ANTIOXIDANT AND ANTITUMOR ACTIVITY OF INDONESIAN HERBAL INGREDIENTS

AKTIVITAS ANTI OKSIDAN DAN ANTI TUMOR KANDUNGAN TANAMAN HERBAL INDONESIA

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ABSTRACT

The present study was to aimed to evaluated and compare in vitro antioxidant activities of 2 Indonesian herbal ingredients (A and B), determined total phenol content., cytotoxic and apoptosis induction activities on HL-60 cells. These data were providing some useful information for people healthy dietary and the new potential application of natural antioxidant containing food materials in functional foods and also as new cancer therapeutics promising candidates. The parameters were total antioxidant activity, amount of total cytotoxic effect on the growth of human promyelocytic leukemia cells (HL-60). Statistical comparison was perform with Student's t-test at $p<0.05$. The correlation coefficient (r^2) between the parameters tested was established by regression analysis. The scavenging effect of extracts herbal on DPPH radicals increased from 0.3-1.5 mg/ml, where is sample A 14.33% to 64.29% and sample B 9.09% to 57.53% was obtained. High content of total phenol compounds were in sample A (21.72 mg GAE/g), lower amounts were in sample B (17.53 mg GAE/g). Apoptosis of HL-60 cells from the morphological changes side (chromatin condensation). Chromatin condensation, a specific and distinct feature of apoptotic cells, was found in the majority of treated cells. The results indicated that the cell death receptor pathway was involved in the apoptosis induced by Indonesian herbal extracts.

Key words : Indonesian herbal, antioxidant, apoptosis of HL-60 cells

ABSTRAK

Penelitian ini ditujukan untuk mengevaluasi dan membandingkan aktivitas antioksidan 2 jenis herbal Indonesia, melalui meneliti kandungan fenol, cytotoksid dan induksi apoptosis terhadap sel HL-60. Data ini merupakan informasi yang berguna untuk manusia dalam menciptakan makanan kesehatan dan aplikasi baru dari antioksidan alami dalam bahan makanan sebagai pangan fungsional dan sebagai bahan untuk obat kanker. Penelitian ini menggunakan 2 buah sampel A dan sampel B. Aktivitas antioksidan total di analisa dengan metode DPPH. Kandungan total fenol di tentukan dengan metode Folin-Ciocalteu dan dihitung sebagai ekuvalensi dari asam gallic dan di ekspresikan sebagai gallic acid equivalent (GAE) dalam mg/g sampel. Pengaruh ekstrak herbal terhadap cytotoksid terhadap pertembuhan sel promyelocytic leukimia (HL-60) dianalisa dengan MTT kalorimeter. Hasil ditampilkan sebagai rata-rata ± penyimpangan baku. Analisis Statistik menggunakan Student t-test, pada tingkat signifikansi $p<0,05$. Koefisien korelasi (r^2) terhadap parameter yang di test di hitung menggunakan analisa regresi. Kandungan ekstrak sampel A kering adalah 242,4mg dan sampel B adalah 256,4mg. Diketahui bahwa efek memakan dari ekstrak herbal Indonesia terhadap radikal DPPH meningkat 0,3 – 1,5mg/ml, dimana sampel A adalah 14,3 - 64,29% dan sampel B adalah 9,09 - 57,53%. Total fenol tertinggi didapat pada sampel A (21,72mg GAE/g) dan kandungan fenol rendah pada sampel B (17,53 GAE/g). Apoptosis dari sel HL-60 dilihat dari perubahan morpologi (kondensasi kromatin). Kondensasi kromatin secara spesifik dan fitur yang berbeda dari sel apoptosis, diketemukan pada sebagian besar sel perlakuan. Hasil ini menunjukan bahwa kematian sel diakibatkan oleh ekstrak herbal Indonesia.

Kata kunci: Herbal Indonesia, anti oksidan, apoptosis sel HL-60

INTRODUCTION

The role of free radicals in many disease conditions has been well established. Antioxidant compound in food play an important role as a health-protecting factor to reduce the risk for chronic diseases including cancer, heart disease, brain dysfunction, cataracts and arthritis. There is an increasing interest in natural antioxidants, e.g., polyphenols, present in medical and dietary plants which might help prevent oxidative damage (Silva *et al*., 2005). Polyphenol possess ideal for free radical scavenging activity. Recently, many of the phenol compounds were identified to possess strong antioxidant activity (Rabah *et al*., 2005). The presence of phenol compounds in the human diet is associated with protective effects against some chronic-degenerative disease related to oxidative stress (Riberio *et al*., 2008).

Apoptosis or programmed cell death is an essential event that plays an important role in organism development and homeostasis (Rabah *et al*., 2004 and Liu *et al*., 2006). Tumor growth is regulated by the balance between cell proliferation and apoptosis. Deregulated cell proliferation and suppressed cell death together provide the underlying platform for neoplastic progression. In turn, one essential strategy for cancer therapy is to target the lesions that suppress apoptosis in tumor cells (Zhang *et al*., 2004). In the search for new cancer therapeutics, the herbs being used in traditional medicines for cancer treatment are promising candidates.

Five Indonesian herbals were chosen for the present experiment including *Curcuma xanthorriza* Roxb., *Curcuma aeruginosa* Roxb., *Piper retrofractum* Vahl., *Zingiber aromaticum*, Val. and *Aegle marmelos*, L. Corr. For generations, Javanese people has used rhizome of *C. xanthorrhiza*. as an anti-stress (Yasni and Imaizumi, 1991) as well as anti-inflammatory agent (Claeson and Panthong, 1993; Claeson and Pongprayoon, 1996), antibacterial and antimicrobial (Hwang and Shim, 2000), antihepatotoxic (Lin and Lin, 1995), and antioxidative (Masuda and Isobe, 1992).

The rhizomes of *C. aeruginosa* have been used as anthelmintic medicine in Indonesia. The water extract from the rhizome of *C. aeruginosa* effectively inhibited on HIV-1 infected MT-4 cells (Otake *et al*., 1995). *P. retrofractum* is a piper species indigenous which is used in the Indonesian medicine. Studies on its crude extracts also exhibited insecticidal properties (Banerji *et al*., 2002). *Z. aromaticum*, belonging to the family Zingiberaceae, is one of the popular traditional medicines extensively used in Indonesia. Some studied reported that *Z. aromaticum* effective as an anticancer agent (Kirana *et al*., 2003), antibacterial (Ficker *et al*., 2003), antifungal and anti-inflammatory (Handayani, 1994). *A. marmelos*, commonly known as bael fruit, is a spinout tree belonging to the family Rutaceae and has an important place in indigenous systems of medicine (Jagetia *et al*., 2005). *A. marmelos* is claimed to be useful in treating pain, fever, inflammation, respiratory disorders, cardiac disorders, dysentery and diarrhea (Arul *et al*., 2005).

The purposes of present study were to evaluate and compare in vitro antioxidant activities of 2 Indonesian herbal, determine total phenolic content., cytotoxic and apoptosis induction activities on HL-60 cells.

These data were providing some useful information for people healthy dietary and the new potential application of natural antioxidant containing food new materials in functional foods and also as cancer therapeutics promising candidates.

METHODOLOGY

Material

This experiment used 2 samples (sample A and sample B) as shown in Table 1, ethanol 90%, methanol 80%, dimethyl sulfoxide (DMSO), 2,2-Diphenyl-1- picrylhydrazyl (DPPH), (S)-(-)-6-hydroxy-2,5,7,8-tramethylchroman- 2-carboxylic acid (Trolox), gallic acid, Na_2CO_3, Folin-Ciocalteu, HL-60 cells, RPMI medium 1640, Fetal Bovine Serum (FBS), MTT solution, 0.04 HCl-isopropanol, PBS, RIPA buffer, 6xSDS buffer, TBST and ECL.

Sample Preparation

Preparation of the samples as were as follows: five Indonesian herbals were purchased from East Java and Central Java, Indonesia. Mature and about full size of each herbal were harvested, washed in flowing water, sliced and dried under the sun until the water content reached less than 10%. The dried slices of each herbal were crushed using 0.2 mm diameter mesh and then mixed together as according to the composition as shown in Table 1.

Sample Extraction

Samples were extracted with ethanol 90% for 48 hour in room temperature. Extracts were evaporated to dryness under reduced pressure at 30^{o}C in a rotary evaporator. Total extractable compound of each sample were weighted and dissolved in DMSO for assessment of antioxidant activity and determination total phenolic content. Samples extracts was stored at -20 ^{o}C before analysis.

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Table I. Percentage of Herbal Ingredients of Sample A and Sample B

Herbal Name	Sample A	Sample B
(a) *Curcuma xanthorriza* Roxb.	-	16 %
(b) Curcuma aeruginosa Roxb.	-	10 %
(c) Piper retrofractum Vahl.	-	8 %
(d) Zingiber aromaticum Vahl.	33.33%	19 %
(e) Aegle marmelos L. Corr.	66.67%	47 %

a. Curcuma xanthorriza Roxb.

b. Curcuma aeruginosa Roxb.

c. Piper retrofractum Vahl.

d. Zingiber aromaticum, Val

e. Bael Fruit (*Aegle marmelos*, L. Corr)

DPPH Radical Scavenging Assay

Total antioxidant activity of the extracts was assessed by DPPH method. Each sample (Concentration : 0.3, 0.6, 0.9, 1.2 and 1.5 mg/ml) and DPPH solution were placed into each well of 96 well micro titer plates and mixed vigorously. The mixture was allowed to react for 30 minutes in the dark and absorbance was measured using microplate reader at 490 nm.

Determination of Phenolic Content

The amount of total phenolic in the extracts was determined by Folin-Ciocalteu method and calculated as gallic acid equivalent and expressed as a gallic acid equivalent (GAE) in milligrams per gram of samples.

Cell Viability Assay

Cytotoxic effect of herbal extracts on the growth of human promyelocytic leukemia cells (HL-60) was assessed by MTT colorimetric assay. The procedures as shown as below: briefly 100 µl of cell suspension (2 x 10^4 cells/ml) were treated with various concentration of herbal extracts (0, 25, 50, 100 and 200 µg/ml) for 48 hour in 96 well micro plate. MTT solution was added into each well and incubated for 4 hour. After dissolving the MTT formazan product, the amount of formazan product was determined by measuring the absorbance at 595 nm with a micro plate reader.

Western Blotting Analysis

The procedures of western blotting analysis for caspase-3, caspase-8, caspase-9 and PARP cleavage as shown as below:

HL-60 cells (2 x 10^6 cells) were cultured on RPMI-1640 medium supplemented with 10% fetal bovine serum for 24 hours at 37°C with 5% CO_2. The cells were treated with samples (0, 200, 400 µg/ml) and then incubated for 12 hours at 37°C with 5% CO_2. The cells were harvested by centrifugation (2500 rpm at 4°C) and rinse with ice-cold PBS. The pellets were lysed with modified

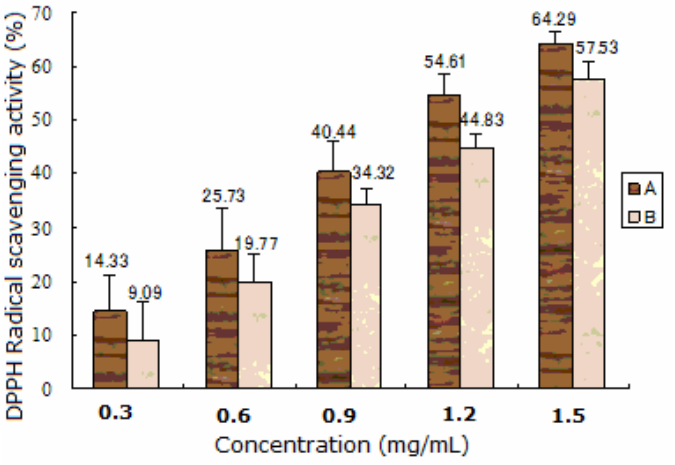

Figure 2. DPPH Radical Scavenging Activity of the Extracts from Indonesian Herbal

RIPA buffer containing 1 M Tris HCl (pH 8.0), 10% NP-40, 10% Na-deoxycholate, 5 M NaCl, 0.5 M EDTA (pH 7.4-8.0), 0.1 PMSF, 200 mM Na_3VO_4, 200 mM NaF, Protein inhibitor cocktail and H_2O. The lysates were then sonicated for 10 second and centrifuged at 14.000 g for 15 minutes at 4°C. The supernatants (100 µl) were boiled in 6xSDS buffer (20 µl) and bromophenol blue solution (2 µl) for 5 minutes at 100°C. Equal amounts of lysates protein were run on SDS-PAGE and electrophoretically transferred to PVDF membrane. The blots were first blocked with TBST buffer containing 5% of non-fat dry milk. The membrane was incubated with specific primary antibody overnight at 4°C. The blots were further incubated for 1 hour with HRP-conjugated secondary antibody. Bounds antibodies were detected by ECL kit with Lumi Vision Image Analyzer.

Statistical Analysis

Results were expressed as mean ± standard error. Statistical comparison was performed with Student's t-test. Differences were considered significant at $p<0.05$. The correlation coefficient (r^2) between the parameters tested was established by regression analysis.

RESULT AND DISCUSSION

Extracts Yield (amount of total extractable compound)

The amounts of extractable were 242.4 mg/gr dry sample A and 256.4 mg/gr sample B. It has been known that almost all of herbal contained essential oils and almost all of the essential oil were terpene or terpenoid. *C. xanthorrhiza* contained 32 of essential oil compound such as sikloisopremmirsen and ptolylmetilcarbinol (ISM, 2007). *C. aeruginosa* contains isocurcumenol 8,25%; curcumenol 9,92%, curcumenone; 1,85% and dihidrocurdione 9,41% (Elfahmi, 2006). Limonen was found in *Z. aromaticum* and eugenol was one of essential oils component *A. marmelos.*

Antioxidant Activity

The DPPH is a stable free radical, which has been widely accepted as a tool for estimating free radical scavenging activities of antioxidants. Figure 2 shows the dose response curve of DPPH radical scavenging activities of Indonesian herbal extracts. The scavenging effect of Indonesian herbal extracts on DPPH radicals increased from 0.3-1.5 mg/ml, where is sample A 14.33% to 64.29% and sample B 9.09% to 57.53% was obtained.

The IC50 was identified as the effective concentration at which the DPPH radicals were scavenged by 50% and it was show that extracts of sample A (1.07±0.14) had the higher DPPH radical scavenging activity than extracts of sample B (1.29±0.08). A higher of DPPH radical scavenging activity is associated with a lower IC50 value. It was evident that the extracts did not show the hydrogen donating ability to act as antioxidants. However from the statistical analysis, IC50 in scavenging abilities on DPPH radicals between sample A and sample B was not significantly different ($P>0.05$).

It has been reported that each herbal from samples has an antioxidant activity. Component such as lignan contained in *C. aeruginosa*, *P. retrofractum* (sasamin), *Z. aromaticum* (limonene) and *A. marmelos* (limonene) might be responsible for its powerful antioxidant capacity (ISM, 2007). Curcumin which contained in *C. xanthorrhiza* and *C. aeruginosa*, xanthorrhizol in *C. xanthorrhiza* also had an antioxidant activity (Pan *et al.*, 1999)Marmelin in *A. marmelos* had been reported

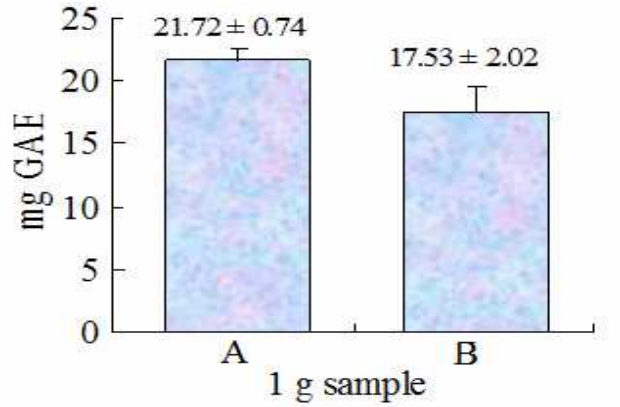

Figure 3. Total Phenolic Content of Indonesian Herbal Extracts (A and B). Amounts are represented as GAE (mg/g)

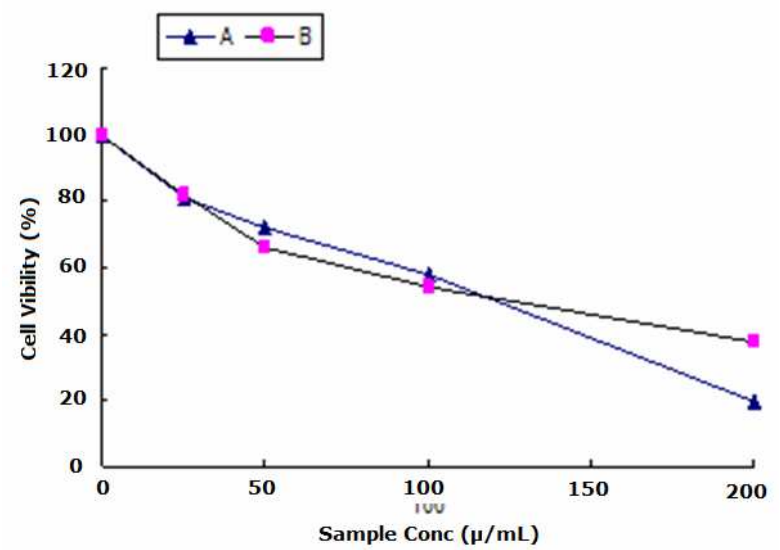

Figure 4. Effect of Indonesian Herbal Extracts on Cell Survival Rate

has an antioxidant activity (Singh *et al*., 2000). Others reports have also showed outstanding antioxidant activity of clove inhibiting lipid peroxidation by marmelin (Rajadurai *et al*., 2005). The active constituents in *A. marmelos* are eugenol and flavonoid glicosida compounds, available to traps free radical such as hydroxyl or super oxide (Singh *et al*., 2000).

Sample A which contained only 2 herbals had an antioxidant activity higher than sample B which contained 5 herbals. The difference of herbal composition of each sample might influence of antioxidant activity.

Total Phenolic Content

The content of phenolic could be used as an important indicator of its antioxidant capacity which may preliminary screen edible medical plant and use as natural sources of antioxidant functional foods (Liu *et al*., 2008). Figure 3 shows total phenolic content of the Indonesian herbal extracts.

High content of total phenolic compounds were found in sample A (21.72 mg GAE/g). Lower amounts of phenolic content were obtained in sample B (17.53 mg GAE/g). These finding suggested that the level of antioxidant activity and the amount of phenolics were closely related to each other. With further data analysis, a significant linear correlation (r^2 = 0.99 for sample A and r^2 = 0.98 for sample B) between antioxidant activity and total phenolic content of each sample was confirmed, which suggested that phenolic compound in these sample provide substantial antioxidant activity.

HL-60 cells which is a valid model for determining the anticancer compounds (Hou *et al*.,

2003), were used to determine the anticancer properties of Indonesian herbal extracts. Figure 4 shows the dose-dependent manner of Indonesian herbal extracts on proliferation of HL-60 as measured with the MTT assay after the incubation times of 48 hours.

The results suggested that both samples, A and B showed significant inhibition of the cell growth of HL-60. The maximum inhibition was observed at the concentration of 200 µg/ml, which caused 80.4% inhibition with sample A and 62.3% inhibition with sample B, respectively.

From the effective concentration of the extract, seen that extracts of sample A (90.91±0.18 µg/ml) had the higher antiproliferation activity than extracts of sample B (113.86±1.08 µg/ml), as shown by the lowest value of IC50. A higher of antiproliferation activity is associated with a lower IC50 value. This findings demonstrate that the antiproliferation activity of Indonesian herbal extracts increased with the increase of samples concentration.

CONCLUSION

The Indonesian herbals extract had antioxidant, cytotoxic and apoptosis induction on HL-60 cells activities. High content of total phenolic compounds were found in sample A (21.72 mg GAE/g) and in sample B (17.53 mg GAE/g).

ACKNOWLEDGEMENT

Thank you for the opportunity to get PAR Program from General Directorate Higher Education, Ministry of Education, Republic Indonesia to visit and doing research in Kagoshima University Japan.

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