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Submission date: 04-Dec-2019 01:56PM (UTC+0800)

Submission ID: 1216636959

File name: The_Efficacy_of_pomegranate.pdf (555.77K)

Word count: 3207

by Anis Irmawati

Character count: 17011

BENZOPYRENE INDUCTION

THE EFFICACY OF POMEGRANATE EXTRACT (*PUNICA GRANATUM L.*) AND ELLAGIC ACID ON THE EXPRESSION OF VEGF AND ORAL CANCER CELLS APOPTOSIS OF *MUS MUSCULUS* DUE TO BENZOPYRENE INDUCTION

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(Received 04 April, 2019; accepted 11 June, 2019)

ABSTRACT

Cancer is one of the leading causes of death throughout the world. In Indonesia in 2013 it reached 1.4% or an estimated 347.792 people and became the cause of death number seven. Squamous cell carcinoma is the most common cancer in the oral cavity, the patient's survival is less than 50%. Until now complete cancer treatment has not been done while every year new cases continue to emerge, despite advances in the field of surgical therapy, radiation, chemotherapy is growing rapidly. Pomegranate (*Punica granatum L.*)/PgL, is one of the plants with the active ingredient Ellagic Acid (EA), which has anti-cancer activity in vitro. There has not known the effectiveness of Pomegranate if given in whole extract or Ellagic acid isolation. Purpose of this study was to prove the effect of Pomegranate extract (*Punica granatum L.*/PgL) and Ellagic Acid on the expression of VEGF and oral cancer cells apoptosis of *Mus musculus* due to benzopyrene induction. 24 mice (Balb / c), males, 5 months old, were divided randomly into 3 groups, namely: K0: not induced by benzopyrene, but not given PgL and EA; K1: benzopyrene induced, not given PgL and EA; P1: benzopyrene induced and given EA; P2: benzopyrene induced and given PgL. Benzopyrene at a dose of 0.04 mg was dissolved in 0.04 mL olivarium oil, given 3 times a week for 4 weeks. The application of PgL and EA at a dose of 75 mg/kg BW/day for 4 weeks. Examination of VEGF expression by immunohistochemical techniques, while cell apoptosis with tunnel assay. There was significant difference in VEGF expression between all groups ($p = 0.006$), also in cancer cell apoptosis there were significant differences between all groups ($p = 0.001$). Pomegranate (*Punica granatum L.*) extract was more effective to decrease VEGF expression and increase apoptosis cancer cell than Ellagic acid on *Mus musculus* which induced by benzopyrene.

KEY WORDS : Pgl Extract, Ellagic Acid, Oral Squamous Cell Carcinoma, VEGF, Apoptosis

INTRODUCTION

Cancer is a disease characterized by uncontrolled growth in multicellular organisms, which occurs due to the transformation of an oncogen. Oncogenes are protoonogens that experience mutations, mutations cause overactive proteins that are encoded by these genes and can develop into cancer (Sudiana, 2008; Kresno, 2012).

Cancer occurs due to loss of growth regulation mechanisms, cell differentiation, decreased number of deaths/apoptosis, vascular growth/increased

angiogenesis/ angiogenesis and wild p53 mutations resulting by no control of CDK in the cell division cycle, regulation of cell death/apoptosis does not occur (Mendelsohn *et al.*, 2008).

Apoptosis is a form of cell death that is needed both for normal cell development and tissue homeostasis. Apoptosis is an important mechanism to prevent the proliferation of cells undergoing cancer, apoptosis is associated with an enzyme called telomerase, and this enzyme is active in embryonal cells whereas in somatic cells it is not, except when the cell is mutated toward cancer

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(Salido *et al.*, 2009).

Handling cancer completely has not been done, whereas every year new cases continue to emerge due to various predisposing factors, one of the predisposing factors is smoking as a cause of oral squamous cell carcinoma (Tanaka *et al.*, 2011). This happens because cigarettes contain benzopyrene which is a carcinogenic substance.

Squamous cell carcinoma is the most common cancer in the oral cavity. Usually it does not cause complaints at an early stage, patients with squamous cell carcinoma of the buccal mucosa 68% and 48% spread to lymph nodes (Wahyuni, 2010). The microscopic description of squamous cell carcinoma shows proliferation of squamous epithelial cells, which experience atypia accompanied by changes in the shape of rete peg process, abnormal formation of keratin, increase in cell basaloid, cell order becomes irregular and forms nest tumors which infiltrate the surrounding tissues (Safriadi, 2008).

Many efforts to manage cancer still face many obstacles, which result in a lack of success in preventing and treating cancer. One treatment effort that has been pioneered since ancient times is the use of phytopharmaca, exploring the chemical constituents in plants that can potentially be used as medicine. One of the medicinal plants is pomegranate (PgL). The main group of pomegranate phytochemicals (PgL) is polyphenols, which consist of flavonoids (flavonols, flavonols and anthocyanins), hydrolyzable tannins (ellagitannins and gallotannins) and condensed tannins (Jurenka, 2008).

Pomegranate extract can increase invitro apoptosis in cultured squamous cell carcinoma of the human tongue at a dose of 250 ug/mL (Lansky and Newman, 2007). Whole pomegranate extract (PgL) contains an active ingredient, one of which is Ellagic acid (EA). Ellagic acid in whole pomegranate extract (PgL) in free form as ellagic acid-glycosides or bound in the form of ellagitannins (Seeram *et al.*, 2005). *In vitro* ellagic acid functions as an anti-cancer but is rarely investigated *in vivo*. The activity and concentration of ellagic acid in plasma is low due to low water solubility, besides that ellagic acid is easily subjected to transformation and degradation before being absorbed (Seeram *et al.*, 2006).

The advantages of whole pomegranate extract (PgL) is it have some active ingredients that are likely to work synergistically, including

polyphenols can increase solubility and absorption of ellagic acid. Therefore this study aims to prove the effect of Pomegranate extract (*Punica granatum* L./PGL) and Ellagic acid on the expression of VEGF and oral cancer cells apoptosis of Musculus due to benzopyrene induction. If the whole effect of pomegranate extract (PgL) on Swiss Webster strain Mice (Balb / c) can be revealed, then the whole pomegranate extract (PgL) can be used as an alternative treatment for squamous cell carcinoma of the oral cavity.

MATERIALS AND METHODS

Ethical approval

This research has obtained ethical approval from the Board for Animal Experiments at the Faculty of Dental Medicine, Universitas Jember, No. 16/ KKEPK.FKG/II/2012.

Samples

The sample of this study was Swiss Webster (Mus musculus) strain (Balb/c), male sex, age around 5 months, and have body weight 30-50 g, obtained from experimental animal unit at Gajah Mada University. The sampling technique in this study was carried out by simple random method. The sample size was 6 for each group. Samples were divided into four groups by randomly, namely negative control group (K0), positive control group (K1), and treatment group P1 & P2.

Material

PgL was obtained by extracting all parts of pomegranate in powder form and has been standardized. Ellagic acid was white crystals, is one of the active components of PGL, which was obtained by isolating PgL extract.

Experimental design

This type of research was laboratory experimental research. The research was conducted in the Biomedical laboratory of the Faculty of Dental Medicine, Jember University. The study sample was divided into 4 groups. K0: mice induced by 0.04 mL olivarum oil, in the right buccal mucosa of the oral cavity, 3 times a week for 4 weeks, the following week given CMC-Na 0.3% per oral, with a dose of 0.1 mL/10 g/BW, once a day for 4 weeks. K1: mice induced by benzopyrene 0.04 mg/0.04 mL olivarum oil, on the right buccal mucosa of the oral cavity, 3 times a week for 4 weeks, the following week given

CMC-Na 0.3% per oral with a dose of 0.1 mL/10 g/BW, once a day for 4 weeks. P1: mice induced by benzopyrene 0.04 mg/0.04 mL olivarium oil, in the right buccal mucosa of the oral cavity, 3 times a week for 4 weeks, the following week given ellagic acid per oral, a dose of 75 mg/kg BW/day dissolved in CMC- Na 0.3% 0.1 mL/ 10 g/BW, once a day for 4 weeks. P2: mice induced by benzopyrene 0.04 mg/0.04 mL olivarium oil, in the right buccal mucosa of the oral cavity, 3 times a week for 4 weeks, the next week given PgL per oral, a dose of 75 mg/kg BW/day dissolved in CMC-Na 0.3% 0.1 mL/10 g/BW, once a day for 4 weeks.

In the end of week 9, all animal samples were anesthetized with ether before being sacrificed to obtain a sample of upper right buccal mucosa tissue. The samples were then fixed in 10% buffered formalin and made into a paraffin block.

Examination of VEGF expression and apoptotic cells

VEGF expression was examined by immunohistochemical methods. Animals of squamous cell carcinoma are mice (Balb/c) that have undergone malignancy in their epithelium due to exposure of benzopyrene with microscopic picture: showing cell proliferation - the atipia squamous epithelium, which is a change in the shape of the rete peg process, abnormal keratin formation, and irregular cell arrangement.

Examination of apoptotic cells using the tunnel assay method, cells expressing apoptosis are blackish brown.

The expression of VEGF and the apoptosis cells were subsequently calculated under a light microscope (Olympus, Tokyo, and Japan) at 10 different visual fields at 400× magnification.

Statistical analysis

The data were analyzed by SPSS version 20 (IBM, New York, and USA) using an Anova test to

Table 1. The difference test result of VEGF expression with Anova.

Group	Mean ± SD	Anova test (p)
Control (-) / K0	0.133 ± 0.103 ^a	0.006*
Control (+) / K1	0.350 ± 0.104 ^c	
P1	0.267 ± 0.103 ^{bc}	
P2	0.150 ± 0.104 ^{ab}	

Note : * significance : $\alpha=0.05$

^{abc}=the same superscript showed no different between group (based on LSD test)

determine the differences between groups on VEGF expression and apoptosis cell, continue with LSD test, with significance 0.05.

RESULTS

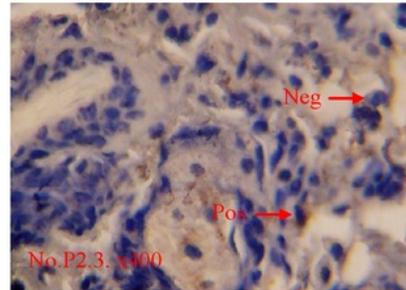


Fig. 1. Expression of VEGF on P2 group. Neg=negative, Pos=positive.

Table 2. The difference test result of apoptosis cell with Anova.

Group	Mean ± SD	Anova test (p)
Control (-) / K0	0.083 ± 0.132 ^a	0.000*
Control (+) / K1	0.050 ± 0.054 ^a	
P1	0.233 ± 0.081 ^b	
P2	0.367 ± 0.196 ^b	

Note : * significance : $\alpha=0.05$

^{abc}=The same superscript showed no different between group (based on LSD test)

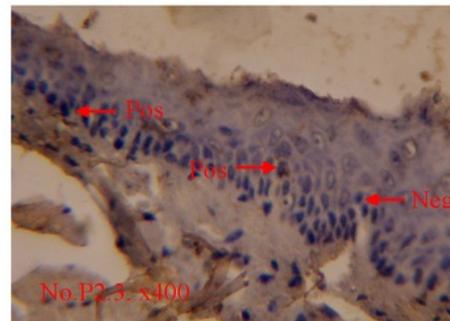


Fig. 2. Apoptosis cell on P2 group. Neg=negative, Pos=positive.

DISCUSSION

The increasing prevalence of oral squamous cell carcinoma is inseparable from the poor lifestyle of the community, among others due to smoking habits, smoking is one of the most risk causes, especially benzopyrene (Tanaka, 2011).

Ellagic acid, one of the active ingredients of

pomegranate (PgL), can inhibit the development of cancer cells, has antiproliferation properties, induce apoptosis, antioxidant as in vitro (Seeram *et al.*, 2006; Jurenka, 2008), but the weakness of ellagic acid is easily transformation and degradation before absorbed. PgL has several active ingredients that make it easier to absorb and there are several active ingredients that can work synergistically as anti-cancer.

The ability of PgL to kill cancer cells compared to EA can be seen through apoptotic expression in the pomegranate group (PgL) compared to groups K0, K1, P1 because natural polyphenols in PGL can stimulate the production of interferon-g (IFN-g) in an immunocyte population, which very important in stimulating the activation of cytotoxic T lymphocytes (CTL) and natural killer cells (NK cells). When CTL and NK cell are active, there will be many cancer cells undergoing apoptosis. Apoptosis can occur due to activation of caspase enzyme, which activation of enzymes can be through various pathways, including through T-cell Receptor (TCR) or from the activity of granzyme that enters cell by pore forming factors perforin (Abbas *et al.*, 2005).

Polyphenols has a function as ligands which will trigger cell apoptosis through fas-receptors. Polyphenols in the green tea can induce apoptosis through TNF- α pathways. The potency of polyphenols in herbal medicine is it's ability to inhibit activation of Nuclear factor kappa B (NF-KB), a transcription factor that plays an important role in molecular regulation (Tuzulakhova, 2001).

Polyphenol compounds contained in herbal medicine have the effect of blocking the Receptor Growth Factor and inhibiting Mitogen-Activated Protein Kinase (MAPK) on the Receptor Tyrosine Kinase signaling pathway (RTKs) (Hiroko *et al.*, 2002).

Ellagic acid is one of the active ingredients in pomegranate invitro in PaCa-2 cells, inhibits the activity of the nuclear factor kappa B (NF-kB) transcription factor. NF-kB is the main transcription factor that usually has an anti apoptotic role in cancer cells. NF-kB inhibits apoptosis by increasing the activity of Bcl-2 (anti apoptosis), Bcl-2 inhibits mitochondrial permeability which inhibits the release of cytochrom C and suppresses Bax. Ellagic acid decreases NF-kB activity, causes Bcl-2 to decrease, so that the activity of the mitochondrial apoptotic pathway progresses, cytochrome C release and caspase activation occur.

Ellagic acid in vitro increases apoptotic receptors by increasing the expression of TRAIL, R2/DR2, DR5 receptors. The expression of this receptor is regulated by wild p53. So that the increase this receptor is in line with the increase of wild p53.

Ellagic acid in invitro studies shows that it can increase the expression of apoptotic receptors, among others: TRAIL R2/DR5. DR5 binds to death stimuli and causes activation of pro-caspase 8, and then cross talk between special lines of extrinsic apoptosis (through death receptors) and intrinsic pathways (via mitochondria (Mohammad, 2002)

The most studies are still invitro, weaknesses in studies in vitro, sensitivity and specific reactions do not reflect actual results and do not always reflect the results of studies *in vivo*. So that encourages researchers to conduct research *in vivo* on animal models. PgL bioavailability is better than ellagic acid, this illustrates the multivector influence and synergistic effects of various compounds contained in PgL. The presence of polyphenols in PgL can increase the solubility and absorption of ellagic acid in the digestive tract (Seeram *et al.*, 2005).

Ellagic acid (EA) increases the expression of apoptotic receptors, including increased TRAILR2/DR2 receptors, expression of DR5 is regulated by wild p53 so that it can be associated with wild p53. The increase in wild p53 will increase pro-caspase and cross talk activation between extrinsic and intrinsic pathways for special apoptosis (Mohammad, 2002). The mechanism of PgL compared to ellagic acid in killing oral cavity cells through wild p53 expression showed the highest wild p53 expression in PgL compared to group K1 (untreated cancer) and P1 (EA), wild p53 was a tumor suppressor gene protein that is a protein as a factor controlling cells and triggering apoptosis. Wild p53 mutates in about 70% of cancers (Safriadi, 2008).

PgL contains several active ingredients that work more potent than ellagic acid so that the expression of wild p53 is higher, some active ingredients that function to inhibit cancer; ellagic acid, caffeic acid, acid luteolin, punical acid; individually shows anti-cancer activity in prostate cancer, when combined, all will show better activity as in this study (Seeram *et al.*, 2006). Increased expression of wild p53 in this study is lower than PgL because ellagic acid is easily to transformed and degradation before absorption, its solubility is low in water and insoluble in intestinal fluid unlike PgL (Seeram *et al.*, 2005).

Effectivity of PGL compared to ellagic acid to killing oral cancer cell of mice through VEGF expression shows: VEGF expression of PgL at P2 group showed the lowest compared to ellagic acid (P1) because pomegranate extract inhibited cyclooxygenase (COX-2) activity reducing prostaglandin so that it did not trigger angiopoietic and inhibit VEGF secretion, so angiogenesis was not formed (Seeram *et al.*, 2006).

Caffeid acid which is one of the active ingredients of pomegranate (PgL) can inhibit the activation of STAT3. STAT3 is an activator of VEGF gene transcription in cancer. STAT3 activity plays a major role in VEGF over production.

VEGF is a stimulant for cancer and neovascular growth. The development of cancer angiogenesis processes will trigger growth factors and stimulate endothelial migration and proliferation of cancer cells.

The results showed that PgL can reduce VEGF expression, by inhibiting VEGF angiogenesis formation will be inhibited so that the nutrient supply to cancer cells will be cut off so that cancer cells will die (Kresno, 2011).

CONCLUSION

Pomegranate (*Punica granatum* L.) extract was more effective to decrease VEGF expression and increase apoptosis cancer cell than Ellagic acid on Mus musculus which inducted by benzopyrene.

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