ANTICANCER ACTIVITY OF ROYAL JELLY *Apis mellifera* AGAINST WIDR CELL LINE AND HEla CELL LINE

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Abstract — Cervical cancer is the most common cause of women’s death after breast cancer in Indonesia, meanwhile colon cancer is the second leading cause of death from cancer among adults. One of the efforts for cancer treatment is to consume natural compounds such as Royal jelly, which has been reported to have anticancer activity. Recent studies also indicated Royal jelly action against breast cancer cell line MCF-7 1 mg/mL and colon cancer cell line CaCo-2 0.5 mg/mL. This research aimed to determine cancer cell inhibition activity by Royal jelly toward cervical cell line HeLa, colon cell line WiDr and normal cell line Chang. The total phenol test, cell culture, WiDr cell preparation, Royal jelly treatments and MTT cytotoxic tests were carried out. Results showed that Royal jelly inhibited HeLa cell proliferation 36.425% and WiDr cell line 16.625% at 125 µg/mL.

Keywords — Royal jelly, antioxidant, widr and hela cell lines
INTRODUCTION

Colon cancer is a cancer that grows and develops in the colon or rectum which causes weight loss, changes in cancer structure and bowel function in digestion, and fatigue which results in decreased productivity (Karim and Huso, 2013). Colon cancer risk factors are related to lifestyle, age and genetic disorders (Yeatman, 2001). According to Haggar and Boushey (2009), colon cancer sufferers in 2005 were 108,100 individuals and in 2008 increased to 148,900 diagnoses. Approximately 49,900 patients die in the United States from colon cancer. The metastatic nature of colon cancer causes this disease to only have a survival rate of less than 5 years at stage 4 (<10%) (Yu et al., 2015).

Cervical cancer is a malignant neoplasm of cervical cells. Cervical cancer generally affects women and is caused by infection with the Human Papillomavirus (HPV) (Jadon and Joshi, 2012). Human Papillomavirus is a DNA virus that infects the basal epithelium such as skin and mucosa causing cervical cancer (Cuts et al., 2007). According to Sreedevi et al. (2015), the incidence of cervical cancer mostly occurs in developing countries (86%). Every year, about 122,844 women are diagnosed with cervical cancer and 67,477 (54.5%) die. The incidence of colon cancer in Indonesia is the third most common type of cancer in Indonesia with 100 cases diagnosed from 100,000 Indonesians in 2006 (Ministry of Health, 2006). The incidence of cervical cancer in Indonesia is around 0.8% with 98,062 patients diagnosed, and the highest prevalence occurs in the Riau Islands Province, North Maluku and DI Yogyakarta (Ministry of Health, 2015).

At least patients who receive medical treatment for cancer in Indonesia is still relatively expensive, so it is necessary to develop affordable cancer therapies for cancer patients, especially from the lower middle class. Chemotherapy is the administration of chemical compounds to reduce and inhibit the proliferation of cancer cells, but this method has side effects such as weakness, nausea, hair loss, dry skin, and drastic weight loss and can damage the formation of normal cells around it. Anticancer herbal drugs that have been approved by the FDA include vinca alkaloids, taxane, podophilotoxin and camptothekin (Safarzadeh et al., 2014).

Royal jelly has been known to have biological benefits such as antioxidant, neurotopic, hypocholesterolemic, anti-aging, antibiotic, anti-inflammatory, anti-immunodulator, and antitumor (Karadeniz, 2011). Royal jelly contains long chain fatty acids, namely 10-hydroxy-2-decanoyl acid which acts as an antitumor (Townsend et al., 1960). Antitumor effect plays a role in the process of cancer cell proliferation. According to Barnutiu et al., (2011), other active components of Royal jelly are low protein fraction (major Royal jelly fraction), namely MRJP 1-MRJP 6, vitamins (L-ascorbate, vitamin D, vitamin E, vitamin B complex), royalisin, apismanin, and albumin.

There has been no research that uses Royal jelly as a substance that inhibits the proliferation of colon cancer cells and cervical cancer cells. Therefore, this study aimed to examine the cytotoxic potential of Apis mellifera Royal jelly on cervical cancer cells (HeLa) and colon cancer cells (WiDr) and liver cells (Chang) as normal control cells. This research is expected to provide data on the antiproliferative activity of Royal jelly against sustainable cancer cells for the development of anticancer drugs in the future.

MATERIALS AND METHODS

Materials

The ingredients used in this study were Apis mellifera Royal jelly from a beekeeping farm in Wonogiri, Central Java and commercial Royal jelly brand Spring Leaf.
Australia, sterile distilled water, NaCl 10% w/v, sodium bicarbonate 7.5% w/v, FeCl3 reagent, Merck Folin Ciocalteu reagent, dimethyl sulfoxide (DMSO) CTCC® 4-X™ Merck, methanol 99% Merck, diphenyl-2-picrylhydrazyl (DPPH) 0.4 mM Sigma Aldrich, L-ascorbic acid Sigma Aldrich, cancer cells colon (WiDr, ATCC®-CCL™ 218), HeLa cancer cells (HeLa, ATCC®-CCL-2™) and normal liver cells (Chang, ATCC®-CCL™ 13) were obtained from the American Type Culture Collection, doxorubicin Kalbe as positive control, Fetal Bovine Serum (FBS) Gibco, Roswells Park Memorial Institute (RPMI) medium 1640 Gibco, Dulbecco’s Modified Eagle (D-MEM) Gibco medium, penicillin-streptomycin, 3-(4,5 dimethylthiazole-2-yl)- 2,5-diphenyl tetrazolium bromide (MTT) Sigma Aldrich, 2-(N-morpholino)-ethanosulphonic acid buffer Sigma Aldrich, 10% trypsin Gibco, 60% ethanol, Axygen T-300 Scientific white tips pipette, Axygen TR-222-Y Scientific yellow tips pipette, Stardec blue tips pipette, Whatman parafilm, Kinpak plastic wrap, and Ansell gloves.

Methods

In this study, the total phenol test was carried out using the Folin Ciocalteu method (Vongsak et al. 2013) and the anticancer activity of Royal jelly using the MTT method in the study (CCRC 2000). Royal jelly was tested in vitro against normal liver cells (Chang, ATCC®-CCL™ 13), colon cancer cells (WiDr, ATCC®-CCL™ 218), and cervical cancer cells (HeLa, ATCC®-CCL-2™). The inhibitory activity and the percentage of the number of cells before and after treatment of cancer cells against Royal jelly showed anticancer activity.

Preparation of Royal jelly Stock Solution (Bramasta 2013 Modification)

Royal jelly was dissolved in various types of solvents, namely distilled water, RPMI medium and D-MEM according to the test to be carried out. Ten (10) mg of Royal jelly was dissolved in 5 mL of solvent, then a sonicator was carried out for 10 minutes until the sample was completely dissolved and then 5 mL of solvent was added to a total volume of 10 mL with a stock solution concentration of 1000 g/mL. The 1000 g/mL Royal jelly solution was then diluted 10 mL to a final concentration of 50 g/mL which would be used for various tests such as total phenolic, and MTT.

Determination of Total Phenolics (Vongsak et al., 2013)

Determination of total phenolic Royal jelly was carried out by modifying the method of Vongsak et al. (2012). A total of 200 L of Royal jelly solution from a stock solution of 100 g/mL Royal jelly, added 500 g/mL Folin Ciocalteu reagent 10% v/v, and added 300 g/mL sodium bicarbonate 7.5% w/v to a final concentration of 0 g/mL, 4 g/mL, 8 g/mL, 12 g/mL, 16 g/mL, and 20 g/mL in test tubes. The test tube was read at room temperature (22 °C ± 1 °C) for 30 minutes. The absorbance of the sample was calculated at a wavelength of 760 nm with distilled water blank. Total phenol is expressed in milligrams equivalent of gallic acid. The standard curve used was gallic acid 0 g/mL, 4 g/mL, 8 g/mL, 12 g/mL, 16 g/mL, and 20 g/mL.

Cell Culture (Haryanti and Katno, 2011)

The sustainable cells of Widr, HeLa and Chang are a collection of the Microbiology and Immunology Laboratory of the Center for Primate Studies, LPPM, Bogor Agricultural University. Cell cultures were grown in RPMI 1640 Gibco growth medium containing 10% v/v FBS and 1% v/v Gibco penicillin-streptomycin. Meanwhile, for Chang and HeLa cells, Gibco’s D-MEM growth medium was used.

WiDr Cell Preparation (Filipic et al., 2015 Modification)

Widr cells were taken from the nitrogen
tank and thawed in a water bath at 37 °C. The ampoules were sprayed using 70% ethanol and put in a laminar air flow. The ampoule was opened and the WiDr cells were transferred to a sterile conical tube containing RPMI 1640 medium. The cell suspension was centrifuged at 1000 g for 3 minutes. New RPMI 1640 medium was added to the cell suspension and centrifuged for 5 minutes. WiDr cell suspension was added with 1 mL of medium containing 10% FBS and resuspended slowly until homogeneous. WiDr cells were added to a small tissue culture flask and incubated in a 37 °C CO2 incubator. WiDr culture medium was replaced after 24 hours and grown until the cell population reached 80% (80% confluent). WiDr cells that had reached 80% population were washed with 3.5 mL of PBS 2 times and 300 µL of Trypsin-EDTA and then incubated for 3 minutes in a CO2 incubator. Five (5) mL of the culture medium was added and the cells were resuspended to separate from the flask wall. WiDr cells were counted using a hemacytometer. A similar way of working was also carried out for Chang cells and HeLa cells by changing the RPMI 1640 Gibco medium to D-MEM Gibco medium.

**MTT Cytotoxic Test (CCRC 2000)**

The results of the 48-hour cell incubation from the previous method were added to the 5 mg/mL tetrazolium salt solution as much as 10 µL per well. The mixed color is yellow. Incubate the microplate for 4 hours in a 5% CO2 incubator at 37 °C. After incubation and formazan crystals formed, the Royal jelly solution was discarded. Formazan crystals were dissolved in 100 µL of 96% ethanol in each well. The color of the solution became purple. The absorbance value was measured on a microplate reader at a wavelength of 595 nm. All treatments were carried out in triples. The data obtained from the proliferation test with MTT is the absorbance value of each well which is converted to % inhibition.

**RESULTS AND DISCUSSION**

Royal jelly samples used came from 2 different places, namely Royal jelly from Wonogiri, Central Java and Melbourne, Australia. Determination of the total phenol content of Royal jelly using gallic acid standard (Figure 1). The concentration of gallic acid on the average absorbance data at a wavelength of 760 nm produces a linear standard line equation of \( y = 0.032x + 0.016 \) with a regression value (R2) of 0.960. Total phenol Royal jelly is expressed in milligrams gallic acid equivalent per milligram Royal jelly (mg GAE/mg). The total phenol content of Royal jelly found in the Wonogiri Royal jelly sample was 116.06 mg GAE/mg ± 0.03 (as much as 1 mg of Royal jelly was equivalent to 116.06 mg of gallic acid, while Australian Royal jelly had a total phenol of 54.81 GAE/mg ± 0.58 mg (as much as 1 mg of Royal jelly is equivalent to 54.81 mg of gallic acid).
Anticancer testing began with the cytotoxicity test of Royal jelly on normal cells using the MTT method. This test aims to determine the selectivity of Royal jelly against normal cells. According to Wang et al. (2010), formazan color intensity correlated with the number of living cells. The normal cells used are Chang cells. According to Otang et al. (2014), Chang cells were derived from normal liver tissue obtained from a boy of Chinese descent, and isolated in 1954. Chang cells were used to model cellular cytotoxicity activity.

The effect of Royal jelly on normal Chang cells was expressed in % proliferation (Figure 2). The percentage of proliferation indicated an increase in the number of cells after adding Royal jelly for 48 hours of incubation. The results of the percentage of cell proliferation of Chang against Royal jelly showed an increase in the percentage of proliferation with an increase in the concentration of the solution. The addition of Royal jelly resulted in an increase in the number of cells. The highest % proliferation was found at a concentration of 375 µg/mL at 42.30% ± 13.01 while the lowest % proliferation was at a concentration of 125 µg/mL at 12.12% ± 8.75 and for a concentration of 250 µg/mL at 21.90% ± 6.80.

The effect of Royal jelly on Chang cells was expressed by % proliferation (Figure 2). Increasing the concentration of Royal jelly did not inhibit Chang’s normal cell growth. Increasing the concentration will further increase the number of normal cell populations in the microplate. Therefore, Royal jelly is non-toxic to normal cells and induces new cell growth.

The results of the induction of Chang’s cell proliferation by Royal jelly in this study are in accordance with the research conducted by Kamakura et al. (2001) which states that the active protein substance of Royal jelly can trigger the proliferation of mouse hepatocytes and increase the formation of blood albumin. Hattori et al. (2007) reported the activity of neurogenesis or the formation of nerve cells by Royal jelly against nerve stem progenitor cells in fibroblast growth factor (FGF-2) medium. Royal jelly can also trigger the differentiation and proliferation of brain nerve cell progenitors into neurons, astrocytes, and oligodendrocytes. Royal jelly can also trigger the growth of MC3T3-E1 cells and bone osteoblasts of mice induced by Royal jelly for 9 weeks (Narita et al., 2014).

The effect of Royal jelly on WiDr colon cancer cells was expressed in % inhibition (Figure 3). The percentage of inhibition indicated a decrease in the number of cells after the addition of Royal jelly with a certain concentration. The results of % inhibition of WiDr cells decreased with the increase in the concentration of Royal jelly solution. The highest percentage decrease in the number
of WiDr cells was found at a concentration of 125 µg/mL at 16.65% ± 4.80 while the lowest % inhibition was at a concentration of 375 µg/mL at 11.51% ± 5.31. The effect of Royal jelly on HeLa cervical cancer cells was also expressed in % inhibition (Figure 3). The percentage decrease in the number of HeLa cells after being treated with Royal jelly at various concentrations decreased along with the increase in concentration, such as WiDr cells. The result of the highest % inhibition of HeLa cells was at a concentration of 125 µg/mL at 36.42% ± 7.90, while the lowest was at a concentration of 375 µg/mL at 31.03% ± 2.70.

A positive control of doxorubicin was used on WiDr cells and HeLa cells. Controls were used to compare the anticancer activity of Royal jelly against WiDr cells and HeLa cells. The results of % inhibition of doxorubicin positive control (Figure 4) showed that HeLa cells were more sensitive to doxorubicin than WiDr cells. Doxorubicin killed more HeLa cells than WiDr cells at the same concentration.

The effect of doxorubicin on WiDr cells showed an increase in % inhibitory activity (Figure 4). The concentrations used were 1 µg/mL, 3 µg/mL, and 6 µg/mL. The highest % inhibition result was at a concentration of 6 g/mL of 91.92% ± 0.76 while the lowest was at a concentration of 1 µg/mL of 73.65% ± 4.00. Positive control concentration of 1 g/mL was able to kill >50% WiDr cells. The positive control effect of doxorubicin on HeLa cells also showed an increase in % inhibition with increasing concentration (Figure 4). The highest % inhibition result was 93.60% ± 1.38 at a concentration of 6 µg/mL, while the lowest was 81.68% ± 2.40 at a concentration of 1 µg/mL. The use of low concentrations of doxorubicin has been able to reduce the HeLa cell population >50%. Based on the % inhibition data in Figure 4, doxorubicin kills HeLa cancer cells more than WiDr cells. This is because at a concentration of 1 g/mL, doxorubicin was able to kill HeLa cells >80% compared to 70% WiDr cells.

Fig. 3. Percentage of Royal jelly inhibition against WiDr cells (⃣) and HeLa cells (◼) at 3 different concentrations.

Fig. 4. Percentage of doxorubicin inhibition against WiDr cells (⃣) and HeLa cells (◼) at 3 different concentrations.

The positive control used in this study was doxorubicin. The drug is a commercial drug for chemotherapy. Chemotherapy is a systematic therapy by giving synthetic drugs to inhibit the growth of cancer cells (Jong, 2002). Doxorubicin is an anthracycline antibiotic that has antineoplastic activity, isolated from Streptomyces peucetius var. caesius (NCI, 2009). The mechanism of doxorubicin in killing cancer cells is binding to DNA and interfering with topoisomerase II activity involved in DNA repair reactions, formation of free radicals that disrupt cellular membranes, DNA and proteins (Thorn et al. 2011), and the formation of excess...
ceramides. Exogenous ceramides can induce cancer cell death through apoptosis (Yang et al. 2014).

Chang’s cell morphology (Figure 5), WiDr cells (Figure 6), and HeLa cells (Figure 7) showed different shapes and numbers. The number of Chang cells after Royal jelly treatment appeared to increase, and live cells were adherent. The morphology of HeLa cells and WiDr cells that have shown an inhibitory effect is characterized by abnormal cell shape compared to normal cells. Abnormal cell shape such as irregular and separated from living cell colonies.

The morphology of Chang cells, WiDr cells, and HeLa cells were observed under a microscope with a magnification of 1430 x 640. Observations were made twice, namely before and after incubation of cells using Royal jelly. Chang and HeLa cells are elongated with tapering ends, while WiDr cells tend to be rounded. The results of observations of Chang’s cells under a microscope showed an increase in the number of cells before and after Royal jelly treatment (Figure 5). The number of cells at a concentration of 0 g/mL was 46.5 x 10^4 cells/100 L, while the number of cells at a concentration of 125 g/mL, 250 g/mL and 375 g/mL respectively were 53.9 x 10^4 cells/100 L, 56.7 x 10^4 cells/100 L, and 66.16 x 10^4 cells/100 L.

Observations of WiDr cells under a microscope showed changes in cell morphology (Figure 6). Changes between concentrations of 0 g/mL as control cells and treatment (125 µg/mL, 250 µg/mL and 375 µg/mL) were abnormal cell shape after Royal jelly treatment, such as irregular cell shape, and dead cells separated from cell colonies. The number of WiDr cells at a concentration of 0 g/mL was 71 x 10^4 cells/100 L, while the number of WiDr cells at a treatment concentration of 125 g/mL, 250 g/mL and 375 g/mL was 59.18 x 10^4 cells/100 L, 61.20 x 10^4 cells/100 L, and 63.12 x 10^4 cells/100 L. Observation of WiDr cells on doxorubicin (Figure 8) showed that the number of dead cells was higher than that of Royal jelly samples. Microscopic observations of WiDr cells before and after Royal jelly treatment showed changes in cell morphology, such as in WiDr cells in the form of abnormal cell shapes (Figure 8).

Abnormal cell shape is indicated by black arrows. The number of HeLa cells at a concentration of 0 g/mL was 48 x 10^4 cells/100 L, while the number of HeLa cells at various concentrations used in this study were 30.52 10^4 cells/100 L, 32.62 10^4 cells/100 L, and 33.05 10^4 cells/100 L. Observation of HeLa cells against
doxorubicin (Figure 9) showed higher inhibitory activity than Royal jelly, irregular cell morphology and lower live cell colonies than dead cells.

The anticancer activity of Royal jelly was tested on colon cancer cells and cervical cancer cells. Colon and cervical cancer cells used were WiDr (ATCC®-CCL™ 218) and HeLa (ATCC®-CCL-2™) cells. WiDr cells were derived from primary rectosigmoid colon adenocarcinoma from a 78-year-old woman in 1971 (Kusuma et al., 2010). WiDr cells are colon cancer cells originating from epithelial tissue with adherent properties that are resistant to chemotherapeutic agents and COX-2 overexpression (Haryanti and Katno, 2011). HeLa cells are human cervical cancer epithelial cells isolated in 1915 from a 31-year-old woman named Henrietta Lacks (Masters, 2002). The anticancer activity of Royal jelly is expressed in % inhibition of WiDr and HeLa which is the percentage of inhibition of cells by Royal jelly.

Data on % inhibition of WiDr cells and HeLa cells showed a decrease in the percentage of inhibition with increasing concentrations of Royal jelly. The decrease in the percentage of inhibition could be due to the content of bioactive compounds such as phenolic compounds and fatty acids found in different Royal jelly at high and low concentrations, and the level of solubility of Royal jelly bioactive compounds with distilled water and cell medium used during the study. HeLa cells are more sensitive to Royal jelly. This was indicated by the higher % inhibition of HeLa cells than WiDr cells at the same concentration (125 µg/mL).

The result of % inhibition of Royal jelly against HeLa cells was 36.425% while WiDr cells were 13.830%, but this value was still lower than doxorubicin of 81.680% for HeLa cells and 73.643% for WiDr cells. HeLa cells were more sensitive to doxorubicin and Royal jelly. The low antiproliferative activity of Royal jelly is in accordance with the study of Filipic et al. (2015). The antiproliferative activity of Royal jelly against CaCo-2 colon cancer cells tends to be low at a concentration of 0.5 mg/mL (Filipic et al., 2015). Royal jelly can also inhibit the growth of MCF-7 breast cancer cells at a concentration of 1 mg/mL (Nakaya et al., 2007).

The mechanism of the proliferation of Chang cells and inhibition of the growth of HeLa cells and Chang cells were not investigated in this study, so the exact mechanism is not yet known. Factors that affect the growth of normal liver cells (hepatocytes) are epidermal growth factor (FPE), insulin, glucagon, hepatocyte growth factor (FPH), pyruvate, lactate, and nicotinamide. According to Kamakura et al. (2001), the fraction of Royal jelly protein (major Royal jelly protein) 57 kDa is an active component of Royal jelly that can increase DNA synthesis and hepatocyte proliferation. The 57 kDa Royal jelly protein fraction has a cytokine factor effect on hepatocytes, and triggers the production of autocrine growth factors such as TGF-GF and fibroblast growth factor to activate intracellular signal transduction and trigger DNA synthesis and albumin production. Meanwhile, the 350 kDa Royal jelly protein fraction functions to maintain the number of hepatocytes so that normal cell growth can be controlled (Fujii et al., 1996).

Another bioactive component of Royal jelly that can inhibit the growth of cancer cells is 10-hydroxy-2-decanoate (10-HAD) which is only found in Royal jelly. According to Li et al. (2013), the mechanism of 10-HAD activity against cancer cells is inhibiting angiogenesis, as a modulator of estrogen receptors and inhibiting cell proliferation at
the S and G2 stages, as well as inducing macrophage activity and increasing the production of antitumor cytokines (tumor necrosis factor) for apoptosis. The activity of 10-HAD is also involved in modulating oxidative stress by decreasing lipid peroxidation and induction of apoptosis (Filipic et al., 2015).

CONCLUSIONS

The highest total phenol Royal jelly was obtained by the Wonogiri Royal jelly sample of 116.06 mg GAE/mg ± 0.03 Royal jelly was non-toxic to normal Chang cells by increasing normal cell proliferation by 42.30% ± 13.01 at a concentration of 375 µg/mL. The anticancer potential of Royal jelly in HeLa cells and WiDr cells at a concentration of 125 µg/mL with inhibitory percentages of 36.42% ± 7.90 and 16.65% ± 4.80 in HeLa cells.

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