Antioxidant Activities of Extracts of Trengguli Stem Bark (Cassia fistula L.)

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Antioxidant Activities of Extracts of Trengguli Stem Bark (Cassia fistula L.)

Hermien Noorhajati , Mulyadi Tanjung , Nanik Siti Aminah and Ami Suwandi J.S.

***Abstract*-- This research is conducted to examine the antioxidant activity on the extract of stem bark *C.fistula*. The antioxidant activities of *C.fistula* stem bark extract were evaluated with lipid peroxides test using ferric thiosyanat method (FTC) and 2.6-di-t-butyl-4-metilfenol (BHT) as standard equivalent antioxidant capacity. *C.fistula* stem bark maceration successively used solvent normal heksane (non polar), ethyl acetate (semi-polar) and methanol (polar).**

The etyl acetate extract (Ea) shows higher antioxidant activity than the n.hexane extract (Hx) and methanol extract (MeOH). Therefore, the sequence of antioxidant activity is as follows ethyl acetate extract > methanol extract > n.hexane extract, with antioxidant activity consecutively at 5 hours: 65.98%, 58.19% and 32.66%. Those amount are equivalent to the standard synthetic antioxidant BHT (100 ppm), which causes 95.7% antioxidant activity (in 5 hours) inhibition of linoleic acid peroxidation.

There is a connection between antioxidant activity of an extract with the content of the total phenol in each extract. From the assay of phenolic extracts with the method of Folin-Ciocalteu reagent (FCR) and also using afzelechin standard as a comparision, we find that the ethyl acetate extract has the highest total phenolic where the entire sequences are as follows: Ea> MeOH> Hx. with total phenol content consecutive 177.55, 123.2167, 7.433333

***Index Term*-- Cassia fistula, Antioxidant, Lipid peroxide, Total Phenolic.**

I. INTRODUCTION

Cassia fistula Linn is known as Aragvadha in Ayurveda is an important medicinal plant belonging to family Caesalpiniaceae. The stembark is anti dysenteric, laxative and diuretic [11][16]. The whole plant possesses medicinal properties useful in the treatment of skin diseases, inflammatory diseases, rheumatism, anorexia and jaundice [12]. It is a medium sized deciduous tree that reaches the height of about 8-15m to 24m. The young stem bark is of greenish grey smooth and rough, dark brown in color when mature. Leaves are alternate, pinnate, 1 to 1.5 feet long and possess pairs of four to eight ovate leaflets. It bears yellow colored flowers. The fruit beared by the amaltas tree is pendulous, cylindrical, brown and septate having a length of 25 to 45 centimeters and possess a diameter of 1 to 3 centimeters. It has within it about 30 to 100 seeds [16].

Recognized by the British Pharmacopoeia [2], *C. fistula*, a member of the family Leguminosae, is widely used for its medicinal properties. It has been reported that the extract *C. fistula* possessed a variety of biological and pharmacological activities, extract *C. fistula* showed antiinflammatory activity and hypoglycaemic activity, also showed antibacterial, antitumor, hepatoprotective, antifertility, antioxidant [22]. In the Indian literature, this plant has been described to be useful against skin diseases, liver troubles, tuberculous glands and its use in the treatment of haematemesis, pruritus, leucoderm and diabetes. It also has been suggested that *C. fistula* extract is used as an anti-periodic agent and in the treatment of rheumatism [2] [12][22]. The leaf extract is also indicated for its anti-tussive and wound healing properties [4] [5].

Antioxidants are needed in food, as food additives. They also served to prolong the shelf life of food and maintain food safety, nutritional quality, functionality and palatability [13]. Antioxidants must be non-toxic, relatively inexpensive, and effective. They should also have a carrythrough effect during processing, and should not alter the quality of the end-product [13]. The process of lipid oxidation in foods is one of the main causes of chemical decomposition, resulting in rancidity and / or damage, the nutritional quality of the color, flavor texture, and safety in food during storage and processing [1]. Other than that antioxidants are substances that the body needs to neutralize free radicals and prevent damage caused by free radicals on normal cells, proteins and fats. Free radicals can cause various diseases.

The lipid oxidation process is involved in oxidative damage occurring at a cellular level leads to aging, because lipids,

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which should keep the skin to stay fresh changes to the lipid peroxide reacts with free radicals that accelerate aging [17]. Oxidative effects may also increase levels of LDL (Low density lipoprotein), which then causes the accumulation of cholesterol in the blood vessel walls. This resulted in the onset of atherosclerosis or more commonly known as coronary heart disease, Free radical mediated peroxidation of membrane lipids and oxidative damage of DNA are believed to be associated with a variety of chronic health problems, such as cancer, atherosclerosis, neurodegenerative diseases and aging [3][7]. Coronary heart disease (CHD) is a disease which killed an estimated 15 million people or about 30% of the total causes of death and is expected to increase to 40% by 2020 (WHO, 2001). There are three stages of work processes of antioxidants in the body, prevent or inhibit the occurrence of fat peroxide, capturing reactive oxygen species (ROS) and also repair damage caused by ROS. Which include reactive oxygen species (ROS) are superoxide (O_2 *), hydroxyl (HO *), peroxyl (ROO *), hydrogen peroxide (H_2O_2), singlet oxygen (O_2), nitric oxide (NO *), peroxy nitrite (ONOO *), and hypochlorous acid (HOCl) [3]. Reactive oxygen species (ROS) are a class of highly reactive molecule derived from the metabolism of oxygen. ROS, including superoxide radicals, hydroxyl radical and hydrogen peroxide molecule, are often generated as by product of biological reactions or from exogenous factors. There is extensive evidence to implicate ROS in the development of degenerative diseases [23].

Antioxidant compounds usually contain phenolic compounds, antioxidant activity becomes much higher if the hydroxy group is substituted on the aromatic compound nucleus. Phenolic compounds in plants that show anti-free radical activity is a flavonoid compound, stilben, anthraquinone, phenyl propanoid, Santon and also alkaloids [10]. Currently more natural antioxidants be an option, because of concerns over side effects and toxic properties of synthetic antioxidants, such as carcinogenesis

The process of formation of free radicals in the human body through the events of cell metabolism and the presence of free radicals in the body can be controlled by the body itself by forming an endogenous antioxidant. But the body does not have excessive antioxidant defense system so that the body requires exogenous antioxidants, which are widely available in plants such as vitamin E, vitamin C, carotenoids [25]. There is overwhelming evidence showing that natural antioxidants play a role in wellness, health maintenance, and the prevention of the chronic and degenerative diseases. Several antioxidant based formulations have been developed for the treatment of diseases like atherosclerosis, stroke, diabetes, Alzheimer's disease and cancer during the last 3 decades [23]. This has attracted a great deal of research interest in natural antioxidants. It is necessary to study the botanicals to screen out for their antioxidant potential. Natural antioxidants can protect the human body from free radicals and retard the progress of many chronic diseases as well as lipid oxidative rancidity in foods. Hence, the studies on natural antioxidants have gained increasingly greater importance [9].

In 2002 had done the research to test the antioxidant in some parts of the plant *C. fistula* using the TEAC (Trolox equivalent antioxidant capacity) and FRAP method (Ferric reducing antioxidant power), it was reported that the order of the antioxidant properties of ethanol extract of *C. fistula* from various parts of the plant is the bark > leaves> flowers> pulp [18][24]. From the literature search, we recognized that *C. fistula* is known as plant which possesses medicinal properties useful in the treatment. In the ilterature search, we further recognize that the highest antioxidant properties exist in the stembark. Based on this, we conducted research on the antioxidant properties of bark extracts *C.fistula* using a variety of solvents which are non-polar to polar, namely normal-hexane, ethyl acetate and methanol. In our research, we tested the antioxidant activity of each extract in C.fistula bark using the FTC method (Ferric thiocyanate).

MATERIALS AND METHODS

Collection of Plant Materials

The fresh stem bark of *Cassia fistula Linn* plants was collected from Purwadadi, Pasuruan district, East Java in February 2009 and we have confirmed the authenticity of the plants from the Botanical Gardens Purwadadi, Indonesia.

Preparation of Plant Extract

C. fistula stem bark collected washed then dried by aerating at room temperature (30 ° C) for several days. Dried stem bark crushed in an electric grinder by using 2kg dry powder and then extracted (maceration) in stages, starting from the non-polar solvent to polar solvent. Plant samples were extracted in a row during 3x24jam up to 3x, ranging from normal hexane (to extract non-polar compounds), ethyl acetate (to extract the semi-polar compounds) and methanol (to extract polar compounds). Solvent in each extract was vacuum evaporated with a rotary evaporator.

Chemical compound

Solvents used for maceration were high quality technical materials that had been distiled first, namely n-hexane, ethyl acetate and methanol. The materials used for this analysis are those with proanalysa (pa) degree and obtained from Merck (Darmstadt, Germany), namely linoleic acid, tween-40 , phosphate buffer, methanol, ammonium thiocyanate, ferrous chloride, hydrochloric acid, ethanol.

Antioxidant activity by linoleic acid peroxidation method

Antioxidant activity of stem bark extracts *C. fistula* with various solvents were determined using ferric thiocyanate method [14]. Ferric thiocyanate method (FTC) is based on the determination of peroxide (lipid) at the primary stage of linoleic acid peroxidation. The peroxide reacts with ferrous chloride to form a reddish ferric chloride pigment which is measured at 500nm.

The linoleic acid emulsion was prepared by homogenizing 0.28 g of linoleic acid, 0.28 g of tween-40 as emulsifier and 50 ml of phosphate buffer (0.2 M, pH 7.0).

Each extract was dissolved in methanol and pipette (0.5 ml) into different test tubes (equivalent to 100 ppm), then they were mixed with 2.5 ml of linoleic acid emulsion, 2.5 ml of phosphate buffer (0.2 M, pH 7.0) and incubated at 37^0 C for 5 h. The mixture prepared as above, without test sample, was served as control. Aliquots (0.1 ml) were drawn from the incubation mixture at intervals of 1 h and were mixed with 5.0 ml of 75% ethanol, 0.1 ml of 30% ammonium thiocyanate and 0.1 ml of 20 mM in ferrous chloride in 3.5% hydrochloric acid and allowed to stand at room temperature for 3 min. The colour developed was measured at 500 nm. The degree of linoleic acid peroxidation was calculated at 5 h using the following formula [21].

Antioxidant activity = [1 - (increase in absorbance of sample / increase in absorbance of control)] · 100.

The control and the standard were subjected to the same procedure as the sample except that for the control, only the solvent was used, and for the standard, 0.5 ml (equivalent 100 ppm), of the sample was replaced by 0.5 ml (equivalent 100 ppm) of 2,6-di-t-butil-4-metilfenol (BHT). BHT was used as standard for comparison. All tests and analyses were carried out in triplicate and averaged.

Preparation of standard curve of total phenolic

The total phenol content of plant extracts was determined by Folin–Ciocalteu reagent (FCR) according to the procedure reported by Singleton, Orthofer, and Lamuela Raventos (1999) with some modifications. Into the respective solution of pure phenolic compounds (Afzelechin) in ethanol with the concentration variation of 0.04: 0.05: 0.06: 0.08, and 0.1 mg / mL plus as many as one mL each with 7, 5 mL of Folin Ciocalteu Reagent (FCR), which was diluted 10-fold with distilled water. After standing at room temperature for 5 min, 7.5 ml of 60 mg/ml of aqueous Na_2CO_3 solution were added. The mixture was kept at room temperature for 2 h and then the absorbance was measured at 725 nm. The results were expressed in afzelechin equivalents, determined utilizing a separately prepared absorbance versus concentration curve for afzelechin [8].

Determination of total phenolic content

C. fistula stem bark extract as much as 0.1 mg dissolved in 1 mL of ethanol. Further into the extract solution was added 7.5 mL of Folin Ciocalteu Reagent (FCR), which has been diluted 10 times with distilled water. After 5 minutes add 7.5 mL of Na_2CO_3 solution of 60 mg / mL, the mixture was shaken on a shaker, allowed to stand for 2 hours, then measured the absorbance at a wavelength of 725 nm. All tests and analyzes carried out in triplicate and averaged.

RESULTS AND DISCUSSIONS

In FTC method, the amount of peroxide at the initial stage of lipid peroxidation was determined. During the linoleic acid oxidation, peroxides are formed, which oxidize Fe^{+2} to Fe^{+3}. The formed Fe^{+3} ions complexes with thiocynate ions (SCN^-), which has a maximum absorbance at 500 nm. The concentration of peroxide decreases as the antioxidant activity increases. The lower the absorbance value exhibited, the higher the antioxidant activity.

The antioxidant activities of extract from stem bark *C. fistula* the peroxidation of linoleic acid, as measured by thiocyanate method, are shown in Fig. 1. Absorbance of control increased from 1.0591 to 1.1136 at 3 hours, and then decreased to 0,8882. This is due to oxidation of linoleic acid, generating linoleic acid hydroperoxides, which leads to many secondary oxidation products [13]. 2,6-di-t-butil-4-metilfenol (BHT) showed an initial absorbance of 0.023 and a maximum absorbance of 0.045 on hour 5. Extract ethyl acetat had absorbance value of 0.3724 initially and gradually raised to 0.3021 on hour 5. The control had the highest absorbance value 0.8882, followed by hexane extract (0,5981), methanol extract (0,3713), ethyl acetate extract (0,3021) on hour 5. Based on the results obtained, the ethyl acetate extract was found to possess antioxidant activity, which is comparable to standard 2,6-di-t-butil-4-metilfenol (BHT), at a concentration of 100ppm.

The oxidized products (i.e, linoleic acid hydroperoxides) react with ferrous chloride to form ferric chloride, then to ferric thiocyanate (blood-red colour) [13]. After the incubation period (3 h), the formation of peroxides is stagnated, due to non-availability of linoleic acid. Also, the intermediate products may be converted to stable end-products. The non-availability of hydroperoxides, results in the retardation of oxidation of ferrous chloride. Hence, the absorbance does not increase. In the presence of extract hexane, ethyl acetate extract, methanol extract and BHT, oxidation of linoleic acid was very slow. Hence, the colour development is slow. The antioxidant activities of the extract of each extract, are shown in Fig. 2., for hexane extract (Hx), ethyl acetate extract (Ea), methanol extract (MeOH) to be 37.45%, 70.04% and 66.24%, respectively, at 3 h; and we found the data of 32.66%, 65.98% and 58.19%, respectively, at 5 h. Those data are equivalent to (100 ppm) BHT as a standard synthetic antioxidant that in 5 hours can cause 95.7% inhibition of linoleic acid peroxidation. In brief, total antioxidant activity of *C.fistula* stem bark extract and positive control are determined by FTC method decreased in the order of BHT > Ea > MeOH > Hx.

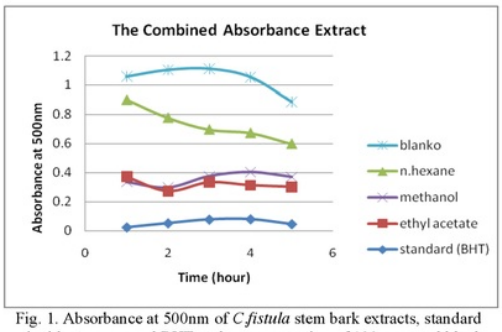

Fig. 1. Absorbance at 500nm of *C.fistula* stem bark extracts, standard antioxidant compound BHT at the concentration of 100 ppm and blanko (BHT: 2.6-di-t-butyl-4-metilfenol). Absorbance at 500nm determined by ferric thiocyanate method (FTC). A low absorbance value represents a high level of antioxidant activity. Values are the average of triplicate experiments.

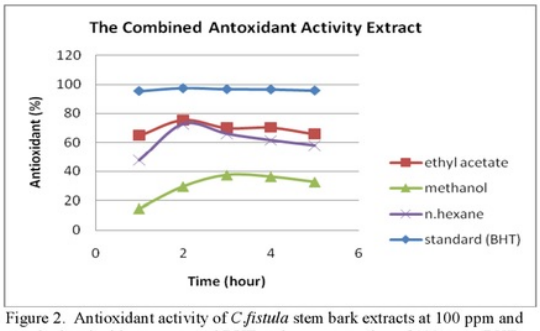

Figure 2. Antioxidant activity of *C.fistula* stem bark extracts at 100 ppm and standard antioxidant compound BHT at the concentration of 100 ppm (BHT: 2.6-di-t-butyl-4-metilfenol). Antioxidant activity determined by ferric thiocyanate method (FTC). Values are the average of triplicate experiments.

The present study reveals that ethyl acetat extract from stem bark *C. fistula* showed antioxidant property which is comparable to the standard BHT. Thus ethyl acetat extract exhibited significant *in vitro* antioxidant activity by inhibiting the oxidation of linoleic acid in FTC method. The activity was comparable with standard BHT .

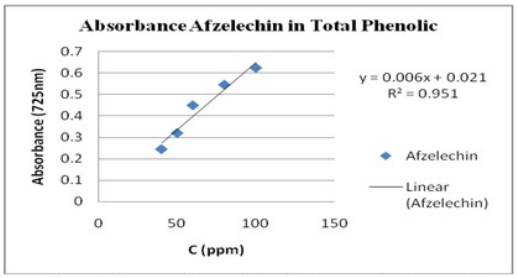

Figure 3. The curve linear relationship between absorbance with concentration afzelechin as a standard test of total phenolic. The regression equation obtained from the calibration curve : $y = 0.006x + 0.021$; $R^2 = 0.951$.

Antioxidant compounds generally contain phenolic group, while extracts are mixture of dozens of compounds with different functional groups, polarity, and chemical behavior. A thorough phenol test is needed to gain further information on the total phenol content of each extract as antioxidants.

A specific reagent of Folin-Ciocalteu reagent (FCR) is used for the early detection of the content of total phenolic compounds in a sample Folin-Ciocalteu reagent is a reagent that has been developed to determine the total phenolic content in a sample of natural material. This reagent is a mixture of phosphomolybdate and phosphotungstate used to analyze phenolic compounds by measuring the ability of test compounds to inhibit the oxidation of the reactants. The principle of this test is the reaction of reduction events Mo (VI) to Mo (V) by a component of antioxidants such as phenolic compounds. Complex Mo (V) is formed which can be detected by UV-Vis spectrophotometry [20].

The assay to determine the total phenolics content employs Folin and Ciocalteu's phenol reagent in which the result will depend on the chemical structure of phenolics (i.e. the higher the number of functional – OH group the higher the total phenolics content) [15]. Therefore afzelechin was used as the standard to represent all phenolics present in *C. fistula* extracts. Fig. 3 exhibits afzelechin equivalents of total phenolic contents of all extracts. As displayed, ethyl acetate extract carried highest total phenolics and the rest of the extracts followed the order of Ea > MeOH > Hx.

Table 1. Total phenolic contents of extracts calculated from regression equation of calibration curve (A_{725nm} = 0.006[C_x]mg + 0.021). Figure 1 through 3 shows a positive relationship exists between the antioxidant activity of each extract as measured by the FTC method and total phenolic content of extracts.

TABLE I
Total phenol in *C. fistula* extract

Extract	C (ppm)	A (at 725nm)	Total Phenol (mg)
Afzelechin	100	0.6166	99.2667
HX	100	0.0656	7.433333
Ea	100	1.0863	177.55
MeOH	100	0.7603	123.2167

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The decreasing order of antioxidant activities in extracts of the stem bark *C.fistula* correlates well with the order of antioxidant capacities found by content of phenolic compounds obtained from measurements total phenolic of each extract.

CONCLUSIONS

This study revealed that stem bark extracts *C.fistula* has the potential to inhibit the oxidation of linoleic acid *in vitro*. Ethyl acetate extract showed significant antioxidant activity *in vitro* by inhibiting the oxidation of linoleic acid in the ferric thiocyanate method (FTC). The antioxidant activity of ethyl

acetate extract of stem bark *C.fistula* is comparable with standard 2.6-di-t-butyl-4-metilfenol (BHT) antioxidant activity. This information, may be useful in drug design and clinical use of antioxidants. Nonetheless, further test on the stem bark *C.fistula* extract is needed to support the feasibility of its use in clinical utilization as an antioxidant. Therefore, the expected outcome of this research could encourage other similar research in the effort of finding the marker compound (chemical marker) for antioxidants as a lipid peroxide.

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