

Phytochemical assay, potential of antimalarial and antioxidant activities of green tea extract and its fraction

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ABSTRACT

Objective : The research objective was to evaluate the phytochemical compounds, the antioxidant and anti-malarial activity of green tea extract and its fraction.

Methods : The antioxidant activity were measured by 1,1-diphenyl-2-picryl-hydrazyl (DPPH) scavenging activity used in vitro laboratories prospective experimental design and completely randomized design, using ethanol extract, hexane, ethyl acetate, butanol fraction, and water fraction of green tea in 7 levels of concentration compared to epicatechin (EC). The data were analyzed using linear regression analysis, and were continued by determined Inhibitory Concentration₅₀ (IC₅₀). The anti-malarial activity of green tea fractions were compared to artemisinin. The data were analyzed using probit analysis, and were continued by determined IC₅₀.

Result: The results showed that the highest antioxidant activity (IC₅₀) was the fraction of ethyl acetate 2,184 µg/ml. The anti-malarial activity IC₅₀ of water and ethyl acetate fraction, ethanol extract and butanol fraction were 0,000090 µg/mL, 0,003 µg/mL, 0,006 µg/mL, 0,011 µg/ml respectively.

Conclusion : Extract and fractions of green tea have potential anti-malarial and antioxidant properties.

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1. Introduction

Malaria is significant health problem in the world both in tropical or subtropical country especially in Asia, Africa, and Latin America, it cause 200 million infections and 500 thousand deaths per year [1,2,3]. 70% population of 1.8 billion people in South-East Asia is in some risk for malaria which reported the incidence was more than 1 case per 1000 population annually. Indonesia is one of South-East Asia country which has high risk for malaria with more than 30% and more than 40% confirmed cases are due to *P. falciparum* in 2011 [4].

Pathophysiology of malaria is related with free radical productions which cause oxidative stress

in host cells [5]. In normal condition, aerobic organisms need molecular oxygen for their life. Reactive Oxygen Species (ROS) is one of molecular oxygen formation which in small amount is very essential for many physiological processes in aerobic organism, but ROS is very toxic at high dose [6]. ROS and Reactive Nitrogen Species (RNS) are associated with oxidative stress. Malaria infections induce OH[•] (one of main ROS in human) production in liver which trigger oxidative stress and apoptosis [7].

Artemisinin is malarial drug which often use. The mechanism of artemisinin involve the heme-mediated decomposition of the endoperoxide bridge to produce carbon-centred free radicals. Free radicals will damage the plasma membrane

of parasite and interfere with the enzyme of parasite and causing parasite death [8]. To overcome the side effects of free radicals needed antioxidants.

However previous study proved that *P. falciparum* showed the resistance to artemisinin derivatives [9]. Artemisinin-based combination therapies (ACTs) are the best anti-malarial drugs. Artemisinin enhances efficacy and has the potential of lowering the resistance emerges [10].

Green tea (*Camellia sinensis* L.) is one of a kind of popular beverage worldwide particularly in Asian countries like China, Korea, Japan and India [11]. Green tea contains catechins, vitamin B, potassium, ascorbic acid, glutamic acid, aspartic acid, caffeine, theanine. The majority tea compounds are catechins, namely : Epigallocatechin - gallate (EGCG), Epigallocatechin (EGC), Epicatechin - gallate (ECG) , Epicatechin (EC), Galocatechin (GC), Catechin (C), and Catechin - gallate (CG) that have potential antioxidant activity [12, 13,14,15,16].

This research was conducted to evaluated phenolics compound, antioxidant activity to decrease the free radicals formation as side effect of artemisinin and host immune responses, and evaluated anti-malarial activity of green tea and fractions to find the new combination for artemisinin (ACTs) which have antimalarial activity with low resistance.

2. Materials and Methods

The used materials were DPPH (Sigma-Aldrich), DMSO (Merck), methanol HPLC grade (Merck), HEPES (Sigma-Aldrich), Aqua bidestillata, Gentamicin Sulfate (Sigma-Aldrich), sodium carbonate (Sigma-Aldrich), Ethylene Diamine Tetra Acetic Acid (EDTA) (Sigma-Aldrich), NaCl (Merck), *P. falciparum* 3D-7 (Pharmacognocny Lab. Faculty of Pharmacy Airlangga University, Surabaya, Indonesia), Giemsa stain (Sigma-Aldrich), RPMI 1640 media (Sigma-Aldrich), Antibiotic Antimycotic Solution (Sigma-Aldrich).

2.1 Plant material

Dried green tea leaves were obtained from Walini Tea Manufacturer (PTPN VIII, Bandung, Indonesia), tea plantation located in Cigaruni, Garut, West Java, Indonesia.

2.2 Extract and fraction preparation

Extraction was done based on maceration method [16,17,18,19], and fractions were done as modified partition [16,17]. Five hundred gram of dried green tea were soaked in distilled 70% ethanol by maceration extraction, filtered and evaporated using rotatory evaporator in 40°C. Our process resulted ethanol extract of green tea 125 g (25%), ethanol extract of green tea was stored at 4°C. One hundred and fifty gram of green tea extract was partitioned with n-hexan and water (1:1) yielded hexane fraction 4.66 g (3.11 %), The residue was partitioned with ethyl acetate and water (1:1) yielded ethyl acetate fraction 9.95 g (6.63 %), the residue was partioned with buthanol and water (1:1) yielded buthanol fraction 21.18 g (14.12 %), the residue was water fraction 74.16 g (25.58 %). Green tea fractions were stored at 4°C.

2.3 Phytochemical assay

The four fractions of green tea extract were tested by phytochemical assay including flavonoid assay, phenolic, saponin, triterpenoid, steroid, terpenoid, tannin, and alkaloid assay based on modified Farnsworth method [20].

2.4 DPPH scavenging activity assay

The DPPH assay was carried out in modified method [16,18,19,21]. 50 µl green tea extract and fractions diluted in methanol HPLC grade with various concentrations (0.19 µg/ml to 100 µg/ml) and were introduced into micro-plate and added by 200 µl DPPH 0.077 mmol in DMSO. The mixture was shaken vigorously and incubated at darkness and room temperature for 30 min, then measured the absorbance values at 517 nm using a micro-plate reader. DPPH 250 µl was used as negative controls and 250 µl methanol was used as blank. The radical scavenging activity of each sample was expressed by the ratio of lowering DPPH absorption (%) and negative control (100%). DPPH antioxidant activity (%) was

$$\text{scavenging \%} = \frac{A_c - A_s}{A_c} \times 100$$

measured with :

Aa: sample absorbance

Ac: negative control absorbance (without sample)

2.5 Collection of serum for *P. falciparum* culture

Collection of human blood based on the guidelines approved by Institutional Ethics Committee (IEC) collaboration between

Maranatha Christian University, Bandung and Immanuel Hospital Bandung. Blood of healthy donor was collected in the tube without anticoagulant, blood was kept in the room temperature for 3-4 hours. Serum was separated from erythrocyte. Serum was inactivated at 56°C for 1 h furthermore serum was persevered in the deep freezer (-20°C).

2.6 Collection of erythrocytes for *P. falciparum* culture

Blood of healthy donor was collected in the tube containing anticoagulant EDTA and the blood was centrifuged at 2,000 rpm for 10 min, the supernatant and buffy coat was discarded. Red blood cells (RBCs) were cleansed with serum free RPMI (1:1) and centrifuged for 3 times, resulted ready RBCs for Plasmodium culture [22].

Table 1. The result of phytochemical assay of green tea extract and its fractions (hexane fraction, ethyl acetate, buthanol, and water fraction)

Sample	Compound content							
	Phenols	Triterpenoids	Steroids	Terpenoids	Saponins	Alkaloids	Flavonoids	Tannins
Green tea extract	+++	++	-	+++	+	++	++	+++
Hexane fraction	+++	+++	-	+++	-	+	-	+++
Ethyl acetate fraction	+++	+++	+	+++	+	++	+++	+++
Buthanol fraction	+++	++	-	+++	-	+++	+	+++
Water fraction	+++	++	-	+++	+++	+++	++	+++

100 µL RBC. The culture was incubated in the candle jar with CO₂ level 5% and placed at the 37°C incubator. When the parasitemia level reached 1-2%, the culture could be treated by green tea extract and its fractions. Green tea extract and fractions (hexane, ethyl acetate, buthanol, water fractions) were dissolved in DMSO 10%.

2.8 In vitro antimalarial assay

Extract and fraction were then tested in 7 serial concentrations include 0 µg/ml for control; 0,01µg/ml; 0,001 µg/ml; 0,0001 µg/ml; 0,00001 µg/ml; 0,000001 µg/ml; and 1x10⁻¹⁰ µg/ml in 6 wells micro-plates, the replication was duplicate every treatment [22,23]. Each well was received 10 µL of parasite-loaded erythrocytes, 5% haematocrit, and 90 µL of the different compound dilutions. The plates were incubated at 37°C for 48 h after confirmation of presence of mature schizonts in control wells. After incubation, contents of the wells were harvested and the red cells were transferred to a clean microscopic slide to form a series of thick films. The films were stained for 10 minutes in 10 % Giemsa solution (pH 7.3).

2.7 Preparation of culture medium and extract, fractions solution

Culture medium was prepared by dissolving 10.43 g RPMI 1640 powder, 5.94 g of HEPES, gentamicine sulfate 50 µg/mL in 1 liter of distilled-deionised water. The medium was filtered using 0.22 µm membrane filter and stored at 4 °C in aliquots of 100 ml. Before cultivation preparing the complete medium, every aliquot 100 ml was supplemented with 2 ml of Sodium bicarbonate 5 % b/v. The complete medium 104.2 ml was added by 11.5 ml serum. One tube of *P. falciparum* was thawed from nitrogen liquid tank and was added by 5 ml complete medium then the supernatant was discarded after centrifugation at 1.500 rpm for 5 min, The pellet or cell of *P. falciparum* was cultivated in the plate culture which supplemented with 2 ml complete medium and

Schizont growth inhibition per 200 asexual parasites was counted in 10 microscopic fields. The control parasite culture freed from compound was considered as 100 % growth. The percentage inhibition per concentration was calculated using the formula: [(% parasitemia in control wells - % parasitemia of test wells) / (% parasitaemia of the control)] x 100 [24,25]. Growth inhibition was expressed as percent of the number of schizonts for each concentration, compared to controls which incubation time was started at 0 h until 48 h later. Each concentration was repeated twice. The percentage inhibition of parasitemia was analyzed by probit analysis to determine the IC₅₀ value [25].

3. Result

3.1 Phytochemical assay

The phytochemical assay showed that green tea extract and fractions contained high phenols (+++), high terpenoids (+++), ethyl acetate fraction contained high flavonoids (+++), buthanol and water fractions contained high

alkaloids (+++), water fraction contained high saponins (+++), hexane fraction and ethyl acetate fractions contained high triterpenoids (+++), high tannins (+++)(Table 1).

3.2 Antioxidant activity of green tea extract and fractions

The antioxidant activity of green tea extract and fractions wasevaluated by DPPH scavenging activity. The DPPH free radical scavenging activity of samples was calculated in median IC (IC₅₀), it is the concentration of antioxidant needed to scavenge 50% of the DPPH free radical. Ethyl acetate fractions and green tea extract

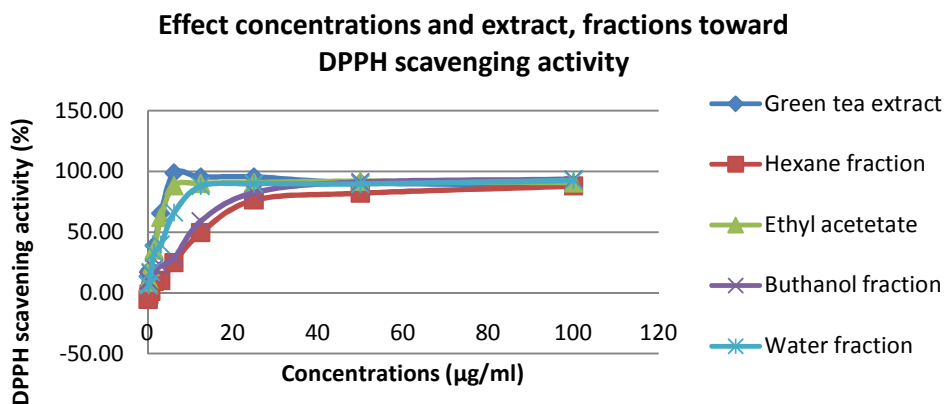


Figure 1. DPPH scavenging activity of green tea extract and fractions diluted in methanol to 100, 50, 25, 12.5, 6.25, 3.125, 1.563, 0.781, 0.391, and 0.195 µg/ml.

Table 2. IC₅₀ DPPH scavenging activity of green tea extract and fractions. The DPPH scavenging activity test was measured triplicate for each catechin compound. [Linear equations, coefficient of regression (R2), and IC₅₀ were calculated.]

Samples	Linear equation	R2	IC ₅₀ (µg/ml)	Average IC ₅₀ (µg/ml)
Green tea extract	y = 19.512x + 7.677	0.973	2.17	2.334 ± 0.30
	y = 19.811x + 7.314	0.984	2.15	
	y = 13.699x + 13.333	0.983	2.68	
Hexane fraction	y = 3.649x - 2.946	0.998	12.89	11.725± 1.66
	y = 3.680x - 4.159	0.996	12.46	
	y = 4.313x - 7.609	0.859	9.83	
Ethyl acetate fraction	y = 24.445x + 0.790	0.998	2.01	2.184 ± 0.15
	y = 19.678x + 5.329	0.937	2.27	
	y = 19.283x + 6.228	0.990	2.27	
Buthanol fraction	y = 3.406x + 10.938	0.943	11.45	11.180 ± 0.25
	y = 3.444x + 11.785	0.987	11.11	
	y = 3.497x + 11.609	0.987	10.98	
Water fraction	y = 8.885x+12.588	0.891	4.21	4.318 ± 0.12
	y = 10.392x+3.951	0.977	4.45	
	y = 8.823x + 12.105	0.915	4.29	

showed the highest antioxidant activity (Table 1, Fig.1).

Based on Table 2. showed that ethyl acetate fraction was the most active antioxidant in

scavenging DPPH free radical with IC₅₀ 2.184 µg /ml and green tea extract at second place with IC₅₀ value was 2.334 ± 0.30 µg /ml

Table 3. Antimalarial activity of green tea extract, fractions and artemisinin[inhibition of parasitemia was measured for 48 h incubation]

Samples	Doses(µg/mL)	Parasitemia degree(%)	Inhibition	Growth rate (%)
Green tea extract	0	7.5	0	100
	1x10 ⁻¹⁰	7.5	0	100
	0.000001	5	33.33	66.67
	0.00001	5	33.33	66.67
	0.0001	5	33.33	66.67
	0.001	4.5	40	60
	0.01	4	46.67	53.33
Hexane fraction	0	4	0	100

	1x10 ⁻¹⁰	4	0	100
	0.000001	4	0	100
	0.00001	3.5	12.5	87.5
	0.0001	3.5	12.5	87.5
	0.001	3.5	12.5	87.5
	0.01	3.5	12.5	87.5
Ethyl acetate fraction	0	8	0	100
	1x10 ⁻¹⁰	8	0	100
	0.000001	6.5	18.75	81.25
	0.00001	6.25	21.88	78.13
	0.0001	5	37.5	62.5
	0.001	5	37.5	62.5
	0.01	3.5	56.25	43.75
Buthanol fraction	0	8	0	100
	1x10 ⁻¹⁰	9	0	100
	0.000001	8	11.11	88.89
	0.00001	7.5	16.67	83.33
	0.0001	6	33.33	66.67
	0.001	5.5	38.89	61.11
	0.01	5	44.44	55.56
Water fraction	0	8	0	100
	1x10 ⁻¹⁰	8	0	100
	0.000001	4	50	50
	0.00001	4	50	50
	0.0001	4	50	50
	0.001	4	50	50
	0.01	3	62.5	37.5
Artemisinin(μ M)	0	8	0	100
	1x10 ⁻¹⁰	8	0	100
	0.000001	2	75	25
	0.00001	1	87.5	12.5
	0.0001	0.8	99	1
	0.001	0	100	0
	0.01	0	100	0

3.3 Antimalarial activity

Average of parasitemia degree and parasitemia growth rate were treated green tea extract fractions can be seen in the Table 3. And table 4.

Table 4 showed that artemisinin as antimalarial drug was the most active antimalarial, ethyl acetate fraction was the most active antimalarial activity compared to the other fractions, hexane fraction was the unactive antimalarial activity. Table 3 and 4 shows that higher dose exhibited a better effect to inhibit parasites growth.

Table 4. The IC₅₀ of green tea extract and fractions in *P.falciparum* after 48 h incubation. [Each sample was measured in duplicate and inhibition of parasitemia proliferation was analyzed using probit analysis].

Samples	IC ₅₀ (μ g/mL)
Green tea extract	0,006
Hexane fraction	850.745
Ethyl acetate fraction	0.003
Buthanol fraction	0,011
Water fraction	0,000090
Artemisinin	0,00000282

green tea extract include EC and EGC [30], the strong antioxidant properties of green tea are attributed to catechins of EGCG and EGC [31], EGCG was active in DPPH scavenging activity

4. Discussion

Green tea contained high antioxidant both extract and fractions. This data was validated with previous research that green tea extract contains several polyphenolic components with antioxidant properties [11,26,27], this data consisten with our data (Table 1), green tea contains high polyphenols. Green tea leaves retain their green color and almost all of their original polyphenol content [28]. Green tea contains mainly flavanols or catechins of EGCG, EGC, ECG, and EC [29], additional active components of

with IC₅₀ 0.505 μ g/ml [16]. EGC was active in DPPH scavenging activity 92.20-95.13% and ECG 84.80-97.86% at concentrations between 3.125 to 100 μ g/ml [30]. Green tea has greater antioxidant

potential than oolong and black teas [26], black tea extract has IC_{50} of DPPH scavenging activity 5.405 $\mu\text{g/ml}$ was lower antioxidant activity [16], compared to green tea with IC_{50} of DPPH scavenging activity 2.334 $\mu\text{g/ml}$. Green tea is processed without fermentation retain their green color and almost all of their original polyphenol content [28]. Green tea extract and fraction contained high tannins (+++) (Table 1), tannins has antioxidation action, like free radical scavenging activity, chelation of transition metals, inhibition of pro-oxidative enzymes and lipid peroxidation [33]. Ethyl acetate fraction exhibited the highest antioxidant activity compared to the other samples, this data was validated with phytochemical assay (Table 1) that ethyl acetate fraction contained high flavonoid (+++), hexane fraction exhibited the lowest antioxidant activity, this data was validated with phytochemical assay that hexane fraction was no flavonoid content (Table 1), flavonoids are group of natural substances with variable phenolic structures and are found in in fruit, vegetables, beverages such as tea and wine [34,35] flavonoids have skeleton with two aromatic rings (ring A and B) interconnected by a three carbon-atom, heterocyclic C ring, and flavonoids classified into six main groups, flavanones, flavones, isoflavones, flavonols, flavanols, and anthocyanins [36], flavonoids exhibit a wide range of biological activities, for the powerful antioxidant activity [37].

Green tea extract, ethyl acetate, buthanol and water fractions exhibited antimalarial activity, this data was validated with previous reserach that catechins in tea possessed antimalarial activity namely EGCG and ECG inhibited *P.falciparum* (strains NF54, K1 and 3D7) with IC_{50} 10 μM -40 μM , the ungalated catechins were lower potency, with IC_{50} values in excess of 100–300 μM [38,39]. Antimalarial activity of green tea extract and fractions due to flavonoids content based on phytochemical assay (Table 1), this data was consistent with previous research that many natural and synthetic flavonoids possess antimalarial activity [36]. Phytochemical compound in plant extracts have antioxidative effect against oxidative stress which caused by malaria parasite [40, 41]. Plant's phytochemical compounds such as tannins, flavonoids, alkaloids, saponins, phenolics, etc. showed antioxidant and antimalarial effect [2, 42, 43, 44]. Plant crude extract with phytochemical compound has been observed as reducing and oxidant scavenging agents with indicate the modification of oxidative character which is induced by parasites [40].

Plant extract may also play a role to prevent FP- Fe III (one of the haemoglobin degradation's product) detoxification by intercalating with iron-carboxylate bond (link with hemozoin) thereby inhibiting their polymerase [1, 42]. Ca^{2+} , Fe^{2+} , and Mg^{2+} act as cofactor of Plasmodium enzyme ribonucleotide reductase (RNR) are chelated by orthodiphenol and carboxyl functions (metal chelator which is one of plant bioactive agent). The plant extract can inhibit plasmodium proliferation by blocking parasites choline extraceluller transport which is important for biosynthesis of essential parasite molecules, phosphatidylcholines [43].

5. Conclusion

Green tea extract and fractions contain high tannins, polyphenols and ethyl acetate fraction contain high flavonoids. Hexane fraction is unactive antimalarial activity, water fraction is the most active antimalarial activity. Ethyl acetate fraction and water fraction are active in antioxidant and antimalarial activities

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Conflict of Interest

The authors report no conflicts of interest

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