RESEARCH COMMUNICATION

Anticlastogenic and Anticarcinogenic Potential of Thai Bitter Gourd Fruits

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Abstract

   Thai bitter gourd fruits (*Momordica charantia* Linn., TBG) has been previously demonstrated to possess phase II detoxificating enzymes inducing properties, as well as the ability to reduce phase I carcinogen activating enzyme activity in rat liver. In addition, it was partially inhibited 7,12-dimethylbenz(a)anthracene (DMBA)-induced mammary gland carcinogenesis in female Sprague-Dawley rats. In this study, we therefore examined the anticlastogenic and anticarcinogenic effect of TBG against clastogens, cyclophosphamide (CYP) and DMBA, in mice using the *in vivo* erythrocyte micronucleus assay and azoxymethane (AOM)-induced colon carcinogenesis in rats, respectively. For anticlastogenicity test, male mice were fed with modified AIN-76 diets containing 6.25% and 12.5% of ground freeze-dried TBG for 2 weeks prior to administration of clastogens till the end of experiment. Blood samples were collected and counted for reticulocytes by using the fluorescent microscope. For anticarcinogeicity test, male Wistar rats were fed with modified AIN-76 diets containing 5% and 10% ground freeze-dried TBG for 2 weeks prior to, during and 1 week after the completion of AOM administration (15 mg/kg once a week for 2 weeks). It was found that TBG at 6.25% resulted in a significant reduction in micronucleated peripheral reticulocytes (MNRETs) induced by only CYP. Study on anticarcinogenic potential demonstrated that rats fed with TBG diets at the concentration tested developed significantly higher incidence as well as the multiplicities of colon tumors than the control group. These results demonstrated that Thai bitter gourd fruits possesses anticlastogenic potential against clastogen in the mouse. Interestingly, it had no preventive potential against AOM-induced colon carcinogenesis in rat, rather increasing the incidence of colonic neoplasm when giving during the initiation stage.

Keywords: Thai bitter gourd - *Momordica charantia* - colon cancer - micronucleus - AOM rodent model

Introduction

   Cancer chemoprevention is a new promising strategy for cancer prevention by the use of either synthetic or naturally occurring chemicals to inhibit, reverse or retard tumorigenesis (Wattenberg, 1985; Greenwald et al., 1990; Hong and Sporn, 1997). There are a large number of phytochemicals present in our diets have shown antimutagenic and anticarcinogenic effects in numerous animal and cell culture systems (Wattenberg, 1983; Steinmetz and Potter, 1991; Dragsted et al., 1993; Surh, 2003). However, epidemiological studies suggest that active agents found in the diet might reduce or increase the relative risk of cancer development (Weisburger, 1991). Chemopreventive agents may function by a variety of mechanisms i.e. directed at all stages of carcinogenesis (Wattenberg, 1997). Induction of phase II detoxification enzymes, such as glutathione *S*-transferase, is one of the mechanisms of protection against the initiation of carcinogenesis (Talalay et al., 1995). Another mechanisms of chemoprevention may be the initiation of phase I activating enzymes, such as cytochrome P450.

   Various kinds of vegetables and spices which commonly consumed in Thailand have diverse medicinal properties and have been previously shown antimutagenic properties in our laboratory such as neem flowers, cabbage, celery cabbage, Chinese kale, Chinese radish and Thai bitter gourd fruits (Rojanapo and Tepsuwan, 1992; 1993; Kusamran et al, 1998a). Some of these vegetables including neem flowers, Asiatic pennywort leaves, cassia leaves, Thai bitter gourd fruits, and Indian mulberry leaves could also increase the activity of some phase II detoxification enzymes while decreasing the activity of some phase I enzymes (Kusamran et al, 1998b, Tepsuwan, 1997). Noteworthy, these vegetables could inhibit some chemically induced carcinogenesis (Kusamran et al, 1998a; Tepsuwan, 1999; 2002) and have anticlastogenic activities against clastogens (Kupradinun et al, 1997; 1998; 2011a,b).

   Thai bitter gourd or bitter melon fruits (*Momordica*

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charantia Linn., TBG) was widely used for food and as herbal medicine for its anti-diabetes, anti-tumor, anti-viral and anti-hypertension activities (Nerurkar and Ray, 2010, and references therein). Several studies have demonstrated the activity of TBG both *in vitro* and *in vivo* studies. *In vitro* studies have demonstrated anti-cancerous and anti-leukemic activities of TBG against numerous cell lines (Kim et al., 2002; Takemoto et al., 1982) and also anti-viral activity against numerous viruses including Epstein-Barr, Herpes and HIV viruses (Sun et al., 2001; Bourinbaiar and Lee-Huang, 1996; Jiratchariyakul, et al., 2001). Several in vivo studies have also demonstrated the antitumorous activity of TBG. Water and ethanol extracts inhibited the development of mammary tumors in mice (Nagasawa, et al., 2002) and aberrant crypt foci formation in the rat colon (Chiampanichayakul, et al., 2001). It has been previously demonstrated in our laboratory that it possesses phase II enzymes, as well as the ability to reduce phase I enzyme activities in rat liver (Kusamran et al., 1998b). In addition, it also partially inhibited DMBA-induced mammary gland carcinogenesis in female Sprague-Dawley rats (Kusamran et al., 1998b).

The present studies were designed to evaluate the inhibitory effect of Thai bitter gourd fruits against micronucleus formation induced by clastogens in male mice and AOM-induced colonic neoplasm in male rats.

Materials and Methods

Chemicals

Cyclophosphamide (CYP) was obtained from ASTA Medica AG (Frankfurt am Main, Germany). 7,12-dimethylbenz(a)anthracene (DMBA) and all vitamins used for the preparation of vitamin mixture were obtained from Sigma Chemicals Co. (St. Louis, MO, U.S.A). Acridine orange (AO) was obtained from E. Merk (Germany), Azoxymethane (AOM) was purchased from Sigma Aldrich (St. Louis, U.S.A). Chemicals used for the preparation of salt mixture were obtained from Fluka Chemicals Co. (Switzerland) and casein (EM HV milk protein) was the product of D.M.V.Co. (The Netherland).

Vegetables

TBG fruits were purchased from local markets in Bangkok and washed with tap and distilled water. After removed seed, chopped into small pieces and then lyophilized. Freeze-dried samples were blended to powder and kept at -20°C until use.

Animals, diets and experimental procedure

Clastogenicity and Anticlastogenicity testing in mice : Male ICR mice, 5 weeks old were obtained from the National Laboratory Animal Center (NLAC), Mahidol University, Nakorn Pathom, Thailand. Animals were maintained at the Laboratory Animal Facility of the National Cancer Institute according to the Institutional Care Guidelines which was approved by the Animal Ethic Committee of the institute. All animals were housed in shoes box stainless steel cages in an air-conditioned room at 23°±2°C and relative humidity 50±20% with 12 h light/dark cycle. For each experiment, animals were

acclimatized for 5-7 days by giving a modified AIN-76 diet (basal diet) before starting the experiment. Animal diets were base on the AIN-76 rat diet with slight modification (Bieri et al., 1976; Reeves et al, 1993). TBG diets were prepared by substituting the freeze-dried TBG for an equal amount of protein, cornstarch, sucrose, and fiber proportional to plant's proximate analysis at 6.25% and 12.5%.

Clastogenicity testing: After acclimation, mice were randomly divided by weight into 3 groups of 8-10 mice each. Group 1 was assigned as the control group that continued to receive the basal diet, while the other groups (groups 2 and 3) were assigned as experimental groups receiving basal diets containing 6.25% and 12.5% of TBG for 2 weeks and continued till the end of experiment. Both control and experimental groups were pair-fed in such a way that described by Kusamran et al., 1998 and water *ad libitum*. The experimental design is summarized in Figure 1. At 2 weeks after feeding the experimental diets, blood samples were collected and subjected to micronucleus assay as previously described (Hayashi et al., 1990, Kupradinun, 2008). The frequencies of MNRETs were recorded based on the observation of all 1,000 reticulocytes per mouse as classified by Vander et al., 1963.

Anticlastogenicity testing : CYP was intraperitoneally injected into mice those have been used previously for clastogenicity test at 50 mg/kg BW just after the blood sample was collected. Then blood samples were collected at 24 and 48 h after CYP injection and analyzed for AO-stained reticulocytes by fluorescent microscope (Figure 1). The remaining groups of the experimental animal for clastogenicity study were administered with DMBA at 40 mg/kg BW (in corn oil, p.o), then at 24 and 48 h after clastogen administration and analyzed for reticulocytes as in the above experiment.

Anticarcinogenicity testing in rats: A total of 90 male Wistar rats, aged 4-5 weeks old were obtained from the
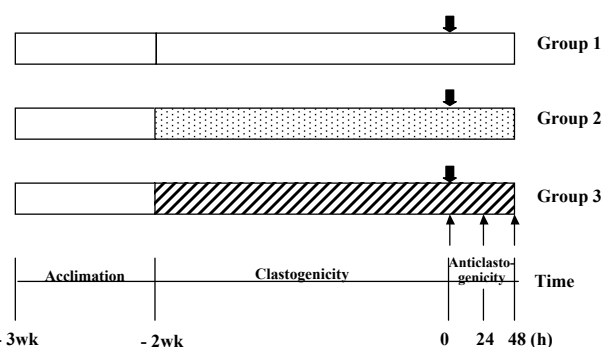
Figure 1. Schematic diagram of experimental design for study the clastogenic and anticlastogenic effect of TBG in mice. Open bar is the control group (group 1). Dot bar and hatched bar are experimental groups (groups 2&3). Arrows indicates blood collection. Black arrows indicate clastogens administration: cyclophosphamide (CYP); 7,12-dimethylbenz(a) anthracene (DMBA).
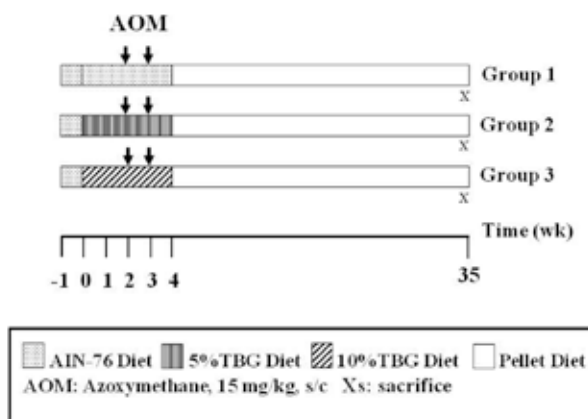
Figure 2. Experimental Protocol for Study the Anticarcinogenic Activity of TBG against AOM-induced Colon Carcinogenesis in Rats. Male Wistar rats in each group were fed with modified AIN-76 diet (dot bar) or 5%TBG diet (cross bar) and 10%TBG diet (hatched bar) for 2 weeks before giving azoxymethane (AOM) 15 mg/kg, s/c, once a week for 2 weeks (arrows) and then 1 week after the completion of AOM. After that rats fed with normal pellet diet (open bar) through the entire period of experiment. All rats were sacrificed (X), the colons were processed for routine histopathology as described in the Materials and Methods.

NLAC, Thailand. Animals using in this study was also approved by the Animal Ethic Committee. All rats were housed in stainless steel mesh cages in Laboratory Animal Facility of the National Cancer Institute as described above. Animals were acclimatized for 5-7 days by giving a modified AIN-76 diet before starting the experiment. In this study, we added ground freeze-dried TBG at 5% and 10% in basal diet.

After acclimation, they were randomly divided by weight into 3 groups of 30 rats each. Group 1 was received the basal diet serving as the control group which continued to receive the basal diet. Groups 2 and 3 were received the 5% and 10% TBG diets for 2 weeks before AOM (15 mg/kg BW, S/C, once a week for 2 weeks) administration, and then 1 week after the completion of AOM (Figure 2), serving as the experimental groups. Due to the bitter taste of TBG, rats did not like to eat this type of vegetable diets. Thus, both control and experimental groups were therefore pair-fed and water *ad libitum*. After that the diet was changed to normal pellet diet (Perfect Companion Group Co. Ltd., Thailand) until the end of experiment at week 30. All rats were sacrificed, the colons were excised, cut open and examined for any tumors, counted and measured for their sizes, and processed for routine histopathological study.

Statistical analysis

The significant difference in the frequencies of MNRETs between the experimental and control groups was analyzed using Kruskal-Wallis H and nonparametric Mann-Whitney U test. While the significant difference in the incidence of tumor was assessed by statistical techniques described by Peto et al. 1980, where as that of the number of tumors per rat, body and liver weights were analyzed using One-Way ANOVA and Kruskal-Wallis H Test.

Results

Clastogenicity and Anticlastogenicity testing in mice

Body weight and food consumption were daily recorded during the experiment. It was found that there were no significant differences between the control and experimental groups (data not shown).

The frequencies of MNRETs in mice treated with CYP was shown in Figure 3A. The number of MNRETs slightly increased at 24 h and maximum at 48 h of both control and experimental groups. Therefore, the percent inhibition was calculated to compare the inhibitory effect of the vegetable against clastogens induced MNRETs at 48h after treatment. Mice fed with TBG at 6.25% in the diets, MNRETs was significantly decreased at both 24 h and 48 h ($P= 0.009$ and $P= 0.047$), respectively. While TBG at 12.5% in the diets, MNRETs was significantly decreased only at 24h ($P= 0.014$). The inhibitory effects at low and high doses were about 40% and 17%, respectively.

Figure 3B shows MNRETs in mice treated with DMBA. The pattern of MNRETs formation is fairly similar to CYP. It was found that feeding of TBG at 6.25% in the diets had no effect to the frequencies of MNRETs. While TBG at 12.5% in the diet, MNRETs
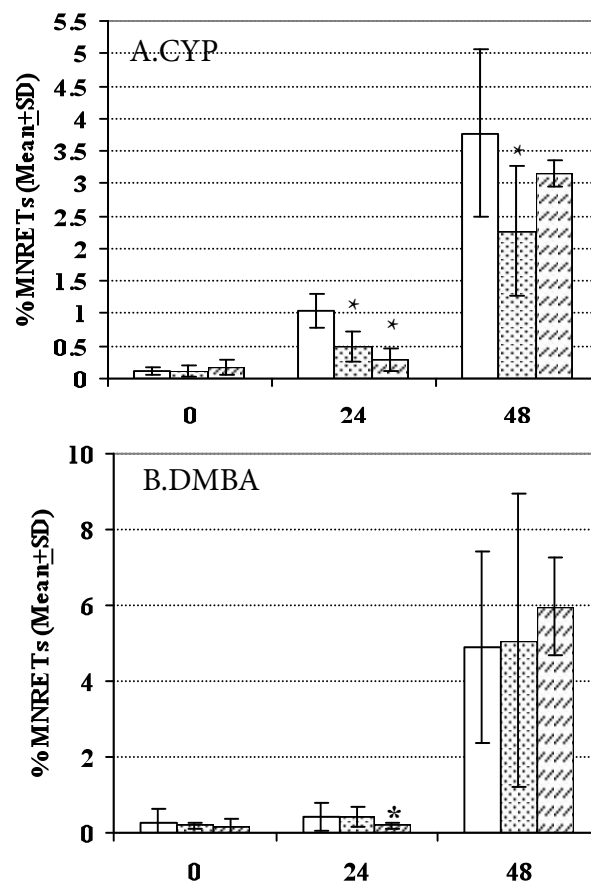
Figure 3. Mean frequencies of MNRETs in mice fed with TBG diets after clastogens, 3A:CYP, 3B: DMBA, administrations. The frequency of MNRETs in the control group received basal diet (open bar) and the experimental groups (dot bar and hatched bar) received TBG at 6.25% and 12.5% in the diets, respectively. * Significant differences at $P< 0.05$.

Table 1. Incidence and Multiplicity of Colonic Neoplasms in Rats

Groups	No. of Rats						No. of Tumors		
	Total	With Tumor (%)	Type of Tumors (%)			Fold of Control	Total	Per Rat (M±SE)	Fold of Control
			ACC	SC-ACC	SCA				
Control	27	19(70.37)	12(63.2)	1(21.1)	4 (14.8)	1.0	44	1.59±0.56	1.0
5%TBG diet	29	27(93.1)*	20 (74.1)*	0(0)	20 (74.1)*	1.32	120	3.97±0.49*	2.7
10%TBG diet	28	27(92.9)*	33 (85.2)*	3(11.1)	7(25.9)	1.32	102	3.57±0.40*	2.3

ACC: Adenocarcinoma ; SC-ACC: Scirrhous adenocarcinoma; SCA: Superficial carcinoma   *Significant difference at P<0.05

was significantly decreased ($P= 0.019$) from those of the control group at 24h. However, MNRETs was slightly increased at 48 h.

Anticarcinogenicity test in rats

The mean body and relative liver weights of rats fed TBG diets either 5% or 10% TBG in diets were not significantly different from those of the control rats.

The incidence of tumors in the control group was about 70% with a multiplicity of 1.6. In groups 2 and 3 which fed 5% and 10% TBG diets, the incidences of tumors were significantly higher than those of the control group (93%), while the multiplicities being 3.97±0.49 in group 2 and 3.57±0.4 in group 3 and also was significantly different ($P< 0.05$). The incidence was increased 1.3 fold and multiplicity was 2.7 fold in low dose group while 1.3 fold and 2.3 fold in high dose group when compared with those of the control group. Macroscopic view of the colons of the rats in all groups showed nodular or polyploidy-like tumors, of varying sizes, at the middle and distal part of colons. Among these tumors formation, simple tubular adenocarcinoma is the most common type and increased according to the concentration of TBG and significantly difference from those of the control group. Superficial carcinoma was the highest type about 74% in the low dose group and was significantly different ($P< 0.05$) while scirrhous carcinoma was not found in the low dose group(Table 1). Moreover, metastases were observed in the high dose group.

Discussion

According to pair feeding of animals either in clastogenicity, anticlastogenicity or anticarcinogenicity test, TBG had no effect on the growth rate of all animals. In addition, it had no effect to liver weight of rats and on the spontaneous formation of MNRETs in mice. TBG at both low and high doses had shown the anticlastogenic activity as it could reduce MNRETs induced by CYP but no activity to DMBA. This result is different from our previous study that TBG either in low or high doses in the diet were significantly decreased the multiplicity of mammary gland tumors induced by DMBA in the rats (Kusamran et al., 1998).

Unfortunately for anticarcinogenic potential, we found that TBG could not inhibit AOM-induced colon carcinogenesis in rats but increased both the incidence and multiplicity of tumors. This study was the same as previous study that diets containing TBG could not inhibit liver carcinogenesis-induced by AFB[1] in Wistar rats (Kupradinun, in preparation). Moreover, recent researches

have been shown that seed extracts of bitter melon could induce apoptosis and inhibit histone deacetylase-1 selectively in prostate cancer cells (Xiong et al., 2009). In addition, they have preventive effects against breast and colon cancers (Lee-Huang et al., 2000; Kohno et al., 2004) and TBG extract decreased AOM-induced aberrant crypt foci (ACF) formation (Chiampanichayakul, et al, 2001). Moreover, it was shown anti-cancer activity in a mouse mammary tumor model (Nagasawa et al, 2002). These phenomena depend on the compounds present in the different parts of this plant. Seed oil of bitter melon contain alpha-eleostearic acid (α-ESA) which suppressed the growth of DLD-1 human colon cancer cells (Tsuzuki et al, 2004) and HL60 human promyelocytic leukemia cells (Kobori et al, 2008) by inducing apoptotic activity (Grossman et al., 2009). However, the acetone extract of bitter melon seed, which is probably rich in α-ESA, did not induce apoptosis in the cells and also did not suppress the colon cancer growth in xenograft model (Tsuzuki et al, 2004). These results are fairly similar to that of neem flowers which were highly significantly increased the incidence of colonic neoplasm induced by AOM (Kupradinun et al., in preparation) while inhibiting AFB[1] and DMBA induced liver and mammary gland carcinogenesis in rats (Tepsuwan et al, 2002). Results of neem flowers indicated organ different in chemopreventive potential.

Previous reports have noted that phytochemical compound such as quercetin, which found mainly in fruits and vegetables, increased the incidence of AOM-induced colon cancer but was weakly effective to mammary tumorigenesis in rats (Piereira et al, 1996). Moreover, quercetin has been shown mutagenicity (Brown, 1980; MacGregor and Jurd, 1978) and also induced chromosome aberrations and sister chromatid exchange *in vitro* (Yoshida et al, 1980). However, this chemical has shown chempreventive potential against mammary gland carcinogenesis in rats (Verma et al, 1988) and colon carcinogenesis in mice (Deschner et al, 1991). From previous studies have shown that chemical compounds may have different effects to chemically induced-colon cancer neoplasm. Some compounds might be the chemoprotective effect from a chemical induced-carcinogenesis or promoting effects from another chemical compounds. For example, chlorophyllin, the water-soluble salts of chlorophylls, which commonly present in green and leafly vegetables had the inhibitory effect to ACF formation and colon carcinogenesis induced by a heterocyclic amine in fried ground beef, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) or 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) (Guo

et al, 1995a,b) but had promoting effect induced by 1,2-dimethylhydrazine (DMH) (Nelson, 1992; Xu et al, 2001). Moreover, there was study shown that indole-3-carbinol (I3C) which found in cruciferous vegetables had inhibitory effect to colon carcinogenesis induced by PhIP or IQ (Guo et al, 1995a; Xu et al, 1996; 2001) but increased cancer incidence which induced by DMH (Pence et al, 1986). From epidemiological studies have shown that high fruits and vegetables consumption might increase the risk of colorectal cancer, particularly in male (Phillips and Snowdon, 1985; Tajima and Tominga, 1985; Shibata et al, 1992; Steinmetz and Potter, 1083). However, the mechanism by which TBG had the promoting effect of AOM-induced colon carcinogenesis in rats was not known.

The recent of our studies were shown that TBG possess phase II detoxificating enzymes inducing property, as well as the ability to reduce phase I carcinogen activating enzyme activities in rat liver (Kusamran et al, 1998a). In the present studies, TBG has anticlastogenic potential against clastogen in mouse peripheral reticulocytes but has promoting effect to colon carcinogenesis in rats induced by AOM. These information indicated that TBG may have chemopreventive effect in many systems except colon carcinogenesis. Based on the present findings and data it is possible for TBG to act as a tumor promoter or anticarcinogen, depending upon the test species, initiating agent and exposure protocol. So further study should be was performed since AOM is not a human carcinogen as PhIP or IQ which present in cooked food, but the problem is PhIP is not commercial available. In addition, further study of the chemopreventive effect of the TBG in mouse colitis-associated colon carcinogenesis model as well as extract or active main compounds of TBG should be performed.

In conclusion, these results demonstrated that Thai bitter gourd fruits had no clastogenic activity in the mouse, but they do contain compound(s) capable of inhibiting the clastogenic activity of some clastogen, indicating that it may have chemopreventive potential against genotoxicants. Noteworthy, Thai bitter gourd fruits had no preventive potential against AOM-induced colon carcinogenesis in rat. On the contrary, it may promote colon tumor development. However, the effect was no dose response relationship. These findings also suggest that Thai bitter gourd fruits may have different chemopreventive potential on different organs because in mammary gland model it could significantly decrease the multiplicity of tumor.

Acknowledgements

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