RESEARCH ARTICLE

Antioxidant and Antiproliferative Activities of an Ethylacetate Fraction of Picria *Fel-Terrae* Lour. Herbs

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Abstract

Background: Excessive production of oxygen free radicals and imbalance in the mechanisms responsible for antioxidant protection may result in the onset of many diseases including breast cancer. Objective: To evaluate antiproliferative and antioxidant activity of an ethylacetate fraction (EAF) of Picria fel-terrae Lour. Herbs in the T47D cell line. Methods: Phenolic and total flavonoid contents in EAF were determined. EAF was tested for cytotoxicity and effects on the cell cycle and apoptosis, as well as antioxidant activity. Results: EAF was found to contain high levels of phenolic agents (92.88 ± 0.50 mg GAE/g), total flavonoid (84.39 ± 0.07 mg QE/g). The EAF of Picria fel-terrae Lour. herbs was found to have an IC50 of 62.98µg/mL, caused accumulation in G0-G1 and S phase and increased early and late apoptosis. Antioxidant activity in DPPH assays gave an IC50 of 166.90 ± 0.10 µg/mL. Conclusions: The results reveal that EAF of Picria fel-terrae Lour. herbs has antiproliferative activity and strong antioxidant potential. Further studies are now needed to isolate the responsible antiproliferative and antioxidant components.

Keywords: Antiproliferative- antioxidant- *picria fel-terrae* lour- ethylacetate fraction

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Introduction

Oxidation is an important process in living organisms. Free radicals arising from metabolism or enviromental sources interact continously with biological system. Reactive species are molecules or atoms that have an electronic instability and highly reactive. The uncontrolled production of oxygen free radicals and the unbalanced mechanism of antioxidant protection results in the onset of many diseases, such as cancer, diabetes, Alzheimer's, heart diseases and aging (Jamuna, et el., 2012; Nagmoti, et al., 2012; Rosidah, et al., 2008; Yang, et al., 2004).

The world health organization (WHO) reported that breast cancer is one of the leading causeof death and the most common cancer type amongst women worldwide in 2012 (WHO, 2015). Moreover,breast cancer ranks as the fifth cause of death from cancer overall (522,000 deaths), is themost frequent cause of cancer death in women in less developed countries (324,000 deaths,14.3% of total), and the second cause of cancer death in developed countries (198,000 deaths,15.4%) after lung cancer. A recent study reported that breast cancer is leading in the estimated new cancer cases, and the second most common death cause of women suffering from cancer in the USA (Siegel, et. al., 2015). Therefore, research and development in cancer detection and therapy isurgently required to solve those problems.

Breast cancer occurs when breast cells start to grow uncontrollably. These cells can invade nearby tissues and spread throughout the body. Each type of tissue in the breast can form a cancer, but the cancer usually arises in the milk ducts or glands. Factors that influence the risk of breast cancer are the length of exposure to hormones (e.g. menstruation at an early age or late menopause), reproductive factors (e.g. no children and first pregnancy at an advanced age), dietary factors and lack of physical activity (e.g. obesity and dietary fat), radiation during breast development, hormone replacement therapy on chronic use, as well as congenital genetic factors associated with breast cancer like the presence of gene mutations (Barnett, et. al., 2008).

Poguntano (Picria fel-terrae Lour.)have been used as drug of colic, malaria, diuretic, fever, and skin disease (Perry, 1980). Modern pharmacological investigations indicated that the extract of Picria fel-terrae Lour. exerts diuretic, antypiretic, hepatoprotective, cardioprotective, antidiabetic, antioxidant, anti-inflammatory, anthelmintic, and analgesic activities (Dalimunthe, et al., 2015; Huang, et al., 1994; Thuan, et al., 2007; Zhong, et al., 1979; Zou, et al., 2005; Harfina, et al., 2012; Sitorus, et al., 2014; Sihotang, et al., 2016; Patilaya and Husori, 2015). Moreover, Picria fel-terrae inhibits hepatitis B (HB) e-antigen excreted by HepG2 2215 cell lines, suggesting to have anti-HB virus activity (Zheng, et al., 2010). It can

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be developed as co-chemotherapeutic regimen for breast cancer by inducing apoptosis and cell cycle arrest and suppressing cyclin D1 and Bcl-2 expression based on the recent studies (Satria, et al., 2015; Lestari, et al., 2013). The aim of this study was to determine total phenolic content, antioxidant and antiproliferative activities of ethylacetate fraction of Picria fel-terrae Lour. Herbs.

Materials and Methods

Plant and chemicals material

Fresh herbs of Picria fel-terrae Lour. was collected from Tiga Lingga village, Dairi regency, Sumatera Utara province, Indonesia. Picria fel-terrae Lour. was identified in Research Centre for Biology, Indonesian Institute of Science, Bogor, and the voucher specimen was deposited in herbarium. Chemicals used were AlCl3.6H2O (Merck), annexin-V (BioLegend), distilled water, DMSO (Sigma), 1,1-diphenyl-2-picrylhydrazyl (DPPH) (Sigma), [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide] (MTT) (Sigma), folin ciocalteu, propidium iodide kit (BioLegend), quercetin (Sigma), sodium acetate (Merck), sodium bicarbonate (Merck).

Preparation of Ethylacetate Fraction (EAF)

The air-dried and powdered herbs of Picria fel-terrae Lour. (1 kg) were repeatedly fractionated by cold maceration with n-hexane (3x3 d, 7.5 L). The powder was dried in the air and fractionated with ethylacetate (3x3 d, 7.5 L) at room temperature with occasional stirring. The filtrate was collected, and then evaporated under reduced pressure to give a viscous fraction and then freeze dried to dry (Satria, et al., 2015; Anggraeni, et al., 2015; Hasibuan, et al., 2015).

Determination of Total Phenol Concentration

The total phenol concentration (TPC) of the sample was determined using folin reagent. Briefly, 100 µL of EAF (500 µg/ml) were mixed with 7.9 mL of distilled water and 0.5 mL of folin-ciocaleu's reagent (1:10 v/v) and mixed with vortex for 1 minute. After mixing, 1.5 mL of 20% aqueous sodium bicarbonate were added, and the mixture was allowed to stand for 90 min within termittent shaking. The absorbance was measured at 775 nm using a spectrophotometer. Total phenolic concentration is expressed as gallic acid equivalent in mg per gram of extract. The methanol solution was use a blank. All assays were carried out in triplicate (Rosidah, et al., 2008 and Jamuna, et al., 2012). The equation to determine total phenolic concentration:

$$C\ (GAE) = \frac{c\ x\ V}{M}\ x\ F$$

C (GAE): Concentration of phenolic as gallic acid equivalent; c: concentration determined from standard curve (µg/mL); V: volume which used in the assay (mL); M: mass of the sample which used in the assay (g); and F: dilution factor.

Determination of Total Flavonoid Concentration

The amount of total flavonoids in the extracts was measured spectrophotometrically as previously reported. Briefly, 2 mL of EAF in methanol was mixed with 0.10 mL of 10% aluminium chloride (AlCl3.6H2O), 0.10 mL of sodium acetate (NaC2H3O2.3H2O) (1 M) and 2.80 mL of distilled water. After incubation of 40 min, absorbance was measured at 432 nm using a spectrophotometer. To calculate the concentration of flavonoids, we prepared a calibration curve using quercetin as standard. The flavonoid concentrasion is expressed as quercetin equivalents in mg per gram of extract. All assays were carried out in triplicate (Diab, et al., 2015 and Jamuna, et al., 2012). The equation to determine total flavonoid concentration:

$$C\ (QE) = \frac{c\ x\ V}{M}\ x\ F$$

C (QE): Concentration of flavonoid as quercetin equivalent; c: concentration determined from standard curve (µg/mL); V: volume which used in the assay (mL); M: mass of the sample which used in the assay (g); and F: dilution factor.

Cytotoxicity assay

The cells were treated with EAF and doxorubicin. In this test, T47D cell line wasgrown in RPMI 1,640 medium, medium containing 10% Fetal Bovine Serum (Gibco), 1% penicillin-streptomycine (Gibco), and fungizone 0.5% (Gibco) in a flask in a humidified atmosphere (5% CO_2) at 37°C. The inoculums seeded at $1x10^4$ cells/mL at an optimal volume of 0.1 mL per well. After 24 h incubation, the medium was discharged and treated by EAF and doxorubicin. After incubation 24 h, the cells were incubated with 0.5 mg/mL MTT for 4 h in 37°C. Viable cells reacted with MTT to produce purple formazan crystals. After 4 h, SDS 10% as stopper (Sigma) in 0.01N HCl (Merck) was added to dissolve the formazan crystals. The cells were incubated for 24 h in room temperature and protected from light. After incubation, the cells were shaken, and absorbance was measured using ELISA reader at λ 595 nm. The data which were absorbed from each well were converted to percentage of viable cells (Hasibuan, et al., 2015; Satria, et al., 2014).

The equation to determine viability of cells

$$Viability = \frac{Abs\ of\ treatment - Abs\ of\ medium}{Abs\ of\ control\ cells - Abs\ of\ medium}\ x\ 100\%$$

Preparation of Cells for Flowcytometry Analysis

T47D cells ($5x10^5$ cells/well) were seeded into 6-well plate and incubated for 24 h. After that, the cells were treated with EAF and then incubated for 24 h. Both floating and adherent cells were collected in conical tube using tripsin 0.025%. The cells were washed thrice with cold PBS and centrifuged 2500 rpm for 5 min. The supernatant was separated, while the sediment was collected (Satria, et al., 2015; Anggraeni, et al., 2015).

Cell Cycle Analysis

Cells were fixed in cold 70% ethanol in PBS at -20°C for 2 h. The cells were washed thrice with cold PBS and resuspended then centrifuged at 3,000 rpm for 3 min and PI kit (containing PI 40 µg/mL and RNAse 100 µg/mL) added to sediment and resuspended and incubated at 37°C

for 30 min. The samples were analysed using FACScan flowcytometer. Based on DNA content, percentage of cells in each of stage in cell cycle (G1, S and G2/M) were calculated using ModFit Lt. 3.0.s.

Apoptosis Analysis

Annexin V kit was added to sediment and suspended and incubated at 37°C for 30 min. The samples were analyzed using FACScan flowcytometer.

Free Radical Scavenging Activity Test

The free radical scavenging activity was measured by 1,1-diphenyl-2-picrylhydrazyl (DPPH•) method of Blois (1958). 0.2mM solution of DPPH• in methanol was prepared and 100µl of this solution wasadded to various concentrations of EAF at the concentrations of 50, 100, 200 and 400 µg/ml. After 60 minutes, absorbance was measured at 516 nm. Quercetine was used as the reference material. All thetests were performed in triplicate and percentage of inhibition wascalculated by comparing the absorbance values of the control andtest samples (Rosidah, et al., 2008 and Jamuna, et al., 2012).

$$Percentage\ of\ inhibition = \frac{Abs\ control - Abs\ test}{Abs\ control}\ x\ 100\%$$

Statistical analysis

Data was expressed as mean ± SD. Analysis of variance (ANOVA) with the Tukey post hoc test was used for multiple comparison. All statistics were analyzed using the SPSS 20 software.

Results

Total Phenolic and Total Flavonoid Contents

Total phenolic content (TPC) was determined according to the Folin Ciocalteau method which is based in the reduction of phosphomolybdic-phosphotungstic acid (Folin) reagentto a blue-colored complex in an alkaline solution (Cicco, et al., 2009). The EAF of Picria fel-terrae Lour. herbs were found to contain high levels of phenolic content 92.88 ± 0.50 mg GAE/g. Phenolic compounds are known as powerful antioxidant (Shahidi and Wansundeara, 1992) and they are very important plant consistuents because of their free radial scavenging ability due to their hydroxyl groups (Hatano, et al., 1989). The solubility of phenolics is governed by their chemical nature which may vary from simple to highly polimerized substance in different quantities, as well as the polarity of the solvent used (Dai and Mumper, 2010; Diab, et al., 2016). Structurally, phenolic compounds have at least one aromatic ring with one or more hydroxyl groups and divided into 10 different classes depending on their basic chemical structure (Heim, et al., 2002).

In the case of total flavonoid contnet (TFC), the EAF was given flavonoid content 84.39 ± 0.07 mg QE/g. Flavonoids are a group of polyphenolic compounds, which exhibit several biological effects such as anti-inflammatory, anti-hepatotoxic, anti-ulcer, anti-allergic, anti-viral and anti-cancer activities (Umamaheswari, et al., 2008). They are capable of effectively scavenging the reactive oxygen species because of their phenolic hydroxyl groups and so they are potent antioxidant salso (Cao, et al., 1997).

Inhibitory Concentration 50% (IC50)

MTT method was used to determine cell viability after incubation for 24 h. In every treatment EAF and doxorubicin was shown to inhibit cells growth. The IC50 value of EAF was 62.98 µg/mL and doxorubicin 0.203 µg/mL.The cytotoxicity estimate of natural product is related to content of active compound in these plants including Picria fel-terrae Lour. Flavonoids and triterpenoids/steroids estimated as active compounds (Yadav, et al., 2012). Doxorubicin is one of chemotherapeutic agent showed strong activity on T47D cell lines with IC50 value of 0.203 µg/mL. T47D cells line underwent resistant to doxorubicin pass through to p53 mutation (Di Leo, et al., 2007; Vassade, et al., 2005).

Discussion

Effect on Cell Cycle and Apoptosis

To evaluate the effect of EAF to increase cell death by modulating cell cycle, we concentrated on it for further studies using flowcytometry method. The effect of EAF is given in Figure 1. Whereas treatment of EAF in 25 µg/mL caused cell accumulation at G0/G1 phase (57.2%) and for control cell (51.7%). At S phase the accumulation after EAF treatment (24.4%) and for control cell (21.0%). This fact was to indicate that EAF can inhibit cell grow that G0/G1 and S phase.In the cell cycle analysis, EAF was exhibited higher G0-G1 and S phase accumulation compared to control cells. This analysis was also showed that cells underwent apoptosis, indicated by occurence of apoptosis during inhibition of cell cycle on G0-G1 phase (Satria, 2015).

Evaluation of apoptosis induction was performed using flowcytometry method with Annexin-V. as shown in Figure 2. As shown in Figure 2, the cells in the upper and lower right quadrants represent late apoptotic/ necrotic and early apoptotic cells, respectively. The percentage

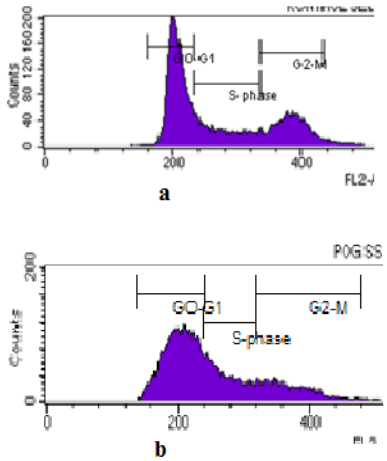

Figure 1. Cell Cycle Analysis Using Flowcytometry. t47d cells were treated by eaf for 24h and stained using propidium iodide. (a) control cells; (b) EAF25 µg/ml. EAF exhibited G0/G1 and S phase and decreased T47D cell population

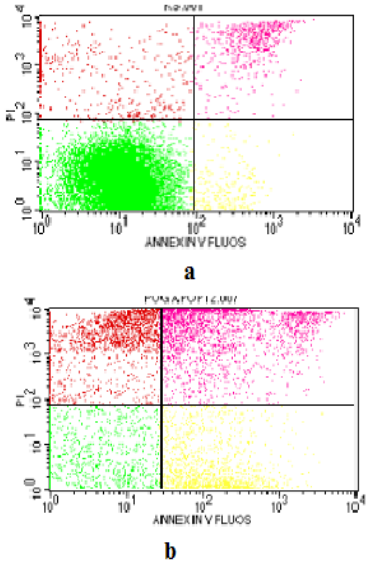

Figure 2. Apoptosis analysis using flowcytometry. T47D cells were treated by EAF for 24h and stained using Annexin-V. (a) control cells; (b) EAF 25 µg/mL.

of control and EAF in early apoptotic 3.9% and 72.7%, in late apoptotic/early necrotic 2.7% and 10.2%, and in late necrotic 2.3% and 9.3%. In apoptotic study, EAF increased the cells undergo apoptosis in early apoptosis and late apoptosis if compared to control T47D cell lines. Apoptosis is a process of programmed cell death with changes on morphology, membrane blebbing and chromatine (Ruddin,et al., 1997). The isolated polyphenols from plants including kaemferol, quercetin, anthocyanins, coumarin acid, and ellagic acid were shown to inhibit the growth (inhibit cell cycle and induce apoptosis) of human breast (MCF-7), oral (KB, Cal-27), colon (HT-29, HCT-116), and prostate (LNCaP, DU-145) tumor cell lines (Zhang, et al., 2008; Daminiaki, et al., 2000; Lim, et al., 2006; Tang, et al., 2007).

Antiradical Activity

Antiradical power of the plant samples was measured in term of hydrogen donating ability using DPPH which is a stable, nitrogen-centered free radical and produces deep purple colour in methanol solution. Antioxidants either transfer an electron or a hydrogen atom to DPPH, thus neutralizing its free radical character (Pan, et al., 2008). DPPH test, which is based on the ability of DPPH, a stable free radical, to decolorize in the presence of antioxidants, is a direct and reliable method for determining radical scavenging action (Hasan,et al., 2009). The DPPH assay has been largely used as a quick, reliable and reproducible parameter to search the in vitro general antioxidant acitivity of pure compounds as well as plant extracts (Koleva,et al., 2002). The reducing capacity of compounds could serve as indicator of potential antioxidant property (Meir,et al.,1995). It is very important to point out that a low IC50 value reflects a high antioxidant activity of the fraction, since the concentration necessary to inhibit the radical oxidation in 50% is low. IC50 for EAF and quercetin in DPPH assay were 166.9 ± 0.1 µg/mL and 4.94 ± 0.05 µg/mL respectively.

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