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Ethnopharmacognosy study, antioxidant activity, and chemical content in chicken bile

Slamet Tuty[a*], Irda Fidrianny[b], Sukrasno[b]

[a]Departement of Pharmacy, Al-Ghifari University, Bandung, West Java Indonesia
[b]Department of Pharmaceutical Biology, School of Pharmacy, Bandung Institute of Technology, Bandung 40132, West Java, Indonesia

ABSTRACT

The aim of this research was to study the ethnopharmacognosy, antioxidant activity, and chemical content of chicken bile. In addition, a bioactivity-guided isolation was also performed to identify compounds in chicken bile having antioxidant activity. Extraction was carried out by reflux method. Each extract was tested for its antioxidant activity using DPPH and CUPRAC methods. Fractionation was performed on selected extract, namely ethyl acetate extract, using vacuum liquid chromatography and subfraction using preparative TLC method and classical column chromatography (CCC). Compound **1** was characterized and identified by NMR, compound **2** was characterized and identified by LC-MS/MS and NMR, subfraction **7** was characterized by GC-MS. The result of the antioxidant activity of ethanol extract of chicken bile using the DPPH method (IC_{50}: 46.64 g/mL), ethyl acetate extract (IC_{50}: 69.99 g/mL), n-hexane extract (IC_{50}: 71.65 g/mL, fraction 15-17 (IC_{50}: 88.77 g/mL) showed strong antioxidant, whereas by CUPRAC method all extracts (EC_{50}: 69.90, EC_{50}: 80.28, EC_{50}: 70.40) and fraction (EC_{50}: 64.53) are strong antioxidants. The result of identification of compound **1** by NMR was identified as cholesterol, identification of compound **2** by LC-MS/MS was (*E*)-hexadecyl-ferulate and subfraction **7** which was identified by GC-MS produced dominant compounds, namely oleic acid, n-hexadecanoic acid, octadecanoic acid and palmitoleic acid. In conclusion, extract, fraction 15-17 and subfraction of chicken bile have antioxidant activity where the active antioxidant compound is a phenol group compound, namely (*E*)-hexadecyl-ferulate. The other chemical constituents found in chicken bile are, oleic acid, n-hexadecanoic acid, octadecanoic acid, and palmitoleic acid.

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*Corresponding authors:
tuti.slamet@yahoo.co.id

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1. Introduction

Antioxidant compounds have a very important role for health (BPOM RI, 2000) and various scientific evidences show that antioxidant compounds reduce the risk of various chronic diseases, such as cancer, coronary heart disease, and diabetes where the main character of antioxidant compounds is their ability to ward off free radicals (Thaipong et al., 2006). Chicken bile empirically used to treat diabetes, malaria, asthma, and increase stamina (Dehpour et al., 2009), but the results are still questionable because they cannot be scientifically justified. Moreover, since the development of chicken bile research are still lacking because of it usually turns into a waste, also until now there has been no specific research on the management of chicken bile waste for the benefit of humans, here we developed the extraction until isolation method to figure out the chemical content of chicken bile which has high antioxidant activity.

2. Materials and methods

2.1. Materials

Chicken bile (Fig. 1), freeze dryer, TLC plate, ethanol 96%, DPPH (2,2-diphenyl-1-picrylhydrazyl), gallic acid, quercetin was purchased from Sigma-Aldrich (MO, USA). Other chemicals used were analytical grade.

Fig. 1. Chicken bile

2.2. Methodology

2.2.1. Sample preparation

Chicken bile was collected from Brebes city, Central Java. It was cut into small size and dried using freeze dryer and then stored in a dry bottle.

2.2.2. Extraction of chicken bile

Three hundred grams of powdered sample was extracted by reflux using different polarity solvents. First, sample was refluxed using n-hexane and repeated three times. The remaining residue was then extracted three times by using ethyl acetate. Finally, the residue was extracted using ethanol. In the end, there were n-hexane extract, ethyl acetate extract, and ethanol extract.

2.2.3. Determination of total phenolic content (TPC)

TPC was evaluated using Folin-Ciocalteu reagent with gallic acid (55 - 120 μg/mL) as a standard solution. The procedure was referred from the other investigation (Pourmorad et al., 2006; Prior et al., 2005). The absorbance of tested samples was observed at λ 765 nm. TPC was stated as gallic acid equivalent per 100 g extract (g GAE/100 g) (Ravipati et al., 2012).

2.2.4. Determination of total flavonoid content (TFC)

TFC was evaluated with minor modifications from another research (Chia-Chi et al., 2002). Quercetin (40 – 100 μg/mL) was utilized as a standard solution to obtain a calibration curve. The absorbance was assessed at λ 415 nm. TFC was expressed as quercetin equivalent per 100 g extract (g QE/100 g).

2.2.5. Antioxidant activity by DPPH assay

The antioxidant activity test was determined by the DPPH method by determining the IC_{50} value. Each chicken bile extract was made of several concentrations, then 1 mL of sample solution was added with 1 mL of 50 μg/mL DPPH solution. The mixture was then incubated for 30 min and the absorbance measured at λ 515 nm. Methanol was used as a blank, DPPH solution of 50 μg/mL as a negative control, and ascorbic acid as a positive control. The IC_{50} value is determined through the linear regression equation from the calibration curve, namely the percentage of curing as the y-axis and the concentration of antioxidants as the x-axis. The IC_{50} value is calculated by entering the 50% value into the regression equation as the y value, then the x value is calculated as the IC_{50} concentration (Sukrasno et al., 2017).

2.2.6. Antioxidant activity by CUPRAC assay

Determination of antioxidant activity using the CUPRAC method was carried out using a modified Apak's method (Apak et al., 2007). The 100 μg/mL CUPRAC solution was prepared by mixing 1705 ppm copper (II) chloride with 1562 ppm neocuproine in a 1:1 ratio to obtain a Cu(II)-Nc solution. Then the Cu(II)-Nc solution was diluted using ammonium acetate buffer pH 7. Each sample was made into several concentrations in methanol for 1 mL and then mixed with 1 mL CUPRAC solution (100 μg/mL). The mixture was then incubated for 30 min in a dark room and the absorbance was measured at λ 450 nm. Ammonia acetate buffer was used as a blank, CUPRAC solution 100 μg/mL as a negative control and ascorbic acid as a positive control. The CUPRAC capacity was measured as the percent increase in the absorbance of CUPRAC after addition of the extract. The EC_{50} value is calculated using the linear regression equation of the calibration curve for each sample.

2.2.7. Calculation of activity antioxidant index (AAI)

DPPH scavenging activity and CUPRAC of chicken bile extract were presented as AAI. The estimation of AAI was conducted by the equation below (Scherer and Godoy, 2009):

AAI = final concentration of radical solutions (μg/mL) / IC_{50} or EC_{50} (μg/mL).

2.2.8. Active compound isolation

The selected extract next were fractionated using vacuum liquid chromatography (VLC) then the TLC profile was monitored and the qualitative antioxidant test by the appearance of DPPH spots. After that, it was sub-fractionated by column chromatography, the chromatography monitored by TLC and sprayed with the appearance of DPPH spots. Further purification was carried out by preparative TLC. The isolates obtained were monitored by TLC with visible DPPH spots. The purity test was carried out by the single development TLC method using 3 different mobile phases of polarity. The results of TLC were observed under UV light λ 254 nm and sprayed with spots of sulfuric acid. Characterization and identification of the active compounds of chicken bile extract were carried out by magnetic resonance spectrometry and mass spectrometry.

2.2.9. Statistical analysis

All study results are expressed as mean ± standard deviation. Analysis of variance was performed using Tukey's one-way ANOVA - post hoc procedure (p value <0.05). Pearson correlation coefficient (r) analysis was used to determine the correlation between treatments. The processing of statistical analysis was carried out using SPSS 16.0 (IBM, USA).

3. Results and discussion

3.1. Antioxidant activity of chicken bile extract using DPPH and CUPRAC methods.

In Table 1, it shows that the n-hexane extract and ethyl acetate extract of chicken bile have moderate antioxidant activity with an AAI value of 0.698 ± 0.001 and 0.714 ± 0.001, while the ethanol extract of chicken bile has strong antioxidant activity with an AAI value of 1.071 ± 0.004 and it can be seen that n-hexane extract, ethyl acetate extract, and ethanol extract of chicken bile has moderate antioxidant activity with AAI CUPRAC values in the range 0.62 - 0.75.

Table 1. 'Total antioxidant capacity' of different extracts of chicken bile

Chicken bile extract	Antioxidant capacity assay by DPPH		Antioxidant capacity assay by CUPRAC	
	IC_{50} attenuation DPPH (μg/mL)	AAI value	EC_{50} attenuation CUPRAC (μg/mL)	AAI value
n-hexane extract	71.65 ± 0.190 ^{a}	0.70 ± 0.001 ^{a}	66.90 ± 0.49 ^{a}	0.75 ± 0.009 ^{a}
Ethyl acetate extract	69.99 ± 0.150 ^{a}	0.71 ± 0.001 ^{a}	80.28 ± 0.02 ^{b}	0.62 ± 0.003 ^{b}
Ethanol extract	46.64 ± 0.200 ^{b}	1.07 ± 0.004 ^{b}	70.40 ± 0.50 ^{a}	0.71 ± 0.001 ^{a}
Fraction 15-17	88.77 ± 0.390 ^{a}	0.56 ± 0.002 ^{a}	64.53 ± 0. 50 ^{a}	0.77 ± 0.005 ^{a}
Ascorbic acid	3.50 ± 0.005 ^{c}	14.26 ± 0.023 ^{c}	4.16 ± 0.10 ^{c}	12.00 ± 0.319 ^{c}

Note: a-c: different letters indicate that there are significant differences (p<0.05). AAI = Calculation of activity antioxidant index. The result is expressed as (mean±SD)

3.2. Determination of total phenol and flavonoid levels

Based on the results of total phenol determination. the highest total phenol was obtained by the ethanol extract of chicken bile (1.96 g ± 0.03 GAE/100 g). Data processing was performed statistically using one-way ANOVA – Tukey. The results showed that the total phenol in the ethyl acetate extract of chicken bile was not significantly different from the ethanol extract of chicken bile, but both were significantly different from the n-hexane extract of chicken bile ($p < 0.05$).

Based on the results of determination of total flavonoids, it was found that the highest total flavonoid levels were found in the ethanol extract of chicken bile (9.48 ± 0.016 g QE/100 g). Data processing was performed statistically using one-way ANOVA – Tukey, and it was found that the total flavonoids in the n-hexane extract of chicken bile were not significantly different from the ethanol extract of chicken bile, but both were significantly different from the ethyl acetate extract of chicken bile ($p < 0.05$).

The results of the determination of total phenol and flavonoid levels summarized in Table 2.

Table 2. Total phenolic and flavonoid content in different extract of chicken bile

Chicken bile extract	Total phenols (g GAE/100g)	Total flavonoids (g QE/100g)
n-hexane extract	1.33 ± 0.01 [a]	8.52 ± 0.08 [a]
Ethyl acetate extract	1.88 ± 0.03 [b]	6.42 ± 0.06 [b]
Ethanol extract	1.96 ± 0.03 [b]	9.48 ± 0.16 [a]

Note: a-b: Different letters in one column indicates significant difference ($p<0.05$). The result is expressed as (mean±SD)

The correlation between total phenol and total flavonoids in chicken bile extract against AAI DPPH and AAI CUPRAC was statistically tested using the Pearson method. The results of statistical tests were summarized in Table 4. The correlation between total phenol and total flavonoids with the AAI value and the correlation between the antioxidant method was indicated by a positive and significant correlation value. The correlation between the two antioxidant test methods, DPPH and CUPRAC, was statistically tested using the Pearson method. The results of statistical tests were shown in Table 3 and Table 4

Table 3. Correlation between total phenol. total flavonoid and AAI DPPH. AAI CUPRAC of chicken bile

Antioxidant parameter	Person Correlation Coefficient (r)	
	Total Phenol	Total Flavonoid
AAI DPPH	0.628*	0.722*
AAI CUPRAC	0.653*	0.816**

Note: * = Significant at $p<0.05$; ** = Signifiacnt at $p<0.01$

Table 4. Correlation between two antioxidant methods

Antioxidant parameter	Person Correlation Coefficient (r)
	AAI CUPRAC
AAI DPPH	1.000**

Note: ** = Significant at $p<0.01$

3.3. Isolation of active compounds

The purity test of compound **1** by single development TLC with 3 mobile phases with different polarity showed a single isolate. Furthermore. compound **1** was characterized and identified by Nuclear Magnetic Resonance (NMR) The ^{1}H and ^{13}C NMR spectra of compound **1** shown in Fig. 2. The ^{13}C NMR of compound **1** was compared with the literature (Kalinowski et al., 1988) as shown in Table 5. The results of the confirmation of ^{13}C NMR compound **1** and ^{13}C NMR of cholesterol showed a match. and it can be concluded that compound **1** was cholesterol.

Table 5. The confirmation of ^{13}C NMR of compound **1** and cholesterol

No	^{13}C NMR of compound 1 (ppm)	^{13}C NMR of cholesterol (ppm) (Kalinowski et al., 1984)
1	37.4	37.5
2	31.8	31.6
3	72.0	71.3
4	42.4	42.4
5	140.8	141.2
6	121.9	121.3
7	32.1	32.0
8	32.1	32.3
9	50.3	50.5
10	36.7	36.5
11	21.2	21.2
12	39.9	40.0
13	42.4	42.4
14	56.9a	56.9
15	24.5	24.3
16	28.4	28.3
17	56.3	56.5
18	12.0	12.0
19	19.6	19.4
20	35.9	35.4
21	18.9	18.8
22	34.3	36.5
23		24.1
24	39.7	39.8
25		28.3
26	23.0	22.8
27	22.7	22.8

The purity test of compound **2** by single development TLC with 3 different mobile phases of polarity showed that the isolate was single and had antioxidant activity. The compound **2** was identified by LC-MS / MS and the result was shown as (E) -hexadecyl-ferulate ($C_{26}H_{42}O_4$) (Fig. 3) which is known as an antioxidant. The results of LC-MS/MS and mass of compound **2** were shown in Fig. 4 and Fig. 5, respectively.

Compound **2** was also characterized and identified by Nuclear Magnetic Resonance (NMR) (Fig. 2). The comparison of ^{13}C NMR compound **2** with the reference (Nadal et al., 2018) were shown in Table 6.

Table 6. The confirmation of ^{1}H and ^{13}C NMR of compound **2** with the reference

No	Compound 2		Reference (Nadal et al., 2018)	
	^{1}H	^{13}C	^{1}H	^{13}C
1	0.88	11.11	0.88	12.50
2	4.05	38.89	3.95	55.93
3	1.72	22.85	1.72	22.70
4	4.22	68.33	4.22	64.62
5		178.7		167.39
6	6.74	114.63	6.29	114.71
7	7.71	130.16	7.63	144.63
8		127.27		127.65
9	7.12	114.21	7.08	109.30
10		139.41		147.90
11		131.02		146.78
12	7.11	114.70	7.05	115.67
13	6.75	114.79	6.94	123.04

(a)

(b)

Fig. 2. Characterization and identification by NMR of compound **1** (a) and compound **2** (b)

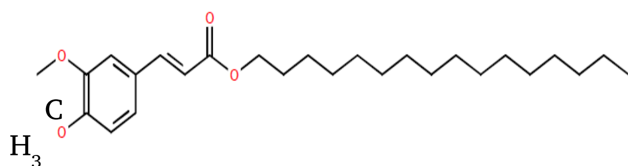

Fig. 3. Chemical formula E-hexadecyl-ferulate (($C_{26}H_{42}O_4$))

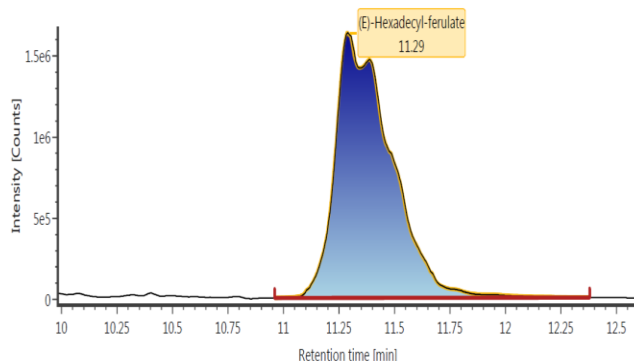

Fig. 4. Chromatogram pattern of isolate Y

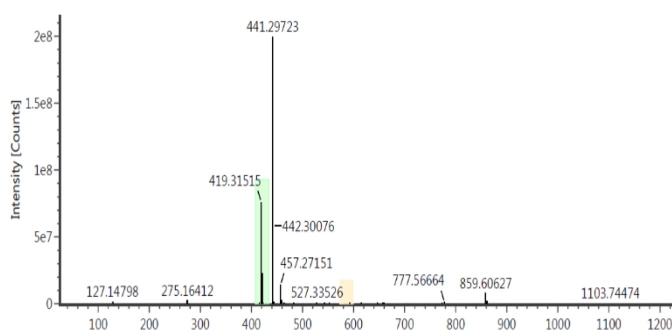

Fig. 5. Mass (m/z) of isolate Y

Peak	R time	%	Name
33	22,400	38,72	Oleic Acid
30	20,596	35,6	N-Hexadecanoic Acid
34	22,574	17,92	Octadecanoic Acid
29	20,328	1,53	Palmitoleic Acid

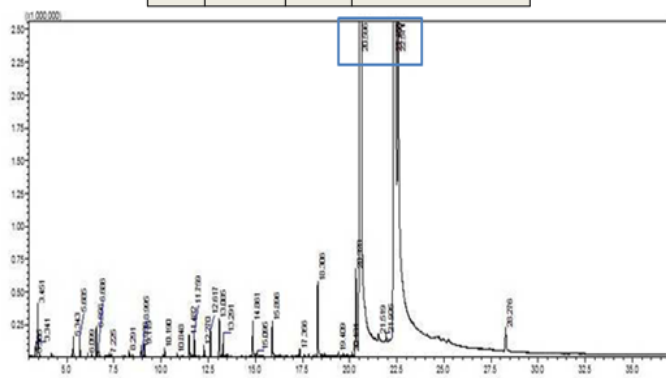

Fig. 6. Characterization and identification of subfraction 7 by GC-MS

In the next fractionation with VLC, there were 20 fractions obtained. Fraction 2-7 were sub-fractionated with VLC to yield 24 subfractions. Of them, subfraction 7 was selected for identification using Gas Chromatography-Mass Spectrometry (GC-MS) (Li et al.. 2015). The results showed that subfraction 7 contained 4 dominant compounds, which are oleic acid (38.72%) at 22.4 min of retention time, n-hexadecanoic acid (35.6%) at 20.596 min, octadecanoic acid (17.94%) at 22.574 min, and palmitoleic acid (1.53%) at 20.328 min (Fig. 6)

4. Conclusion

The extracts of n-hexane. ethyl acetate. ethanol of chicken bile have antioxidant activity. Among the fractions of chicken bile. fractions 15-17 have antioxidant activity. Chicken bile subfraction has antioxidant activity and contains active antioxidant compounds. The active antioxidant compound is a phenol group compound, namely (E)-Hexadecyl-ferulate. Whereas other chemicals found in chicken bile are cholesterol, oleic acid, n-hexadecanoic acid, octadecanoic acid, and palmitoleic acid.

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Conflict of interest

The authors declare that there is no conflict of interest.

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