

CYTOPROTECTIVE ACTIVITY OF ETHANOL FRACTION OF *COLEUS AMBOINICUS* LOUR. LEAVES AGAINST VERO CELLS INDUCED BY H₂O₂

RENY HARYANI¹, URIP HARAHAP^{1*}, MASFRIA², DENNY SATRIA³

¹Departement of Pharmacology, Faculty of Pharmacy, Universitas Sumatera Utara, Medan, Indonesia. ²Departement of Pharmaceutical Chemistry, Faculty of Pharmacy, Universitas Sumatera Utara, Medan, Indonesia. ³Departement of Pharmaceutical Biology, Faculty of Pharmacy, Universitas Sumatera Utara, Medan, Indonesia. Email: urip.hrp@gmail.com

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ABSTRACT

Objective: The objective of this study is to assess the cytoprotective activity of *Coleus amboenicus* Lour. leaves ethanol fraction (EtF) in Vero cells which induced by hydrogen peroxide (H₂O₂).

Methods: Cytoprotective activity was determined using 3-(4,5-dimethylthiazol-2)-2,5-diphenyltetrazolium bromide (MTT) method, apoptosis activity was analyzed by flow cytometry, and expression of radical spesies oxygen (ROS) was observed by imunocytochemistry.

Result: The EtF (100 µg/mL) was showed the highest percent viability 74.42±0.28% using MTT method. Furthermore, the EtF was increased viability of Vero cell after induced with 0.8 mM H₂O₂ and EtF was decreased the expression of ROS.

Conclusion: Based on the description, EtF has cytoprotective activity towards Vero cells which induced by 0.8 mM H₂O₂.

Keywords: Cytoprotective activity, *Coleus amboenicus* Lour., Ethanol Fraction, H₂O₂, Vero cells.

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INTRODUCTION

Oxidative stress arises due to an imbalance between the production of free radicals or reactive oxygen species (ROS) and antioxidants, so free radical levels are higher than antioxidants [1]. Excessive ROS damages cellular lipids, proteins, and DNA and inhibits the normal function of cells [2]. Oxygen free radicals are formed as by-products of aerobic cellular metabolism and are produced under the conditions of oxidative stress. However, increased production of ROS which are unable to be neutralized by antioxidants in cells (such as superoxide dismutase and catalase) and lead to oxidative stress may disrupt cellular homeostasis and impair cell components including membrane lipids, proteins, and DNA [3].

Antioxidants are molecules that can reduce the effects of free radicals to stabilize the harmful molecules, neutralize free radicals by donating electrons, or accept it by eliminating the conditions of unpaired electrons in radicals [4]. The use of antioxidants that act as free radical scavenger is a strategy to prevent the initiation of cancer cells induced by ROS and the early stages of carcinogenesis and thus can reduce the incidence of cancer [5]. Antioxidants cause resistance to oxidative stress by reducing free radicals, inhibiting lipid peroxidation, and other mechanisms so as to prevent the disease [6].

Coleus amboenicus Lour. A plant that has been used ethnobotany in Indonesia for empirical by the people of North Sumatra, especially mothers who breastfeed. Many pharmacological properties have been reported in *C. amboenicus* Lour. including urolithiasis, antiepileptic, antitumorogenic, antimutagenic, radioprotective effect, antiviral, antifungal, and neuropharmacological properties [7]. Several studies of this plant have conducted a research on the antioxidant activity and cytotoxic leaf extract of *C. amboenicus* Lour. [8]. The results showed that the ethyl acetate extract and ethanol extract have antioxidant activity with IC₅₀ values of each 350.74 µg/mL and 281.26 µg/mL with DPPH. This study aimed to assess cytoprotective effect of *C. amboenicus* Lour. in Vero cells which induced by H₂O₂.

MATERIALS AND METHODS

Material

The n-hexane, ethyl acetate, and ethanol were purchased from Merck (Darmstadt, Germany), DMSO (Merck), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma Chemical, St. Louis, MO), H₂O₂ from Merck Darmstadt, Germany, and M199 media and phosphate-buffered saline (PBS) 10% v/v (Gibco, Grand Island, NY, USA).

Plant material

C. amboenicus Lour. was obtained from Tanjung Pura, Langkat, North Sumatra, Indonesia. The plant was identified at Herbarium Meandense, University of Sumatera Utara.

Preparation of Ethanol Fraction (EtF)

The leaves of *C. amboenicus* were dried at 45°C and ground into powder. The dried leaves powder (1 kg) was extracted with n-hexane by maceration method (3×3 d, 7.5 L) at room temperature with occasional stirring. The same procedure was applied to ethyl acetate and ethanolic fraction. Fraction from each solvent was concentrated by a rotary evaporator (Heidolph VV-200) and the concentrated fraction was dried by freeze-dryer (Edwards).[9,10].

Cytoprotective assays

MTT assays

Cytoprotective activity was determined by the MTT colorimetric assay. Vero cell lines were plated at 1×10⁴ cells/well in a 96-well plate was grown in M199 medium, containing 10% fetal bovine serum (Gibco), 1% penicillin-streptomycin (Gibco), and fungizone 0.5% (Gibco). The culture cells were incubated in a humidified incubator at 37°C at atmosphere of 5% CO₂ and 95% air for 24 h. After incubation for 24 h at 37°C, the medium was discharged and cells were treated by EtF with different concentrations and incubated for 24 h. After that, cell was induced with 0.8 mM H₂O₂ for 3 h. MTT 0.5 mg/mL solution was added to each well and further incubated for 4 h at 37°C. Viable cells react with MTT to produce purple formazan crystals. After 4 h, the stopper 10% SDS (Sigma) in 0.01

N HCl (Merck) was added to dissolve the formazan crystal. The cells were then incubated for 24 h at room temperature and protected from light. After incubation, the cells were shaken. Optical density was read with an ELISA reader at λ 595 nm. The experimental data were absorbance of each well and then converted to a percentage of viable cells [11-13].

The equation to determine viability of cells is:

$$\text{Viability} = \frac{\text{Absoftreatment} - \text{Absofmedium}}{\text{Absofcontrolcells} - \text{Absofmedium}} \times 100\%$$

Flow cytometry analysis

Vero cells (5×10^5 cells/well) were seeded into 6-well plate and incubated for 24 h. After that, were induced with 0.8 mM H_2O_2 for 3 h. And then, the cells were treated with EtF (100 $\mu\text{g}/\text{mL}$) and then incubated for 24 h. Both floating and adherent cells were collected in conical tube using trypsin 0.025%. The cells were washed thrice with cold PBS and centrifuged 2500 rpm for 5 min. The supernatant was separated, while the sediment was collected and fixed in cold 70% ethanol in PBS at -20°C for 2 h. The cells were washed thrice with cold PBS and suspended, then centrifuged 3000 rpm for 3 min and annexin V kit added to sediment and suspended and incubated at 37°C for 30 min. The samples were analyzed using FACScan flow cytometer [11-13].

Immunocytochemistry assays

Vero cells (5×10^4 cells/well) were seeded on coverslips in 24-well plate and incubated for 24 h. After that, Vero cell Line were induced with 0.8 mM H_2O_2 for 3 h. The cells were treated with ethanol extract and then incubated for 24 h. After incubation, the cells were washed with PBS and then fixed with cold methanol at 4°C for 10 min. After that, the cells were washed with PBS and blocked in hydrogen peroxide (H_2O_2) blocking solution for 10 min at room temperature, incubated using primary antibody ROS for 1 h, then washed thrice with PBS, and then incubated with secondary antibody for 10 min. The cells were washed with PBS, then incubated in 3,3-diaminobenzidine (DAB) solution for 10 min, and washed with Aquadest. Afterward, the cells were counterstained with Mayer Hematoxylin for 5 min, and the coverslips were taken and washed with Aquadest and then immersed with xylol and ethanol 70%. Protein expression observed by a light microscope (Nikon YS100). Cells that express a particular protein will provide the brown color, while the cells that do not give a specific protein will provide blue color [11].

RESULT AND DISCUSSION

MTT methods are a quantitative test. This test is based on measuring the intensity of the color (colorimetric) that occurs as a result of metabolism of a substrate by living cells into a colored product. This assay is based on the assumption that dead cells or their products do not reduce tetrazolium. The assay depends both on the number of cells present and on the mitochondrial activity per cell [14,15]. The reduction of yellow tetrazolium MTT is reduced by metabolically active cells, in part by the action of dehydrogenase enzymes, to generate reducing equivalents such as NADH and NADPH. The resulting intracellular purple formazan can be solubilized quantified by spectrophotometric means. The extract showed cytoprotective activities in a concentration-dependent manner (Fig. 1).

Fig. 1 shows that H_2O_2 as the oxidant source in this study at a concentration of 0.8 mM has induced cell damage and a decrease in live cell count by 5% compared with Vero cell control without H_2O_2 treatment. The EtF of *C. amboinicus* was found to be $76.08 \pm 0.91\%$ at 100 $\mu\text{g}/\text{mL}$ compared with control cell with H_2O_2 treatment. These results indicate that ethanol extract has cytoprotective activity.

Evaluation of cell viability was performed using flow cytometry assay with annexin V and PI as shown in Fig. 2 and Tabel 1. The percentage of cell treatment by EtF in viability cell was 49.59 %, in early apoptotic was

30.32 %, in late apoptotic/early necrotic was 3.91%, and in late necrotic was 16.27%. Percentage of cell count with early apoptosis for cell control was 9.42%, cell control+ H_2O_2 was 10.93%, and EtF was 30.23%. Percentage of cell count with late apoptosis or early necrosis for control was 2.97%, cell control+ H_2O_2 was 45.72%, and EtF was 3.91%. The final apoptosis signifies that the cell has fragmented DNA and undergoes phagocytosis. Percentage of cell number late necrosis for cell control 7.04%, cell control+ H_2O_2 33.03%, and EtF 16,27%. Decreased necrotic cells show that EtF has no cytotoxic ability. The number of viability cells on the control of cells+ H_2O_2 is very small because many cells experience death from apoptosis and necrosis. Apoptosis and necrosis will increase as the percentage of viability cells decreases. H_2O_2 is a non-radical ROS with weak oxidizing activity. It diffuses through cell membranes rapidly and interacts with Fe^{2+} and possibly Cu^{2+} ions to form hydroxyl radicals and other free radicals [14].

Immunocytochemistry assay with ROS antibody is negative characterized by blue stained nuclei in the cells (Fig. 3). The results obtained that the cells can be protected by EtF, so there are few cells that express ROS. This shows that EtF has protective activity against cells that have been induced with H_2O_2 because it can decrease ROS expression.

As seen in Fig. 3, the control cell showed strongly brown positive reactions in the cell cytoplasm, many giant cells, irregular cell shape, and cell nuclei shrink, as seen in the cell control+ H_2O_2 . Negative reactions show blue in the cytoplasm, as shown in the cell control and ethanol extract. The results showed that the cells can be protected by ethanol extract, so there are few cells that express ROS. Thus, it is possible that ethanol extract has protective activity against cells that have been induced with H_2O_2 because it can decrease ROS expression.

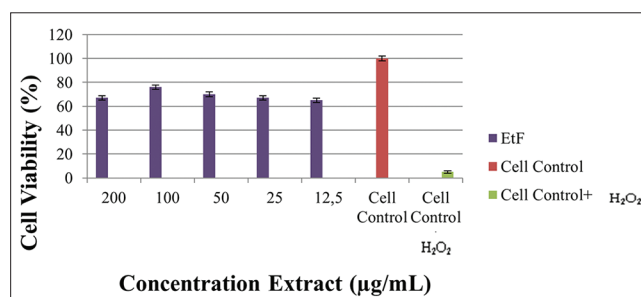


Fig. 1: Viability of ethanol fraction on 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide assay

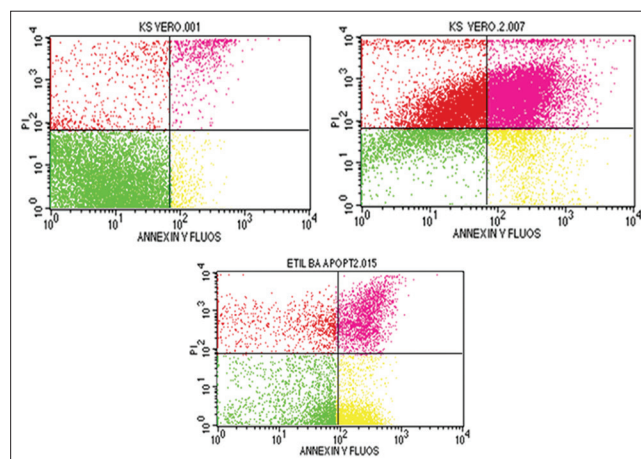


Fig. 2: Apoptotic analysis of ethanol fraction on Vero cell line. (a) control cells; (b) control cells+ H_2O_2 ; (c) ethanol extract (100 $\mu\text{g}/\text{mL}$)

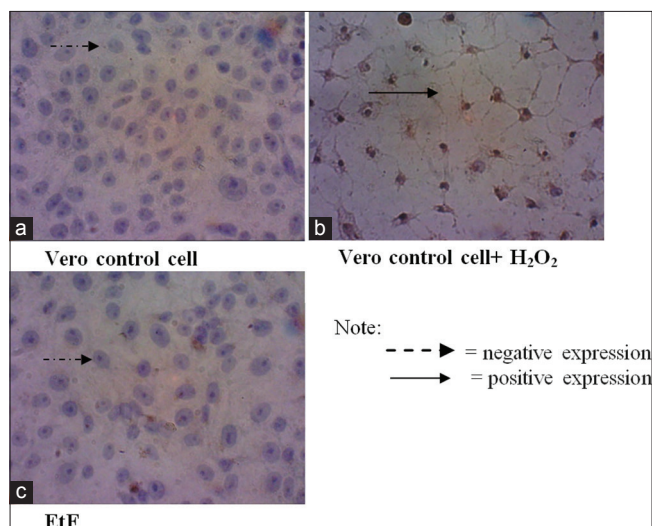


Fig. 3: Expression of radical species oxygen antibody with immunocytochemistry assay. (a) Vero control cell, (b) Vero control cell+H₂O₂, (c) ethanol fraction

Table 1: Evaluation of cell viability was performed using flow cytometry assay with annexin V and PI

Treatment	LL (%)	LR (%)	UR (%)	UL (%)
Control cells	80.56	9.42	2.97	7.04
Control cells+H ₂ O ₂	10.32	10.93	45.72	33.03
EtF	49.59	30.23	3.91	16.27

LL: Lower left: The percentage of viability cells, LR: Lower right: The percentage of early apoptotic, UR: Upper right: The percentage of late apoptotic/early necrotic, UL: Upper Left: The percentage of late necrotic

C. amboinicus Lour. contains flavonoids. Flavonoids contribute to antioxidant activity in vitro by flavonoids can chelate metal ions prevent them in participation to form free radicals and protect against oxidative stress. Antioxidant flavonoids seem to be a combination of direct reaction with free radicals and chelating properties responsible for the production of ROS. It is believed that antioxidants exert their protective effect by decreasing oxidative damage to DNA and by decreasing abnormal increases in cell division [16-17]. Flavonoids inhibit the enzymes responsible for superoxide anion production, such as xanthine oxidase and protein kinase C. A number of flavonoids efficiently chelate trace metals, which play an important role in oxygen metabolism. Free iron and copper are potential enhancers of ROS formation, as exemplified by the reduction of H₂O₂ with the generation of the highly aggressive hydroxyl radical [18].

CONCLUSION

From these result, it is concluded that the EtF of *C. amboinicus* Lour. has cytoprotective activities on injured Vero cell line. The results also indicate that plant extract is a significant source of natural antioxidant, which might be helpful in preventing oxidative stress from H₂O₂ 0.8 mM. EtF with MTT assay showed the viability cell by 76.08±0.91 % at 100 µg/mL, increased viability cell by flow cytometry, and decreased ROS expression with immunocytochemistry.

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CONFLICT OF INTEREST

The authors have no any conflicts of interest in this work.

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