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Investigating the antioxidant and anticytotoxic activities of propolis collected from five regions of Indonesia and their abilities to induce apoptosis

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Abstract

Propolis is a resinous substance collected by stingless bee or honey bee from various plant sources. The substance is known to contain beneficial properties for human. The geographical origin of propolis determines its biological properties. In this study, propolis were collected from five regions of Indonesia with the objective of determining the yield, their total flavonoid content, their capacity to induce apoptosis, and their toxicity to the Michigan Cancer Foundation-7 (MCF-7) cell line. The inhibition of antioxidant 1,1-diphenyl-2-picrylhydrazyl (DPPH), the induction of apoptosis to Saccharomyces cerevisiae and the anticytotoxic ability were determined. Propolis from Pekanbaru region had higher yield than other regions with value of 19.97%; propolis from Kendal had higher quantity with value of 46.60%, total flavonoid content; propolis from Pandeglang was higher in DPPH oxidation capacity with value of 68.94 $\mu g.ml^{-1}$; propolis from Kendal, expressed petite cell induction in *S. cerevisiae* cells with value of 81.44%, and the anticytotoxic to MCF-7 breast cancer cell line were best observed in propolis from Makassar region with a value of 47.71% life cells. All of the propolis extracted from the stingless bee hive *Trigona* spp from five regions in Indonesia contained flavonoids.

Key words: Propolis, *Trigona* sp, flavonoids, anticancer, MCF-7, MTT-assay

Introduction

Propolis is a resinous hive product collected by honeybees or stingless bees and is used to make a nest as well as for defense. Propolis (in Greek) means defense of the city or the bee hives. It thus implies a product involved in the defense of the bee community (Salatino et al., 2005). Honey bees belonging to the *Trigona* sp do not have a sting for defense which likely causes them to produce relatively more propolis than stinging bees. Their are capable of producing a chemical for defense (Caron 1988).

One of the functions of propolis is to protect the beehive from bacterial contamination and insect attack. Propolis has been used in folk medicine since ancient times and recent studies have been conducted which reveal an advantage of propolis as antibacterial, antifungal, antiviral, antiinflammation, local anesthesia, hepatoprotective, immuno-stimulant, antiparasitic, and antitumor functions (Yousef and Salama, 2009; Fearnley 2005; Woo, 2004). More than 180 active compounds in propolis are known (Kasahara et al., 2004; Khismatullina, 2005), among which the flavonoids, aromatic acids, terpenoids, phenylpropanoids, and fatty acids (Lustosa et al., 2008). For the last 40 years, many studies have focused on the chemical composition, biological activity, pharma-cological, and therapeutical uses of propolis (Khismatullina,2005).

Nunes et al. (2013) stated that the composition of the propolis depends on the season, the vegetation, and the area of collection. Fernandes-Silva et al. (2013) also stated that the chemical composition of propolis depends on the plant or plants from which the resin is collected and, consequently, on the geographic location of the hive. According to Franchi et al. (2012), the

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difference in the quality of propolis could be seen from the location and color of a beehive. However, the composition of propolis is more complex and unpredictable than previously assumed (Teixiera et al., 2005). Nowadays, most of the propolis used for commercial production to improve health and prevent illnesses are from Europe and America (especially Canada and Brazil), and very little from Asia.

The objective of this study was to compare the properties of the *Trigona* propolis extracted from five regions of Indonesia by determining the total flavonoid content, antioxidant activity, apoptosis induction, and the anticytotoxic activity to MCF-7 breast cancer cells line.

Materials and Methods

Materials and Equipment

The materials used in this study include: *Trigona* bee hives from Pandeglang, Kendal, Banjarmasin, Makassar, and Pekanbaru, 70% ethanol as solvent, Roswell Park Memorial Institue (RPMI) media, MCF-7 cancer cell line from LAPTIAB The Agency for The Assessment and Applicantion of Techno-logy, *S. cerevisiae*, DPPH, and $AlCl_3$. Equipment used in this study were an autoclave, an incubator with 5% CO_2, microwave processor, a laminar air flow, UV-Vis Spectrophotometer, and an orbital shaker.

Methods

Propolis Extraction

Propolis was extracted by using a modification (in time) of the method reported by Trusheva et al. (2006) and Jang et al. (2009). Extraction was conducted by using a combined method of maceration in 70% ethanol and microwave heating (Kriss Microwave Oven, 2450 MHz and 800 Watts). Aliquots of 2.0 g of crude propolis were obtained from each regions and combined with 18 ml of 70% ethanol. Each sample was mixed for 18 h at 200 rpm using an orbital shaker. After that, the samples were heated with microwave oven for 10 minutes. The extract was filtered and the filtrate was evaporated in a water bath at temperature of 50±2^{o}C for three hours or until the water and ethanol evaporated.

Total flavonoid determination (Chang et al., 2002)

Total flavonoid content was determined by the colorimetric method using aluminium chloride. Aliquots of 2.0 ml of the extract solution, or standard solutions of quercetin (60, 50, 40, 20, 15, 10, and 5 $\mu g.ml^{-1}$), then were added to 100 µl of 10% $AlCl_3$, 0.1 ml of 1 M Na-Acetate and 2.8 ml of distilled water. The mixtures produced a yellow solution which was , then shaken vigorously until homogeneous and then left for 30 mins. The absorption was measured at a wave length of 415 nm. Total quercetin expressed as total flavonoid equal weight (g) of each dried sample weight (100 g).

DPPH Antioxidant Assay

The antioxidant activity assay was carried out according to Chang et al. (2002) and Chang et al. (2007). The propolis extract was redissolved in 70% ethanol and prepared at various concen-trations of 500, 250, 125, 62.5, 31.25, 15.5, and 7.75 $\mu g.ml^{-1}$. These were then mixed with 125 µmoles DPPH. The mixtures were the shaken vigorously and left to stand at room temperature for 30 mins in the dark. The absorbance at 515 nm of the reaction solutions was measured. The percentage of DPPH decolorization of the sample was cal-culated according to the following equation:

$$\text{DPPH radical scavenging (\%)} = (1 - A/A_o) \times 100$$

where A_o is the absorbance of the mixture without a sample and A is the absorbance of the mixture with sample after 30 mins. The IC_{50} of antioxidant activity was calculated as the concentrations of sample that inhibited by 50% the scavenging activity of DPPH radicals under these conditions.

Cell Apoptosis Test

Cell apoptosis assays were carried out according to Laun et al. (2001). YEPD agar plates were added to the propolis extract (50 $\mu g.ml^{-1}$) and poured with 200 µl of *S. cerevisiae* culture and then incubated at room temperature for 24 h. The growth of *S. cerevisiae* was measured by direct colony counting.

MCF-7 Cancer Cell Line Anticytotoxic Assays

Anticytotoxic activity assays were performed on cultured MCF-7 cancer cell line using the methylene blue test method that has been reported by Lin and Hwang (1991). The extract was dissolved in a viscous solvent of DMSO to make a 10% (v/v) substock solution, and then diluted in the RPMI 1640 medium to make a 1% (v/v) substock solution. Dissolution samples stratification was conducted to obtain a final concentration of test solutions of 100 $\mu g.ml^{-1}$. Then 20 µl of the test solution was added to the well plate which contained 100 µl cancer cell line and incubated for 24 h at 37^{o}C in an incubator with 5% CO_2. The number of living cells was calculated using an ELISA (enzyme linked immunosorbent assay) reader, i.e. a serological test

that is commonly used in various immunology laboratories at a wavelength of 515 nm.

Analysis of biologically active compounds in Propolis

The methods for analysis of biologically active compounds reported by Harborne (1987) were employed for conducting the tests.

a) Alkaloids. A total of 100 mg of propolis extract was put into the test tube and combined with two drops of ammonia and 5 ml of chloroform and then filtered. The filtrate was added to 1 ml of 2.0 M H_2SO_4, and the acid fraction was taken then added by Dragendorf, Meyer and Wagner reagents. The presence of alkaloid was charac-terized by the formation of a red precipitate with Dragendorf rea-gent, white precipitate on Meyer reagent, and a brown precipitate on Wagner reagent.

b) Triterpenoids and Steroid. A total of 100 mg of extract was put into the test tube, then added by 5 ml of hot ethanol, and filtered. The filtrate obtained was evaporated, and then combined with 1 ml of diethyl ether. After mixing by vortex, then added by 1 ml of concentrated H_2SO_4 and 1 ml CH_3COOH. Red or purple color indicates the presence of triterpenoids and green or blue showed the presence of steroid.

c) Flavonoids. A total of 100 mg of extract was put into the test tube, then combined with 5 ml distilled water, and filtered. The filtrate obtained was combined with Mg powder, 1 ml of concentrated HCl, and 1 ml of amyl alcohol. The mixture then was shaken to allow the separation. Color (yellow-red) that forms at the interface of amyl alcohol showed the presence of the flavonoids.

d) Tannins. A total of 100 mg of extract was put into the test tube, then combined with 5 ml distilled water, and then filtered. The filtrate obtained was combined with 3 drops of 1% $FeCl_3$. The formation of a blue or greenish black color indicated tannin.

e) Saponins. A total of 100 mg of extract was put into the test tube, then combined with 5 ml distilled water and filtered. The filtrate obtained was vigourously shaken and left for 10 minutes. The formation of stable foam showed the presece of saponin compounds.

Results and Discussion

Propolis Extraction

Propolis is produced from beehives *Trigona* spp extracted using microwave treatment (Microwave-assisted extraction, MAE), which can increase the contact between solvent and sample (Jang et al., 2009). It is hoped that the desired compounds can be extracted properly. However, before the extraction by using a microwave, stinglessbees hive was macerated in ethanol. The 70% ethanol was capable to extract the flavonoid as the important compounds in propolis. The 70% ethanol is semipolar solvent which can extract all active components with different polarity in the propolis (Cunha et al., 2004; Hasan et al.,2006) and the optimum conditions for extracting flavonoid for *Alpinia pricei* rhizome (Hseu et al., 2011).

The chemical composition of propolis as well as colors and odor was strongly influenced by the source materials and the bee hives age. Filtrate colors of propolis produced from five different regions were light-yellow, yellow-black to brown and black. The compositions of each propolis were different from nest to nest, location to location, season to season and because bees take resin from the plants around the nest, then the composition of propolis vary depending on the locations or regions. Color, indicates the level of lightness and chromaticity of an object (Suparno et al., 2007). The chemical ingredient of a material or the existence of oxidized products (Suparno et al., 2009), can be used to predict yield of propolis in a propolis extract. Propolis extract having a darker color indicate a higher extractable yield compared with a brighter color. A higher flavonoid content is indicated by darker color (Woo, 2004). The yields of propolis extract obtained from five regions in Indonesia were different. Table 1 shows that the yield of propolis from Pekanbaru was significantly different from that from Makassar, but for other locations yields were almost the same. According to Paviani et al. (2011), the difference in the origin of propolis and solvent polarity resulted in differences in yield and lead to differences in the types and amounts of flavonoids.

Total Flavonoid

Trigona Spp. propolis produces large quantities of flavonoids compared to other types of bees. The higher concentration of flavonoid was indicated the color of the solution when reacted with $AlCl_3$. A high concentration was shown by color changes from yellow to the darker color and indicated by higher absorbance value which means that the extract contains high levels of flavonoid (Surendra et al., 2012; Woo, 2004).

Table 1. Yield, total flavonoid, antioxidant activity, and anticytotoxic activity of the propolis extracted from five regions in Indonesia.

Character	Regions				
	Makassar	Pekanbaru	Kendal	Pandeglang	Banjarmasin
Yield, %	1.85±0.51^{c}	19.97±2.19^{a}	7.28±1.59^{b}	11.05±3.20^{b}	8.38±0.70^{b}
Total Flavonoid, µg. ml^{-1}	38.78±1.62^{b}	16.90±0.537^{e}	46.60±0.78^{a}	30.62±1.50^{c}	24.60±0.73^{d}
Antioxidant Activity (IC_{50}), µg.ml^{-1}	1125.56±133^{b}	308.88±12^{c}	144.06±52.53^{d}	68.935±5.63^{e}	4162.61±845.9^{a}
Anticytotoxic Activity at 100 µg.ml^{-1}, % Live Cell	47.71±9.31^{c}	76.35±1.48^{a}	50.26±2.70^{c}	70.64±1.21^{b}	75.79±1.33^{a}

Remark: Result (number) followed by the same superscript letter on the same row shows that the results were not significant at α=0.05.

Table 2. Phytochemicals comprizing the propolis extracted from five regions in Indonesia.

No.	Class of Compounds	Regions				
		Makassar	Pekanbaru	Kendal	Pandeglang	Banjarmasin
1.	Alkaloids	-	-	-	-	-
2.	Flavonoids	+	+	+	+	+
3.	Saponins	-	+	+	-	-
4.	Tannins	-	+	+	+	-
5.	Steroids	-	-	-	-	-
6.	Triterpenoids	-	-	-	-	-

Remarks: + = positive results, - = negative results

Table 1 shows that propolis from Kendal contained the highest flavonoid content, and this was followed by propolis from Makassar, Pandeglang, Banjarmasin and Pekanbaru. Syamsuddin et al. (2010) reported that propolis originated from three regions of Java Island, each containing different types and values of flavonoids. Similarly, Daugsch et al. (2008) and Paviani et al. (2011) reported that for propolis from Brazil the differences in the quantities and types of flavonoids depended on bee-hive and solvent polarity. Antioxidant activities derived from plant sources are often associated with the content of flavonoids (Table 2). Flavonoids as antioxidants can capture free radicals capture by providing a hydrogen atom to the radical (Ratnam et al., 2006). The relationship between total flavonoid extract and antioxidant activity can be seen from the results of the antioxidant test. The flavonoid from propolis have antioxidant capacities that are much stronger than those of vitamins C and E (Prior and Cao, 2000).

Antioxidant Activity

The antioxidant test was conducted to determine the antioxidant potential of propolis extract, using the DPPH scavenging method. DPPH usage to scavenge radicals has some advantages, i.e. it is easy to use, it has high sensitivity, and it enables the analysis of large numbers of samples in a short time.

The parameters used for this test is IC_{50}, i.e. the concentration of extract required to capture the DPPH free radical by 50%. IC_{50} values were obtained from an equation which relates the concentration of the extract to the percent age of captured radicals. A smaller IC_{50} value of the extract means higher capturing activity of the DPPH radical, and therefore, increased activity as an antioxidant. Potential propolis extracts as an with high antioxidant activity were the propolis extracts from the Pandeglang with an IC_{50} value of 68.935 µg.ml^{-1}. That propolis extracts originated from Pekanbaru, Makassar, and Banjarmasin were not active as antioxidants is shown by their IC_{50} values wich were more than 150 µg.ml^{-1}. The extract originating from Kendal was moderately active antioxidant, as having an IC_{50} value was less than 150 µg.ml^{-1}.

The difference in the antioxidant activity is mainly due to the flavonoid content of propolis extracts or other compounds, which are potential antioxidants. The composition of propolis was influenced by the type and age of the bee-hive and existing vegetation around the hive of *Trigona* spp (Table 2). Total flavonoid content shows that the highest value was from Kendal, but its antioxidant properties was lower. This is in accordance with the

statement of Bankova et al. (2000) that the active ingredient in natural propolis is variable, depending on the resin plant origin, climate, and the time collecting resin plant by the bees. This difference is caused by the influence of different types of flavonoids (Jang et al., 2009). Antioxidant activity has a good correlation with flavonoid content (quercetine, apigenine, and kaempferol) and caffeic acid concentration (Coneac et al., 2008). The presence of techtochrysine (Lee et al., 2003) or propoline (Chen et al., 2004) increases enzymes that contributing in the antioxidant activity.

S.cerevisiae Apoptosis Induction

Apoptosis, or programmed cell death, is a normal development in the health of multicellular organisms. Cell death is a response to various stimuli and during apoptosis; the organisms are in a self-regulated and controlled state. This makes apoptosis distinct from another forms of cell death called necrosis, which is uncontrolled cell death caused by the lysis of cells, inflammatory responses and, potentially, to serious health problems (Granot, 2003). After receiving specific signals which instruct cells to undergo apoptosis. Typically a number of changes occur in the cell. Proteins known as *caspase* are normally activated. Bhatia-Kissova and Camougrand (2010) stated that the apoptosis mechanism in yeast caused by the addition of rifampicin or lactate originated in the mitochondria to form the mitochondria of *caspase* 1 (Yca1). This is the case also with the presence of propolis in this study. Many chemicals cause apoptosis in *S. cerevisiae* such as glucose, acetic acid and propolis (Sukhanova et al., 2011). The process of apoptosis occurring in *S. cerevisiae* has been described by Lotti et al. (2011). Cytochrome *c,* but not *endonuclease G* (Nuc1p), was involved in the propolis-mediated cell death in *S. cerevisiae* (de Castro et al., 2011).

This study shows that the induction of apoptosis on *S. cerevisiae* cell caused by propolis originated from Pekanbaru, Banjarmasin, Pandeglang, Makassar, and Kendal gave apoptosis induction potential of 50.94, 65.08, 67.75, 71.09 and 81.43%, respectively (Figure 1).

This difference was caused by the type of flavonoids contained in the propolis (Jang et al. 2009). In the research reported by Umthong et al. (2011), Thailand *Trigona* propolis contained compounds with antiproliferative activity in-vitro on cancer, but not on normal cell lines. The differences of type and number of flavonoids distinguished the mechanism of propolis in apoptosized cancer cell.

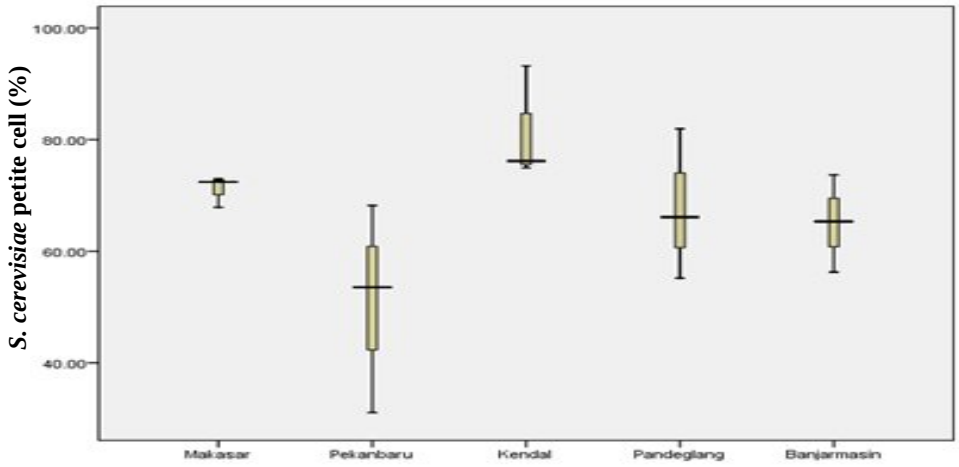

Figure 1. The persentage of *S. cerevisiae* petite cell with 50 µg.ml^{-1} propolis from five regions in Indonesia.

MCF-7 Cancer Cell Line Anticytotoxic

The results shows that propolis originating from Pekanbaru provided an opportunity to live MCF-7 cells more than others, the opposite is the originated from Makassar that has better capacity as an anticancer agent (Table 1). This shows that the location of the beehive affected the quality of propolis produced. Data in Table 1 demonstrates the inhibition of cancer cell growth inhibition by propolis. Research conducted by Syamsuddin et al. (2010) suggests that there are differences in the results of MCF-7 cell IC50 of ethanol extract of propolis originated from Batang, Lawang and Sukabumi. The difference in the results is shown by the effect of the location of the hive on antibacterial activity has been done by Daugsch et al. (2008). Similarly, when compared between Fatoni (2009) and Hasan et al. (2011) showed differences in the antibacterial activity of propolis from different locations (Bukittinggi and Pandeglang) with different origin plant. The difference in effect between propolis origin may be due to the differences of propolis's chemical content (Zhu et al., 2011). Inhibition of cancer cells by propolis is caused by the activation of the caspase enzyme pathway and protein transcription pathways (Madeo et al., 2004).

Huang et al. (2012) reported that there were propolis components controlling cancer cell proliferation and maintaining or upregulating the function of tumor suppressor genes in normal cells. Research reported by Umthong et al. (2011) stated that *Trigona propolis* originating from Thailand contained compounds with antiproliferative activity in-vitro on cancer but not on normal cell lines. This difference is caused by the influence of type and number of flavonoids in the propolis (Jang et al., 2009), such as the chrysin and cafeic acid which inhibits the growth of cancer cells directly (Sawicka et al., 2012). In addition, different types of flavonoids would affect inhibitory mechanisms (Sawicka et al., 2012; Watanabe et al., 2011). The difference in propolin amounts, especially D, C, E, A, and B, will cause a difference in strengths of propolis as antitumor agents (Chen et al., 2004).

Conclusions

Propolis from Pekanbaru region had higher yield than other regions with value of 19.97%; propolis from Kendal had higher quantity with value of 46.60%, total flavonoid content; propolis from Pandeglang was higher in DPPH oxidation capacity with value of 68.94 $\mu g.ml^{-1}$; propolis from Kendal, expressed petite cell induction in S. cerevisiae cells with value of 81.44%, and the anticytotoxic to MCF-7 breast cancer cell line were best observed in propolis from Makassar region with a value of 47.71% life cells. All of the propolis extracted from the bee hive *Trigona* spp. from five regions in Indonesia contained flavonoids.

Therefore, propolis from Indonesia indicating may have the potency as anticancer agents and has potential in anticancer drug research.

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