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Expression of p53 and Bcl2 Mammary Adenocarcinoma in C3H Mice administered with *Salvia miltiorrhizae Bunge* root extract.

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ABSTRACT

Background : Breast cancer is on the first or second most malignancy in many places around the world. The incidence of breast cancer was increasing, also in Indonesia. Prevention and treatment of breast cancer have not been satisfactory, since patients and medical practitioners are searching for other therapeutic and preventive agents. *Salvia miltiorrhizae Bunge* roots is one of the therapeutics agent used in traditional medicine have been studied in foreign country.
Objective : To prove the p53 and Bcl2 expression in C3H mice mammary adenocarcinoma using multilevel doses of the Salvia miltiorrhizae Bunge roots extract.
Methods : The study is a randomized post-test only controlled group design. A total of 18 adenocarcinoma mice, divided into 3 groups. The first group was administered with 60mg/KgBW/day and the second group was given by 180mg/KgBW/day, for 21 days. All mice were terminated on day 22nd. The p53 and Bcl2 expressions were analyzed using Allred score, statistically analized by Kruskal Wallis followed by *Post-Hoc* Mann Whitney U test.
Results : Immunohistochemistry staining shows the p53 expressions have been increased between groups, but Bcl2 were decreased. The Kruskal-Wallis and *Post-Hoc* Mann Whitney U test are significantly difference between groups.
Conclusion : All of the p53 and Bcl2 expressions between groups are significantly differences.
Key words : mammary adenocarcinoma, Salvia miltiorrhizae Bunge, p53, Bcl2

INTRODUCTION

In 2012, International Agency for Research on Cancer, World Health Organization found that 1,7 million womans have been diagnosed with breast cancer and there are 6,3 million womans alive that have been diagnosed with breast cancer in the 5 years before. Since 2008, the estimated incidence of breast cancer has been increasing by more than 20%, while the mortality rate has been increasing by 14%. Breast cancer is also the most common caused of cancer death among womens (522.000 deaths in 2012) and also the most frequently diagnosed cancer in womens in 140 of the 184 countries all around the world. [1-3]

There are no accurate statistical data in Indonesia, but the data that have been collected from the hospital showed that breast cancer ranked first than the other cancer that can be found in woman. [4]

Breast abnormalities in Indonesian woman 96% of it take a form of a tumor are being identified by the patient itself making it easier for the doctor to detect breast cancer. Unlike in the western country where every childbearing age woman are demanded by the Health Insurance company to check their breast periodically so the number of early stages breast cancer is much higher than in the developing countries where there are no necessity for childbearing age woman to check their breast..[5,6]

Medical therapy for breast cancer until today still didn't have a satisfactory results.WHO data in 2008, explained that the people of Asia and Africa, 80% of them used treatment with plants substance so its being called herbal medicine.[7] Herbal medicine that most being used and distributed in the world are the Chinese medicine. [8] Some of the Chinese herbal medicine has been used widely in many developing countries, where such therapies is acceptable and affordable. [8]

One of the many Chinese herbal medicine that is widely used is the extract of the dried roots of *Salvia miltiorrhizzae Bunge,* called Danshen. Danshen is a plant that is said being a native plants from China, but it has been tried to plant this plants in other countries, such as Korea, Poland and Japan. Danshen that growing outside of China, has been studied , have a different content with Danshen that growing in the China.[9, 10] The highest content of the active substance is found in Danshen that grows in China. Danshen had been included in the list of standarized herbal medicine in China [9],and has been prescribed in many countries in the world. [10]

Research on the active subtance contained in the extract of *Salvia miltiorrhizae Bunge* in the medical world had been done substanstially, with the development of knowledge about the chemical composition of the *Salvia miltiorrhizae Bunge* has been studied and more widely known. [11,12]

Composition of the roots extract *Salvia miltiorrhizae Bunge* divided into 2 major part, there are the fat-soluble subtance (*lipofilik*) and the water-soluble subtance (*hidrofilik*). Contents of the extract of *Salvia miltiorrhizae Bunge* are diterpene quinon and phenolic acid derivative, including tanshinone I, IIA, IIB, cryptotanshinone, isocryptotanshinone, miltirone, tanshinol I, II and salviol. Those active components that compose the extract of *Salvia miltiorrhizae Bunge*, based on the previous studies, it is known that Tanshinone I, IIA, IIB, Crypotanshinone, Isocryptotanshinone, and various derivative of Tanshinone, compose about 5% of 100mg *Salvia miltiorrhizae Bunge* extract.[9-12]

Previous experimental study, used 20mg/Kg BW Tanshinone i.v., in this study will be proposed dose of 60mg/Kg BW per day orally, and 180mg/Kg BW per day orally, which take a part on the role of malignant cell apopthosis pathway.[13] One of the content of Danshen have a protective effect against lipid peroxidation in vitro and in vivo. 11 Tanshinone and its derivatives are components of *Salvia miltiorrhizae Bunge* extract that plays an active role in the mechanism of carsinogenesis, which has been proved through previous experimental studies both in vitro and in vivo.[12]

In the pharmacopoeia of the Chinese said that the recommended dosage is 9-15 grams per daily dose in the form of boiled medicine. In some case, a high dose was given, up to 20 grams per day, in the case of an inflamatorry diseases including viral hepatitis. According to *Materia Medica Cina Langka*, doses up to 30-60 grams can be used in cases of angina and athritis. Relatively high dose of Salvia, compared to other Chinese herbs (typical dosage recommendation is 3-9 grams for many herbs) may be associated with relatively low levels of active constituents and low solubility in water. [13]

At higher dose, salvia can caused dry mouth, dizziness, fatigue, numbness, shortness of breath, and other symptoms that will usually dissapear spontaneously without interupting treatment. *Materia Medica Cina Langka* noted that salvia isn't suitable for patients who have blood deficiency accompanied with cold, or with a tendency of bleeding. However, most of the guide in *materia medica* didn't warned this. Salvia has a very low acute toxicity, with LD50 injection of 40-80 g/Kg BW.[13]

Suggestion of using salvia are for poor blood circulation treatment, it can't be combined with coumadin (warfarin), because there is a possibility of increased anticoagulant effect. In a literature survey conducted in October 2000, Dalam sebuah survei literatur dilakukan melalui Oktober 2000, it have been found three cases of increasing anticoagulant activity.In the literature, people take Salvia along with Warfarin. Such effect may be rare and tend to be dose dependent, as the mechanism appears to be a simple addictive effect of the anticoagulant activity of Salvia along with that being produced by Warfarin. Therefore, people who use Coumadin should either avoid using Salvia, or use it in a relatively low dose (no more than equivalent of 6-9 gram per day in the boiled dose) with attention to the routine blood clotting tests for those who taking the drug. [13]

Toxic doses of the *Salvia miltiorrhizae Bunge* root extract hasn't widely known. Oral dose given to mice for 90 days at 400 times of the recommended human doses (2.500 mg/Kg BW), was reported to be toxic. Lethal dose of the *Salvia miltiorrhiza Bunge* extract that soluble in the water are being reported 25g/Kg BW in mice.[12]

Salvia miltiorrhizae Bunge root extract is known to have an effect that primarily related with

cardiovasculer, angina pectoris, cerebrovascular, antiplatelet, anticoagulant and thrombolytic , antibiotic, hepatoprotective, and CNS suppresant/ sedative effect. [11] Data from United States Patent Application Publication, stated that pharmacological activity of *Salvia miltiorrhizae Bunge*, as mentioned above, as well as anti-neoplastic or anti-cancer.[13] Experimental studies have been conducted on the anti-cancer mechanism of Tanshinone as one of the active ingredients of Danshen, on human breast cancer. [13,14]

Cytotoxic effects, anti-neoplastic of the active subtance of *Salvia miltiorrhizae Bunge* root extract has been studied previously, in the malingnant cells of the colon, ovary, lung, mouth, breast, and leukemia. [13-17]

Breast cancer study as an example, the results showed that Tanshinone have a stronger inhibitory effect than Tamoxifen, which Tanshinone effectively induce apoptosis in breast cancer cell which had positive and negative estrogen receptor.[13,14] In this study use only one of the active subtances contained in the *Salvia miltiorrhizae Bunge* root extract. [12] Previous research states that more complex form of the herbal preparations would have a better effect than just the active substance, therefore in this research is very interesting is to study *Salvia miltiorrhizae Bunge* root extract in the raw form, without purification. [18,19]

A statement that Tanshinone from *Salvia miltiorrhizae Bunge* root extract has a stronger effect on breast cancer than tamoxifen as an standart hormonal anti-neoplastic agent, it is necessary to know more about the effect of *Salvia miltiorrhizae Bunge* root extract, that is the complex compound form in the living system of the carsinoma mammae cell up to apoptosis stage.[13,14,18,19]

Carcinogenesis process involves 4 things, which is: 1. Oncogene, 2. Tumor Suppresor Gene, 3. DNA Mismatch-Repair Genes, 4. Apoptosis.[20]

1. Oncogene

Proliferation in normal cells is controlled by proteins in cell membrane, which will affect the cascade of biochemical signals , a signal transduction process. Those proteins are growth factor and cytokines (protein mediation). The signals control genes that regulate cell growth and division. Oncogene is converted into normal cellular genes called proto-oncogene which is involved in this cascade events. Proto-oncogene mutations predicted spontaneously, through interaction with virus, or by chemical or physical agents. When proto-oncogene converted to oncogene, cellgrowth and proliferation pathways changed. This can cause the abnormal cells growth (neoplastic transformation). More than 100 oncogenes has been identified. Genes are the medium by which a cell produce proteism, that each of it has a very spesific role. A mutated gene can caused to over production of a protein, or lower production of a protein, or a change in the protein that may not be able to carry out its objectives. Onkogene usually produce more certain proteins when mutated, while tumor suppressor gene normally produce less proteins product that is needed to suppress the growth when mutated[20]

2. Tumor Suppressor Gene

Activation of oncogene and inactivation of tumor suppressor gene is needed in the process of cancer . Tumor suppressor gene normally associated with cell growth and differentiation and programed cell death (apoptosis). More than 12 tumor suppressor genes have been identified. Proteins produced by tumor suppressor genes normally inhibit proliferating cells or splitting during the period when growth and not when the DNA repairing occured. Tumor suppressor gene is like a “brake” of a cell. Mutation that inactive tumor suppressor gene as an example is the p53 gene mutation, which is the most common mutation seen in human cancer, found about 50%. Breast carcinoma, colon, abdomen, urinary bladder and testicle, melanoma, and soft tissue sarcoma all associated with the mutations in the p53 gene. P53 protein is found in the nucleus of cell and regulate cell functions such as cell growth, DNA repair, and apoptosis. The most important role of p53 is to stop the growth of a cell , allowing the cell to have time to repair the damaged DNA. If the p53 gene mutated, then the p53 gene loss the function to repaired damaged DNA, then apoptosis doesn’t occured, and the results is unregulated cell growth. [20]

3. DNA Mismatch-Repair Genes

Dna Mismatch-Repair Genes found to be associated with cancer susceptibility and genetic instability of cancer cells that allows multiple mutations to occured. This instability accelerated the progress of the cancer. The normal functions of this gene is to repair damaged DNA. Mutations in the DNA mismatch-repair genes most important is in the hereditary non-polyposis colorectal cancer (HNPCC). [20]

4. Apoptosis

Apoptosis is programmed cell death, or cell suicide program , this programs refers to to the

death of the damage cell. It is not random, but occurs in cells with damaged DNA. When cell mutated and can't repair itself, then that cell can be sacrificed to prevent worse mutation that are inherited to the next generation of cells. Inhibition of apoptosis might be an important step in carcinogenesis. 2 genes involved in the apoptosis are tumor suppressor gene p53 and Bcl2 proto-oncogene.[20]

Research problems emerge after knowing the role of p53 and Bcl2 both cellular and molecular. Are there differences in the expression of p53 and Bcl2 in breast adenocarcinoma in C3H mices that have been given *Salvia miltiorrhizae Bunge* root extract compared the group who weren't given *Salvia miltiorrhizae Bunge* root extract? This study aims to prove increase expression of p53 and decrease expression of Bcl2 in the breast adenocarcinoma of C3H mices that have been given multilevel doses of *Salvia miltiorrhizae Bunge* root extract. This study is expected to provide concrete evidence, increasing knowledge and can be applied practically in the medical practice, that there is drugs besides synthetic chemical based drugs including phytopharmaca or herbal drugs that also can be used for breast cancer treatment or as a combination. The results of this study are expected to provide a role in the development of medical science in the future so that it can show the benefit of phytopharmaca or herbal medicine, also it can be base for future research , and complement prior knowledge, so it will obtain more complete and right knowledge about herbal medicine to a disease. For the community, the results of this study expected add knowledge for general public, about the use of phytopharmaca.

METHOD

Laboratory experimental research with *randomized post test only controlled group design* on 18 and 1 C3H mice that have induced breast adenocarcinoma which were obtained from the Laboratory of Experimental Pathology , University of Indonesia in Jakarta, with the inclusion criteria : C3H female mice that have tumor and appeared to be healthy and active, aged 6-8 weeks ,with body weight between 20-30 grams and no anatomical abnormalities, while the drop out criteria is when the mice died during treatment. Tumor C3H mice were then divided into 3 groups, controls , treatment 1 (were given *Salvia miltiorrhizae Bunge* extract 60mg/Kg BW/day dissolved in water, then given per soner) and treatment 2 (180mg/Kg BW/day), for 21 days. One tumor C3H mice was terminated at day 1 of the study to determine the type of the tumor breast adenocarcinoma.

Before the experiments started the mice has been weighed with weight scale and mass measured using calliper, as well as 21 days after treatment. On the 22th day, all mice were terminated using ether chloride, then the growing tumor mass being excised. The tumor mass preparation the being fixated with 10% buffered formalin and being processed with routine histopathology tissue, paraffin blocks were made then thinly slices using a microtome for each block as 3 slides. One slide is attached to normal object glass then given routine staining using hematoxyllin eosin (HE). Other slides attached to object glass that contains poly-L-lysine layer (PolysineTM Microslides; MENZEL-GLASER) then being process for immunohistochemical staining for p53 and Bcl2 proteins. Evaluation of p53 and Bcl2 expression used Allred score [67] and read by 2 anatomical pathologist.

Data that has been collected are primary data results of the scoring of p53 and Bcl2 expression based on Allred score and dan processed with computer program SPSS 15.0 for Windows. Statistical analysis were being performed with significance difference between 2 groups, using Mann-Whitney test and Kruskal-Wallis test was used to test differences more than 2 groups. P values was considered significant if $p \leq 0,05$ with a 95% degree of confidence and 80% power.

RESULTS

Up to 21th day, there are no mice that fall into drop out criteria. Interpretation of p53 and Bcl2 expression using Allred score, perfomed by 2 experts in Anatomical Pathology with Kappa test results that showed a correlation coefficient close to 1, specifically 0,704 for p53 expression and 0,832 for Bcl2 expression with significancy of 0,000 which means that 2 assessors mutually consistent

Mean descriptive analysis of p53 expression showed the highest result were in the 3rd group, the group of tumor C3H mices that was given 180mg/Kg BW/day *Salvia miltiorrhizae Bunge* root extract. Mean of the lowest p53 expression was showed by the 1st group, the group of tumor C3H mices that wasn't given *Salvia miltiorrhizae bunge* root extract.

Table 1. Mean Differences of p53 expression using Allred score

Group	N	Mean	Median	Standart Deviation	*Kruskal Wallis*	
					Df	*p*
C	30	3,3667	3,0000	0,66868	2	0,000
T1	30	3,8333	4,0000	0,74664		
T2	30	4,8000	5,0000	1,03057		

Uji *Kruskal Wallis p*=0,000 (p≤0,05), significant

Mann Whitney test was performed to determine significant differences between treatment group , and the results is significant in all comparisons between.

Table 2. Expression of p53 Difference test between treatments groups

Group I	Group J	Z (I-J)	*P*
Control	Treatment 1	-2,711	0,007
Control	Treatment 2	-5,017	0,000
Treatment 1	Treatment 2	-3,604	0,000

While the mean descriptive analysis of Bcl2 expression showed the lowest results were in the 3rd group the group of tumor C3H mices that was given 180mg/Kg BW/day *Salvia miltiorrhizae Bunge* root extract. Mean of the highest Bcl2 expression was showed by the 1st group, the group of tumor C3H mices that wasn't given *Salvia miltiorrhizae bunge* root extract.

Table 3. Mean Differences of Bcl2 expression using Allred score

Groups	N	Mean	Median	Standart Deviation	*Kruskal Wallis*	
					Df	*p*
C	30	6,3000	6,0000	0,46609	2	0,000
T1	30	5,3333	6,0000	0,80230		
T2	30	4,4667	4,0000	0,97320		

Uji *Kruskal Wallis p*=0,000 (p≤0,05), significant

Mann Whitney test was performed to determine significant differences between treatment group , and the results is significant in all comparisons between.

Microscopic Description

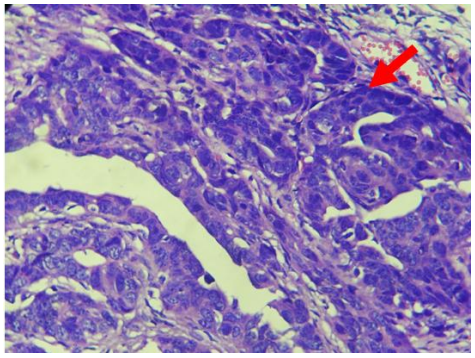


Figure 1. First day termination,

Hematoxyllin Eosin (HE) staining, 400x. Cells appeared to be round, ovale, hard pleiomorfik, rough chromatine (red arrow), mitotic appearance ± 20/10 HPF, partially in groups, forming glandular structures ± 20%, invaded swollen stroma fibromixoid connective tissue surrounded by lymphocytes histiocytes inflammatory cells, and invasion of the blood vessels, and necrotic appearance in the center. The description above was suitable with Breast adenocarcinoma in C3H Mice.

Hematoxyllin Eosin Staining, 400x magnification

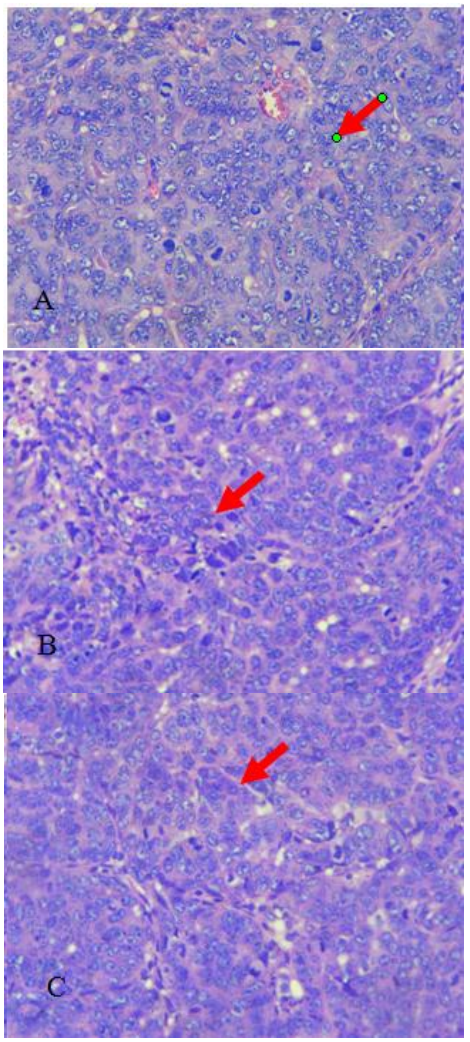


Figure 2. A. Control Group; B. Treatment Group 1; C.Treatment Group 2

p53 Imunohistochemical staining, 400x magnification

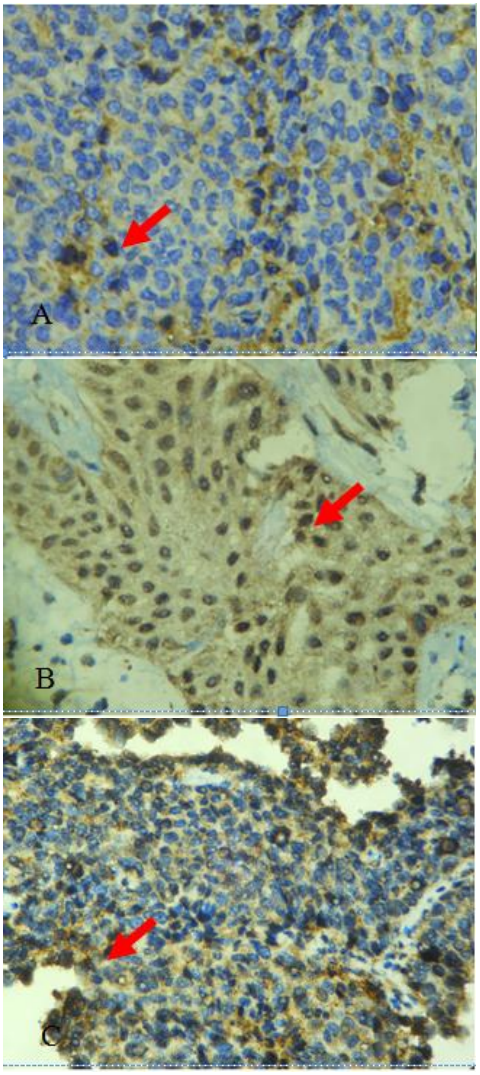
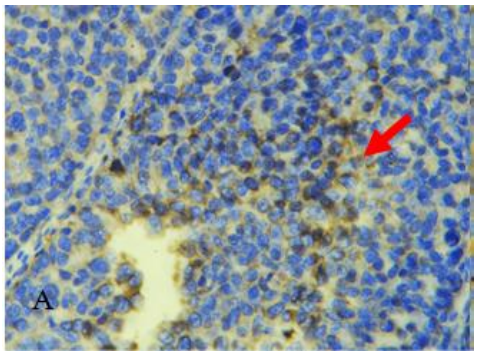


Figure 3. p53 expression showed with the brown color in the nucleus of the breast adenocarcinoma cells .
A.Control Group , *Allred score* 3; B.Treatment Group 1, *Allred score* 4; C.Treatment Group 2, *Allred score 5*



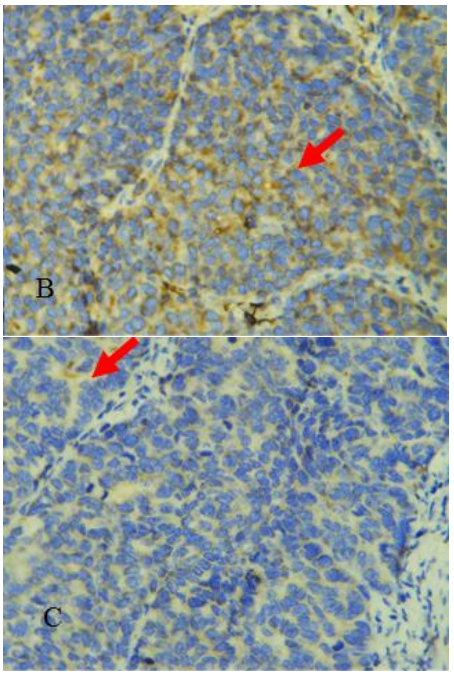


Figure 4. Bcl2 expression showed with the brown color in the cytoplasm and nucleus membrane of the breast adenocarcinoma cells.
A.Control Group, *Allred score* 5; B. Treatment Group 1, *Allred score* 4; C.Treatment Group 2, *Allred score* 2

DISCUSSION

Breast carcinoma is a malignancy that ranked 1st or 2nd in many places around the world, with an increasing incidence, as well as in Indonesia. Preventive and curative measure hasn't been satisfying. Herbal medicine used by eastern medicine, like *Salvia miltiorrhizae Bunge* root extract that contain active subtance tanshinone and its derivatives, which has been previously studied abroad and are known to have anti carcinogenic effects. Until now it has been known many path/pathways of carcinogenesis, including Tumor Suppresor Gene inactivation or activation of proto oncogene. P53 is a protein with an important role as a protector of the genes, so it was called " the guardian of the genome", "guardiang angel gene", or "master watchman". In the apoptotic pathway, p53 transcribes a large number of proteins that involved intrinsic and extrinsic apoptosis pathways, it plays an important role in protecting cells from genetic mutations because of damaged DNA.

P53 expression has many varieties, its depends on the variety of conditions and cellular functions, regulation, apoptosis, DNA replication, proliferation, etc. In the normal conditions, concentration amount of p53 (p53 null) in the cytoplasm are very low and only activated when cells experienced stress, where this p53 null will

transform to p53 wild, which will protect cells from many genetic mutations into p53 mutant. In malignancy conditions, p53 wild concentration are decreasing because of the genetic mutations, so that previous journal said that wild p53 protein can be one of the prognostic factor, in the incidence of malignancy.

In this study the results were consistent with the previous studies, that in the control group, it was found that p53 expression was significantly lower than treatment group 2.

Bcl2 protein has an important function as key regulator of the mitochondrial apoptotic pathway. These proteins control mitochondrial outer membrane (MOM) permeability which release cytochrome c and other apoptotic factor into cytosol. This leads to activation of the caspase cascade that considered as the point of no return in programmed cell death. Apoptotic regulation by the Bcl2 protein is essential for the tissue homeostasis, embryonic development and maturation of blood cells. [48] One important thing, the deregulation of Bcl2 protein has a major role in the formation of tumors and in the cellular response to anticancer therapy. Bcl2 family are also involved in other diseases, such as autoimmune, infectious, and neurodegenerative disorders. On the other hand, there is increasing evidence that Bcl2 family proteins also have other additional function in the celullar processes, such as in the mitochondria and metabolism, which remain mostly yet unknown. [49] Over the last 25 years, Bcl2 family proteins widely studied because of the biological relevance and potential as theurapetic target. 20 or more members of the Blc2 family proteins have been identified and classified according to its function in apoptosis.

The result of this study showed that the expression of Bcl2 in the group that has been given *Salvia miltiorrhizae Bunge* root extract seem significantly lower than the group which weren't given the treatment. The findings of this studies indicate that, *Salvia miltiorrhizae Bunge* root extract give a function to fix anti apoptotic condition, which is expected to become a new hope both for breast cancer patient and clinicians.

The weakness of this study are, the dose used for the calculations was using the base dose for the cardiovascular problems, and there has been no definite dose for malignancy, so further researchs is needed to determine the appropriate doses for malignancy conditions which possible to be different with the dose for cardiovascular problems. It also makes *Salvia miltiorrhizae Bunge* root extract dose has yet to be applied to humans, so its suggested also for futher research in human dose adjusment.

CONCLUSIONS

From the results of the study, it can be concludes that there is meaningful difference in the increased expression of p53 and decrease expression of Bcl2 of the breast adenorcarcinoma in the C3H mices that were given multilevel doses of *Salvia miltiorrhizae Bunge* root extract.

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