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Antioxidant and Anti-malarial Properties of Catechins

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Authors' contributions

This work was carried out in collaboration between all authors. Author IB designed the study and performed the statistical analysis. Author RT managed the result and wrote the protocol. Author WW managed and prepared the sample, equipment and laboratory. Author FR managed the literature searches. Author MM managed to collect, calculate, and analyze the data. Author NF wrote the first draft of the manuscript. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Aims: This research was performed to evaluate the antioxidant and anti malarial activities of various catechins including *catechin* (C), *epicatechin* (EC), *catechin-gallate* (CG), *gallocatechin-gallate* (GCG), *epigallocatechin* (EGC), *epicatechin-gallate* (ECG), epigallocatechin gallate (EGCG).
Study Design: The antioxidant activity was measured by 1,1-diphenyl-2-picryl-hydrazyl (DPPH) scavenging activity and anti-malarial activity was determined by *In vitro* assay against *P. falciparum* culture, antioxidant activity was analyzed using linear regression analysis, and was continued by determining Inhibitory Concentration 50 (IC_{50}). The anti-malarial activity was analyzed by probit analysis and IC_{50} determination.

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Place and Duration of Study: Medical Research Center, Faculty of Medicine, Maranatha Christian University, Bandung. Pharmacognicy Laboratory, Airlangga University, Surabaya. Biomolecular and Biomedical Research Center, Aretha Medika Utama, Bandung, Indonesia from March 2013 to October 2013.

Results: The results showed that EC has the highest antioxidant activity with IC_{50} = 0.41µg/ml while ECG and GCG with IC_{50} = 0.52 µg/ml. For anti-malarial activity, CG has the highest anti malarial activity (IC_{50} = 0.37 µM).

Conclusion: Catechins have high antioxidant activity and CG has highest anti-malarial activity.

Keywords: Antioxidant; anti-malarial; catechins; free radical.

1. INTRODUCTION

Malaria is one of the most harmful parasitic diseases in the world and also responsible for the death of many people. This disease is caused by Plasmodium and spread out by female *Anopheles* mosquitoes as a vector, this disease is easily spread among human in certain conditions. *Plasmodium* that can infect and cause a disease in humans are *P. falciparum*, *P. malariae*, *P. ovale*, and *P. vivax*. *P. falciparum* has the highest prevalence, virulence, drug resistance and potential to cause high mortality [1,2].

Every year more than 500 million people worldwide are infected by malaria. Mortality due to malaria is between 1.5 million to 2.7 million deaths per year. One of the countries which have higher risk of Malaria is Indonesia. In 2007, 1.75 million clinical cases of malaria has been found and the number of malaria positive patients is 331,000 [3].

Cerebral malaria (CM) is the most severe complication and the major cause of death.Out of all cases of *P. falciparum* malaria (10%) and fatal cases malaria (80%) are caused by CM [3]. When parasite infected humans, parasitized red blood cells (PRBCs) will trigger constant oxidative stress. PRBC-induced apoptosis in endothelial cells is mediated through an oxidative stress pathway [4]. The oxidative stress caused by exogenous free radicals which are reactive oxidant species (ROS) and reactive nitrogen species (RNS), both are produced by the immune system of the host. Endogenous production of ROS generated during host's cell haemoglobin digestion with simultant reactions [5,6,7]. PRBCs stimulates the release of T helper 1 (Th1) which will produce interferon gamma (IFN-γ). IFN-γ stimulates the release of monocytes and macrophages which produce tumor necrosis factor-alpha (TNF-α). These events led to nitric oxide (NO*) formation by *inducible nitric oxide synthetase* (iNOS). TNF-α can induce or upregulate various cell adhesion molecules (CAM) on endothelial cells [3,4].

These free radicals are highly reactive, causing lipid peroxidation, trigger disfunction and deleterious of red blood cells [6,8]. Malarial pathogenesis associate with free radicals formation and decrease of antioxidant level [5,6,7]. While antioxidant can decrease parasite number and severe infection [7,8]. The free radical generated by malarial parasites are counter acttacked by cytoprotective enzymes and antioxidants [9].

The malarial drug which often used is artemisinin, It can involve heme-mediated decomposition of endoperoxide bridge by produce carbon-centred free radicals. Free radical will damage parasites' plasma membrane, and interfere with parasites' enzyme causing parasite death [10]. Many studies proved that *P. falciparum* is resistant to artemisinin derivatives [11]. Artemisinin based combination therapies (ACTs) are the best anti-malarial drugs. Artemisinin enhances efficacy and has the potential of lowering the resistance emerges [12].

This research was conducted to find the new ACTs which have high antimalarial activity with low resistance effect and have antioxidant activity to decrease the free radicals formation as side effect of malaria pathogenesis. Various studies have been conducted to find alternative malarial treatment using compounds that have antioxidant and anti-malarial activity. Flavonoids from green tea namely cathecins have high antioxidant activity but there are still limited data for antimalarial activity [13].

2. MATERIALS AND METHODS

The material in this study were DPPH (Sigma-Aldrich), DMSO (Merck), methanol HPLC grade (Merck), the various catechins including (+)-

catechin hydrate (C), epicatechin (EC), cathecingallate (CG), gallocatechin (GC), (–)-epicatechin gallate (ECG), epigallocatechin (EGC), gallocatechingallate (GCG) (–)-epigallocatechin gallate (EGCG) purchased from Biopurify Phytochemicals Ltd, Hepes (Sigma-Aldrich), Aquabidestillata, Gentamicin Sulfate (Sigma-Aldrich), sodium carbonate (Sigma-Aldrich), Ethylene Diamine Tetra Acetic acid (EDTA) (Sigma-Aldrich), NaCl (Merck), *P. falciparum* 3D-7 (obtained from Pharmacognocy Lab. Faculty of Pharmacy Airlangga University, Surabaya, Indonesia), Giemsa stain (Sigma-Aldrich), RPMI 1640 Media (Sigma-Aldrich), Antibiotic Antimycotic Solution (Sigma-Aldrich).

2.1 DPPH Scavenging Activity Assay

The DPPH assay was carried out by method from Frum et al. and Widowati et al. [14,15]. Fifty µl catechins in DMSO 10% (8 compounds) with various concentrations (0.195 µg/ml to 100 µg/ml) were introduced in microplate and added 200 µl DPPH 0.077 mmol in DMSO. The mixture was shaken vigorously and incubated at room temperature for 30 min, and then the absorbance values measured at 517 nm using a microplate reader. Negative controls used DPPH 250 µl, blank used 250 µl DMSO 10%. The radical scavenging activity of each sample was expressed by the ratio of lowered DPPH absorption (%), relative to the absorption (100%) of DPPH solution in the absence of test sample (negative control). DPPH antioxidant activity (%):

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

As: Sample absorbance
Ac: Negative control absorbance (without sample)

2.2 Antimalarial Activity

2.2.1 Collection of blood samples for in vitro sensitivity test

Collection of human blood conducted 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℃ for 1 h then presevated in the deep freezer (-20℃). Blood of healthy donor was collected in the tube contaning anticoagulant EDTA and the blood was centrifuged for 10 minute at 2,000 rpm, the supernatant and buffy coat was discarded. Red blood cells (RBCs) was cleansed with serum free RPMI (1:1) and was centrifuged again. Centrifuging and cleansing the blood was repeated 3 times, resulted ready RBCs for treatment [16].

2.2.2 Preparation of culture medium and extracts solution

Culture medium was prepared by dissolving 10.43 g RPMI 1640, 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. The medium was stored at 4℃ in 100 ml aliquots. Before complete medium preparation,every 100 ml aliquot was supplemented with 2 ml Sodium bicarbonate 5% b/v [16]. 104.2 ml complete medium was added by 11.5 ml serum. One tube of *P. falciparum* from nitrogen liquid tank was thawed and added by 5 ml complete medium. The mixture then centrifuged at 1,500 rpm for 5 minutes, the supernatant was discarded. The pellet or cell of *P. falciparum* was cultivated in the plate culture which supplemented with 2 ml complete medium and 100 µl RBCs. The culture was incubated in the *candle jar* with 5% CO_2 level, and was placed at 37℃ incubator. When the paracitemia level reached 1-2%, the culture could be treated by catechins. Catechins (C, EC, CG, GC, ECG, EGC, EGCG) were dissolved in DMSO 10%. Catechins were then tested in 6 serial concentrations include 0,01µM; 0,001 µM; 0,0001 µM; 0,00001 µM; 0,000001 µM; and $1x10^{-10}$µM in 6 wells microplates, the replication was duplicate every treatment [16,17]. Briefly 10 µl of parasite-loaded erythrocytes, 5% haematocrit, and 90 µl of catechins dilutions were introduced in well plate. The plates were incubated at 37℃ for 48 h, after confirmation of mature schizonts presence in control wells (without compounds). After incubation, contents of the wells were harvested and the red cells weretransferred to a clean microscopic slide to form a series of thick films. The films were stained for 10 minutes in 10% Giemsa solution of pH 7.3. 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: [(% parasitaemia in

control wells – % parasitaemia of test wells)/(% parasitaemia of the control)] x 100 [18,19]. Growth inhibition was expressed as percent of the number of schizonts for each concentration, compared with controls. Each concentration was repeated twice. Percentage parasitaemia incubation was started at 0 h and 48 h later, the percentage inhibition of parasitemia calculation was analyzed by probit analysis to determine the IC_{50} value [19].

3. RESULTS

3.1 Antioxidant Activity of Catechins

The antioxidant activity of *catechins* was assayed by DPPH scavenging activity. The DPPH free radical scavenging activity of Catechins (C, EC, CG, GC, ECG, EGC, EGCG) was calculated in median IC (IC_{50}). It is the concentration of antioxidant needed to scavenge 50% of the DPPH free radical. EC, GCG and ECG showed the highest antioxidant activity and the lowest was C (Table 1, Fig. 1).

(Fig. 1) shows that antioxidant activity of catechins was highly similar between 20-100 µg/ml and shows low activity in less concentration. Table 1 shows that catechins has high DPPH free radical scavenging activity, the highest antioxidant activity was EC and followed by GCG and ECG.

3.2 Anti-malarial Activity of Catechins

The percentage inhibition of parasitaemia treated with catechins. Catechins killed *P. falciparum* and decreased parasitaemia proliferation in a concentration-dependent manner, higher concentration exhibited stronger anti-malarial activity or inhibit parasitaemia proliferation better (Fig. 2).

Table 1. IC_{50} DPPH scavenging activity of catechins. The DPPH scavenging activity test was measured triplicate for each catechin compound. [Linear equations, coefficient of regression (R2), and IC_{50} were calculated.]

Samples	Linear equation	R2	IC_{50} (µg/ml)
C	y=6.6934x + 2.9987	0.992	7.02
EC	y = 67,102x + 18,527	0,922	0,41
CG	y = 28.28x + 34.31	0.994	0,56
GC	Y=1.5805X + 46.221	0.884	2.39
GCG	y = 65.36x + 15.99	0,997	0,52
ECG	y = 62.09x + 18.03	0,845	0,52
EGC	y = 23.87x + 20.76	0,913	1,23

The IC_{50} of various Catechins in *P. falciparum* after 48 h incubation exhibited that catechins had high anti-malarial activity (Table 2).

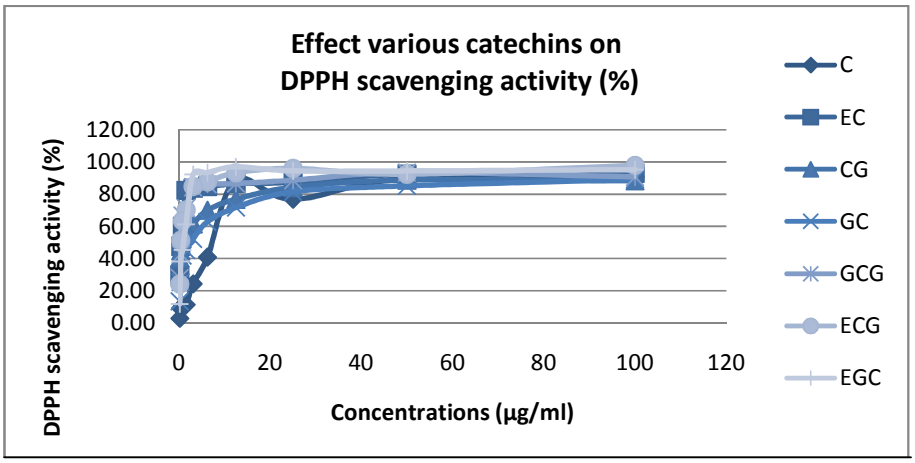

Fig. 1. DPPH scavenging activity of catechins diluted in DMSO 10% to 100, 50, 25, 12.5, 6.25, 3.125, 1.563, 0.781, 0.391, and 0.195 µg/ml

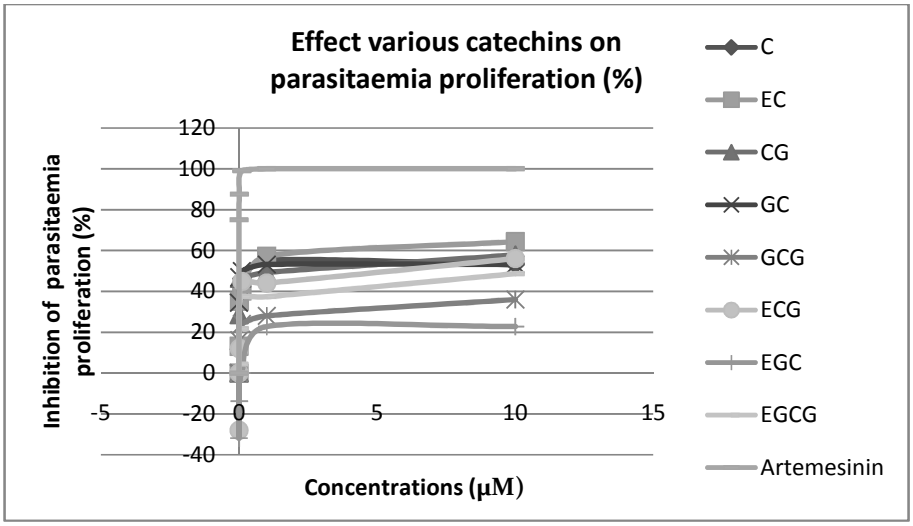

Fig. 2. Anti-malarial activity of catechins diluted in DMSO 10% to 10, 1.0, 0.1, 0.01, 0.001 and 0.0000001µM/mland incubated for 24 h. Inhibition of parasitaemia proliferation was interpreted as antimalarial activity

(Table 1) shows that catechins (C, EC, CG, GC, GCG, ECG, EGC) have high antioxidant activity, this result was consistent with previous studies [20,21,22,23,24]. There are several isomers of catechins namely catechin (C), catechin-gallate (CG), gallo-catechin (GC), gallocatechin-gallate (GCG), epicatechin (EC), epicatechin-gallate (ECG), epigallo-catechin (EGC), and epigallo-catechin-gallate (EGCG) [25,26,27,28,29].

Table 2. The IC_{50} of catechins in *P. falciparum* after 48 h incubation. [Each catechin was measured in duplicate and inhibition of parasitaemia proliferation was analyzed using probit analysis]

Samples	IC_{50} (µM)
C	0.734
EC	0.456
CG	0.366
GC	7.457
GCG	50.777
ECG	2.049
EGC	98.145
EGCG	5.633
Artemisinin	0,00000282

Catechins group are derivative of condensed tannins,also known as polyphenols. Catechins have many functional hydroxyl (OH) groups which differed in the number and position of the hydroxyl groups in the molecule [26]. Catechins and polyphenols were effective scavengers of free radicals *in vitro* and antioxidant acitivities through their effects on transcription factors and enzyme activities [30]. Catechins as a free radical scavenging activity is related to its standard one-electron reduction potential ($E^{o'}$), a measure of the reactivity of an antioxidant as H^+ or electron donor under standardized conditions. A lower $E^{o'}$ of compund indicates that less energy is required for H^+ or electron donation [28]. All catechins demonstrated high antioxidant activity and EC exhibited the lowest IC_{50} compared with the other compounds. A material has a strong antioxidant activity when having IC_{50} values < 200 ppm or < 200 µg/ml [31]. According to Widowati et al. [23] DPPH scavenging activity of EGCG with IC_{50} was 0.505 µg/ml. The order antioxidant activity of catechins are: EC > EGCG> GCG=ECG>CG > EGC >GC> C. Differences in free radical scavenging ability of catechins is related to difference of ability to transfer hydrogen (H) atoms toward DPPH free radicals to form stable of 1,1-diphenyl-2-picrylhydrazine [32,33].

The data in (Table 2) shows that the most active anti-malarial activity was CG ($IC_{50=}$ 0.366 µM) and the lowest antimalarial activity was EGC ($IC_{50=}$ 98.145 µM). C, EC, CG grouped as active antimalarial activity; GC, ECG, EGCG as moderate active anti malarial activity; GCG and EGC as very weak antimalarial activity. This data was validated with previous study that classifying an extracts as very active (IC_{50} value less than 0.1 µg/ml), active (0.1-1.0 µg/ml), good to

moderate active (1.1-10 µg/ ml), weak (11-25 µg/ ml), and considered very weak (26-50 µg/ml) while more than 100 µg/ml is inactive [34]. The previous study reported that EGCG and ECG were able to inhibit the growth of *P. falciparum* (strains NF54, K1 and 3D7) with IC_{50} value was 10 µM-40 µM, while the ungallated catechins had less potent anti-malarial activity with IC_{50} values in excess of 100–300 µM. The anti-malarial mechanisms of catechins were not yet able to be explained [35,36].

4. CONCLUSION

Catechins have high free radical scavenging and EC is the most active antioxidant. Catechins are classified active, moderate active, very weak anti-malarial activity and CG is the most active both antioxidant and anti-malarial activities.

CONSENT

All authors declare that 'written informed consent was obtained from Institutional Ethics Committee (IEC), collaboration between Maranatha Christian University, Bandung and Immanuel Hospital Bandung for publication of this case report and accompanying images.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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