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Potential of *Coleus tuberosus* as an antioxidant and cancer chemoprevention agent

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Abstract: *Coleus tuberosus* is classified as a local vegetable of the family *Lamiaceae*, the subfamily *Nepetoideae* and tribe *Ocimeae*. Ethanolic extract peel of *Coleus tuberosus* (EEPC) and ethanolic extract flesh of *Coleus tuberosus* (EEFC) were evaluated for their antioxidant activities by using ferric thiocyanate (FTC) and thiobarbituric acid (TBA) method. Cancer chemopreventive activities were evaluated by using human breast cancer MCF 7 cancer cells based on the MTT assay. The result showed that antioxidant activity of EEPC was higher than that of EEFC. Cancer chemoprevention of EEPC was higher than EEFC. The antioxidant and antiproliferative activities were in a dose-dependent manner. The antioxidant activities and cancer chemoprevention were related with oleanolic acid and ursolic acid content of both peel and flesh of *Coleus tuberosus*. These result indicated that EEPC and EEFC might be used as potential source of natural antioxidants and as cancer chemopreventive agents.

Keywords: Peel, flesh, *Coleus tuberosus*, antioxidant, cancer, chemopreventive

Introduction

Every day human cells are exposed to reactive oxygen species (ROS) of both endogenous and exogenous sources. When excessive amount of ROS is produced, a disturbance in pro-oxidat/antioxidant balance in favour of the pro-oxidant state may occur, which may lead to oxidative stress and cell damage (Ramos *et al*., 2008). To prevent cell damage, human cells need intake diets that contain antioxidant compounds such as polyphenols, phenolic acid, flavonoids and triterpenoids. These antioxidants are to prevent chronic complication in part through their interactions with ROS and ability to scavenge free radicals (Seifried *et al*., 2007).

ROS include free radicals such as superoxide anion radicals (O_2^{*}), hydroxyl radicals (OH^{*}) and non free-radical species such as H_2O_2 and singlet oxygen ($^1O_2$) (Manda *et al*., 2009). As a result increased levels of these ROS which has the potential to lead to DNA damage and subsequently promote the mutation that initiate to tumor progression (Waris and Ahsan, 2006). Human tumor cell lines *in vitro* have been shown to produce ROS at a greater rate than that of non-transformed cell lines.

Breast cancer is the most common malignancy in the world. Approximately one-third of the women with breast cancer developed metastases and ultimately died of the disease. In 1970, the estrogen receptor-positive MCF-7 cell line was derived from patient with metastatic breast cancer. Since then 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 preventive or treatment of number of major solid tumors, particularly metastatic and drug-resistant breast cancer (Spencer *et al*., 1994).

Coleus tuberosus is tuber vegetables of the family *Lamiceae*. One of the characteristics of bioactive compounds belonging to the family *Lamiaceae* mainly in members of the subfamily *Nepetoideae* is the presence of triterpenic acid. Two kinds of triterpenic acid in *Coleus tuberosus* were ursolic acid and oleanolic acid (Nugraheni *et al*., 2010). There is growing interest in the elucidation of the biological and pharmacologic roles of the plant-derived triterpenic acid compound in term of antioxidant (Yang *et al*., 2007), hepatoprotectory and analgesic (Fai and Tao, 2009), antitumor (Feng *et al*., 2008) and immunodulatory effect (Martin *et al*., 2007).

Investigation of *Coleus tuberosus* extracts, in mediating cancer cells and antioxidant are very limited. *Coleus tuberosus* shown strong *in vitro* anti-tumor promoting activity when assayed using Raji cells (Mooi *et al*., 1999). The content of bioactive compounds are mainly triterpenic acid with their functional ability as inhibition of activation of EBV early antigen in Raji cells which induced by

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tumour promoter phorbol 12-myristate 13-acetate (PMA) and sodium n-butyrate (Hsum *et al.*, 2008). Antioxidant activity of flavonoid preparation from Solenostemon rotundifolius enhanced activities of antioxidant enzyme in hyperlipidemic rats (Sandhya and Vijayalaksmi, 2000). The active anti-tumor promoting compounds identified are maslinic acid, stigmasterol, β-sitosterol and campesterol attributed to their ability as inhibition of the expression of EBV early-antigen in Raji cells (Mooi *et al*., 2010).

However, research on the determination triterpenic acid in different parts of *Coleus tuberosus* mainly in peel and flesh, antioxidant activity and potential as chemopreventive agent in human breast cancer has not been done. The objectives of this work were to investigate the antioxidant and cancer chemopreventive activities of ethanolic extract of flesh and peel of *Coleus tuberosus*.

Materials and Methods

Materials

Linoleic acid, 2-thiobarbituric acid, butylated hydroxytoluene (BHT), L-ascorbic acid, and ammonium thiocyanate were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Ursolic Acid was from Santa Cruz Biotech Inc., 3-(4,5-Dimethylthiazol-2)-2,5-diphenyltetrazolium bromide (MTT), oleanolic acid, Dulbecco's modified eagle medium (DMEM), were purchased from Sigma-Aldrich. Fetal bovine serum (FBS) was purchased from Gibco NY., MCF-7 was obtained from ATCC. Ethanol and acetic acid were of HPLC grade. All other reagents and solvents were of analytical reagent grade.

Preparation of crude extract

Coleus tuberosus obtained from a farmer in Bantul district, Yogyakarta Special Region, Indonesia at commercial maturity, 3 month. Peel (±1 mm) of *Coleus tuberosus* obtained by peeling using potato peeler. Peel and flesh of *Coleus tuberosus* were cut into small pieces and dried with a cabinet dryer at 40°C for 24 h, then ground into powders. Preparation of the crude extract (Mooi *et al*., 1999), sample was macerated with ethanol (1:5) at room temperature for a week. The crude extract were then filtered and dried with water bath and evaporated in a vacuum rotary evaporator at 45°C. The extract was stored at -20°C for further analysis. The ethanol peel and flesh from *Coleus tuberosus* were named EEPC (ethanol extract peel of *Coleus tuberosus*) and EEFC (ethanol extract flesh of *Coleus tuberosus*).

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

Identification of triterpenic acid in *Coleus tuberosus* was carried out as described by (Du and Chen (2009) with minor modification. HPLC analysis was performed on HPLC apparatus (Shimadzu Corporation, Kyoto Japan) equipped with an Eurospher 100-5 photodiode array detector. Separation was carried out at 30°C on Eurosphere 100-5 C-18 column (5 micron, 250x4, 6 mm). The mobile phase consisted of a mixture, MeOH : 0.15% CH3COOH (90:10), monitored by wavelenght 210 nm, and the flow rate was 1 ml/min. The extract was dissolve in 1 ml methanol, then filtered with millex filter 0.45 µM and injected into an HPLC. The samples and the standards were injected at a volume of 20 µl each. Ursolic acid and oleanolic acid 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 ursolic acid, oleanolic acid content in EEFC and EEPC based on standard curve of ursolic acid and oleanolic acid.

Antioxidant assays

Ferric thiocyanate (FTC) method

The standard method described by Zahin *et al.* (2009) was used. A mixture of (100-400 µg/ml) ethanolic extract of *Coleus tuberosus*, BHT, and L-ascorbic acid (10-40 µg/ml) in absolute ethanol, 4.1 ml of 2.5% linoleic acid in absolute ethanol, 8.0 ml of 0.05 M phosphate buffer (pH 7.0) and 3.9 ml of water was placed in a vial with a screw cap and then placed in an oven at 40°C in the dark. To 0.1 ml of this solution was added 9.7 ml of 75% ethanol and 0.1 ml of 30% ammonium thiocyanate. Three min after the addition of 0.1 ml of 0.02 M ferrous chloride in 3.5% HCl, the absorbance was measured at 500 nm, each 24 h until 7 day. The mixture without plant sample was used as the negative control. All measurements were carried out in triplicate. The inhibition of lipid peroxidation was calculated as follows:

$$\% \text{ Inhibition} = 100 - [(A_1/A_o) \times 100]$$

Where A_o is the absorbance of the control reaction and A_1 is the absorbance in the presence of the sample extracts.

Thiobarbituric acid (TBA) method

The TBA method was referred from Zahin *et al*. (2009). Two milliliters of 20% trichloroacetic acid

and 2 ml of 0.67% 2-thiobarbituric acid were added to 1 ml of sample solution, as prepared in FTC method. The mixture was placed in a boiling water bath and, after cooling, the mixture was centrifuged at 3000 g for 20 min. The absorbance of the supernatant was measured at 552 nm. Antioxidant activity was based on the absorbance on the final day of FTC method. All measurements were carried out in triplicate.

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 humidified 37°C, 5% CO_2 incubator.

Cell viability assay

An MTT assay was performed according to the method by Hogan *et al*. (2010) with slight modification. MCF-7 cells were plated into 96-well microtiter plates at a density of $1.5x10^5$/well in the final volume of 100 µl culture medium per well. The cells were treated with EEPC and EEFC (700, 800, 900 and 1000 µg/ml), ursolic acid (5, 10, 15 and 20 µg/ml) and oleanolic acid (40, 50, 60,and 70 µg/ml) and maintained at 37°C with CO_2 for 24 h. After the incubation period, 10 µl of MTT labeling reagent (5 mg/ml) was added to each well. The microtitter plate was then incubated again 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°C 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 wells containing cell culture medium without samples was calculated using A_{550nm}(sample)/A_{550nm} (control) x 100. The concentration of the extract causing 50% cell death known as the inhibition concentration (IC_{50}) was determined from a graph of percentage of cell death versus log concentration of samples.

Statistical analysis

The result were 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 was used to compare the mean values at $P<0.05$. A significant difference was considered to be $p<0.05$. SPSS Version 16.0 was used.

Result and Discussion

Determination ursolic acid and oleanolic acid

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

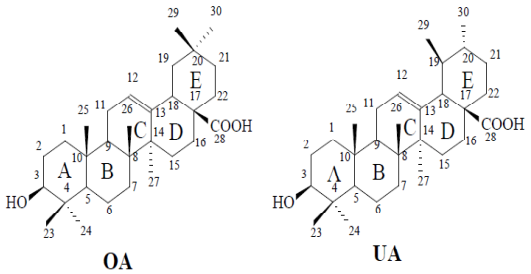

Figure 1. Chemical structure of oleanolic acid (OA) and ursolic acid (UA)

Antioxidant activity assay in linoleic acid emulsion by ferric thiocyanate (FTC) method

The ferric thiocyanate method measures the peroxide value at the initial of the lipid peroxidation, where ferric ion is formed upon reaction of peroxide with ferrous chloride. The ferric ion then unites with ammonium thiocyanate producing ferric thiocyanate, a red-coloured substance. The darker the colour, the higher is the absorbance.

The effect of various concentration of EEPC and EEFC on lipid peroxidation of linoleic acid emulsion are shown in Figure 2A and B, ursolic acid and oleanolic acid are shown in Figure 3 A and B. Overall, peroxide value were the highest in the control sample when compare to in the other samples at all time points. Although peroxide value increased with increasing storage time in all samples. Other have also reported increased peroxide value over time in products with or without antioxidant added. Increased peroxide value of duct meatballs treated with *Cosmos caudatus*, *Polygonum minus*, and BHT over 21 days of storage (Nurul *et al*., 2010). Increased peroxide value of water extract of selected Malaysian herbs (*Cosmos caudatus*, *Centella asiatica*, *Murraya koenigir*, *Polygonum minus*, *Oenanthe javanica*, and BHT over 7 days of storage and peroxide value were higher in the control (Huda-faujan *et al*., 2007).

Inhibition of lipid peroxidation of EEPC at the concentrations 100, 200 and 400 µg/ml were

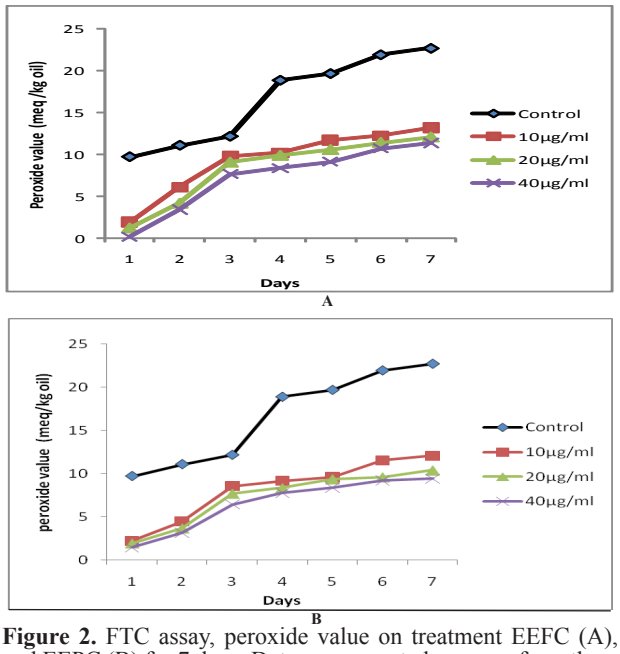

Figure 2. FTC assay, peroxide value on treatment EEFC (A), and EEPC (B) for 7 days. Data are presented as mean from three independent experiments

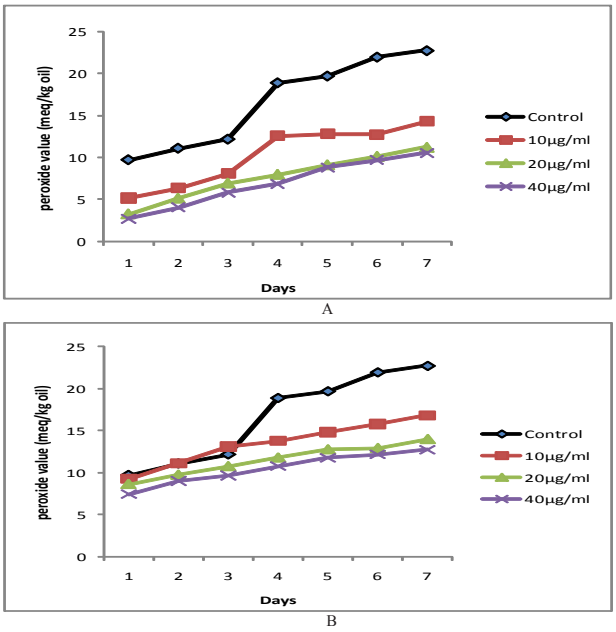

Figure 3. FTC assay, peroxide value on treatment ursolic acid (A), and oleanolic acid (B) for 7 days. Data are presented as mean from three independent experiments

36.74%, 42.51%, and 43.40%, respectively. EEFC at concentration 100, 200, and 400 µg/ml were found to be 36.90%, 36.80%, and 39.17%, respectively. On the other hand, inhibition on peroxidation of linoleic acid emulsion of BHT and L-ascorbic acid at concentration 10, 20, and 40 µg/ml: 50.82%, 52.85%, and 56.02%, respectively and 36.01%, 39.80%, and 44.43%, respectively (Figure 4).

The results clearly showed that EEPC had stronger antioxidant activity than EEFC, but lower than BHT and ascorbic acid. This result showed that the inhibition on peroxidation of linoleic acid emulsion was in a dose-dependent manner. The

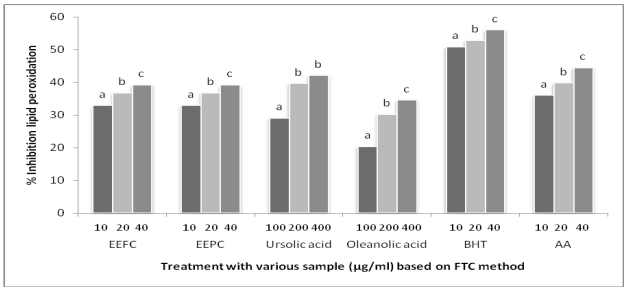

Figure 4. Percentage of inhibition of lipid peroxidation of different concentration of EEFC, EEPCT, ursolic acid, oleanolic acid, BHT and AA by FTC method. Data are presented as mean from three independent experiments. Means with different letters within the same sample are significantly different at level of $p < 0.05$

higher antioxidant activity of peel than flesh from *Coleus tuberosus* was related with the higher content of bioactive compound such as ursolic acid and oleanolic acid in the peel than the flesh.

Ursolic acid had stronger antioxidant activity than oleanolic acid, but lower than BHT and ascorbic acid. This result showed that the inhibition on peroxidation of linoleic acid emulsion a dose-dependent manner. Since both compounds are region-isomers, the differences in their potency may be attributed to their structural arrangement of the substituents. It is the methyl group at positions 19 and 20, which makes a difference in potencies of these compounds (Senthil *et al*., 2007).

Ursolic acid and oleanolic acid has a good ability to inhibit peroxidation of linoleic acid compared with standard antioxidant compound that is quercetin, AA, and BHT. UA and OA are likely to lipophilic so that the evaluation of antioxidants using linoleic acid, showed that UA and OA can inhibit the occurrence of lipid peroxidation. This is consistent with research Adhikari *et al*. (2006) which reported that the ursolic acid and oleanolic acid and other triterpenoids is an efficient protector of lipid peroxidation.

OA and UA significantly retarded oil oxidation in sunflower oil. UA and OA, examined at a concentration 0.05% w/w oil, exhibit significant antioxidant activity, with UA presenting higher antioxidant activity than OA (Assimopoulou *et al*., 2005). OA and UA have protective effect against iso proterenol-induced myocardial ischemia in rats. This activity is related with the capability of OA and UA to block the induction of lipid peroxidation. Presence of different substituent at different position of molecule alters the antioxidant potency. Since both compounds were region-isomers, the difference in their potencies may be attributed to their arrangement of the substituents. It is the methyl group at position 19 and 20, which makes a difference in potencies of these compounds (Senthil *et al*., 2007).

The higher antioxidant activity on EEPC than

EEFC agreed with related researches that investigated and compared antioxidant activities in the peel and the flesh of fruits and vegetables. Extract of the peel of red pitaya have greater antioxidant activities than the flesh. (Wu *et al.*, 2006). The peel of apple has significantly higher antioxidant activities than the flesh (Wolfe *et al*., 2003). The mango peel extract exhibited stronger antioxidant activity than mango flesh extract (Kim *et al*., 2010). Difference antioxidant activity in peel and flesh from related researches associated with bioactive compounds content.

Coleus tuberosus contains triterpenic acid (maslinic acid), phytosterol (stigmasterol, β-sitosterol dan campesterol) Mooi *et al*. (2010). Bai *et al.* (2007) states that treatment using some of root extract of *A. deliciosa* rich in oleanolic acid on rat liver injury by CCl_4 suppress the occurrence of lipid peroxidation by decrasing numbers of peroxide. Buthanol extracts which has the highest OA levels have the greatest inhibitory capacity compared with other solvent extracts.

The content of secondary metabolites such as maslinic acid, and phytosterol have an ability to inhibit the occurrence of lipid peroxidation. UA, OA and maslinic acid included in the triterpenoid compounds, which in the study Adhikari *et al*. (2006) sshows that the triterpenoid is an efficient protector against lipid peroxidation. Ferreti *et al*. (2010) reported that phytosterol (β-sitosterol, stigmasterol and campesterol) with various concentration (5-50 μM) can inhibit LDL lipid peroxidation induced by Copper.

Ethanol extract of peel of *Coleus tuberosus* can inhibit lipid peroxidation by decreasing the peroxide value greater than the ethanol extract of flesh of *Coleus tuberosus*. This capability is related to the content of triterpenic acid on the peel is greater than the flesh (Table 1) and other bioactive compounds. A difference in the ability of antioxidants is related to differences in content of bioactive compounds found in the peel and the flesh. Research by Cetkovic *et al*. (2007) reported that the extracts n-buthanol *Satureja montana* L. subsp. *Kitaibelii* can inhibit lipid peroxidation in sunflower oil which dinduksi 4,4 '-azobis (4-cyanovaleric acid) and ACVA-induced liposome 2,2'-azobis (2-amidino-propane) dihydrochloride) AAPH. Inhibition of n-buthanol extract is higher than inhibition by ethyl acetate extract; this is related to differences in content of bioactive compounds, especially phenol compounds. Levels of phenol compounds in n-buthanol 1358.14 μg/g while the ethyl acetate 969.43 μg/g. This is correlated with differences in content of bioactive compounds (phenol), ethyl acetate extract of 0127 μg/mg of GAE and chloroform 0089 μg/mg GAE.

Antioxidant activity determination in linoleic acid emulsion by thiobarbituric acid (TBA) test

FTC is used to measure the production of peroxide compounds at the initial stage of oxidation while TBA test is used to measure the secondary product of oxidation such as aldehyde and ketone (Huda-faujan *et al*., 2007). The TBA test is a colorimetric technique in which the absorbance of the red chromogen formed between TBA and malondialdehyde is measured (Hanachi, 2009). The first step in lipid oxidation is the abstraction of a hydrogen atom from a fatty acid and subsequent oxygen involvement gives a peroxy radical. Peroxide then gradually decomposes to lower molecular compound; mainly malondialdehyde during oxidation process and malondialdehyde can be determined by TBA method. At low pH and high temperature, malondialdehyde binds TBA to form a red complex that can be measured at 532 nm. Generally, antioxidants suppress the hydrogen atom abstraction from fatty acid, which leads to the decrease of hydroperoxide formation. It is well known that phenolic compound, triterpenic acid acts as hydrogen donors in that reaction mixture and therefore, the formation of hydroperoxides were decreased.

The control sample (no antioxidant addition) had higher TBA level than the sample that were subjected to treatment with EEPC and EEFC, BHT, and L-ascorbic acid. Figure 5 shows the percentage of inhibition of lipid peroxidation of EEPC, EEFC, UA, OA, BHT and ascorbic acid. All samples exhibit a dose-dependent antioxidant activity. It shows that the antioxidant activity of EEPC was greater than EEFC. UA had stronger antioxidant activity than OA. The antioxidant activity in these studies increased, as concentrations of the extract samples increased. (Nagai *et al*., 2003). These results were in concordant with related research that investigated and compared the antioxidant activities in peel and flesh of fruits and vegetables. Nara *et al*. (2006) reported that the free and bound phenolics in the peel showed high antioxidant activity, while those in the flesh showed low activity. The apple peel extract exhibited stronger antioxidant activity than apple flesh extracts from growing regions in Chile (Yuri *et al*., 2009).

Inhibition of lipid peroxidation of EEPC at the concentrations of 100, 200 and 400 μg/ml were 35.05%, 40.71% and 43.40%, respectively. EEFC at the concentrations of 100, 200, and 400 μg/ml were found to be 30.78%, 34.99%, and 36.92%, respectively. On the other hand, inhibition on peroxidation of linoleic acid emulsion of BHT at the concentrations of 10, 20, and 40 μg/ml were 48.13%,

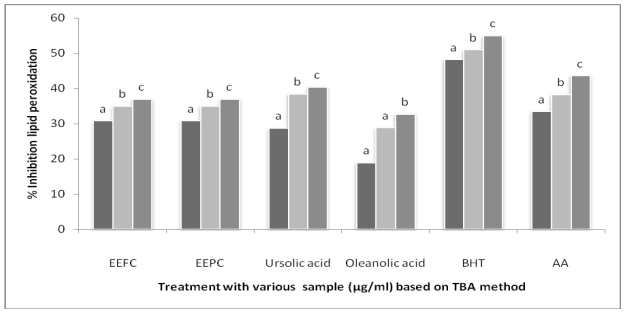

Figure 5. Percentage of Inhibition of lipid peroxidation of different concentration of EEFC, EEPC, ursolic acid, oleanolic acid, BHT and AA by TBA method. Data are presented as mean from three independent experiments. Means with different letters within the same sample are significantly different at level of $p < 0.05$

50.93%, and 54.91%, respectively. The inhibition on peroxidation of linoleic acid emulsion of L-ascorbic acid at the concentrations of 10, 20 and 40 µg/ml were 33.41%, 38.20%, and 43.57%, respectively (Figure 5). The result of this study confirm that EEPC, EEFC and its bioactive compounds (UA and OA) can significantly delay lipid oxidation and reduce the risk presented by lipid peroxidation products. Antioxidant activity related with presence of the ursolic acid and oleanolic acid in the peel and flesh of ethanolic extract of *Coleus tuberosus*.

Ethanol extract of flesh and peel of *Coleus tuberosus* can inhibit lipid peroxidation. The ability is related to the content of bioactive compounds contained in the ethanol extract. Sadasivan *et al.* (2006) reported that methanol extract of *Hedyotis corymbosa* (L.), which contain oleanolic acid, ursolic acid and sitosterol can significantly reduce the MDA in rat liver induced by $FeCl_2$-AA. This extract can reduce the accumulation of lipid peroxidation *in vitro* and depend on the concentration used, where at 25 µg/ml and 50 µg/ml concentrations can show the inhibition of 72.25% and 80%.

Coleus tuberosus besides containing oleanolic acid, and ursolic acid, according to Mooi *et al*. (2010) contains maslinic acid and phytosterol. The content of secondary metabolites such as maslinic acid, phytosterol have an ability to inhibit the occurrence of lipid peroxidation. Other secondary metabolites in *Coleus tuberosus* such as beta-sitosterol contribute to the ability of ethanolic extracts of flesh and peel of *Coleus tuberosus* in inhibiting lipid peroxidation. Shanmugarajan *et al*. (2008) stated that methanol extract of *Ficus hispida* Linn. rich in oleanolic acid and β-sitosterol has a cardioprotective effect of cyclophosphamide in mice induced by lowering the level of MDA. Oleanolic acid and β-sitosterol has the ability sebagaai reported anti-lipid peroxidation. Research Shanmugarajan and Devaki, 2008 reported that methanolic extract of leaves of *Ficus hispida* Linn containing oleanolic acid, β-sitosterol, hispidin can inhibit the activation of lipid peroxidation

Ethanolic extract of peel of *Coleus tuberosus* has the ability to suppress lipid peroxidation inhibition of MDA formation is greater than the ethanolic extract of flesh of *Coleus tuberosus*. This is related to the content of triterpenic acid on the peel than the flesh one (Table 1). Differences in antioxidant capability is related to differences in content of bioactive compounds found in the skin and the flesh. Azrina *et al*. (2010) demonstrated that the methanol extract of the fruit peel of *Canarium odontophyllum* have the greater ability to inhibit lipid peroxidation is than the parts of meat, (47.9 ± 0.00% and 11.61 ± 1.14%). These differences are associated with higher levels of bioactive compounds in the peel compared to the flesh (387.5 ± 33.23 mg and 267.0 ± GAE/100 g GAE/100 g 4:24 mg.)

Inhibition of human breast cancer cells proliferation

The anti-tumor promoting properties of *Coleus tuberosus* have been widely reported by various research group, but antiproliferative activities of *Coleus tuberosus* on cancer cells has not been done. Significant anti-tumor promoting activities displayed by *Coleus tuberosus* suggested its potential to be developed as chemoprevention. Ethanolic extract of *Coleus tuberosus* (6.25-200 µg/ml) had strong inhibition activity toward Eipstein Bar Virus (EBV) activation in Raji cells induced by phorbol 12-myristate 13-acetate and sodium-n-butirate by dose-dependent manner (Mooi *et al*., 1999). Ethanolic extract of *Coleus tuberosus* (12.5-400 µg/ml) were able to suppress both early antigen diffuse (EA-D) and early antigen restricted (EA-R) by performing western blotting of treated Raji cells with human sera of nasopharyngeal carcinoma patients by dose-dependent manner (Ali *et al*., 2000). Other related research also showed that antiproliferative activity or cytotoxicity were in a dose-dependent manner (Rahmat *et al.*, 2003; Huang *et al*., 2005; Yuan and Walsh, 2006).

This study measured the amount of cell viability with the micro-culture Tetrazolium Salt (MTT) assay. The effect of EEPC and EEFC on the proliferation of human breast cancer cells was determined. The percentage of cell viability was measured by comparing the optical density (OD) against the control. The antiproliferative activity of EEPC and EEFC presents as a percentage of the cell viability versus concentration. Cells grown in 96-well tissue culture plate were incubated with yellow MTT solution for approximately 4 h. After this incubation period, purple formazan salt crystals were formed. These salt crystal were insoluble in aqueous solution,

but may be solubilised by adding the solubilisation solution and incubating the plates overnight in a humidified atmosphere (37°C, 5% CO_2). The solubilised formazan product was quantified spectrophotometrically using an ELISA Reader. An increase in number of living cells resulted in an increase in the total metabolic activity in EEPC and EEFC sample. This increase directly correlated with the amount of the purple formazan crystal formed, as monitored by the absorbance.

The antiproliferative activity of ethanolic extract of peel and flesh of *Coleus tuberosus* toward growth of MCF 7 human breast cancer cells *in vitro* are presented in Figure 6. The ethanolic extract of peel of *Coleus tuberosus* showed relatively high antiproliferative activities toward MCF 7 cells in a dose-dependent manner. The antiproliferative activity of EEPC and EEFC expressed as the median effective dose (EC_{50}), with a lower EC_{50} value signifying a higher antiproliferative activity. The ethanolic extract of peel of Coleus tuberosus exhibited the highest antiproliferative effect (P<0.05) toward MCF 7 with the lowest EC_{50} : 1124.11 ± 35.19 µg/ml, followed by ethanolic extract of flesh of *Coleus tuberosus* 1512.97 ± 50 µg/ml. Bioactive compounds, ursolic acid has EC_{50} = 9.02 ± 0.12 , oleanolic acid EC_{50} 136.27 ± 7.19. These results showed that EEPC had the antiproliferative effect higher than EEFC at all concentrations which corresponds with its lower IC_{50}. Its bioactive compound, ursolic acid had antiproliferative effect higher than oleanolic acid.

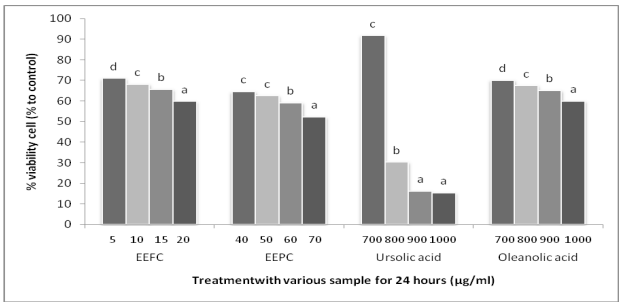

Figure 6. Percentage of viability cells treatment with EEFC, EEPC, ursolic acid and oleanolic acid at variation of concentration, incubation 24 hours. Data are presented as mean from three independent experiments. Means with different letters within the same sample are significantly different at level of $p < 0.05$

Ursolic acid and oleanolic acid as bioactive compounds in *Coleus tuberosus* had strong inhibitory the proliferation of MCF-7 cells in dose-dependent manner compare with oleanolic acid, which corresponds its lower $IC_{50.}$ Previous studies showed that ursolic acid had strong inhibitory the proliferation and induce apoptosis in cancer cells lines (Shan *et al*., 2009), but oleanolic acid, which the isomer of ursolic acid have lower antiproliferative effect. Shyu *et al.* (2010) indicated that oleanolic acid and ursolic acid could inhibit the growth of HuH7 human hepatocellular carcinoma cells with IC_{50} 100 µM and 75 µM, respectively. Ursolic acid and oleanolic acid had antiproliferative effect on Jurkat cell line (T cell lymphoma) were suggest that ursolic acid and oleanolic acid have significant anti-tumor activity. Ursolic acid is most effective than oleanolic acid with IC_{50} value : 75 µM and 150 µM.

The inhibition of cancer cell proliferation by ethanolic extract of peel and flesh of *Coleus tuberosus* can be partially explained by the triterpenic acid content mainly oleanolic acid and ursolic acid. Oleanolic acid and ursolic acid was responsible for their antiproliferative activities. Presence of different substituents at different position of molecule alters the cancer chemopreventive potency (Sun *et al*., 2006). Neto (2007) reported that ursolic acid in the peel of cranberry fruits and apple was responsible for their antiproliferative activity and capability to inhibit growth of several leukemia cell lines and A.549 human lung carcinoma.

He and Liu (2007) reported that that ursolic acid, one of thirteen triterpenoid isolated from apple peels show high potential anticancer activities against human HepG2 liver cancer cells, MCF-7 breast cancer cells, and Caco-2 colon cancer cells. These results showed ursolic acid isolated from apple peels have potent antiproliferative activity and may be partially responsible for the anticancer activity of whole apples. Ursolic acid and oleanolic acid are ubiquitous triterpenoids in plant kingdom, medicinal herbs, and are integral part of the human diet. OA and UA have similar chemical structures but differ only in the position of the methyl group in ring E. OA has two methyl groups at its C-20 position while UA has a respective methyl group at C-19 and C-20 position. (Feng *et al*., 2009). OA and UA possesses as anticancer in many cancer lines (Zhang *et al*., 2007; Zhang *et al*., 2007)

Ethanol extract of flesh and peel of *Coleus tuberosus* can inhibit proliferation in MCF-7 cell dose and time dependent manner. This ability related to the content of bioactive compounds, such as ursolic acid and oleanolic acid on flesh and peel of *Coleus tuberosus*. Zhang *et al*. (2007) that treatment of extract *Fructus Ligustri Lucidi* (FLL) rich oleanolic acid and ursolic acid inhibit the proliferation of leukemia cells HL-60. FLL extract inhibited the growth of dose and time dependent manner. Changes in morphology indicate the occurrence of DNA fragmentation indicating apoptotic cell at concentration of 20 mg/ml.

In addition to UA and OA, ethanolic extract of flesh and peel of Coleus tuberosus have other bioactive

compounds such as maslinic acid, phytosterol: stigmasterol, β-sitosterol and campesterol (Mooi *et al*., 2010). Maslinic acid which is included in the bioactive compounds contained in Coleus tuberosus has an ability as anti-tumor by inhibiting nuclear factor-k B. Li *et al*. (2010) explains that the treatment with maslinic acid on pancreatic cancer cells can inhibit proliferation and invasion depending on concentration and treatment time (dose and time dependent manner) 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. Reyes-zurita *et al*. (2009) reported that acid treatment of *Olea europaea* L. which content maslinic acid can inhibit proliferation and induce apoptosis in colon cancer cells HT-29 dose dependent manner.

Phytosterol was reported to possessed the ability as an anti-cancer (Bardford and Award, 2007). Treatment with phitosterol can inhibit tumor growth, reducing cell cycle progression, induces apoptosis and inhibition of tumor metastasis. Jayaprakasha *et al.* (2007) reported that treatment of HT29 colon cancer with beta-sitosterol from the fruit of *Poncirus trifoliate* at concentration of 0.63 μM for 48 h to inhibit proliferation and induce apoptosis. Similar research that evaluated and compare antiproliferative activity in different part of vegetables and fruits showed that peel extract of vegetables and fruits have higher antiproliferative activity than the flesh. Peel extract of mango (*Mangifera indica* L.) exhibited significant antiproliferative effect in human hepatoma cell line, HepG2, compares to that of the flesh extract, in a dose-dependent manner (Kim *et al*., 2010). Apple peels were also shown to more effectively inhibit the growth of HepG2 human liver cancer cells than the other apple component (Wolfe *et al*., 2003). The antiproliferative study on B16F10 melanoma cells revealed that the peel component was a stronger inhibitor of the growth of B16F10 melanoma cancer cells than the flesh (Wu *et al*., 2006). This activity associated with different content of the bioactive compounds in peel and flesh. This study showed that the growth of the MCF-7 cells was inhibited with EEPC, EEFC and its bioactive compounds (ursolic acid and oleanolic acid).

Conclusion

The result from this study showed the level of natural antioxidant and cancer chemopreventive on ethanolic extract of peel and flesh *Coleus tuberosus*. Ethanolic extract of peel of *Coleus tuberosus* showed higher antioxidant activities and antiproliferatives activities than ethanolic extract of flesh of *Coleus tuberosus* in a dose-dependent manner. Ursolic acid and oleanolic acid that presence in *Coleus tuberosus* may be partially responsible for the antioxidant and cancer chemopreventive agent of *Coleus tuberosus*.

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