High Antitumor Activity of Ethanolic Extracts of Papua's Ant Nest Plant (*Myrmecodia tuberosa*) on an Oral Carcinoma (KB) Cell Line

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Abstract: *Papua's ant nest (Myrmecodia tuberosa) is a traditional plant that is known to have the strong anticancer compound. Efficacy of ant nest was shown by the content of active substances include flavonoids, polyphenols, tannins and glycosides. Ant nest plant has been used to treat various diseases traditionally and safely without any side effects. Aims of the study were to examine the high antitumor activity of ethanolic extracts of Papua's ant nest plant on an oral carcinoma cells through the growth inhibition and apoptosis induction of KB cells. In the present study, KB cells were treated with various concentration of ethanol extract of Papua's ant nest plant. MTT assay was conducted to determine the growth inhibition of KB cells. The double-staining analysis was carried out to evaluate the induction of cells apoptosis. The results revealed the growth of KB cells treated with ant nest extract was remarkable suppressed. The percentage of cell inhibition was known at 84.2%. Therefore, apoptosis of KB cells was markedly induced characterized by an increase in death cells. Percentage of apoptosis was detected at 88%. In addition, the fifty-inhibitory concentration (IC_{50}) of Papua's ant nest was reported at 215 μg/ml in KB cell line. These results indicated that Papua's ant nest plant exhibited a high potential antitumor activity in oral carcinoma KB cells.*

Keywords: Papua's ant nest plant, ethanolic extract, growth inhibition, apoptosis, KB cells.

1. Introduction

Cell growth is controlled through an intricate network of extracellular and intracellular signaling pathways which process both negative and positive growth signals. The ultimate recipients of many of these signals are cyclin-dependent kinases (CDKs), a family of enzymes which catalyze events required for individual cell-cycle transitions. CDKs require association with cyclins for activation, and the timing of CDK activation is dependent largely upon the timing of cyclin expression.[1] The activity of cyclins–CDKs complexes is affected by two groups of CDK inhibitors (CKIs) which have an inhibitory effect on cell cycle progression. Both families have a conserved amino-terminal domain that is sufficient for both binding to cyclin/CDK complexes and inhibiting CDK catalytic activity. [2]

The KB cell line was established from a removal of human mouth epidermal carcinoma cells. These cells were derived from human carcinoma of the nasopharynx. KB cells exhibit an epithelial cell morphology and are known to produce keratin. It was reported that HeLa cell line marker chromosomes have identified in the KB cell line, and through DNA fingerprinting, KB cells are now thought to have been produced due to HeLa cell contamination. [3]

Myrmecodia tuberosa (M. tuberosa) are plants that are attached to other plants (epiphyte) and thrived in the highlands of Wamena, Papua, Indonesia. Originally, M. tuberosa was introduced in the inland of Papua, and commonly used as a medicine by local residents. M. tuberosa was used by the people of Papua since the 1950s for a mixture porridge and boiled drink to enhance the body immunity [4]. Interestingly, M. tuberosa has been used to treat various diseases traditionally and safely without any side effects by some tribes in Wamena inland for a long time. It was reported that M. tuberosa can inhibit various types of human cancers including brain, breast, lung, colon, liver, uterus, prostate, skins, cervical and blood cancers [5,6]. In addition, the fifty-inhibitory concentration (IC50) of extract of M. tuberosa was reported at 33.28 μg/ml in cervix cancer cell line (HeLa cell), and 353.18 μg/ml in breast cancer cell line (MCF-7 cell). [5] Recently reported that M. tuberosa could treat a variety of systemic diseases such as leukemia, heart diseases, tuberculosis, kidney and prostate, various allergies, migraine, rheumatism, hemorrhoid, and infection diseases [7]. Although *M. tuberosa* can inhibit various types of human cancers, its antitumor effect on KB cell is still unclear. In the present study, the high antitumor activity of *M. tuberosa* extract through growth inhibition and apoptosis induction on KB cell line was examined.

2. Materials and Methods

Cell and cell culture
KB cells (ATCC CCL-17) were delivered from LPPT-UGM, Yogyakarta, Indonesia, and maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO, USA) supplemented with 10% fetal calf serum (FCS; Moregate BioTech, Bulimba, Australia), 100 μg/ml streptomycin, and 100 U/ml penicillin (Invitrogen Corp., Carlsbad, CA, USA). Cells were incubated at 37^0C with 5% CO_2.

Preparation of crude ethanol extract

Fresh Papua's ant nest plants were collected from Wamena inland, Papua, Indonesia, and identified at the Department of Biology Pharmaceutical, Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta, Indonesia. Plants were cut into small pieces and dried inside the incubator at 55^0C. Five hundred gram of pieces were soaked in 500 ml of 80% ethanol and put on the shaker at 150 rpm for 3 days at ambient temperature. The mixtures were then filtered. The filtrate were evaporated using vacuum rotary evaporator (BÜCHI Rotavapor R-200/205, Model R205V800). Stock solutions of crude ethanol extracts were prepared by diluting the extracts with suspending agent (200 μl of dimethyl sulfoxide) followed with aquabidest solution to obtain a final concentration of 100 mg/ml.

Suppression of cell growth (MTT assay)

KB cells were seeded on 96-well plates (Falcon, NJ, USA) at 2×10^4 cells per well in DMEM containing 10% FBS. After 24 h, the culture medium was replaced with new medium containing various concentrations (0, 62.5, 125, 250, 500, and 1000 μg/ml) of ethanol extract of ant nest plants and 12.5 μg/ml of docetaxel hydrate (Aventis Pharma, Japan) as a positive control. After 24 h, 20 μl fresh 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide [MTT, 5 mg/ml phosphate-buffered saline (PBS)] (Sigma-Aldrich, USA) was added to each well. After 4 h of incubation, the culture media were discarded, 100 μl of dimethyl sulfoxide (DMSO: Nacalai tesque, Inc., Kyoto, Japan) was added to each well and vibrated to dissolve the MTT formazan. The optical density was measured at 540 nm with a BioRad microplate reader (BioRad Laboratories, Hercules, CA, U.S.A.). Each determination was performed in triplicate.

Induction of apoptosis using double-staining analysis

Acridine orange (AO) and ethidium bromide (EB) double staining were carried out in this study. DNA-binding dyes AO and EB (Sigma, USA) were used for the morphological detection of apoptotic cells. AO is taken up by both viable and non-viable cells and emits green fluorescence if intercalated into double stranded nucleic acid (DNA). EB is taken up only by non-viable cells and emits red fluorescence by intercalation into DNA. After treatment with different concentrations of ant nest extract for 24 h, the cells were detached, washed by cold PBS and then stained with a mixture of AO (100 μg/ml) and EB (100 μg/ml) at room temperature for 10 min. The stained cells were observed by a fluorescence microscope (Zeiss, Germany) at 100 × magnifications. The cells were divided into three categories as follows: living cells (normal green nucleus), early apoptotic (bright green nucleus with condensed or fragmented chromatin), late apoptotic (orange-stained nuclei with chromatin condensation or fragmentation). In each experiment, more than 100 cells/sample were counted.[8]

Statistical Analysis

Statistical differences between the means for the different groups were evaluated with Stat View 4.5 (version 5.0J, SAS Institute Inc, Cary, NC, USA) using one-way ANOVA and a post hoc *t-test*. The significance level was set at 5% for each analysis.

3. Results

Cell growth *in vitro*

KB cells treated with various concentrations of Papua's ant nest extract were examined by the MTT [(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. Relative cell number was evaluated by comparing the absorbance in each cell. It was detected that the more increasing concentration of the extract, the more decreasing the number of viable KB cell based on the colorimetric absorbance values. Data indicated the negative control has the highest absorbance at 1.416 ± 0.05, whereas the positive control (Docetaxel IC_{50} = 12.5 μg/ml) has an absorbance at 0.514 ± 0.20. Concentration of 500 to 1000 μg/ml of extract has the lower absorbance at 0.587 ± 0.04 and 0.365 ± 0.03, respectively (Table 1).

Table 1: Mean and standard deviation of KB cell growth after treatment with the various concentration of ant nest plant extract for 24 hours.

Negative control (Aquadest)	62.5 μg/ml	125 μg/ml	250 μg/ml	500 μg/ml	1000 μg/ml	Positive control (Docetaxel) IC_{50} = 12.5 μg/ml
1.416 ± 0.05	0.909 ± 0.03	0.863 ± 0.01	0.732 ± 0.02	0.587 ± 0.04	0.365 ± 0.03	0.514 ± 0.20

The percentage increase in KB cell growth inhibition by extracts concentration of 62.5, 125, 250, 500 and 1000 μg/ml was known at 35.5%, 39.5%, 58.3%, 68.5% and 84.2% compared to the negative control, whereas 12.5 μg/ml of docetaxel was detected at 62.4% (Figure1). In addition, IC_{50} of ant nest extract was found at concentration 215 μg/ml. These data showed that extract of Papua's ant nest was effective and has the strong potential to inhibit the growth of KB cells.
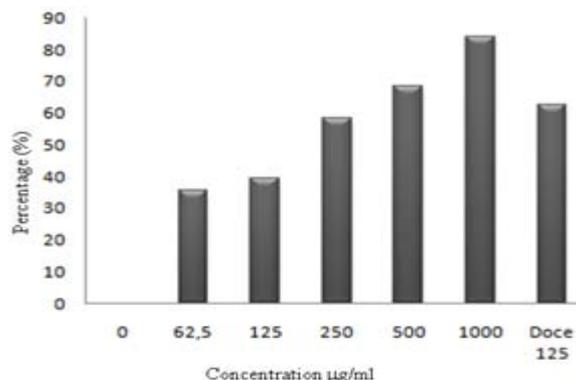

Figure 1: The percentage increase in growth inhibition of

KB cells after treated with various concentrations of extract for 24 hours

In the culture cell, the growth of KB cell was inhibited with the various concentration of ant nest plant extract. The greater concentration of the extract was appeared the greater the KB cell growth inhibition. In addition, the morphology of KB cells that turn into long, flat, oval and irregular form (Figure 2).
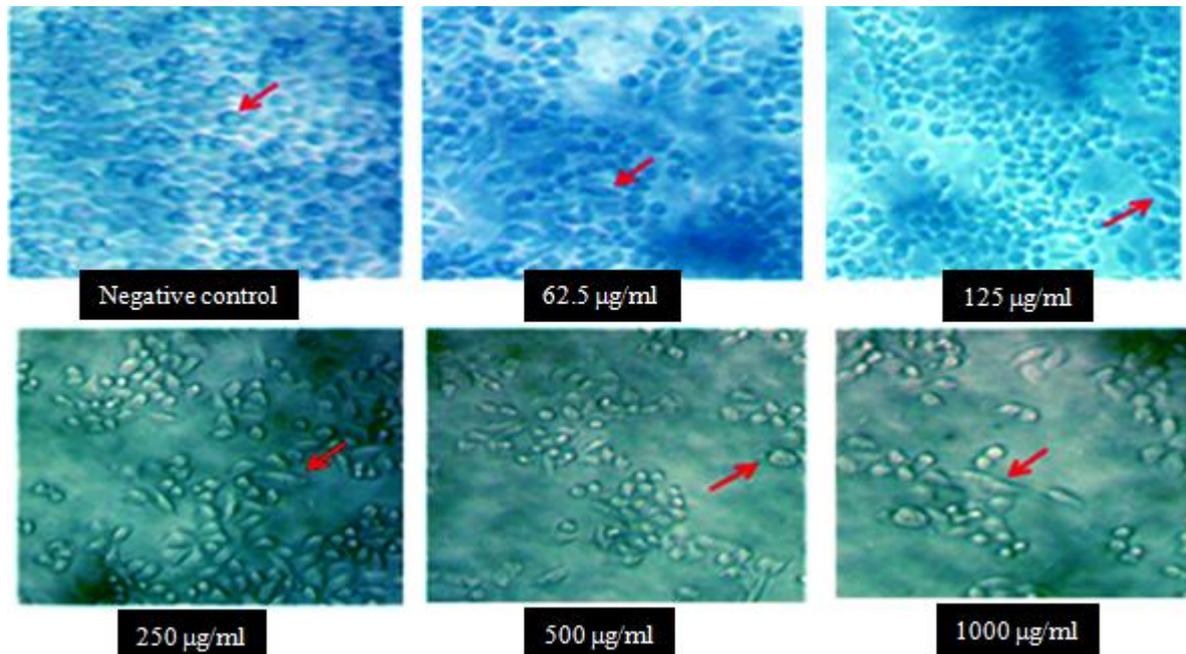

Figure 2: Differences in the cell number and changes in KB cell morphology incubated with different concentrations of the extract for 24 hours.

Linear regression between the extract concentrations of ant nest plant with the percentage of KB cell viability were shown in Figure 3.
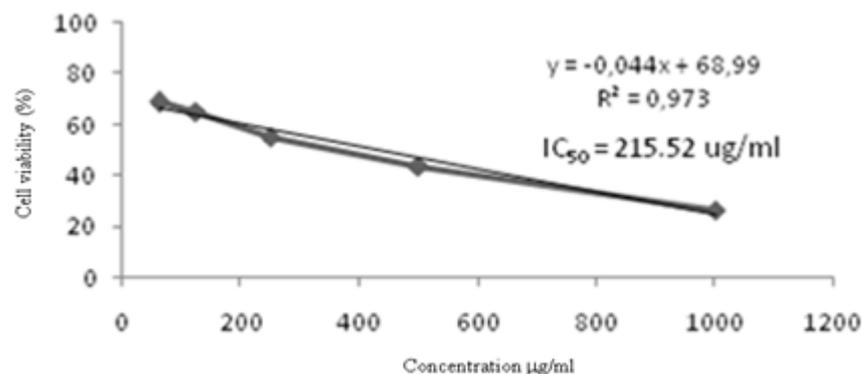

Figure 3: Linear regression between extract concentrations of ant nest plant with KB cell viability

Results of linear regression analysis of the extract concentrations of ant nest plant with the percentage of KB cell viability has a regression coefficient r = 0.973. These results indicated a very strong relationship between the concentrations of the extract with KB cell viability. Given the linear regression equation y = 0,044x + 68.99.

Induction of apoptosis
In this study, the acridine orange-ethydium bromide (AO-EB) double-staining test was used to evaluate the apoptotic potential of Papua's ant nest extract on an oral carcinoma KB cells. These dyes emit different shades of fluorescence and possess a different ability to penetrate cells. AO penetrates into living cells, emitting green fluorescence after intercalation into DNA. The second dye, EB emits red fluorescence in the cells with an altered cell membrane. Morphological features of apoptosis such as chromatin condensation, nuclear fragmentation, alterations in the size and the shape of cells, as revealed by fluorescence microscopic analysis, were observed predominantly after ant nest extract treatment in 24 h.
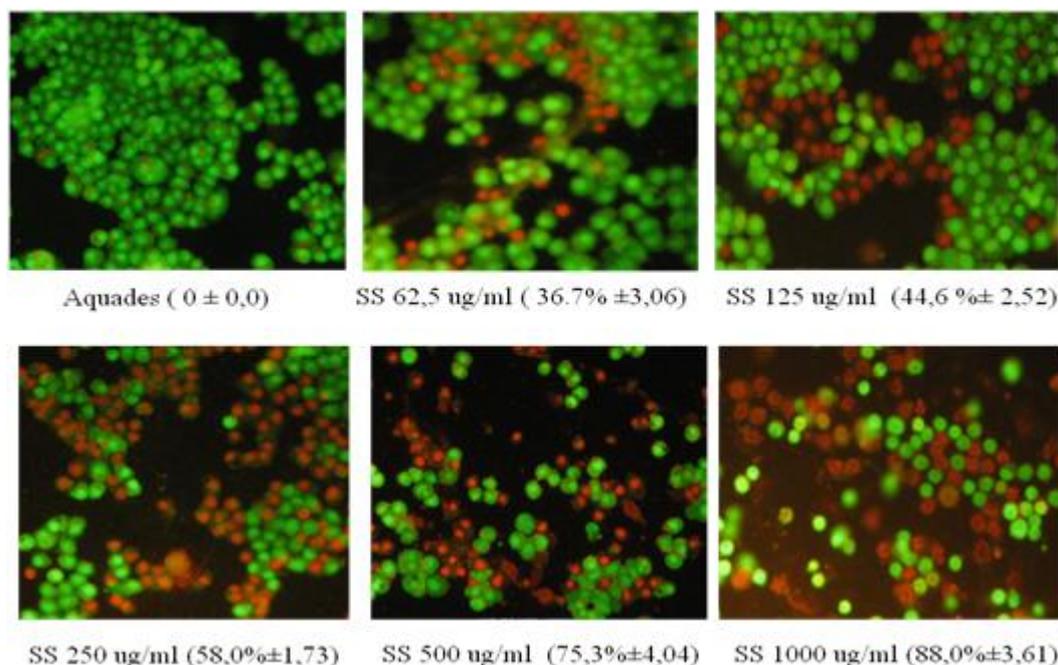
Figure 4: KB cell apoptosis after incubation with ethanol extract of ant nest plant for 24 hours using a double-staining method magnification 40x. Normal cells (green) and cell apoptosis (orange-red).

As shown in Figures 4, ant nest extract treatment in the different concentrations (62.5, 125, 250, 500 and 1000 μg/ml) has an effect on apoptosis, while the maximum percentage of apoptotic cells was observed in 1000 μg/ml concentration of extract (88.00 ± 3.61%). However, the minimum percentage of apoptotic cells was detected in 62.5 μg/ml extract concentration (36.67 ± 3.06%) [F = 356.607; P < 0.000].

4. Discussion

The normal operation of cell cycle requires a delicate balance between positive and negative regulatory factors. Any alteration in this balance can result in abnormal cell proliferation, which may lead to malignant transformation. Loss of normal cell cycle control plays a crucial role in the genesis of most types of cancer [1]. The cell cycle is modulated by the interaction of multiple cell cycle molecules, including cyclins, cyclin-activating kinase (CAK), cyclin-dependent kinases (CDK) and cyclin-dependent kinases inhibitors (CDIs) [9]. Disruption of the cell cycle can cause cell growth abnormalities. In the present study, concentration-dependent of Papua's ant nest extract has strong capability to suppress the growth of KB cells. These result may originate from secondary metabolite of ant nest plant include flavonoid, polyphenol, tocopherol and tannin compound contained in extract. Flavonoid is a class of natural compound which exhibits activity as reductor to hydroxyl, superoxide and peroxyl radicals [10]. Flavonoids and polyphenols have been proved to inhibit proliferation and angiogenesis of human tumor cells in vitro [11], as well as in experiment animals [12]. Engidaa *et al*. [13] reported that Papua's ant nest plant has five flavonoid compounds through HPLC examination, namely kaempferol, luteolin, rutin, quercetin and apigenin. Interestingly, kaempferol was found to have the highest concentration (13.77 mg/ g). It was reported that kaempferol has strong antitumor activity proved by inhibition of various types of human cancer cells

through suppressed the angiogenesis, signal transduction and decreased the expression of a wide variety of protein growth factors, including ovarian cancer [14], kidney [15], breast [16], colon [17] and lung [18]. In the present study, ant nest plant extract inhibited the KB cells growth by 68.5% to 84.2% . In addition, IC_{50} of ant nest extract in this study was found exactly at 215 μg/ml. These suggest that ant nest plant has sufficient cytotoxic properties by category active [19]. Recent study reported that flavonoids would increase p53 protein characterized by apoptosis induction [20], and tannins would augment p27 protein that caused the cell cycle arrest [21].

Since components of apoptotic programs represent promising targets for anticancer therapy, increasing cell cycle arrest by ant nest plant extract could be a useful apoptosis-modulating strategy for treatment of oral cancers. High levels of apoptosis in cancer cells are strongly associated with chemotherapeutic sensitivity [22]. Therefore, the main purpose of double staining AO-EB is to detect apoptosis. Multiple methods, such as PI, *in situ* nick translation, terminal deoxynucleotidyl, acidic denaturation, and thermal denaturation assays, have been developed to detect apoptosis by monitoring changes in cell morphology and surface markers [23]. These methods identify cell death; however, they also have drawbacks. These methods involve multi-step procedures, including diversion, washing, and transfer of samples, using both time and materials [24]. In our study, increased apoptosis KB cells marked with orange-red color, appropriate with the increased in the extract concentration. Ant nest plant extract at 1000 μg/ml of concentration has induced apoptosis by 88%. Liu *et al*. [25] reported AO penetrated normal and early apoptotic cells with intact membranes, fluorescing green when bound to DNA. EB only entered cells with damaged membranes, such as late apoptotic and dead cells, emitting orange-red fluorescence when bound to concentrated DNA fragments or apoptotic bodies [24]. Fluorescent staining using AO alone has been used in the past; however, detection of cell apoptosis

Paper ID: NOV1531391   1622

using AO-EB is a relatively new approach, and few papers have reported its use [23]. In comparison to AO staining, the AO/EB method improves the detection of apoptosis and can distinguish between late apoptotic and dead cells [25].

In conclusion, extract of Papua's ant nest plant exhibited a high potential antitumor activity in oral carcinoma KB cells. In addition, IC_{50} of ant nest extract was detected at 215 μg/ml.

5. Acknowledgments

References

[1] Supriatno. Antitumor activity of Papua's Myrmecodia pendans in human oral tongue squamous cell carcinoma cell line through induction of cyclin-dependent kinase inhibitor p27Kip1 and suppression of cyclin E. J Cancer Res Ther 2014, 2(3): 48-53.

[2] Harada K, Supriatno, Kawaguchi S, Onoue T, Kawashima Y, Yoshida H and Sato M: High antitumor activity using intratumoral injection of plasmid DNA with mutant-type p27[Kip1] gene following *in vivo* electroporation. *Oncol Rep* 2005; 13: 201-206.

[3] Hseu YC, Chen CS, and Wang SY. Alpinia pricei rhizome extracts induce cell cycle arrest in human squamous carcinoma KB cells and suppress tumor growth in nude mice. Evidence-Based Compl Alt Med 2011, 1:1-11.

[4] Subroto, MA, Saputro H (2006) Gempur Penyakit dengan Sarang Semut, Penebar Swadaya Press, Depok, Indonesia, p. 15–31.

[5] Fatmawati D, Puspitasari PK, Yusuf I. (2011). Cytotoxic Effect of Ethanolic Extract of Sarang Semut (Myrmecodia pendens) on HeLa Cervix Cancer Cell Line. In Vitro Experimental Study. Sains Med 3:112–120.

[6] Soeksmanto A, Subroto MA, Wijaya H, Simanjuntak. (2010). Anticancer Activity test for Extract of Sarang Semut Plant (Myrmecodya Pendens) to HeLa and MCM-B2 Cell. Pak J Biol Sci 13:148–151.

[7] Hertiani T, Sasmito E, Sumardi, Ulfah M. (2010). Preliminary Study on Immunomodulatory Effect of Sarang-Semut Tubers Myrmecodia tuberosa and Myrmecodia pendens. J Bio Sci 10:136–141.

[8] Attari F, Sepehri H, Delphi L, Goliaei B. (2009). Apoptotic and Necrotic Effects of Pectic Acid on Rat Pituitary GH3/B6 Tumor Cells. Iranian Biomed J 13(4):229-236.

[9] Supriatno, Harada K, Yoshida H and Sato M. (2005) Basic investigation on the development of molecular targeting therapy against cyclin-dependent kinase inhibitor p27[Kip1] in head and neck cancer cells. Int J Oncol 27: 627-635.

[10] Harun N and Syari W. (2002). Antioxydant activity of *daun dewa* extract in suppress of halotan hepatotoxyc with sub anasthesi dose in Rattus Novergiccus. J. Sains Tech. Pharmasy, 7: 63-70.

[11] Fotsis T, Pepper MS, Aktas E, Breit S and Rasku S. (1997). Flavonoids, dietary inhibitors of cell proliferation and angiogenesis in vitro. Cancer Res., 57: 2916-2921.

[12] Hertog MGL, Holmann PCH, Katan MN and Kromhout D. (1993). Intake of potentially anticarcinogenic flavonoids and their determinants in adults in the Netherlands Nutr Cancer, 20: 21-29.

[13] Engidaa AM, Kasima NS, Tsigiea YA, Ismadjib S, Huynhc LH and Jua YH. (2013). Extraction, identification and quantitative HPLC analysis of flavonoids from sarang semut (Myrmecodia pendan). Industrial Crops and Products 41: 392–396

[14] Luo H, Rankin GO, Liu L, Daddysman MK, Jiang BH and Chen YC. (2009). Kaempferol inhibits angiogenesis and VEGF expression through both HIF dependent and independent pathways in human ovarian cancer cell Nutr Cancer 4: 554-563.

[15] Song W, Dang O, Xu D, Chen Y, Zhu G, Wu K, Zeng J, Long O, Wang X, He D and Li L. (2014). Kaempferol induced cell cycle arrest and apoptosis in renal cell carcinoma through EGFR/P38 signaling. Oncol Rep 31(3): 1350-1356.

[16] Choi EJ and Ahn WS. (2008). Kaempferol induced the apoptosis via cell cycle arrest in human breast cancer MDA-MB-453 cells. Nutr Res Pract 2(4): 322-325.

[17] Li W, Du B, Wang T, Wang S and Zhang J. (2009). Kaempferol induces apoptosis in human HCT 116 colon cancer cells via the ataxia-teleangiectasia mutated-p53 pathway with the involvement of p53 upregulated modulator of apoptosis. Chem Biol Interact 177(2): 121-127.

[18] Nguyen TT, Tran E, Ong CK, Lee SK, Do PT, Huynh TT, Nguyen TH, Lee JJ, Tan Y, Ong CS and Huynh H. (2003). Kaempferol induces growth inhibition and apoptosis in A549 lung cancer cells is mediated by activation of MEK-MAPK. J Cell Physiol 197(1): 110-121.

[19] Tanamatayarat P, Limtrakul P, Chunsakaow S, Duangrat C. (2003). Screening of some rubiaceous plants for cytotoxic activity against cervix carcinoma (KB-3-1) cell line. Thai J Pharm Sci 27 (3-4): 167-172.

[20] Ren W, Qiao Z, Wang H, Zhu L, Zhang L. (2003). Flavonoids: promising anticancer agents. Med res Rev 23(4): 519- 534.

[21] Nam S, Smith DM, Dou QP. (2001). Tannic acid potently inhibits tumor cell proteosome activity increase p27 and Bax expression, and induces G1 arrest and apoptosis. Cancer Epid Biomarker Prev 10: 1083-1088.

[22] Yamamoto M, Maehara Y, Oda S. 1999. The p53 tumor suppressor gene in anticancer agent-induced apoptosis and chemosensitivity of human gastrointestinal cancer cell lines. Cancer Chemother Pharmacol 43:43–49.

[23] Lecoeur H. 2002. Nuclear apoptosis detection by flow cytometry: influence of endogenous endonucleases. Exp Cell Res 277:1–14.

[24] Ribble D, Goldstein NB, Norris DA. 2005. A simple technique for quantifying apoptosis in 96-well plates. BMC Biotechnol 5:12-16.

[25] Liu K, Liu PC, Liu R, Wu X. 2015. Dual AO/EB staining to detect apoptosis in osteosarcoma cells compared with flow cytometry. Med Sci Monit Basic Res 21:

Paper ID: NOV1531391

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