Full Length Research Paper

In vitro antioxidant, antiproliferative and apoptosis effect of *Coleus tuberosus* L.

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Coleus tuberosus (Blume) Benth. is classified as a local vegetable of the family *Lamiaceae*, subfamily *Nepetoideae* and tribe *Ocimeae*. Ethanolic extract the flesh of *C. tuberosus* and ethanolic extract of the peel of *C. tuberosus* evaluated antioxidant properties based on 1,1-Diphenyl-2-picryl hydrazyl radical assay, antiproliferative based on 3-(4,5-dimethylthiazol-2)-2,5-diphenyltetrazolium bromide assay and morphological change apoptosis based on staining with acridine orange and ethidium bromide using human breast cancer Michigan Cancer Foundation 7 (MCF 7) cells. Results showed that antioxidant and antiproliferative activity of ethanolic extract of the peel of *C. tuberosus* was higher than ethanolic extract of the flesh of *C. tuberosus*. Based on morphological changes with acridine orange and ethidium bromide staining, ethanolic extract of the flesh of *C. tuberosus*, ethanolic extract of the peel of *C. tuberosus*, ursolic acid and oleanolic acid have antiproliferative activity through induced apoptosis in MCF-7 cells. The antioxidant and antiproliferative activities were in a dose-dependent manner. The antioxidant activities and antiproliferative activity of ethanolic extract of the the flesh of *C. tuberosus* and ethanolic extract of the peel of *C. tuberosus* related with oleanolic acid and ursolic acid content. These result indicated that ethanolic extract of the flesh of *C. tuberosus* and ethanolic extract of the peel of *C. tuberosus* might be used as a potential source of natural antioxidants and antiproliferative agents.

Key words: Antioxidant, antiproliferative, apoptosis, *Coleus tuberosus*, ursolic acid, oleanolic acid.

INTRODUCTION

Free radicals can lead to a variety of physiological and biochemical lesions and induce degenerative diseases such as coronary artery disease, aging, alzheimer, and cancer (Lin et al., 2010). Natural source of food that are believed to have a high antioxidant and antiproliferative activity on tumor cells have become an important element of cancer prevention and treatment strategies. Three decades of research have revealed that cancer prevention is easier than to treat. Consumption of certain fruits and vegetables may reduce the risk of cancer (Aggarwal et al., 2004).

Approximately more than 60% of currently used anticancer chemotherapeutics are derived from natural sources, including plants. The major categories of plant-derived compounds that have medicinal properties are terpenoids, flavonoids, and alkaloids (Wicaksono et al., 2009). These natural inhibitors of carcinogenesis are apparently non toxic as markedly less toxic and other many drugs. While it is generally accepted that a diet of large amount of vegetables, fruits, and other plant products lowers cancer incident, there is still a need to identify the most effective constituents of the diet, as well as to elucidate their mechanisms of action (Nanasombat et al., 2009).

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Approximately one-third of the women with breast cancer developed metastases and ultimately died of the disease. MCF-7 cell has become a prominent model system for the study of breast cancer as it relates to the susceptibility of the cells to apoptosis. Further, it has become increasingly important in the prevention or treatment of a number of major solid tumors, particularly metastatic and drug-resistant breast cancer (Spencer et al., 1999). Data based on Hospital Information System in Indonesia 2007, the incidence of breast cancer 8227 cases or 16.85% and cervical cancer 5786 cases or 11.78%. This showed breast cancer cases is still dominating in Indonesia. Therefore early detection and search for potential antitumor compounds are important in the control of breast cancer (Mukherjee et al., 2005).

C. tuberosus is a vegetable, member of the family Lamiceae. 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 derivates (Janicsak et al., 2006). There is growing interest in the elucidation of the biological and pharmacological roles of plant-derived triterpenic acid compounds such as ursolic acid, oleanolic acid, maslinic acid, in terms of antioxidant, hepatoprotective, analgesic, antitumor, anti inflammatory, and immunmodulatory effect (Yoon et al., 2008; Feng et al., 2008; Fai et al., 2009).

Investigations of *C. tuberosus* extracts, in mediating cancer cells and antioxidant activity are very limited. *C. tuberosus* shows strong *in vitro* anti-tumor promoting activity when assayed using Raji Cells (Mooi et al., 1999; Hsum et al., 2008). The active anti-tumor promoting compounds are identified as maslinic acid, stigmasterol, β-sitoterol and campesterol attributed to their ability as inhibition of the expression of EBV early-antigen in Raji cells (Mooi et al., 2010). The active compounds identified in peel and flesh *C. tuberosus* were ursolic acid and oleanolic acid (Nugraheni et al., 2010). Ursolic acid and oleanolic acid may occur as aglycones of saponins and as free acids. Both OA and UA have many important pharmacological effects, which are rather similar because of the closeness of their chemical structures (Janicsak et al., 2006). Several studies have shown that ursolic acid and oleanolic acid has the ability as free radicals scavenger (Ozgen et al., 2011; Donfack et al., 2010).

Research on antioxidant, antiproliferative and apoptosis effect of different parts of *C. tuberosus* mainly in flesh and peel in human breast cancer has not been done. The objective of this work was to investigate antioxidant, antiproliferative and apoptosis effect of ethanolic extract of the flesh and peel of *C. tuberosus* ursolic acid and oleanolic acid on MCF-7 cell lines.

MATERIALS AND METHODS

Material

Ursolic acid originated Santa Cruz Biotech Inc. (Santa Cruz, CA USA), DPPH, 1,1-diphenyl-2-picryl hydrazyl radical, 3-(4,5-dimethylthiazol-2)-2,5-diphenyltetrazolium bromida (MTT), Dulbecco's Modified Eagle Medium (DMEM), oleanolic acid, ethidium bromide, acridine orange, ethanol and acetic acid were of HPLC grade. All other reagents and solvents were of analytical reagent grade from Sigma-Aldrich (Sigma Aldrich USA), Fetal Bovine Serum (FBS) from Gibco (Invitrogen Inc. CA USA). MCF-7 were obtained from ATCC (LGC Standards, Middlesex UK).

Preparation of *C. tuberosus* extracts

C. tuberosus was obtained from farmer in Bantul District, Yogyakarta Special Region, Indonesia at commercial maturity of 3 month. Cultivation conditions such as low temperatures of 15 to 20°C, enough sunlight, humidity of 80 to 90%, rainfall of 2500 - 3300 mm per year and grow well in soil acidity (pH) of 4.9 to 5.7). The peel and flesh of *C. tuberosus* were cut into small pieces and dried with cabinet dryer at 40°C for 24 h, then blended into powders. Preparation of crude extract refers to Mooi et al. (1999) (17), sample was macerated with ethanol (1 kg of peel or flesh powder of *C. tuberosus*: 5 L ethanol) at room temperature for a week. The crude extract were then filtered through Whatman No.1 filter paper (United Kingdom), dried with waterbath and evaporated in vacuum rotary evaporator at 45°C. The yield extract is 20% (1 kg dried flesh of *C. tuberosus* yielded 220 g dried extract and 1 kg dried peel *C. tuberosus* yielded 200 g dried extract). The extract was stored at -20°C for further analysis. Stock solution for EEFC and EEPC was prepared from 10 mg and dissolved in 200 μl DMSO, OA and UA prepared from 5 mg was dissolved in 100 μl DMSO.

Determination of triterpenic acid by high performance liquid chromatography (HPLC) analysis

Identification of triterpenic acid in C. tuberosus (Du and Chen, 2009)

HPLC analysis was performed on HPLC apparatus equipped with Eurospher 100-5 photodiode array detector (Shimadzu Corporation, Kyoto Japan). Separation was carried out at 30°C on Eurosphere 100-5 C-18 column (5 micron, 250 × 4, 6 mm). The mobile phase consisted of a mixture, MeOH : 0.15% CH_3COOH (90:10), monitored by wavelenght 210 nm, and the flow rate was 1 ml/min. Extract dissolve in 1 ml methanol, then filtered with millex filter 0.45 μM and injected into HPLC. The samples and the standards were injected at a volume of 20 μl each. UA and OA were identified by comparison of their retention time (t_R) values and UV-visible spectra with those of known standards and were quantified by peak areas from the chromatogram. Determination of UA, OA content in EEFC and EEPC based on standard curve of UA and OA (Table 1).

DPPH scavenging assay

The extracts and bioactive compounds were measured in terms of hydrogen donating or radical scavenging ability using the stable radical DPPH following the method described by Singh et al. (2009). To 2 ml of DPPH (0.1 mM in methanolic solution), added 300 μl of EEFC, EEPC (1, 2 and 4 × 10^{-1} mg/ml), OA and UA (1, 2 and 4 × 10^{-2} mg/ml). The color change of the reaction mixture was then read at 517 nm against the blank, which did not contain the extract/bioactive compounds. The L-ascorbic acid, BHT were used as the positive control. Sample without treatment was used as negative control. The percent DPPH decolourization of the sample was calculated as:

Table 1. Triterpenic acid content in EEFC and EEPC.

Sample	UA (10^{-3} mg/g sample)	OA (10^{-3} mg/g sample)
EEFC	3.41 ± 0.04^b	3.71 ± 0.06^b
EEPC	13.78 ± 0.15^a	19.75 ± 0.30^a

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

$$\% \text{ Inhibition} = \frac{B_0 - B_1}{B_0} \times 100$$

Where B_0 is the absorbance of the control reaction and B_1 is the absobance in the presence of EEFC, EEPC, UA, OA, L-ascorbic acid and BHT.

Antiproliferation assay

Cell culture

Human breast cancer MCF-7 cells were obtained from ATCC. Cells were cultured in the DMEM, supplemented with 10% heat-inactivated Fetal Bovine Serum and penicillin (100 units/ml-streptomycin (100 µg/ml), using 75 cm^2 flasks in a 37^oC in humidified 5% CO_2 incubator.

Cell viability assay

An MTT assay was performed according to the method of Hogan et al. (2010), with slight modification in density cancer cells. MCF-7 cells were plated into 96-well microtiter plates at a density 1.5 × 10^4/well in a final volume of 100 µl culture medium per well. The cells were treated with EEFC and EEPC (7, 8, 9, and 1 × 10^{-1} mg/ml), UA (5, 10, 15 and 20 × 10^{-3} mg/ml), OA (4,5, 6 and 7 × 10^{-2} mg/ml) and maintained at $37°C$ with CO_2 for 24, 48 and 72 h. Sampel without treatment was used as negative control. After the incubation period, 10 µl of MTT labeling reagent (5 mg/mL) was added to each well. The microtiter plate was then incubated agains for 4 h at $37°C$ with 5% CO_2. Then, 100 µl of the solubilisation solution was added into each well. The plate was allowed to stand overnight in the incubator at 37^oC and 5% CO_2. The cell viability was measured using ELISA reader at 550 nm. The relative cell viability (presented as percent) relative to control (negative control) wells containing cell culture medium without samples was calculated using:

A_{550nm}(sample)/A_{550nm} (control) × 100

The concentration of extract causing 50% percent cell death known as the inhibition concentration (IC_{50}) was determined by a graph of percentage of cell death versus log concentration of samples. The experiment was carried out in three replicates.

Acridine orange / ethidium bromide staining methods

MCF-7 Cells were cultured on cover slips at 1 × 10^5 cells/well onto 12 wells plate until 50 % confluent. The medium was replaced with fresh medium containing sample with concentration EEFC, EEPC (0.9 mg/ml), ursolic acid (7 × 10^{-3} mg/ml), oleanolic acid (7x10^{-2} mg/ml), and negative control (without treatment). Cells then were in-cubated for 24 h in humidified atmosphere at $37°C$ in CO_2 5%. The

medium was removed and added by Working Solution, ethidium bromide/acridin orange (a mixture of acridine orange and ethidium bromide 1:1, 4 mg/ml) for 5 min. Cover slip, containing cells was removed and covered on the object glass. Then assessment can be carried out under fluorescence microscope (Meiyanto et al., 2007). Acridine Orange/Ethidium Bromide Staining uses combination of two dyes to visualize cells with aberrant chromatin organization. Acridine Orange was used to visualize the number of cells which has undergone apoptosis, but it cannot distinguish viable from non-viable cells. To achieve this, a mixture of Acridine Orange and Ethidium Bromide was used. The differential uptake of these two dyes allows the identification of viable and non-viable cells.

Statistical analysis

The results are presented as the average and standard deviation of three experiments. One-way ANOVA was used to analyze the mean differences between sample followed by Duncan's multiple comparison test were used. A significant difference was considered to be p<0.05. SPSS Version 16.0 was used.

RESULTS AND DISCUSSION

Determination UA and OA

UA and OA are ubiquitous triterpenoids in plant kingdom, medicinal herbs and are integral part of the human diets. In the present study, the quantitative determination of UA and OA in ethanolic extracts from *C. tuberosus* was performed by HPLC. Using a standard curve of UA and OA, the amount of UA and OA in EEPC were calculated to be 13.78 ± 0.15, 19.75 ± 0.30, for UA and OA, respectively. EEFC: 3.41 ± 0.04, 3.71 ± 0.06 µg/g sample, for UA and OA, respectively (Table 1).

DPPH radical scavenging

The free radical scavenging activity of EEFC, EEPC, UA, OA, L-ascorbic acid, BHT was assessed by DPPH assay, as shown in Figure 1. EEFC, EEPC, ursolic acid and oleanolic acid demonstrated ability to inhibit the DPPH radical. Percentage of radical scavenging activity at the highest concentration, EEFC (0.4 mg/ml) : 12.17 ± 0.10, EEPC (0.4 mg/ml) : 60.22 ± 0.19, ursolic acid (4 × 10^{-2} mg/ml) : 22.46 ± 0.33, oleanolic acid (4 × 10^{-2} mg/ml) : 19.44 ± 0.35, positive control BHT (4 × 10^{-2} mg/ml) : 36.5 ± 0.35 and AA (4 × 10^{-2} mg/ml) : 91.44 ± .53.   Inhibitory
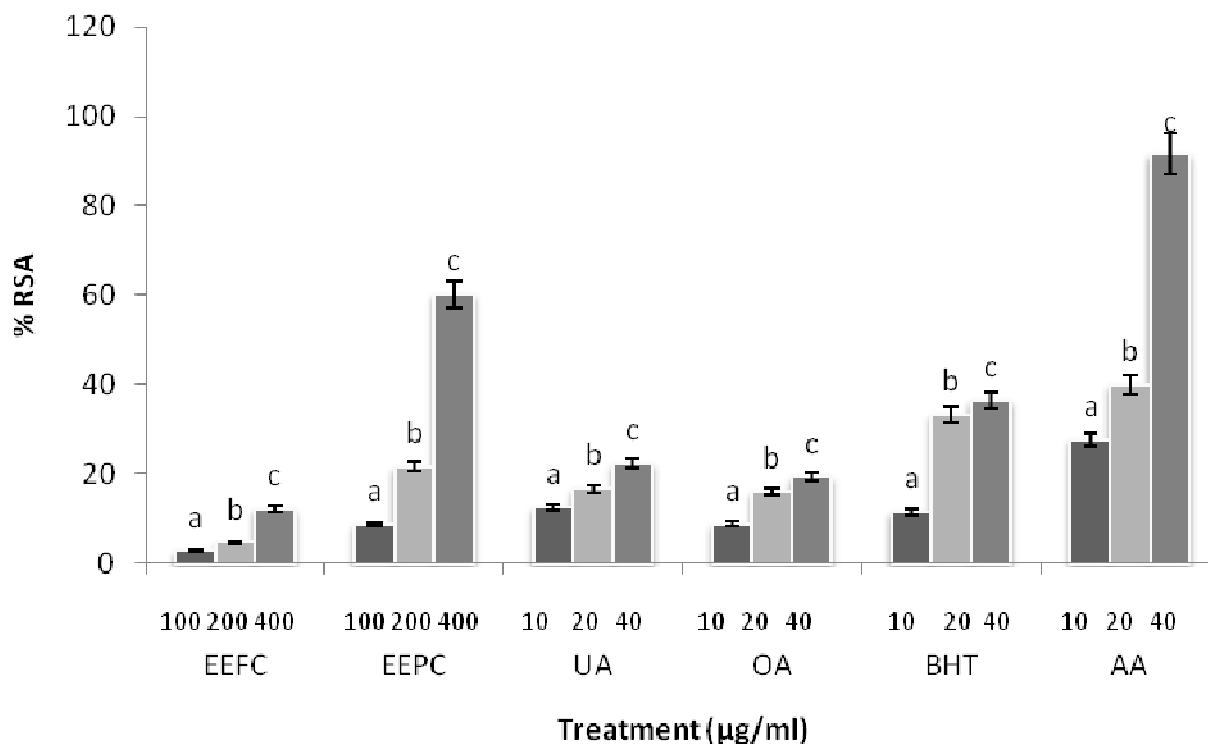
Figure 1. DPPH radical scavenging activity of EEFC, EEPC, ursolic acid, oleanolic acid compared with commercial antioxidant (BHT and AA). Data presented from three independent experiments. Different letters are significantly different (P<0.05) in the same sample.

activity of EEFC, EEPC, UA and OA lower than positive control BHT and L-ascorbic acid.

## Effect of EEPCT, EEFCT, UA and OA on proliferation human breast cancer MCF-7 cells

Figures 2A and B, shows the effects of EEPC and EEFC on proliferation of human breast cancer MCF-7 cells. The antiproliferative activity of EEPCT and EEFCT on MCF-7 cells were characterized by conducting MTT assay. Cells were treated with 0.7; 0.8; 0.9 and 1 mg/ml of extracts and incubated for 24, 48 and 72 h. These results indicated EEFC at highest concentration: 1 mg/ml at 24, 48 and 72 h, MCF-7 cells viability were 60.321, 44.264 and 41.457, respectively. Treatment with EEPC at highest concentration: 1 mg/ml at 24, 48 and 72 h, viability of MCF-7 cells were 52.184, 30.316 and 29.742%, respectively.

It was also shown that at all treatments, the percentage of growth inhibition activity increased gradually with advancement of time. The antiproliferative activity of EEPC and EEFC were expressed as the inhibitory of concentration (IC_{50}). Since the lower the IC_{50} value indicated the higher antiproliferative effect of the sample. IC_{50} growth inhibition after incubation 24, 48, and 72 h of EEFC 1512.97 ± 50.8, 965. 31 ± 3.39, 829.86 ± 5.73 (10^{-3} mg/ml),

respectively. EEPC: 1124.11 ± 35.19, 812.22 ± 5.72, 698.23 ± 1.61 (10^{-3} mg/ml), respectively (Table 1). This study showed that EEPC had higher antiproliferative effect than EEFC at all concentrations which corresponds with its lower IC_{50}.

Figure 3 shows the effects of treatment with UA (5.10^{-3}, 1.10^{-2}, 15.10^{-3} and 2.10^{-2} mg/ml) and OA (4.10^{-2}, 5.10^{-2}, 6.10^{-2} and 7.10^{-2} mg/ml) and incubated for 24, 48 and 72 h on proliferation of human breast cancer MCF-7 cells. These results indicated that UA at highest concentration : 2.10^{-2} mg/ml at 24, 48 and 72 h, MCF-7 cells viability were 15.375; 12.756 and 11.543%, respectively. Treatment with oleanolic acid at highest concentration: 7.10^{-2} mg/ml at 24, 48 and 72 h, viability of MCF-7 cells were 59.379; 42.458 and 39.828%, respectively. Thus the detailed analysis of the results clearly indicated that EEPC, EEFC, UA, and OA caused significant growth inhibition *in vitro* of MCF-7 cells in dose and time-dependent manner. This means that increasing the concentration used and the longer time of incubation with compounds, have an impact on increasing the ability of inhibition of proliferation. This is indicated by the declining number of living cells with increasing concentrations and incubation time of MCF-7 cells.

The antiproliferative activity of oleanolic acid, and ursolic acid expressed as the inhibitory of concentration (IC_{50}). The sensitivity of MCF-7 breast cancer cells to UA,
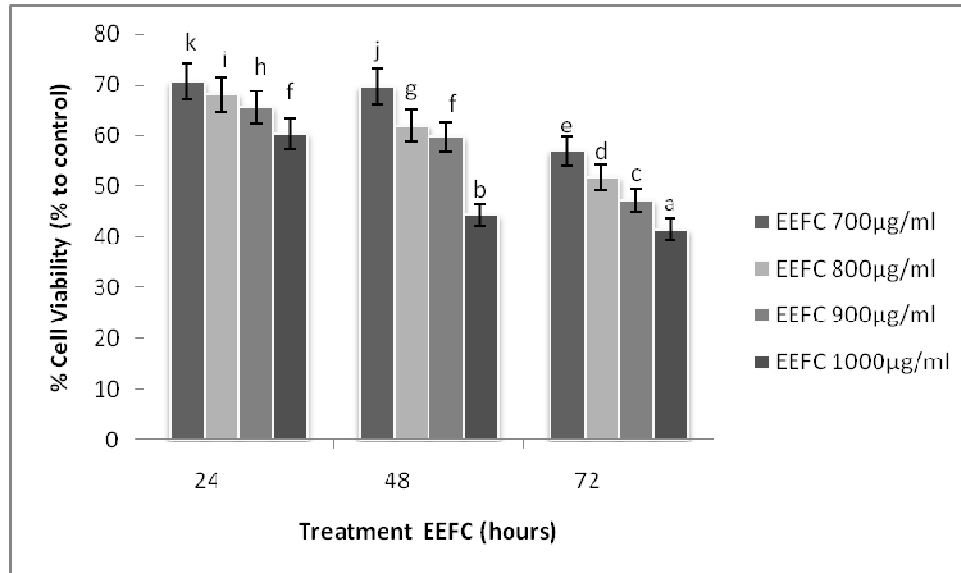
A
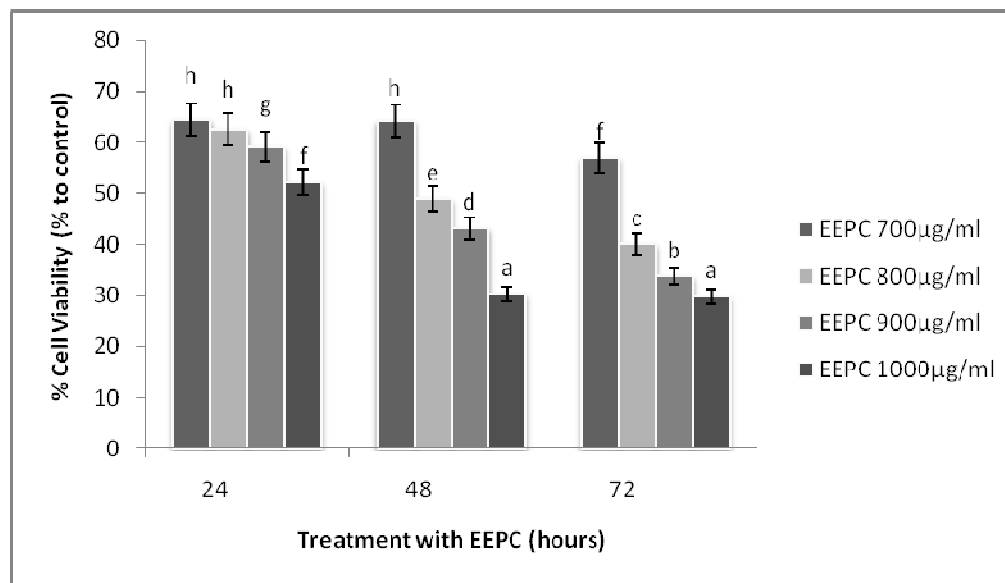
B

Figure 2. Effect of EEFCT, and EEPCT on the proliferation of MCF-7 cells. EEFC inhibited the proliferation of MCF-7 cells in dose-and time-dependent manners, [a-k] different letters are significantly different (P<0.05) (a) and EEPC inhibited the proliferation of MCF-7 cells in dose-and time-dependent manners, [a-h] different letters are significantly different (P<0.05). (b). Data are presented as mean±SD from three independent experiments.

and OA is characterized by IC_{50} value. The lower the IC_{50} value indicated the higher antiproliferative effect of the sample. IC_{50} growth inhibition after incubation 24, 48, and 72 h for UA: 9.02 ± 0.12, 3.81 ± 0.18, 0.64 ± 0.03 (10^{-3} mg/ml), respectively. OA: 136.27 ± 7.19, 48.61 ± 0.90, 39.66 ±0.55 (10^{-3} mg/ml), respectively (Table 2). These results indicate that elevated antiproliferative effects strengthened with time of exposure.

Staining acridine orange-ethidium bromida on MCF-7 cells treated with EEFC, EEPC, UA and OA

The phenotypic characteristics of cells treated with EEFC, EEPC, UA and OA were evaluated by microscopic inspection of overall morphology. Treatment of EEFC, EEPC, UA and OA showed a significant evidence of cell death even after 24 h. The ability of all treatment to

Table 2. IC_{50} of treatment with ursolic acid, oleanolic acid, EEFC, and EEPC of MCF-7 cells after incubation for 24, 48 and 72 h.

Compound	IC_{50} (10^{-3} mg/ml)		
	24 h	48 h	72 h
Ursolic acid	9.02 ± 0.122^a	3.81 ± 0.184^a	0.64 ± 0.03^a
Oleanolic acid	136.27 ± 7.19^b	48.61 ± 0.9^b	39.66 ± 0.55^c
EEFC	1512.97 ± 50.80^c	965.31 ± 3.39^c	829.86 ± 5.73^e
EEPC	1124.11 ± 35.19^d	812.22 ± 5.72^d	698.23 ± 1.61^d

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

induce apoptosis was initially screened by using acridine orange/ ethidium bromide staining. The treated cells showed obvious nuclear condensation after 24 h treatment. Control cells showed bright green nucleus (Figure 4a) with uniform intensity and had not taken up ethidium bromide, where the apoptotic cells appeared orange in color (Figures 4b, c, d and e).

DISCUSSION

Many Researches on family Lamiaceae showed that plants of Lamiaceae family contain triterpenic acid derivatives such as UA and OA (Zou et al., 2008). Triterpene derivatives have diverse biological/ pharmacological effects as they show beneficial properties at several points of tumor genesis and promoting apoptosis of formed invasive cells (Feng et al., 2009).

DPPH assay was carried out to measure the ability to remove or scavenge free radicals is classified as primary antioxidant. The higher antioxidant activity of peel than flesh from *C. tuberosus* was related with the higher content of bioactive compound such as UA and OA in the peel than the flesh (Table 1) and another bioactive compounds in *C. tuberosus* such as maslinic acid and phytosterol (stigmasterol, beta-sitosterol and campesterol) (Mooi et al., 2010).

The higher antioxidant activity of EEPC than EEFC agrees with related research that investigated and compared the antioxidant activity in peel and flesh of fruits and vegetables. Extract of peel of red pitaya, apple and mango have greater antioxidant activity than flesh one and its associated with bioactive compounds content (Wu et al., 2006; Kim et al., 2010).

The result showed that UA, OA, EEFC and EEPC have ability as an antioxidant *in vitro* assay. This research undertaken using *in vitro* chemical that has limitation in describing the whole mechanism and antioxidant potential in biological systems. However, some research suggests that bioactive compounds found in *C. tuberosus* when evaluated using biological systems showed activity as a cellular antioxidant. Gayatri et al. (2010) reported that UA can act as radical scavenger in rat liver cancer

induced by Diethylnitrosamin. Capability is done by restoring antioxidant enzyme activity (SOD, CAT, and GPX). OA has the ability as a radical scavenger by stimulating the cellular antioxidant defense in QZG cells induced by tert-Butyl hidroperoxida (t-BHP) to increase the production of glutathione (GSH) (Wang et al., 2010). Maslinic acid as one of the bioactive compounds in *C. tuberosus* also have the ability to modulate the activity of cellular antioxidant both enzymatic and non enzymatic (Ramos et al., 2010). Phytosterol as bioactive compound in *C. tuberosus* has the ability as an antioxidant by modulating antioxidant enzyme in RAW 264.7 macrophages by increasing SOD and GPX (Vivancos and Moreno, 2005).

The micro-culture tetrazolium salt (MTT) assay was used in this study to measure the amount of cell viability. The potential antiproliferative effect of EEFC, EEPC, UA, and OA was investigated, determining their effects on the viability of a human breast cancer cell line, MCF-7. The results indicated that EEFC, EEPC, UA and OA caused significant growth inhibition of MCF-7 cells in dose and time-dependent manner. EEPC showed higher antiproliferative activities than EEFC toward MCF 7 cells in a dose- and time- dependent manner. The sensitivity of MCF-7 breast cancer cells to EEFC, EEPC, UA, and OA is characterized by IC_{50} value (Table 2). These results indicate that elevated antiproliferative effects strengthened with time of exposure.

The higher antiproliferative effect of EEPC compare EEFC was related with the higher of UA and OA content in the peel than the flesh (Table 2). Antiproliferative activity of EEFC and EEPC is also supported by the presence of other bioactive compounds found in *C. tuberosus*, such as maslinic acid and phytosterol such as stigmasterol, β-sitosterol and campesterol (Mooi et al., 2010). Some studies indicate that the bioactive compounds contained in *C. tuberosus* such as UA,OA, and maslinic acid (Li et al., 2010; Reyes-zurita et al., 2009) and phytosterol were stigmasterol, β-sitosterol and campesterol (Jayaprakasha et al., 2007) have an ability as antiproliferative in cancer cells.

Previous studies showed that UA had strong inhibitory the proliferation and induce apoptosis in a number of cancer cells lines, but OA, which the isomer of UA have
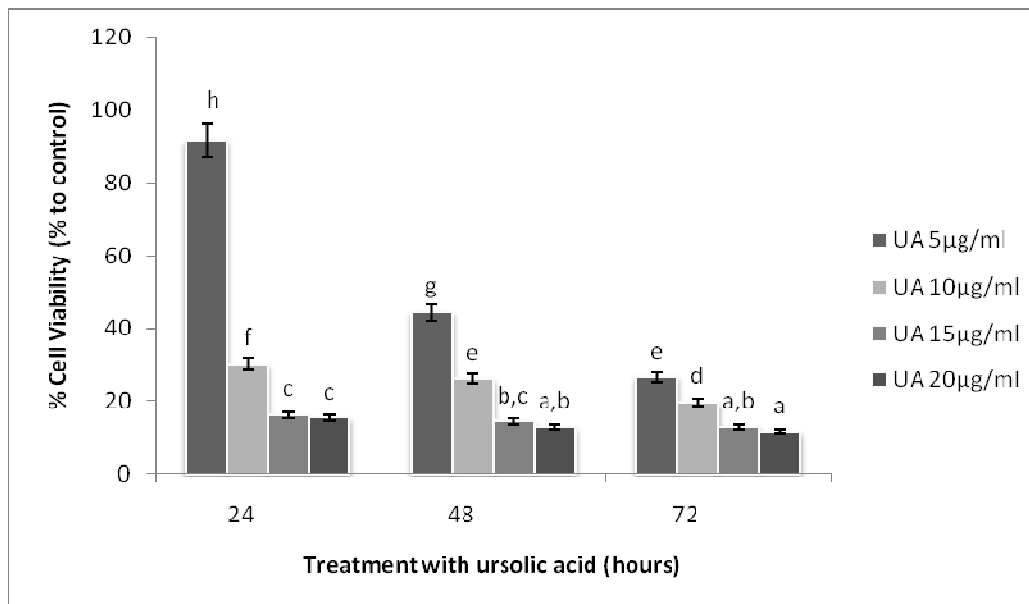
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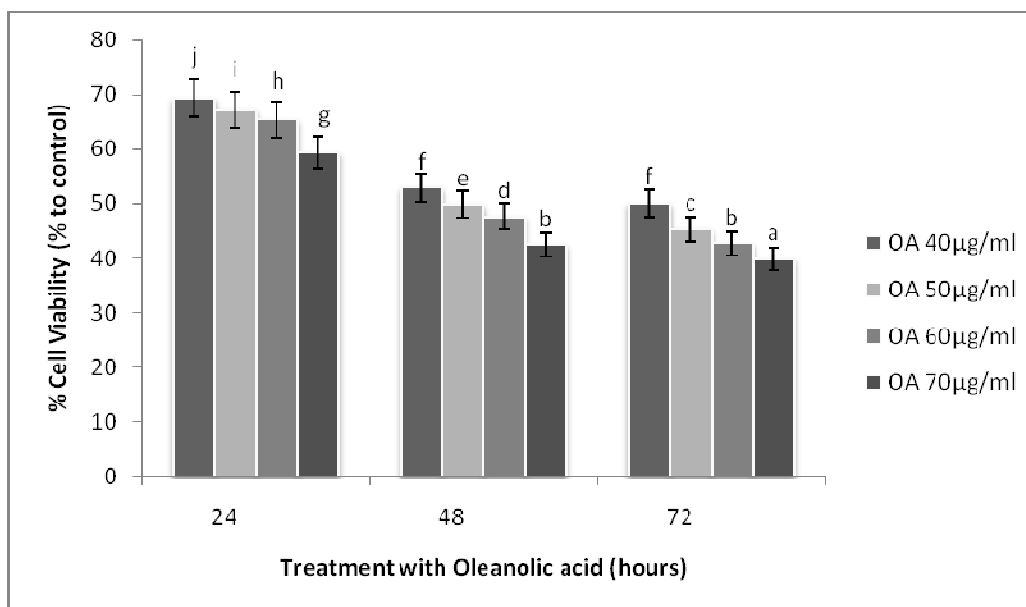
B

Figure 3. Effect of UA and OA on the proliferation of MCF-7 cells. UA inhibited the proliferation of MCF-7 cells in dose-and time-dependent manners, [a-h] different letters are significantly different (P<0.05) (A), OA inhibited the proliferation of MCF-7 cells in dose-and time-dependent manners [a-j] different letters are significantly different (P<0.05) (B). P<0.05 compared to control. Data are presented as mean±SD from three independent experiments.

lower antiproliferative effect. Shyu et al. (2010) indicated that OA and UA could inhibit the growth of HuH7 human hepatocellular carcinoma cells with IC_{50} 100 and 75 µM, respectively. UA and OA had antiproliferative effect on Jurkat cell line (T cell lymphoma) were suggest that UA is most effective than OA with IC $_{50}$ value: 75 and 150 µM

most effective than OA with IC $_{50}$ value: 75 and 150 µM. Presence of different substituents at different position of molecule alters the antiproliferative effect. Since both the compounds are region-isomers, the difference in their potency may be attributed to their structural arrangement of the substituents. It is methyl group at positions 19 and
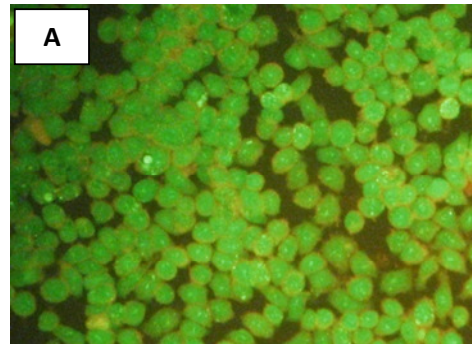
A. No treatment
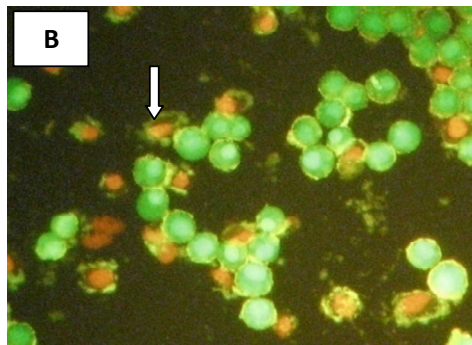
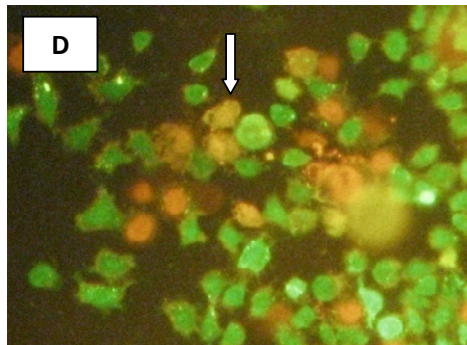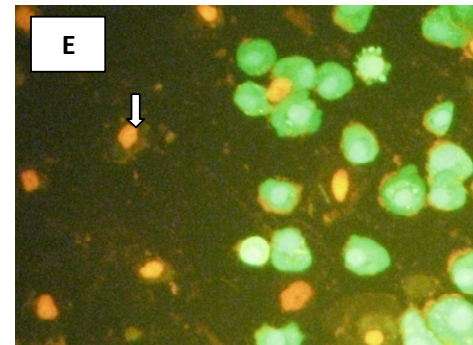EEFC 0.9 mg/ml

EEPC 0.9 mg/ml

UA 7 x 10^{-3} mg/ml
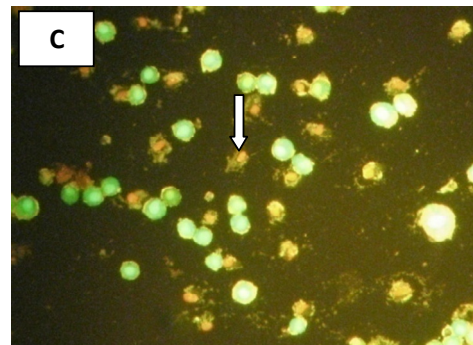
OA 7 x 10^{-2} mg/ml

Figure 4. Cellular and nuclear morphological changes of MCF-7 cells following exposure to various concentrations of EEFC, EEPC, UA and OA for 24 h. Cells were distinguished according to the fluorescence emission and the morphological aspect of chromatin condensation in 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 White arrows indicate apoptotic.

20, which makes a difference in potencies of these compounds (Senthil et al., 2007; Liu et al., 1995).

UA and OA may act as a radical scavenger. It shows that UA and OA may act as antioxidants. The ability of UA and OA as antiproliferation related to the ability of UA and OA as an antioxidant in reducing oxidative stress in cancer cells. Reducing of oxidative stress by the UA and OA possible mediated through the inhibition of DNA-binding activity of NF-kB. Decreased on the expression of NF-kB causes the inhibition proliferation and induced

apoptosis on cancer cells. OA and UA inhibit proliferation and induce apoptosis in HuH7 cells through decrease NF-kB, 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 (Shyu et al., 2010). Shisodia et al. (2005) reported decreased of expression of NF-kB caused inhibited proliferation lymphoma cells. Li et al. (2010) reported maslinic acid can inhibit proliferation and invasion of pancreatic cancer cells in dose dependent manner and through the inhibition of NF-KB. Maslinic acid also inhibit the expression of genes associated with cancer cell proliferation is cyclin D1, COX-2, Bcl-2 and Bcl-xl. Phytosterol which one of the bioactive compounds in *Coleus tuberosus* have antiproliferative activities in cancer cells. Treatment with phytosterol can inhibit proliferation, reduce cell cycle, induce apoptosis. Award et al. (2000) reported that treatment with β-sitosterol, especially at concentrations 16 μmol/ml can stimulate apoptosis six times higher than the control in breast cancer cells MDA-MB 231 and LNCap 4. Jayaprakasha et al. (2007) reported that treatment with β-sitosterol from the fruit of *Poncirus trifoliate* at concentration 0.63 μM for 48 h may inhibit proliferation and induced apoptosis in colon cancer HT-29. Mechanism of UA, OA, EEFC, EEPC, maslinic acid and phytosterol inhibits cancer cell proliferation and induced apoptosis possible through decreased the expression of NF-kB and its gene expression influenced by NF-kB, such as COX-2, Bcl-2, Bcl-xl.

Staining of apoptotic cells with fluorescent dyes such as Acridine orange (AO) and ethidium bromide (EB) is considered the correct method for evaluating the changed nuclear morphology as demonstrated in Figure 4. AO/EB are double staining cell morphological analysis AO is taken up by both viable and dead cells. 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. EB was excluded from living cells. 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).

Mechanism of UA and OA inhibit proliferation dose dependent manner by increasing DNA fragmentation in Huh7, HepG2 and Hep3B cell lines (Yan et al., 2010). UA and OA increasing activation of AIF (apoptosis inducing factor) that caused chromatin condensation and DNA fragmentation. DNA fragmentation in MCF-7 cells treated with UA, OA, EEFC and EEPC possible through the AIF, because cancer cells MCF-7 from ATCC do not express caspase-3 as an executor of apoptosis that causes DNA fragmentation (Janicke, 2009). It can be seen on the results of staining with AO and EB, which indicates the occurrence of apoptosis (Figure 4), which the characteristics of apoptosis were chromatin condensation

and fragmentation of DNA.), UA increased the expression of apoptosis inducing factor (AIF) in RC-58T/h/SA#4 cells and R-HepG2 cells (Kwon et al., 2010; Yang et al., 2010).

Analysis of apoptosis can also be performed using flow cytometry. Several studies have shown that treatment with UA, and OA, on cancer cells can cause apoptosis and characterized by cell cycle arrest. Treatment with UA lead to accumulation of cells in G0/G1 phase indicating the occurrence of apoptosis (Chen et al., 2010; Syu et al., 2010). Treatment with OA led to accumulation of cells in G0/G1 phase (Martin et al., 2007).

Conclusion of this research the ethanolic extract of peel and flesh *C. tuberosus* and its bioactive compounds (UA and OA) are able to inhibit proliferation in human breast cancer MCF-7 cells at a dose and time-dependent manner. Growth and proliferation inhibition is through induction of apoptosis. UA, OA and other bioactive compounds that presence in *C. tuberosus* may be partially responsible for the anticancer activities of whole *C. tuberosus.*

Abbreviations: EEFC, Ethanolic extract flesh of C. tuberosus; **EEPC,** ethanolic extract peel of *C. tuberosus*; **MTT,** 3-(4,5-dimethylthiazol-2)-2,5-diphenyltetrazolium bromide; **DMEM,** Dulbecco's modified eagle medium; **FBS,** fetal bovine serum; DPPH, 1,1-diphenyl-2-picryl hydrazyl radical; **AO/EB,** acridine orange/ethidium bromide; **MCF 7,** Michigan cancer foundation 7; **OA**, oleanolic acid; **UA,** ursolic acid.

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