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Quantitative Phytochemical Analysis and Determination of Anti-Cholesterol Activity of Sungkai (*Paronema canescens* Jack.) Leaf Extracts

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ABSTRACT

Sungkai (*Paronema canescens*) has been used traditionally to cure fever, malaria, wounds, ringworm, and toothaches. The community of Musi Banyuasin in South Sumatra, Indonesia, uses this plant to treat hypertension. One of the possible causes of hypertension is an abnormal level of cholesterol. This research was aimed at conducting quantitative phytochemical analysis and investigate the anti-cholesterol activity of Sungkai leaves extracts. Fresh leaves of Sungkai were extracted using ethanol and methanol by the maceration method. The Sungkai leaves were also fractionated using solvents with step-wise gradient of increasing polarity; n-hexane, ethyl acetate, and methanol followed by an anti-cholesterol activity test. The results showed that the ethanol and methanol extract of Sungkai leaves had total flavonoid content of 8.23±1.41 and 2.671±2.64 mg Quercetin Equivalent (QE)/g, total phenolic content of 13.00±1.48 and 4.47±1.41 mg Gallic Acid Equivalent (GAE)/g, and steroid content of 108.29±0.03 and 82.23± 0.39 mg Cholesterol Equivalent (CE)/g, respectively. The ethanol extract had higher flavonoid and steroid contents than the methanol extract, but the total phenolic content was lower, although the difference was not significant. The ethanol extract had higher anti-cholesterol activity than the methanol extract with IC_{50} values of 272.14 and 374.26 µg/mL, respectively on the one hand, whereas ethyl acetate fraction, on the other hand, showed the highest anti-cholesterol activity among other fractions with an IC_{50} value of 156.43 g/mL. The finding from this work suggests that the ethyl acetate fraction of Sungkai leaf extract has excellent potential as a cholesterol-lowering drug.

***Keywords*:** Anti-cholesterol, Leaf extract, *Paronema canescens*, Phytochemical analysis.

Introduction

Cholesterol is unsaturated alcohol of the steroid family of compounds and is a fundamental component of cell membranes. It is also a precursor of various critical substances such as adrenal steroid hormones and bile acids. Cholesterol is needed in the body as it plays an essential role in human life.[1] The human body requires an average amounts of cholesterol, but a disease condition results if the amount is excessive. High cholesterol levels in the blood, known as hyperlipidemia, will lead to the deposition of cholesterol and other fatty substances in the blood vessels.[2] Hyperlipidemia is one of the most common diseases in the world.[3] High cholesterol is one of the triggers for hypertension.[4] The high cholesterol in the blood will cause narrowing and stiffness in the walls of the blood vessels, which can cause atherosclerosis that leads to an increase in blood pressure, blockage of the heart arteries, and risk of heart disease. Synthetic drugs, such as simvastatin, gemfibrozil, cholestyramine, and niacin, have been used to lower cholesterol, but the use of synthetic drugs can cause side effects. There is now emerging interest in developing drugs using natural compounds, affecting multiple targets with reduced side effects. Natural compounds offer a great opportunity for the discovery of novel therapeutic candidates for the treatment of hyperlipidemia.[5] Several medicinal plants have been reported to show hypolipidemic activity, such as *Lagenaria siceraria*,[6] *Stevia rebaudiana*,[7] *Cassia occidentalis*,[8] and *Rumex obtusivolius*.[9]

Furthermore, the Sungkai (*Paronema canescens*) plant has been used as a traditional antihypertension remedy. It is a traditional medicine used in South Sumatra, particularly by the Musi Banyuasin ethnic group, to treat hypertension and hypercholesterolemia.[10] Some scientific information about the chemical content and biological activity of the *P. canencens* plant have been reported. The methanol extract of *P. canencens* leaves contained alkaloids, terpenoids, steroids, flavonoids, and tannins.[11] Steroids, triterpenoids, and phenolic in plants have the effect of lowering cholesterol levels.[12,13] Furthermore, the flavonoid class of compounds have also been reported to reduce cholesterol levels in the blood.[14] The extract of *P. canescens* leaves have been said to have weak anti-plasmodial and cytotoxic activities.[15] Muharni *et al*.[16] reported that betulinic acid from the extract of *P. canescens* leaves showed anti-cholesterol activity with IC_{50} value of 60.53 µg/mL, whilst the standard anti-cholesterol compound simvastatin had an IC_{50} value of 24.68 µg/mL. Based on the IC_{50} value obtained, betulinic acid is active for cholesterol reduction, but the activity is weaker compared to simvastatin. The aim of this study was to analyze the phytochemicals of *P. canescens* leaf extracts quantitatively and investigate the anti-cholesterol activity of the extracts and fractions of *P. canescens* leaves.

Materials and Methods

Sample collection

Fresh leaves of *P. canescens* were collected from the Musi Banyuasin Regency of South Sumatra (-3^{0}34'1.434"N 104^{0}77'18.19"E), Indonesia,

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in January 2021. The plant was identified as *Paronema canescens* Jack at the Herbarium Bogoriense, Research Center for Biology Indonesian Institute of Sciences Bogor Indonesia, an herbarium specimen with voucher number B-134/IV/D1-01/1/2021 was deposited in the center.

Extraction using methanol and ethanol [17]
The fresh leaves of *P. canescens* (0.5 kg each) were extracted with ethanol and methanol at room temperature (3×24 hours) and then filtered. The procedure of maceration was repeated three times. The filtrate was then concentrated using a vacuum rotary evaporator (DRAGONLAB RE100-pro) at 60°C to obtain crude extracts of ethanol and methanol.[17] Quantitative phytochemical analysis (total steroid, phenol, and flavonoids) of each extract was determined.

Quantitative phytochemical analyses of P. canescens leaf extracts
Determination of total steroid content
The total steroid content in *P. canescens* leaves extracts was determined by the photometric method,[18] using standard cholesterol solution. The cholesterol standard curves made at 5 different concentrations (20, 40, 60, 80, and 100 µg/mL), by pipetting 0.2, 0.4, 0.6, 0.8, and 1.0 mL of 1000 µg/mL from stock solution of cholesterol and added to chloroform and made up to 10 mL in a volumetric flask. The flask was covered with aluminium foil to protect it from light. An aliquot of 2.5 mL of each standard solution and the sample were taken and added to 2 mL acetic anhydride and 0.1 mL sulfuric acid and allowed to stand for 16 minutes. The absorbance of the solution was measured using a spectrophotometer (Shimadzu) at λ_{max} 630 nm. The steroid concentration in the test sample was calculated from the standard cholesterol calibration curve and was expressed in mg cholesterol equivalent per gram dry weight (mg CE/g sample).

Determination of total phenol content [19]
The total phenol content in *P. canescens* leaf extract was determined by the spectrophotometric method using Folin Ciocalteu reagent according to Santoso *et al*.,[19] with slight modifications. Gallic acid was employed as a standard phenol with 10, 20, 30, 40, and 50 g/mL concentrations. An aliquot of 1 mL gallic acid and the sample were added to 2.5 mL of Folin Ciocalteu reagent. The mixture was shaken, and after 5 minutes, it was added to 1.5 mL of 7.5% Na_2CO_3, incubated for 30 min at room temperature. Absorbance was measured at λ_{max} 736 nm. The total phenol content was expressed as mg gallic acid equivalents per gram of dry weight (mg GAE/g sample) from a gallic acid calibration curve. All the samples were analyzed in three replicates.

Determination of total flavonoid content
Total flavonoid content in *P. canescens* leaf extracts was measured by spectrophotometry according to the method described by Amalich *et al.*,[20] with slight modifications. As a standard, quercetin was used at concentrations of 1, 2.5, 5.0, 7.5, and 10 g/mL. An aliquot of 1 mL diluted sample of the extract and standard quercetin solution was added to 0.3 mL of 5% $NaNO_2$ solution. The solution was mixed thoroughly, incubated for 5 min, and 0.3 mL $AlCl_3$ (10%) was added. Then, after 5 min, 2 mL of 1.0 M NaOH was added and homogenized for 5 min. Absorbance was determined at λ_{max} 510 nm. The total flavonoid content was expressed as mg of quercetin equivalent per gram of dry weight (mg QE/g sample). All the samples were analyzed in triplicate.

Extraction of P. canescens using solvents of step-wise increased[21]
The fresh leaves of *P. canescens* (0.5 kg) was extracted by maceration method intially using n-hexane for 72 h. The residue was further extracted using solvent with increased polarity; ethyl acetate and finally extracted using highly polar solvent; methanol. Each of the extraction step was repeated three times. Filtrates from each solvent were concentrated by vacuum evaporator at 60°C to obtain fractions of n-hexane, ethyl acetate and methanol.

Anti-cholesterol activity test
The anti-cholesterol activity of ethanol and methanol extracts, as well as each fraction (n-hexane, ethyl acetate, and methanol), was determined using the photometric method where cholesterol is reacted with the Liebermann-Burchard reagent.[22, 23] Simvastatin was used as a positive control. The concentrations of the extracts used were 50, 100, 200, 300, 400, and 500 µg/mL, while the concentrations of the simvastatin used were 10, 20, 30, 40, 50, 75, and 100 µg/mL. The samples (2.5 mL) were tested by the addition of 2.5 mL cholesterol standard (100 µg/mL), then shaken for 2 min, and 2 mL of acetic anhydride and 0.1 mL of sulfuric acid were added. After incubation in the dark for 16 min at room temperature, the absorbance of the solution was measured at 620 nm. The experiments were repeated three times and the percentage of inhibition was calculated using the following formula:

$$\% \text{ Inhibition} = \frac{\text{A control} - \text{A sample}}{\text{A control}} \text{ x } 100$$

From the percentage inhibition values, a curve was plotted between the sample concentration against the per cent inhibition.

Statistical Analysis
Measurements were made in triplicate. Data are provided as mean ± SD. Data were analyzed statistically using ANOVA (α 0.05), followed by the Duncan New Multiple Range Test (DNMRT) at $P < 0.05$. The IC_{50} value was determined based on the linear regression curve, showing the relationship between concentration and inhibition value of extracts and fractions performed using Microsoft excel.

Results and Discussion

Total phenolic, flavonoid, and steroid contents in P. canescens leaves extracts.
The extraction of active compounds in plants can be carried out by the maceration method. In the selection of solvent type, several factors, including selectivity, ability to extract, toxicity, and ease of solvent evaporation are considered.[24] Ethanol and methanol are polar solvents that dissolve in water and other organic solvents, hence can dissolve water-soluble components. Extracts of plant compounds are always a mixture of different classes of secondary metabolites that are selectively soluble in various solvents. The use of an alcoholic solution as solvent provides satisfactory results in the extraction process. Total steroid, phenolic, and flavonoid contents and the anti-cholesterol activity of each extract were determined.
Total steroid content was determined based on a standard curve of cholesterol (Figure 1). The total steroids in the ethanol extract and methanol extract of the leaves were 108.29 ± 0.03 and 82.23 ± 0.39 mg CE/g sample, respectively, according to the linear regression equation y = 0.0066x + 0.0497 (Table 1). These results indicate that the total steroid content in the ethanol extract was higher than in the methanol extract. This can be explained by the fact that ethanol has a slightly lower polarity than methanol, which improves the suitability of steroid class compounds for use in ethanol as a solvent. Steroids were reported to have antiinflammatory,[25] antiviral,[26] antimalarial, [27] antibacterial,[28] and inhibitory activity on cholesterol synthesis.[29]
The total flavonoid content was determined using the quercetin standard calibration curve. Based on the linear regression curve of quercetin, y = 0.033x + 0.0616 (Figure 1), the total flavonoid contents of ethanol and methanol extracts of *P. canescens* leaves were 8.23 ± 1.41 and 2.671±2.64 mg QE/g of the sample, respectively. Flavonoids are polyphenol compounds that are widely distributed in the plant, both in the form of polar glycosides and less polar flavonoid aglycones such as isoflavones, flavanones, and flavones, and flavanols.[30] The result indicates a higher total flavonoid content in the ethanol solvent than in the methanol. The total phenolic content was determined by the Folin Chiocalteu reaction using gallic acid as a standard (Figure 1). Total phenolic content is measured in milligrams of gallic acid equivalent per gram of sample (mg GAE/g). Based on the linear regression curve of gallic acid, y = 0.0316x - 0.0024, the total phenolic contents of ethanol and methanol extracts of Sungkai leaves were 13.00±1.48 and 14.47±1.41 mg GAE/g sample, respectively. The total phenolic content was higher in the methanol extract compared to the ethanol extract.

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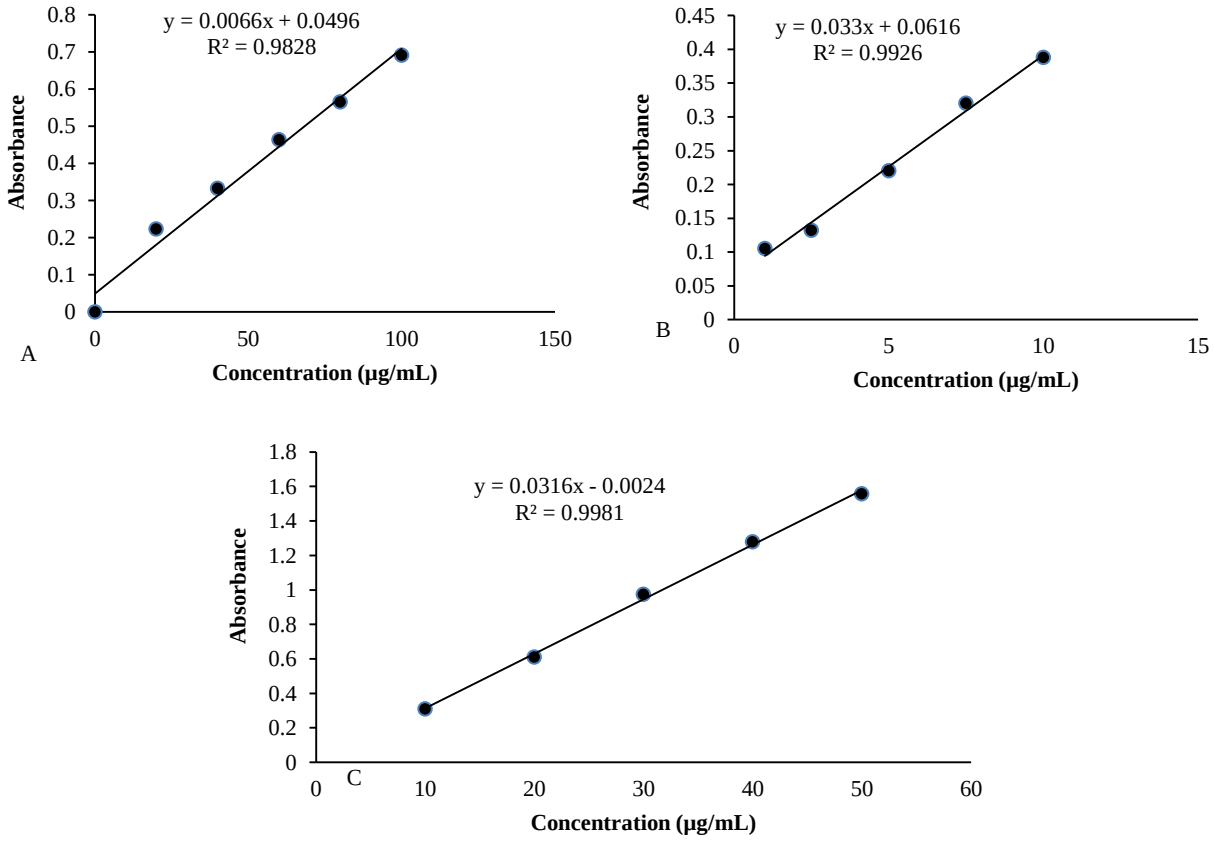

Figure 1: Standard curves of cholesterol (A), Quercetin (B) and Gallic acid (C)

Table 1: Quantitative phytochemical Analysis of ethanol and methanol extracts.

Compounds	Ethanol extract	Methanol extract
Steroid	108.29 ± 0.03	82.23 ± 0.39
Phenolic	13.00 ±1.48[a]	14.47 ± 1.41[a]
Flavonoid	8.23 ± 1.41	2.671 ± 2.64

The data were presented as mean ± SD; n=3; Number followed by the same superscript indicated a non-significantly difference according to the Duncan New Multiple Range Test (DNMRT) at 0.005.

This observation can be explained by the fact that phenolic group of chemicals are extremely polar, making them more soluble in methanol than in ethanol. Statistical analysis showed that there was no significant (p > 0.05) difference among the solvents. Based on literature study the crude extract is usually reported in methanol solvents, but the application to *in vivo* tests uses ethanol solvents because ethanol has low toxicity compared to methanol solvent.[31-34] Ethanol was considered a better filter since it is more selective, moulds and yeasts are difficult to grow in ethanol, it is non-toxic, neutral, and has good absorption. Alcohol, such as ethanol and methanol, is an organic solvent that is widely used in foods, pharmaceuticals, chemical synthesis, and other applications. Both are becoming a growing public health concern; however, methanol is extremely toxic and unsafe for human consumption.[35] Medini *et al*.,[26] also obtained higher levels of total phenolics in the methanol extract of *Limonium delicatulum* flowers (46.5 ± 1.81) than in the ethanol extract (29.58 ± 7.21). Also, Koffi *et al*. [37] reported that when extraction using the solvents ethanol and methanol was carried out, ethanol had a higher total phenolic content than methanol from 23 Ivorian plants.

Anti-cholesterol activity test result

The anti-cholesterol activity was determined based on the calculation of percentage inhibition (% I), as presented in Table 2. For both the ethanol and methanol extracts, the lower the absorbance value, the higher the percent inhibition value. A higher percent inhibition value indicates a higher anti-cholesterol activity. Also, the percent inhibition value of methanol extract at concentrations of 300 and 200 µg/mL based on the statistical analysis were not significantly (P> 0.05) different. The ethanol extract had a higher percent inhibition than the methanol extract at the same concentration. Statistical analysis revealed that the % inhibition value of the ethanol and methanol extracts differed significantly (P>0.05) at the same concentration. The anti-cholesterol activity of ethanol extract was higher than methanol extract. Simvastatin, a typical anti-cholesterol, had a percent inhibition of 57.6 ± 2.3 at a concentration of 50 g/mL (Figure 2), while the ethanol extract of *P. canescens* leaves had a percent inhibition of 15.18 ± 0.36. These observations indicated that the anti-cholesterol activity of ethanol extract was weaker compared to standard simvastatin. The anti-cholesterol activity of ethanol extract is weaker than standard simvastatin because the crude extract of ethanol extract contains various compounds. These other compounds are causing weak activity because the amount of active anti-cholesterol compounds is lower than simvastatin. To increase its activity, the mixture was subjected to further extraction using solvents with increase polarity (n-hexane, ethyl acetate and methanol) to obtain fractions. In the anti-cholesterol activity of fractions (Table 3), the n-hexane fraction demonstrated that at various concentrations, there was no significant (P > 0.05) difference in the percent inhibition with increasing sample concentration.

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Table 2: Anti-cholesterol activity of *Pcanescens* leaf extracts

Concentration (µg/mL)	Absorbance		% Inhibition	
	Extract methanol	Extract ethanol	Extract methanol	Extract ethanol
50	0.678 ± 0.001	0.648 ± 0.001	11.25 ± 0.64^{e}	15.18 ± 0.36^{i}
100	0.537 ± 0.001	0.522 ± 0.022	29.71 ± 0.07^{d}	31.68 ± 2.92^{d}
200	0.438 ± 0.002	0.468 ± 0.001	42.67 ± 0.26^{c}	38.72 ± 0.13^{h}
300	0.413 ±0.002	0.308 ± 0.002	45.94 ± 0.20^{c}	59.69 ± 0.28^{a}
400	0.377 ±0.001	0.267 ± 0.003	50.65 ± 0.08^{b}	65.05 ± 0.33^{g}
500	0.322 ± 0.008	0.163 ± 0.002	57.85 ± 0.99^{a}	78.66 ± 0.35^{f}

The data were presented as mean ± SD; n = 3; Number followed

Table 3: Anti-cholesterol activity of *P. canescens* leaf extract fractions

Concentration (µg/mL)	Absorbance ± SD			% Inhibition ± SD		
	n-Hexane ftaction	Ethyl acetate fraction	Methanol fraction	n-Hexane fraction	Ethyl acetate fraction	Methanol fraction
50	0.658 ± 0 .002	0.488 ± 0.010	0.565 ± 0.232	19.16 ± 0.19^{d}	44.48 ± .03^{h}	30.59 ± 0.20^{b}
100	0.634 ± 0.007	0.446 ± 0.017	0.515 ± 0.221	22.11 ± 0.89^{cd}	49.26 ± 1.91^{g}	36.73 ±0.22^{a}
200	0.620 ± 0.002	0.427 ± 0.001	0.477 ± 0.117	23.82 ± 0.08^{c}	51.42 ± 0.06^{g}	41.40 ± 0.24^{h}
300	0.588 ± 0.001	0.415 ± 0.004	0.452 ± 0.143	27.76 ± 0,07^{b}	52.78 ± 0.47^{g}	44.47 ± 0.16^{h}
400	0.583 ± 0.003	0.305 ± 0.001	0.375 ± 0.122	28.37 ± 1.81^{b}	65.30 ± 0.11^{f}	53.93 ± 0.24^{g}
500	0.544 ± 0.190	0.217 ± 0.032	0.315 ± 0.011	33.17 ± 0.19^{a}	75.31 ± 3.68^{e}	61.30 ± 0.17^{f}

The data were presented as mean ± SD; n = 3; Number followed by the same superscript indicated a non-significantly difference according to the Duncan New Multiple Range Test (DNMRT) at 0.005.

The ethyl acetate fraction showed a higher percentage inhibition value than the other fractions at the same concentrations. Based on the percentage inhibition data, the IC_{50} value was determined (Figure 3).
The IC_{50} value is a concentration that can inhibit 50% cholesterol, and it was determined based on the linear regression equation between concentration (X) and percentage inhibition value (Y). Also, the ethanol extract had a smaller IC_{50} value (274.14 µg/mL) than the methanol extract (374.26 µg/mL). Meanwhile, the inhibition of the ethyl acetate fraction (IC_{50} 156.43 g/mL) was lower than that of the n-hexane fraction (IC_{50} 1116.22 g/mL) and the methanol fraction (IC_{50} 340.84 g/mL). The IC_{50} value of 24.68 g/mL was calculated by linear regression using conventional simvastatin (Figure 2) as a positive control. A higher anti-cholesterol activity was observed in the ethanol extract compared to the methanol and other fractions. Ethyl acetate fraction showed the highest anti-cholesterol activity, but the activity was weaker in relation to the anti-cholesterol standard, simvastatin, which had an IC_{50} value of 24.68 µg/mL.
These findings indicate that the semi-polar components in the plant determines the anti-cholesterol activity. Muharni *et al*.[15] reported that the pure compound isolated from the n-hexane fraction of Sungkai leaves, namely betulonic acid, had an IC_{50} value of 60.53 g/mL.
Betulonic acid belongs to a type of triterpenoid that tends to be a polar component, so it was suspected that betulonic acid was also present in the ethyl acetate fraction since the fractionation was carried out by maceration using solvent with step-wise increasing polarity instead of partition method. Hence the anti-cholesterol activity of the ethyl acetate fraction is higher than other fractions. This finding indicates that the simpler the form of the component mixture, the higher the activity. The secondary metabolites tannins, saponins, and flavonoids lower cholesterol levels.[38] The compounds are able to scrape cholesterol deposits off the inside walls of blood vessels, so it prevents the occurrence of hypertension, stroke, and heart disease. Cholesterol can interact with carbonyl and hydroxyl groups of flavonoid compounds.[14] The carbonyl group on the flavonoid will generate hydrogen bonds with the hydroxyl groups on cholesterol to form hydrogen bonds.

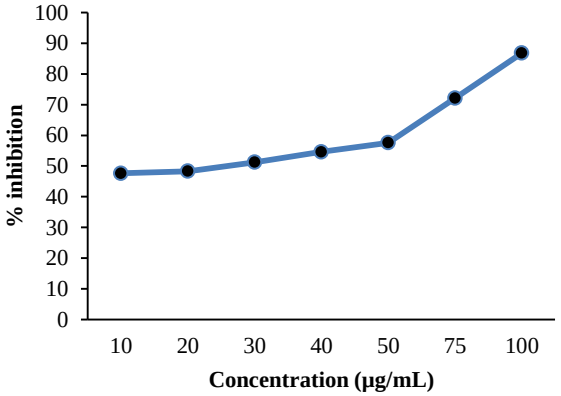

Figure 2: Percentage inhibition of Simvastatin cholesterol

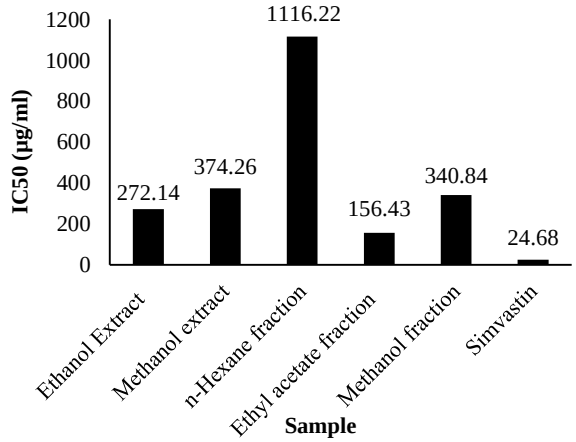

Figure 3: IC_{50} value of extracts and fractions

Cholesterol will react with anhydrous acetic acid and sulfuric acid in its free state.[39] Studies using animal models of atherosclerosis showed that ingestion of dietary flavonoids delays the development of atherosclerotic plaques.[40] Black tea, which is rich in polyphenols and flavonoid compounds, is also reported to prevent atherosclerosis. The increased anti-cholesterol activity of ethanol extract over methanol extract was assumed to be due to the higher flavonoid content of ethanol extract. This observation is in agreement with the study of Masfria *et al*.,[38] who reported that secondary metabolites such as tannins, saponins, and flavonoids have the ability to lower cholesterol levels *in vitro.* This is in line with the assertion of Masfria *et al*.,[38] that secondary metabolites such as tannins, saponins, and flavonoids have the ability to lower cholesterol levels *in vitro*. In a study involving the potential of triterpenoid compounds to lower cholesterol, Bachmid *et al*.,[41] found that an ethanol extract of patikan leaves containing triterpenoid compounds had anti-cholesterol efficacy at doses of 30 mg/kg BW, resulting in a 71 percent reduction in cholesterol levels. Musa *et al*., [23] reported two triterpenoid compounds from *Saurauia vulcani* Korth, which demonstrated an exceptional anti-cholesterol activity. The anti-cholesterol properties of this compound are influenced by the presence of hydroxyl groups, double bonds, and carboxylic acid. In previous studies, it has also been reported that betulonic acid from an n-hexane extract of Sungkai leaves had an anti-cholesterol activity with a value of IC_{50} 60.53 µg/mL. However, the anti-cholesterol activity of the extract was significantly lower than that of the pure substance. However, for the extract, the anti-cholesterol activity was much weaker than the pure compound. This shows that the anti-cholesterol properties are much higher in the pure compound than in the mixed condition. The decreased activity of extracts compared to fractions or pure compound specifies that other components in the mixture lower their anti-cholesterol activity.

Conclusion

In this study, the findings indicated that the ethanol extract of *P. canescens* leaves has higher anti-cholesterol activity than the methanol extract, whereas the anti-cholesterol activity of ethyl acetate fraction was higher than that of n-hexane and methanol fractions. The anti-cholesterol activity of *P. canescens* leaves has revealed its potential as a natural source of anti-cholesterol agents.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article are original and that any liability for claims relating to the content of this article will be borne by them.

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