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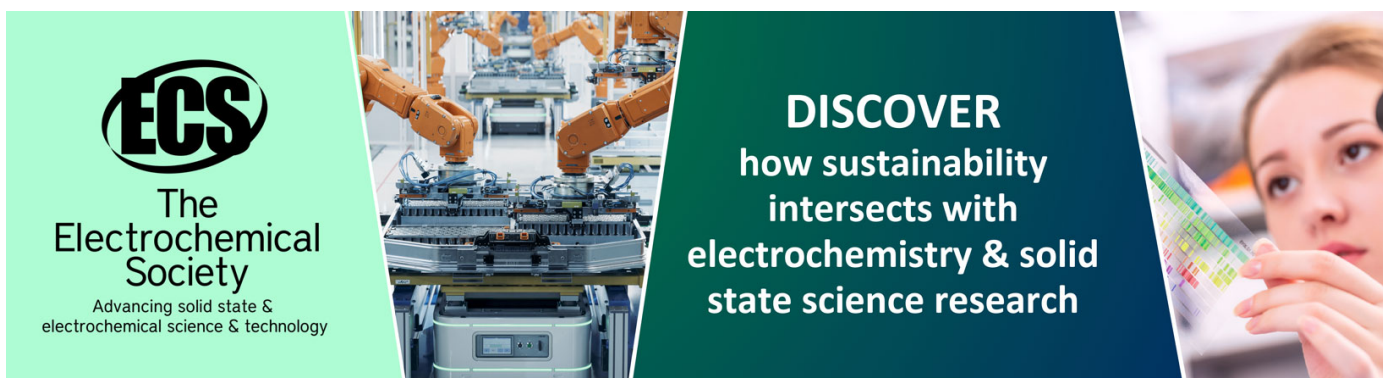
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In vitro anti-cholesterol and anti-hypertensive activity of stem bark the *Flacourtia rukam*

M Muharni^{1*}, J Julinar¹, H Yohandini¹, F Fitrya² and R Melati²

¹ Department of Chemistry, Faculty of Mathematics and Natural Sciences, University of Sriwijaya. Jl. Raya Palembang Prabumulih KM 32 Indralaya 30662 South Sumatera Indonesia.

² Department of Pharmacy, Faculty of Mathematics and Natural Sciences, University of Sriwijaya. Jl. Raya Palembang Prabumulih KM 32 Indralaya 30662 South Sumatera Indonesia

*E-mail: muharnimyd69@gmail.com

Abstract. *Flacourtia rukam* belongs to family Salicaceae and Indonesia is known as rukem. *F. rukam* is popular among people especially Musi Banyuas in South Sumatera, Indonesia for the treatment of hypertensive. Investigated effect anti-cholesterol and anti-hypertensive activity from extracts the stem bark of *F. rukam* have been done. The anti-cholesterol activity was measured by the photometric method by reaction cholesterol with Liebermann-Burchard reagent and anti-hypertensive activity using Angiotensin Converting Enzyme (ACE) inhibitory method. The crude ethanol extract showed the highest anti-cholesterol activity compared to the fractions with IC₅₀ value of 157.88 mg/L. Crude ethanol extract also contained the highest phenolic and flavonoid content compared to fractions. The anti-cholesterol activity of extracts is equivalent to the total phenolic and flavonoid contained. In an anti-hypertensive activity study, the crude ethanol extracts exhibited the percentage of ACE inhibitory activity with IC₅₀ 119.82 mg/L. The results indicated that the stem bark of *F. rukam* might reduce or control the cholesterol levels and blood pressure. Anti-cholesterol and anti-hypertensive activity the stem bark of *F. rukam* is higher in extract form compared to fractions (synergistic).

1. Introduction

The use of traditional medicinal plants has been long carried out by the community. The use of these traditional medicinal plants based on habits from generation to generation without clearly the right dosage and was not officially registered at an institution, so was not known what compounds that are providing properties in the treatment of disease [1]. Hypertension is a type of subchronic disease that is commonly found in the community. Hypertension is experienced by many people especially aged 40 years and over. Hypertension is related to cholesterol levels in the blood. People with high cholesterol have a great chance of hypertension. High lipids in the blood cause various diseases that can even result in death [2].

Plants are one source of new drug compounds anti-hypertensive potent. Exploration of the potential of traditional medicinal plants which is efficacious in the treatment of hypertension needs to be done to obtained new sources of natural ingredients to be developed into a source that is officially registered hypertension medicine. Several extracts and compounds derived from plants have been proven in vitro as an effective natural ACE inhibitor because of the presence of flavonoid molecules, triterpenoids, polyphenols, peptides [3,4].



One of the traditional medicinal plants used for the treatment of hypertension is *F. rukam* belongs to family Salicaceae known in Indonesia as rukem [5]. *F. rukam* is popular among local people especially in Musi Banyuasin south of Sumatra Indonesia for treatment of hypertensive, the *F. rukam* claimed to show hypertension activity by lowering blood pressure [6]. According to some literature, the leaves, roots, and fruit have been used to treat many diseases [5], and the stem bark especially in south Sumatera used to treat hypertension disease. In previous research, three compounds such as friedelin, steroid glycoside, and phenol compound have been isolated from the stem bark [7]. The efficacy of a medicinal plant was related to secondary metabolite compounds contained in the plant.

Many compounds have been reported to be active as antihypertensive or anticholesterolemia phenolic compounds. (8). This study is a continuation of our previous research in order to prove the efficacy of rukam plants used as traditional medicine to treatment hypertension. In a previous study one phenol compound from ethyl acetate fractions and identified as poliothryoside which showed antihypertensive activity with IC_{50} 226.87 mg/L [9]. Phenolic compounds were known to have the ability to reduce levels of LDL and total cholesterol. The decreasing cholesterol, triglyceride, and LDL levels in the blood will reduce the risk of hypertension disease. The activity of a compound sometimes found to be higher in extract or fractions compared in pure form. In this paper I want to report anticholesterol and antihypertension properties the stem bark of *F. rukam*.

2. Materials and Method

2.1. Chemicals

Chemicals and reagents used for the study of analysis anti-cholesterol and antihypertensive activity were purchased from Sigma Aldrich, while other chemicals reagent used were purchased from Merck (Egypt) and solvents using in extraction were analytical reagent grade.

2.2. Plant material

Samples were taken from South Sumatra Indonesia. The sample has been identified by Dr. Laila Hanum (number specimen VIC 2702) Botanical as Head of Laboratory Department of Biology University of Sriwijaya. The stem bark of *F. rukam* (3 kg) was dried in room temperature until weight constant and then made into a fine powder (1500 g).

2.3. Extraction

Samples were divided into 2 parts, 750 g was extracted by maceration with ethanol for 24 h, after evaporated by rotary evaporator at 50°C an ethanol crude extract was obtained. The other 750 g was macerated using solvents with increasing polarity from -hexane, ethyl acetate, and methanol and after concentrating with a rotary evaporator under reduced pressure at 50°C obtained fraction n-hexane, ethyl acetate fraction, and methanol fraction. and the percentage of yield was calculated.

2.4. Measurement of total phenolic

Measurement of the total phenolic carried out by spectrometry analysis using the Folin-Ciocalteu method [10,11].

2.5. Measurement of flavonoid content

Measurement of the total phenolic carried out by spectrometry analysis using the colorimetric method using $AlCl_3$ reagents [11].

2.6. Anti-cholesterol assay

The anticholesterol activity the extract and fractions were determined using the photometric method. Cholesterol reacted with Liebermann-Burchard reagent. The crude extract, and fractions used over concentration range of 25, 50, 100, 150, 200 mg/L. The sample was tested (5 mL) by addition of 2.5 mL cholesterol standard 100 mg/L then shaken 2 min, added 2 mL acetic anhydride and sulfuric

acid 0.1 mL [12]. The mixture was left until 16 min at room temperature. The measurement with 3 repetitions. As a blank, using Ethanol, negative control cholesterol 100 mg / L, and positive control using simvastatin with concentrations of 10, 20, 30, 40, 50 mg/L. The amount of residual cholesterol was determined based on absorbance data and was measured at λ_{max} 420 nm using spectrophotometer. The % inhibition was calculation with formula:

$$\% \text{ Inhibition} = \frac{A_0 - A_s}{A_0} \times 100$$

Where, A_0 : Absorbance control and A_s : Absorbance sample.

2.7. Anti-hypertensive assay using ACE method

The sample (80 μ L) added 50 μ L of HHL, and 15 μ L BSA, incubation for 5 min at temperature 37°C. after that added 50 μ L (ACE (0.05 units/mL), which then kept at 37°C for 30 min. The reaction is quenched by pouring 250 μ L 0.5 M HCl. In the addition of 1.7 mL ethyl acetate, then hippuric acid was formed. The mixture is then centrifuged at 10000 rpm for 10 min, the supernatant (800 μ L) dried in the oven at 95°C for 75 min. The hipuric acid product was dissolved into 1000 μ L distilled water. Absorbance was measured at λ_{max} 228 nm using a UV-Vis spectrophotometer instrument [13]. The activity of ACE inhibitors is calculated using the formula:

$$\% \text{ ACE inhibitory activity} = \frac{(A - B) - (C - D)}{(A - B)} \times 100$$

Where, A: Absorbance control B: Absorbance blank control; C: Absorbance sample, D: Absorbance blank sample.

3. Results and Discussion

3.1. Extraction yield

Extraction 750 g the sample using ethanol after concentrated to obtained crude ethanol extract 22 g with a yield percentage 2.93%. Extraction 750 g other of the stem bark with increased polarity to obtained n-hexane fraction (0.52 g, yield percentage 0.07%), ethyl acetate fraction (7.5 g, 1%) and methanol fraction (10.7 g, 1.43%). yield percentage obtained is relatively low. Yield percentage of the extract can be influenced by several factors including plant varieties, plant age, plant maintenance processes, and environmental factors where plants grow. The yield value also depends on the content of the secondary metabolite, and the maceration method used.

3.2. Quantity of total phenolic and flavonoid content

Determination of total phenolic content in the sample base on the reaction of the Folin-Ciocalteu reagent with extracts. This method is based on the reduction power of the hydroxy group of phenolic compounds which reduce phosphomolybdate-phosphotungstate into blue molybdenum (Lee, 2003). The total phenolic content of extract and fractions were analyzed by spectrophotometry method using gallic acid as standard at λ_{max} 674 nm. The calibration curve of gallic acid (10; 12.5; 25; 50; 75; 100 mg/L) represented by regression linear equation $y = 0.028x + 0.027$ provided that $R^2 = 0.998$. The total phenolic was as the weight of gallic acid (mg) per gram extract (mg GAE/g) and total flavonoid as the weight of quercetin (mg) per gram extract (mg QE/g). The total phenolic contained within the crude extract of ethanol is 9.799 ± 0.027 mg GAE/g. The total phenolic content methanol fraction > ethyl acetate fraction > n-hexane fraction.

The Flavonoid content of extract and fractions were calculated by using a quercetin standard curve at λ_{max} 435 nm that has been obtained from previous experiments (the regression linear equation is $y = 0.0558x - 0.0312$, coefficient correlation $R^2 = 0.9997$). The crude ethanol extract contained flavonoid 3.066 ± 0.027 mg QE/g, while data for fraction while the highest phenol infraction is found in the methanol fraction 2.909 ± 0.048 2.909 ± 0.048 (Table 1). Total flavonoids in ethanol extract and fraction showed the same data pattern with total phenolic data. Phenolic and flavonoid compounds

have multiple biological effects and oxidative stress-related disorders such as antioxidant, anticancer, antidiabetic, anticholesterol, antihypertension, and anti-inflammatory properties. Flavonoid compounds also include in a group of phenolic compounds. The data show the quantitative of flavonoid is lower compared to phenolic others. The same data pattern was found both in crude extract and in all fractions.

Table 1. Total phenolic (TP) and flavonoid (TF) content

Sample		TP (mg GAE/g $\pm$ SD)	TF (Mg QE/g $\pm$ SD)
Fraction	n-hexane	0.746 $\pm$ 0.008	0.713 $\pm$ 0.015
	Ethyl acetate	5.783 $\pm$ 0.129	2.195 $\pm$ 0.069
	Methanol	8.061 $\pm$ 0.111	2.909 $\pm$ 0.048
Crude extract	Ethanol	9.799 $\pm$ 0.027	3.066 $\pm$ 0.027

3.3. *In vitro* anti-cholesterol activity

The anticholesterol activity was determined by reacting cholesterol with Lieberman-Burchard (LB) reagent. Simvastatin used as positive control in this experiment. The anti-cholesterol activity was determined based on the amount of residual cholesterol (Tables 2) using the standard cholesterol 1 curve, the calculation made base on the inhibition percentage of cholesterol using equation 1.

Table 2. Residual cholesterol after reaction of extract and fraction.

Concentration (mg/L)	Residual cholesterol $\pm$ SD (mg/L)			
	Crude ethanol extract	Fraction		
		Methanol	Ethyl acetate	n-hexane
25	67.66 $\pm$ 3.99	75.19 $\pm$ 0.95	78.29 $\pm$ 33.34	83.00 $\pm$ 0.94
50	65.28 $\pm$ 6.21	69.28 $\pm$ 5.45	75.34 $\pm$ 0.14	80.38 $\pm$ 1.32
100	56.57 $\pm$ 7.26	59.04 $\pm$ 0.44	63.34 $\pm$ 0.08	78.28 $\pm$ 1.13
150	47.99 $\pm$ 1.36	51.57 $\pm$ 2.62	62.00 $\pm$ 0.29	72.57 $\pm$ 0.51
200	41.99 $\pm$ 6.32	49.85 $\pm$ 1.68	59.54 $\pm$ 1.24	70.43 $\pm$ 0.94

Table 3. Residual cholesterol after reaction of simvastatin

Concentration (mg/L)	Residual cholesterol $\pm$ SD (mg/L)
10	55.90 $\pm$ 0.73
20	42.81 $\pm$ 0.36
30	33.29 $\pm$ 0.42
40	23.76 $\pm$ 0.93
50	11.87 $\pm$ 0.29

Tables 2 and 3 showed the Residual cholesterol from reactions is inversely related to concentration. When the cholesterol residue gets higher, the anti-cholesterol activity is getting lower. Residual cholesterol by n-hexane fraction is higher compare to ethyl acetate fraction and methanol fraction. when compared with crude ethanol extract the residu cholesterol is lower compared to all fractions. Base on level cholesterol can be calculated percentage inhibition (Figures 1 and 2). It appears that high inhibition percentage corresponds to the low activity of anticholesterol. A similar concentration (50 mg/L) crude ethanol extract showed higher inhibition percentage compared to other fractions, meanwhile, methanol fraction showed the highest anticholesterol activity compared to other

fractions (methanol > ethyl acetate > n-hexane). At the same concentration simvastatin as Positive control having inhibition percentage of cholesterol 87.3 ± 0.55 mg/L.

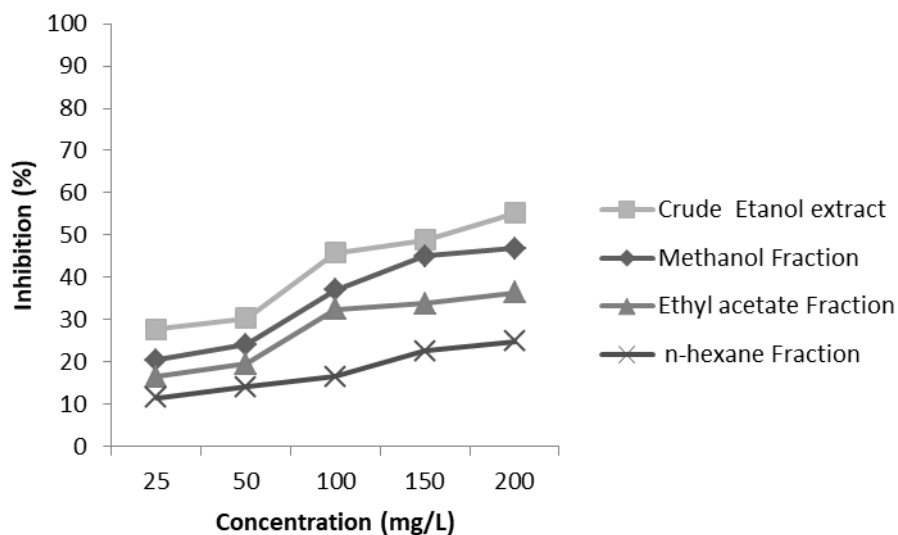


Figure 1. Percentage inhibition cholesterol of the crude ethanol extract and fractions

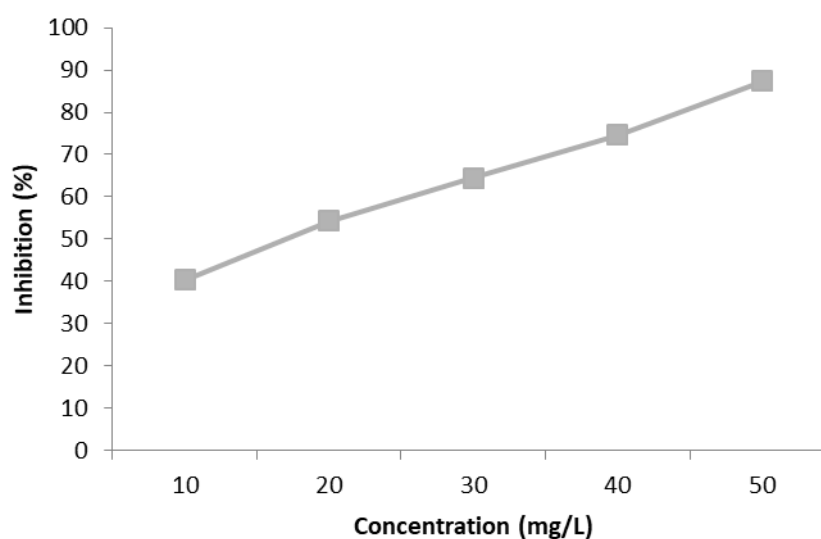


Figure 2. Percentage inhibition cholesterol of Simvastatin

The IC_{50} value calculated using the linear regression method provided that the concentration of the compound (x) and percent of cholesterol decrease (y) (Figures 1 and 2). The smaller the IC_{50} value means the stronger the anti-cholesterol power [14,15]. The result of the calculation shows IC_{50} values for the n-hexane, ethyl acetate, and methanol fraction were obtained 516.67; 294.23; and 201.94 mg/L respectively while the value of total ethanol extract is 157.88 mg/L and simvastatin shown IC_{50} 17.58 mg/L (Figure 2). The crude ethanol extract has higher anti-cholesterol activity compare to fractions. This data shows that the activity of bioactive compounds in extracts is synergistic.

Reduction of cholesterol-related to the chemical compound in extract and fraction. Phenolic compounds were known to have the ability to reduce levels of LDL and total cholesterol so that triglyceride levels in the blood also decrease. Beside phenolic compound, β -sitosterol glycoside

compounds work by inhibiting the absorption of cholesterol in the digestive tract. We have also succeeded in identifying the presence of steroid glycoside compounds in methanol fraction of stem bark of *F. rukam* [7]. Based on data, the anticholesterol properties of the extract are thought to be due to the phenolic and steroid compounds.

The results showed anticholesterol properties of extract and fraction of stem bark *F. rukam* fraction were proportional to total phenolic and flavonoid contents. The crude ethanol extract showed higher anti-cholesterol activity compared to fractions and also had the highest total phenolic and flavonoid contents compared to other fractions. Flavonoids reduce cholesterol levels by reducing LDL [16, 17]. Flavonoids also play a role in reducing triglycerides and increasing HDL. Based on anticholesterol activity data, the highest activity was shown by crude ethanol extract so that for antihypertensive activity test was only done for crude ethanol extract.

Table 4. IC₅₀ value of anti-cholesterol

Sample	IC ₅₀ (mg/mL)
n-Hexane	516.67
Fraction Ethyl acetate	294.23
Methanol	201.94
Crude extract Ethanol	157.88
Positive control Simvastatin	17.58

3.4. *In vitro* anti-hypertensive activity

The determination of antihypertension activity conducted by measuring inhibitory activity against *Angiotensin-Converting Enzyme* (ACE) [13]. The ACE inhibitory activity is an effective method in the antihypertensive test. The activity expressed as a percentage of inhibition ACE, measured based on the rate of formation of hippuric acid from Hippuryl- Histydl-Leucine (HHL) using equation 2. Hippuric acid produced by ACE hydrolysis was measured at λ_{max} 228 nm. The higher inhibitory activity ACE corresponds to the low concentration of hippuric acid formation [18].

Table 5. % inhibitory ACE activity crude ethanol extract

Concentration (mg/L)	% ACE Inhibition $\pm$ SD
62.5	40.81 $\pm$ 3.16
125	53.86 $\pm$ 0.73
250	62.79 $\pm$ 3.22
500	66.74 $\pm$ 0.53
1000	77.64 $\pm$ 0.57

Table 6. % inhibitory ACE activity of standard Captopril

Concentration (mg/L)	% ACE Inhibition $\pm$ SD
10	45.67 $\pm$ 2.80
20	63.24 $\pm$ 0.65
30	85.54 $\pm$ 0.57
40	88.07 $\pm$ 0.77
50	90.33 $\pm$ 0.75

The anti-hypertensive activity of ethanol extract and standard captopril were shown in Tables 5 and 6. Based on the data obtained, it is shown that the higher the sample concentration, percent the lower amount the hippuric acid formed, which indicates high inhibition percentage of ACE activity. At concentration test (1000 mg/L) crude ethanol extract, shown the inhibitory percentage 77.64 %.

Captopril is used as a positive control and at concentration 50 mg/L showed an inhibition percentage of 90.33%. The IC₅₀ value of ethanol extract and captopril was determined base on the linear regression method using data concentration (x) and inhibitory percentage of ACE (y). Crude ethanol extract has IC₅₀ alue 119.82 mg/L, whilst captopril as an antihypertensive standard gives an IC₅₀ value of 12.55 mg /L. The IC₅₀ value of the crude ethanol extract was higher than pure compound polyotrisoside IC₅₀ 226.87 mg/L [9]. This shows that the antihypertensive properties the extract stem bark of rukam also synergistic. ACE inhibitor compounds reported so far including flavonoids, phenolics [19–21]. Medicinal plants generally do not only have a single specific property for the treatment of a disease but generally have multiple properties, so they can also treat other diseases. The compound proofed inhibits the activity of ACE effectively.

Based on the findings, we concluded that antihypertensive and anticholesterol properties of secondary metabolite compounds within ethanol extracts probably have a synergistic effect hence it has activity higher than fractions. The antihypertension properties of extracts and fractions shown a linear correlation with anticholesterol properties. Antihypertensive activity of extract the stem bark of *F. rukam* related to flavonoid and total phenol content whereas the ethanol extract of *F. rukam*.

4. Conclusions

The crude extract ethanol contained total phenolic 9.799 mg QAE/g and flavonoid 3.066 mg QE/g. anti-cholesterol and antihypertensive activity the stem bark of *F. rukam* is higher in extract form compared to fractions (synergistic) with IC₅₀ 157.88 and 119.82 mg/L respectively.

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