

Antioxidative, antiproliferative, and apoptosis effect of *Coleus tuberosus* flesh and peel ethanol extracts on cervical cancer cell lines

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ABSTRACT

The purpose of this study is to determine the antioxidant activity, antiproliferation, apoptosis, and cell cycle arrest induced by *Coleus tuberosus* flesh and peel extracted with ethanol. The antioxidant activity was determined using the 1,1-diphenylpicrylhydrazyl and cellular antioxidant assays, and the antiproliferative activity was determined using the 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Flow cytometry was used to assess cell cycle arrest, and acridine orange-ethidium bromide staining was used to assess apoptosis-induced ability. The results show that the *C. tuberosus* peel extract has higher antioxidant activity than the flesh extract. *Coleus tuberosus* flesh ethanol extract (CFEE) and *C. tuberosus* peel ethanol extracts' (CPEE) antioxidant activities inhibitory concentration 50 (IC₅₀) were 1290.00±1.58g/ml and 310.97±0.32µg/ml, respectively. The *C. tuberosus* peel extract has greater antiproliferative activity than the flesh extract. The *C. tuberosus* flesh and peel extracts had antiproliferative activities (IC₅₀) of 651.35±4.24 and 366.41±3.52µg/ml, respectively. The flesh and peel extracts cause apoptosis in HeLa cells. Cell cycle arrest in sub-G1 (M) and cell cycle inhibition in G0-G1 are caused by the *C. tuberosus* peel extract. According to this study, the CFEE and CPEEs have the potential to be a source of natural antioxidants and antiproliferation of cervical cancer.

1. INTRODUCTION

Cervical cancer is one of the cancers which kills women in developing countries every year [1]. This is in line with the incidence of cervical cancer in Indonesia, the second cause of women's death at 23.4 per 100,000, an average death rate of 13.9 per 100,000 [2]. The known stage of cancer and the rate at which treatment is administered determine a person's life expectancy. Assume the cancer is discovered and treated in its early stages. In that case, the life expectancy is approximately 70%–75%, 60% in the second stage, and 25% in the third stage, and it is difficult to survive in stage four sufferers.

Eating foods high in biologically active compounds and antioxidants can help prevent cancer because diet can prevent

30%–40% of cases. *Coleus tuberosus* is one plant that has antitumor properties. *Coleus tuberosus* belong to the tuber vegetable species, belonging to the same nation as *Solanum tuberosum* and classified in the Family Lamiaceae and the subfamily Nepetoideae. *Coleus tuberosus* belongs to a group of plants used as food and medicine based on ethnobotany and phylogeny in *Plectranthus* [3].

Some research suggests that *C. tuberosus* can potentially have an antitumor, antioxidant, and antiproliferation effects in cancer cells [4–6]. Triterpenic acid compounds with antioxidant and antiproliferative properties include ursolic acid, oleanolic acid, and maslinic acid. Triterpenic acids are among the biologically active compounds found in *C. tuberosus* (ursolic acid, oleanolic acid, and maslinic acid) [6,7]. Ursolic acid, oleanolic acid, and maslinic acid are triterpenic acid compounds with antioxidant and antiproliferative properties [8–11].

HeLa cells are cervical cancer cells that are often used in research. A distinctive feature of cancer cells is rapid proliferation. Proliferation in cancer cells is uncontrolled growth and infinite

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cleavage. Cancer is caused by the imbalance of active oxygen and antioxidant defense system in the body, which can cause oxidative stress and promote the damage of protein, DNA, and RNA, leading to abnormal cell growth [12,13]. Prevention of cancer through bioactive compounds of plants can stop carcinogenesis and inhibit proliferation. One way to achieve that is by consuming phytochemical compounds contained in foods that have the ability as cellular antioxidants. Inhibition of cancer cell proliferation due to bioactive compounds can interact with cancer processes at the genetic level and modify the detoxification side of the drug, DNA repair, and cell proliferation. Dietary interventions containing phytochemical compounds that have the ability as cellular antioxidants can be a key strategy to prevent the development of cancer.

The study aims to determine the potential of different parts of the *C. tuberosus* ethanol extract on antioxidative, antiproliferative, and apoptosis effect and cell cycle arrest in HeLa cell lines.

2. MATERIALS AND METHODS

2.1. Preparation of *C. tuberosus* Flesh Ethanol Extract (CFEE) and *C. tuberosus* Peel Ethanol Extracts (CPEE) Through a Maceration Process

Coleus tuberosus was taken from a 3-month-old plant. A peeler was used to separate the tuber and peel. *Coleus tuberosus* flesh and peel were dried for 24 hours in a drying cabinet at 40°C. The dried flesh and peel were then size-reduced and sifted through a sieve Tyler size 80 mesh. The flesh and peel flour was then frozen (−20°C) until needed. For 7 days, the *C. tuberosus* tuber and peel maceration process employed ethanol at a 1:5 ratio. Following that, the solution was filtered through Whatman paper and dried on a rotary evaporator at 45°C. The extract that resulted was kept at 20°C.

2.2. Analysis of Antioxidant Activity by the 1,1-Diphenylpicrylhydrazyl (DPPH) Method

The ability of free radical scavengers was measured to assess antioxidant activity [14]. The hydrogen supply capacity of the CFEE or CPEE to capture radicals was measured using DPPH. Two milliliters of a 0.1 mM DPPH methanol solution, plus 100–2,000 µL of ethanol extract from *C. tuberosus* flesh or peel of varying concentrations, was added. After 30 minutes, the reaction mixture discolored, and it was read at 517 nm against blanks. The inhibitory concentration 50 (IC₅₀) of the CFEE or CPEE required to capture 50% DPPH was used to demonstrate antioxidant ability. The lower the IC₅₀, the greater the antioxidant capacity. The IC₅₀ values are calculated by using linear regression between extract concentrations and radical capture percentages.

2.3. Cell Culture

For evaluation of the activity of cellular antioxidants, antiproliferation, cell cycle, and apoptosis, frozen cells (HeLa cells from American Type Culture Collection (ATCC): CCL-2TM) from liquid nitrogen storage were laid at room temperature until partially melted, then inserted in a conical tube 15 ml, plus 10 ml of washing media, and shaken. The pellets were

centrifuged at 750 g for 7 minutes and then added to the culture medium [Roswell Park Memorial Institute (RPMI)] in a flask. RPMI culture media were used to culture HeLa cells. In laminar airflow, all activities were carried out aseptically. The cell was then incubated at 37°C with a 5% CO₂ flow. Cell development was monitored on a daily basis, and if the media began to turn yellow, it was replaced with new media. Media on HeLa cells were removed and washed with sufficient Phosphate Buffer Saline (PBS). After 4–5 weeks of incubation, cells reached confluence, and then they were detached after trypsinization [0.05% trypsin with Ethylenediaminetetraacetic acid (EDTA) in PBS] and transferred to 75 cm² flasks for continued growth. The culture medium was renewed once needed until cells reached 80%–90% confluency. The 7th–15th passages of HeLa were used in the experiment.

2.4. Analysis of Cellular Antioxidant Activity

The antioxidant activity of *C. tuberosus* on HeLa cells was studied [15,16]. HeLa cells were grown in a microplate with 96 wells and an RPMI medium. RPMI plus fetal bovine serum 10% (v/v), penicillin 100 U, and streptomycin 100 mg/ml, CO₂ at 5%, and temperature 37°C are the conditions. The growth medium was processed and washed with PBS after being placed on the microplate for 20 hours. *Coleus tuberosus* tuber and peel ethanol extracts 100–800 µg/ml in Dimetil sulfoksida (DMSO) was used in various concentrations.

Three wells are treated for 20 minutes with different concentrations of 100 µl CFEE or CPEE, followed by 30 minutes with 25 mM Dichlorodihydrofluorescein diacetate (DCFHDA) dissolved in medium and 100 ng/ml Phorbol 12-myristate 13-acetate (PMA) DMSO. A Becton Dickinson FACS (BD FACS) Calibur flow cytometer with a 535 nm wavelength was used to fluoresce 20,000 HeLa cells. The percentage of reactive oxygen species reduction was used to calculate cell antioxidant activity:

$$\text{Percentage of ROS reduction} = (\text{Fit}_0 - \text{Fit}_1) \times 100 / (\text{Fit}_0 - \text{Fit}_2),$$

where Fit₀ are cells subjected to oxidative stress, Fit₁ are cells subjected to extract treatment, and Fit₂ are cells subjected to no oxidative stress [17].

2.5. Evaluation of Antiproliferation

Incubating HeLa cells with CFEE or CPEE resulted in antiproliferative activity [18]. HeLa cells are used in research (ATCC). Cell concentrations (1.5 × 10⁴ cells/ml) were plated in 96-well plates. At 5% CO₂ and 37°C, 10% (v/v) fetal bovine serum, 100 U penicillin, and 100 mg/ml streptomycin were added to Dulbecco's modified eagle medium (DMEM). It was left for 24 hours. A 100–800 µg/ml ethanol extract of *C. tuberosus* flesh or peel was added to HeLa cancer cells. The extract contained 0.5% of ethanol which was applied as control. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to assess cancer cell viability. The concentration of HeLa cells in the solution was 1.5 × 10⁴ cells/ml. The HeLa was washed with HBBS after 24 hours of incubation. The cells were then incubated for 4 hours in 50 µl of an MTT solution (0.5 mg/ml in DMEM). The next step was to add the stop solution, let it sit overnight, and

then measure the absorbance at 570 nm with a multilabel plate reader. The following formula calculates the number of viable cells using the MTT assay:

$$\text{HeLa cells viability (\%)} = \left(\frac{\text{treatment absorbance}}{\text{without treatment absorbance}} \right) \times 100.$$

2.6. Apoptosis Induction Evaluation

Acridine orange and ethidium bromide (AO-EB) staining was used to assess morphological changes caused by apoptosis induction in HeLa cells treated with the CFEE or CPEE. On the coverslip, HeLa cells were plated at 10^5 cells per well. After that, the growth medium was replaced with one containing the CFEE or CPEE at a concentration of 15.875 or 31.25 g/ml, respectively. This was incubated for 24 hours at 5% CO_2 and 37°C. The next step was to remove the growth medium and incubate for 5 minutes with acridine orange-ethidium bromide at a concentration of 100 g/ml PBS and place the coverslip over the glass object, and then this was observed with a fluorescence microscope (Zeiss MC 80) [19].

2.7. Cell Cycle Arrest

In a 6-well plate, 10^6 cells per well were distributed and cultivated at 37°C for cell adaptation. For 24 hours, HeLa cells were exposed to the CFEE or CPEE at concentrations of 7.8125, 15.625, 31.25, 62.5, and 125 g/ml. The cultivated cells were removed after 24 hours and washed twice with ice-cold PBS. The cells were fixed and permeabilized for 1 hour at 4°C with ice-cold 70% ethanol. The HeLa cells were then rinsed in PBS and resuspended in a solution containing 50 ml/ml propidium iodide dye and 250 mg/ml RNase A. The cell suspension was incubated at room temperature for 30 minutes before being utilized for fluorescence-activated cell sorting with 10,000 cells per group (FACS; Caterplus Flow Cytometry; Becton Dickinson Co., Germany) [20]. The ModFit LT Cell Cycle 3.0 analysis software was used to determine the percentage of HeLa cells in the G0-G1, S, and G2/M phases (Becton Dickinson).

2.8. STATISTICAL ANALYSIS

The data displayed are the mean standard deviation (\pm SD) in triplicate. Analyzing variance and the least significant difference test were used to test *in vitro* antioxidant activity, cellular antioxidant activity, and antiproliferation.

3. RESULTS AND DISCUSSION

3.1. Antioxidant Activity Evaluation

The DPPH method was used to assess antioxidant activity. The DPPH method was used to demonstrate that the CFEE or CPEE has antioxidant activity *in vitro* (Table 1). The antioxidant activity was expressed as an IC_{50} , indicating that the free radical scavenger (DPPH) has a 50% capacity. The higher the antioxidant activity, the lower the IC_{50} . As a result, the peel ethanolic extract has higher antioxidant activity than the flesh extract *C. tuberosus*. The antioxidant activity of the CFEE or CPEE was supported by the presence of biologically active compounds such as oleanolic

Table 1: IC_{50} CFEE and CPEE by the DPPH method.

Compounds	IC_{50} ($\mu\text{g/ml}$)
CFEE	$1,290.00 \pm 1.58^b$
CPEE	310.97 ± 0.32^a

Difference notation: difference significant at $p < 0.05$.

acid, maslinic acid, ursolic acid, phenol, and flavonoids, with the peel having a higher concentration than the flesh [6,7,21,22]. Ursolic acid, oleanolic acid, maslinic acid, and phytosterols acted as free radical scavengers [23–29]. The difference in the number of bioactive compounds was thought to have an impact on its antioxidant activity.

3.2. Cellular Antioxidants Activity Evaluation

Figure 1 demonstrated that the CFEE or CPEE could reduce PMA-induced reactive oxygen species in HeLa cells in a dose-dependent manner. The Reactive Oxygen Species (ROS) reduction percentages in HeLa cells incubated with the CFEE or CPEEs at various concentrations of 100, 200, 400, and 800 $\mu\text{g/ml}$ were 44.32 ± 0.95 , 52.52 ± 0.20 , 72, 77 ± 0.64 and 80.135 ± 0 , 52, respectively. The percentages of ROS reduction in HeLa cells incubated with the CFEE or CPEE at different concentrations of 100, 200, 400, and 800 $\mu\text{g/ml}$ were 25.67 ± 0.95 , 42.98 ± 0.20 , 59 , 12 ± 0.64 and 66.23 ± 0.07 , respectively.

The exceeding level of reactive oxygen species in cells will attack DNA, lipids, or proteins that will induce oxidation reactions and damage cell membranes to activate key enzymes on some signaling pathways [30]. The ability of the CFEE or CPEE to reduce reactive oxygen species is related to eliminating reactive oxygen species that attack cell membranes. Ursolic acid, oleanolic acid, maslinic acid, phytosterols, phenols, and flavonoids can maintain the cell membrane fluidity by scavenging reactive oxygen species so that communication signals at the cellular level continue to function normally, including signals related to the activation of antioxidant enzymes [Nuclear erythroid 2-related factor 2 Antioxidant Response Element (Nrf2ARE)], transcription factors that regulate the genes encoding antioxidant defense system [31,32]. Higher expression of Nrf2ARE can enhance the antioxidant defense system in cells.

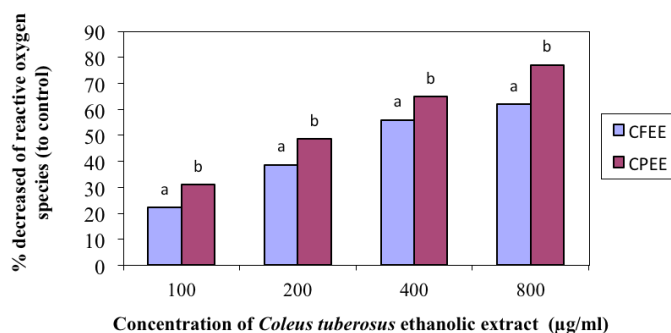


Figure 1: At 24 hours incubation, the percentage decrease in reactive oxygen species of HeLa cells with various concentrations of the CFEE and CPEE. The data displayed is the mean of the experimental triplicates. There are significant differences between the letters ($p < 0.05$).

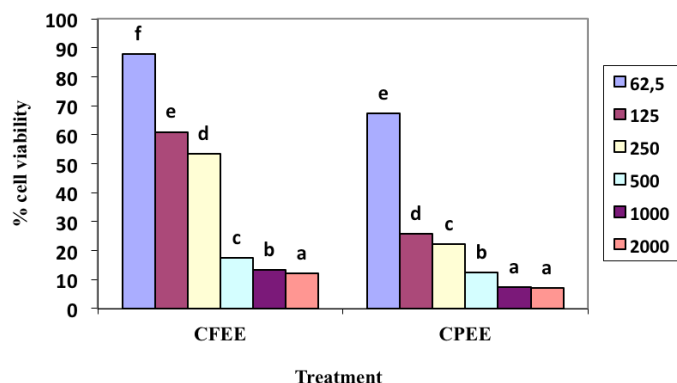


Figure 2: Percentage of cell viability in the treatment of various concentrations of the CFEE and CPEE at 24 hours incubation. The data were presented as the average of the triplicate experiments. Different letters indicate a significant difference ($p < 0.05$).

The ability to neutralize singlet oxygen (1O_2), superoxide anion radicals ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($^{\cdot}OH$) will be affected by PMA induction. The *Coleus* ethanol extract contains biologically active compounds that can boost cells' antioxidant defense systems [Catalase (CAT), Superoxide dismutase (SOD), Glutathione peroxidase (GPx), vitamin E, vitamin C, carotene, and Glutathion (GSH)] and reduce oxidative stress [33–36]. The antioxidants in the CFEE or CPEE work as free radical scavengers thus reducing reactive oxygen species in PMA-induced HeLa cells.

3.3. Antiproliferation Activity

The MTT method was used to assess the antiproliferative activity of the CFEE or CPEE in HeLa cells. The cancer cells were treated with a concentration range of 62.5–2,000 g/ml and incubated for 24 hours (Fig. 2). Figure 2 shows that, depending on the dose, incubating HeLa cells with the CFEE or CPEE can reduce the percentage of viable cells. The IC_{50} of the CFEE or CPEE treatment is shown in Table 2.

The interaction of biologically active compounds in the CFEE or CPEE provides a synergistic effect and determines its antiproliferative capacity. The CFEE or CPEEs can both inhibit HeLa cell proliferation. The *C. peel* extract has superior antiproliferative properties to the flesh extract. This is consistent with the discovery that the peel contains more biologically active compounds than the flesh. *Coleus* contains the following biologically active compounds that support antiproliferative activity: Ursolic Acid (UA), Oleanolic Acid (OA), maslinic acid, phytosterols, phenols, and flavonoids that have antiproliferative activity in some cancer cells [37–39]. These biologically active compounds can reduce Nuclear Faktor k B (NFkB) regulatory genes, which play a key role in developing and progressing specific cancer cells, such as proliferation, migration,

and apoptosis [40–43]. Low NFkB expression results in a decrease in cyclin D1 and an increase in tumor suppressor factors (such as p53, p21, and p27), which inhibits HeLa cell proliferation. As a result, the possible antiproliferation mechanism of the CFEE or CPEE reduces oxidative stress by lowering reactive oxygen species. It can control the cell cycle and gene expression that regulates cell proliferation cyclin D1 [44–48].

3.4. Apoptosis-Induced Activity

The CFEE or CPEE at a concentration of 15.625 or 31.25 μ g/ml can induce apoptosis in HeLa cells (Fig. 3). The induction of HeLa cell apoptosis can be detected using Acridine Orange (AO)-EB staining. Evaluation for live and dead cells undergoing apoptosis can use the combination of AO-EB. When AO-EB binds to living cells' double-stranded DNA, it emits green fluorescence. Simultaneously, when AO-EB binds to single-stranded DNA in dead cells, the fluorescence turns red. Cells that enter the early stage of apoptosis turn green, and cells that enter the late stage of apoptosis have their DNA broken and turn red. Meanwhile, in the final apoptosis process, DNA is fragmented and will be red [49].

Due to changes in cell membrane permeability, cells undergo apoptosis. Changes in cell membrane permeability cause AO-EB to enter and embed in cells with the orange-character DNA [50]. While bright green cells show cells have early apoptosis, chromatin condensation causes chromatin to absorb more color than control, and the cells are bright green. However, the cells are still alive, so only AO can color living cells [51]. *Coleus tuberosus* contains bioactive compounds such as oleanolic acid, ursolic acid, maslinic acid, and phytosterols, which are apoptosis inducers [52–55].

3.5. Evaluation of Cell Cycle Arrest

Flow cytometry was used to assess cell cycle arrest in HeLa cells (Beckton Coulter, USA). HeLa cells were induced by various concentrations of the CFEE or CPEE, i.e., 7.812, 15.625, 31.25, 62.5, and 125 μ g/ml (Fig.4 and Fig.5).

The CFEE did not significantly inhibit HeLa cell cycles in the M1 phase, G0-G1 phase, S-phase, or G2-M phase. The CPEE can inhibit the G2-M phase and induce cell death, as evidenced by an increase in the percentage of cells in the sub-G1 (M1) phase. Increased concentrations of the CPEE impact cell death, as evidenced by an increase in the percentage of sub-G1 (M1) cells and an increase in cell cycle arrest G0-G1 phase. This finding is consistent with previous research indicating that increasing the concentration of extracts of a cell-induced product increases cell death by increasing sub-G1 (M) and G0-G1 cell cycle arrest [56,57]. The CPEE could inhibit the cell cycle, causing cell accumulation in a hyperdiploid, and then undergo apoptosis.

4. CONCLUSION

The CPEE and CFEEs have antioxidant, antiproliferative, apoptotic, and cell cycle arrest activity. In terms of antioxidant activity, antiproliferative activity, and ability to induce cell cycle arrest, the *C. tuberosus* peel extract outperformed the flesh extract. According to this finding, the CFEE and CPEEs may be a source of natural antioxidants and cancer-preventive agents.

Table 2: IC_{50} of antiproliferation CFEE and CPEE on HeLa cells.

Compound	IC_{50} (μ g/ml)
CFEE	651.35 \pm 4.24 ^b
CPEE	366.41 \pm 3.52 ^a

Difference notation: difference significant at $p < 0.05$.

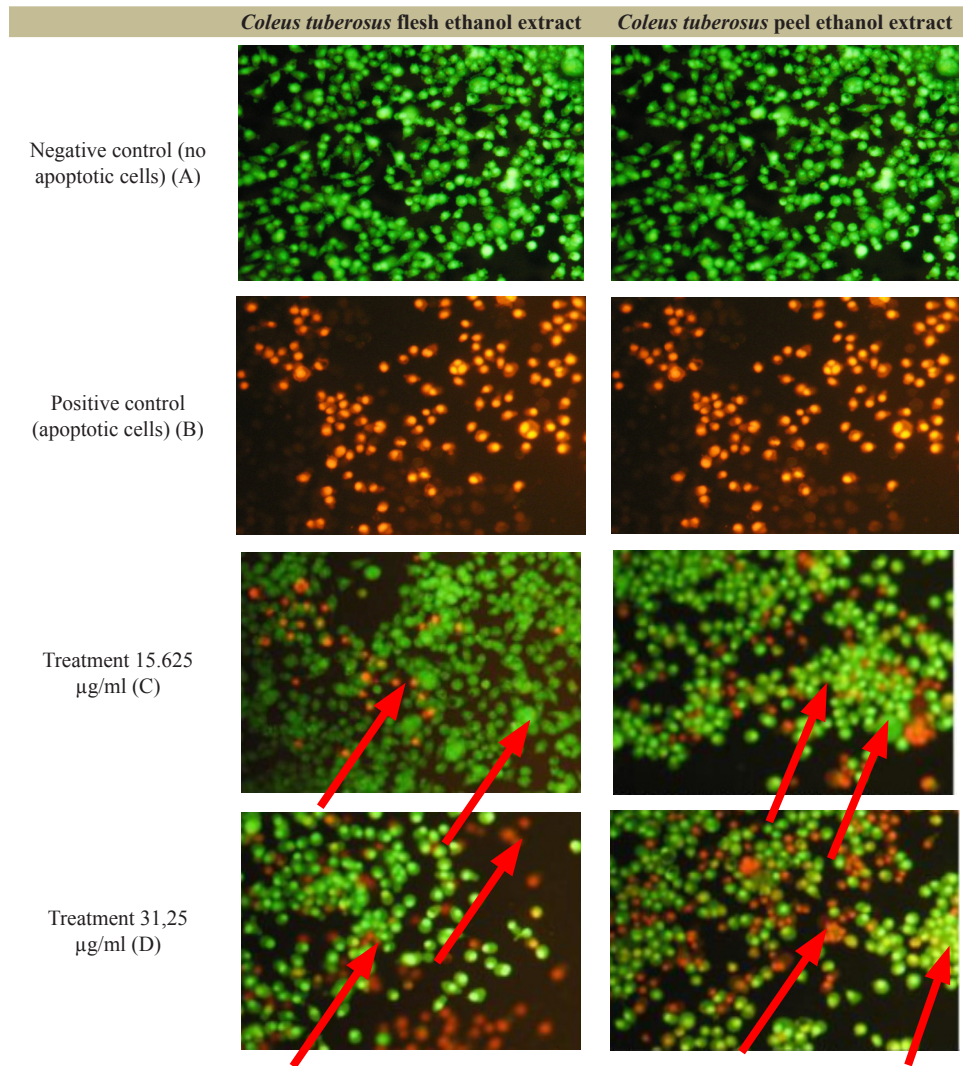


Figure 3: Induction of apoptosis in HeLa cells with the CFEE or CPEE. (A) Negative control; (B) positive control; (C and D) flesh or peel ethanolic extract of *C. tuberosus*. Morphological observations of apoptotic HeLa cells were made using acridine orange-ethidium bromide staining and a fluorescent microscope. Negative control (A) of living cells, normal shape, and green color; positive control (B), cell apoptosis; (C and D) induction of apoptosis, and some cells have died and deformed, and the color of the cells changed from light green to orange. Initial apoptosis (light green color), final apoptosis (orange color).

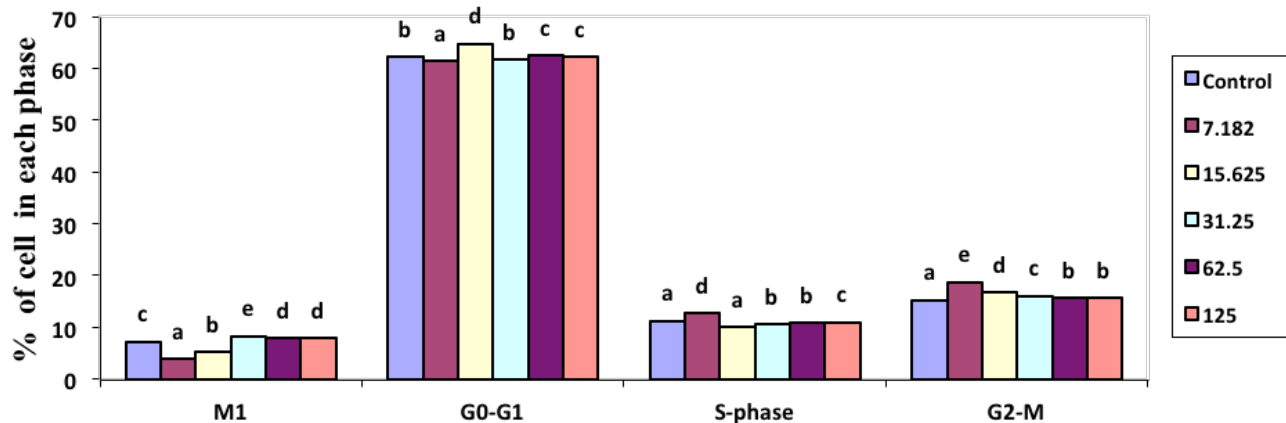


Figure 4: The effect of the CFEE on cell cycle arrest. HeLa cells were treated for 24 hours with various extract concentrations, namely, 7.8125, 15.625, 31.25, 62.5, and 125 µg/ml, and without extract (as a control). Propidium Iodide (PI)-stained cells were analyzed with a flow cytometer. The use of a different letter demonstrates a significant difference ($p < 0.05$).

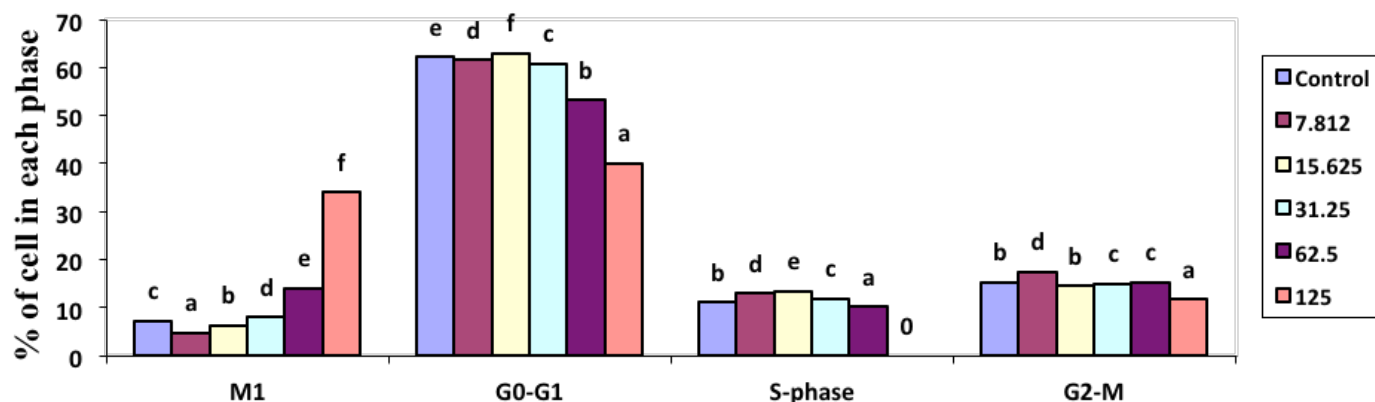


Figure 5: The effect of the CPEE on cell cycle arrest. HeLa cells were treated for 24 hours with various extract concentrations, namely, 7.8125, 15.625, 31.25, 62.5, and 125 µg/ml, and without extract (as a control). PI-stained cells were analyzed with a flow cytometer. The use of a different letter demonstrates a significant difference ($p < 0.05$).

5. AUTHORS' CONTRIBUTIONS

MN participated in the concept and research design, data collection, data analysis, interpretation of analysis results, and writing the manuscript. W and BL participated in designing the research, statistical analysis, and revising the important content of the manuscript. The manuscript has been approved by all of the authors.

6. ACKNOWLEDGMENTS

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7. CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

8. ETHICAL APPROVAL

There were no animal or human subjects in this study.

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