Phytochemical screening and antioxidant activity of ethanolic extract and ethyl acetate fraction from basil leaf (*Ocimum basilicum* L.) by DPPH radical scavenging method

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**Abstract.** Basil leaf (*Ocimum basilicum* L.) contains various compounds such as flavonoid, alkaloid, phenol and essential oil, so it needs to be fractionated to find out the flavonoid compound with the greatest potential as an antioxidant. This research was aimed to know the chemical compound, antioxidant potential of ethanolic extract and ethyl acetate fraction from basil leaf. The basil leaf was extracted by maceration using ethanol 70 %. The crude extract was fractionated with ethyl acetate. The ethanolic extract and ethyl acetate fraction were screened of phytochemical content including identification of flavonoids, alkaloids and polyphenolics. The antioxidant activity of the ethanolic extract and ethyl acetate fraction were tested qualitatively with 2,2-diphenyl-1-picrylhydrazyl (DPPH) and phosphomolybdate. Its antioxidant activity was determined quantitatively using DPPH radical scavenging method. Phytochemical screening test showed that ethanolic extract and ethyl acetate fraction from basil leaf contain flavonoids, polyphenolics, and alkaloids. The qualitative analysis of antioxidant activity of ethanolic extract and ethyl acetate fraction from basil leaf showed an antioxidant activity. The IC$_{50}$ value of ethanolic extract, ethyl acetate fraction and quercetin were 1,374.00±6.20; 389.00±1.00; 2.10±0.01 μg/mL, respectively. The research showed that antioxidant activity of the ethyl acetate fraction more potential than the ethanol extract of the basil leaf, but less than quercetin.

**Keywords:** antioxidant, *Ocimum basilicum* L., basil leaf, ethanolic extract, ethyl acetate fraction, DPPH

1. **Introduction**
The life style changes lead to changes in dietary food. Fast food and instant food with preservative become favourite food. Preservatives and dyes contained in these foods can trigger the formation of free radicals which can cause various diseases. Free radicals are molecules with an unpaired electron. The presence of an unpaired electron, caused this molecule to become highly reactive [1]. Free radicals are also formed in natural processes in aerobic organism. Therefore, the body need antioxidant to neutralize free radicals. Antioxidant can stop the formation of free radicals and the chain reaction, which would result in cell damage. Antioxidant will transferred electrons or hydrogen to reduce its reactivity [2].

The body has a mechanism of defense against to free radicals, such as antioxidant enzymes (primary antioxidant), among are: superoxide dismutase (SOD), glutathione peroxidase (GPx) and
glutathione reductase (GR). But if the amount of free radicals are excessive, the body can not against it. Thus the supply of antioxidants (secondary antioxidant) are needed to against these free radicals. The synthetic antioxidant such as butyl hydroxyl anisol (BHA) and butyl hydroxy toluene (BHT) are very effective and widely used for the processing industry, but have some side effects on human health, such as carcinogenesis promoters [3, 4].

Therefore, there has been much interest in the antioxidant of naturally occurring substances and develop it to replace synthetic antioxidant. Most plant derived foods have an antioxidant compounds that include vitamin C, vitamin E, polyphenols, carotenoids and ubiquinols [5]. One of the vegetables that has potential as a natural antioxidant is basil leaf (O. basilicum L. family Lamiaceae). It was reported that basil contains of flavonoids as naturally antioxidant [6]. The previous research reported on ethanolic extract of various kinds of vegetables including O. basilicum L., Cichorium endivia L.var.crispum Lam, Amaranthus lividus L.sensu Thell, and Sauropus androgynus L. Merr as antioxidants, and basil has the greatest an antioxidant potential [7]. The study of an antioxidant activity from water extract and ethanolic extract from O. basilicum L. which grown in Turkey were reported [8]. Another study was reported that basil extract contains phenolic compounds such as caffeic acid, dihydrokaempferol-3-O-glucoside, luteolin acetyl-glucuronide, dihydroxykaempferol-glycoside, caffeoyl ester, rosmarinic acid, carnosic acid, catechin, caffeoyl-3-O-rutinioside, rosmarinic acid, ferulic acid, apigenin and chlorogenic acid. These compounds potential as an antioxidant [9]. Basil also contains of essential oil which consist of eugenol, chavicol, linalool and a-terpineol. These compounds was reported as an antioxidant [10].

The purpose of this study was to identify of flavonoids, polyphenolics and alkaloids that contain in ethanolic extract and ethyl acetate fraction from basil leaf and evaluate its antioxidant activity. The antioxidant activity test in this study was performed using free radical scavenging method. The compound of 2,2-diphenyl-1-picrilhidrazil (DPPH) was used as free radical model.

2. Materials and Method

2.1. Chemicals

DPPH and quercetin were purchased from Sigma (Aldrich. Co, USA). All chemicals used were analitcal grade.

2.2. Plant materials, extraction and fractionation procedures

Basil leaf was obtained from local market at Muntilan Central Java, Indonesia. 1.7 kg wet basil leaf was dried in oven at 50°C until dry. For preliminary extraction, 200 grams powder of basil leaf was mixed with 800 mL petroleum ether (1:4) by magnetic stirred during 3 hours. The sample was left for 24 hours with periodic shaking. Then it was filtered over Whatman No.1 paper using Büchner funnel. The filtrate was removed, while the dregs was dried in the acidic wardrobe until dry and did not smell petroleum ether.

For ethanolic extraction, the residue was maceration using ethanol 70 % (1:4), then stirred by magnetic stirred during 30 minutes. The sample was left for 24 hours with periodic shaking. The extracts was filtered over Whatman No.1 paper using Büchner funnel. The residue was remacerated two times. The filtrate was collected and ethanol was removed by a Büchi rotary evaporator at 60°C. Then evaporation of extract was continued on waterbath at 60°C until became viscous.

For ethyl acetate fractionation, 5 g of viscous extract was inserted into a mortar with warm distilled water then stirred to form a suspension and transferred into a separating funnel. The extract suspension was added with ethyl acetate (1:1) and shaken to formed two layers. The ethyl acetate-soluble fraction that above was taken, then filtered over Whatman No.1 paper as filtrate I using Büchner funnel. While ethyl acetate unsolved fraction was refractionated with ethyl acetate two times
until obtained filtrate II and III. The ethyl acetate soluble fraction were collected then evaporated by a Büchi rotary evaporator at 60°C. Then evaporation of fraction was continued on waterbath at 60°C until became viscous.

2.3. Phytochemical screening

2.3.1. Flavonoids identification

One drop of ethanolic extract and ethyl acetate fraction were dropped on filter paper and allowed to dry. The dry droplets of the samples on filter paper were steamed with ammonia. When the color change from dimly yellow to intense yellow showed that samples positive contain flavonoids.

Identification of flavonoids from ethanolic extract and ethyl acetate fraction using thin layer chromatography (TLC) were carried out on silica gel GF60 plates in a presaturated chamber. The solvent system was the mixture butanol-acetic acid-water (BAW) (4: 1: 5). The identification of compounds using UV lamp at 254 and 366 nm. The silica gel GF60 plates was sprayed with AlCl3, then observed under UV lamp at 254 and 366 nm.

2.3.2. Alkaloids identification

A volume of 1.0 mL ethanolic extract and ethyl acetate fraction were added 3 drops of Mayer’s reagent. The positive samples with alkaloids contain would give the white precipitate.

2.3.3. Polyphenolics identification

The ethanolic extract and ethyl acetate fraction as much as 1.0 mL were added 3 drops of FeCl3 reagent. The samples positive contain polyphenols when a dark black colors appear.

2.4. Qualitative antioxidant activity

For this analysis, ethanolic extract and ethyl acetate fraction each 1.0 mL were added with 1.0 mL DPPH 0.15 mM reagent. The presence of antioxidant activity was characterized by the change of color from purple to yellow.

For the another analysis, ethanolic extract and ethyl acetate fraction each 1.0 mL were added 1.0 mL phosphomolybdate reagent. The presence of antioxidant activity was indicated by the discoloration from yellow to bluish green.

2.5. Quantitative antioxidant activity

2.5.1. Preparation of DPPH

The DPPH 1 mM solution was prepared by dissolving of 19.716 mg powder of DPPH with ethanol up to 50.0 mL in a volumetric flask. For DPPH 0.15 mM, 15.0 mL DPPH 1mM was added ethanol up to 100.0 mL.

2.5.2. Timing of operation determination

A volume of 1.0 mL samples were added 1.0 mL DPPH 0.15 mM in the test tube. The mixture were homogenized using a T 570 sonikator ultrasonic. The absorbance were measured using a Pharmaspec Shimadzu1800 UV-Vis spectrophotometer at 517 nm for 90 minutes.

2.5.3. Maximum absorption wavelength determination and negative control

The DPPH 0.15 mM as much as 1.0 mL was added with 1.0 mL of ethanol in the test tube. The mixture was homogenized using a T 570 sonikator ultrasonic and left to time of operation. The absorbance was measured using a Pharmaspec Shimadzu 1800 UV-Vis spectrophotometer at 450-600 nm. This data also was used as the absorbance of negative control.

2.5.4. Preparation of stock 0.2 mg/mL and series of quercetin

The quercetin 1 mg/mL was prepared by dissolving of 10.0 mg quercetin with ethanol up to 10.0 mL in a volumetric flask. For quercetin 0.2 mg/mL, 10.0 mL quercetin 1 mg/mL was added with ethanol up to 50.0 mL. The concentration series of quercetin were: 3, 2, 1.75, 1.5, 1.25 and 1 μg/mL.

2.5.5. Preparation of stock 2 mg/mL and series of ethanolic extract

The ethanolic extract 2 mg/mL was prepared by dissolving of 200.0 mg ethanolic extract with ethanol up to 100.0 mL in a volumetric flask. For series of ethanolic extract, each of 5.0, 4.375, 3.75, 3.125, 2.5 and 1.875 mL ethanolic extract 2 mg/mL were added ethanol up to 5.0 mL to obtain the solution with levels of 2, 1.75, 1.5, 1.25, 1 and 0.75 mg/mL, respectively.
2.5.6. Preparation of stock 1 mg/mL and series of ethyl acetate fraction
The ethyl acetate fraction 1 mg/mL was prepared by dissolving of 50.0 mg ethyl acetate fraction with ethanol up to 50.0 mL in a volumetric flask. For series of ethyl acetate fraction, each of 5.0, 2.5, 1.25, 0.5, 0.25 and 0.05 mL ethyl acetate fraction 1 mg/mL were added ethanol up to 5.0 mL to obtain the solution with level of 1, 0.5, 0.25, 0.1, 0.05 and 0.01 mg/mL, respectively.

2.5.7. The measurement of sample
The sample as much as 1.0 mL were added with 1.0 ml of DPPH 0.15 mM in the test tube. The mixture were homogenized using a T 570 sonicator ultrasonic and left to time of operation in the dark. The absorbance were measured using a Pharmaspec Shimadzu 1800 UV-Vis spectrophotometer at maximum wavelength against blank (2.0 mL ethanol). All tests were run in five times. The data were expressed as mean ± SD. Quercetin was used as a positive control.

2.6. Data analysis
The antioxidant capacity was expressed by % inhibition. The inhibition percentage of the DPPH radical was calculated according to the following equation:

\[
\% \text{Inhibition DPPH} = \left( \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \right) \times 100 \%
\]

The IC50 is defined as the concentration of the sample required to cause a 50 % decrease in initial DPPH radical absorbance. The IC50 was found after making the relationship between concentration and % inhibition. The IC50 value of ethanolic extract, ethyl acetate and quercetin were analysed statistically by SPSS 16 with 95% confidence level.

3. Results and Discussion
3.1. Extraction and fractionation
The active compounds of basil leaf were extracted by maceration method using ethanol 70% as solvent without heating process (Table 1). Therefore, it can prevent the occurrence destruction of thermolable active compounds. The deficiency of mazeration method is the process of extraction takes a long time and saturation can occur. Stirring process can prevent any saturation. This extraction aims to attract the compounds that present in the simplicia.

| Process      | Initial Weight (gram) | Results (gram) | Yield (%) |
|--------------|-----------------------|----------------|-----------|
| Drying       | 1,700.00              | 342.21         | 20.13     |
| Extraction   | 200.20                | 125.21         | 62.54     |
| Fractionation| 5.00                  | 0.85           | 17.00     |

The advantage of using ethanol 70% is the compounds from less polar to polar can be withdrawn from simplicia, including flavonoids compounds [11]. The principle of extraction by maceration method is the diffusion from high to low concentration, the liquid of solvent can penetrate to the lining of the cell wall, then enter into the cell cavity that containing the active compounds. The active compounds in the cell were dissolved due to the difference in concentration between the solutions inside the cell (high concentration) with outside the cell (low concentration). The extract had characteristics viscous, brown color and typical smell as basil.

3.2. Phytochemical screening
3.2.1. Flavonoids identification
The results of flavonoids identification of ethanolic extract and ethyl acetate fraction with ammonia were the colors change from dimly yellow to intense yellow. It showed that samples contain flavonoids. Flavonoids were reacted with ammonia to form quinoid in this test. The yellow color occurs because of the formation of quinoid in \( \beta \)-ring that contain a longer conjugated double bond.
[12]. The results of identification of flavonoids with ammonia are shown in Table 2. While the reaction of flavonoid with ammonia is shown in Figure 1.

**Table 2.** The results of flavonoids identification with ammonia

| The samples          | The samples solution | The samples with ammonia | Flavonoids content |
|----------------------|----------------------|--------------------------|--------------------|
| Ethanol extract      | Dimly yellow         | Intense yellow           | Positive           |
| Ethyl acetate fraction | Dimly yellow       | Intense yellow           | Positive           |
| Quercetin            | Dimly yellow         | Intense yellow           | Positive           |

![Figure 1. Reaction of flavonoid with ammonia [12]](image)

The another test of flavonoids from ethanolic extract and ethyl acetate fraction were TLC method. The results of these tests obtained spots that were visually yellow after being sprayed using AlCl$_3$ reagents and read on 254 nm UV rays and 365 nm. Flavonoids were reacted with AlCl$_3$ to form flavonoid-Al$_3^{+}$ complex. The Rf of quercetin, ethanolic extract and ethyl acetate fraction were 0.81, 0.78 and 0.78, respectively. The Rf value are almost the same among the three samples. These results show that ethanolic extract and ethyl acetate fraction contains flavonoids. The identification of flavonoids with TLC are shown in Figure 2.
3.2.2. Alkaloids identification

The results of alkaloids identification of ethanolic extract and ethyl acetate fraction with Mayer’s reagent were white precipitate for all of the samples. The Mayer's reagents is a mixture of mercury with potassium iodide.

| The samples | The samples solution | The samples with Mayer’s reagent | Alkaloids content |
|-------------|----------------------|----------------------------------|-------------------|
| Ethanolic Extract | Brown-black | White precipitate | Positive |
| Ethyl acetate fraction | Green-brown | White precipitate | Positive |

Alkaloids contain nitrogen atom with free electron pairs. This pair of free electrons reacts with the metal to form a covalent bond coordinate. The reaction of alkaloid test using this reagent may occur between the electrons of the nitrogen atom reacts with K⁺ ion of dipotassium tetraiodomercurat so that forming a potassium-alkaloid complex which characterized by white precipitate [13]. The results of identification of alkaloids with Mayer’s reagent are shown in Table 3. While the reaction of alkaloid with Mayer’s reagent is shown in Figure 3.
3.2.3. Polyphenolics identification

The addition of FeCl$_3$ to the ethanolic extract and the ethyl acetate fraction that the colors change from brown-black and green brown respectively to black-brown. It indicates that the samples contains of polyphenolics compounds. Polyphenolics compounds are containing OH groups. Therefore, it react with FeCl$_3$ to form flavonoid-Fe$^{3+}$ complex [13]. The results of identification of polyphenolics with FeCl$_3$ are shown in Table 4. While the reaction of polyphenolics with FeCl$_3$ is shown in Figure 4.

Table 4. The results of polyphenolics identification with FeCl$_3$

| The samples        | The samples solution | The samples with FeCl$_3$ | Polyphenolics content |
|--------------------|----------------------|--------------------------|-----------------------|
| Ethanolic extract  |                      |                          | Positive              |
| Brown-black        |                      | Black-brown              |                       |
| Ethyl acetate fraction |                  |                          | Positive              |
| Green-brown        |                      | Black-brown              |                       |

Figure 3. Reaction of alkaloid with Mayer’s reagent [13]
3.3. Qualitative antioxidant activity

The qualitative antioxidant activity with DPPH were resulted the color change from purple to yellow-brown. It was showed that ethanolic extract and ethyl acetate fraction positive as an antioxidant. The results of qualitative antioxidant activity analysis with DPPH are shown in Table 5.

Table 5. The results of qualitative test antioxidant activity with DPPH

| The samples            | The samples solution | The samples with DPPH | Antioxidant activity |
|------------------------|----------------------|-----------------------|----------------------|
| Ethanolic Extract      | Purple               | Yellow-Brown          | Positive             |
| Ethyl acetate fraction | Purple               | Yellow-Brown          | Positive             |

The another test of an antioxidant activity is reductive to phosphomolybtae. The results of this test, both of the ethanolic extract and ethyl acetate fraction positive as an antioxidant. It was characterized by the change of color from yellow to green-blue. The reaction mechanism of this method was drawn by reaction of flavonoid with phosphomolybtae. Flavonoids which contain in the samples were oxidized by phosphomolybtae/ MO (VI) and formed MO (V). The oxidized flavonoids form react with \( H^+ \) in acidic condition (Figure 5). Antioxidant activity analysis qualitatively with phosphomolybdate are shown in Table 6.
Table 6. The results of qualitative antioxidant activity with phosphomolybdate

| The samples            | The samples solution | The samples with phosphomolybdate | Antioxidant activity |
|------------------------|----------------------|-----------------------------------|----------------------|
| Ethanolic extract      |                      |                                   | Positive             |
|                        | Yellow               | Green-Blue                        |                      |
| Ethyl acetate fraction |                      |                                   | Positive             |
|                        | Yellow               | Green-Blue                        |                      |

3.4. Quantitative antioxidant activity

The quantitative antioxidant activity analysis was performed using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method [15]. Quercetin was used as a standard because it has been shown have as free radical scavenging activity. The compound has phenolic (OH) groups at 3', 4', 3, 5, and 7 positions. The reaction mechanism of this method was illustrated by the flavonoid structure following the pattern of BHT reaction mechanisms with DPPH [15]. The reaction of type 1, flavonoid donate electron to DPPH and forming flavonoid radical species. Then flavonoid undergo resonance and quinoid structure are formed. While DPPH is forming DPPH₂ that stable and yellow color. The reaction of type 2, flavonoid after donating their electron to DPPH was formed a complex with DPPH (Figure 6).
Figure 6. Reaction of flavonoid with DPPH [16]

The results of timing of operation determination from ethanolic extract, ethyl acetate fraction and quercetin were 72\textsuperscript{nd} until 83\textsuperscript{nd}, 76\textsuperscript{nd} until 90\textsuperscript{nd} and 68\textsuperscript{nd} until 81\textsuperscript{nd} minutes, respectively. The results of measurement of maximum absorption wavelength determination was obtained 516 nm. The quantitative antioxidant activity analysis with DPPH are shown in Table 7.

### Table 7. The results of quantitative antioxidant activity analyzed with DPPH method

| Sample                  | IC\textsubscript{50} (µg/mL) | CV (%) |
|-------------------------|------------------------------|--------|
| Ethanolic extract       | 1,374.00 ± 6.20              | 0.45   |
| Ethyl acetate fraction  | 389.00 ± 1.00                | 0.26   |
| Quercetin               | 2.10 ± 0.01                  | 0.48   |

The results of IC\textsubscript{50} value analysis statistically in normality test of ethanolic extract, ethyl acetate fraction and quercetin were obtained significance of 0.200>0.05; 0.200>0.05 and 0.072>0.05. It showed that data is normally distributed. The homogeneity test showed that data were homogeneous (p: 0.081>0.05). The results of analysis antioxidant activity showed that potential antioxidant activity of ethyl acetate fraction was higher than ethanolic extract of the basil leaf, but less than quercetin. The IC\textsubscript{50} value of ethanolic extract, ethyl acetate fraction and quercetin were significantly different from each other (p: 0.000<0.05).

*O. basilicum* also active as antioxidant which performed with other methods, including ferric thiocyanate method, reductive capability by Fe\textsuperscript{3+}-Fe\textsuperscript{2+} transformation, ferrous ions chelating capacity and hydrogen peroxide scavenging activity [8].

4. Conclusions

Phytochemical screening test showed that ethanolic extract and ethyl acetate fraction from basil leaf contain flavonoids, polyphenolics, and alkaloids. The qualitative analysis of antioxidant activity of ethanolic extract and ethyl acetate fraction from basil leaf showed an antioxidant activity. The antioxidant activity of the ethyl acetate fraction more potential than the ethanolic extract of the basil leaf, but less than quercetin.
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