

RESEARCH COMMUNICATION

Suppressive Effects of *Moringa oleifera* Lam Pod Against Mouse Colon Carcinogenesis Induced by Azoxymethane and Dextran Sodium Sulfate

Sirintip Budda¹, Chaniphun Butryee¹, Siriporn Tuntipopipat¹, Anudep Rungsipipat², Supradit Wangnaithum², Jeong-Sang Lee³, Piengchai Kupradinun^{4*}

Abstract

Moringa oleifera Lam (horseradish tree; tender pod or fruits) is a major ingredient in Thai cuisine and has some medicinal properties. Previous studies have shown potentially antioxidant, antitumor promoter, anticlastogen and anticarcinogen activities both *in vitro* and *in vivo*. The present study was conducted to investigate chemopreventive effects on azoxymethane (AOM)-initiated and dextran sodium sulfate (DSS)-promoted colon carcinogenesis in mice. Male ICR mice were divided into 8 groups: Group 1 served as a negative control; Group 2 received AOM/DSS as a positive control; Groups 3-5 were fed boiled freeze-dried *M. oleifera* (bMO) at 1.5%, 3.0% and 6.0%, respectively supplemented in basal diets for 5 weeks; Groups 6-8 were fed with bMO diets at the designed doses above for 2 weeks prior to AOM, during and 1 week after DSS administration. At the end of the study, colon samples were processed for histopathological examination. PCNA indices, iNOS and COX-2 expression were assessed by immunohistochemistry. The results demonstrated the incidences and multiplicities of tumors in Groups 6-8 to be decreased when compared to Group 2 in a dose dependent manner, but this was significant only in Group 8. The PCNA index was also significantly decreased in Group 8 whereas iNOS and COX-2 protein expression were significantly decreased in Groups 7 and 8. The findings suggest that *M. oleifera* Lam pod exerts suppressive effects in a colitis-related colon carcinogenesis model induced by AOM/DSS and could serve as a chemopreventive agent.

Keywords: *Moringa oleifera* - chemoprevention - colon carcinogenesis - azoxymethane - dextran sodium sulfate

Asian Pacific J Cancer Prev, 12, 3221-3228

Introduction

Colon cancer is one of the important causes of morbidity and mortality in developed countries, and is increasing in number among Thai populations, especially in major cities like Bangkok (Khuhaprema et al., 2008). In Thailand, colon cancer is the 3rd ranking of cancer incidence in male and the 5th ranking in female (Khuhaprema et al., 2010). This is caused by the change of Thai dietary habits to a more Western style. Thai cuisine has been known and popular in worldwide, especially among the health-conscious consumer due to the values and functional ingredients contained in a variety of herbs, spices, and vegetables. Some of them have been proved that they are relevant to prevent many diseases, however, only a few have been scientifically approved of their potential for health, particularly their protective effects and/or molecular mechanisms on colon cancer.

Moringa oleifera (MO) belonging to the family Moringaceae is found in Asia, Africa, Latin America,

including Thailand (Fahey, 2005). The leaves, fruits, flowers and immature pods of this tree are used as a highly nutritive vegetable in many countries, particularly in Philippines, Pakistan, India, Hawaii and Africa (D'Souza and Kulkarni, 1993; Anwar and Bhangar, 2003; Anwar et al., 2005). The medicinal values of different parts of this plant have long been recognized in folklore medicine (Faizi et al., 1995). In Thailand, the tender pods (fruits), and leaves have been consumed as vegetables for more than 100 years (Mokkhasmit et al., 1971). An analysis of utilization of different parts of MO indicated that the highest crude proteins and fat were shown in mature MO seeds. High oleic acid (18:1) contents were shown in MO seeds (Amaglo et al., 2010). Several bioactive compounds were isolated from MO pod and seed such as niazirin, niazimicin, niazicin A, benzyl-isothiocyanate, benzyl-thiocarbamate, and glucomoringin (Francis et al., 2004; Brunelli et al., 2010; Cheenpracha et al., 2010). Oral administration of MO pods extract could increase detoxification enzymes in liver involved in reactions

¹Institute of Nutrition, Mahidol University, Nakhon Pathom, ²Department of Veterinary Pathology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand, ³Department of Pharmacology, Yale University School of Medicine, New Haven, USA, ⁴Section of Animal Laboratory, Research Division, National Cancer Institute, Bangkok, Thailand *For correspondence: pkupradi@yahoo.com, pkupradi@health.moph.go.th

of phases I and II. Furthermore, it could inhibit skin papillomagenesis in female Swiss albino mice (Bharali et al., 2003). A previous study showed that male ICR mice that received diet containing freeze-dried boiled MO had potentially anti-clastogenic activity (Promkum et al., 2010). The ethanol extracts of MO seeds showed potential activity as an antitumor promoting agent both *in vitro* and *in vivo* assays (Guevara et al., 1999). The isothiocyanate from glucomoringin, an active compound found in MO seeds, was found to reduce myeloma growth in nude mice (Brunelli et al., 2010). Moreover, benzyl-isothiocyanate could decrease 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced leukocyte infiltration and inhibit excessive superoxide generation in inflammatory leukocytes in mouse dermis (Nakamura et al., 2004). However, there has been no report regarding their effects on human health benefit, particularly in term of anti-colon carcinogenesis. Determination of the effective dose of anti-colon carcinogenesis based on animal model, particularly the effect and molecular mechanisms of suppression, should be elucidated. The results from our study will be useful for promoting Thai foods containing MO pods as a chemopreventive agent.

Materials and Methods

Chemicals

Azoxymethane (AOM) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dextran Sodium Sulfate (DSS) was purchased from MP Biomedicals Co. (Solon, California, USA). The 3-aminopropyltriethoxysaline and proliferating cell nuclear antigen (PCNA) antibody (Dako, Denmark) was kindly provided by Assoc. Prof. Dr. Anudep Rungsipat, Faculty of Veterinary Science, Chulalongkorn University. Cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) antibodies were purchased from Abcam Cambridge (MA, USA). Envision™ Polymer Detection system was purchased from Dako Denmark A/S (Glostrup, Denmark). For preparing animals diets, AIN-76 minerals mixture was purchased from MP Biomedicals, Solon, California, USA, and vitamins mixture products of Clea Japan Inc. (Osaka, Japan), was provided by Prof. Dr. Tadashi Okamoto, Kobe Gakuen University, Japan. Vitamin K1 was kindly provided by DSM Nutritional Products Ltd. (Sisles, Switzerland) and cellulose (SOLKA-FLOC® 200 FCC) from FS&D Corp. (St. Louis, MO, USA). Sodium caseinate was the product of Erie Foods International, Inc (Erie, Illinois, USA). Other reagents were mostly of analytical grade and obtained locally.

Sample and animal diet preparations

M. oleifera Lam pod (MO) was purchased from a central distributor market located in Bangkok representing MO cultivated in Northern, Eastern, Central and Western areas of Thailand. MO was prepared in traditional fashion as previously described (Promkum et al., 2010). All boiled MO together with boiled water were blended, dried and ground in an electric grinder and called as bMO. Then aliquots of bMO were pooled, packed in vacuum bags

and stored at -20 °C until use.

The amount of bMO given to animals were based on the human serving size per person at the 97.5 percentile reported from the consumption data in persons aged more than 3 years old which the serving size was 1.98g/kg body weight/day (Food Consumption Data of Thailand, 2006). The experimental diets were prepared by mixing bMO with basal AIN-76 diet at 1.5%, 3.0% and 6.0%. The preparation for animal diets was done with slight modification from Bieri et al. and Reeves et al. (Bieri et al., 1977; Reeves et al., 1993) and the content of bMO in basal AIN-76 diet was approximately equivalent to 10, 20 and 40 times of human consumption.

Animals and experimental design

Sixty four male ICR mice, aged 3 weeks old, weighing 15 ± 3 grams, were purchased from the National Laboratory Animal Center, Mahidol University, Thailand. The animals were maintained at the Laboratory Animal Facility of the National Cancer Institute according to the Institute Care Guidelines which were approved by both institutes, the Animal Ethics Committee of the National Cancer Institute and Mahidol University. The animals were acclimatized for 5 days in a clean conventional room maintained at $23 \pm 2^\circ\text{C}$ with 12 h light/dark cycle and controlled relative humidity at $50 \pm 20\%$ and were housed in filtered top plastic cages. The mice were given modified AIN-76 diet (basal diet) and water *ad libitum*. After acclimatization, all mice were randomly distributed into 8 Groups (8 mice each). Mice were given basal diet and diets supplemented with bMO at various doses in pair-fed form. Group 1 was assigned as negative control group and fed with basal diet. Group 2 was assigned as positive control for colitis-associated colon cancer. AOM (10 mg/kg BW) was intraperitoneal injected to the mice at the 3rd week of experiment and followed by 2% DSS in drinking water for one week at the 4th week of experiment. Therefore, the mice were fed with basal diet until the end of experiment. Groups 3-8 were assigned as the experimental groups in the initiation phase of colon carcinogenesis. Groups 3-5 were fed with basal diet supplemented with bMO at 1.5%, 3.0% and 6.0%, respectively for 5 weeks and were continued to be fed with basal diet until the end of experiment at the week 20th. Groups 6-8 were fed with bMO diets at 1.5%, 3.0% and 6.0%, respectively for 2 weeks prior to giving AOM, during and 1 week after DSS administration. Then the mice were fed basal diet until the end of experiment (Figure 1). At the end of the study, the mice were sacrificed and necropsied. The colons were excised, cut open longitudinally along the main axis, washed with phosphate buffer saline (PBS) and then examined for the presence of tumors. The tumors were counted, measured for their sizes, and fixed with 10% buffered formalin prior to performing histopathological and immunohistochemical examinations.

Immunohistochemical examination

For proliferating cell nuclear antigen (PCNA), colon samples were determined with slight modification (Birchall et al., 1997; Kawabata, 1999). To antigen retrieval, colon samples were pretreated in PBS (pH

6.0) in a microwave oven at 65 °C for 10 min. To block endogenous peroxidase, the sections were washed in PBS and incubated in 3% H₂O₂ in CH₃OH for 10 min at room temperature (RT). 1% Bovine Serum Albumin (BSA) was dropped on slides and they were incubated in a humidified chamber for 60 min at RT. Then, the colon samples were incubated overnight at 4°C with anti-PCNA mouse monoclonal antibody (dilution 1:200, Abcam). After primary antibody incubation, the sections were washed with PBS, incubated with a biotinylated secondary antibody for 45 min (Envision™ polymer detection system, DAKO) and washed with PBS. The reaction occurring between antibody and protein expression were developed with diaminobenzidine (DAB) for 3 min and counterstained with Mayer's hematoxylin. Intensity of immunoreactivity nuclear positive cell was examined and cell numbers were recorded using a light microscope (40x, Olympus BX 40, Japan). Duplicated slide were used for each colon sample in a mouse per group. PCNA Index was calculated from the percentage of immunohistochemical staining positive cells in 1000 cells counted (Kawabata, 1999).

For detecting inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), colon samples were determined with slight modification method (Tanaka et al., 2003). Briefly, the colon samples were pretreated by Tris buffer (pH 8.0) for iNOS and citrate buffer (pH 6.0) for COX-2 in an autoclave at 121°C for 5 min in order to antigen retrieval. Thereafter, they were washed in PBS and incubated in 3% H₂O₂ in deionized water to block endogenous peroxidase for 10 min at RT. Then, all colon sample incubations were humidified with 1% BSA for 60 min in plastic chamber at RT. All colon sections were washed with PBS and incubated overnight at 4°C with anti-iNOS or anti-COX-2 mouse monoclonal antibody (dilution 1:250 or 1:100, respectively, Abcam). After primary antibody incubation, the sections were washed with PBS and incubated for 45 min with a biotinylated secondary antibody (Envision™ polymer detection system, DAKO). Then all sections were washed with PBS, developed with DAB for 2 min and counterstained with Mayer's hematoxylin. Cytoplasmic staining was examined using light microscope (40x, Olympus BX 40, Japan), positive tumor staining area were captured in slide section area at various locations (40 pictures/group) by using data from H&E diagnosis and data manipulation was done by Image Pro Analysis 6.0 Program (Carl Zeiss, Germany). The percentage of iNOS and COX-2 protein expression was calculated from the percentage of average positive tumor staining area in total area.

Data and statistical analysis

All measurements were statistically evaluated using the SPSS software (version 18.0; SPSS, Inc, Chicago, IL, USA). The normality of data distribution was evaluated by Kolmogorov-Sminov test and the significant differences between groups were determined by Student's *t* test. The χ^2 test or Fisher's exact probability test were used for determination of the significant difference between groups in tumor incidence. P-value < 0.05 was considered statistically significant.

Results

General observations

During the experimental period, diarrhea and bloody stools were observed at the 3rd-4th week of experiment. Diarrhea was observed in almost of the AOM/DSS treated groups while bloody soft or watery stools were found in some mice. However, no significant clinical signs were subsequently observed. Disappearances of abnormal symptom were shown after the 4th week of experiment. After 12th week, some mice in the AOM/DSS treated groups had anal prolapsed.

Effect of bMO on the body weight and food consumption

In AOM/DSS treated groups, the mean body weight was slightly decreased in the 6th-7th week of experiment while food consumption was slightly decreased in the 4th-5th week of experiment. Then the mean body weight increased in normal curve and was not significantly differences (p>0.05) from those of the negative control group (Group 1) (data not shown).

Effect of bMO on the incidence and multiplicity of tumors

As shown in Table 1, there was no evidence of tumor nodules in Group 1, and Groups 3-5 which fed basal

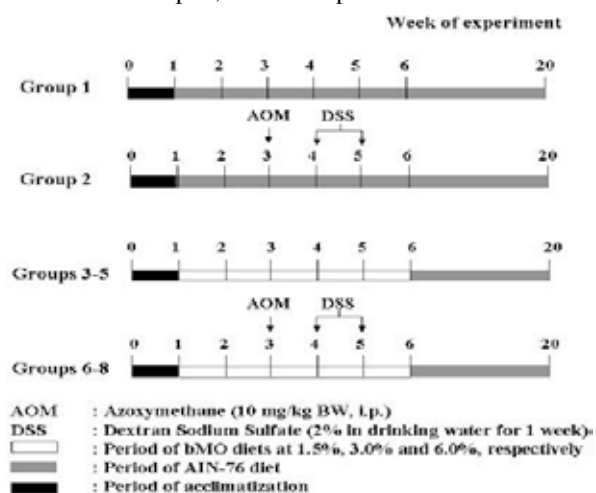


Figure 1. Experimental Designs to Study the Effect of bMO Against Mouse Colon Carcinogenesis.

Table 1. Incidence, Multiplicity and Histological Types of Colonic Neoplasm in Mice.

Gr.	Treatments	No. of Mice				No. of Tumor/mice (Mean±SD)
		With tumor (%)	Adenoma (%)	Adenocarcinoma (%)	Superficial Tubular (%)	
1	Control	0	0	0	0	0
2	AOM+DSS	7 (87.5)	0	0	7 (87.5)	9.5 ± 6.1
3	1.5% bMO	0	0	0	0	0
4	3.0% bMO	0	0	0	0	0
5	6.0% bMO	0	0	0	0	0
6	1.5% bMO+AOM/DSS	5 (62.5)	0	2 (25.0)	4 (50.0)	5.1 ± 5.2
7	3.0%bMO+AOM/DSS	4 (50.0)	1(12.5)	0	3 (37.5)	4.5 ± 4.9
8	6.0%bMO+AOM/DSS	3 (37.5)*	0	1 (12.5)	3 (37.5)	2.8 ± 4.2

diet supplemented with 1.5%, 3.0% and 6.0% bMO, respectively for 5 weeks. The highest incidence of tumor (87.5%) were detected in the positive control group (Group 2) which was fed basal diet and given AOM/DSS and significantly different ($p < 0.05$) from those of Group 1. The number of tumor per mice were decreased in Groups 6, 7, and 8 but significantly decreased only in Group 8. Tubular adenocarcinomas were diagnosed in Groups 6-8 with the incidence of 50.0%, 37.5%, and 37.5%, respectively (Table 1). Superficial adenocarcinomas were also diagnosed in Groups 6 (25.0%) and 8 (12.5%) while adenoma was detected only in Group 7 (12.5%). The tumor multiplicities decreased in Groups 6 (5.1 ± 5.2), 7 (4.5 ± 4.9), and 8 (2.8 ± 4.2) in comparison to Group 2, but significant decrease was found only in Group 8 (Table 1). Gross examination of colon tumor nodules formation was shown in Figure 2A. Most of the tumor nodules were detected in the middle to distal colon of mice. Multiple tumors more than 1cm in diameter were observed in all AOM/DSS treated groups. Macroscopic findings revealed the multiple tumor nodules size more than 1cm in diameter (Figure 2A) and were well-differentiated tubular adenocarcinomas (Figures 2B-2C). The highest tumor multiplicity (9.5 ± 6.1) was shown in Group 2 and significantly increased in comparison to Group 1.

Effect of bMO on PCNA, iNOS and COX-2 protein expressions

The results demonstrated that there was no significant difference between Groups 3-5 and Group 1. The highest PCNA index (56.5 ± 16.2) was shown in Group 2. A significant difference was shown in comparison with Group 1 (18.6 ± 4.1). PCNA index was decreased in Groups 6 (48.7 ± 17.7), 7 (40.9 ± 17.5), and 8 (37.7 ± 15.1), respectively and significantly decreased ($p < 0.05$) was shown in Group 8 in comparison to Group 2. (Figure 3a) Immunohistochemical staining of PCNA index were shown in Figures 3b-3e which correlated with percentage

of bMO in the diets. The strong PCNA index was seen in Group 2 (Figure 3b) while weak expression were seen in Group 6, 7 and 8, respectively (Figures 3c-3e).

The iNOS protein expression shown in Figures 4. Loss of iNOS and COX-2 protein expressions detected by immunohistochemistry were shown in Group 1 and Groups 3-5. The highest percentage of iNOS and COX-2 protein expressions were shown in Group 2 (39.8 ± 30.2 , 15.9 ± 26.1 , respectively) and a significant difference was shown in comparison with Group 1. The percentage of iNOS protein expression was decreased in Groups 6 (35.2 ± 28.0), 7 (27.4 ± 24.6), and 8 (27.2 ± 24.7) but significantly decreased was shown only in Groups 7 and 8 in comparison with Group 2 (Figure 4a). Whereas the percentage of COX-2 protein expression was decreased in Group 6 (10.7 ± 22.7) and loss of COX-2 protein expression was found in Groups 7 and 8. The immunoreactivity against iNOS was found in all colonic lesions in Groups 6-8. However, the intensity of dark brown reaction product was faded only in Groups 7 and 8 (Figures 4d-4e). A strongly positive reaction of COX-2 was found only in Group 6 while a loss of expression was found in Groups 7 and 8.

Discussion

The results from this study showed that the food consumption and growth rate of mice were not significantly different among the control and the experimental groups. Many studies reported that *M. oleifera* (MO) pods and seeds contained high nutrients, non-nutrients and phytochemical compounds such as protein, amino acid, vitamins, minerals, essential fatty acid, β -carotene, various phenolic compound, glucosinolates and benzyl-isothiocyanates (Bennett et al., 2003; Farooq et al., 2007; Amaglo et al., 2010; Cheenprapa et al., 2010). However, the anti-nutrients such as trypsin and amylase inhibitors were also found in raw MO seeds and they could interfere

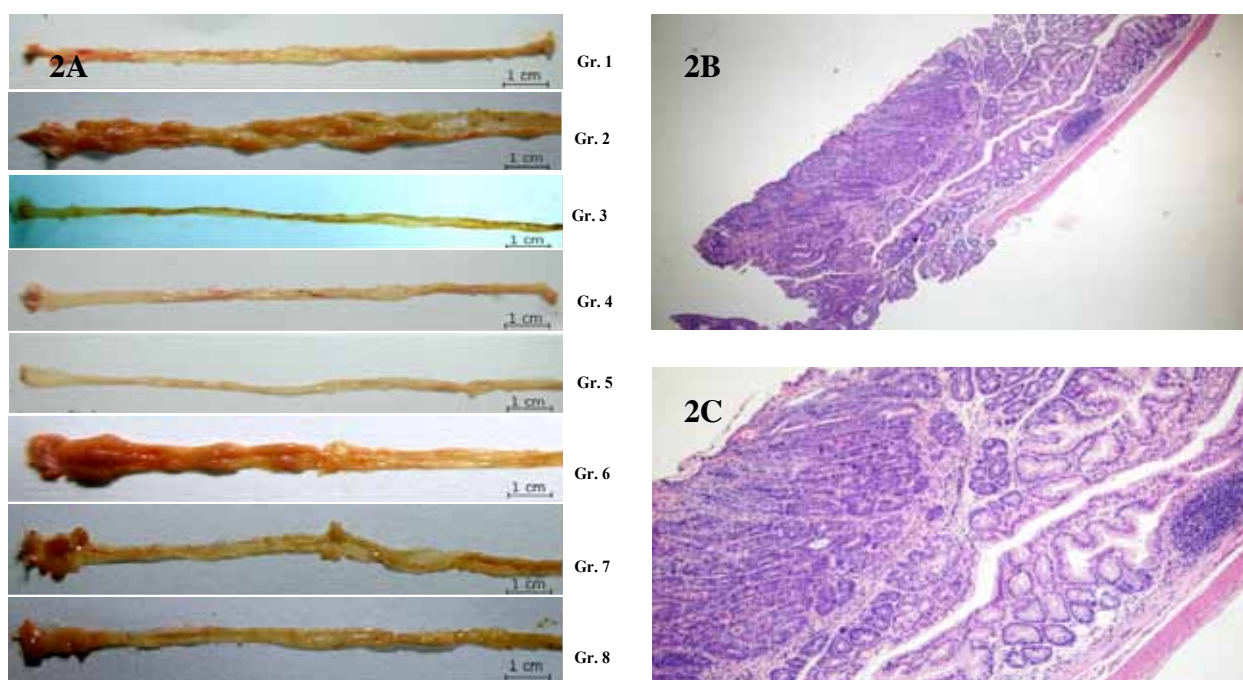


Figure 2. Macroscopic and Microscopic Views of Mouse Colons in the Different Groups

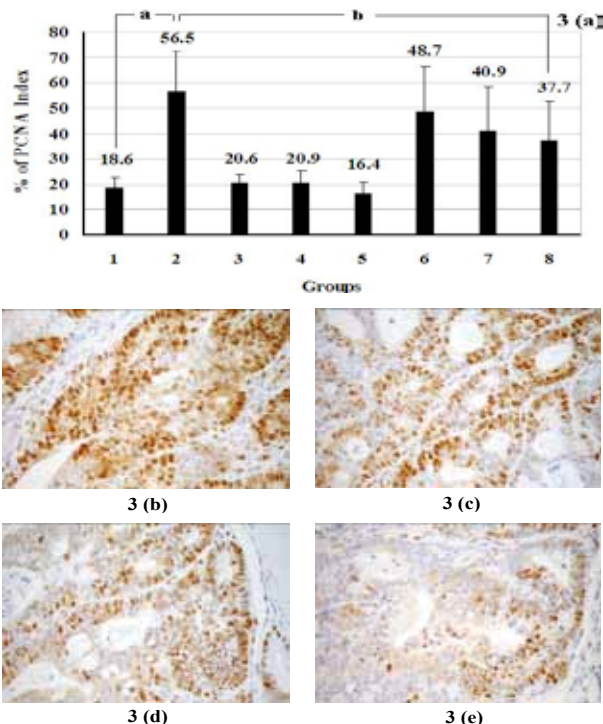


Figure 3. Effect of bMO on the PCNA Index.

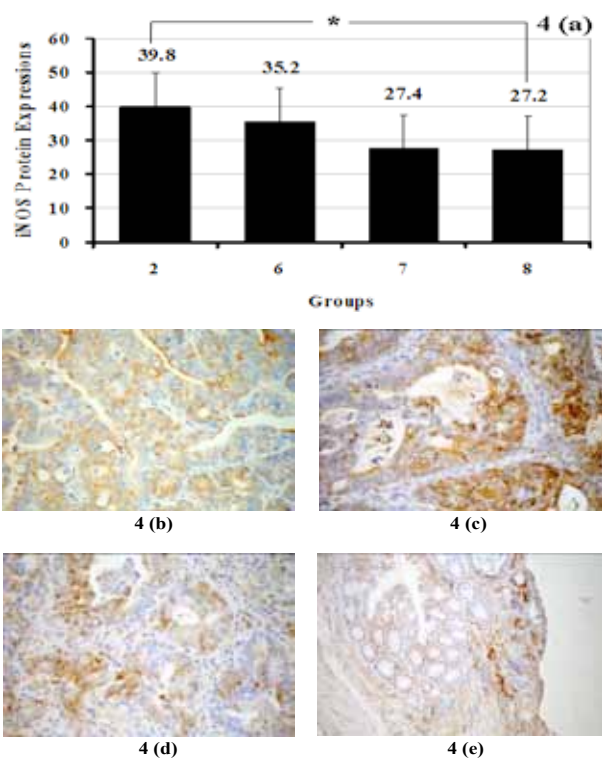


Figure 4. Effect of bMO on the iNOS Protein Expression.

with nutrients digestion and absorption by negatively affecting the metabolic process in the human body (Sharaf et al., 2009). This may explain why the mice's body weight in our study was not affected by the boiled MO samples, whose anti-nutrient compounds were decreased or detoxified by various processing such as soaking and heat processing (Emiola and Ologhobo, 2006). However, the body weight and food consumption of the mice in the experimental groups were slightly decreased during the

period of AOM/DSS administration as compared with the negative control group (Group1). This might be the effect of carcinogen administration. However, their body weights were increased to normal level within 1 week after AOM/DSS administrations.

According to Tanaka and coworkers (Tanaka et al., 2003), we use 2% DSS in drinking water for 1 week after AOM administration in the mice. The result demonstrated that the colonic neoplasm could develop within 20 weeks, which was shorter than rat carcinogenesis models (36-40 weeks) (Davidson et al., 1999). Thus, a two-stage mouse colon carcinogenesis model initiated with AOM and promoted by DSS used in our experiment has more potentially model to determine the pathogenesis and chemoprevention of colitis-related colon carcinogenesis of dietary phytonutrients. The percentage of tumor incidence (87.5%) was less than other studies (Bennett et al., 2003; Tanaka and Yasui, 2008; Yohei et al., 2008; Rosenberg et al., 2009). This may be cause by the carcinogenic risks from exposure to exogenous chemical carcinogens depending not only on the intrinsic nature and each chemical's dose but also may depend on inter-individual variability in sensitivity to the carcinogens (Lazarus et al., 1998). An individual difference in the susceptibility to chemical carcinogens is one of the most important factors to estimate human cancer risk (Suzuki et al., 2004). Furthermore, it might be caused by the strain and stock of mice used in this study.

Regarding the colon and anti-colon carcinogenesis activity of bMO, there were no adverse effects of bMO at dose levels of 1.5%, 3.0%, and 6.0% in the basal diet that represents 13, 26 and 52 times of human serving (estimated from actual amount of mice consuming diet containing bMO) when they were administered to young mice. It can be concluded that bMO at the tested doses did not possess acute and sub-acute toxicity by macroscopic examination. No data supported the toxicity of MO pods (fruits) in animal model. Only the acute toxicity study of water extract of MO seeds in Swiss mice showed that it had an LD50 of 446.5 mg/kg.BW which was determined its low toxicity (Paulo et al., 2009). Moreover, the effect of the aqueous extract of MO leaf (300 mg/kg BW) on the gastric ulcer model in Holtzman strain albino rats didn't show any toxic effect (Debnath et al., 2010). For the effect of bMO on colon carcinogenesis in our study, the incidence and multiplicity of tumors were decreased by bMO in the dose dependent manner and significantly decreased at the highest dose (6.0%). It can modulate the histological type and the number of colon tumors which was confirmed by histopathological examination. The number of tubular adenocarcinomas was reducing in accordance with the increasing number of superficial adenocarcinomas when compared to the lower dose of bMO groups. The high contents of omega-9 oleic (18:1) fatty acid (more than 70% of total fatty acid) were found in MO mature pods (Amaglo et al., 2010; Ayerza, 2011). We speculated that the chemopreventive effect of bMO arose from fatty acids present in MO which might modulate cell proliferation and/or apoptosis and anti-inflammation which plays an important role in colon carcinogenesis. It has been

reported that human colon tumor growth is promoted by oleic acid through mechanisms that comprise an increase in fatty acid oxidation and disturbance of membrane enzymes (Suzuki et al., 1997; Calder et al., 1998). In contrast, olive oil, an important source of omega-9 oleic fatty acid, may prevent against the development of colorectal cancer through its influence on secondary bile acid patterns in the colon. Bile acids, in turn, might influence polyamine metabolism in colonic enterocytes in ways that reduce the progression from normal mucosa to adenoma and carcinoma (Stoneham et al., 2000). Other studies showed that omega-9 rich diets could decrease the development of aberrant crypt foci (ACF) and colon carcinomas in rats induced by AOM. These effects may be partly due to modulation of arachidonic acid metabolism and local PGE2 synthesis (Bartolí et al., 2000). Moreover, oleic acid can reduced an anti-inflammatory effect by inhibiting reactive oxygen species (ROS), p38 MAPK, and Akt/IKK/NF signaling pathways in LPS-stimulated BV2 microglia cell (Oha et al., 2009). For health effects, MO seeds oil has low saturated fatty acid content and high mono-unsaturated fatty acid content similarly to olive oil which has beneficial health effects (Amaglo et al., 2010). Moreover, the negative association between high intakes of mono-unsaturated and poly-unsaturated fatty acids, derived chiefly from olive oils, and colorectal cancer risks were reported in 1953 Italian colorectal cancer subjects (Franceschi & Favero, 1999). In addition, epidemiological data have confirmed a lower colon cancer incidence in Mediterranean countries, where olive oil was consumed (Alarcón et al., 2001).

Another hypothesis for chemopreventive effect of MO pods may be due to the modulation of detoxification enzyme. It has been shown that MO pods extract has the potential for modulating phase I and II enzymes such as cytochrome b5, cytochrome P450, catalase, glutathione-peroxidase, reductase and S-transferase in mice (Bharali et al., 2003). Moreover, the diet containing bMO showed potentially anti-clastogenic activity against both direct and indirect-acting clastogens in male ICR mice (Promkum et al., 2010). In the present study, a potent colon carcinogen, AOM, was used to induce colon carcinogenesis, so bMO in the diet might act via the carcinogenesis processes through metabolic activation (Fiala et al., 1977).

Regarding the role of non-nutrient bioactive compounds in MO pods, niazimicin, an alkaloids isolated from MO seeds, was found to have a potential antitumor promoting activity in the two-stage mouse skin carcinogenesis using 7,12-dimethylbenz(a)anthracene (DMBA) as initiator and TPA as a tumor promoter (Guevara et al., 1999). Therefore, it was possible that anti-colon carcinogenic activity of bMO might be due to this bioactive compound. Another bioactive compound reported to inhibit the tumor cell proliferation is glucomoringin. This compound is an isothiocyanate that is found in MO seeds and it is easily extracted from the seeds of MO about 8-10% of the whole weight and it can reduce myeloma growth in nude mice (Brunelli et al., 2010). Moreover, MO pod also contains high portion of benzyl-isothiocyanate which shows anti-inflammatory activity on macrophage 264.7 cells (Cheenpracha et al., 2010).

Cell proliferation has long been suspected to play a significant role in the carcinogenesis (Terada et al., 2001). PCNA is a biomarker, which is closely related to invasion and metastasis of malignant neoplasms and their prognosis (Vane et al., 1994; Shen et al., 2003). The presence of inflammation is associated with up-regulation of iNOS and COX-2 (Maeda and Akaike, 1998), resulting in an increased risk of cancer development (Cohen and Ellwein, 1990). The inhibitory effect of bMO may be in part due to modification of colon tumor cell proliferation. To explain the mechanism of PCNA protein expression, we found that the anti-colon carcinogenesis activity of bMO induced by AOM/DSS administration can potentially to inhibit colon cancer cell growth. PCNA protein expressions were decreased in the bMO dose dependent manner. The effect of bMO on PCNA protein expression may due to omega-9 oleic (18:1) fatty acid in MO pods. To support our study, olive oil supplemented in Caco-2 and HT-29 colorectal cancer cells were induced cell apoptosis and cell differentiate by omega-9 oleic and omega-6 linoleic acid contents (Llor et al., 2003). The extra virgin olive oil rich in oleic acid could decrease the PCNA levels and induced tumors cell apoptosis by modulation effects on breast cancer through a different combination of Ras signaling pathways, a different proliferation-apoptosis balance, and presumably distinct levels of DNA damage in DMBA-induced mammary cancer in rats (Solanas et al., 2010). We can conclude that bMO has a chemopreventive effect via down-regulated of PCNA gene expression.

Among the several types of cancer, particularly gastric carcinoma and colon adenoma, COX-2 is up-regulated generating protumorigenic eicosanoids, in particular, prostaglandins that can promote cell growth, angiogenesis and suppression of immunity. Contrastly, iNOS produces large amounts of nitric oxide (NO) implicated in initiation, promotion and progression of tumors. Besides, NO has been shown to stimulate COX-2 activity and increase p53 mutations in chronic inflammation, contributing to clonal cellular expansion and genomic instability (Wu, 1996). Another mechanism of chemoprevention of the diet containing bMO may be due to their anti-inflammatory effect. Our study determined the mechanism of gene expression by iNOS and COX-2 protein expression. We found that bMO potentially reduced the mediators in inflammatory process via down-regulated iNOS and COX-2 gene expression. We found that iNOS protein expression decreased in the dose dependent manner whereas COX-2 protein expression decreased only in the low dose group (1.5% bMO). However, COX-2 protein expression was not found in the medium (3.0%) and high (6.0%) doses of bMO. The effect of bMO on iNOS and COX-2 gene expression may be due to omega-9 oleic acid in MO pods. To support our study, it has been reported that oleic acid reduces lipopolysaccharide-induced expression of iNOS and COX-2 in BV2 murine microglia cells. Moreover, olive oil with abundant omega-9 fatty acids could inhibited COX-2 expressions and decreases the risk of neoplasm associated with chronic colitis in IL-10 knockout mice (Hegazi et al., 2006). Additionally, extra-virgin olive oil-enriched diet was significantly decreased COX-2 and iNOS expression in DSS-colitis-associated colon

carcinogenesis in C57BL/6 mice (Fidalgo et al., 2010).

Another postulation, Cheenpracha and coworkers reported that benzyl-isothiocyanate; a bioactive compound that found in MO pods extract reduced lipopolysaccharide-mediated iNOS expression in murine macrophage RAW 264.7 cell. Moreover, benzyl-isothiocyanate could inhibit the initiation of N-nitrosobis (2-oxopropyl) amine induced pancreatic carcinogenesis by decreasing COX-2 expression (Kuroiwa et al., 2006). It was possible that the decreased in COX-2 expression might be caused by benzyl-isothiocyanate contained in MO pods. Also, benzyl-isothiocyanate could decrease TPA-induced leukocyte infiltration and inhibit excessive superoxide generation in inflammatory leukocytes in mouse dermis (Nakamura et al., 2004). Topical application of benzyl-isothiocyanate inhibited TPA-induced mouse skin inflammation (Miyachi et al., 2004). In addition, other pathways in gene expression of MO studied by Mischiati and coworkers could modulate the carcinogenesis in cell cycle by up-regulated the expression of genes involved in the survival mechanism, such as the checkpoint suppressor 1 and the basic leucine zipper transcription factor E4BP4, or in the detoxification mechanism, such as cytosolic superoxide dismutase (Mischiati et al., 2006).

Acknowledgements

This project was financially supported by a grant from the National Center for Genetic Engineering and Biotechnology, Ministry of Science and Technology, Thailand. The authors would like to thank Ms. Wantana Youngnoi for taking care of our experimental animals and technical assistance, Ms. Ankana Heng for preparing animal diets and Mr. Noparat Sinthabundit for technical assistance. We also thank Dr. Aikkarach Kettawan (Institute of Nutrition, Mahidol University) for kindly provide AIN-76 vitamin mixture and diet containment. Special thank to Prof. Dr. Young-Joon Surh, Director and Professor Tumor Microenvironment Research Center, College of Pharmacy, Seoul National University for his valuable suggestions.

References

- Alarcón LC, Barranco MD, Motilva V, Herrerias JM (2001). Mediterranean diet and health: biological importance of olive oil. *Curr Pharm Des*, **7**, 933-50.
- Amaglo NK, Bennet RN, Rosario BLoC, et al (2010). Profiling selected phytochemicals and nutrients in different tissues of the multipurpose tree *Moringa oleifera* L. grown in Ghana. *Food Chem*, **122**, 1047-54.
- Anwar F, Bhanger MI (2003). Analytical characterization of *Moringa oleifera* seed oil grown in temperate regions of Pakistan. *J Agric Food Chem*, **51**, 6558-63.
- Anwar F, Ashraf M, Bhanger MI (2005). Interprovenance variation in the composition of *Moringa oleifera* oil seeds from Pakistan. *J Am Oil Chem Soc*, **82**, 45-51.
- Ayerza R (2011). Seed yield components, oil content, and fatty acid composition of two cultivars of moringa (*Moringa oleifera* Lam.) growing in the Arid Chaco of Argentina. *Ind Crop Prod*, **33**, 389-94.
- Bartolí R, Bañare FF, Navarro E, et al (2000). Effect of olive oil on early and late events of colon carcinogenesis in rats: modulation of arachidonic acid metabolism and local prostaglandin E2 synthesis. *Gut*, **46**, 191-9.
- Bennett RN, Mellon FA, Foidl N, et al (2003). Profiling glucosinolates and phenolics in vegetative and reproductive tissues of the multipurpose trees *Moringa oleifera* L. (horseradish tree) and *Moringa stenopetala*. *J Agr Food Chem*, **51**, 3546-53.
- Bharali R, Tabassum J, Azad MR (2003). Chemomodulatory effect of *Moringa oleifera* Lam on hepatic carcinogen metabolizing enzymes, antioxidant parameters and skin papillomagenesis in mice. *Asian Pac J Cancer Prev*, **4**, 131-9.
- Bieri JG, Stoewsand GS, Briggs GM, et al (1977). Report of the American institute of nutrition ad hoc committee on standards for nutritional studies. *J Nutr*, **107**, 1340-8.
- Birchall MA, Schock EBV, Harmon BV, Gobé G (1997). Apoptosis, mitosis, PCNA and bcl-2 in normal, leukoplakic and malignant epithelia of the human oral cavity: prospective, in vivo study. *Oral Oncol*, **6**, 419-25.
- Brunelli D, Tavecchio M, Falcioni C, et al (2010). The isothiocyanate from glucomoringin inhibits NF-kB and reduces myeloma growth in nude mice in vivo. *Biochem Pharmacol*, **79**, 1141-8.
- Calder PC, Davis J, Yaqoob P, et al (1998). Dietary fish oil suppresses human colon tumour growth in athymic mice. *Clin Sci*, **94**, 303-11.
- Cheenpracha S, Park EJ, Yoshida WY, et al (2010). Potential anti-inflammatory phenolic glycosides from the medicinal plant *Moringa oleifera* fruits. *Bioorg Med Chem Lett*, **8**, 6598-602.
- Cohen SM, Ellwein LB (1990). Cell proliferation in carcinogenesis. *Science*, **249**, 1007-11.
- D'souza J, Kulkarni AR (1993). Comparative studies on nutritive values of tender foliage of seedlings and mature plants of *Moringa oleifera* Lam. *J Econ Taxon Bot*, **17**, 479-85.
- Davidson LA, Lupton JR, Jiang Y, Chapkin RS (1999). Carcinogen and dietary lipid regulate ras expression and localization in rat colon without affecting farnesylation kinetics. *Carcinogenesis*, **20**, 785-91.
- Debnath S, Biswas D, Ray K, Guha D (2010). *Moringa oleifera* induced potentiation of serotonin release by 5-HT3 receptors in experimental ulcer model. *J Phytomed*, **18**, 91-5.
- Emiola IA, Ologhobo AD (2006). Nutritional assessment of raw and differently processed underutilized legume seed in broiler diet. *J Anim Vet Adv*, **5**, 96-101.
- Fahey JW (2005). *Moringa oleifera*: a review of the medical evidence for its nutritional, therapeutic, and prophylactic properties. Part 1. Trees for Life Journal: a forum on beneficial trees and plants. 1:5 <http://www.TFLJournal.org/article.php/20051201124931586>.
- Faizi S, Siddiqui BS, Saleem R, et al (1995). Fully acetylated carbamate and hypotensive thiocarbamate glycosides from *Moringa oleifera*. *Phytochem (Oxford)*, **38**, 957-63.
- Farooq A, Sajid L, Muhammad A, Anwarul HG (2007). *Moringa oleifera*: a food plant with multiple medicinal uses. *Phytother Res*, **21**, 17-25.
- Fiala ES, Bobotas G, Kulakis C, Wattenberg LW, Weisburger JH (1977). Effects of disulfiram and related compounds on the metabolism in vivo of the colon carcinogen, 1, 2-dimethylhydrazine. *Biochem Pharmacol*, **26**, 1763-68.
- Fidalgo SS, Villegas I, Cárdeno A, et al (2010). Extra-virgin olive oil-enriched diet modulates DSS-colitis-associated colon carcinogenesis in mice. *Clin Nutr*, **29**, 663-73.
- Franceschi S, Favero A (1999). The role of energy and fat in cancers of the breast and colon-rectum in a southern European population. *Ann Oncol*, **10**, 61-3.
- Francis JA, Jayaprakasam B, Olson LK, Nair M (2004). Insulin

- secretagogues from *Moringa oleifera* with cyclooxygenase enzyme and lipid peroxidation inhibiting activities. *Helv Chim Acta*, **87**, 317-26.
- Guevara AP, Vargas C, Sakurai H, et al (1999). An antitumor promoter from *Moringa oleifera* Lam. *Mutat Res*, **440**, 181-8.
- Hegazi RA, Saad RS, Mady H, et al (2006). Dietary fatty acids modulate chronic colitis, colitis-associated colon neoplasia and COX-2 expression in IL-10 knockout mice. *Nutr*, **22**, 275-82.
- Kawabata K (1999). Paraganglia of the gallbladder: a report of two cases with an immunohistochemical study. *Pathol Res Pract*, **195**, 781-6.
- Khuhaprema T, Srivatanakul P (2008). Colon and rectum cancer in Thailand: an overview. *Jpn J Clin Oncol*, **38**, 237-43.
- Khuhaprema T, Srivatanakul P, Attasara P, et al (2010). Cancer in Thailand (Vol 5). 2001-2003. Bangkok; Bangkok Medical Publisher, Thailand.
- Kuroiwa Y, Nishikawa A, Kitamura Y, et al (2006). Protective effects of benzyl isothiocyanate and sulforaphane but not resveratrol against initiation of pancreatic carcinogenesis in hamsters. *Cancer Lett*, **241**, 275-80.
- Lazarus P, Sheikh SN, Ren Q, et al (1998). p53, but not p16 mutations in oral squamous cell carcinomas are associated with specific CYP1A1 and GSTM1 polymorphic genotypes and patient tobacco use. *Carcinogenesis*, **19**, 509-14.
- Llor X, Pons E, Roca A, et al (2003). Fish oil, olive oil, oleic acid and linoleic acid on colorectal neoplastic processes. *Clin Nutr*, **22**, 71-9.
- Maeda H, Akaike T (1998). Review: nitric oxide and oxygen radicals in infection, inflammation, and cancer. *Biochemistry (Mosc)*, **63**, 854-65.
- Mischiati C, Sereni A, Khan MTH, Lampronti I, Gambari R (2006). Effect of plant extracts on gene expression profiling: from macroarrays to microarray technology. *J Nat Prod*, **2**, 21-33.
- Miyachi K, Fritzler MJ, Tan EM (2004). Benzyl isothiocyanate inhibits excessive superoxide generation in inflammatory leukocytes: implication for prevention against inflammation-related carcinogenesis. *Carcinogenesis*, **25**, 567-75.
- Mokkhasmit M, Swasdimongkol K, Ngarmwathana W, Permiphath U (1971). Pharmacological evaluation of Thai medicinal plants. *J Med Assoc Thai*, **54**, 490-504.
- Nakamura Y, Miyoshi N, Takabayashi S, Osawa T (2004). Benzyl isothiocyanate inhibits oxidative stress in mouse skin: involvement of attenuation of leukocyte infiltration. *Biofactors*, **21**, 255-7.
- Oha YT, Jung YL, Jinhwa L, et al (2009). Oleic acid reduces lipopolysaccharide-induced expression of iNOS and COX-2 in BV2 murine microglial cells: Possible involvement of reactive oxygen species, p38 MAPK, and IKK/NF-kB signaling pathways. *Neurosci Lett*, **464**, 93-7.
- Paulo F, Ana C, Davi F, et al (2009). Larvicidal activity of the water extract of *Moringa oleifera* seeds against *Aedes aegypti* and its toxicity upon laboratory animals. *An Acad Bras Cienc*, **81**, 207-16.
- Promkum C, Kupradinun P, Tuntipopipat S, Butryee C (2010). Nutritive evaluation and effect of *Moringa oleifera* pod on clastogenic potential in the mouse. *Asian Pac J Cancer Prev*, **11**, 627-32.
- Reddy BS, Tokumo K, Kulkarni N, Aligia C, Kelloff G (1992). Inhibition of colon carcinogenesis by prostaglandin synthesis inhibitors and related compounds. *Carcinogenesis*, **13**, 1019-23.
- Reeves PG, Nielsen FH, Fahay Jr GC (1993). AIN-93 Purified diet for laboratory rodents: Final report of the American Institute of Nutrition Ad Hoc Writing Committee on the reformulation of AIN-76 rodent diet. *J Nutr*, **123**, 1939-51.
- Rosenberg DW, Giardina C, Tanaka T (2009). Mouse models for the study of colon carcinogenesis. *Carcinogenesis*, **30**, 183-96.
- Sharaf AM, Ebrahium ME, Ammar MS, Ghany ME (2009). Influence of using *Moringa* meal flour as meat extender on quality characteristics of beef burger patties during frozen storage. *World J Dairy Food Sci*, **4**, 32-40.
- Shen LJ, Zhang HX, Zhang ZJ, et al (2003). Detection of HBV, PCNA and GST-pi in hepatocellular carcinoma and chronic liver diseases. *World J Gastroenterol*, **9**, 459-62.
- Solanas M, Grau L, Moral R, et al (2010). Dietary olive oil and corn oil differentially affect experimental breast cancer through distinct modulation of the p21Ras signaling and the proliferation-apoptosis balance. *Carcinogenesis*, **31**, 871-9.
- Stoneham M, Goldacre M, Seagroatt V, Gill L (2000). Olive oil, diet and colorectal cancer: an ecological study and a hypothesis. *J Epidemiol Community Health*, **54**, 756-60.
- Sugie S, Mori Y, Okumura A, et al (1994). Promoting and synergistic effects of chrysazin on 1, 2-dimethylhydrazine-induced carcinogenesis in male ICR/CD-1 mice. *Carcinogenesis*, **15**, 1175-79.
- Suzuki I, Iigo M, Ishikawa C (1997). Inhibitory effects of oleic acid and docosahexaenoic acids on lung metastasis by colon-carcinoma-26 cells are associated with reduced matrix metalloproteinase-2 and -9 activities. *Int J Cancer*, **73**, 607-12.
- Suzuki R, Kohno H, Sugie S, et al (2004). Sequential observations on the occurrence of preneoplastic and neoplastic lesions in the mouse colon treated with azoxymethane and dextran sodium sulfate. *Cancer Sci*, **95**, 721-7.
- Tanaka T, Kohno H, Suzuki R, et al (2003). A novel inflammation-related mouse colon carcinogenesis model induced by azoxymethane and dextran sodium sulfate. *Cancer Sci*, **94**, 965-73.
- Tanaka T, Yasui R (2008). Preclinical animal studies on chemoprevention of colorectal cancer. *J Clin Gastroenterol*, **23**, 1669-76.
- Terada R, Yasutake T, Nakamura S, et al (2001). Evaluation of metastatic potential of gastric tumors by staining for proliferating cell nuclear antigen and chromosome 17 numerical aberrations. *Ann Surg Oncol*, **8**, 525-32.
- The National Bureau of Agricultural Commodity and Food Standards, Ministry of Agriculture and Cooperatives, Thailand (2006). The amount of food consumption at 95 percentile per capita and eater only. In: Food Consumption Data of Thailand, The Agricultural Co-operative Federation of Thailand, Bangkok, pp. 173.
- Ugwu FM, Oranye NA (2006). Effects of some processing methods on the toxic components of African breadfruit (*Treculia africana*). *Afr J Biotech*, **5**, 2329-33.
- Vane JR, Mitchell JA, Appleton I, et al (1994). Inducible isoforms of cyclooxygenase and nitric-oxide synthase in inflammation. *Proc Natl Acad Sci USA*, **91**, 2046-50.
- Wu KK (1996). Cyclooxygenase-2 induction: molecular mechanism and patho-physiologic roles. *J Lab Clin Med*, **128**, 242-5.
- Yohei S, Shimizu M, Tsurumi H, et al (2008). EGCG and polyphenon E attenuate inflammation-related mouse colon carcinogenesis induced by AOM plus DSS. *Mol Med Report*, **1**, 355-61.