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Research Article

Antioxidant activity assay and determination of phenolic and flavonoid content of Libho (*Ficus Septica* Burm. F) fruits

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Keywords: ABTS radical; Antioxidant; CUPRAC radical; *Ficus Septica* Burm. F; Phenolic content; Flavonoid content

Abstract

Background: One of the plants used in Indonesian traditional medicine Libho (*Ficus Septica* Burm. F) is traditionally used to treat some diseases, including malaria, diarrhea, diabetes, analgesic, antifungal, dysentery, anthelmintic, antioxidant, hemostatic and anti-inflammatory.

Purpose: The purpose of the study is to investigate the potential *in vitro* antioxidant activity assay and phytochemical content of Libho fruits.

Methods: Libho leaves powder was extracted with the maceration method Antioxidant activity was evaluated using ABTS cation and CUPRAC radicals. Total phenolic content was determined using the Folin-Ciocalteau method. Meanwhile, the total flavonoid content was determined using the aluminum chloride complex colorimetric method;

Results: Extract and fraction of *Ficus Septica* Burm. F fruits have the strongest antioxidant potential. The ethyl acetate fraction showed the strongest antioxidant activity on both ABTS and CUPRAC radicals with IC50 values of 6.33 ± 0.01 µg/mL and 11.64 ± 0.28 µg/mL, respectively. Ethyl acetate fraction also showed high phenolic and flavonoid content with values of 28 ± 0.05 mg GAE/100 mg sample and 43.08 ± 0.48 mg QE/100 mg sample, respectively.

Conclusion: Ethyl acetate fraction has the potential to be used as a source of natural antioxidants and has the potential to be used as a nutraceutical.

Background

Free radicals are molecules or compounds with one unpaired electron in their outer shell, making them relatively unstable and highly reactive. Because of their reactive nature, free radicals are always trying to attract electrons from other molecules or other cells in the body that are around them. Its ability to oxidize other molecules can result in oxidative damage in the body. One example of free radicals is reactive oxygen species (ROS). ROS can react and disrupt macromolecules, such as proteins, lipids and nucleic acids in the body. If it cannot stop the damage caused by ROS, it causes oxidative stress [1,2]. Oxidative stress is an essential factor in various types of pathogens in chronic disease. As a result, oxidative stress is the causative agent of various diseases such as arthritis, cancer, asthma, atherosclerosis, diabetes and Parkinson's and Alzheimer's diseases [3,4].

Antioxidants are molecules or compounds that play a role in preventing and stabilizing damage caused by free radicals by donating electrons or protons from antioxidant molecules to damaged cells. Antioxidants can also convert free radicals into waste products, which are excreted from the body [5-7]. Antioxidant compounds such as phenolics, flavonoids, and polyphenols can scavenge free radicals such as hydroperoxides, peroxides, or lipid peroxyl so that they can

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inhibit oxidative mechanisms that cause degenerative diseases [7-9]. Natural sources have been considered good antioxidants since ancient times. Plants in traditional medicine are used to treat chronic and infectious diseases [10,11]. The abundance of plant materials that exist in nature causes the interest of researchers to seek new medicinal sources from plants to increase. The plant contains chemical components that can provide certain physiological effects on the human body, such as alkaloids, flavonoids, tannins and other phenolic groups [12,13]. Traditional medicines from plants and their extracts, usually used as a treatment for various infectious diseases, are being modified and refined into modern formulations that can be used in various diseases such as ischemic heart disease, diabetes, atherosclerosis, liver disease and initiation carcinogenesis [14] Libho (*Ficus Septica* Burm. F) is one of the plants used in traditional medicine, among others, as an effective therapy in treating hypertension, liver, and diabetes. Degenerative diseases such as cancer and aging are caused too often exposed to free radicals [15],. This plant reported secondary metabolites, including alkaloids, tannins, saponins, terpenoids, and flavonoids [16]. The flavonoid group is found in E. odoratum L plants, is flavonols, flavanone and chalcones. In addition, it also contains phenolic acids, such as ferulic acid and protopathic acid [17]. The presence of phenolic compounds in the Libho plant allows it to have the potential as an antioxidant. Because the antioxidant activity in plants is generally related to the total phenolic and flavonoid content [18]. The ability of phenolic and flavonoid compounds as antioxidants is due to the ability of flavonoid and phenolic compounds to donate hydrogen atoms both in the form of glycosides and in the free state or aglycones [19]. This study aimed to evaluate the ability of phenolics and total flavonoids as antioxidants.

Materials and methods

Plant materials

Fresh Libho (*Ficus Septica* Burm. F) fruits collected from Kabangka District, Muna Regency, Southeast Sulawesi Province, Indonesia in September - October 2020. Fruits material was authenticated at the Laboratory of Biology, FKIP, University of Halu Oleo, Indonesia by Mrs. Murni Sabilu and deposited in the herbarium of the same laboratory with a voucher specimen number BIO 268 (Figure 1), The fresh fruits (10 kg) were washed under running tap water to remove sand and debris and were airdried under shade for 10 days. The final weight after drying was 1.4 kg and powdered using a mechanical grinder. DPPH (Sigma-Aldrich®, USA), ABTS (Sigma-Aldrich®, USA), Galic Acid (Sigma-Aldrich®, USA), Quercetin (Sigma- Aldrich®, USA), methanol (E. Merck, Germany), ethyl acetate (E. Merck, Germany), chloroform (E. Merck, Germany), n-hexane (E. Merck, Germany) and equates.

Extraction

Seven hundred and fifty grams of Libho fruit powder was macerated with 10 L methanol for 72 hours. The extract obtained was concentrated with a rotary evaporator to obtain crude extract. The crude extract (105 g). after the 90-gram crude extract was partitioned with the aid of a separating funnel into hexane-soluble (24 g), chloroform soluble (17.2 g), ethyl acetate soluble (16.6 g) and water fractions (35.9 g). the extraction and fractionation scheme is shown in Figure 2.

Antioxidant activity test by ABTS cation method

Determination of the antioxidant properties of the sample using the ABTS method, the ABTS cation radical was first prepared following the method [20,21], namely, making seven mM ABTS solutions, namely by dissolving 18 mg ABTS in 5 mL solution, then added 5 mL of potassium persulfate solution, incubated in a dark room at 23 °C for 16 hours before use, resulting in ABTS with a dark blue color. Measure the antioxidant capacity of samples with ABTS radicals was carried out by adding 3 mL of methanol to 1 mL of sample and 1 mL of ABTS cation radical solution. Shake until homogeneous and incubated in the dark for 10 minutes at room temperature. The absorbance of the solution was measured at 745 nm.

The formula calculates radical scavenging activity

$$\%inhibition = \frac{A_{blank} - A_{sampl}}{A_{sampl}} 100\%$$

The IC50 value is obtained by replacing y with 50 in the linear regression equation y = bx + a so that the value of x is obtained. the value of x is the IC50 value of the sample.

(a) (b)

Figure 1: (a) Plant libho (Ficus Septica Burm. F) and (b) Libho (Ficus Septica Burm. F) fruits.

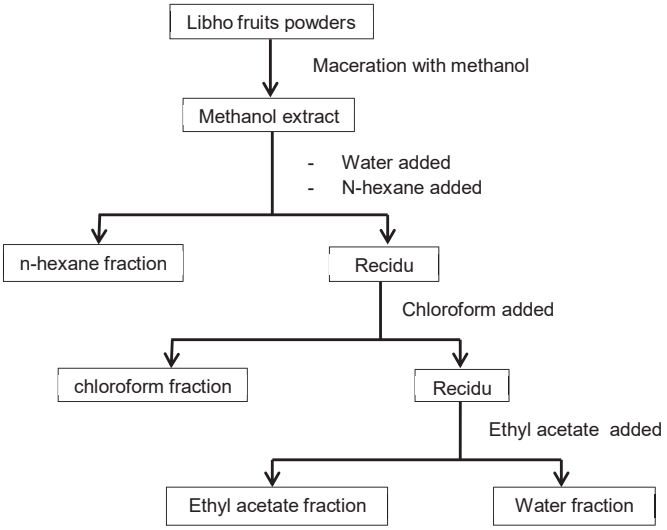

Figure 2: Extraction and fractionation schema of Libho fruits powder.

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Antioxidant activity assay by CUPRAC method

Cupric Reducing Antioxidant Capacity (CUPRAC) was evaluated by following the method [22,23] with some modifications, using copper(II)- neocuproine reagent as the chromogenic oxidizing agent and ascorbic acid as the standard. This assay carefully mixed 1 ml of the sample with 1 ml of neocuproine (7.5 mM) and CuCl2 (10 mM) reagents, and the absorbance was measured at 450 nm after 30 min.

Measurement of total phenolic content

Extracts and fractions were analyzed for TPC by the Folin-Ciocalteau assay as described by [24-26] using gallic acid as standard. Briefly, put 1 ml of the sample in a test tube, and then 0.4 mL of Folin- Ciocalteau reagent was added, then allowed to stand for 5-8 minutes. Then 4 mL of 7% Na2CO3 solution was added and then shaken until homogeneous. Then it was allowed to stand for 30 minutes at room temperature, and then the absorbance was measured using a UV-Vis spectrophotometer at 750 nm. The total phenolic content was calculated as milligrams of gallic acid equivalent (mg GAE/gram sample).

Determination of total flavonoids

Measurement of flavonoid content using the aluminum chloride method, as described by [27-29], with minor modifications. Briefly, added as much as 1 ml of the sample and 3 mL of methanol p.a, then 0.2 mL of 10% AlCl3 and 0.2 mL of 1 M potassium acetate. Then made up 10 ml with distilled water. Shake until homogeneous and incubated at room temperature for 30 minutes. Then the absorbance was measured using a spectrophotometer at 417 nm. The total flavonoid content was calculated as milligrams of quercetin equivalent (mg QE/gram sample).

Data analysis

All data were represented as mean and standard deviation of three parallel measurements and analyzed with SPSS version 16.0 (SPSS, Chicago, IL, USA) and Microsoft Excel (American corps). The difference between the means was calculated by one-way analysis of variance and was considered significant if $p < 0.05$. The antioxidant activity test was carried out in at least three replications. Pearson correlation coefficient (r) was determined to determine the relationship between phenolic and flavonoid content and antioxidant activity.

Results and discussion

Antioxidant activity assay by ABTS method

Determination of antioxidant strength using the ABTS method is obtained from the oxidation of potassium persulfate with ABTS salt [30], as shown in Figure 3. This method measures the relative ability of antioxidants to scavenge ABTS. The ABTS method has greater sensitivity in detecting antioxidant capacity because its response to antioxidants is higher than the DPPH method and has faster reaction kinetics [31]. The principle of the ABTS method is based on the ability of antioxidant compounds to stabilize radicals by donating protons to radical compounds. The process of capturing protons by radical compounds is characterized by a change in the color of the radical solution from blue to colorless. The speed of decreasing color intensity indicates the strength of antioxidant compounds in reducing free radicals [2,32,33]. ABTS assay can be carried out on lipophilic and hydrophilic compounds observed at 750 nm. Based on the results of the ABTS method, showed that the extract and fraction of Libho fruits had very strong radical scavenging abilities, as shown in Table 1.

Table 1 shows that the ethyl acetate fraction has the strongest antioxidant activity compared to the methanol extract, chloroform, n-hexane, and water fractions, with IC50 values of 6.33 ± 0.01 µg/mL (ethyl acetate fraction), 6.76 ± 0.04 µg/mL (methanol extract), 6.83 ± 0.02 µg/mL (chloroform fraction), 7.17 ± 0.02 µg/mL (n-hexane fraction), and 10.24 ± 0.05µg/mL (water fraction), respectively. Ascorbic acid was used as standard. This is in line with several studies showing that the ethyl acetate fraction has very strong antioxidant potential, including the ethyl acetate fraction of *Clerodendrum cyrtophyllum* Turcz leaves [34], and the ethyl acetate fraction of Rambutan (*Nephelium lappaceum* L) peel [20].

Antioxidant activity assay with the CUPRAC method

Another method used to measure the antioxidant activity of Libho fruits is the CUPRAC method. This method uses copper neocuproine (2,9-dimethyl-1,10-phenanthroline) chelate abbreviated (Cu(II)-Nc) as a chromogenic oxidant [35,36]. An antioxidant reduces Cu (II) to Cu (I) in this test. The chromophore Neocuproine (Nc) reacts with CuCl2 to form a Cu(I)–Nc complex at pH 7 at a wavelength of 450 nm. The reaction of chromogenic reagents with n-electron reductant (AO) of antioxidant compounds produces a stable yellow-orange color within 30 minutes.

The chromogenic oxidizing reagent of the developed CUPRAC method, namely, Cu(II)-Nc, reacts with an n-electron reducing antioxidant (AOX) [37], as shown in Figure 4.

The data in Table 1 shows that the extract and fraction of Libho fruits have a very strong ability to reduce Cu (II) to Cu (I). The data in Table 1 shows that the ethyl acetate fraction has

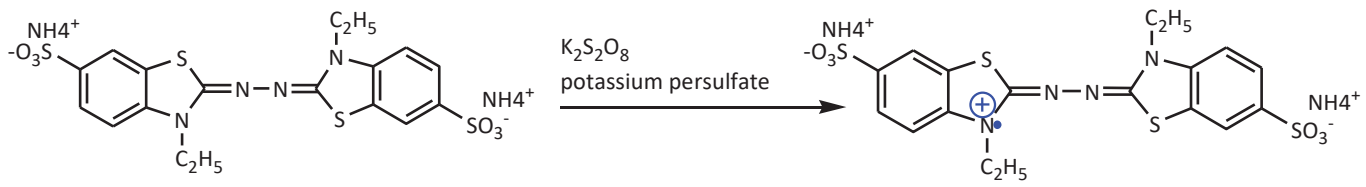

Figure 3: Formation of stable ABTS radical from ABTS with potassium persulfate [30].

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stronger antioxidant activity than methanol extract, chloroform fraction, n-hexane fraction and water fractions, with IC50 values of 5.05 ± 0.02 g/mL (ethyl acetate fraction), 6.09 ± 0.06 g/mL (methanol extract), 7.71 ± 0.05 g/mL (chloroform fraction), 9.01 ± 0.12 g/mL (n-hexane fraction), and 11.91 ± 0.3 (water fraction), respectively, Ascorbic acid was used as standard. This is in line with several studies showing that the ethyl acetate fraction has very strong antioxidant potential, including the ethyl acetate fraction of *Nicotiana glauca* leaves [38].

Determination of total phenolic and flavonoid contents

The flavonoid compounds in the fruits of Libho were measured by adding hydrochloric acid and aluminum powder. This addition aims to reduce the benzopyrene core in the flavonoid structure so that the color changes to orange and red [39]. Changes in color to red and orange indicate the formation of a complex between the hydroxyl group and the ketone or hydroxyl group in the ortho position. Meanwhile, the addition of sodium acetate to detect the presence of a 7-hydroxy group indicates a flavonol group [40]. Therefore, in this study, quercetin was used as a standard.

Based on the data, Table 2 shows that the flavonoid content at extract and fraction of Libho fruit with value, 43.08 ± 0.48 mg QE/100 mg sample (ethyl acetate fraction), 28.46 ±

0.08 mg QE/100 mg sample (methanol extract), 23.67 ± 0.42 mg QE/100 mg sample (chloroform fraction), 22.13 ± 0,47 mg QE/100 mg sample (n-hexane fraction) and 14.41 ± 0.42 mg QE/100 mg sample (water fraction), respectively.

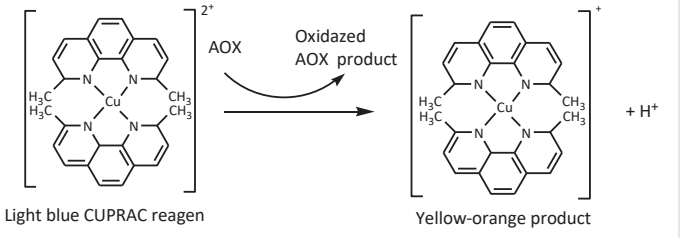

Figure 4: The CUPRAC reaction and chromophore: Cu(I)-Nc chelate cation [7,48].

Table 1: Antioxidant data of Libho fruits.

Sample	IC50 value (µg/mL)	
	ABTS	CUPRAC
Methanol extract	6.76 ± 0.04	6.09 ± 0.06
n-hexane fraction	7.17 ± 0.02	9.01 ± 0.12
Chloroform fraction	6.83 ± 0.02	7.71 ± 0.05
Ethyl acetate fraction	6.33 ± 0.01	5.05 ± 0.02
Water fraction	10.24 ± 0.05	11.91 ± 0.3
Ascorbic acid	4.64 ± 0.03	4.43 ± 0.01

Table 2: Total phenolic and flavonoid content of Libho (*Ficus Septica* Burm. F) fruits.

Sample	Flavonoid content (Mean ±SD) (mg QE/100 mg sample)	Phenolic content (Mean ±SD) (mg GAE/100 mg sample)
Methanol extract	28.46 ± 0.08	23.32 ± 0.11
Hexane fraction	22.13 ± 0,47	23 ± 0.19
Chloroform fraction	23.67 ± 0.42	26.05 ± 0.19
Ethyl acetate fraction	43.08 ± 0.48	28 ± 0.05
Water fraction	14.41 ± 0.42	7.6 ± 0.12

Determination of phenolic content in Libho fruits using the Folin-Ciocalteau method. The principle of this method is the formation of complex compounds that are blue. The formation of this blue color occurs because the Folin-Ciocalteau reagent oxidizes the phenolic group (alkali salt) or the phenolic group reduces heteropoly acid (phosphomolybdate-phosphotungstic) into a molybdenum-tungsten complex. The reaction between phenol compounds and the Folin-Ciocalteau reagent can only occur in an alkaline environment. As a result, protons dissociate in phenolic compounds into phenolic ions. The greater the concentration of phenolic compounds in the sample, the more phenolic ions that will reduce heteropoly acid (phosphomolybdate phosphotungstic) to a molybdenum-tungsten complex so that the resulting blue color is more intense [40].

Table 2 shows that the phenolic content in Libho fruit with value, 28 ± 0.05 mg GAE/100 mg sample (ethyl acetate fraction), 26.05 ± 0.19 mg GAE/100 mg sample (chloroform fraction), 23.32 ± 0.11 mg GAE/100 mg sample (methanol extract), 23 ± 0.19 mg GAE/100 mg sample (n-hexane fraction) and 7.6 ± 0.12 mg GAE/100 mg sample (water fraction), respectively.

The high content of phenolic and flavonoids in an ordinary sample correlates with the antioxidant activity of natural compounds. This is due to the presence of hydroxy groups in flavonoids and phenolics. The hydroxy group contributes to scavenging radicals or reducing radicals because the hydroxy group compounds in phenols and flavonoids have redox properties and high hydrogen mobility capabilities in their molecular structure [41-43]. In addition, the presence of hydroxyl groups, and o-hydroxy groups in phenolic and flavonoid compounds, greatly influences free radical scavenging. In addition, polyphenolic compounds such as flavonoids, an ortho-dihydroxy structure in ring B, 2,3 double bonds in conjugation with 4-oxo in ring C, hydroxy groups at positions 3 and 5 on ring A, or the angle between the rings in the compound structure causes the antioxidant effect [44-47].

The correlation between the phenolic and flavonoid content in the sample shows that the phenolic and flavonoid levels simultaneously provide a very strong relationship in ABTS cation radical scavenging. Based on the analysis results using SPSS 16.0, partially flavonoid compounds did not significantly influence the stabilization of ABTS cation radicals. As indicated by the significance value of 0.934 > 0.05. Meanwhile, the total phenolic content partially contributed to the radical stabilization of ABTS. This contribution is shown by the statistical analysis results, where the significance value is 0.024 < 0.05. Meanwhile, the simultaneous contribution of phenolic compounds and flavonoids in stabilizing ABTS radicals, based on the results of data analysis, showed that phenolic compounds and flavonoids simultaneously gave the strongest contributions to stabilizing ABTS cation radicals with a value of R2 = 0.959. This indicates that the contribution of phenolic compounds and flavonoids simultaneously in

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stabilizing ABTS radicals contributes 95.9%. While other compounds besides phenolic and flavonoid influenced 4.1%. Meanwhile, phenolic compounds and flavonoids did not show a significant correlation in reducing CUPRAC radicals. This is indicated by the results of statistical analysis either partially or simultaneously. Partially, the correlation between phenolic compounds in reducing CUPRAC radicals was 0.46 > 0.05, and the correlation between flavonoid compounds in reducing CUPRAC radicals was 0.33 > 0.05. while simultaneously, the correlation of phenolic compounds and flavonoids in reducing CUPRAC radicals was 0.82 > 0.05.

Conclusion

Extracts and fractions of Libho (*Ficus Septica* Burm. F) fruits showed antiradical activity using the ABTS and CUPRAC radical methods. Among the samples evaluated, the n-hexane fraction of Libho fruits showed very strong antioxidant activity and very high phenolic and flavonoid content Libho fruits can be developed as a source of antioxidants from natural ingredients.

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