

Apoptosis of human Breast Cancer Cells induced by Ethylacetate Extracts of Propolis

¹Syamsudin, ²Partomuan Simanjuntak, ³Ratna Djamil and ⁴Wan Lelly Heffen

¹Department of Pharmacology, Faculty of Pharmacy, Pancasila University,
Jl. Srengseng Sawah, Jagakarsa, Indonesia

²Department of Natural Product Chemistry, Research Centre for Biotechnology,
Indonesian Institute of Science, Jln. Raya Bogor Km 46, Cibinong 16911, Indonesia

³Department of Research and Development, Dharmais Cancer Hospital,
Jl. Letjen. S. Parman. Kav 84-86, Jakarta, Indonesia

⁴Department of Biological Pharmacy, Faculty of Pharmacy, Pancasila University,
Jl. Srengseng Sawah, Jagakarsa, Indonesia

Abstract: Problem statement: Propolis has been ethno medically claimed to possess a wide array of biological activities including anticancer activity. The purpose of this research was to verify the folklore claim. **Approach:** This study was performed in a human breast carcinoma cell, MCF-7. Extract of propolis from different solvent, ethylacetate and n-butanol showed induced apoptotic cells was detected by flow cytometry. **Results:** The results demonstrated that ethylacetate extract of propolis can induce apoptosis in MCF-7 as large as 13.21% during the 24 h incubation. On the other hand, doxorubicin is able to induce apoptosis as large as 18.89% during the 24 h incubation. **Conclusion:** The extracts of propolis ethylacetate had cytotoxic activity and triggers apoptosis on MCF-7 cells.

Key words: Apoptosis, propolis, human breast cancer cell

INTRODUCTION

Apoptosis is cellular suicide or programmed cell death which is mediated by activation of an evolutionary conserved intracellular pathway. Recently the relation of apoptosis and cancer has been emphasized and increasing evidence suggests that the processes of neoplastic transformation, progression and metastasis involve alterations of normal apoptotic pathway (Bold *et al.*, 1997). Apoptotic also gives some clues about effective anticancer therapy and many chemotherapeutic agents were reported to event their anti-tumor effect including apoptosis of cancer cells.

Propolis (bee glue) is a natural resinous product of honey bees. The cancer inhibitory effects of phenolic compounds in propolis have been confirmed on a variety of culture cell lines and animal tumor models (Aso *et al.*, 2004; Scheller *et al.*, 1989). Epidemiological and preclinical evidence suggest that phenolic and polyphenolic photochemical in propolis possess cancer chemo preventive properties (Kwon *et al.*, 2007; Tsao *et al.*, 2004; Orsolich *et al.*, 2005). This has

led to an increased emphasis on cancer prevention strategies in which propolis is used as dietary supplement as the richest source of plant phenolics and polyphenolics). The chemical composition of propolis varies according to the plants that can be found in as pefic regions (Ghisalberti, 1979). Although numerous researches have been reported the biological activities of propolis collected in Europe, Brazil and Egypt, information about Indonesian propolis are still limited. Previous study previous study shows that the methanol extracts of propolis from Batang (Central Java) has selectivity on breast cancer cells, MCF-7 and T47D compared to other cancer cells. The objective of this study is to evaluate the ant proliferative effect of propolis and the mechanism of apoptosis in MCF-7 human breast cancer.

MATERIALS AND METHODS

Sample collection: Propolis samples were collected from Batang (Central Java, one of province of Indonesia). Hand collected propolis samples were kept dessicated in the dark up to their processing.

Corresponding Author: Syamsudin, Department of Pharmacology, Faculty of Pharmacy, Pancasila University,
Jl. Srengseng Sawah, Jagakarsa, Indonesia

Sample extraction: Four hundred gram of each sample was cut into small pieces and extracted at room temperature with 1 Lt of 70% ethanol (twice after 24 h). The ethanolic extracts was evaporated under vacuum at 50°C until dryness. The extracts was fractionated using the mixture of ethylacetat and water (1:1) inside a separating funnel. Later, the ethyl acetate layer was separated from water layer. The water layer was refractionated using n-buthanol inside a separating funnel. The n-buthanol layer was separated from the water layer. The ethylacetate, n-buthanol and water phases were concentrated in a vacuum evaporated and dried until the weights were stable.

Morphological studies of MCF-7 cells: Morphological studies by using the normal inverted microscope out in order to observe the morphological changes of cell death in MCF-7 cells elicited by the ethylacetate and n-buthanol extracts. The concentration of the IC₅₀ value of the respective extracts of propolis was used for the morphological studies.

Cell culture and assay for cytotoxic activity: The human breast cancer, MCF-7 cell lines were supplied by Cancer Hospital Center Dharmais, Jakarta, Indonesia. The human tumor cytotoxicities were determined following protocols established by the NCI (Monks *et al.*, 1991). 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) exponentially growing cells were harvested and a 50-iL 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% CO₂ to allow cell attachment, the cells were treated with varying concentrations of test specimens in their respective medium (100 iL) 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, IC₅₀ which is the drug concentration that inhibits 50% of cell lines growth, was determined from the graph. The experiment was conducted in triplicates.

Annexin/propidium iodide-staining: The number of apoptotic cell death induced by ethylacetate and n-buthanol extracts of propolis was measured by flow cytometry. The treated and untreated MCF-7 cells were harvested and washed with cold PBS. An aliquot (10⁵ cells/100 µL) of cell suspension was added with 1 µL

Fluorescein Isothiocyanate (FITC)-conjugated annexin-V and 2.5 µL Propidium Iodide (PI; 250 µg mL⁻¹). After 10 min incubation on ice, the cells were measured immediately.

Data analysis: All data are presented as mean ± SD. The mean values were calculated from obtained by no less than three independent experiments.

RESULTS

Prior to measurement of cytotoxic effects of different extracts of propolis to MCF-7 cells, we analyzed the pattern of MCF-7 cell growth. Since the cells were exponentially growing during 5 days. MCF-7 cells were incubated for 48 h after treatment with ethylacetate, n-buthanol and aqueous extracts of propolis or doxorubicin. Following appropriate incubation live cells were analyzed by MTT assay. The effect of the propolis extracts on the growth of MCF-7 cells is shown in Fig. 1, where the ethylacetate-extract-induced-MCF-7 cells growth depended on the doses. The IC₅₀ value of the ethylacetate extract is stronger than those of n-buthanol and water extracts, having IC₅₀ >200 µg mL⁻¹ (Fig. 1).

According to the results of cytotoxicity measurements IC₅₀ of ethyl acetate extract of propolis was 47.45 µg mL⁻¹. The occurrence of morphological change of the treated cells indicated the cytotoxicity effect of the fraction Fig. 1. To understand the effect of the fraction on growth inhibition and apoptosis induction, the proliferation kinetic and apoptosis assay were carried out at inhibitory.

Many chemical compounds can inhibit the growth of tumor cells, but not all of them can trigger apoptosis. To determine whether apoptosis was induced by the compounds mentioned above, we performed flow cytometric analysis with Annexin V-FITC conjugated to Propidium Iodide (PI).

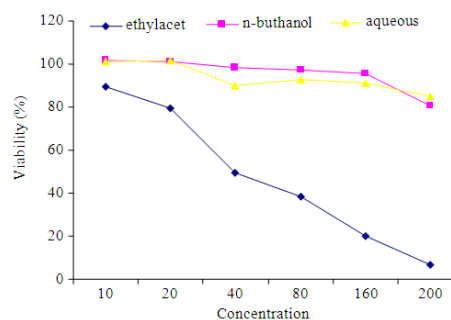


Fig. 1: Effect of propolis extract on the survival rate of MCF-7 cells

DISCUSSION

Natural products play an important role in chemotherapy, having contributed considerably to approximately 60 available cancer chemotherapeutic drugs. The need to develop more effective anti-tumor drugs has prompted investigators to explore new sources of pharmacologically active compounds, especially from propolis. Propolis (bee glue) is a natural resinous product of honey bees. The cancer inhibitory effects of phenolic compounds in propolis have been confirmed on a variety of culture cell lines and animal tumor models (Chen *et al.*, 2003; Suzuki *et al.*, 2002). Epidemiological and preclinical evidence suggest that phenolic and polyphenolic phytochemicals in propolis possess cancer chemo preventive properties (Nada *et al.*, 2007). This has led to an increased emphasis on cancer prevention strategies in which propolis is used as dietary supplement as the richest source of plant phenolics and polyphenolics.

Apoptosis also seems to be a reliable marker for the evaluation of potential agents for cancer prevention. A wide variety of natural compounds appear to possess significant cytotoxic as well as chemo preventive activity. Flowcytometry was used to evaluate the behaviour of cells treated with apoptotic agents. Annexin V-FITC-positive, PI-negative (Annexin V-FITC (+) PI (-)) cells were considered to be in an early apoptotic stage, while Annexin V-FITC-positive, PI-positive (Annexin V-FITC (+) PI (+)) cells were considered to be late apoptotic or necrotic (Fig. 2). The Fig. 3 shows that the ethyl acetate extract of propolis at the concentration of 47.45 $\mu\text{g mL}^{-1}$ can induce apoptosis on MCF-7 cells as large as 13.21% using flow cytometry method, whereas the doxorubicin at the concentration of 145.57 nM is able to induce apoptosis as large as 18.89% during the 24 h incubation. The morphological changes observed using the normal inverted microscope show characteristic rounding off of dying cells on treatment at IC_{50} concentration with EEP and doxorubicin for 24 h compared to untreated control (Fig. 4).

The major active components of propolis are flavonoids and phenolic acids present in EEP, they have many biological and pharmacological activities including immunopotential and antitumor effects. Phytochemical screenings reveal that moderate concentration of flavonoids, saponins, terpenes and steroids some of which chemical compounds have been associated to cytotoxic activities. This reveals moderate concentration of flavonoids, saponins, terpenes and steroids some of which chemical compounds have been associated to cytotoxic activities. The study by Li *et al.*

(2007) showed that propolis inhibits cellular proliferation and induces apoptosis in prostate cancer cells. The flavonoids and phenolic components found in propolis are known to affect the apoptosis of prostate cancer cells and may play an important role in cancer chemoprevention (Bankova, 2005).

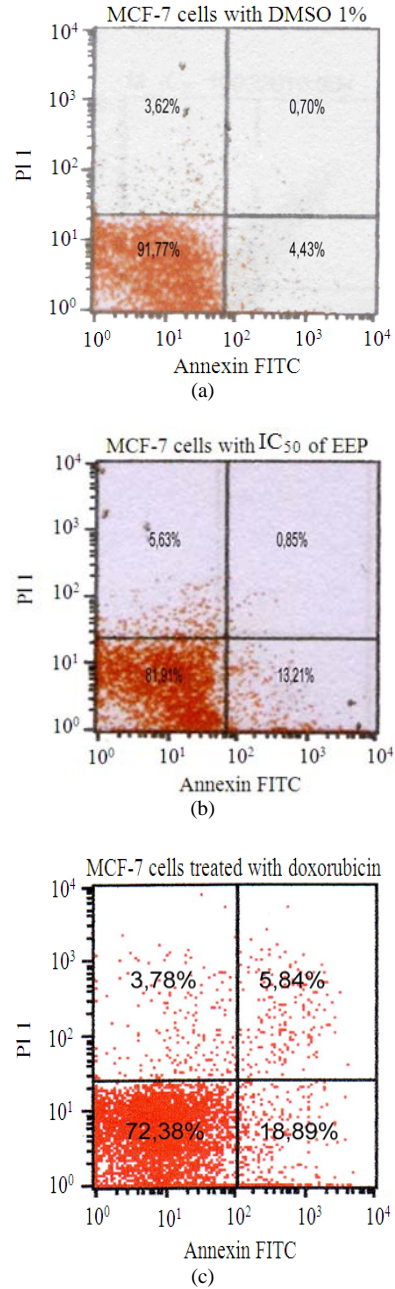


Fig. 2: Ethylacetate Extract of Propolis (EEP) induction of apoptosis of MCF-7 cells demonstrated by annexin V staining

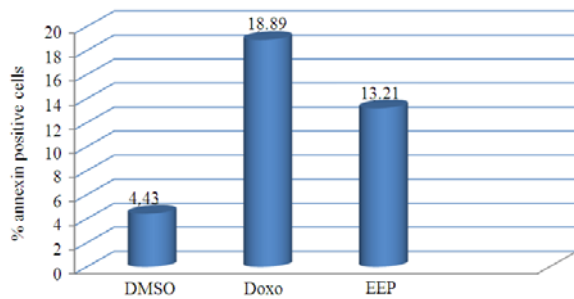
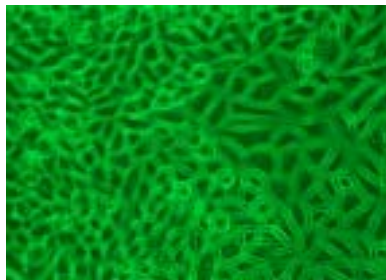
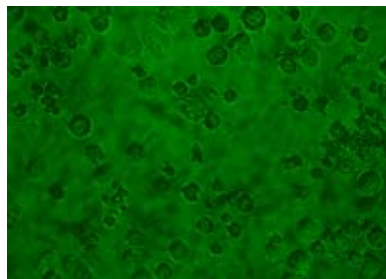


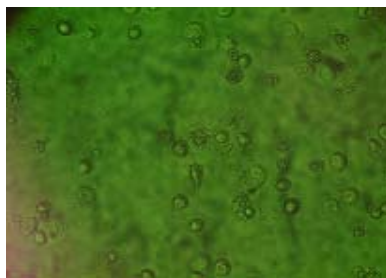
Fig. 3: MCF-7 cells were treated with DMSO, doxorubicin, EEP with IC₅₀ of EP for 24 h, apoptotic cells were demonstrated by determining the number of annexin V positive cells



(a)



(b)



(c)

Fig.4: (A) Morphological features of MCF-7 (B) The changes observed after 24 h treatment at IC₅₀ concentration with EEP (C) The changes observed after 24 h treatment at IC₅₀ concentration with Dox (100 x magnification)

This research shows the potential of the ethyl acetate extract of propolis as an anticancer agent to inhibit cell proliferation and trigger apoptosis. Even though the EEP cytotoxic activity is lower than that of conventional chemotherapy agent such as doxorubicin, the EEP IC₅₀ value is quite promising to be developed as a chemoprevention agent if we consider that this research used MCF-7 known to have high resistance to several chemotherapy agents. MCF-7 cells have low sensitivity against doxorubicin with IC₅₀ 148 nM. The resistance of MCF-7 against doxorubicin is due to the increase of Pgp expression which pumps doxorubicin out of cells. This also happens in other cancer cells (Patel and Tannock, 2009).

CONCLUSION

The ethylacetate extract of propolis shows sufficiently strong cytotoxicity on MCF-7, with the IC₅₀ 47.45 µg mL⁻¹ and triggers apoptosis of MCF-7 cells.

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