Antiproliferation, apoptosis induced by ethanolic extract of *Coleus tuberosus* in cervics cancer cells

ARTIKEL

HIBAH BERSAING

Antiproliferation, apoptosis induced by ethanolic extract of *Coleus tuberosus* in cervics cancer cells

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Antiproliferation, apoptosis induced by ethanolic extract of *Coleus tuberosus* in cervics cancer cells

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Abstract

Coleus tuberosus is classified as a local vegetable of the family *Lamiaceae*, subfamily *Nepetoideae* and tribe *Ocimeae*. Ethanolic extract Flesh of Coleus tuberosus and Ethanolic extract of Peel of *Coleus tuberosus* evaluated antiproliferative based on MTT assay. Morphological change apoptosis based on staining with acridine orange and ethidium bromida using human cervical cancer HeLa cells. Cell cycle arrest evaluated with flow cytometry The result showed that antiproliferative activity of EEPC was higher than EEFC. EEFC, EEPC induced apoptosis in MCF-7 cells. The antiproliferative activities were in a dose-dependent manner and IC_{50} EEPC and EEFC were 366.41 µg/ml, 651.35µg/ml, respectively. These result indicated that EEPC and EEFC might be used as a potential source of antiproliferative agent based on apoptosis induction mechanism.

Keywords : antiproliferative, apoptosis, *Coleus tuberosus*

Introduction

Cancer is a disease caused by disruption of the normal regulation of cell growth control (Balasubramanian , 2007) . In the world , every 2 minutes , a woman dies from cervical cancer , in Indonesia, every 1 hour (Ferlay J et al . GLOBOCAN 2002. IARC 2004). According to a survey of 5,423 women conducted in Asia and 9 countries , including Indonesia , proved to be only 2 percent of women knew that HPV infection is a cause of cervical cancer . So women's knowledge about cervical cancer still low. Cervical cancer is the most common cancer found in Indonesia (34.4 % of all cancers in women). Nearly 70 % of them were known to be at an advanced stage (> stage IIB) . In 15,000 new cases , 8,000 deaths . Every day 40-45 new cases are found, with 20-25 people died. Indonesian Cancer Foundation explained that the highest mortality rate of cervical cancer among other types of cancer among women. Estimated, 52 million Indonesian women at risk for cervical cancer, while 36 percent of women of all patients with cancer is cervical cancer patients. There are 15,000 new cases per year with 8,000 deaths per year. Five- year survival rate if the cancer is detected and treated at stage 1 is 70-75 percent , in stage 2 is 60 percent, at 25 percent live stage 3 , and stage IV patients are expected to survive difficult .

Based on the study, 30-40 % of all cancer cases can be prevented by diet . Based on the concept of cancer prevention by incorporating foods that contain bioactive compounds and cancer prevention functions as an antioxidant necessary. One of the plants showed antitumor potency is *Coleus tuberosus*. Coleus tuberosus are tubers which become carbohydrate sources and research Hsum Wie Yap et al (2008) shows chloroform extract of Coleus tuberosus has antitumor

potential in the promotion stage of Raji cells, GC - MS analysis showed triterpenic acid compounds. According to Min Yang (2007) and Yosra et al , (2010), the benefits of the group triterpenoid compound is an antioxidant and antitumor .

Coleus tuberosus is a plant vegetables included in the same nation with potato (Solanum tuberosum) is Solanales, one tribe but different genera namely Lamiaceae. Sunarjono (2009) classifies *Coleus tuberosus* in the tuber vegetables group. Some secondary metabolites in the family Lamiaceae is an assortment of mainly terpenoids , mono - , sesqui - , di- and tri - terpenes who have the ability as an anti- cancer (Pistelli , 2006) .

Nugraheni et al (2011) showed that extracts of peel and flesh of *Coleus tuberosus* have the ability to prevent proliferation and as an antioxidant to reduce oxidative stress in breast cancer cells (MCF-7). Further research anti-proliferation activity of peel and flesh *Coleus tuberosus* extract and the mechanism of inhibition of cell proliferation and cancer needs to be done on other types of cancer cells, so the research is expected to be a reference to the use of *Coleus tuberosus* extract as a nutraceutical for cancer prevention .

HeLa cell line is a continuous cell line derived from epithelial cells of cervical cancer (cervix) a women with cervical cancer named Henrietta Lacks who died of cancer in 1951 (Anonymous, 2006a) . HeLa cells are cervical cancer cells from infection Human Papillomavirus (HPV 18) so as to have different properties with normal cervical cells . Cervical cancer cells infected by HPV are known expressed two oncogene, namely E6 and E7. E6 and E7 proteins shown to cause the nature of immortal human keratinocytes in primary culture, but these

immortal cells are not tumorigenic to a genetic process occurs . Thus, the viral oncogenes are not directly induce tumor formation, but induces a series of processes that ultimately can lead to cancer properties (Goodwin and DiMaio , 2000) . The majority of cervical cancer cells, including HeLa cells , and the p53 gene have p105Rb in the wild-type form . Thus, growth regulatory genes are active in normal cells is also present in cervical cancer cells. However, its activity is inhibited by the expression of E6 and E7 proteins of HPV (Goodwin and DiMaio , 2000) .

One of mechanisms plant bioactive compounds ability to inhibit proliferation of cancer cells is by inducing apoptosis (Liu and Finley, 2005). Apoptosis is a process of programmed cell death, is genetically regulated, active nature, characterized by chromatin condensation, cell fragmentation and the cell phagocytosis by phagocytic cells or by macrophages. Deregulation of apoptosis resulting in pathological conditions, including the occurrence of uncontrolled cell proliferation such as cancer .

The purpose of this study was to determine the selectivity of the anti-proliferation in vitro meat extract and black potato skins against HeLa cancer cells, determine the ability of extracts of black meat and potato skins induces apoptosis in HeLa cancer cells and determine the ability of extracts of black meat and potato skins in inducing cell cycle arrest in cells HeLa cancer .

Materials and Methods

Materials

ethanolic extract peel and flesh of *Coleus tuberosus*, HeLa cancer cell lines. Chemicals : RPMI (Sigma Aldrich), 3 - (4,5- Dimethylthiazol - 2 - yl) -2,5 - diphenyltetrazolium bromide (MTT) (Sigma Aldrich), DMSO (Sigma Aldrich), Phosphate buffered saline (PBS) (Gibco) . Equipment : 96 - well plate , elisa reader and brands Leitz microscope , laminar air flow (LAF) class II , Acridine orange - ethidium bromide

Preparation of extracts

Peel of *Coleus tuberosus* separated with the flesh. *Coleus tuberosus* peeled using a peeler with a thickness of 1 mm. Then dry with a cabinet dryer for 12 hours, crushed and extracted by maceration with 95 % ethanol . Thus obtained ethanolic extract peel and flesh extract of *Coleus tuberosus*. Extracts stored in frozen condition during the study period .

Evaluation of anti-proliferation of HeLa cancer cell lines

The principle is a bioactive compound reacting with the cancer cells into cells tested . 3 - (4,5- dimethylthiazol - 2 - yl) -2,5 - diphenyltetrazolium bromide (MTT) assay was used to evaluate the anti - proliferative activity of the extract were tested with the cancer cells . Tests based on the tetrazolium salt (MTT) into a blue formazan by the mitochondrial enzyme succinate dehydrogenase . Conversion is only found in living cells and the amount of formazan produced is proportional to the number of living cells there . So the MTT assay was used to test potential anti - proliferative activity of the extract material (Osama et al , 2009) .

Evaluation are used reference to Hogan et.al (2010) . Cervic cancer cell lines HeLa (ATCC) . Cell ($1.5x10^4$cells/ml) in 96 -well plated in RPMI plus 10 % (v / v) fetal bovine serum , 100 U Penicillin and Streptomycin 100mg/ml . 37 ° C, 5 % CO_2. allowed to stand for 24 hours . Cancer cells were treated for 24-72 hours in media experiments with ethanolic extract peel and flesh of *Coleus tuberosus* at a concentration of 5-40 □ g / ml. Cell viability was determined by MTT assay. After incubation for 24-72 hours , cells were washed with HBSS and then treated with the ethanolic extract peel and flesh of *Coleus tuberosus* in the growth media were tested in 96 - well plate . Each treatment was repeated 3 times. Cells were then incubated for 72 hours at 5 % CO_2, 37°C in the incubator. Media were treated eliminated at the end of each incubation period, washed with HBSS and then cells were incubated for 4 hours with 50 mL solution of MTT reagent (0.5 mg/ml in RPMI) was added to each of the wells, plus stop solution, let it overnight then absorbance was recorded at 570 nm with a multilable plate reader . Absorbance data required for cell viability were expressed by the percentage of control (number of living cells in control cells) in the experiment . After each treatment , the MTT assay .

Cell viability (%) = (Abs treatment / Abs without treatment) x 100 .

Induction of apoptosis

Apoptosis observations with DNA Painting Cover slips (Nunc) were implanted into the 6 well plate (Nunc) and cells ($2x10^4$) distributed on it, and then incubated for 24 hours in a CO_2 incubator and test fraction was added to the concentration 150 µg/ml. Incubation was continued for 24 hours. At the end of incubation, the

culture medium was taken, then the cover slip removed from the wells and placed on a glass object and then sprinkled with acridine orange/ethidium bromide (100 µg/ml acridine orange (Bio-Rad) in PBS and 100 µg/ml ethidium bromide (bio-Rad) in PBS. Cell morphology observations made with a fluorescence microscope (Zeiss MC 80) .

Statistical analysis

The experiments were conducted with three replications . Data shown are mean ± SD of three replications . Testing performed by ANOVA antiproliferasi one lane , if there is a real difference followed by Duncan's Multiple Range Test (DMRT) .

Result and Discussion

Result

Effect of EEPC and EEFC on proliferation human cervic cancer HeLa cells.

The treatments were given in T47D and HeLa cells by ethanol extract of peel and flesh of Coleus tuberosus with various concentration showed that HeLa cells sensitive to treatment with the ethanol extract peel of Coleus tuberosus as indicated by the low IC_{50} compared with the treatment of ethanol extract flesh of Coleus tuberosus (Table 1).

Staining Acridine orange-Ethidium Bromida on HeLa cells treated with EEFC, EEPC,

The phenotypic characteristics of cells treated with EEFC, EEPC were evaluated by microscopic inspection of overall morphology in Hela cells. Treatment of EEFC, EEPC showed a significant evidence of cell death even after 24 h. The ability of all treatment to induce apoptosis was initially screened by using acridine

orange/ ethidium bromide staining. The treated cells showed obvious nuclear condensation after 24 h treatment (Figure 1 and Figure 2). Positive control cells showed bright green nucleus, negative control showed orange-red with uniform intensity, where the apoptotic cells appeared orange in color.

Discussion

Coleus tuberosus is tuber vegetables of the family *Lamiceae,* and the member of subfamily *Nepetoideae* and tribe *Ocimeae*. One of the characteristics of bioactive compounds in plants belonging to the family *Lamiaceae* mainly in members of the subfamily *Nepetoideae* is the presence of triterpenic acid. Many Research on family *Lamiacea*e showed that plant belonging in family *Lamiaceae* presence triterpenic acid such as ursolic acid and oleanolic acid (Zou et al., 2008; Abdel-Mogib et al., 2002; Bariceviv et al., 2001; Pistelli, 2006; Janicsak et al., 2006). The objective of the current study were investigate antiproliferatives and apoptosis effect of EEFC, EEPC on HeLa cells.

The micro-culture Tetrazolium Salt (MTT) assay was used in this study to measure the amount of cell viability. The potenstial antiproliferative effect of EEFC, and EEPC, was investigated, determining their effects on the viability of a human cervical cancer cell line, HeLa. MTT is cleaved by tetrazolium ring by succinate dehydrogenase in active mitochondria. Metabolically active cells cleave MTT and generate a formazan product, which forms purple crystals and colour developed is directly proportional to cell number. The amount of dye produced is proportional to the number of metabolically live cells.(Ruso et al., 2009).

The anti-tumor promoting properties on *Coleus tuberosus* have been widely reported by many research group (Mooi et al., 1999; Ali et al., 2000) , but antiproliferatives activities of *Coleus tuberosus* on cancer cells has not been done. The results indicated that EEFC, and EEPC caused significant growth inhibition of HeLa cells in dose dependent manner. EEPC showed higher antiproliferative activities than EEFC toward HeLa cells in a dose- and time- dependent manner. The sensitivity of HeLa cancer cells to EEFC and EEPC is characterized by IC_{50} value (Table 1).

The inhibition of cancer cell proliferation by EEFC and EEPC can be partially explained by the triterpenic acid content mainly ursolic acid and oleanolic acid. Ursolic acid and oleanolic acid were responsible for their antiproliferative activities. The higher antiproliferative effect of EEFC compare EEPC was related with the higher of ursolic acid and oleanolic acid content in the peel than the flesh.

Extensive cell death was observed in proliferating human breast cancer cells after treatments with EEFC, and EEPC. To determine if the treatment-induced cell death occurred through cytotoxic necrosis and/or apoptosis, cells were harvested and assayed for apoptosis induction with acridine orange/ethidium bromide. Staining of apoptotic cells with fluorescent dyes such as AO and EB is considered the correct method for evaluating the changed nuclear morphology As demonstrated in Figure 1 and 2.

Acridine orange (AO)–ethidium bromide (EB) double staining cell morphological analysis Acridine orange is taken up by both viable and deadcells. It would fluoresce green when bound to double stranded DNA in living cells and

fluoresce red when bound to single stranded DNA which dominates in dead cells. Ethidium bromide was excluded from living cells. The results obtained from the acridine orange–ethidium bromide double staining are shown in Figure 4. Viable cells with intact DNA and nucleus give a round and green nuclei. Early apoptotic cells will have fragmented DNA which gives several green colored nuclei. Late apoptotic cells DNA would be fragmented and stained orange and red.(Ho et al., 2009).

AO/EB staining, based on nuclear morphology (perinuclear chromatin condensation, nuclear collapse and eventual fragmentation), allows us to distinguish several subpopulations of apoptotic cells from viable cells. The addition of the light microscopy appearance of these AO/EB cells, for example cell rounding, surface blebbing and shrinkage, provide a complete morphological profile of an apoptotic cell.

Mechanism of EEPC, EEFC induced apoptosis in HeLa cells has not been done, however the mechanism of apoptosis EEFC and EEFC possible have in common with one of the mechanisms of apoptosis in cancer cells treated with ursolic acid and oleanolic acid on other cancer cells, based on the identification bioactive compounds in EEFC and EEPC were ursolic acid and oleanolic acid.

There were many research supported that ursolic acid and oleanolic acid as a bioactive compounds in Coleus tuberosus induces apoptosis in a number of cancer cells lines. Treatment with oleanolic acid A and ursolic acid induced a dramatic loss of the mitochondria membrane potential and interfered with the ratio of expression levels of pro- and antiapoptotic Bcl-2 family members in HuH7 cells. Oleanolic acid and ursolic acid-induced apoptosis involving the release of

mitochondria cytochrome *c* into the cytosol and subsequently induced the activation of caspase-9 and caspase-3, followed by cleavage of poly (ADP-ribose) polymerase (PARP). Moreover, HuH7 cells treated with OA and UA suppressed the activity of NF-κB and modulated the mRNA expression of X-linked inhibitor of apoptotic protein (XIAP) as compared with untreated cells. These results demonstrate that oleanolic acid and ursolic acid induce apoptosis in HuH7 cells through a mitochondria-mediated pathway and downregulation of XIAP. (Shyu et al., 2010)

Conclusion of this research the ethanolic extract of peel and flesh *Coleus tuberosus* were able to inhibit proliferation in human cervical cancer HeLa cells at a dose and time-dependent manner. Growth and proliferation inhibition is through induction of apoptosis.

Acknowledgment

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Table 1. IC_{50} of treatment EEFC, and EEPC on HeLa cells after incubation for 24 hours.

Compound	IC_{50} (10^{-3} mg/ml)
	24 hours
EEFC	651.35 ± 3.80^{b}
EEPC	366.41 ± 4.19^{a}

Values were expressed as mean ± standard deviation (n=3). Means with different letters were significantly different at level of $p < 0.05$.

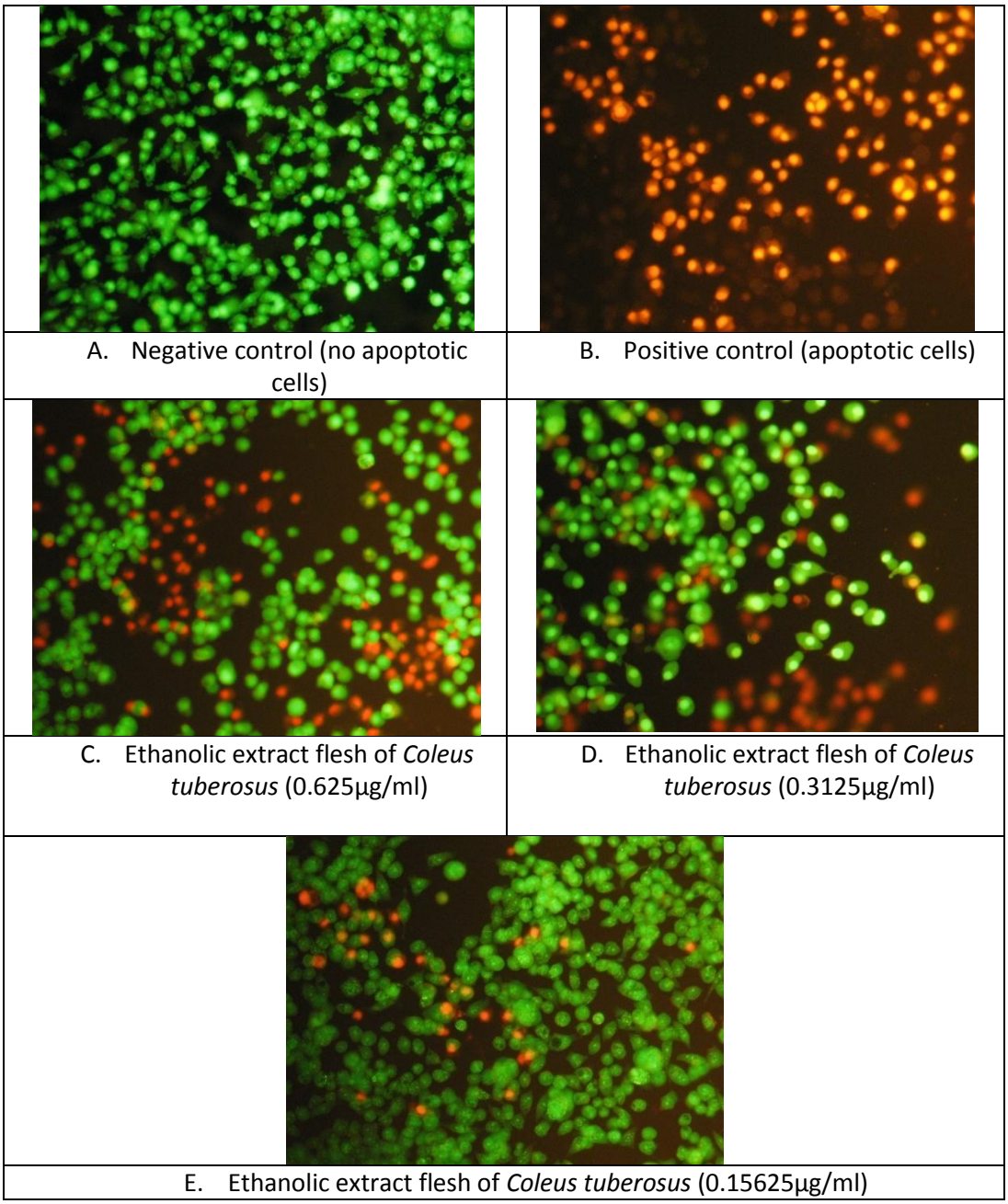
Figure Legends

Figure 1 - Cellular and nuclear morphological changes of MCF-7 cells following exposure to various concentrations of EEFC for 24 h. Cells were distinguished according to the fluorescence emission and themorphological aspect of chromatin condensationin the stained nuclei. (1) Viable cells have uniform bright green nuclei with organized structure. (2) Early apoptotic cells have green nuclei, but perinuclear chromatin condensation is visible as bright green patches or fragments.(3) Late apoptotic cells have orange to red nuclei with condensed or fragmented chromatin

Experimental conditions were as follows: A = untreated cells, B. = treated cells with doxorubin, C= EEFC : 0.625 mg/ml, D. EEFC : 0.3125 mg/ml, E. EEFC : 0.15625 mg/ml,.

Figure 2 - Cellular and nuclear morphological changes of MCF-7 cells following exposure to various concentrations of EEPC for 24 h. Cells were distinguished according to the fluorescence emission and themorphological aspect of chromatin condensationin the stained nuclei. (1) Viable cells have uniform bright green nuclei with organized structure. (2) Early apoptotic cells have green nuclei, but perinuclear chromatin condensation is visible as bright green patches or fragments.(3) Late apoptotic cells have orange to red nuclei with condensed or fragmented chromatin

Experimental conditions were as follows: A = untreated cells, B. = treated cells with doxorubin, C= EEPC : 0.625 mg/ml, D. EEPC : 0.3125 mg/ml, E. EEPC : 0.15625 mg/ml,.



A. Negative control (no apoptotic cells)

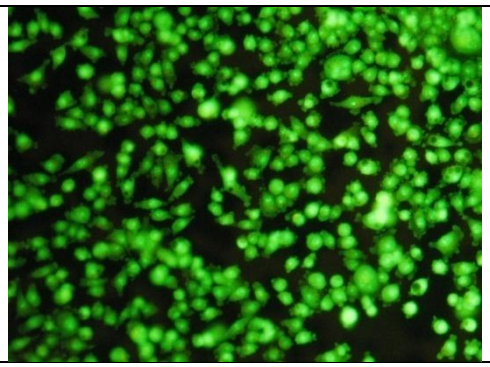
B. Positive control (apoptotic cells)

C. Ethanolic extract flesh of *Coleus tuberosus* (0.625µg/ml)

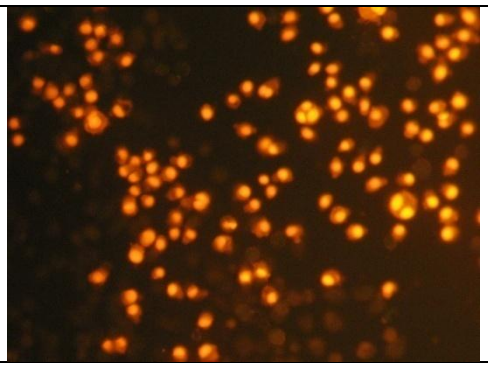
D. Ethanolic extract flesh of *Coleus tuberosus* (0.3125µg/ml)

E. Ethanolic extract flesh of *Coleus tuberosus* (0.15625µg/ml)

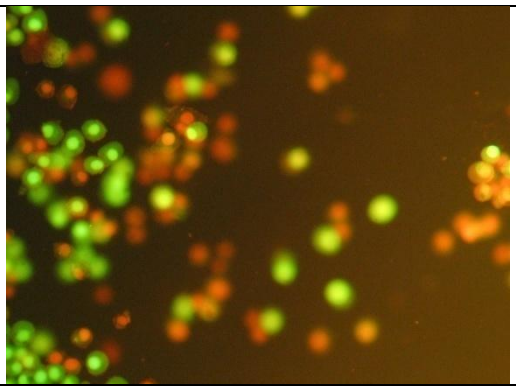
Figure 1



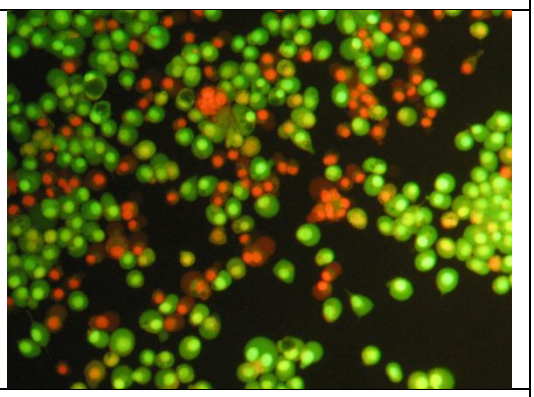
Negative control (non apoptotic cells)



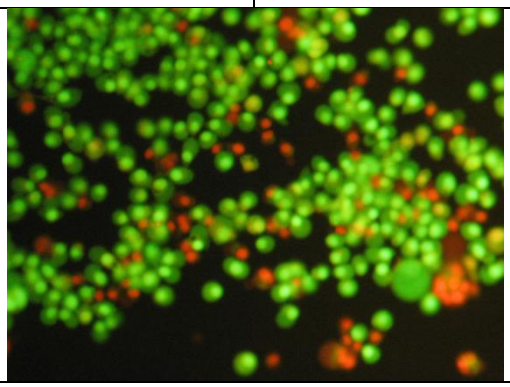
Positive control (apoptotic cells)



Ethanolic extract peel of *Coleus tuberosus* (0.625µg/ml)



Ethanolic extract peel of *Coleus tuberosus* (0.3125µg/ml)



Ethanolic extract peel of *Coleus tuberosus* (0.15625µg/ml)

Figure 2

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