

POTENCY OF ANTIOXIDANT, ANTICHOLESTEROL AND PLATELET ANTIAGGREGATION OF BLACK TEA (*Camelia sinensis*)

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ABSTRACT

*Epidemiological studies have shown an inverse correlation between diets rich in polyphenols, flavonoids and reduced risk of cardiovascular disease. These associations were mainly ascribed to the antioxidant, antiaggregation and anticholesterol capacity of polyphenols. This association has been explained that atherogenesis is initiated by hypercholesterol, lipid peroxidation and hyper aggregation platelet. The research was carried out to evaluate antioxidant, anticholesterol and antiaggregation activities of methanol extract and fractions of black tea (*Camelia sinensis*). To evaluate antioxidant activity of methanol extract and fractions were compared with (-)-Epigallocatechine 3-gallate (EGCG), anticholesterol activity were compared with simvastatin and antiaggregation activity were compared with aspirin. Antioxidant activity using the 1,1-diphenyl-2-picryl-hydrazyl (DPPH) free radical scavenging activity at two concentrations 100 µg/mL, 50 µg/mL and anticholesterol was cholesterol oxidase activity at two concentrations 0.005 µg/mL, 0.0025 µg/mL, the antiaggregation activity used epinephrine (EPN)*

agonist at two concentrations 300 µM, 75 µM and one concentration 37.1 µg/mL of methanol extract and fractions of black tea. To know the difference of treatment, the data were analysed using Analysis of Variance (ANOVA) and be continued Duncan's new multiple range test. The results showed that DPPH scavenging activity were high (88.59-93.556%), the anticholesterol showed high activity (93.663-97.434%) and the antiaggregation showed low up to high activity (79.967-4.31%). Using Duncan's new multiple range test showed that anti-oxidant activity were not different among extract and fractions of black tea, the highest anticholesterol activity was black tea extract 0.005 µg/mL (97.434%) and the highest antiaggregation activity was ethyl acetate fraction on 75 µM EPN agonist (4.310 %), water fraction 37.1 µg/mL with 300 µM EPN agonist (21.833%).

Key words : *Black tea, Camelia sinensis, antioxidant, anticholesterol, antiaggregation, flavonoids, cardiovascular disease*

ABSTRAK

Potensi Antioksidan, Antikolesterol dan Antiagregasi Platelet Teh Hitam (*Camelia sinensis*)

Berdasar studi epidemiologi menunjukkan adanya hubungan terbalik yaitu antara makanan yang kaya polifenol, flavonoid dan pengurangan risiko penyakit kardiovaskuler. Hubungan ini terutama disebabkan oleh aktivitas antioksidan, antikolesterol dan antiagregasi dari polifenol, serta aterogenesis yang dipicu oleh hiperkolesterol, peroksidasi lipid dan hiper agregasi platelet. Penelitian ini dilakukan untuk mengetahui aktivitas antioksidan, antiagregasi dan antikolesterol dari ekstrak metanol dan fraksi-fraksi teh hitam (*Camelia sinensis*). Untuk mengetahui aktivitas antioksidan ekstrak metanol dan fraksi-fraksi teh hitam dibanding (-)-*Epigallocatechine 3-gallate* (EGCG), aktivitas antikolesterol dibandingkan simvastatin dan antiagregasi dibandingkan aspirin. Aktivitas antioksidan adalah penghambatan radikal bebas *1,1-diphenyl-2-picryl-hydrazyl* (DPPH) dilakukan pada 2 konsentrasi yaitu 100 µg/mL, 50 µg/mL dan antikolesterol adalah aktivitas kolesterol oksidase dilakukan pada 2 konsentrasi yaitu 100 µg/mL, 50 µg/mL. Aktivitas antiagregasi menggunakan agonist epinephrine (EPN) pada konsentrasi agonist 300 µM, 75 µM dan konsentrasi 37,1 µg/mL ekstrak metanol dan fraksi-fraksi teh hitam. Untuk mengetahui perbedaan perlakuan, data dianalisis menggunakan *Analysis of Variance* (ANOVA) dilanjutkan uji jarak berganda Duncan. Hasil penelitian menunjukkan pemerangkapan DPPH memiliki aktivitas antioksidan tinggi sebesar 88,59-93,556%, antikolesterol menunjukkan aktivitas kolesterol oksidase tinggi sebesar 93,663-97,434%, antiagregasi menunjukkan aktivitas rendah sampai tinggi sebesar 79,967-4,31%. Berdasarkan hasil uji jarak berganda Duncan aktivitas antioksidan tidak berbeda nyata antar ekstrak dan fraksi-fraksi teh hitam, aktivitas antikolesterol tertinggi adalah ekstrak teh

hitam 0,005 µg/mL sebesar 97,434% aktivitas antiagregasi tertinggi pada agonist EPN 75 µM sebesar 4,310% dan fraksi air 37,1 µg/mL pada agonist EPN 300 µM sebesar 21,833%.

Kata kunci : Teh hitam, *Camelia sinensis*, antioksidan antikolesterol, antiagregasi, flavonoid, penyakit kardiovaskuler

INTRODUCTION

High plasma cholesterol is one of the greatest risk factors that contribute to the prevalence and severity of cardiovascular disease (CVD) (Sasazuki *et al.* 2000; Yung *et al.* 2008). Variety of factors contribute to the beneficial effects of functional foods, much attention has been addressed to plant polyphenols (Grassi *et al.* 2008). Epidemiological studies have shown an inverse correlation between diets rich in polyphenols and reduced risk of CVD (Hertog *et al.* 1996; Mukamal *et al.* 2002; Yung *et al.* 2008). Tea (*Camelia sinensis* L) is one of the most popular beverages in the world because of its attractive flavor and aroma. Polyphenols are the most significant group of tea components, especially flavonoids (Willson 1999; Cabrera *et al.* 2006).

Studies using animal models of atherosclerosis indicate that dietary flavonoid consumption delays atherosclerotic plaque development (Grassi *et al.* 2008). In a long-term study of a Dutch cohort the highest tea consumption was associated with a lower risk of death from coronary heart disease (Geleijnse *et al.* 2002; Yang and Landau 2000). Black tea is a major source of flavonoids, with antioxidant effects that may help to retard atherosclerosis (Sesso *et al.* 1999). The potential protective effect of flavonoids

has been attributed to antioxidant, antithrombogenic and antiinflammatory properties (Hertog 1996; Middleton 1998; Leenen *et al.* 2005; Geleijnse *et al.* 2002). Flavonoids may also improve vascular function in animal experiments (Duffy *et al.* 2001; Geleijnse *et al.* 2002). The relative risk of incident myocardial infarction was lower in tea drinkers with a daily intake >375 mL than in non tea drinkers. An increased intake of tea and flavonoids may contribute to the primary prevention of ischemic heart disease (Geleijnse *et al.* 2002). Fresh tea leaves are rich in flavanol monomers known as catechins. The principal catechins found in tea are (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG) and (-)-epigallocatechin gallate (EGCG). EGCG is the most abundant catechin in tea (Graham 1992; Frei and Higdon 2003). In manufacturing black tea, the tea leaves are crushed to allow the polyphenol oxidase to catalyse the oxidation, leading to polymerization of catechins. The remaining catechins account for 3-10% of the solids in brewed black tea. Theaflavins (TF), which include theaflavin (TF1), theaflavin-3-gallate (TF2), theaflavin-3'-gallate (TF2B) and theaflavin-3,3'-digallate (TF3), are key to the characteristic color and taste of black tea, and account for 2-6% of the solids in brewed black tea. The major fractions of black tea polyphenols, accounting for >20% of the solids in brewed black tea, are known as thearubigens (Yang and Landau 2000).

This research was to evaluate the antioxidant, anticholesterol and antiaggregation platelet of methanol extract, fractions of black tea by *in vitro* assay.

MATERIALS AND METHODS

Plant and chemical material

Dried black tea leaves obtained from Walini Tea Company, Indonesia. DPPH (1,1-diphenyl-2-picrylhydrazyl) (Sigma Chemical Co.); HPLC grade methanol (Merck); (-) epigallocatechin gallate (EGCG) (Sigma Chemical Co.); dimethyl sulfoxide (Merck); Cholesterol KIT (Randox) reagent contains 4-Aminoantipyrine, phenol, peroxidase, cholesterol esterase, cholesterol oxidase; Simvastatin (Kimia Farma); Cholesterol (Sigma Chemical Co.), Epinephrine (EPN) (Helena Laboratories 2008).

Extraction and fractionation

The dried black tea leaves were extracted by maceration technique, produced 7.8% methanol extract. The fractionation used liquid partition method. The first step used n-hexane: water (7:3) as solvent produced hexane fraction 20.45%. The second step used ethyl acetate : water (9:1) produced ethyl acetate fraction 10.93% and the third step used n-butanol : water (9:1) as solvent produced butanol fraction 3.30% and the remain was water fraction 14.14%.

DPPH radical scavenging activity assay

The DPPH assay was carried out as described by Frum and Viljoen (2006). Pipette 50 µL of sample (two concentrations) enter at the microtitre plate and then were added 200 µL of 0.077 mmol/L methanol solution of DPPH and the reaction mixture was shaken vigorously and kept in the dark for 30 min at room temperature, DPPH was determined by microplate reader at 517 nm.

Anticholesterol activity assay

The anticholesterol assay was carried out as described by Iswantini *et al.* (2005) and Cholesterol (Chol) Enzymatic Endpoint Method (Randox Laboratories Ltd. 2009). Cholesterol was dissolved in chloroform until achieving 25 mg/10 mL. Anticholesterol assay used Cholesterol manual (Randox 2009). Pipette 5 μ L of sample (two concentrations) enter at the microtitre plate and then were added 1000 μ L Randox reagent and 5 μ L cholesterol as sample. Blank solution comprised of 10 μ L distilled water and 1000 μ L Randox reagent; negative control comprised of 10 μ L cholesterol and 1000 μ L Randox reagent; standard comprised of 10 μ L Randox standard and 1000 μ L Randox reagent. Mixed and incubated for 10 minutes at room temperature, measured the absorbance by microplate reader at 500 nm against reagent blank.

Platelet antiaggregation activity assay

Aggregation activity was measured by Platelet Aggregation Chromogenic Kinetic System (PACKS-4). Pipette 450 μ L of platelet poor plasma (PPP) into a cuvette. Pipette 450 μ L platelet rich plasma (PRP) and 40 μ L sample into cuvettes with stir bar, incubate the cuvettes at 37^o C for 3-5 minutes. Insert the PPP cuvette into appropriate channel and set the instrument, insert the PRP cuvette into the appropriate channel. Add 50 μ L of EPN at level 300 μ M, 75 μ M to the PRP cuvette and record the percent aggregation (Helena Laboratories 2008).

Statistic analysis

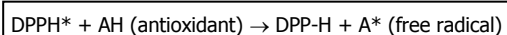
To verify the statistical significance of the parameter, the data were calculated including the values of means and standard deviation ($M \pm SD$) and 95% confidence interval (CI) of means. To compare among treatments, used analysis of variance (ANOVA) with completely randomized designs. P-values of less than 0.05 were considered as statistically significant. Furthermore, to know the difference of levels among treatments and to know the best treatment used Duncan's new multiple range test 95% confidence interval. Statistical analysis used SPSS 16.0 program.

RESULTS AND DISCUSSIONS

The DPPH scavenging activity

There were no different among concentrations and among antioxidant agents. All treatment (extract, fractions of black tea and two concentrations) had similar and high antioxidant activity ($p = 0.07 > 0.05$), the highest antioxidant activity was butanol fraction at level 100 μ g/mL (93.56%), the lowest antioxidant was ethyl acetate fraction at level 100 μ g/mL (88.59%).

The DPPH assay base on the scavenging of DPPH radicals is followed by monitoring the decrease in absorbance at 517 nm which occurs due to reduction by the antioxidant (AH) or reaction with free radical species (R^*) :



Base on DPPH scavenging activity test, sample contains antioxidant

Which hydrogen (H) captured by DPPH free radical or antioxidant donates hydrogen (H) was indicated purple color (DPPH free radical) become 1,1-diphenyl-2-picrylhydrazyl yellow color (Kikugawa *et al.* 2001; Gordon 2001). When DPPH^{*} reacts with an antioxidant compound, which can donate hydrogen, it is reduced. The changes in colour (from deep-violet to light-yellow) were measured at 517 nm (Miliauskas *et al.* 2003).

Black tea had high antioxidant activity due to it contains flavonoids, there are EC, ECG, EGC, EGCG, TF1, TF3, TF2B, Thearubigins, Kaempferol, Myricetin, Quercetin (USDA 2003). The most widely recognized properties of tea polyphenols are their antioxidant activities, arising from their ability to scavenge reactive oxygen species (Yang and Landau 2000). Dietary antioxidants, especially tea and tea flavonoids have attracted increasing interest due to their relatively potent radical scavenging (Nanjo *et al.* 1996; Mandel *et al.* 2004).

The anticholesterol activity

Anticholesterol activity among extract, fractions of black tea and concentrations treatment were significant ($p=0.00<0.05$) (Table 1.)

All of anticholesterol candidate agents from black tea including methanol extract and four fractions showed high anticholesterol activity >93.66% although were lower than simvastatin. All of anticholesterol agents except butanol fraction showed that high concentration (0.005 µg/mL) were more active than lower concentration.

Previous study some catechins have been shown to inhibit a key enzyme (squalene epoxidase) in the pathway of cholesterol biosynthesis. Theaflavin was found to be twice as effective in blocking the activity of squalene epoxidase (Leung *et al.* 2001; Wang *et al.* 2001; Cooper *et al.* 2005). Black tea extract contains polyphenols, flavonoids, catechins (USDA 2003), by *in vitro* and *in vivo* studies that tea or catechins inhibit the intestinal absorption of dietary lipids. Studies *in vitro* indicate that catechins, particularly EGCG, interfere with the emulsification, digestion, and micellar solubilization of lipids, critical steps involved in the intestinal absorption of dietary fat, cholesterol, and other lipids. Tea or its catechins lower the absorption and tissue accumulation of other lipophilic organic compounds so can be used as safe and effective lipid-lowering therapeutic agents (Koo and Noh 2007). In animals, catechins reduce the solubility of cholesterol in micelles an action consistent with the observation that high doses of tea modulate cholesterol levels in animals fed high cholesterol (Yang *et al.* 2001; Cooper *et al.* 2005).

Based on the Table 1 showed that simvastatin had high anticholesterol activity, this data was validated with previous study that statins as 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (*i.e.*, simvastatin) have been accepted as the first choice for lowering low-density lipoprotein (LDL), cholesterol levels (Hubacek and Bobkova 2006). These inhibitors act primarily by inhibiting the cellular biosynthesis of cholesterol in the liver (Risovic *et al.* 2006).

Table 1. Means, standard deviation and Duncan's new multiple range test of extract and fractions of black tea on anticholesterol activity (%)

Concentrations ($\mu\text{g/mL}$)	Samples					
	Methanol extract	Hexane fraction	Ethyl ace-tate fraction	Butanol fraction	Water fraction	Simvastatin
0.005	97.43 $\pm$ 0.15 d	95.85 $\pm$ 0.29 c	96.88 $\pm$ 0.31 d	95.40 $\pm$ 0.14 bc	96.88 $\pm$ 0.17 d	99.35 $\pm$ 0.51 e
0.0025	95.40 $\pm$ 0.34 bc	95.29 $\pm$ 0.27 b	95.81 $\pm$ 0.24 bc	95.45 $\pm$ 0.20 bc	93.66 $\pm$ 0.22 a	95.40 $\pm$ 0.25 bc

Note : The data showed mean and standard deviation. The same small letters (among extract, fractions and simvastatin at two level concentrations) show no significant at the 5 % (Duncan's Post Hoc test)

According the antioxidant activity showed that methanol extract and fractions of black tea had high antioxidant activity and according to Table 1 showed that extract and fractions of black tea indicated high anticholesterol activity. This results were validated with previous research that the positive effects of natural antioxidant nutrients on cardiovascular dysfunction, resulting from multiple factors, such as atherosclerotic plaques, low-density lipoprotein (LDL) oxidation, triglycerides, and cholesterol metabolism (Mandel *et al.* 2004).

The platelet antiaggregation activity

Antiaggregation activity among black tea extract, fractions and agonist concentrations treatment were significant ($p=0.00<0.05$) (Table 2). All of antiaggregation agents, except hexane fraction were capable to lower aggregation compared with negative control (without antiaggregation agent) both in two concentrations of EPN agonist.

The ethyl acetate fraction was more active than aspirin at the 75 μM EPN agonist, water fraction, methanol fraction had same antiaggregation ac-

tivity as aspirin at the 75 μM EPN agonist. Methanol extract, ethyl acetate, butanol, water fraction of black tea and aspirin had same antiaggregation activity at the 300 μM EPN agonist. The methanol extract, ethyl acetate, butanol and water fraction of black tea except hexane fraction showed promising anti-aggregation activity.

This research result was validated with previous research that based on *in vitro* studies have shown that isolated flavonoids at high physiological concentrations can reduce platelet aggregation and markers of platelet activation (Rein *et al.* 2000; Hodgson 2008). Several *in vitro* studies show that flavonoids component inhibit platelet aggregation, a major process contributing to both the development of atherosclerosis and acute platelet thrombus formation followed by embolism-producing cyclic flow reduction in stenosed arteries. Flavonoid antiaggregation effects may be attributed to inhibition of thromboxane formation, thromboxane receptor antagonism, blunting hydrogen peroxide production, or inhibition of phospholipase C (Tzeng *et al.* 1991; Pignatelli *et al.* 2000; Buck *et al.* 2003). Flavonoids inhibit platelet aggregation because

Table 2. Means, standard deviation and Duncan's new multiple range test of extract and fractions of black tea on antiaggregation activity (%)

Concentrations (µM) of EPN	Samples						
	Negative control	Methanol extract	Hexane fraction	Ethyl acetate fraction	Butanol fraction	Water fraction	Aspirin
300	45.83±4.31 e	23.47±3.31 bc	76.50±3.15 f	28.33±3.70 bcd	25.90±2.80 bc	21.83±2.35 b	22.97±3.01 bc
75	88.27±3.31 g	34.40±3.05 d	79.97±6.35 f	4.31±1.81 a	40.73±3.26 e	26.90±2.49 bc	29.10±4.40 cd

Note : The data showed mean and standard deviation. The same small letters (among extract, fractions, negative control and aspirin on two level concentrations of agonist) show no significant at the 5 % (Duncan's Post Hoc test)

of their antioxidant activity, either by inhibiting the formation of endogenous mediators derived from phospholipid peroxidation, by blocking enzymatic free radical production, or by reducing platelet sensitivity to agonists by preventing lipid peroxidation (Murphy *et al.* 2003). Previous research that flavonoids (quercetin and catechin) had antioxidant activity significantly inhibit the release of platelet H_2O_2 , whose production is associated with collagen-induced platelet aggregation. Quercetin and catechin reduced phosphatidylserine (PS) exposure, thrombin formation, phosphatidylinositol 4,5-bisphosphate (PIP2) level and resynthesis after platelet activation with collagen, thrombin or calcium ionophore. Flavonoids also prevented $[Ca^{2+}]$ increase induced by collagen inducer (Buck *et al.* 2003). Quercetin and catechin are flavonoids antioxidant, synergistically inhibited platelet function (*in vitro* assay) by blunting the release of hydrogen peroxide (H_2O_2) from platelets, subsequently reducing phospholipase C activation, calcium mobilization, and inositol phosphate synthesis. Aspirin

as antiplatelet agent on all levels concentration inducer by inhibiting the production of TXA_2 , inhibiting the enzyme COX (Hyun-Jung *et al.* 2006).

These results suggest that the antiplatelet activity of methanol extract, ethyl acetate, butanol and water fractions of black tea may be mediated by TXA_2 receptor blockade with TXA_2 synthase inhibition and suppression of cytosolic $[Ca^{2+}]$ mobilization, inhibited the activation of phospholipase C, followed by inhibition of protein kinase C activity and TXA_2 formation, thereby leading to inhibition of the phosphorylation of P47 and intracellular Ca^{2+} mobilization, finally resulting in inhibition of platelet aggregation (McKenzie 2004; Sheu *et al.* 2004). TXA_2 formation cause the bond between platelets is weak and platelet aggregation is reversible (primer aggregation), secondary aggregation need longer time and produces irreversible aggregation platelet (McKenzie 2004). Epinephrine in the presence of fibrinogen and Ca^{2+} (*in vitro*) induces both primary and secondary aggregation, potentiates aggregation (Kjeldsen *et al.* 1995).

CONCLUSIONS

Methanol extract, fractions of black tea had high antioxidant activity, were potential to inhibit lipid peroxidation, high anticholesterol activity were potential to lower cholesterol level, antiplatelet activity were potential to inhibit aggregation platelet in hyperaggregation individu.

Black tea was promising beverage to inhibit and reduce cardiovascular disease risk.

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