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Chemical Composition of Propolis from Different Regions in Java and their Cytotoxic Activity

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Abstract: Problem statement: Propolis samples from tropical zones, such as Java (Indonesia) with its vast biodiversity, have become a subject of increasing scientific and economic attention. The association of the chemical composition of propolis from different geographic regions with cytotoxic activities lead to the identification of active principles, a fundamental tool to achieve standardization of this bee product. **Approach:** The purpose of this research was evaluate the quality of propolis collected at different places in Java (Indonesia) based on cytotoxic activity. The ethanolic extracts of propolis from different areas in Java were tested for cytotoxicity against tumor cell lines (T47D, MCF-7, Hela, Myeloma and Vero) using MTT assay. Propolis samples were collected from Batang (Central Java), Lawang (East Java) and Sukabumi (West Java). **Results:** The extract of propolis from Batang showed the most potent activity of T47D and MCF-7 with IC_{50} 34.67±8.3 and 37.8±2.5 µg mL^{-1}. The extract of propolis from Sukabumi showed the most potent activity of Hela cell with IC_{50} 147.34±8.9. However, all propolis extract did not show activity of myeloma and Vero cells. **Conclusion:** Ethanolics extract of three propolis samples from Batang (Central Java), Lawang (East Java) and Sukabumi (West Java) regions in Java were investigated using GC-MS. From 37 compounds identified, 7 among of them were found for the first time in propolis. This indicated that the secondary metabolite extract of propolis from Batang (Central Java) obtained in the study has antiproliferative activity of breast carcinoma cells (T47D and MCF-7).

Key words: Propolis, Java, cytotoxicity activity, GC-MS, MTT assay

INTRODUCTION

Propolis is a resinous hive product collected by honeybees (*Apis mellifera*, L.) from various plant sources. Bees use it as a construction material, to smooth internal walls of the hive and preserve it form extreme moisture and drought condition. They also use it to embalm dead invaders and in such way, prevent the development and spread of microbial diseases. Propolis is widely used in traditional medicine and is reported to have of pharmacological activities. Besides its traditional uses, it has recently gained popularity as a food supplement in numerous countries, claimed to improve health and prevent diseases[2,3]. Various biological activities, such as anticancer, antioxidant, antibiotic effects have been reported for propolis and its components[10].

The composition of the propolis depends on the place and time of collection. As a consequence, more than 160 components have been identified so far, among which phenolic compounds, including flavonoids, are major components[4]. Due to the increasing interest in the characteristics of Indonesian propolis, we undertook a study using samples collected in different regions. We evaluated the quality of propolis collected at different places in Java (Indonesia) based on cytotoxic activity.

MATERIALS AND METHODS

Propolis: Propolis samples were collected from different regions in Indonesia in April 2007 Sukabumi (West Java), Batang (Central Java) and Lawang (East

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Java). Hand-collected propolis samples were kept desiccated in the dark up to their processing.

Extraction: One gram of each sample was cut into small pieces and extracted at room temperature with 50 mL of 70% ethanol (twice after 24 h). The alcoholic extract was evaporated under vacuum at 50°C until dryness. The percentage of extracted matter was as follows: Batang propolis 0.56% w/w, Lawang propolis 0.43% w/w and Sukabumi propolis 0.67% w/w.

GC-MS-MS analysis: The GC-MS-MS analysis was performed using temperature programming. The column oven temperature was initially held at 100°C for 2 min, then programmed to rise to 280°C at a rate of 20°C min^{-1} and held for 5 min. The total run-time was 16 min. The temperatures of the injector port and the interface were set at 250 and 280°C, respectively. The carrier gas (helium) flow rate was 1.0 mL min^{-1}. The ionization energy was set at 70 eV. The mass spectra were collected by scanning from m/z 50-550 at 2 sec intervals.

Identification of compounds: Peaks were identified using computer searches in commercial reference libraries. Reference compounds were co-chromatographed when possible to confirm GC retention times.

Cell culture and assay for cytotoxic activity: The human cervix carcinoma (HeLa), Myeloma, MCF-7, T47D and Vero cell lines were supplied by Cancer Hospital Center Dharmais, Jakarta, Indonesia. The human tumor cytotoxicities were determined following protocols established by the NCI[11]. Cellular viability in the presence and absence of experimental agents was determined using the standard 3-(4,5-dimethylthiazol-2-yl)-2,5-dimethyltetrazolium bromide (MTT, Sigma, St. Louis, MO) assays as described previously. In brief, exponentially growing cells were harvested and a 50 íL suspension containing 2500 cells was plated in 96-well microtiter plates (Falcon, Becton Dickinson, NJ). After 24 h of incubation at 37°C under 5% CO_2 to allow cell attachment, the cells were treated with varying concentrations of test specimens in their respective medium (100 íL) and incubated for 4 days under the same conditions as above. After adding a solution of MTT for 4 h, the amount of formazan formed was measured spectrophotometrically at 590 nm using Immuno Mini NJ-2300 plate reader. The inhibition Concentration, IC_{50} which is the drug concentration that inhibits 50% of cell lines growth, was determined from the graph. The experiment was conducted in triplicates.

Data analysis: The absorbance of the formazan solution at 590 nm was determined on the spectrophotometer. IC_{50} values (dose of drug that produces a 50% reduction in the absorbance compared to the control) were determined from the dose-response cytotoxic curves. Data obtained were analyzed using the 2-way Analysis Of Variance (ANOVA) and Duncan′s Mean Separation Test using the percentage of cell viability and IC_{50} values as parameters.

RESULTS

Propolis is a resinous product collected by bees (*Apis mellifera*) from tree exudates, mainly resins of leaf buds mixed with beeswax to form a sealing material in their honeycombs, smooth out the internal walls and protect the entrance from intruders[5,7]. Propolis samples from tropical zones, such as Indonesia with its vast biodiversity, have become a subject of increasing scientific and economic attention. The association of the chemical composition of propolis from different geographic regions with biological activities lead to the identification of active principles, a fundamental tool to achieve standardization of this bee product[14]. Propolis cytotoxicity on cultures of human and animal tumor cells, including breast carcinoma, melanoma, colon and renal carcinoma cell lines[6].

Table 2 lists cytotoxicity activity of propolis of cancer cell lines, i.e., T47D, MCF-7, Hela, Myeloma and Vero. The results indicated that the extract of propolis from Batang showed the most potent activity of T47D and MCF-7 with IC_{50} 34.67±8.3 and 37.8±2.45 µg mL^{-1}. The extract of propolis from Sukabumi showed the most potent activity of Hela cell with IC_{50} 147.34±8.9. However, all propolis extract did not show activity of Myeloma and Vero cells. This indicated that the secondary metabolite extract obtained in the study has antiproliferative activity of breast carcinoma cells (T47D and MCF-7). T47D and MCF-7 cell containing Estrogen Receptor (ER) were used as a model for studying the events associated with response to chemotherapy of breast cancer cell[12].

DISCUSSION

Although the components of propolis responsible for its cytotoxic action were not investigated in this research, Present study opens a new perspective for further investigation. A large amount of work dealing with antitumor action of propolis and its components indicates their promising usefulness and claims for new Propolis samples were collected from three regions in Java area (Indonesia), Batang (Central Java), Lawang

(East Java) and Sukabumi (West Java), each them characterized by some types of pre dominant trees or shrubs. The chemical composition of the three Java samples was investigated using GC-MS after silylation. Each individual substance is presented in Table 1.

The peak numbers in the Table 1 are given according to the retention time only to the major peaks. The following compounds were identified for the first time in propolis: 1,3-bis(trimethylsilylloxy)-5,5-proylbenzene, 3,4-dimethylthioquinoline, 4-oxo-2-thioxo-3-thiazolidinepropionic acid, D-glucofuranuronic acid, dofuranuronic acid, patchoulene and 3-quinolinecarboxamine. Four new sugar and sugar derivates have been identified: arabinofuranose, D-ribose, threitol and arabinitol. Silanol is also new compound which is found in propolis. The variability of constituents of propolis in three samples showed that they were collected by the honeybee from different plants depending on the geographic location[9]. The specific composition of plant population varies as a function of elevation, soil type and moisture. The plant population include many native and introduced species of flowering trees, shrubs and herbs[13]. The plant origin of propolis determines its chemical diversity. Bee glue′s chemical composition depends on the uniqueness of the local flora at the site of collection and thus on the geographic and climatic characteristics of this site. This fact results in the strikling diversity of propolis chemical composition, especially of propolis originating from tropical regions[1].

The three samples of propolis in the research have one thing in common. All of them contain phenolic acids. Phenolics acids are considered to play a positive role in the prevention of human diseases. As a popular traditional medicine, propolis is a rich source of caffeic acid. The active components of the propolis responsible for its clinical usages were extensively studied[8]. The Batang sample showed the presence of groups of aromatics acids, terpenes and quinoline. The 3,4-dimethylthioquinoline and 3-quinolinecarboxamine were identified in the ethanolic extracts and also new in propolis. The existence of quinoline as 3,4-dimethylthioquinoline and 3-quinolinecarboxamine in Batang Propolis indicated that there could be another plant source for propolis which need more investigation.

In contrary to Batang sample, The Lawang sample contains very high amounts of aromatic acids, where the dominant vegetation was native plant and *C. petandra* and *E. longam* trees. The 4-oxo-2-thioxo-3-thiazolidinepropionic acid and glucofuranuronic acid were found for the first time in propolis. It also contained terpene which is also new to propolis: patchoulene.

Table 1: Chemical composition of ethanolic extracts of Batang, Lawang and Sukabumi propolis samples (percentage of total ion current, GC-MS)

Compound	Batang	Lawang	Sukabumi
Aliphatic acids			
Hexadecanoic acid	-	-	0.72
Aromatic acids			
Benzoic acid	-	0.41	-
Phenylic acid	94.22	95.62	94.51
Dofuranuronic acid	-	-	0.32
D-glucofuranuronic acid	-	0.56	-
1,3-bis(trimethylsilyloxy)-5,5-propilbenzene	2.40	-	-
4-oxo-2-thioxo-3-thiozolidinepropionic acid	-	0.79	-
Terpenes			
Abietic acid			
1-Naphtalenemethanol	3.76	95.62	-
Patchoulene	-	0.27	-
	-	0.27	-
Quinoline			
3,4-dimethylthioquinoline			
3-quinolinecarboxamine	0.53	-	-
Sugars and alcoholic sugars			
D-mannopyranose			
D-xylose	0.31	-	-
Arabinofuranose	0.24	-	-
D-ribose	0.23	-	-
D-galactose	0.15	-	-
D-mannitol	-	0.51	-
D-glucitol	-	-	1.62
Erythritol	-	-	1.62
Threitol	0.81	0.86	0.88
Arabinitol	-	-	0.86
	-	-	0.81
Others			
Glycerol	0.81	0.86	0.88

Table 2: IC_{50} values (µg mL^{-1}) of ethanolic extract of propolis from different area in Java in Hela, myeloma, Vero, MCF-7 and T47D cell lines. Values are mean of 3 separate determinations and errors represent the SD values

	IC_{50} of ethanolic extract of propolis		
Cell lines	Batang (Central Java)	Lawang (East Java)	Sukabumi (West Java)
T47D	34.670±8.3	267.67±9.3	367.81±8.7
Hela	589.31±4.1	674.35±3.8	147.34±8.9
Myeloma	>1000	>1000	>1000
Vero	>1000	>1000	>1000
MCF-7	37.8±2.5	178.45±6.3	276.45±9.8

The Sukabumi propolis sample was gathered from a bee hive situated near the mountains at about 700 m altitude, temperature 20-26°C and the humidity in the ranges of 85-96%. In this area there are relatively high number *C. petandra* and *H. brasiliensis* trees. It contained very low amounts of aromatic acids. From those samples, there is also silanol compound which is found for the first time in propolis. Silanol is polysiloxanes which are the most common and one of the most important polymer chemistry. Probably this is

because the propolis sample was taken from the area where rubber plants grow. The extract latex product from Hevea rubber plants is polydimathylsiloxane elastomer.

CONCLUSION

From the results of the GC-MS-MS analysis we conclude that Ethanolics extract of three propolis samples from Batang (Central Java), Lawang (East Java) and Sukabumi (West Java) regions in Java were investigated using GC-MS. From 37 compounds identified, 7 among of them were found for the first time in propolis. A large amount of work dealing with cytotoxic activity of propolis and its claims for new investigations. In order to explore propolis's potential as a cancer chemopreventive and chemotherapeutic agent, our laboratory is investigating propolis action in breast carcinoma cells.

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