethanol extract fraction dissolved in 5 ml methanol obtained a concentration of 10000 ppm. The quercetin standard solution and sample was taken 1.0 ml then add 3 ml methanol, 0.2 ml $AlCl_3$ 10%, 0.2 ml anhydrous sodium acetate 20% and 5.6 ml aquadest then homogenized. It was incubated for 9 minute in a place at room temperature. Absorbance was measured with a double beam UV-Vis spectrophotometer at 442 nm[11].

2.6 Cholesterol Reduction Activity Test

100.0 mg cholesterol standard was dissolved with 100 ml chloroform. The concentration 1000 ppm was diluted to a concentration of 250 ppm. 50.0 mg of saffron ethanol extract fraction dissolved in 50 ml chloroform obtained a concentration of 1000 ppm then made in series with concentrations of 300, 400, 500, 600 and 700 ppm. The sample solution was taken 2.0 mL add 5.0 ml cholesterol control standard 250 ppm homogenized with a vortex, then add 2.0 ml anhydrous acetic acid and 0.1 ml intense H_2SO_4. The solution was homogenized then covered with aluminum foil and left in dark place at room temperature for 21 minutes. The absorbance was measured with a double beam UV-Vis spectrophotometer at 671.70 nm[2].

2.7 Data Analysis

Measurement of total flavonoids was carried out by first analyzing the data using a standard curve, linear regression y = b x + a, which y is the absorbance, and x is the concentration of the standard solution. The total flavonoids content (mgQE/g extract) were calculated using equation :

$$\text{TFC} = \text{Sanple Concentration (mg/L)} \times \frac{\text{Sample Volume (L)}}{\text{Sample Weight (g)}}$$

Calculation the percentage of decreased levels cholesterol using the following formula:

$$\% \text{ Cholesterol Reduction Activity} = \frac{\text{Cholesterol Standard Control Absorbance} - \text{Sample Absorbance}}{\text{Cholesterol Standard Control Absorbance}} \times 100\%$$

2.8 Statistical Analysis

All the experiments were done in triplicates. The experimental results are expressed as mean ± SD of triplets. Statistical analysis was performed using Statitical Product and Srvice Solution, Version 23.

III. RESULTS AND DISCUSSION

This research was conducted by measuring total flavonoid content and the maximum concentration of saffron ethanol extract fraction to reducing cholesterol levels in vitro. Saffron were collected from the International Gold Star supplier for quality-controlled Quality in the Saffron Quality Control Lab Department of the Republic of Afghanistan. Researchers obtained LoA from supplier Dr. Safron.

Saffron flower pistil samples were extracted by remaceration method until a clear filtrate was obtained and the solvent was changed every 24 hours. The saffron remaceration method occurs when the solvent is added repeatedly after filtering. The solvent will attract more compounds because of the long extraction time. The principle of remaceration is the dissolution of secondary metabolites based on their solubility in the solvent. There is a diffusion process that occurs until there is a balance of concentrations inside and outside the cell [7]. Extraction using ethanol 50% because it is more polar than 70% or 96% which is can more attracting flavonoid compounds which are generally polar[9]. Ethanol with a concentration of more than 20% can prevent the growth of microorganisms[15]. The filtrate was concentrated with a rotary evaporator and a water bath at a temperature of 60°C because it is below the boiling point of ethanol which is 78°C to protect the compound[4]. In this study, the yield value of saffron extract was 23.66%.

The results of phytochemicals test showed that saffron extract contains phenolics, flavonoids, saponins, alkaloids, terpenoids, steroids and tannins. Identification of stains and selection of the optimal mobile phase to separate several compounds of saffron extract before proceeding with fractionation by preparative thin layer chromatography method was carried out by TLC test using ethyl acetate: isopropyl alcohol: aquadest (65:25:10) as mobile phase and spot appearance using $AlCl_3$. The use of the mobile phase and the spectrophotometer is expected to attract and detect flavonoid compounds that are semi-polar to polar. TLC test results obtained 6 spots can be seen in appendix 11 and the calculation of the Rf value is presented in table 1.

Table 1. TLC Test for Identification of Saffron Extract Stains (*Crocus sativus*) for TLCP

Stain	Rf	Color
1	0.92	Blue
2	0.63	Blue
3	0.45	Yellow
4	0.23	Blue
5	0.20	Blue
6	0.05	Blue

The saffron extract that has been analyzed qualitatively will then be fractionated using the preparative thin layer chromatography method. The printed plate was then dried in an oven at 110ºC for 30 minutes to remove water molecules that could interfere with the compound separation process. Fractionation is carried out by spotted saffron extract on the plate, the staining was repeated up to 3 times so that the resulting band was clear then eluted with the mobile phase of ethyl cetate: isopropyl alcohol: aquadest (65:25:10) which had been saturated[6]. The results of the saffron extract TLC obtained 6 bands. The Rf value of the TLC result band is presented in table 2 and the TLCP result shown in 254 nm UV light is shown in Figure 1.

Table 2. Results of TLC Saffron Extract (*Crocus sativus*)

Fraction	UV light 254 nm	
	Rf value	Color
1	0.96	Blue
2	0.63	Blue
3	0.45	Yellow
4	0.26	Blue
5	0.20	Blue
6	0.07	Blue

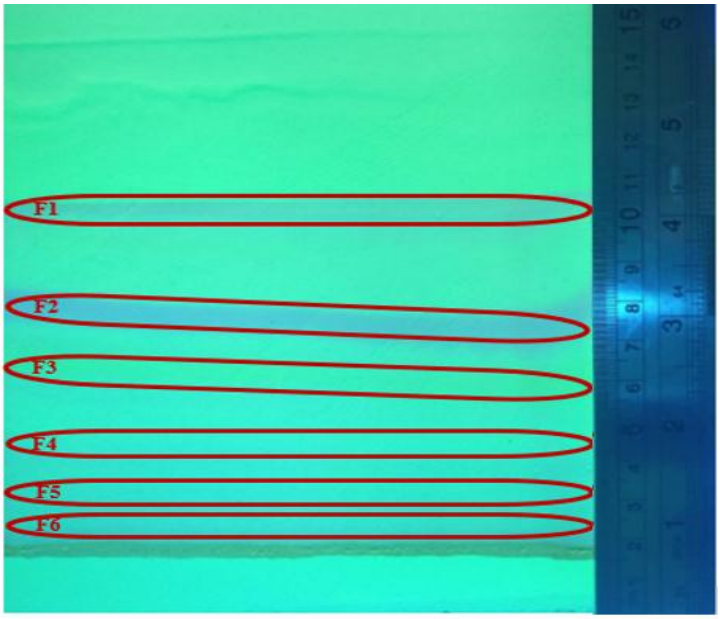

Figure 1. Results of TLCP Saffron Extract (*Crocus sativus*) at 254 nm UV Light

3.1 Total Flavonoid Content Test

Based on the results of the TLCP test, 4 fractions were selected which will be continued to quantitative tests, They are fractions 2, 3, 4 and 5, because fraction 1 is located at the end of the elution limit and fraction 6 is located near the highlight of the extract. for quantitative test. Fractions 2, 3, 4 and 5 were then measured quantitatively total flavonoid content using a UV-Vis spectrophotometer. Quercetin is used as a standard for comparison because it is a flavonoid compound from the flavonol group which has a keto group at C-4 and a hydroxy group at C-3 or C-5 which is besides from flavones and flavonols that can form complexes with $AlCl_3$[3]. $AlCl_3$ reagent can form a yellow complex due to the reaction between Al^+ metal and hydroxyl groups which can cause a shift in wavelength towards the visible (visible) so that the absorbance of the complex formed can be read on the spectrophotometer. The more concentrated the yellow color, the higher the flavonoid concentration in the sample[5]. The reaction for the formation of complex compounds between quercetin and $AlCl_3$ can be seen in Figure 2.

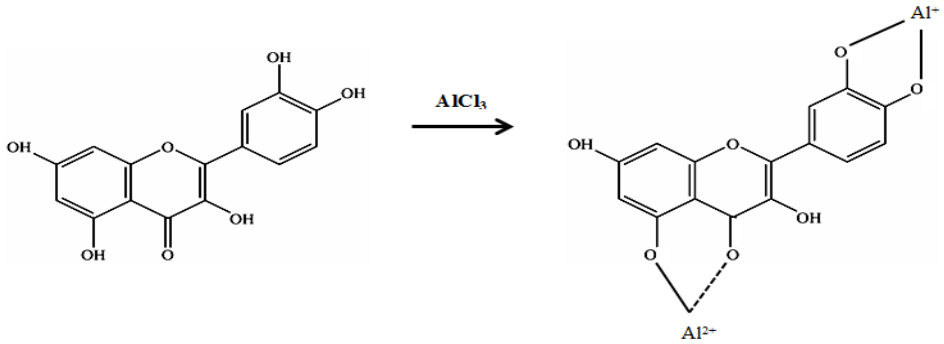

Figure 2. Reaction for the Formation of Quercetin Complex Compounds with AlCl3

The determination of total flavonoid content in the saffron ethanol extract fraction begins with determining the operating time to determine the stable measurement time of the sample when it reacts completely with the reagent and search the maximum wavelength aims to obtain the maximum absorbance value, resulting in maximum sensitivity and a small error rate. The results showed that the operating time was 9 minutes and the maximum wavelength was 442 nm. The regression equation is obtained y = 0.0076x + 0, 0114. In the measurement of total flavonoid levels, the absorbance results obtained a linear relationship between absorption and concentration, this is indicated by the correlation coefficient (r) of 0.9994.

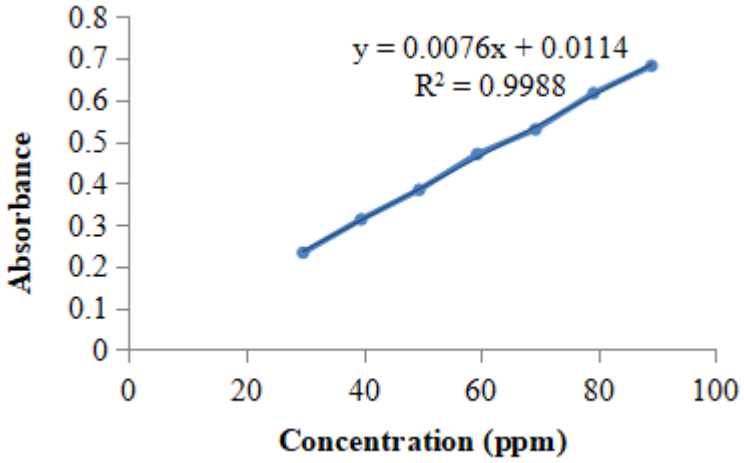

Figure 3. Linear Graph of Quercetin Concentration vs Absorbance

The results of the determination total flavonoid content in the ethanol extract fraction saffron were different. The highest total flavonoid content was produced by fraction 5 at 7.2282 ± 0.07 mgQE/g, fraction 4 at 6.2040±0.17 mgQE/g, fraction 3 at 6.0802 ± 0.18 mgQE/g and fraction 2 at 3.8518±0.43 mgQE/g. The results of the determination of the total flavonoid content of the ethanol extrtact fraction saffron can be seen in table 3. The results of the calculation showed that fraction 5 had a total flavonoid content greater than fractions 2, 3 and 4. From these results, it can be concluded that fraction 5 was selected to be tested for cholesterol reduction activity.

Table 3. Total Flavonoid Content of Ethanol Extract Fraction Saffron (*Crocus sativus*)

Fraction	Total Flavonoid Level (mgEQ/g)			Average (mgEQ/g)
	Replication 1	Replication 2	3 replication	
2	3.4094	3.8853	4.2608	3.8518±0.43
3	6.1882	6.1781	5.8744	6.0802 ± 0.18
4	6,0202	6.2262	6,3657	6.2040±0.17
5	7.2738	7.2575	7.1532	7.2282 ± 0.07

3.3 Cholesterol Reduction Activity Test

The test of cholesterol level reduction activity used the Liebermann-Burchard method because the process was simple and only requires the addition of anhydrous acetic acid and concentrated sulfuric acid. In this method, it is necessary to add anhydrous acetic acid to extract cholesterol, ensure that the media is free of water and form acetyl derivatives and the addition of concentrated sulfuric acid to accelerate the formation of color complexes in cholesterol, the solution will change color to blue-violet and then blue-green[12]. The resulting color will be read the absorbance with a UV-Vis spectrophotometer.

Fraction 5 ethanol extract saffron is the highest flavonoid total which is this fraction had activity in reducing cholesterol levels. This study requires a standard cholesterol solution to control cholesterol. The standard concentration of cholesterol control used in this study based on the results of the orientation carried out was 250 ppm. The operating time obtained was 21 minute and the maximum wavelength was 671.70 nm. The absorbance results obtained can be calculated by calculating the percentage reduction in cholesterol levels as shown in table 4 and the graph of the percentage reduction in cholesterol levels in fraction 5 ethanol extract saffron is presented in Figure 4.

Table 4. Percentage Reduction in Cholesterol Levels Saffron (Crocus sativus) Ethanol Extract Fraction

Concentration (ppm)	Decreased Cholesterol Levels (%)			Average (%)
	Replication I	Replication II	Replication III	
300	26.13	15.88	7.03	16.35
400	37.35	21.82	21.10	26.76
500	45.83	34.81	36.97	39,20
600	50.07	42.68	50.07	43.24
700	62.93	50.41	60.69	58.01

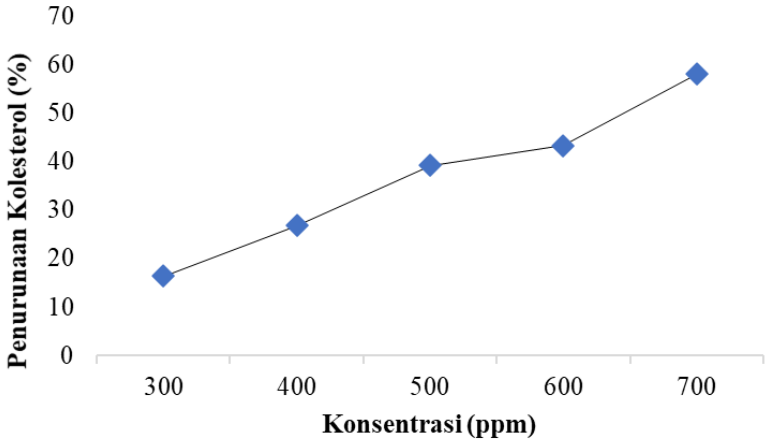

Figure 4. Graph of Percentage Decrease in Cholesterol Levels Fraction 5 Ethanol Extract Saffron (*Crocus sativus*)

Based on the table presented, it shows that the highest reduction in cholesterol levels in the sample of fraction 5 ethanol extract saffron at 700 ppm is obtained 58.01%. The mechanism of quercetin can reduce cholesterol levels due to the presence of a hydroxy group that reacts with cholesterol to form a quercetin-cholesterol complex. The strongest hydroxy group in the quercetin compound is at hydroxy group position 3-4. The hydroxy group in cholesterol reacts with quercetin, so that cholesterol can be bound to quercetin and cholesterol levels are reduced. Cholesterol that does not form a complex or so-called free cholesterol will react with sulfuric acid to form a blue-green color. If the color of the solution is greener, more light is absorbed, resulting in a high absorption value from the results of the UV-Vis spectrophotometer analysis which causes the percentage value of cholesterol reduction to be smaller[2]. The reaction for the formation of the quercetin-cholesterol complex can be seen in Figure 5.

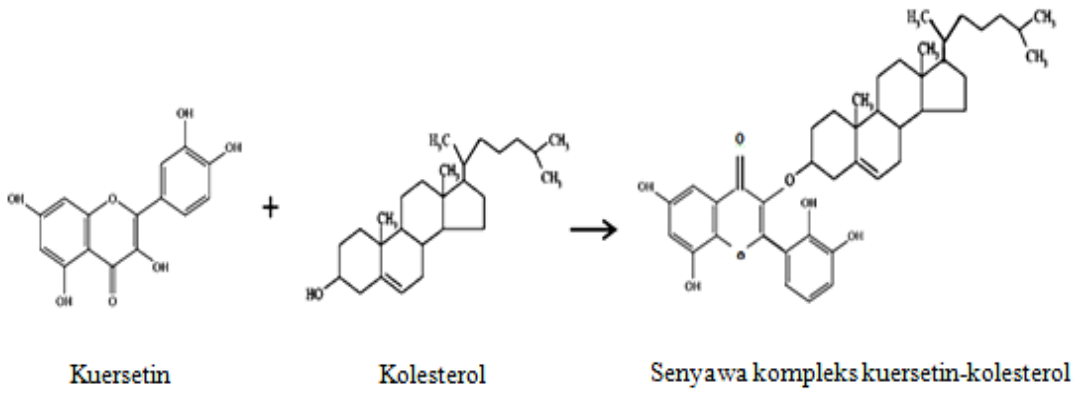

Figure 5. Reaction of Quercetin-Cholesterol Complex Formation

Data analysis for total flavonoid content is obtained normally distributed and homogeneous with a significance value > 0.05. The data was continued to the 1-way ANOVA test is obtained that there was a difference between groups with a significance value < 0. Based on the 1-way ANOVA test, the total flavonoid fraction of the saffron ethanol extract showed that there was a significant difference between groups with a significance value of <0.05. So it can be concluded that each fraction has a significant difference in the total flavonoid test in vitro. In the data analysis of the percentage reduction in cholesterol levels activity, it was found that the data were normally distributed and homogeneous with a significance value > 0.05. The test was continued by using the 1-way ANOVA test is obtained a significance value <0.05, meaning that there was a difference in each sample concentration, then a post-ANOVA test was carried out to determine the location of the difference between the sample concentrations. Based on the 1-way post ANOVA test, the percentage of cholesterol level reduction activity in fraction 5 ethanol extract saffron showed that there was a significant difference between the sample concentrations with a significance value (<0.05), so it can be concluded that each concentration has a significant difference in vitro cholesterol reduction activity test.

IV. CONCLUSION

The ethanol extract fraction of saffron (*Crocus sativus*) had the highest anticholesterol activity value at a concentration 700 ppm with an average percentage of cholesterol reduction of 58.01%. There is a significant difference between the groups of saffron ethanol extract fractions so that the one with the highest flavonoid content is fraction 5. There are differences in the activity of reducing cholesterol levels in each concentration of fraction 5 ethanol extract of saffron using the *Liebermann-Burchard* method.

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