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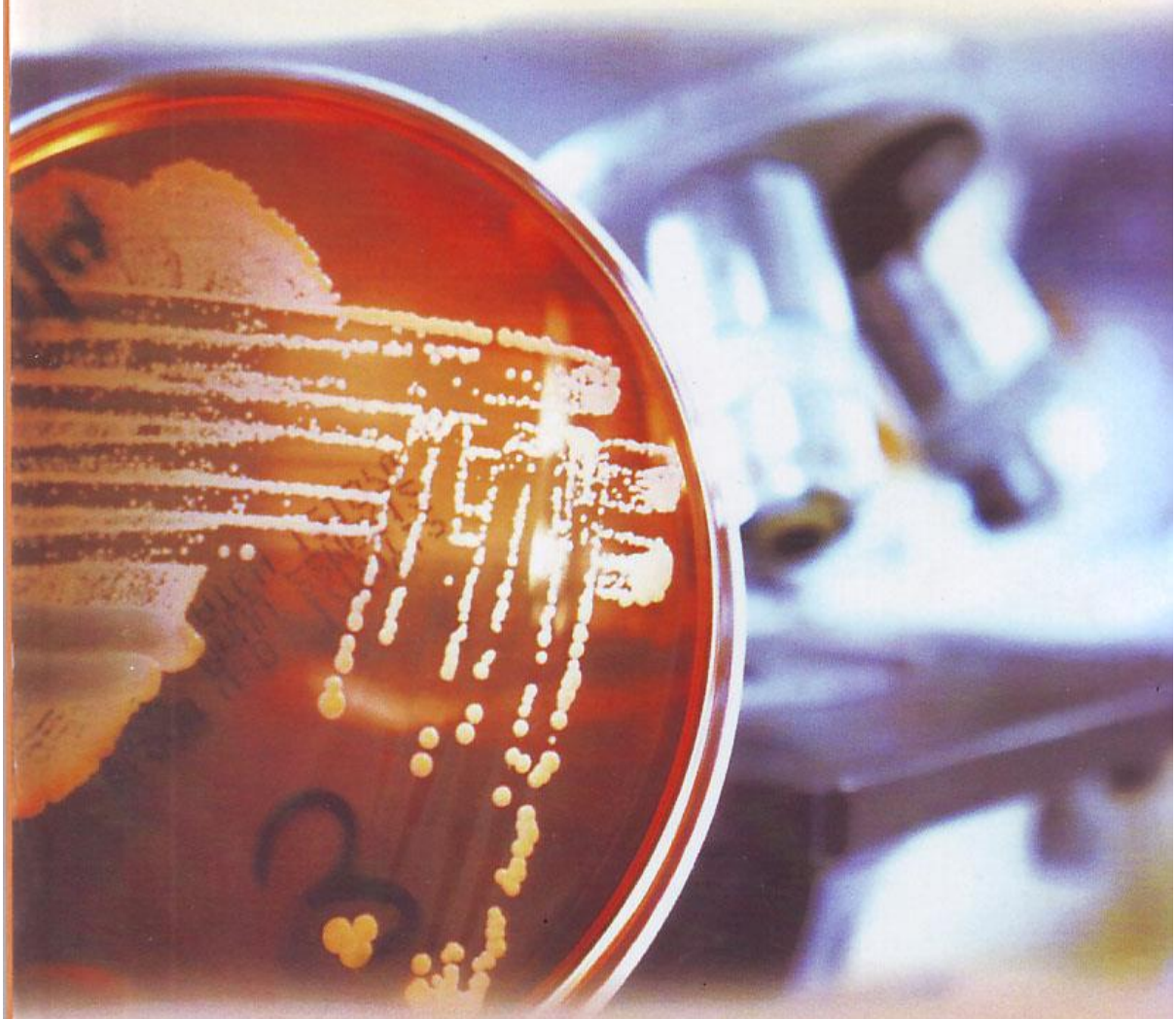

- Antitumor activity of antisense oligonucleotide p45^{Skp2} in soft palate carcinoma cell squamous in vitro
- Evaluation of local muscle soreness treatment with anterior bite splint made of soft putty impression material
- Unidentified angular recurrent ulceration responsive to antiviral therapy

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Research Report

The inhibition of malignant epithelial cells in mucosal injury in the oral cavity of strains by pomegranate fruit extract (*Punica granatum linn*) through Bcl-2 expression

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ABSTRACT

***Background**: Squamous cell carcinoma of oral cavity is a malignant neoplasms derived from epithelia. The malignant neoplasms are cells that have changed their structure and function, and their number becomes increasing abnormally, invasive, and metastatic. Carcinoma can be caused by the resistance of malignant cell apoptosis. Bcl-2 is a proto-oncogene of Bcl family that inhibits the process of cell apoptosis and suppresses Bax protein (pro-apoptotic). The management efforts of cancer diseases, however, still have many obstacles. Thus, the researcher was triggered to explore more herbal plants, namely pomegranate. Pomegranate as a medicinal plant is accessible and cheap. Ellagic acid (EA) is a single active compound derived from whole pomegranate fruit extract (PGL), which has anti-cancer activity as in vitro, but EA is low concentration in plasma, low water solubility, and insoluble in intestinal. These facts prompted the researcher to compare between pomegranate extract, which consists of several active compounds, and that, which only consists of ellagic acid. Thus, this research is expected to know how some active compounds can work synergistically in the PGL, so the effect can be more potent. **Purpose**: The purpose of this research, therefore, was to compare EA with PGL in reducing the expression of Bcl-2. **Methods:** This laboratory experimental research was used 32 male mice (Balb/c) in the age of 5 months. They were randomly divided into 4 groups: 2 control groups (K0: which was not exposed with benzopirene and also untreated and K1: which was exposed with benzopirene and also untreated), 2 treatment groups (P1: which was exposed with benzopirene and also treated with EA and P2: which was exposed with benzopirene and also treated with the PGL). Next, an examination was conducted by using immunohistochemical techniques. **Results**: The results then showed that the provision of the PGL could decrease the expression of Bcl-2 significantly higher than that of EA in the malignant epithelial cells of the oral mucosa of those mice. **Conclusion**: It may be concluded that the provision of the PGL can kill malignant cells in the oral cavity of mice by increasing apoptosis through decreasing Bcl-2 expression that was higher than the provision of EA.*

***Key words**: Pomegranate fruit extract, malignant cells, Bcl-2, ellagic acid*

ABSTRAK

***Latar belakang**: Karsinoma sel skuamosa rongga mulut adalah istilah yang digunakan menyebut neoplasma ganas berasal dari epitel. Neoplasma ganas adalah sel yang telah berubah struktur dan fungsi, sehingga mengalami peningkatan jumlah secara abnormal,invasif dan metastasis. Terjadinya karsinoma salah satu disebabkan oleh karena hambatan apoptosis terhadap sel ganas. Bcl-2 adalah protoonkogen keluarga Bcl yang berperan menghambat proses apoptosis sel dan bekerja menekan protein Bax (pro apoptosis). Berbagai upaya penatalaksanaan penyakit kanker masih banyak menemui kendala,sehingga peneliti menggali tanaman obat yaitu buah delima. Buah delima sebagai tanaman obat, mudah didapat dan harganya murah. Ellagic acid (EA) senyawa tunggal bahan aktif dari ekstrak buah delima yang memiliki aktivitas sebagai anti kanker secara in-vitro tetapi EA aktivitas dan konsentrasinya dalam plasma rendah, kelarutan dalam air rendah, metabolisme (EA) tidak larut dalam intestinal. Fakta ini mendorong peneliti untuk membandingkan dengan whole ekstrak delima (PGL) yang terdiri dari beberapa senyawa bahan aktif, tidak hanya ellagic acid, memungkinkan beberapa senyawa bahan aktif pada PGL bisa bekerja sinergis, sehingga efeknya lebih poten. **Tujuan:** Tujuan penelitian ini membandingkan antara EA, dengan whole ekstrak buah delima (PGL) dalam menurunkan ekspresi Bcl-2. **Metode**: Metode penelitian yang digunakan*

adalah eksperimental laboratories, 32 ekor mencit (Balb/c), jantan, umur 5 bulan dibagi secara random menjadi 4 kelompok, 2 kelompok kontrol (K0: tidak dipapar benzopirene dan tidak diberi perlakuan, K1: dipapar benzopirene dan tidak diberi perlakuan), 2 kelompok perlakuan (P1: dipapar benzopirene dan diberi EA, P2: dipapar benzopirene dan diberi PGL). Pemeriksaan dengan menggunakan teknik imunohistokimia. ***Hasil****: Hasil penelitian menunjukkan bahwa pemberian PGL dapat menurunkan ekspresi Bcl-2 lebih tinggi dibandingkan EA pada sel epitel ganas mukosa rongga mulut mencit.* ***Kesimpulan****: Kesimpulan penelitian ini adalah pemberian PGL dapat membunuh sel ganas pada rongga mulut mencit dengan jalan meningkatkan apoptosis melalui penurunan ekspresi Bcl-2 lebih tinggi dibandingkan pemberian EA.*

Kata kunci*: Ekstrak buah delima, sel ganas, Bcl-2, ellagic acid*

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INTRODUCTION

Cancer or malignant neoplasms is still a major health problem in industrialized countries and also in developing countries. In 2005 in the United States there were approximately 1.372 million people diagnosed with cancer, and 570,280 people died of cancer.[1] Oral squamous cell carcinoma is cancer ranked sixth in the world. In India there are 75,000–80,000 new cases reported every year. In Singapore and other Asian countries the number of the same cases is also high.[2] Several studies in Southeast Asia, moreover, also show that an area of buccal mucosa is the most common area of squamous cell carcinoma, that is equal to 50–72%. Squamous cell carcinoma of the oral cavity usually causes no complaints at an early stage. Thus, among 68% patients with squamous cell carcinoma, 48% of them spread to lymph node.[3] It is also known that patient survival index continues to decline over the improvement in the diagnosis and treatment of cancer.[4]

Microscopic picture of squamous cell carcinoma showed that the proliferation of cells - squamous epithelial cells, got atipia then followed with the changes of rete peg processus, the formation of abnormal keratin, the increasing of basaloid cells, the irregular structure of the cells, and the formation of tumor nest infiltrating into the surrounding tissue.[5] The growth of squamous cell carcinoma in the oral cavity was actually influenced by exogenous and endogenous factors, which can make protein function abnormal due to gene mutation. The failure of apoptosis also contributes significantly to the growth and development of squamous cell carcinoma.[6]

Apoptosis can occur physiologically and pathologically. Physiological apoptosis is a cell death process in order to maintain the overall integrity of the body and also to maintain homeostasis. Meanwhile, pathological apoptosis is to limit cell proliferation required, including malignant cells. Pathological apoptosis is an efficient mechanism to eliminate cells that are unnecessary and harmful. On malignant cells, the apoptosis will usually get interference or obstacles.[7] One of proteins that plays an important role in the malignant process is Bcl-2 (protooncogene). Bcl-2 will act as an anti-apoptosis, so the increasing of Bcl-2 protein will inhibit Bax (pro-apoptotic).[8] Bcl-2 is a member of the Bcl family that has a function as an apoptosis inhibitor. Thus, if the number of Bcl-2 protein is decreased, then the pro-apoptotic protein (Bax) will increase and induce apoptosis.[6]

However, various management efforts of cancer diseases have still got many obstacles, which cause the lack of success in preventing and treating malignancies. One treatment that has been initiated is the use of phytopharmaca, in which the contents of chemical elements in plants that can potentially be used as drugs are explored. One of the medicinal plants that has been used is pomegranate *Punica granatum Linn.* (PGL). The main phytochemical group contained in PGL is polyphenol, which consists of flavonoids (flavonols, flavonols and anthocyanins), hydrolyzable tannins (ellagitannins and gallotannins), and condensed tannins (proanthocyanidins). Based on the previous researches, it is also known that pomegranates have therapeutic efficacy, such as anti-bacterial, anti-viral, anti-cancer, and anti-inflammatory.[9] Similarly, based on other researches, the PGL with standardized ingredients involving 40% ellagic acid (EA) can both inhibit the growth of cancer cells, anti-proliferation, and induce apoptosis and anti-oxidants in vitro.[10] Therefore, it is believed that the pomegranate extract can increase apoptosis in vitro in cultured human tongue squamous cell carcinoma with a dose of 250 ug/ml.[11] Thus, the standardized ingredient with 40% EA can indicate that 40% could describe the strength of the pomegranate extract, which is responsible for the pharmacological activity.[12]

The PGL used in this research contained the active ingredient EA. Ellagic acid in the whole pomegranate fruit extract is in the free form as ellagic acid-glycosides or bounded in the form of ellagitannins.[13] Ellagic acid in vitro has a function as an anti-cancer, but still rarely studied in vivo. Actually, the low activity and concentration of EA in plasma is caused by a low solubility in water, in addition, EA can easily transformate and degrade before being absorbed.[13] Thus, the advantage of the PGL is that it has several active ingredients that are likely to work synergistically, such as polyphenol that can improve the solubility and absorption of ellagic acid, as a result, the PGL has potential anti-cancer effects.[13] For the reasons, if the effects of the PGL on Strain Swiss Webster (Balb/c)

can be revealed, then the PGL can be used as an alternative treatment for squamous cell carcinoma of the oral cavity. So, the aim of the study was to compare EA with PGL in reducing the expression of Bcl-2.

MATERIALS AND METHODS

This research was considered as an experimental laboratory research. Animals used in this research were five month male Strains Swiss Webster (Balb/c) with the weight of 30–50 gram. Those animals were obtained from the unit of animal testing, Universitas Gajah Mada, Yogyakarta.

Moreover, those animals were divided into 4 groups, which were 2 control groups, K0 (not exposed with benzopirene and also not treated with EA and PGL) and K1 (exposed with benzopirene and also not treated with EA and PGL), and 2 treatment groups, P1 (exposed with benzopirene and also treated with EA) and P2 (exposed with benzopirene and also treated with PGL). For each group, there were eight strains. PGL used in this research was obtained by extracting all parts of pomegranate fruit into powder and then standardized its ingredient by using 40% of EA, produced by late Biof Xi Biotechnology Co. Ltd. (Room 1–1111, High-tech Venture Park, N0. 69 Jinye Distric of Rood Gaoxin Xi'an, People Republic of China). EA is a white crystalline used as one component of the active ingredient of PGL derived from the same company with PGL.

Next, those strains were exposed with benzopiren (0.04 mg)/olium olivarum (0.04 ml) orally 3 times a week for 4 weeks on the right buccal mucosa in oral cavity of those strains. At the end of the 9 weeks, the oral mucosal tissues of those strains were biopsied, and then sacrificed. Those which were considered to have squamous cell carcinoma were those (Balb/c) which had suffered from malignancy in their epithelia due to benzopirene exposure with the microscopic picture showing cell proliferation-atypical squamous epithelium followed with the changes of rete peg processus shape, the formation of abnormal keratin, and the irregular structure of the cells.

Afterwards, the PGL, EA was given orally every day for 4 weeks. The dose of the PGL, EA used was 75 mg/kg/bw/day dissolved in 0.3% CMC-Na. Then, the examination for the expression of Bcl-2 was conducted by using Immunohistochemical. The procedure of Immunohistochemical conducted on the expression of Bcl-2 involved (1) reagent preparation: the fixation stage of the working solution, DAB, (2) staining, (3) washing, (4) labeling, and (5) reading.

Materials used for immunohistochemical examination in this research were 3% H202, 0.025% trypsin, PBS, aquadestilata, substrate buffer, xylol, absolute ethanol, methanol, water, anti-Bcl-2 (mouse anti- rat) antibodies, enzymes, glass poly L-lysine object, formalin buffer, labeled antiglobulin, secondary antibodies, and streptavidin. The procedures of immunohistochemical examination involved preparation of reagents, staining, washing, labeling, and reading.

The results of immunohistochemical examination showed that cells that did not express Bcl-2 protein did not have brown color (transparent). Afterwards, the calculating of the cells was conducted on 10 fields of view with a microscope using 400x magnification, presented their own means. Finally, analysis of the research data was tested by using normality test, homogeneity test, ANOVA test, and LSD test. Analysis result between treatment groups was conducted by using LSD test.

RESULTS

The results of preparation examination by using immunohistochemical techniques on the expression of Bcl-2 can be seen Table 1. The results of preparation examination with immunohistochemical techniques showed that the administration of standardized PGL (P2/benzopirene + PGL) could decrease the expression of Bcl-2 in malignant squamous epithelial cells of those strains. The decreasing of the expression of Bcl-2 in group P2 (benzopirene + PGL) was about 0.016 ± 0.040 higher than that in group P1 (benzopirene + EA), which was about 0.083 ± 0.075).

DISCUSSION

The results of this research indicate that the standardized PGL could decrease the expression of Bcl–2, which was higher than EA. Bcl-2 gene, an anti- apoptotic, that encodes protein is considered as protooncogene group. It is because Bcl-2 can suppress the function of Bax or pro- apoptotic, and can also inhibit c–myc, which function is to induce apoptosis. Thus, the increasing of the expression of Bcl-2 has a very important role in resisting the apoptosis of malignant cells.[14] The results showed that the decreasing of the expression of Bcl-2 by PGL was stronger than that by the standardized EA. It indicates that there were additional or synergistic effects of the other active ingredients in the pomegranate extract.[15]

One of the possible mechanisms of malignant cells to increase the expression of Bcl-2 is by a process in which Bcl-2 forms a pore in the membrane where it steps on,

Table 1. The mean and standard deviation of cells expressing Bcl-2 on those strains

Group (n = 6)	The number of cells expressing Bcl-2 ($\bar{X}$ ± SD)
Control (–)/K0	0.001 ± 0.001^{a}
Control (+)/K1 (*benzopirene* + CMC)	0.367 ± 0.103^{c}
P1/(*benzopirene* + EA)	0.083 ± 0.075^{b}
P2/(*benzopirene* + PGL)	0.016 ± 0.040^{ab}

*) Different superscripts in the same column were significantly different ($p < 0.05$)

and then interacts with various types of other intracellular proteins that are directly or indirectly involved in apoptotic process. This interaction shows one of the roles of Bcl-2 protein in providing a place for others, so the cellular activity of those proteins stop (eg. Bax, its activities will be stopped). Bcl-2, furthermore, also control checkpoint of caspase activation pathway, so the possibility of Bcl-2 to control apoptosis pathway is either dependent or independent of caspase (intrinsic pathway and extrinsic pathway).[6]

BH3 protein is a protein that has a function to receive stimulus from outside the cell like a drug. The stimulus then makes BH3 proteins active and work directly to free bond between Bax and Bcl-2 proteins, and also decreases the expression of Bcl-2.[17] If the expression of Bcl-2 decreases, Bax protein then has a greater chance of binding to BH3, which is the initiation of apoptosis.

The decreasing of the expression of Bcl-2 then can cause the increasing of Bax activity (pro- apoptotic of malignant epithelial cells). Next, Bax has a role in opening Pt-pore so that cytochrome-c can be out of mitochondria. Afterwards, cytochrome-c activates Apaf - 1, and then Apaf-1 activates caspase cascade, so it causes cell death (apoptosis).[6]

Biovaibilitas of the whole pomegranate fruit extract was better than that of the single compound one. This illustrates the multifactorial and synergistic effects of various compounds in the PGL.[16] The presence of polyphenols in the PGL can actually improve the solubility and absorption of EA in the digestive tract. Besides, polyphenols contained in the whole pomegranate fruit extract also has an ability to inhibit the metabolism of EA by intestinal microflora through antibacterial activity possessed by the PGL so that the PGL has the ability to decrease the expression of Bcl -2 stronger than EA.[15]

Polyphenols found in the PGL has a function as an antibacterial so that EA in the intestinal tract is not metabolized by intestinal microflora. Ellagic acid, as a result, is not subject to degradation and transformation before being absorbed. Thus, this condition would provide a stronger effect in reducing the expression of Bcl-2. Low activity and concentration of EA in the plasma was due to its low solubility in water. Besides that, it is allegedly caused by the fact that EA was susceptible to transformation and degradation before absorbed.[13]

Finally, the results in group K0 (not exposed with benzopirene and also not treated with EA and PGL) showed that the expression of Bcl-2 (0.001) was not expressed. This was due to a physiological system in an individual cell growth that is regulated by a balance system between apoptosis and proliferation. The balance between Bcl-2 and Bax was occured.[8]

Based on the results, it may be concluded that the whole pomegranate fruit extract can increase apoptosis of malignant epithelial cells through a decreased expression of Bcl-2 and it was higher than the provision of EA.

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