Hiroshima J. Med. Sci.
Vol. 67, Special Issue, May, 2018

The Effectiveness of Phaleria Macrocarpa and Chemotherapy in Increasing Caspase 3 and Apoptotic Index in Epidermoid Carcinoma

Helmi SASTRIAWAN[1)], Muhamad Thohar ARIFIN[2) *)], Djoko HANDOJO[2)]

1. *Medical Education Program, Faculty of Medicine Diponegoro University, Semarang, Central Java, Indonesia, 50275*
2. *Department of Surgery, Faculty of Medicine, Diponegoro University, Semarang, Central Java, Indonesia, 50275*

ABSTRACT

Chemotherapy management of epidermoid carcinoma of the skin is relatively expensive and has toxic effects on vital organs. *Phaleria macrocarpa* is a medicinal plant that is often used as an anti-cancer. This study aims to prove the effectiveness of the *Phaleria marcocarpa* extract*, paclitaxel-cisplatin* chemotherapy and the combination of both as a *neoadjuvant* to increase caspase-3 expression and apoptotic index in epidermoid carcinoma of the skin in Swiss male mice. This study used male mice of the Swiss strain, after induction of epidermoid carcinoma cells, 4 group randomization, K (control), P1 (Phaleria macrocarpa 0.0715 mg/ day), P2 (paclitaxel 175 mg/m^2 and cisplatin 50 mg/m^2), P3 (the combination of Phaleria macrocarpa and chemotherapy). The caspase-3 expression was examined with immunohistochemical stain and the apoptotic index examination was done by staining the Tunel. The research shows that there is an increase in caspase-3 expression; the order of the increase (from the most significant to the less significant) is P3, P2, and P1 with significant result ($p<0.05$) compraed to the control group. There is a significant correlation between caspase-3 expression and apoptotic index with a very strong positive correlation. It can be concluded that the *Phaleria marcocarpa* extract*, paclitaxel-cisplatin* chemotherapy and the combination of both increased the caspase-3 expression and the apoptotic index of epidermoid carcinoma cell; there was no significant differences between P1 and P2 but there was a synergy effect from the administration of P1 and P2 combination, and there was significant correlation between the caspase-3 expression and apoptotic index with a very strong positive correlation.

Keywords: *Phaleria marcocarpa,* caspase 3, apoptotic index, epidermoid carcinoma of the skin.

Epidermoid carcinoma (squamous cell carcinoma) is a malignant proliferation of epidermal keratinocytes. It is one of the most common skin cancers occur after basalioma. The incidence is estimated at 25% of all skin malignancies. Epidermoid carcinoma is more common in white skin than in color and occurs more in males than in females, particularly people over the age of 40. Incidence of epidermoid carcinoma is elevated with increasing age.(1)(2) The incidence of metastasis as a whole is 2% - 3%. Predisposing factors of epidermoid carcinoma include ultraviolet radiation, arsenic, hydrocarbon, temperature, chronic radiation, scarring, virus.(2)(3)

According to the National Cancer Institute study, the local recurrence rate after primary therapy from epidermoid carcinoma reaches 3% - 23% depending on the location of the anatomy. Approximately 58% of local recurrences manifested in 1 year, 83% in 3 years, and 95% in 5 years .(4) According to the American Cancer Society, the rate of recurrence of epidermoid carcinoma is still high at 2% and 8.9% after extensive excision with excision limit at 2cm from tumor edge, post radiotherapy 7% - 50% and 20% after curettage and electrodesection.(5)

Modality of epidermoid carcinoma therapy is surgery with wide excision and if KGB (+) is found, KGB dissection is performed. Primary radiation is indicated in an inoperable case. Radiation adjuvant is performed on the condition: the boundary incision is not free from tumor, the incision limit is located near the tumor, there is a contamination of the surgical field by tumor cells, lymph nodes contain more than one metastases, and the diameter of the

*Corresponding author: Muhamad Thohar Arifin. Address: Faculty of Medicine Diponegoro University, Jl. Prof. H. Soedarto, SH Tembalang Semarang,Indonesia. Email: thohar@fk.undip.ac.id

KGB > 3cm, and there is a high-grade malignancy.(2)(3)

Primary chemotherapy is performed in cases with indication of distant metastases, inoperable or patients who fail to be treated surgically and radiotherapy. Currently there is a tendency to use neoadjuvant chemotherapy before surgery or radiotherapy to reduce tumor volume and optimize healing. The chemotherapy commonly used is cisplatin, 5-fluorouracil, bleomicin, doxorubicin and paclitaxel. The rate for partial response is 40%-50% and for the complete response is 28%-31% depending on the given regimen.(2)(3)

The management of epidermoid carcinoma of the skin is relatively expensive and there are often toxic effects that can impair the function of some human vital organs.(6) Currently, many alternative therapies are developed in the form of immunotherapy, namely by modulating the immune system against tumors that are expected to kill cancer cells spread systemically after the local definitive treatment.(7) A number of substances which are tested for cancer therapy influence the expression factor and or activity that regulates apoptosis. In the process of cell apoptosis, caspase 3 is the main implementer that can be activated through extrinsic and intrinsic signal pathways.(8)(9)(10)

Phaleria macrocarpa (*mahkota dewa*) is an Indonesian medicinal plants that have been widely used as an anti-cancer drug plants which have been sold on the open market at 5 gram dose per day. *Phaleria macrocarpa* contains *alkaloids, flavonoids, polyphenols, resins, tannins* and others substances that are efficacious as antihistamines, antioxidants, and medicine for rheumatism, diabetes, high blood pressure, and cancer (cytotoxic). Empirical evidence of its usefulness has been found in many societies, but the scientific proof is still very limited and further studies are required.(11)(12)

The purpose of this study is to prove the effectiveness of the *phaleria marcocarpa* extract, *paclitaxel-cisplatin* chemotherapy and the combination of both as a *neoadjuvant* for caspase-3 expression and apoptotic index of epidermoid carcinoma in Swiss mice.

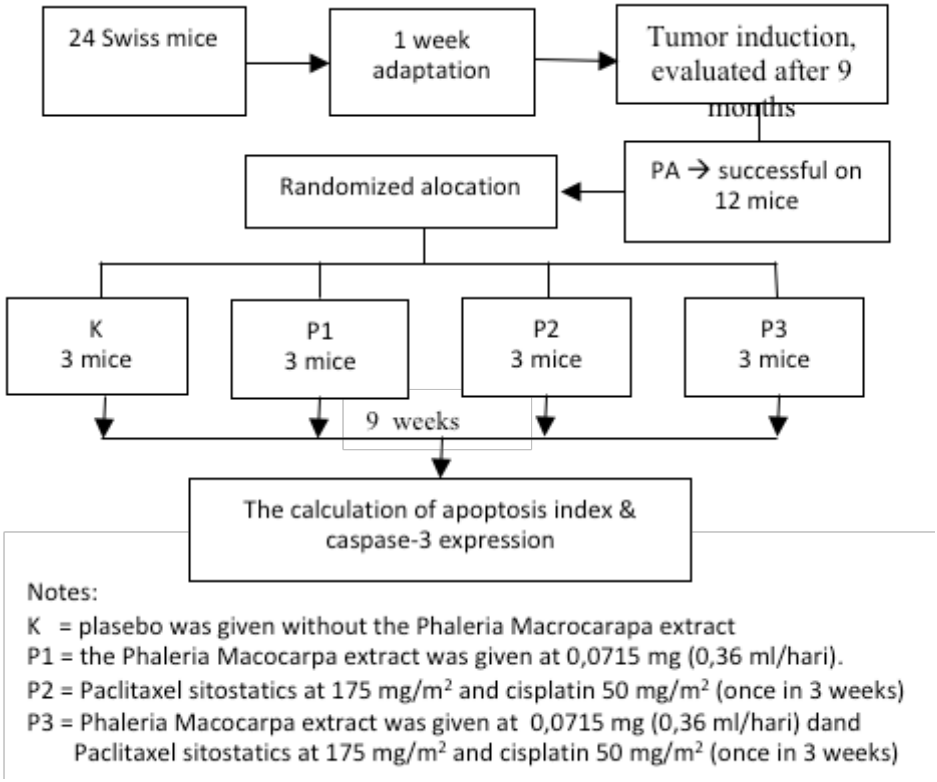
MATERIALS AND METHODS

Twenty four male Swiss mice aged 3 months and weighed 20-30 grams were individually stacked and fed with standard feed for one week with *ad libitum* (at the mice's pleasures) system. Epidermoid carcinoma was induced in the mice's skin by clean shaving the hair in interscapular area for 1.5 x 1.5 cm. The carcinogen used was 9. 12-dimethyl-1.2-benzanthracene (DMBA) 100 nmol (0.025 mg), dissolved in 0.1 ml of acetone reagent per mouse. DMBA was applied 2 times per week for 2 weeks, then continued with topical 12-o-tetradecanoylphorbol-13-acetate (TPA) application in the interscapular region at a dose of 1.7 nmol (0.001 mg) in 0.1 ml aceton per mice tail 2x per week for 22 weeks.

The mice which successfully inoculated and performed PA biopsy with epidermoid carcinoma results, was divided into 4 groups, then they were given treatment for 3 weeks. After the treatment was completed, the mice in anaesthesia with ether and then the mice were exterminated by means of cervical dislocation and the tumor tissues were taken and processed into histologic preparations. Furthermore, Tunel was stained to examine the index of apoptosis and immunohistochemical staining to observe the caspase-3

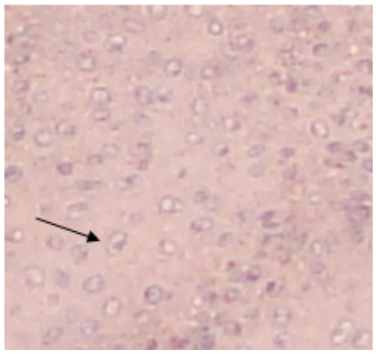
RESULTS

Figure 1. Consolidated research report

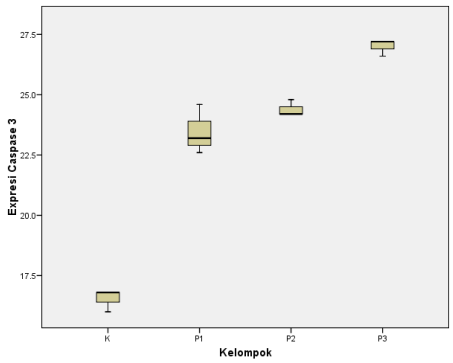


After the treatment, the tumor tissue was taken, and histological examination with immunohistochemical staining with *Caspase 3 (CPP32) Ab-4 rabbit polyclonal antibody RB-1197-PO, NeoMarkers* was conducted to calculate the index of caspase 3 and Tunel staining was performed to calculate apoptotic index (13,14). With immunohistochemical staining on caspase 3, cellular features are called positive if there is a dominant brown apoptotic cell in the cytoplasm and some in the cell nucleus, as well as compared with positive control.(13) Cell provides an overview of apoptosis microscopically on Tunel staining in the form of the apoptotic cells will turn into green fluorescent if observed using a fluorescence microscope with magnification of 400x.(14) Histological examination results are as follows

Figure 2. Histological features of caspase expression 3 (arrow)



Grafic 1. *Box plot* of caspase-3 expression



The normality test using *the Shapiro-Wilk* test shows abnormal data. Furthermore, the *Kruskal Wallis* test shows p value <0.05 or significant, then to observe the difference between groups, *Mann Whitney* test was performed

Table 1. *Mann Whitney* caspase 3 expression

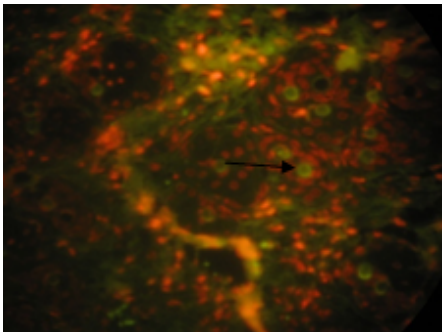
Group	P1	P2	P3
K	0.046*	0.043*	0.043*
P1	–	0.268	0.046*
P2		–	0.043*

note:

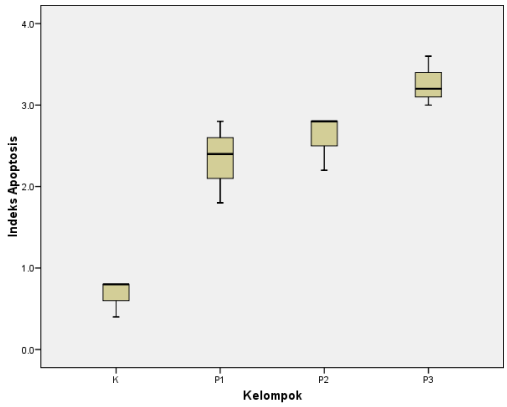
* Significant p<0.05

From table *Mann Whitney* test, it was found that a significant difference (p <0.05) exists between the control group (K) and treatment 1 (P1), treatment 2 (P2) and treatment 3 (P3), between the treatment group 1 (P1) and treatment 3 (P3), between treatment 2 (P2) with treatment 3 (P3)

Figure 3. Histologic picture of apoptosis (arrow)



Grafic 2. *Box plot* of apoptotic index



From the data normality test of apoptotic index with *the Shapiro-Wilk* test, it was found that the obtained data are not normal. Then, *Kruskal Wallis* test shows p value = 0.022 (p <0.05) or significant to determine the difference between groups, *Mann Whitney* test was conducted.

Table 2. *Mann Whitney* Test of Apoptotic Index

Group	P1	P2	P3
K	0.046*	0.043*	0.046*
P1	–	0.487	0.049*
P2		–	0.046*

Note:

* Significant p<0,05

The Mann Whitney test shows a significant difference (p <0.05) exist between the control group (K) and treatment 1 (P1), treatment 2 (P2) and treatment 3 (P3), between the treatment group 1(P1) and treatment 3 (P3), between treatment 2 (P2) and treatment 3 P3).

Meanwhile, regarding with the correlation between the caspase-3 expression and the apoptotic index, the data normality test with *Shapiro Wilk* test shows that caspase 3 expression has p value = 0.025 (p <0.05), which means that the data distribution is not normal, so that for the next test, non-parametric *spearman* correlation test was used

Table 3. *Spearman* correlation of caspase 3 expression and the apoptotic index

Variable	r	P
Expresi Caspase 3 Indeks Apoptosis	0.961	< 0.001*

note :
* Significant p<0,05

The *Spearman* correlation test table shows p value = <0.001 (p <0.05) and the value of r = 0.961, so it can be concluded there is a significant correlation between caspase 3 expression and the apoptotic index with a very strong positive correlation.

DISCUSSION

Phaleria macrocarpa is one of Indonesia's traditional medicinal plants that have anti-cancer effects. However, there is relatively few scientific reference in terms of pharmacology and phytochemical regarding this plant. M*acrocarpa Phaleria* was used as a medicinal plant for anti-cancer and anti-microbial. The empirical evidence of its usefulness has been found in many societies, but the scientific proof is still very limited, so further evidences are needed.(11)(12) As an attempt to prove the effects of *Phaleria macrocarpa* as anti-cancer drugs particularly on the epidermoid carcinoma, this study was conducted. This study aimed to prove the effect of *Phaleria macrocarpa* with indicator caspase 3 expression and apoptotic index of epidermoid carcinoma cells in Swiss mice.

The result shows that there was an increase in caspase 3 expression in all treatment groups which were given *Phaleria macrocarpa* extract*, Paclitaxel-cisplatin* chemotherapy, and the combination of both. The order of increased caspase-3 expression from the most significant increase to the least significant increase is the group which was given a combination of *Phaleria macrocarpa* extract and chemotherapy *paclitaxel-cisplatin* (group P3), followed by the group which was given the chemotherapy *paclitaxel-cisplatin* (group P2) and the group which was given the extract of *Phaleria macrocarpa* (P1). The significant result *(p <0.05)* occurred in all treatment groups compared to the control group (K).

The significant results in the treatment group which were given *Phaleria macrocarpa* extract are closely related to *polyphenols* contained in medicinal plants; *polyphenols* are reported to have the effect of inducing apoptosis through TNF-α (extrinsic pathway*/ extrinsic pathway)* .(10)(15) *Pholyphenol, which constitute as* active substances, contained in *Phaleria macrocarpa* such as *gallic acid* and *flavonoids* play a role in inducing apoptosis; A study using esophageal cancer cells (TE-1) shows that the *gallic acid (GA: 3,4,5-trihydroxybenzoic acid)* would increase the protein pro apoptosis Bax and will decrease Bcl-2 anti-apoptotic protein.(16) Meanwhile, *flavonoids* work by inhibiting the activity of DNA topoisomerase I/II, modulation of signaling pathways, decreasing gene expression of Bcl-2 and Bcl-XL (anti-apoptotic), increasing gene expression of Bax and Bak (pro-apoptotic) as well as the activation of endonucleuses.(17) Topoisomerase, an enzyme that serves to cut the tight winding of DNA is as a result of the opening of *double strand* DNA by enzyme helicase, a U-turn and then connect it again. The enzyme works on the extension of DNA replication. If there is inhibition of topoisomerase activity, there will be stabilization of the cut topoisomerase-DNA complex, resulting in a permanent damage of *double-stranded* DNA which then activates p53.(18)(19) Activation of p53 in response to DNA damage would stop the cycle of mitosis in the G1 phase, so that the cell cannot enter S phase when DNA damage is not repaired, and if the DNA damage cannot be repaired, the p53 will activate protein pro-apoptotic *Bcl-2 Family* as Bid, Bax and Tub which will subsequently lead to caspase-3 inactivation which eventually leads to cell apoptosis.(20)(21)(22)

Bax, Bak, Bcl-2 and Bcl-xl is a Bcl-2 *family* proteins. Bax and Tub are proapoptotic proteins whereas Bcl-2 and Bcl-xl are antiapoptotic proteins. Bcl2 attaches to the outer membrane of the mitochondria thus blocking the release of cytochrome c whereas Bcl-xl binds to Apaf-1. Cytochrome c and Apaf-1 are required in the process of apoptosis through an intrinsic pathway by activating caspase-9. The function of survival is offset by the cell death function mediated by Bax and Bak. Bax may bind to the outer membrane of the mitochondria thus inducing cytochrome c out of mitochondria while Bak may bind with Bcl-xl to release Apaf-1. If the expression of Bax or Bak is raised and Bcl-2 or Bcl-xl is lowered, cell regulation towards the cell death by caspese 9 activation will lead to caspase-3 inactivation leading to cell apoptosis.(15)(16)(21)(22)(23)

Phaleria macrocarpa extract, *Paclitaxel-cisplatin* chemotherapy, and the combination of both is also shown to enhance the apoptotic index when compared with controls. The increase in apoptotic index in the order from large to small were seen in the group which were given a combination of *Phaleria macrocarpa* extracts and *Paclitaxel-cisplatin* chemotherapy (group P3), given *Paclitaxel-cisplatin* chemotherapy (group P2) and the group given the extract *Phaleria macrocarpa* (P1). The significant result *(p <0.05)* occurred in all treatment groups compared to the control group.

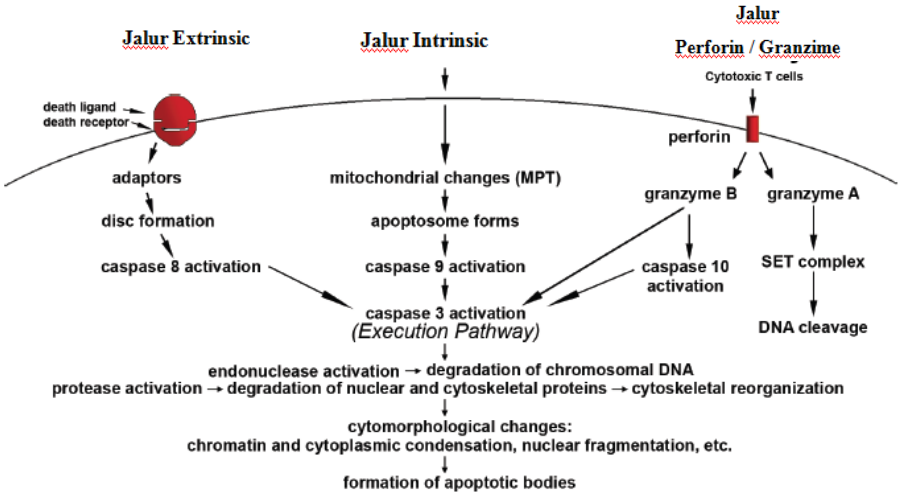
The increase in apoptotic index in the treatment group, apart from the *gallic acid* and *flavonoids* to increase the caspase-3 expression as described above, *gallic acid* and *flavonoids* can also stimulate the production of interferon-γ (IFN-γ) in a population of immunosit, which is very important in promoting the activation of CTL and NK cells in the immune system of immune cells against cancer cells. When CTL and NK cell is active and a lot more going on the *killing* of the tumor cells that cause a lot of apoptosis of tumor cells.(10)(22) Besides the effector function of NK cells to kill cancer cells by secreting *perforin* and *Granzyme* which then induce apoptosis of target cells; releasing IFN-γ which enhances macrophage phagocytosis work; bonding on *the target-cell death receptor,* such as FAS (CD95) or FAS ligand (FasL) in opsonized cancer cells that causes cancer cell to be programmed as apoptosis; As well as breaking/ hydrolyze substrate specific proteins including caspase causing target cells to undergo apoptosis.(10)(15)(16)

Paclitaxel, in addition to inhibit cell proliferation, may also increase apoptosis by reducing expression of Bcl-2 and Bcl-xl, and raise proapoptosis Bax protein.(24)(25) Meanwhile, in addition to inhibit the cell cycle, *cisplatin* also increase apoptosis by inducing P53, P53 will induce proapoptosis Bax protein that will ultimately lead to cell apoptosis. Therefore, a synergistic effect between the extract *Phaleria macrocarpa* and *Paclitaxel-cisplatin* chemotherapy is obtained.(16)(19)(26)(27)

The result of correlation test which was performed to analyze the correlation between the caspas-3 expression and the apoptotic index using *Spearman's* test shows a significant correlation with p = <0.001 (p <0.05) and the value of r = 0.961, so it can be concluded that there is a significant correlation between caspase-3 expression and apoptotic index with a very strong positive correlation. This is in accordance with the three mechanisms of apoptosis:

1. Extrinsic pathway where there is a *death receptor* activation (DR) on the surface of the cell membrane by ligands which then activates caspase 8 and eventually cause the activation of caspase 3.
2. intrinsic pathway where there is *cellular stress* (oxidative stress, radiation, cytotoxic drugs) which will cause the mitochondria to synthesize *cytocrom c* binding to Apaf-1 and pro-caspase 9 forms *apoptosome* which will cause the activation of caspase 9 and in the end causing caspase 3 to be activated. Intrinsic pathway can also be activated by caspase 8*)* through protein breakdown Bid.
3. *Granzime B pathway*s that are sensitive to the target cell .(15)(21)(28)

Figure 4. Apoptosis path (26)



CONCLUSION

Based on this study, it can be concluded that *Phaleria marcocarpa* extract, chemotherapy *paclitaxel* *cisplatin* and the combination of both increased caspase-3 expression and the apoptotic index of epidermoid carcinoma cell; it was not found significant differences between P1 and P2; a synergy effect was obtained from the administration of the combination of P1 and P2; there is a significant correlation between caspase-3 expression and apoptotic index with a very strong positive correlation.

REFERENCES

1. Schawart R, Stoll H. Squamous Cell Carcinoma. In: Fitzpatrick T, Eizen A, Wolff K, Freedberg I, Auten K, editors. Dermatology in General Medicine. 4th ed. New York: Mcgraw Hill; 1993. p. 821–35.
2. Suyatno, Pasaribu E. Kanker Kulit : Bedah Onkologi, Diagnostik Dan Terapi. Jakarta: Sagung Seto; 2010. 149-182 p.
3. Manuaba TW, editor. Panduan Penatalaksanaan Kanker Solid PERABOI 2010. Sagung Seto; 2010. 153-160 p.
4. Rowe D, Carroll R, Day CJ. Prognostic Factors For Local Recurrence, Metastasis, And Survival Rates in Squamous Cell Carcinoma of The Skin, Ear, And Lip. Implications for Treatment Modality Selection. J Am Acad Dermatol [Internet]. 2012;26(6):976–90. Available from: Www.Cancer.Gov
5. Cancer Facts and Figure. American Cancer Society. 2003.
6. Baretta G. Cancer Treatment Medical Guide. 11th ed. Milan: FARMITALIA Carlo Erba – Erbamont; 2001. 174 –17 6 p.
7. Rowinsky E. The Development and Clinical Utility of The Taxane Class of Antimicrotubule Chemotherapy Agent. Annu Rev Med. 1997;48:353–74.
8. Ganju L, Karan D, Chanda S, Srivastava KK, Sawhney RC, Selvamurthy W. Immunomodulatory Effects of Agents of Plant Origin. Biomed Pharmacother. 2003;57(7):296–300.
9. Ghavami S, Hashemi M, Ande S, Yeganeh B, Xiao W, Eshraghi M, et al. Apoptosis and Cancer : Mutations within Caspase Genes. J Med Genet. 2009;46:497–510.
10. Abbas A, Lichtman A, Pillai S. Cellular And Molecular Immunology. 6th ed. Philadelphia: Elsevier-Saunders; 2010. 4-15, 22-3, 65-80, 81-103, 182-7, 247-53, 258-9, 2 p.

11. Winarto W. Mahkota Dewa : Budi Daya Dan Pemanfaatan Untuk Obat. Jakarta: Penebar Swadaya; 2004.
12. Selamat B, Benny I, Djoko H, Dwi P, Riwanto I. Pengaruh Ekstrak Mahkota Dewa (Phaleria Macrocarpa) Terhadap Skor Ekspresi Perforin CTL dan Sel-NK Serta Indeks Apoptosis pada Adenokarsinoma Mamma Mencit C3H. J Kedokt Media Med Indones. 2007;42(1):37–40.
13. Kaur J, Sharma M, Sharma P, Bansal M. No Antitumor Activity of Lantadenes In DMBA/TPA Induced Skin Tumors in Mice: Expression of Transcription Factors. Am J Biomed Sci. 2009;79–90.
14. Stites D, Terr A, Parslow T. Medical Immunology. 9th ed. London: Apleton And Lange A Simon Co; 2002. 65-69-637 p.
15. Disaia P, Creasman W. Tumor Immunology, Host Defense Mechanism And Biologic Therapy. In: Clinical Gynecology Oncology. 4th ed. Philadelphia: Mosby; 1997. p. 534–75.
16. Yeap S, Rahman M, Alitheen N, Ho W, Omar A, Beh B, et al. Evaluation of Immunomodulatory Effect : Selection of The Correct Targets for Immunostimulation Study. Am J Immunol. 2011;7(2):17–23.
17. Bast C. Principles Of Cancer Biology : Tumor Immunology. In: Devita V, Rosenberg S, Hellman W, editors. Cancer Principles and Practice of Oncology. 8th ed. Philadelphia: Lippincott Williams& Wilkins; 2008. p. 267–83.
18. Zou W, Restifo N. Th 17 Cells In Tumour Immunity and Immunotherapy. Nat Rev Immunol. 2010;10(4):248–56.
19. Alshaker H, Matalka K. IFN-Γ, IL-17 And TGF-ß Involvement in Shaping The Tumor Microenvironment : The Significance of Modulating Such Cytokines in Treating Malignant Solid Tumors. Cancer Cell Int. 2011;11:33.
20. Wong R. Apoptosis in Cancer : From Pathogenesis to Treatment. J Exp Clin Cancer Res. 2011;30:87.
21. Cerretti DP, Kozlosky CJ. lecular Cloning of The Interleukin-1 Betaconverting Enzime. Science (80-). 1992;256(5053):97–100.
22. Fan T, Han L, Cong R, Liang J. Caspase Family Proteases and Apoptosis. Acta Biochem Biophys Sin. 2005;37(11):719–27.
23. Ollson M, Zhivotovsky. Caspases And Cancer : Cell Death and Differentiation. 2011;18:1441–9.
24. Zhang N, Hartig H, Dzhagalov I, Draper D, He Y. The Role of Apoptosis in The Development And Function of T Lymphocytes. Cell Res. 2005;15(10):749–69.
25. Amundson S, Myers T, Fornace A. Roles for P53 in Growth Arrest and Apoptosis: Putting on The Brakes After Genotoxic Stress. Natl Institutes Heal. 1998;
26. Soini Y, Paakko P, Lehto V. Histopathological Evaluation of Apoptosis in Cancer. Am J Pathol. 1998;153:1041–53.
27. Hadjiloucas I, Gilmore A, Bundred N, Streuli C. Assesment of Apoptosis in Human Breast Tissue Using An Antibody Against The Active Form of Caspase 3 : Relation to Tumour Histopathological Characteristics. Br J Cancer. 2001;85(10):1522–6.
28. Ghavami S, Hashemi M, Ande S, Yeganeh B, Xiao W, Eshraghi M, et al. Apoptosis and Cancer : Mutations Within Caspase Genes. J Med Genet. 2009;46:497–510.