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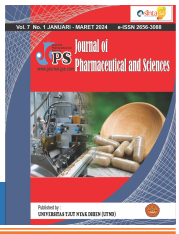

Cytotoxicity of Ethanol Extract of Robusta Coffee Leaves (*Coffea canephora*) in Vero cells

Sitotoksisitas Ekstrak Daun Kopi Robusta (*Coffea canephora*) terhadap Sel Vero

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Abstract

Robusta coffee leaves (*Coffea canephora*) contain various bioactive compounds that may serve as natural antioxidants and potential phytopharmaceutical agents. The objective of this research to evaluate the antioxidant activity and cytotoxicity of the ethanol extract of robusta coffee leaves and to determine the relationship between antioxidant activity and cell viability. Antioxidant activity was assessed using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, while cytotoxicity was evaluated on Vero cells using the MTT assay over a concentration range of 31.25-500 µg/mL. Vitamin C and doxorubicin were used as positive controls. The ethanol extract exhibited very strong antioxidant activity with an IC_{50} value of 22.456 µg/mL, while vitamin C showed an IC_{50} value of 4.217 µg/mL. Cytotoxicity testing demonstrated low toxicity toward Vero cells, with an IC_{50} value of 1512.857 µg/mL, whereas doxorubicin exhibited an IC_{50} value of 60.052 µg/mL. Pearson correlation analysis revealed a strong negative correlation between antioxidant activity and Vero cell viability ($r = -0.93$, $p = 0.024$). Despite this correlation, cell viability remained above 100% at all tested concentrations, indicating the absence of cytotoxic effects on normal mammalian cells. These findings suggest that the ethanol extract of robusta coffee leaves possesses potent antioxidant activity while maintaining a favorable safety profile, supporting its potential to be futher development as a source of natural antioxidants and phytopharmaceutical candidate.

***Keywords*: Antioxidant activity, Plant extract, Cell viability, Oxidative stress, Bioactive compounds.**

Abstrak

Daun kopi robusta (*Coffea canephora*) mengandung berbagai senyawa bioaktif yang berpotensi dikembangkan sebagai sumber antioksidan alami dan kandidat fitofarmaka. Penelitian ini bertujuan untuk mengevaluasi aktivitas antioksidan dan sitotoksisitas ekstrak etanol daun kopi robusta serta mengetahui hubungan antara aktivitas antioksidan dan viabilitas sel. Aktivitas antioksidan diuji menggunakan metode 2,2-difenil-1-pikrilhidrazil (DPPH), sedangkan sitotoksisitas dievaluasi terhadap sel Vero menggunakan metode MTT dengan rentang konsetrasi 31,25-500 µg/mL. Vitamin C dan doksorubisin digunakan sebagai kontrol positif pada uji antioksidan dan sitotoksisitas. Hasil penelitian menunjukkan bahwa ekstrak etanol daun kopi robusta memiliki aktivitas antioksidan yang sangat kuat dengan nilai IC_{50} sebesar 22,456 µg/mL, sedangkan vitamin C memiliki nilai IC_{50} sebesar 4,217 µg/mL. Uji sitotoksisitas menunjukkan bahwa ekstrak bersifat tidak toksik terhadap sel Vero dengan nilai IC_{50} sebesar 1512,857 µg/mL, sementara doksorubisin memiliki nilai IC_{50} sebesar 60,052 µg/mL. Analisis korelasi Pearson menunjukkan adanya korelasi negatif yang kuat antara aktivitas antioksidan dan viabilitas sel Vero ($r = -0,93$; $p = 0,024$). Meskipun demikian, seluruh nilai viabilitas sel berada di atas 100%, yang menunjukkan bahwa aktivitas antioksidan ekstrak tidak disertai efek sitotoksik terhadap sel normal. Hasil penelitian ini menunjukkan bahwa ekstrak etanol daun kopi robusta memiliki aktivitas antioksidan yang kuat dengan profil keamanan yang baik, sehingga berpotensi dikembangkan lebih lanjut sebagai sumber antioksidan alami dan kandidat fitofarmaka.

***Kata Kunci*: Aktivitas antioksidan, ekstrak tanaman, viabilitas sel, stres oksidatif, senyawa bioaktif.**

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Introduction

The use of plant-derived bioactive compounds as complementary therapeutic agents and phytopharmaceutical candidates has gained growing attention. This is due to the growing demand for safe, effective, and sustainable natural medicines. Robusta coffee (*Coffea canephora*) is one promising yet underutilized source of such compounds. While coffee beans have been well studied and widely commercialized, coffee leaves also contain a range of secondary metabolites [1]. These include flavonoids, polyphenols, terpenoids, alkaloids, tannins, saponins, and steroids [2–6]. These compounds have been reported to possess several biological activities. They exhibit antioxidant and anti-inflammatory [4,7], antibacterial [8], antifungal properties [3], and may help protect cells against oxidative damage [4,7].

Antioxidant activity is a crucial parameter in the development of phytopharmaceutical products as oxidative stress and excessive free radical production are linked to cellular damage, aging, inflammation, and various degenerative diseases [9–12]. Bioactive compounds present in robusta coffee leaves may protect cells from oxidative injury and help maintain normal cellular function [6]. Therefore, evaluation of antioxidant activity is necessary to determine the extract's ability to scavenge and neutralize free radicals and to support its potential therapeutic applications [13]. Previous studies have reported significant antioxidant activity of robusta coffee leaf extracts using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay [14,15].

Besides biological efficacy, safety evaluation is also required in developing phytopharmaceutical and complementary therapeutic products. Cytotoxicity assessment provides essential information on the potential toxic effects of plant extracts on normal cells before further development [14,16]. Most previous studies on robusta coffee leaf extracts have focused on their effects against cancer cell lines, such as HeLa, MCF-7, and HepG2 [8,17]. Others have used the Brine Shrimp Lethality Assay (BSLT) as a preliminary toxicity screening method [18]. However, little information is available about their effects on normal mammalian cells [19].

Therefore, this study evaluated both the antioxidant activity and cytotoxicity of the ethanol extract of robusta coffee leaves using Vero cells as a model of normal mammalian cells [19]. IC_{50} values for antioxidant and cytotoxic activities were determined to assess the extract's biological efficacy and safety profile. The findings of this study are expected to provide scientific evidence supporting the development of robusta coffee leaves as a safe and effective phytopharmaceutical candidate and complementary therapeutic agent derived from local natural resources.

Method

Materials and Apparatus

The materials used in this study included robusta coffee leaves (*Coffea canephora* Pierre ex A. Froehner), 96% ethanol, 2,2-diphenyl-1-picrylhydrazyl (DPPH), Minimal Essential Medium (MEM), distilled water, $NaHCO_3$, NaOH, Dulbecco's Modified Eagle Medium (DMEM), 10% dimethyl sulfoxide (DMSO), fetal bovine serum (FBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), phosphate-buffered saline (PBS), vitamin C, sodium dodecyl sulfate (SDS) stopper solution, penicillin–streptomycin, 0.25% trypsin–EDTA, HCl, doxorubicin, and Vero cells.

The equipment used included glass beakers, maceration vessels, 0.2 µm membrane filter, 100-mesh sieve, blender, pH meter, analytical balance, freezer, autoclave, vacuum rotary evaporator, laminar airflow (LAF), water bath, hemocytometer, CO_2 incubator, inverted microscope, 96-well microplates, microplate reader (ELISA reader), vortex mixer, conical tubes, cryotubes, cell counter, culture dishes, and centrifuge.

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Plant Identification

Plant identification was performed to ensure the authenticity of the robusta coffee plant and to prevent errors in sample collection. Morphological characteristics, including leaf shape and size, stem, flowers, and fruits, were examined and compared with botanical descriptions and taxonomic references [20,21].

Preparation of Plant Material

Fresh robusta coffee leaves were collected from Liwa, West Lampung, Indonesia. Healthy and undamaged leaves free from fungal contamination were selected. Leaves were harvested in the morning from the third to fifth leaf positions counted from the petiole base. The collected leaves were washed with running water, sliced into small pieces, and air-dried for five days [22]. The dried material was then ground into powder and sieved through a 100-mesh sieve to obtain a uniform particle size.

Extraction Procedure

A total of 500 g of powdered simplicia was extracted with 10 L of 96% ethanol by the maceration method.The first maceration was performed with 5 L of 96% ethanol for 3 × 24 h, with agitation every 8 h. The residue was subsequently re-macerated with 5 L of 96% ethanol for 2 × 24 h. All filtrates were combined and concentrated using a vacuum rotary evaporator at 40°C, followed by evaporation in a water bath at 40°C until a viscous extract was obtained [2,3].

Antioxidant Activity Assay

Antioxidant activity was evaluated using the DPPH radical scavenging assay. A DPPH solution (50 µg/mL) was prepared by dissolving 2.5 mg of DPPH powder in 50 mL of ethanol [23,24]. Briefly, 100 µL of sample solution at concentrations of 1000, 500, 250, 125, 62.5, 31.25, and 15.625 µg/mL was mixed with 100 µL of DPPH solution in a microplate. The mixture was incubated in the dark for 30 min at room temperature. Vitamin C (0–20 µg/mL) was used as the positive control, while ethanol served as the blank solution. Absorbance was measured at 517 nm using a microplate reader [24]. The percentage of DPPH inhibition was calculated using the following formula [25,26] and IC_{50} values were determined by linear regression analysis.

$$\% \text{ DPPH inhibition} = \frac{\text{Standard Absorbance} - \text{Sample Absorbance}}{\text{Standard Absorbance}} \times 100\%$$

Cytotoxicity Assay

Cell Culture

Vero cells were obtained from the Primate Research Center, IPB University, Indonesia. Cells were cultured in DMEM supplemented with 10% FBS and incubated at 37°C in a humidified 5% CO_2 atmosphere until approximately 50% confluence was achieved. Cells were harvested using PBS washing followed by treatment with 0.25% trypsin–EDTA.

MTT Assay

Harvested Vero cells were counted and seeded into 96-well plates at a density of 5×10^3 cells/well in 100 µL culture medium. Following a 4 h incubation period at 37°C, the culture medium was removed and replaced with extract solutions at concentrations of 500, 250, 125, 62.5, and 31.25 µg/mL. Each concentration was tested in triplicate. Doxorubicin at concentrations ranging from 6.25 to 50 µg/mL was used as the positive control, while Vero cells cultured in medium containing ethanol without the addition of the extract or doxorubicin, served as the negative control. The viability of the negative control cells was designated as 100%. Although doxorubicin was evaluated at lower concentrations (6.25–50 µg/mL) than the extract (31.25–500 µg/mL), the marked difference in IC_{50} values clearly demonstrates that the extract possesses considerably lower cytotoxicity toward Vero cells.

After incubation for 24–48 h, 100 µL of MTT reagent was added to each well and incubated for 2–4 h until purple formazan crystals formed. Subsequently, 100 µL of SDS stopper solution was added, and the plates were incubated for 24 h in the dark at room temperature to dissolve the crystals. Absorbance was measured at 595 nm using a microplate reader [14,27]. Cell viability was calculated from the absorbance values, and IC_{50} values were determined by regression analysis [28].

$$\% \text{ Inhibition} = \frac{\text{Absorbance of control cells} - \text{Absorbance of treatment}}{\text{Absorbance of control cells}} \times 100\%$$

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Statistical Analysis

Pearson correlation analysis was performed to evaluate the relationship between antioxidant activity and Vero cell viability. Correlation coefficients (r) were calculated using the percentage inhibition values obtained from the DPPH assay and the percentage viability values obtained from the MTT assay.

Results and Discussion

The antioxidant activity assay demonstrated that the ethanol extract of robusta coffee leaves exhibited concentration-dependent free radical scavenging activity. An increase in extract concentration resulted in higher percentages of DPPH inhibition, indicating enhanced antioxidant capacity. The highest inhibition percentage was observed at a concentration of 500 µg/mL (85.974%), whereas the lowest inhibition percentage was obtained at 31.25 µg/mL (52.406%). Linear regression analysis yielded an IC_{50} value of 22.456 µg/mL, indicating very strong antioxidant activity. According to the antioxidant classification proposed by Molyneux, compounds with IC_{50} values below 50 µg/mL are categorized as very strong antioxidants. Vitamin C, used as the positive control, also demonstrated very strong antioxidant activity with an IC_{50} value of 4.217 µg/mL. The lower IC_{50} value of vitamin C compared with the extract indicates its higher antioxidant potency, which is expected because vitamin C acts as a pure antioxidant capable of directly donating electrons to neutralize free radicals [29]. Nevertheless, the robusta coffee leaf extract remained within the same antioxidant category, highlighting its potential as a natural antioxidant source.

Table 1. Antioxidant Activity of Robusta Coffee Leaf Extract and Vitamin C Based on Average Inhibition Percentage and IC_{50}

Sample	Concentration (µg/mL)	$\bar{x}$ absorbance±SD	$\bar{x}$ inhibition (%)±SD
Robusta Coffee Leaf Extract	500	0.080±0.001	85.974±0.153
	250	0.090±0.002	84.154±0.423
	125	0.145±0.002	74.529±0.492
	62.5	0.212±0.003	62.677±0.413
	31.25	0.270±0.001	52.406±0.174
	Standar	0.568±0.003	0.000
IC_{50} (µg/mL)			**22.456**
Vitamin C	10	0.140±0.002	75.292±0.441
	5	0.261±0.004	54.108±0.631
	2.5	0.379±0.001	33.274±0.328
	1.25	0.494±0.002	13.087±0.236
	0.625	0.522±0.001	8.038±0.530
	Standar	0.568±0.003	0.000
IC_{50} (µg/mL)			**4.217**

The strong antioxidant activity observed in this study is likely associated with the presence of bioactive secondary metabolites, particularly phenolic compounds, flavonoids, alkaloids, tannins, and chlorogenic acid in robusta coffee leaves [2,4,7], but quantitative analysis is needed for confirmation. Phenolic and flavonoid compounds possess hydroxyl groups capable of donating electrons or hydrogen atoms to stabilize free radicals and terminate oxidative chain reactions [9]. In addition, chlorogenic acid has been reported to scavenge reactive oxygen species (ROS), inhibit lipid peroxidation, and protect cellular membranes against oxidative damage [2]. In the DPPH assay, antioxidant activity occurs through electron transfer or hydrogen atom donation to DPPH radicals, resulting in decreased absorbance values [25]. These findings are consistent with previous studies reporting significant antioxidant activity in robusta coffee leaves. Putri et al. [5] reported that ethanol extract of robusta coffee leaves contains higher total phenolic content than the n-hexane fraction, resulting in greater antioxidant activity. Similar findings were reported by Welis et al. [6], who demonstrated that phenolic and flavonoid compounds contribute substantially to the antioxidant capacity of robusta coffee leaves through their radical-scavenging properties and their ability to maintain cellular redox homeostasis.

The cytotoxicity assay using the MTT method demonstrated that the ethanol extract of robusta coffee leaves exhibited low toxicity toward Vero cells. Cell viability remained above 100% at all tested concentrations, ranging from 103.383% to 136.261%, indicating that the extract did not suppress cell growth or induce cytotoxic effects within the concentration range evaluated. The calculated cytotoxic IC_{50} value was 1512.857 µg/mL, suggesting that the extract can be classified as non-toxic toward normal mammalian cells. Generally, extracts

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with IC_{50} values greater than 1000 µg/mL are considered to possess very low toxicity [19]. The high viability percentages observed in this study indicates that cellular metabolic activity remained intact following treatment with the extract. Although viability values exceeding 100% indicate the absence of cytotoxicity, they may also reflect a potential proliferative effect on Vero cells or interference from naturally occurring colored compounds in the extract with the MTT assay. Therefore, confirmation using complementary methods, such as the Crystal Violet Staining (CVS) assay or direct cell counting, is recommended to validate these findings [16].

The low cytotoxicity observed may be associated with the antioxidant properties of the extract. In the MTT assay, viable cells reduce tetrazolium salts into purple formazan crystals through mitochondrial dehydrogenase activity. The amount of formazan produced is proportional to the number of viable and metabolically active cells [30]. Therefore, the increased absorbance values obtained in this study indicate that cellular metabolism remained active after treatment. Furthermore, antioxidant compounds are known to suppress excessive ROS generation, which can otherwise induce membrane damage, mitochondrial dysfunction, DNA damage, and apoptosis [9,11]. Consequently, the antioxidant constituents present in robusta coffee leaves may contribute to preserving cellular integrity and maintaining normal cellular functions. This interpretation is supported by Rani et al. [19], who reported that phenolic-rich plant extracts generally exhibit low toxicity toward Vero cells. Similarly, Asma et al. [31] suggested that natural bioactive compounds with strong antioxidant activity often display lower cytotoxicity than synthetic cytotoxic agents. Comparable observations were also reported by Utami et al. [17], who demonstrated that bioactive compounds derived from *Coffea canephora* possess promising biological activities while maintaining relatively low toxicity toward normal cells.

Table 2. Cytotoxicity of Robusta and Doxorubicin Coffee Leaf Extracts Based on Average Percent Inhibition, Average Percent Viability, and IC_{50}

Sample	Concentration (µg/mL)	$\bar{x}$ absorbance±SD	$\bar{x}$ inhibition (%)±SD	$\bar{x}$ viability (%)±SD
Robusta Coffee Leaf Extract	500	0.603±0.035	-3.383±5.753	103.383±5.753
	250	0.632±0.009	-8.253±1.938	108.252±1.938
	125	0.638±0.034	-9.380±8.344	109.380±8.344
	62.5	0.676±0.013	-15.894±4.694	115.894±4.694
	31.25	0.794±0.039	-36.261±10.044	136.261±10.044
	Control cells	0.584±0.015	0.000	100.000±0.000
IC_{50} (µg/mL)				**1512.857**
Doxorubicin	50	0.444±0.034	23.870±7.504	76.130±7.504
	25	0.507±0.010	13.125±0.607	86.875±0.607
	12.5	0.534±0.013	8.563±0.262	91.437±0.262
	6.25	0.576±0.042	1.178±8.309	98.822±8.309
	Control cells	0.584±0.015	0.000	100.000±0.000
IC_{50} (µg/mL)				**60.052**

As expected, doxorubicin, used as the positive control, exhibited substantially greater cytotoxic activity than the robusta coffee leaf extract, with an IC_{50} value of 60.052 µg/mL. The cytotoxic mechanism of doxorubicin involves DNA intercalation, inhibition of topoisomerase II activity, and increased ROS production, ultimately leading to apoptosis and cell death [32]. The considerable difference between the IC_{50} values of doxorubicin and the extract highlights the favorable safety profile of robusta coffee leaf extract toward normal mammalian cells.

To further investigate the relationship between antioxidant activity and cytotoxicity, Pearson correlation analysis was performed using DPPH inhibition (%) and Vero cell viability (%) data. The analysis revealed a strong negative correlation between antioxidant activity and Vero cell viability ($r = -0.93$, $p = 0.024$), indicating a statistically significant inverse relationship between the two parameters. The p-value below 0.05 confirms that the observed correlation was statistically significant and unlikely to have occurred by chance. These findings suggest that variations in antioxidant activity were significantly associated with changes in cell viability across the tested concentrations. However, all viability values remained above 100%, indicating that the strong antioxidant activity of the extract was not accompanied by cytotoxic effects on normal cells. This observation may be attributed to the presence of phenolic compounds, flavonoids, and chlorogenic acid, which contribute to antioxidant activity while simultaneously protecting cells from oxidative damage through the scavenging of reactive oxygen species [9,33,34]. Similar findings were reported by Kok et al. [35] , who demonstrated a significant association between phenolic content, antioxidant capacity, and cytotoxicity in

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plant-derived extracts. Therefore, the combination of strong antioxidant activity and low toxicity toward Vero cells supports the potential application of robusta coffee leaf extract as a safe natural antioxidant and a promising phytopharmaceutical candidate.

The present study provides additional scientific evidence regarding the biological activity and safety profile of robusta coffee leaves, a plant material that has received considerably less attention than coffee beans despite its rich phytochemical composition. While previous studies have primarily focused on the antioxidant activity of robusta coffee leaves or their cytotoxic effects against cancer cell lines [6,17], information regarding their safety toward normal mammalian cells remains limited. The findings of this study demonstrate that the ethanol extract of robusta coffee leaves exhibits very strong antioxidant activity while maintaining low cytotoxicity toward Vero cells. This combination of biological efficacy and cellular safety is an important consideration in the development of phytopharmaceutical products and complementary therapeutic agents. Overall, the results suggest that robusta coffee leaves may serve as a promising source of natural bioactive compounds for the prevention of oxidative stress-related disorders and warrant further investigation through compound isolation, mechanistic studies, and in vivo evaluations to support their future development as phytopharmaceutical products [2,4].

Conclusions

The present study demonstrated that the ethanol extract of robusta coffee leaves possesses very strong antioxidant activity and low toxicity toward normal mammalian cells. The extract exhibited a low antioxidant IC_{50} value and maintained high Vero cell viability across all tested concentrations, indicating that its free radical scavenging activity was not accompanied by cytotoxic effects. A significant negative correlation was observed between antioxidant activity and Vero cell viability ($r = -0.93$, $p = 0.024$), indicating a potential association between the extract's antioxidant properties and cellular response. However, this finding should be interpreted cautiously due to the limited number of concentration points evaluated ($n = 5$), and further studies with a broader concentration range and independent replicates are needed to validate this relationship. Overall, this findings provide scientific evidence supporting the safety and potential utilization of robusta coffee leaves as a source of natural antioxidant compounds. These findings suggest that the ethanol extract of robusta coffee leaves is a promising source of natural antioxidant compounds. Nevertheless, further investigations involving active compound isolation, in vivo studies, and chronic toxicity evaluations are necessary before its potential as a phytopharmaceutical candidate can be fully established.

Conflict of Interest

The authors declare no conflict of interest regarding the publication of this article.

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