

Oil Fraction from *Kaempferia galanga* Alcoholic Extract Increases Apoptosis Activity in Mice Colon Cancer

Bilal Subchan A. Santoso,^{1*} Junaidi Khotib, Sukardiman

¹ Pharmacy Academy of Putra Indonesia Malang, Malang, Indonesia

*Corresponding author, email: bilalsas67@yahoo.com

Abstract

Ethyl p-methoxycinnamate effectively inhibit the growth of cancer through a mechanism related to cell cycle regulation and apoptosis induction. We determined Kaempferia galanga that containing ethyl p-methoxycinnamate can increase the apoptosis activity in colon cancer cells. This study was designed to investigate effect of oil fraction from Kaempferia galanga alcoholic extract on apoptosis activity in mice colon cancer. Dimethylbenz(a)anthracene (DMBA)-induced colon cancer in mice is a useful model to investigate the changes of epithelial cells that occur during colon cancer progression. Colon cancer model was induced six times once a week by oral DMBA 50 mg/kg body weight. Administration of three different doses ethyl p-methoxycinnamate of oil fraction are 7.8 mg/kg, 23.4 mg/kg and 78 mg/kg which given to models during 30 days. Data analysis of immunohistochemical preparations was obtained and processed with one-way ANOVA statistical test, followed with post hoc test. Results showed that mice which given samples test increases apoptosis activity in colon cancer epithelial cells. The most effective dose of oil fraction increases apoptosis activity is containing ethyl p-methoxycinnamate 23.4 mg/kg body weight of mice.

Key words: *Kaempferia galanga, ethyl p-methoxycinnamate, dimethylbenz(a)anthracene, colon cancer, apoptosis.*

INTRODUCTION

Cancer is a disease characterized by a change of control mechanisms that regulate cell proliferation and differentiation so that cells undergo abnormal rapid and uncontrolled growth, and can spread to other body tissues. In Indonesia, colorectal cancer included malignant disease of the gastrointestinal tract and often found in the top 10 most cancer types, ranks sixth of existing malignant disease (Sutadi, 2003).

Cases of cancer are most common include lung cancer, prostate, breast and colorectal (Balmer et al., 2006). Colon cancers (large intestine) is aimed at the malignant tumor found in the colon and rectum, or colorectal (Siregar, 2007). One effort of therapy to treat cancer is by

chemotherapy. Anticancer drug or chemotherapy is an ideal drug if it kills cancer cells without harming normal cells.

Ethyl p-methoxycinnamate obtained from *Kaempferia galanga* can restrain the growth of gastric cancer through inhibition of angiogenesis that can inhibit tumor metastasis in gastric cancer (Liu and Wei, 2005). In addition, the composition in *Kaempferia galanga* is effectively inhibited the growth of gastric cancer through a mechanism related to cell cycle regulation and induction of apoptosis (Xiao et al, 2006). In the previous study, 2002 and Ying Hang researching anti-carcinogenic effects of ethyl p-methoxy-cinnamate which apparently showed that ethyl p-methoxycinnamate is a very powerful anti-carcinogenic. Studies on transgenic mice

showed a decrease in apoptosis in breast tumor cells as indicated by the decreased expression of Bax protein pro-apoptosis (Liu et al., 2001).

Based on the description above, then the research needs to uncover the problems associated with the activity of apoptosis in mice colon cancer that induced by *dimethylbenz(a)anthracene* (DMBA) using immunohistochemistry methods, which are expected to explain the role of oil fraction from *Kaempferia galanga* ethanolic extract in apoptosis induction as anticancer activity, through the examination of apoptosis in colon cancer epithelial cells of mice.

MATERIAL AND METHODS

Material

Kaempferia galanga is used in this study were obtained from local farmers in Malang City.

Methods

Extraction and Preparation of Powder

Kaempferia galanga powder weight 500 grams was extracted by maceration method. Maceration is done with 2 liters of 96% ethanol for 24 hours. Maceration was performed four times and the filtrate each maceration were collected and evaporated using an evaporator at low pressure to obtain concentrated extract $\pm 2\%$ of initial volume. The concentrated extract was added with water in 3:1 ratio, and then homogenized and idled for 24 hours until there was a separation between oil fractions and ethanol extract. Oil fraction was in the bottom separated from ethanol extract. The oil fraction was then dried into powder form by adding dryer, lactose: cabo-sil (4:1). The powder was prepared in suspension dosage forms with the addition of suspending agent CMC-Na 0.5% w/v before being given to the mice.

Treatment in Mice

The treatment group was divided into 5 groups. Group I was a normal group and other groups II, III, IV and V were induced by DMBA with doses 1 mg/mice orally once a week for six weeks, and then

incubated for 30 days. After that, one mice each was taken from the normal and DMBA-induced groups, sacrificed and examined to verify that there was no cells morphology changes occurred in the normal group, whereas in other groups there were indeed changes in cells morphology towards the occurrence of colon cancer. After that, group I was given mucilago CMC-Na 0.5% w/v per oral daily for 30 days. Groups II, III, and IV were given suspension containing ethyl p-methoxycinnamate 7.8 mg/kg, 23.4 mg/kg BW, and 78 mg/kg orally each day for 30 days. Group V was given mucilago CMC-Na 0.5% w/v per oral daily for 30 days. After that all experimental animals were sacrificed by dislocation to take the colon.

Histopathology

Histopathological examination was used to see the growth of cancer cells based on morphological changes of cells in tissues. Characteristics of cancer cells in tissues are an irregular arrangement of cells, dense cellularity, there are many cells and different cell sizes, enlarged cell nuclei, chromatin thickened, rough, uneven, hyperchromatic and basophilic, nucleus looks sharp and often stand out with varying sizes and a lot of mitosis (Banerjee et al., 2004). Histopathologic examination was performed with staining colon using hematoxylin-eosin (HE). The results shown that cell nuclei is blue-black, cytoplasm is pink, and red blood cells appear orange or red.

Immunohistochemistry

Immunohistochemical examination was done to see the cells undergoing apoptosis by TUNEL assay methods because this method shows that cells undergoing apoptosis can be found even if there is no change morphologically and very easily distinguish that the cells are not undergoing apoptosis. In this research methylgreen was used as a counterstain that can color nucleus, such that the nucleus that are not undergoing apoptosis

are going to look green (Cotran et al, 2005). This will facilitate the observation and counting the number of cells undergoing apoptosis.

RESULTS AND DISCUSSION

The Results of Histopathology Examination on Mice Colon

Examination of colonic epithelium cells before treatment. Histopathologic examination of colonic epithelium cells of mice has been done before given a different treatment in each of group to ensure that there has been damage to the colonic epithelium cells of mice.

After six weeks of DMBA-induction, incubation was performed to allow an initiated cell develop in a promotion and progression stages which is expected to form colon cancer in the organ. Incubation was carried out for six weeks because an incubation time that is too long can cause death of mice whereas if the incubation time is too short then the malignancy of colon organ, which is not expected, will occur. One way which can be used to determine the development (differentiation) of a tumor in mice is by sacrificing mice at four weeks (the incubation period) after the last DMBA induction and performing microscopic examination on slices of colonic histopathology. The results showed that there was no morphological changes observed in colonic epithelium cells (Figure 1A), whereas there was a change of colonic epithelium cells morphology that characterized by the number of hyperchromatic and mitotic nuclei (Figure 1B). These results are similar to the results in previous studies that DMBA is an inducer of carcinogenesis in the colon in addition to the ovary, skin, breast, lung, and leukemia (Sugiyama et al., 2002; Nicol et al., 2004).

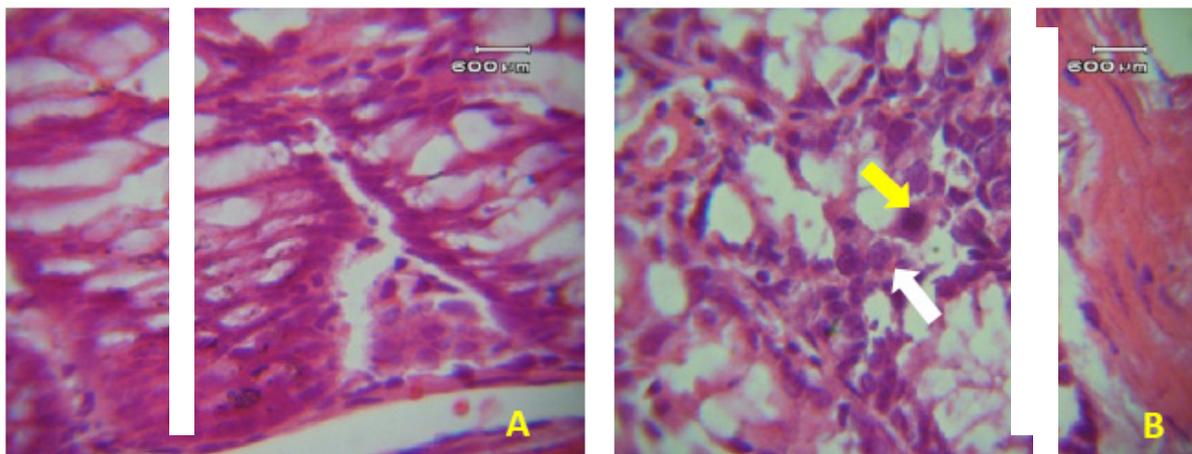
Examination of colonic epithelium cells after treatment. It can be seen in Figure 2A, 2B, 2C, and 2D that all groups have undergone changes, i.e. pleomorphisms, mitosis, and hyperchromasia. Figure 2A is a picture of DMBA-induced colonic epithelial cells which later on were given

oil fraction containing EPMS 7.8 mg/kg. Here, a lot of damages to the cells can be seen. Figure 2B is a picture of DMBA-induced colonic epithelial cells that were then given oil fraction containing EPMS 23.4 mg/kg showing a little movement toward better cells. Figure 2C is a picture of DMBA-induced colonic epithelial cells that were then given oil fraction containing EPMS 78 mg/kg, showing a lot of more damaged cells than Figure 2B. Figure 2D presents colonic epithelium cells induced by DMBA without given oil fraction, having a lot of damage to colonic epithelium cells.

Results of mean pleomorphic scoring in each of ten viewing fields of pleomorphic cells can be seen in Table 1. Actual counts of pleomorphic figures were used. Ten viewing fields of invasive cancer (starting at the location with the highest density of pleomorphic figures) were analyzed. Areas with the least amount of pleomorphic were selected for cell counting. The slide was randomly moved until 10 adjacent fields were counted.

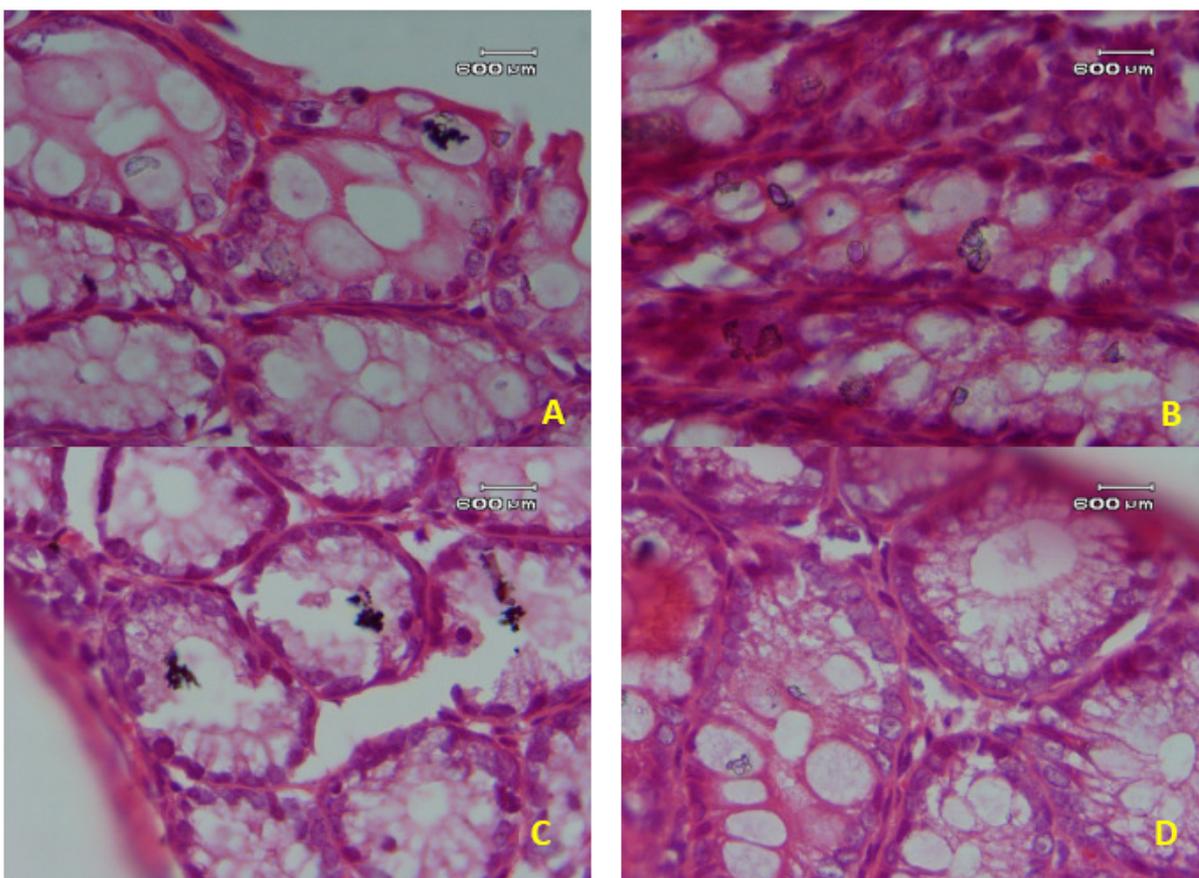
Data analysis was performed to determine whether there is an effect of giving the oil fraction to pleomorphic colonic epithelium cells of mice in each treatment group using one-way ANOVA statistics using SPSS 18 program application.

Data analysis shows that $p = 0.000$ ($p < 0.05$). It can be concluded that there were real differences in mice colonic epithelium cells pleomorphic between treatment groups. Data processing followed by Post Hoc test was performed to determine differences among treatment groups. Post hoc test results showed that there was no significant difference between the treatment group given DMBA without oil fraction treatment (group V) with the treatment groups given oil fraction containing EPMS (groups II and IV) and there was significant difference between the treatment group given DMBA without oil fraction treatment (group V) with the treatment group given oil fraction



A. Group I without given DMBA, colon cells that do not change into cancer; B. Group II, III, IV, and V were given DMBA, colon cells that have changed towards cancer. Sections were counterstained with HE (original magnification, x400).

Figure 1. Transverse slices of colonic epithelium cells before treatment.

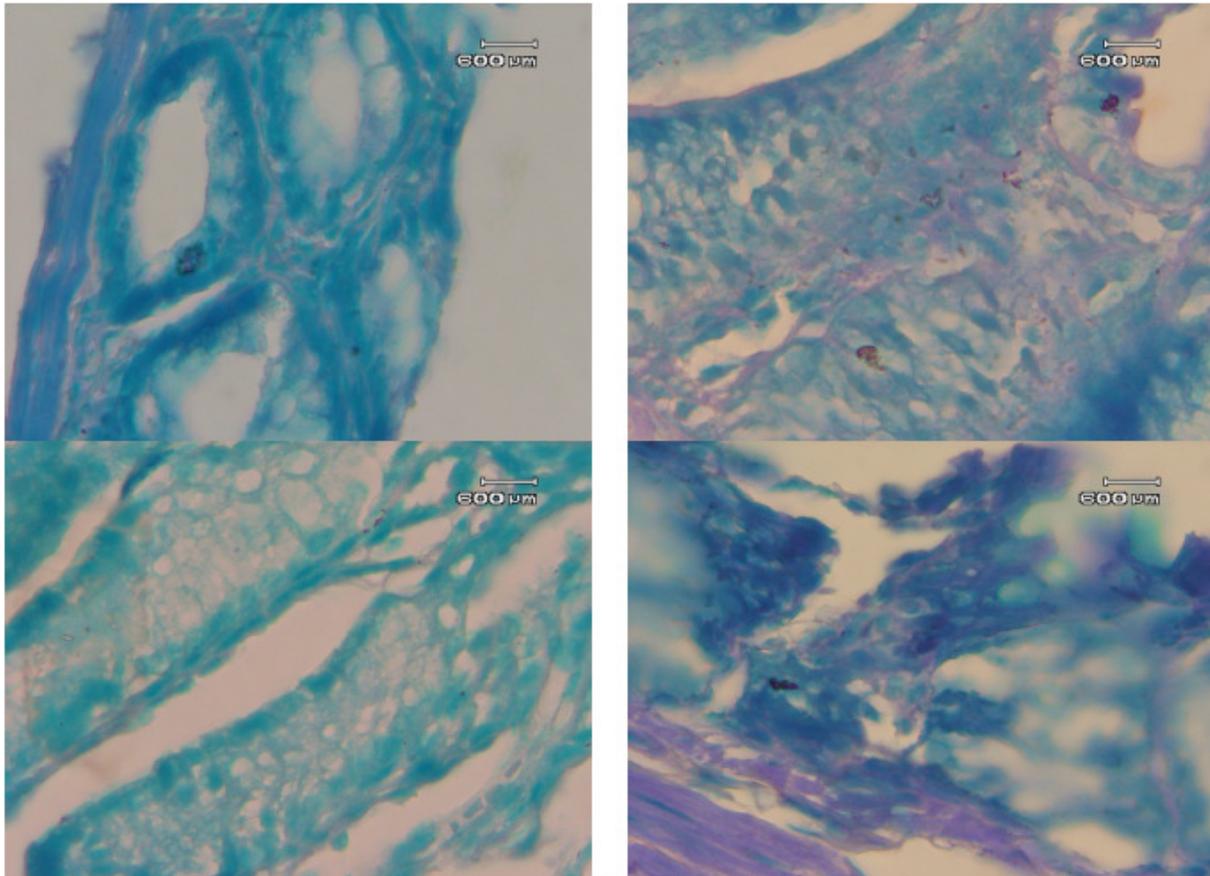


Morphology of colonic epithelium cells after induced by DMBA, then given oil fraction. A. EPMS 7.8 mg/kg; B. EPMS 23.4 mg/kg; C. EPMS 78 mg/kg; D. Without given oil fraction. Sections were counterstained with HE (original magnification, x1000).

Figure 2. Transverse slices of colonic epithelium cells after treatment.

Table 1. Pleumorphic scoring

Groups	Treatment	Mean ± SD
II	DMBA + EPMS 7.8 mg/kg	2.60 ±0.55
III	DMBA + EPMS 23.4 mg/kg	1.40±0.55
IV	DMBA + EPMS 78 mg/kg	2.00 ±0.00
V	DMBA	2.67 ±0.58



Apoptosis activity in colonic epithelium cells after induced by DMBA, then given oil fraction. A EPMS 7.8 mg/kg; B. EPMS 23.4 mg/kg; C. EPMS 78 mg/kg; D. Without given oil fraction Sections were counterstained with IHC (original magnification, x1000).

Figure 3. Apoptosis activity in transverse slices of colonic epithelium cells.

Table 2. Apoptotic counting

Groups	Treatment	Mean ± SD
II	DMBA + EPMS 7.8 mg	2.80 ± 2.49
III	DMBA + EPMS 23.4 mg	9.40± 2.07
IV	DMBA + EPMS 78 mg	4.40 ± 2.07
V	DMBA	1.67 ±0.58

the cells became inflamed. The inflammation can cause damage to proteins

(DNA repair, caspase) that would cause DNA damage and mutations that can

induce tumor cells to develop into malignant cells or proliferation disorder (Liu et al., 2001).

Examination of apoptotic activity in colon cells. Immunohistochemical examination in mice colonic epithelium cells was done to see the apoptosis activity by using apoptag antibodies. Apoptosis activity of this study can be seen in Figure 3.

Immunohistochemical examination is intended to determine the number of colonic epithelium cells death by apoptosis. The method used in this investigation was a quantitative method in which the number of apoptotic cells in each sample is determined by summing all the apoptotic cells found in five viewing fields at 1000 times magnification. In this examination, apoptotic cells showed brown colored (to black) in nucleus whereas normal cells bluish green. The results of counting cells undergoing apoptosis through a calculation on each of the preparations in five viewing fields can be seen in Table 2.

The highest rates of apoptotic cells was present in the group that was induced by DMBA with treatment oil fraction containing EPMS 23.4 mg/kg afterwards and the lowest was found in the DMBA treatment group without oil fraction given.

Data analysis was performed to determine whether there was an effect of giving the oil fraction to apoptosis activity of colonic epithelium cells in each treatment group using one-way ANOVA statistics using SPSS 18 program application.

Data analysis shows that $p = 0.000$ ($p < 0.05$). Hence, it can be concluded that there were real differences in mice colonic epithelium cells apoptosis activity between treatment groups. Data processing followed by Post Hoc test were performed to determine differences among treatment groups. Post hoc test results showed that there was significant difference between the treatment group given oil fraction containing EPMS (groups III) with treatment groups given oil fraction

containing EPMS (groups II and IV) and the treatment group given DMBA without oil fraction treatment (group V).

The balance of cells in tissues (tissue homeostasis) affected the balance of cells proliferation and apoptosis. Each cell has ability to maintain the number of cells in normal circumstances. However, if there is an imbalance of proliferation and apoptosis there will be tumor progression. Apoptosis activity observed in the results of this study indicated that oil fraction containing EPMS 23.4 mg/kg had a lot of apoptotic cells. In the group which was not given oil fraction a little apoptotic cells were present because the colonic epithelium cells were still trying to remove the cells that developed through a programmed cell death (Liu et al., 2001).

CONCLUSION

Apoptosis activity was present in this study. It is known that *Kaempferia galanga* contain ethyl p-methoxycinnamate that may reduce the number of colonic epithelium cells that change towards cancer cells by the examination of pleomorphism of colonic epithelial cells using histopathological methods as well as by examination of apoptosis activity using immunohistochemistry methods. Finally, this study concluded that *Kaempferia galanga* can increase apoptotic cells and the highest apoptosis activity shown by oil fraction containing ethyl p-methoxycinnamate (EPMS) 23.4 mg/kg body weight of mice.

REFERENCES

- Balmer, C.M., Valley A.W. and Iannucci, A. 2006. Cancer Treatment and Chemotherapy. In: J.T. DiPiro, R.L. Talbert, G.C. Yee, G.R. Matzke, B.G. Wells, and L.M. Posey (Eds.). *Pharmacotherapy: A Pathophysiologic Approach*, Sixth Edition, New York: McGraw-Hill Companies, Inc.
- Banerjee, S., Ramos, C.B., Aggrawal, B.B., 2002. Suppression of 7, 12-Dimethylbenz(a)anthracene-induced

- Mammary Carcinogenesis in Rats by Resveratrol: Role of Nuclear Factor B, Cyclooxygenase 2 and Matrix Metalloprotease 9. *Cancer Research*.
- Cotran RS, Kumar V, Robbins SL.,2003. *Robbins Pathologic Basis of Disease*, pp. 1067 Philadelphia: WB Saunders Company.
- Liu, S., Edgerton SM., Moore DH.,*et al.* 2001. Measures of Cell Turnover (Proliferation and Apoptosis) and Their Association with Survival in Breast Cancer. *Clin Cancer Res*.
- Liu, YF.,Wei, PK. 2005. Effect on the volatile oil of *Kaempferia galanga* L. inhibiting the growth and metastasis of human gastric carcinoma. *Journal of Nanjing Railway Medical College*.
- Nicol, C.J., Yoon, M., Ward, J.M., Yamashita, M., Fukamachi, K., Peters, J.M. and Gonzales, F.J., 2004. PPAR γ Influences Susceptibility to DMBA-Induced Mammary, Ovarian and Skin Carcinogenesis. *Carcinogenesis*.
- Siregar, GA. 2007. *Deteksi dini dan penatalaksanaan kanker usus besar*. in: Pidato Pengukuhan Jabatan Guru Besar Tetap dalam Bidang Ilmu Penyakit Dalam pada Fakultas Kedokteran, Sumatera Utara.
- Sugiyama, T., Osaka, M., Koami, K., Maeda, S., and Ueda, N., 7,12-DMBA Induced Rat Leukemia: A Review with Insights into Future Research. *Leukimia Research*.
- Sutadi, S.M., 2003. *Pola Keganasan Saluran Cerna Bagian Atas dan Bawah Secara Endoskopi* di H. Adam Malik-Medan, Medan: Fakultas Kedokteran Bagian Ilmu Penyakit Dalam Universitas Sumatera Utara.
- Xiao, Y., Wei, PK., Li, J., Shi, J., Yu, ZH.,Lin, HM. 2006. Effects of *Rhizoma kaempferiae* volatile oil on tumor growth and cell cycle of MKN-45 human gastric cancer cells orthotopically transplanted in nude mice. *Journal of Chinese Integrative Medicine*.
- Ying, X., Hang, C. 2002. Study on the anti-carcinogenic effects of three compounds in *Kaempferia galanga* L. *Journal of Hygiene Research*.