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Antioxidant profile of chicken bile extract and correlation with chemi cal content

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ABSTRACT

Component in chicken bile may be similar to bear bile, which contains an immunostimulant agent ursodeoxycholic acid and related to antioxidant effect. The purposes of this study were to evaluate the antioxidant potential of chicken bile extract by the DPPH method, analyze the correlation between total phenolic and flavonoid content with IC_{50} of DPPH scavenging activities. Chicken bile was cut and dried using freeze dryer, then extracted by reflux using different polarity solvents. A rotary evaporator was used to concentrate the extracts. Calculation of total flavonoid and phenolic content, antioxidant potential using DPPH assay were carried out by UV-visible spectrophotometry, and Pearson's method was used to analyze their correlation. IC_{50} of DPPH of ethanolic chicken bile extract was 46.64 μg/ml, while ethyl acetate and n-hexane extracts were 69.99 μg/ml and IC_{50} 71.65 μg/ml, respectively. The highest phenolic content and flavonoid content were given by ethanolic chicken bile extract. The total phenolic and flavonoid content in chicken bile extracts had a significant and negative correlation with IC_{50} of DPPH. All chicken bile extracts are potential antioxidants by DPPH assay. Phenolic and flavonoid compounds in chicken bile extracts contributed together in antioxidant capacity by DPPH assay.

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INTRODUCTION

Bear bile has been widely used in traditional Chinese medicine for thousands of years. The research showed that bear bile had pharmacological benefits with minor side effects and consisted of a pure compound which used to cure liver and bile abnormalities. In Chinese medicine formulas, bear bile can also be used for diabetic, nephritis, hemorrhoids, chronic hepatitis (Feng *et al.*, 2009). Bear bile mainly contained bile acids, amino acids, bile pigments, fats and some phospholipids (Feng *et al.*, 2009). The other studies expressed that bear bile contained ursodeoxycholic acid (UDCA) (Bachrach and Hofmann, 1982). UDCA is relatively low content in bear bile and can't be detected by chromatography (Qiao *et al.*, 2011; Wang *et al.*, 2011). UDCA has been used in the treatment of gallbladder stones as an alternative to cholecystectomy (Tint, 1982). However, due to the wide consumption of bear bile, the bear species was endangered. In 2008 the Chinese government ended bear farming for its bile. They provided care and protection for the remaining bears and prohibited the using bear bile in traditional medicine products. The Chinese government will continue to maintain its policy to ban the export of bear bile. At the same time, research must be conducted to look for other alternatives.

The present study has chosen chicken bile which was a useless waste product. Therefore, it has additional value. Components in chicken bile might be similar to bear bile. UDCA had many effects, such as immunomodulator (Yoshikawa *et al.*, 1992) and cytoprotection (Heuman *et al.*, 1991). Two types of immunomodulators are immunostimulant and immunosuppressant. Immunostimulant is related to antioxidants. Antioxidant compounds have a very important role in health and associated with many degenerative diseases such as cancer, heart disease, and diabetic. The major property of antioxidants is its capacity to scavenge free radicals (Prakash *et al.*, 2014). tudy on antioxidant activity of various chicken bile extracts (which are n-hexane, ethyl acetate, and ethanol) from Central Java chicken farms - Indonesia using DPPH, have not been reported yet. This study aims to evaluate the antioxidant potential of chicken bile extracts and correlation with its chemical content.

MATERIALS AND METHODS

Materials

Gallic acid, quercetin, 2,2-diphenyl-1-picrylhydrazyl (DPPH) was purchased from Sigma-Aldrich (MO, USA). Other chemicals used were analytical grade.

Preparation of sample

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

Extraction

Powdered sample 300 g was extracted using increasing polarity solvents by reflux method, repeated three times for each solvent. The first, the sample was extracted using n-hexane. The remaining residue was then continued using ethyl acetate. Finally, the residue was extracted using ethanol. Therefore, there were: n-hexane extract, ethyl acetate extract, and ethanol extract.

DPPH scavenging activity

Each extract was prepared in various concentrations. DPPH assay was used to modified Blois's method (Blois, 1958). The analysis was done in triplicate for each extract and standard. Extract 2 ml was added into DPPH (50 μg/ml) 2 ml. The absorbance was seen at l 515 nm after incubation 30 min. DPPH 50 μg/ml was used as a control. Methanol was used as a blank, meanwhile ascorbic acid as standard. The percentage of DPPH scavenging activity of each concentration was investigated using a reduction of DPPH absorbance (Bedawey *et al.*, 2010). The calibration curve was prepared between the percentage of DPPH scavenging activity versus the concentration of extract. Inhibitory concentration 50 (IC_{50}) of each extract or standard is concentration which can reduce 50% of DPPH absorbance, and can be determined using its calibration curve.

Total phenolic content (TPC)

Gallic acid was used as standard and standard solution 80-170 μg/ml was prepared to obtain a calibration curve. Each extract was analyzed in triplicate. Folin-Ciolcalteu reagent was used to determine total phenolic content (Pourmorad *et al.*, 2006). The absorbance was seen at wavelength 765 nm using UV-Vis spectrophotometer Hewlett Packard 8435. Total phenolic content was exposed as g gallic acid equivalent per 100 g extract.

Total flavonoid content (TFC)

Each extract was prepared triplicate, then analyzed. The method from (Chang *et al.*, 2002) was used to calculate total flavonoid content. The absorbance was measured at wavelength 415 nm. Quercetin was chosen as standard and series of standard solution (20-140 μg/ml) was prepared. The flavonoid content was figured as g quercetin equivalent per 100 g extract.

Statistical Analysis

All results were exposed as means $\pm$ standard deviation. Statistical analysis was conducted using one-way ANOVA and posthoc Tukey using SPSS 16 for Windows, which mean while Pearson's method was used to analyze the correlation between total phenolic, flavonoid, and antioxidant potential.

RESULTS AND DISCUSSION

Determination of animal was done in Herbarium Bandungense - School of Life Science and Technology- Bandung Institute of Technology, showed that the animal used is native chicken from Indonesia, Phylum: Chordata, Class: Aves, ordo: Galliformes, family: Phasianidae, genus: Gallus, species: *Gallus gallus* Lin.

Characterization of chicken bile crude drug exhibited that water content 24%, loss on drying 27%, total ash content 3%, acid insoluble ash 0.5%, water-soluble extractable matter 6% and ethanol soluble extractable matter 15%. Chicken bile was cut into small size and dried using freeze dryer to reduce water content in chicken bile crude drug. Then extraction of the crude drug was performed using three polarities solvents which were n-hexane, ethyl acetate and ethanol to separate compounds in three polarities. The most nonpolar compound will be extracted in n-hexane solvent, the most semi-polar

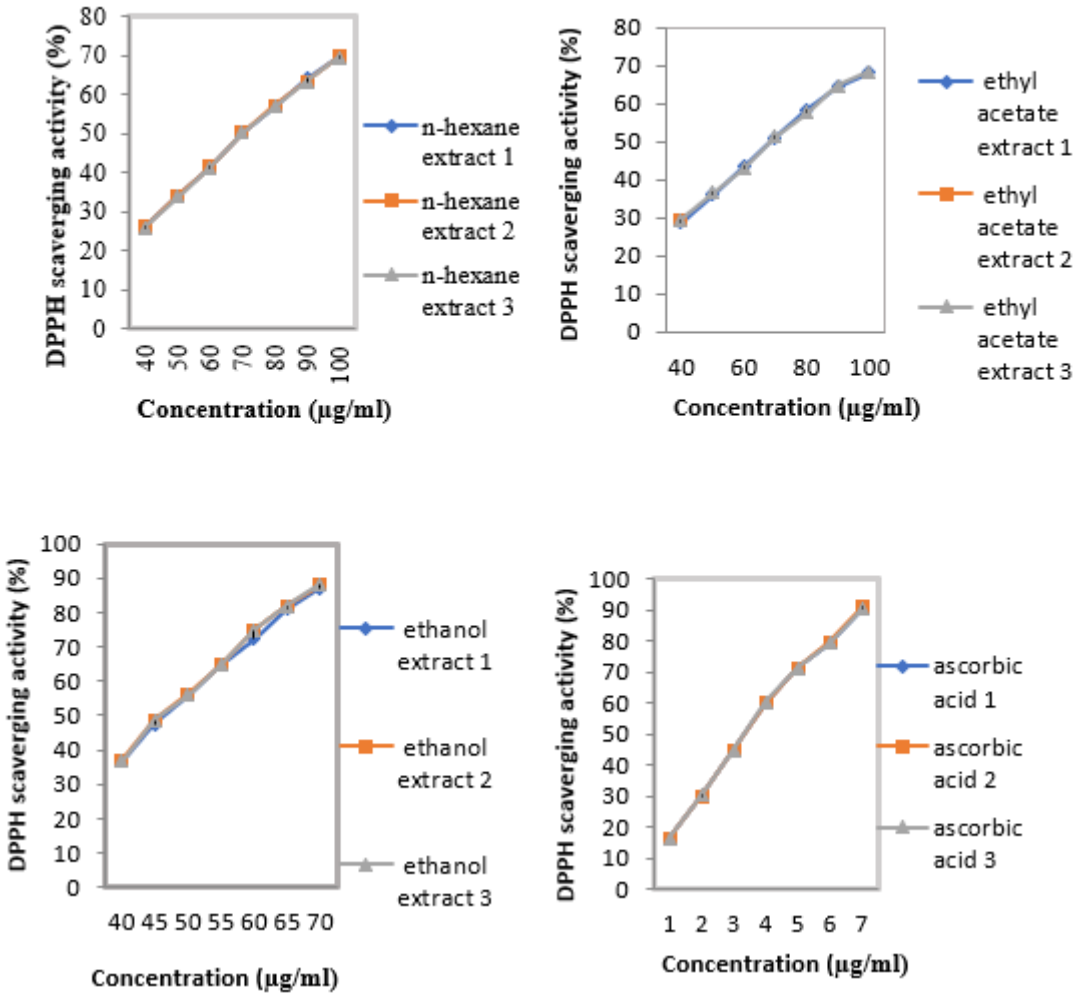

Figure 1: Calibration curve of antioxidant potential of n-hexane, ethyl acetate, ethanol extracts and ascorbic acid standard

compound will be separated in ethyl acetate solvent and finally most polar compound in ethanol.

It was important to prepare the similarity density of all extracts of chicken bile. Therefore the chemical content and antioxidant potential among extracts can be compared. The density of chicken bile extracts was presented as 1% extract and showed similar density which were density (g/ml) of n-hexane extract 0.61, ethyl acetate extract 0.88 and ethanol extract 0.77.

The chemical screening was performed in crude drugs and extracts to find out the presence of secondary metabolites such as alkaloids, flavonoids, tannins, phenols, quinones, saponins, steroids/triterpenoids in crude drug and extracts. The results of the chemical screening can be seen in Table 1. Previous studies (Chen *et al.*, 2012; Gu *et al.*, 1994; Wang, 2014) reported that chicken bile contained taurocholate, taurochenodeoxycholate, and tauroallocholate. Taurochenodeoxycholate is conjugation chenodeoxycholate with taurine. Chenodeoxycholate has pharmacological activity in hypertriglyceridemia, congenital liver diseases, rheumatoid arthritis, and constipation (Broughton, 1994) meanwhile, taurine has antioxidant activity (Jong *et al.*, 2012).

DPPH is one method that is quite popular and often done. The purple colour of DPPH would be changed when antioxidant scavenged the free radicals (Li, 2011) to pale yellow hydrazine compound. The degree of colour change correlated with the concentration of antioxidants in the sample. The antioxidant activity of the sample by DPPH assay can be seen in decreasing of absorbance (Prior *et al.*, 2005). The concentration of sample which can reduce 50% of the absorbance of DPPH free radical was stated

Table 1: Chemical screening of crude drug and chicken bile extract

Sample	Alkaloid	Flavonoid	Phenol	Quinone	Saponin	Tannin	Steroid/Triterpenoid
Crude drug	-	+	+	-	+	+	+
n-Hexane extract	-	+	+	-	-	-	+
Ethyl acetate extract	-	+	+	-	+	-	+
Ethanol extract	-	+	+	-	+	+	+

+ = detected, - = not detected

Table 2: Antioxidant activities in chicken bile extracts

Sample	IC50 of DPPH (μg/ml)
n-Hexane extract	71.65 $\pm$ 0.18 a
Ethyl acetate extract	69.99 $\pm$ 0.15 a
Ethanol extract	46.64 $\pm$ 0.33 b
Ascorbic acid	3.50 $\pm$ 0.005 c

a-c = different subscription letters in a column means significant differences ($p<0.05$)

Table 3: Total phenolic content in chicken bile extracts

Sample	Total phenolic content (g GAE/100 g)
n-Hexane extract	1.33 $\pm$ 0.01 a
Ethyl acetate extract	1.88 $\pm$ 0.03 b
Ethanol extract	1.96 $\pm$ 0.03 b

a-b = different subscription letters in a column means significant differences ($p<0.05$)

Table 4: Total flavonoid content in chicken bile extracts

Sample	Total flavonoid content (g QE/100 g)
n-Hexane extract	8.52 $\pm$ 0.08 a
Ethyl acetate extract	6.42 $\pm$ 0.06 b
Ethanol extract	9.48 $\pm$ 0.16 a

a-b = different subscription letters in a column means significant differences ($p<0.05$)

Table 5: Correlation of phenolic and flavonoid content with antioxidant potential

Parameter	Pearson's correlation coefficient (r) IC50 of DPPH
Total phenolic content	-0.641*
Total flavonoid content	-0.697*

* = significant at $p<0.05$

as $IC_{50.}$ The IC_{50} value is determined through a linear regression equation from the calibration curve, namely the percentage of DPPH scavenging activity as they and the concentration of sample as the x. Various concentrations of each extract were prepared, and each concentration was performed triplicate. Therefore there were three calibration curves. IC_{50} value of each calibration curve was calculated by entering a 50% value into the regression equation as a y value, then calculated the value of x as the concentration of $IC_{50.}$

Based on a calculation using the calibration curve (Figure 1), it can be seen that ethanol extract of chicken bile presented the highest antioxidant activities compared to the other extracts, which presented by the lowest IC_{50} of DPPH (Table 2). The sample was stated as a very strong antioxidant if had IC_{50} lower than 50 mg/ml and strong antioxidant IC_{50} 50-100 mg/ml (Blois, 1958). Based on the classification, the ethanol chicken bile extract was a very strong antioxidant. Meanwhile, n-hexane and ethyl acetate chicken bile extracts were a strong antioxidant.

Statistical analysis using one-way ANOVA- post hoc Tukey showed that IC_{50} DPPH in n-hexane extract of chicken bile was not significantly different with its TFC in ethyl acetate extract, but both were significantly different from ethanol bile chicken extract ($p < 0.05$). IC_{50} of DPPH of all chicken bile extracts were significantly different with IC_{50} DPPH of ascorbic acid. Based on the results in can be seen that the antioxidant activity of ethanol chicken bile extract was one-fifteenth of antioxidant activity of ascorbic acid.

In the present study, total phenolic content (TPC) was investigated by Pourmorad's method (Pourmorad *et al.*, 2006). A sodium carbonate solution was added to provide an alkaline condition. The principle of total phenolic content determination using Folin-Ciocalteu reagent is based on the capacity of the phenolic compound to reduce the phosphotungstate-phosphomolibdenum complex in Folin-Ciocalteu reagent, which occurs in alkaline condition. Incubation 15 min was needed; therefore, the reduction runs completely. The blue chromophore which formed from the reaction was measured using UV-visible spectrophotometry. The alkaline condition and concentration of phenolic compounds in extracts associated with the maximum absorption of the chromophore (Prior *et al.*, 2005).TPC among the various extracts of chicken bile were revealed in term of gallic acid equivalent (Ravipati *et al.*, 2012) using the standard curve equation $y = 0.022 x + 0.033$, $R^2 = 0.997$. The Various extracts of chicken bile showed varied result TPC in the range of 1.33 - 1.96 g GAE/100 g extract (Table 3), while the highest TPC was presented by ethanol extract of chicken bile (1.96 g GAE/100 g), while n-hexane extract exposed the lowest TPC (1.33 g GAE/100 g).

Statistical analysis using one-way ANOVA- post hoc Tukey showed that TPC in ethyl acetate extract of chicken bile was not significantly different from its TPC in ethanol extract, but both were significantly different from n-hexane bile chicken extract ($p < 0.05$).

In total flavonoid content (TFC) assay, quercetin standard was dissolved in methanol. Then aluminium chloride solution was added to form a complex with flavonoids, then cause a bathochromic shift of the ultraviolet spectrum on flavonoids. Aluminium chloride can form complex if flavonoid has OH in C3 and keto in C4 or OH in C5 and keto in C4 or ortho-di OH in C3'-C4'. The complex that occurs between aluminium chloride and ortho di OH in C3'-C4' will be broken in acid condition (Markham and Flavonoids, 2006).

TFC among various extracts of chicken bile were revealed in term of quercetin equivalent (Karabegović *et al.*, 2011) using the standard curve equation $y = 0.009 x + 0.113$, $R^2 = 0.999$. Various extracts of chicken bile gave different results of TFC ranged from 6.42 to 9.48 g QE/100 g extract (Table 4). The ethanol chicken bile extracts presented the highest TFC (9.48 g QE/100 g), followed by n-hexane extract (8.52 g QE/100 g). Statistical analysis using one-way ANOVA- post hoc Tukey denoted that TFC in n-hexane extract of chicken bile was not significantly different from its TFC in ethanol extract, but both were significantly different from ethyl acetate bile chicken extract ($p < 0.05$).

TPC and or TFC of extracts might be correlated with its antioxidant activities. Phenolic acid showed lower antioxidant activity than flavonoids (Heim *et al.*, 2002). The flavonoid aglycones would expose higher antioxidant activity than flavonoid glycosides. The TPC and or TFC value will correlate their antioxidant activities, if sample with higher value of TPC and or TFC also gave higher antioxidant activities which expose by lower IC_{50} of DPPH scavenging activities, therefore TPC and or TFC of extract was significant and negative correlation with IC_{50} DPPH value (Fidrianny *et al.*, 2015).

In Table 5 it can be seen that the TPC and TFC in chicken bile extracts had a significantly negative correlation with its IC_{50} of DPPH scavenging activities. Based on this research, it can be stated that phenolic and flavonoid compounds in chicken bile extracts contributed together in their antioxidant activities

by DPPH assay.

CONCLUSIONS

All chicken bile extracts had antioxidant activities. Ethanol chicken bile extract was a very strong antioxidant by DPPH assay. Phenolic and flavonoid compounds in chicken bile extracts contributed together in antioxidant activities using the DPPH assay. Chicken bile extracts which useless waste products were potential to be developed as a source of natural antioxidant.

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