

ANTIOXIDANT ACTIVITY OF TAUCO ETHANOL EXTRACT AND ITS FRACTIONS

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ABSTRACT

Objective: The objective of this study is to investigate antioxidant activity and phytochemical screening of ethanol extract, fractions of water, ethyl acetate, and n-hexane from tauco.

Methods: Two types of tauco were extracted using soxhletation methods, followed by fractionation using liquid-liquid extraction methods, and phytochemical screening. Antioxidant activity test was carried out using 1,1-diphenyl-2-pikrilhiradzil with ascorbic acid (vitamin C) as a reference. Modified method of Farnsworth was applied for phytochemical screening.

Results: It was found that extracts of ethanol and ethyl acetate fraction containing flavonoids, monoterpenoid, and sesquiterpenoids whereas the water fraction and a fraction of n-hexane only contain monoterpenes and sesquiterpenoids. The inhibition concentration 50 (IC₅₀) value for the ethanol extract, water, ethyl acetate, and n-hexane fractions of two taucos in a row were 1192.71 ppm, 1746.01 ppm, 722.38 ppm, 1845.45 ppm and 1190.15 ppm, 1740.30, 710.46, for tauco A and B, respectively.

Conclusion: It was unexpected that tauco ethanol extract and fractions showed much weaker antioxidant activity than vitamin C, which had the IC₅₀ value of 4.41 ppm.

Keywords: Antioxidant, Flavonoid, Free radicals, 1,1-diphenyl-2-pikrilhiradzil, Tauco, Ascorbic acid.

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INTRODUCTION

Taucu is one of the traditional food items that come from Indonesia and are found especially in the area of West Java. Taucu paste form, can be found, in the form taucu dry and wet. Taucu is processed soy products obtained from fermentation techniques. This fermentation taucu aims to improve taste, but it also can improve the nutritional content contained in soy. Taucu society generally used as food flavoring agent [1]. Taucu generally made of yellow soybeans. Soybean crops including a group of natural flavonoid that isoflavone group. Soybeans are known to have biological activity as an antioxidant, estrogenic effects, antiosteoporosis, and anticancer [2]. Isoflavones are antioxidants that are needed by the body to stop the reaction of free radical formation [3]. Isoflavone glycosides are a major component in soybean and non-fermented soy products, while the soybean fermented product is found free isoflavones (aglycone) in large quantities [4]. Based on research that has been done Hutriadi [5], taucu compounds containing daidzein and genistein. Genistein and daidzein are included in the free isoflavone compounds formed from the fermentation process soybeans [6]. Genistein is the most powerful antioxidant in soy, followed by daidzein [7].

Antioxidants are compounds that have the ability to protect cells and tissues from damage caused by the threat of the presence of free radicals that are reactive [8]. Antioxidants react with free radicals to form non-radical compounds are relatively stable [9]. Whether or not strong antioxidant activity can be seen from the inhibition concentration 50 (IC₅₀) value. IC₅₀ is a number that indicates the concentration of extract that can inhibit the oxidation of 50%. The smaller the IC₅₀ value, the higher the antioxidant activity [10]. Research into the antioxidant activity in soybean has been conducted, but research on the antioxidant activity in soybean fermented product has not been done, particularly in taucu. Therefore, it, in this study, will be conducted research on the antioxidant activity assay method taucu with 1,1-diphenyl-2-pikrilhiradzil (DPPH) with ascorbic acid as a reference. Part of this study has been published in Indonesia [11].

METHODS

Collecting materials and pre-treatment sample

Two well-known brands taucu sample used in this study were obtained from a supermarket in the area of Bandung, West Java. Taucu dried using an oven at 60-70°C until constant weight.

Extraction and fractionation taucu

The extraction method used in this research was the method soxhletation using ethanol 96%. The extraction followed standard soxhletation method [12-14]. It was done approximately 4 hrs or until almost colorless solvent droplets. Viscous liquid extract obtained was then evaporated to obtain a thick extract with constant.

$$\% \text{ Yield} = \frac{\text{Extract weight}}{\text{Sample weight}} \times 100\%$$

Water content of the obtained extract was determined to make sure fulfill Farmakope Herbal requirement, in which the water content in the extract should not be more than 10% to avoid the rapid growth of fungi and microorganisms in the extract [15].

Hydrolysis of isoflavones

Hydrolysis aims to separate the intermediate compounds with glycosides aglycone. 10 g taucu extract dissolved in a mixture of methanol and 2N hydrochloric acid in the ratio 1:1, refluxed in water bath for 2 hrs at a temperature of 40°C. After refluxing, the mixture was evaporated with a rotary evaporator at a temperature of 40°C to remove methanol.

Fractionation

Fractionation performed using liquid-liquid extraction. Extracts taucu hydrolyzed then fractionated using a solvent of water, ethyl acetate, and n-hexane. The results of the water fraction, fraction of ethyl acetate and n-hexane fraction were then evaporated with a rotary evaporator.

Phytochemical screening

Phytochemical screening was conducted on a sample tauco to determine the content of secondary metabolites contained therein based on Farnsworth method [16] which includes:

- Identification of alkaloids
- Identification of compounds polifenolat
- Identification tanin
- Identification flavonoids
- Identification monoterpenoid and sesquiterpenoids
- Identification of Steroids and triterpenoid
- Identification quinone
- Identification of saponin.

Thin layer chromatography (TLC)

TLC plates (stationary phase) used were silica gel GF254. TLC plates prepared with a size of 10×4 cm. Based on research conducted by Irianti *et al.* [17], the developer used is toluene:ethyl acetate:acetic acid at a ratio of 5:4:1. Subsequently observed in ultraviolet (UV) 254 nm and 366 nm. Spotting observed marked for the calculated value of Rf. To test qualitatively antioxidants which had been spotted TLC plate tauco extracts and fractions sprayed with DPPH. DPPH solution was prepared by dissolving reagent DPPH as much as 4 mg in 50 ml of 96% ethanol. Furthermore, the observed color staining formed.

Antioxidant activity test

Test of antioxidant activity with DPPH based on Molyneux method [18,19] with ascorbic acid as a reference. The test includes:

- Preparation of the test solution.
- Determination of the wavelength (λ) maximum DPPH
DPPH solution of 3 ml of ethanol was added 2 ml, homogenized, and observed absorbance at a wavelength of 450-650 nm. The maximum wavelength of absorption characterized by the greatest. Blank of ethanol was used.
- Determination of operating time DPPH solution in ethanol.
- Determination of the sample incubation time.
- The determination of IC_{50} values of samples.

A total of 2 ml of test solution in various concentrations added about 3 ml of DPPH solution, homogenized, and then allowed to stand. Absorbance was read at maximum wavelength was 517 nm. Used as a blank test solution. For testing the reference solution, as many as 2 ml of vitamin C in various concentrations added about 3 ml of DPPH solution, homogenized, and then allowed to stand. Absorbance is read at maximum wavelength is 517 nm. As a form of ascorbic acid solution was used in various concentrations as much as 2 ml and 3 ml of ethanol as much. IC_{50} values were calculated from a linear regression curve between the % inhibition of uptake with various concentrations of extracts of the test solution with the following formula:

$$\% \text{ Inhibition} = \left[1 - \left(\frac{A_{\text{test}}}{A_{\text{control}}} \right) \right] \times 100\%$$

Where

A_{test} = absorbance of DPPH solution in the sample

A_{control} = absorbance of DPPH solution in ethanol (3 ml DPPH and 2 ml of ethanol).

Data were obtained as percent inhibition values that were plotted against the concentration of test solution. IC_{50} describe extract concentration required to inhibit 50% of DPPH radical activity. IC_{50} value obtained from the graph the concentration of the extract of the percent inhibition of DPPH.

RESULTS AND DISCUSSION

Collection results materials and pre-treatment samples

Two samples tauco soybeans, namely, Tauco A and B, from two well-known commercially brands obtained from Bandung, West Java. The water content in tauco was high enough so that samples need to be

dried to remove the water content contained therein. Two taucos as much as 2.25 kg sample dried using an oven at a temperature of 70°C until constant weight was obtained, which was 832.55 and 830.00 g. The water content contained in tauco 37%.

Extraction and fractionation

Taucu dried samples were extracted using methods soxhletation. The soxhletation method will occur repeatedly so that the extraction process will be more perfect. Soxhletation method was done because it was known that the active compounds contained in tauco were thermostable. In addition, the soxhletation method's number of samples and solvents needed just a little so that the extraction process will be more efficient.

A total of 300 g of dried tauco obtained viscous extract of 88.45 and 90 g for Taucu A and B, respectively. We found that the water content in both tauco samples fulfilled Farmakope Herbal Indonesia requirement that was below 10% [15].

The viscous extract obtained was then hydrolyzed to break down into its component isoflavone glycosides and isoflavone glycosides free (aglycone). A total of 10 g of viscous extract hydrolyzed using HCl and methanol at a ratio of 1:1. Hydrolysis process is carried out for 2 hrs at a temperature of 40°C with the aim of accelerating the termination reaction force of the component isoflavone glycosides. Hydrolysis was then evaporated using a rotary evaporator to remove the methanol solvent. The residue was then fractionated to attract the active compound contained in the extract based on the level of polarity. Fractionation was conducted using liquid-liquid extraction using a solvent of water, ethyl acetate, and n-hexane.

Phytochemicals screening results

Phytochemical screening was conducted to determine the content of secondary metabolites contained in the extract and the fractions tauco. The phytochemical screening results are shown in Table 1.

Table 1 showed that the tauco samples, extracts of ethanol and ethyl acetate fraction containing flavonoids, monoterpenoid and sesquiterpenoids, and saponin. While the water fraction and a fraction of n-hexane only contain monoterpenes and sesquiterpenoids. This result comparable to photo screening result of soybean (*Glycine soja* Sieb. and Zucc.) [20], except for tannin. In this result, we did not find tannin content in our samples. It might be due to soybean used and or mixed soybean and other beans were used in making the tauco.

Results of TLC

TLC was carried out using the developer toluene:ethyl acetate:acetic acid (5:4:1). Extracts and fractions tauco observed in visible light, UV 254 nm, 366 nm UV, and spotting DPPH solution. Some TLC results are shown in Fig. 1 and Table 2.

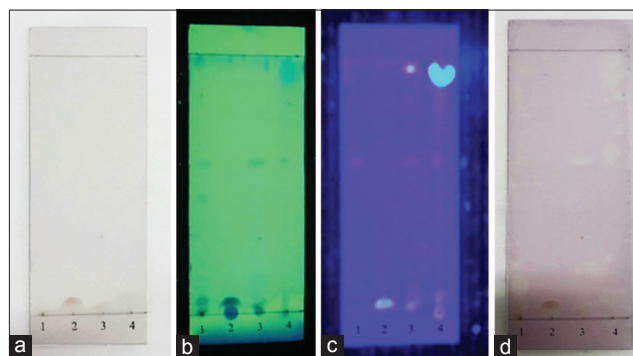


Fig. 1: Thin layer chromatography by the developer toluene:ethyl acetate:acetic acid (5:4:1) of Taucu A. 1 - Ethanol extract, 2 - Water fraction, 3 - Ethyl acetate fraction, 4 - N-hexane fraction. a - visible light, b - ultraviolet 254 nm, c - ultraviolet 366 nm, d - DPP solution

Table 1: Phytochemical screening results

Secondary metabolites	Tauco samples		Ethanol extract		Water fraction		Ethyl acetate fraction		n-heksana fraction	
	Tauco A	Tauco B	Tauco A	Tauco B	Tauco A	Tauco B	Tauco A	Tauco B	Tauco A	Tauco B
Alkaloids	-	-	-	-	-	-	-	-	-	-
Polyphenols	-	-	-	-	-	-	-	-	-	-
Tannin	-	-	-	-	-	-	-	-	-	-
Flavonoids	+	+	+	+	-	-	+	+	-	-
Monoterpenoid and sesquiterpenoid	+	+	+	+	+	+	+	+	+	+
Steroids	-	-	-	-	-	-	-	-	-	-
Triterpenoid	-	-	-	-	-	-	-	-	-	-
Quinone	-	-	-	-	-	-	-	-	-	-
Saponin	+	+	+	+	+	+	+	+	-	-

+: Detected, -: Not detected

Table 2: TLC of ethanol extract and its fractions of Tauco A and B

Sample	Rf	Visible light	UV 254 nm	UV 366 nm	Spotting DPPH
Ethanol extract	0.57	-	Blue	Green	Yellow
	0.91	-	-	Green	Yellow
Water fraction	0.06	-	Blue	Yellow	Yellow
Ethyl acetate fraction	0.57	-	Blue	Green	Yellow
	0.85	-	-	Green	-
	0.95	-	Blue	Yellow	Yellow
N-hexane fraction	0.58	-	Blue	Green	Yellow
	0.94	-	Blue	Yellow	Yellow

DPPH: 1,1-diphenyl-2-picrylhydrazyl, UV: Ultraviolet, TLC: Thin layer chromatography, -: Not detected

TLC carried out as an early detection of antioxidant activity in the sample. DPPH solution was used as spotting patches to determine whether or not the compounds that had antioxidant activity in extracts and fractions of tauco. Their antioxidant activity characterized by the formation of patches of yellow with a purple background. Both Tauco A and B found to have this color.

Determination of wavelength maximum DPPH

DPPH solution color change time is reduced by antioxidants can be measured by using UV-visible spectrophotometry at a wavelength of 515-520 nm (Molyneux, 2004). For this work, it was found the DPPH solution maximum absorption at a wavelength of 517 nm.

Determination of time operating results DPPH solution in ethanol

DPPH solution is unstable, so it is necessary to determine the operating time DPPH. Determining operating time was done to determine the best working time of DPPH solution. Graph DPPH operating time can be seen in Fig. 2.

Fig. 2 showed that the optimum time of DPPH work is in the 35th to 50th minutes where at this time there was no significant increase or decrease in absorbance.

Determination of sample incubation time

Determination of the sample incubation time was needed to see the time it took the test sample, the ethanol extract, the water fraction, fraction of ethyl acetate and n-hexane fraction of tauco be reacted with DPPH, characterized by a steady uptake. The timing of incubation was also done on samples of the comparison, namely, vitamin C. The timing of incubation samples was done in 5 minutes intervals for 2 hrs at a wavelength of 517 nm. It found that the incubation time the ethanol extract tauco, namely, in the 70th minutes to the 80th minutes, the incubation time the water fraction of tauco, namely, in the 100th minutes until the 110th minutes, the incubation time ethyl acetate fraction of tauco, namely, in the 85th minutes to the 95th minutes, and the incubation time n-hexane fraction of tauco, namely, in the 75th minutes to the 85th minutes. As for ascorbic acid C incubation period was from the 30th minutes until the 40th minutes.

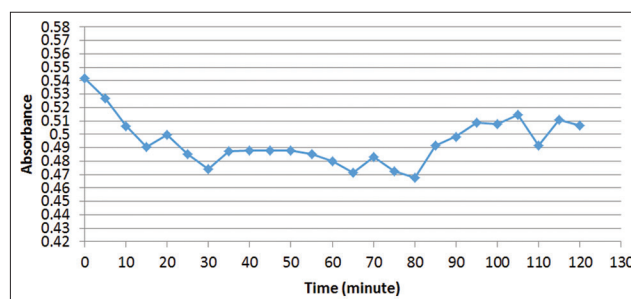


Fig. 2: 1,1-diphenyl-2-picrylhydrazyl operating time

Determination of IC₅₀ sample

In this study, each sample weighed 50 mg and dissolved in 50 ml volumetric flask, so we get the mother liquor with a concentration of 1000 ppm. The mother liquor was then diluted to 100, 200, 300, 400, and 500 ppm. DPPH solution was prepared by weighing 2 mg DPPH dissolved in 50 ml volumetric flask, so we get a DPPH solution with a concentration of 40 ppm. As a reference used ascorbic acid as vitamin C is known to have very powerful antioxidant activity. Vitamin C can donate a hydrogen atom to stabilize free radicals and can prevent a chain reaction.

The parameters used in the test the antioxidant activity by DPPH method were IC₅₀, i.e., the concentration where the sample was able to reduce 50% of the initial concentration of DPPH activity. IC₅₀ value of the samples obtained using the linear regression equation that states the relationship between the concentrations on the X axis the percent inhibition on the Y axis. Testing the absorbance of each sample at various concentrations carried out at a wavelength of 517 nm is then calculated percent inhibition values of each sample. The greater the concentration used, the greater the reduction of free radical activity (% inhibition). Samples which act as antioxidants will donate hydrogen atoms to the radical DPPH. DPPH radical reduction will experience characterized by a color change from purple to yellow. When the color changes, then there will be a decrease in the absorbance of DPPH. The greater the

concentration of the sample used, the greater the ability of antioxidants to reduce DPPH. Measured absorbance decreases with an increase in concentration of the sample. Hence, the greater the concentration of the sample is used, then the greater the percent inhibition of the sample.

Value percent inhibition of each test solution and reference solution at various concentrations is used to create the linear regression equation. IC_{50} value of ethanol extract, the water fraction, fraction of ethyl acetate, and n-hexane fraction of each tauco in a row was 1192.71 ppm, 1746.01 ppm, 722.38 ppm, 1845.45 ppm and 1190.15 ppm, 1740.30, 710.46, for Taucho A and B, respectively. IC_{50} value tauco sample is much larger than its peers, namely, vitamin C. Comparison of IC_{50} values ethanol extract tauco A and B, tauco water fraction, the fraction tauco ethyl acetate, n-hexane fraction tauco, and vitamin C can be seen in Fig. 3.

Ethyl acetate fraction had the smallest IC_{50} values between ethanol extract, the water fraction, and the fraction of n-hexane of tauco so that it could be said ethyl acetate fraction had good antioxidant activity than the ethanol extract, the water fraction, and the fraction of n-hexane from tauco. Tauco was fermented from soybeans, where soy was known to contain secondary metabolites that isoflavone class of flavonoids. Isoflavones in soy can be found in bound form with sugar (isoflavone glycosides) or in free form (aglycone isoflavones). In the process of fermentation of soy, isoflavone aglycone compound to be transformed into compounds isoflavone aglycone [6]. Therefore, the product of fermentation of soy, isoflavone aglycone is found in large quantities [4]. The antioxidant activity of ethanol extracts tauco not as good as the antioxidant activity of ethyl acetate fraction tauco. When the ethanol extract of hydrolyzed tauco, there will be a breakdown of compounds isoflavone glycosides into isoflavone aglycone and glikosidanya. Compounds isoflavone aglycone this hydrolysis results are less polar solvent that will dissolve in ethyl acetate which is semi-polar. Based on research conducted by Jun *et al.* [21], the aglycone isoflavones compounds provide better antioxidant activity than the isoflavone compounds in the form of glycosides. Based on research conducted by Hutriadi [5], the aglycone isoflavones compounds contained in tauco are genistein and daidzein, which both of these compounds are known to have antioxidant activity.

The antioxidant activity was shown by IC_{50} values. An effective antioxidant shown with IC_{50} values is low. IC_{50} value is inversely proportional to the antioxidant activity. The smaller the IC_{50} value, then the ability of antioxidants to reduce free radicals, the better because in low concentrations can inhibit free radicals by 50%. When compared with vitamin C, which have IC_{50} value of 4.41 ppm, the antioxidant activity of ethyl acetate fraction over 163 times weaker than vitamin C. Meanwhile tauco ethanol extract has antioxidant activity 270 times weaker than vitamin C, the fraction of water 395 times weaker compared to vitamin C, and the fraction of n-hexane 418 times weaker than vitamin C. These results, unfortunately, were unexpected. At first place, due to the fermentation process and raw materials of tauco mainly soybeans, it was expected the antioxidant activities of tauco should have high-antioxidant activities. Fidrianny *et al.* [22] reported in their study of shells extracts of four legumes, soybean, red kidney

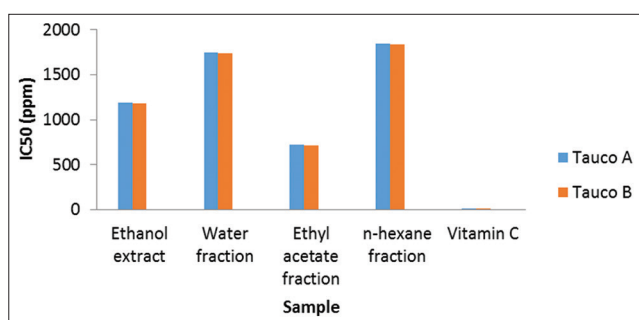


Fig. 3: Comparison inhibition concentration 50 tauco

bean, Bogor peanut, peanut, (except n-hexane shells extract of soybean) had IC_{50} of DPPH scavenging capacities <50 ppm. Most likely, the low antioxidant activities of this report due to tauco process and that soybeans used in these two tauco were mixed beans, not soybeans only.

According to the Jun *et al.* [21], a compound considered to have a very strong antioxidant activity when have IC_{50} values of <50 ppm, strong category if you have IC_{50} values of 50-100 ppm, when the medium category have 101-250 ppm IC_{50} value, a category feeble have IC_{50} values 251-500 ppm, and was listed inactive when the IC_{50} values above 500 ppm. Based on test results, extracts and fractions of tauco have the ability to neutralize DPPH radical, but the antioxidant activity of extracts and fractions tauco included into the category of inactive because it had IC_{50} values above 500 ppm.

CONCLUSION

Based on this research, it is known that extracts and fractions of tauco have antioxidant activity with the ability to neutralize free radicals DPPH. Best antioxidant activity of extracts and fractions tauco is shown by ethyl acetate fraction with IC_{50} value of 722.38 ppm (inactive), followed by ethanol extract with IC_{50} value of 1192.71 ppm (inactive), the fraction of water with IC_{50} value of 1746.01 ppm (inactive), and the fraction of n-hexane with IC_{50} value of 1845.45 ppm (inactive), while vitamin C have IC_{50} values are 4.41 ppm (very strong). These results, however, was unexpected that tauco ethanol extract and fractions showed much weaker antioxidant activity than vitamin C. Further research is needed to test the antioxidant activity of tauco using other methods. This will strengthen the scientific data about the antioxidant activity of tauco.

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