RESEARCH COMMUNICATION

Chemopreventive Potential of Neem Flowers on Carcinogen-Induced Rat Mammary and Liver Carcinogenesis

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Abstract

We have previously reported that dietary neem flowers (*Azadirachta indica* A. Juss var. *siamensis* Valeton) caused a marked increase in glutathione *S*-transferase (GST) activity in the liver, while resulting in a significant reduction in the activities of some hepatic P450-dependent monooxygenases. These results strongly indicate that neem flowers may have chemopreventive potential. In the present study, we examined the inhibitory effects of neem flowers on 9,10-dimethyl-1,2-benzanthracene (DMBA)-induced mammary gland carcinogenesis in female Sprague Dawley rats and on aflatoxin B$_1$ (AFB_1)-induced hepatocarcinogenesis in male Wistar rats. Young animals were fed with AIN-76 purified diets containing either 10-12.5% ground freeze-dried neem flowers for 1 week prior to, during, and for 1 week after the administration of each carcinogen. Interestingly, it was found that neem flowers resulted in a marked reduction of the incidence of mammary gland (about 35.2%) and liver tumors (61.7% and 80.1% for benign and malignant tumors, respectively). Furthermore, the multiplicity of tumors per rats was also lower in the neem flower groups, i.e. those for mammary gland tumors and benign and malignant liver tumors were reduced to 44.0%, 87.9% and 88.9%, respectively. These results clearly demonstrated that neem flowers contain some chemopreventive agents capable of inhibiting AFB_1 and DMBA induced liver and mammary gland carcinogenesis in rats.

Key Words: Neem flowers - Chemopreventive potential - DMBA - AFB_1- Mammary gland carcinogenesis - Liver carcinogenesis

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Introduction

Cancer chemoprevention is a new promising strategy for cancer prevention. It is defined as the use of chemicals (natural or synthetic) or dietary components (daily foods enriched with cancer preventive components) to block, inhibit, or reverse the development of cancer in normal or preneoplastic tissue (Wattenberg, 1985; Greenwald et al., 1990; Weinstein, 1991; Morse and Stoner, 1993; Hong and Sporn, 1997). Chemopreventive agents may function by a variety of mechanisms, directed at all major stages of carcinogenesis (Wattenberg, 1997). Induction of phase II detoxification enzymes, such as glutathione *S*-transferase (GST, EC 2.5.1.18) or NAD(P)H:quinone oxidoreductase (QR, EC 1.6.99.2), is one of the major mechanism of protection against initiation of carcinogenesis (Talalay et al., 1995). The well known GST inducers that possess chemopreventive property include oltipratz (Kensler et al., 1987; Wattenberg and Bueding, 1986), isothiocyanates (Zhang et al., 1992; Zhang et al., 1994) and organosulfur compounds (Sparnins et al., 1988), in which some of them can also induce QR in Hepa 1c1c7 cells (DeLong et al., 1986). Several chemopreventive agents have been identified solely on the basis of their ability to induce phase II enzymes (Wattenberg and Bueding, 1986; Zhang et al., 1994; Lam et al., 1982). Moreover, some vegetables, particularly cruciferous vegetables, that have been shown to decrease the risk of various types of cancers in humans (Armstrong and Doll, 1975; Correa, 1981; Graham, 1983; Howe et al.. 1990; Steinmetz and Potter, 1991; Block et al., 1992) as well as in experimental animals exposed to some chemical carcinogens (Stoewsand et al., 1978; Boyd et al., 1982;

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Wattenberg, 1992), also induced phase II detoxification enzymes such as GST and QR (Sparnins et al., 1982; Bradfield et al., 1985; Ansher et al., 1986). Another mechanism of chemoprevention may be the inhibition of phase I activating enzymes [cytochrome P450 (P450) dependent monooxygenases). Disulfiram, for example, inhibits dimethylhydrazine bioactivation and colon neoplasia (Fiala et al., 1977; Wattenberg, 1979), diallyl sulfide inhibits carcinogen activation and tumorigenesis in animal models (Hong et al., 1991; Wargovich, 1988; Sparnins et al., 1988; Wargovich et al., 1988; Tadi et al., 1991). Another example is sulforaphane, the anticarcinogenic constituent from broccoli (Zhang et al., 1994), inhibits P450 isozyme 2E1 and the genotoxicity of dimethylnitrosamine (Barcelo et al., 1996)

Neem tree (*Azadirachta indica* A. Juss var. *siamensis* Valeton) is the evergreen tree growing throughout Thailand. Neem tree is the magic tree, almost every part of the tree has been used for a long time in agriculture and traditional medicine (van der Nat et al., 1991). Flowers and young leaves are also among the common vegetables eaten in Thailand especially in the winter. We have recently reported that the extracts of neem flowers and young leaves exhibited antimutagenicity against indirect mutagens/carcinogens, namely, aflatoxin B_1 (AFB_1) and benz(a)pyrene [B(a)P] towards *Salmonella typhimurium* (Rojanapo and Tepsuwan, 1992; Kusamran et al., 1998b). In addition, dietary neem flowers could inhibit the formation of micronucleated erythrocytes in mice induced by 9,10-dimethyl-1,2-benzanthracene (DMBA) (Kupradinun et al., 1997). It has also been clearly demonstrated in our laboratory that dietary neem flowers caused a marked increase in the activity of GST in the liver, while resulting in a significant reduction in the activities of some hepatic P450-dependent monooxygenases, namely, aminopyrine demethylase (CYP2C11), aniline hydroxylase (CYP2E1), as well as the capacity to metabolically activate the mutagenicities of AFB_1 and B(a)P towards *Salmonella typhimurium* (Kusamran et al., 1998a). Recently, leaves of Indian neem tree (*Azadirachta indica* A. Juss), which is the same species as our neem tree, has been shown to effectively suppress hamster buccal pouch carcinogenesis initiated by DMBA and also induce GST level in the oral mucosa of animals bearing tumors (Balasenthil et al., 1999). Therefore, it is of great interest to pursue the chemopreventive potential of young leaves and flowers of neem tree. In the present communication, we report the chemopreventive property of Thai neem flowers against AFB_1-induced liver carcinogenesis in male Wistar rats and DMBA-induced mammary gland carcinogenesis in female Sprague Dawley rats.

Materials and Methods

Chemicals

Aflatoxin B_1 (AFB_1) was purchased from Makor Chemicals Ltd. (Jerusalem, Israel). 9,10-dimethyl-1,2-benzanthracene (DMBA) and all vitamins used for the preparation of vitamin mixture were obtained from Sigma Chemicals Co. (St. Louis, MO). Chemicals used for the preparation of salt mixture were obtained from Fluka Chemical Co. (Switzerland), and casein (EM HV milk protein) was the product of D.V.M. Co., (The Netherlands). All other reagents were mostly of analytical grade and obtained locally.

Vegetables

Neem flowers were obtained from local markets in Bangkok. Flowers were removed from the stems, washed successively with tap water and distilled water, and then lyophilized. Freeze-dried materials were blended to powder and kept at -20 ^{o}C until use.

Animals and diets

Both male Wistar and female Sprague Dawley rats were obtained from the National Laboratory Animal Center, Mahidol University, Salaya, Nakornpathom, Thailand. Animals were housed in stainless-steel cages using white paper as bedding material, in a room maintained at 25$\pm$ 2 ^{o}C and with a 12-hr light/dark cycle. For each experiment, they were acclimatized for 5-7 days on a modified AIN-76 purified diet (basal diet, Table 1) before starting the experiment.

Since neem flowers have bitter taste, animals did not like to eat the diet containing this vegetable (only about 15 g/day instead of about 20 g/day for normal basal diet) even at 10-12.5 %. Both control and experimental groups were therefore pair-fed in such a way that the former was given the same amount of food eaten by the latter on the previous day. Animal diets were based on the AIN-76 rat diet (Bieri et al., 1976) with slight modification according to Reeves et al.(Reeves et al., 1993). Vegetable diet was prepared by substituting the ground freeze-dried, vegetable for an equal amount of protein, cornstarch, sucrose and fiber proportional to plant's proximate analysis (Nutrition Division, Department of Health, Ministry of Public Health, 1992). The composition of basal AIN-76 purified diet and diets containing 10-12.5% neem flowers is shown in Table 1.

Effect of neem flowers on DMBA-induced mammary gland carcinogenesis in female rats

Thirty-eight female Sprague Dawley rats, 6 weeks old, were acclimatized for 7 days and randomly divided by weight into 2 groups. One group was assigned as the control group which continued to receive the basal diet, while the other was assigned as the experimental group receiving diet containing 10 % neem flowers (Table 1).

One week after feeding the experimental diets (animals were 55 days old), animals were administered DMBA at 65 mg/kg body weight by gastric intubation. Animals were maintained on experimental diets for one more week and then changed to normal pellet diet (Charoen Pokaphand Co. Ltd., Bangkok) until the end of the experiment. Each rat was examined for the presence of mammary gland tumors

Table 1. Composition of Basal AIN-76 Purified Diet and Diets Containing Neem Flowers

Ingredient	Amount (g/kg) in diet		
	Basal	10% Neem flowers	12.5% Neem flowers
Casein	200	185	180
Cornstarch	500	440	435
Sucrose	150	133	120
Corn oil	50	50	50
DL-Methionine	3	3	3
AIN Mineral mix	35	35	35
AIN Vitamin mix	10	10	10
Choline bitartrate	2	2	2
α-Cellulose	50	42	40
Vitamin K_1	0.002	0.002	0.002
Freeze-dried neem flowers	-	100	125

every week starting from 4 weeks after the administration of DMBA. Twenty weeks later, all animals were sacrificed and autopsied. The tumors were removed, measured for the actual size and processed for routine histopathological examination.

Effect of neem flowers on AFB_1 –induced liver carcinogenesis in male rats

A total of 58 male Wistar rats weighing 47-50 g were randomly divided by weight into 2 groups as above experiment, i.e. control group receiving AIN-76 basal diet while experimental group receiving diet containing 12.5 % neem flowers (Table 1). One week after, all animals were given AFB_1 (75 μg/day and 5 days/week for 4 weeks) by gastric intubation. They were continued to receive experimental diets until one week after completion of AFB_1 treatment and then given normal pellet diet *ad libitum* until the end of the experiment.

Animals were weighed once a week and they were necropsied when they died or became moribund. A total of 14 rats (6 and 8 rats in control and experimental groups, respectively) died during the first 6 weeks of the experiment and were therefore excluded from this study. All surviving animals were sacrificed 78 weeks after starting the first AFB_1 treatment. The livers were removed, weighed and grossly examined for the presence of foci, nodules and tumors. After that, livers as well as other organs, including lungs, kidneys and spleens were fixed in formalin/sodium acetate solution and processed for routine histopathological examination. The classification of both preneoplastic and neoplastic liver lesions was based on the criteria described by Bannasch and Zerban (Bannasch and Zerban, 1990).

Determination of serum gamma-glutamyltranspeptidase (GGT) activity

Serum GGT activity was determined by the method essentially described by Tamaoki et al. (Tamaoki et al., 1975) using L-γ-glutamyl-*p*-nitroanilide as a substrate and glycylglycine as an acceptor. The enzyme activity was expressed as international units per litre of serum (IU/L).

Statistical Analysis

The significant difference in the incidence of mammary gland and liver tumors between experimental and control groups were assessed by the statistical techniques described by Peto et al. (Peto et al., 1980), whereas that of the number of tumors per rat and GGT activity was analysed using Student's *t*-test.

Results

Chemopreventive effect of neem flowers on DMBA-induced mammary gland carcinogenesis in female Sprague-Dawley rats

The chemopreventive effect of neem flowers on DMBA-induced mammary gland carcinogenesis in female Sprague Dawley rats was determined by feeding the AIN-76 diet containing 10% ground freeze-dried neem flowers 1 week prior to and 1 week after the administration of DMBA. Table 2 shows the survival, body weights (initial or prior to giving the experimental diets, after completion of experimental diets and terminal or at the time of sacrifice) and the tumor onset time in both neem flowers and control groups. The results demonstrated that all animals in both groups were survived until the end of the experiment. However, the body weights of rats fed diet-containing neem flowers were slightly lower, but significantly, than those of rats fed control diet, both at 2 weeks after feeding neem flowers diet and at the time of sacrifice (20 weeks after DMBA administration). Interestingly however, the onset time or the latent period until the appearance of the first palpable tumor of mammary gland was slightly longer, although not significantly, in animals fed neem flowers compared to that in the control group (62.7 ± 18.5 days vs. 53.3 ± 8.8 days). Histopathological study revealed that about 80-90% of mammary gland tumors in both groups was adenocarcinoma.

Results in Fig. 1 clearly demonstrated that 10% dietary neem flowers could prevent or inhibit mammary gland carcinogenesis induced by DMBA. The first rat in both groups developed tumors 6 weeks after DMBA administration. However, the incidence of mammary gland

Table 2. Effect of Dietary Neem Flowers on the Body Weight, Survival and Tumor Onset Time in Female Sprague Dawley Rats Treated with DMBA

Group	Survival	Body weight (g)			Tumor onset time (days)
		Initial[a]	2 weeks after[b]	Terminal[c]	
Control	18/18	170.0 ± 1.6	204.8 ± 5.2	303.8 ± 20.3	53.3 ± 8.8
Neem flowers-10%	20/20	170.0 ± 0.0	197.0 ± 2.6 *	285.5 ± 16.1**	62.7 ± 18.5

Results are expressed as means ± SD [a]Body weight before giving the experimental diet [b]Body weight at 2 weeks after giving the experimental diet [c]Body weight at the time when sacrifice
*,** Significantly different from control group at $P < 0.05$, <0.001, respectively

tumors in neem flowers group increased slower than that in the control group. The tumor incidence in neem flowers and control groups reached the plateau at about week 15th (70%) and 10th (90%), respectively. The difference in the tumor incidence between both groups was statistically significant at week 9th, 10th, 12th and 14th (Fig. 1A). The multiplicity (the number of tumors per rat) of mammary gland tumors was also lower in neem flowers group, and the difference was statistically significant at 14-17 weeks after the administration of DMBA (Fig. 1B).

Chemopreventive effect of neem flowers on AFB_1–induced liver carcinogenesis in male Wistar rats

The body and liver weights as well as relative liver weights of rats fed neem flowers diet (12.5% in the diet) were slightly lower than those of control animals. However, the difference was not statistically significant (Table 3). Interestingly, results in Table 4 clearly demonstrated that the incidences of both malignant and benign liver tumors induced by AFB_1 in neem flowers fed group (9.5% and 33.3%) were markedly and significantly lower than those in control group (47.8% and 87.0%). The malignant tumors developed in neem flowers fed group (2 rats) were haemangiosarcoma and clear cell sarcoma, while most of those in control group (8/10 rats) were hepatocellular carcinoma. It is noteworthy that the tumors in the former group were much less aggressive than those in the latter; there was no distant metastasis observed in the former, while in the latter group 3 rats developed lung metastasis and another 2 rats developed primary carcinoma in the other organs. Furthermore, the multiplicity of both malignant and benign tumors in neem flowers group (0.1 and 0.4 tumor/rat) was significantly lower than that in the control group (0.9 and 3.3 tumors/rat). The size of tumor in neem flowers group was also much smaller comparing to that in the control group.

Since GGT has been demonstrated to be activated by several liver carcinogens, including AFB_1 and the activity is significantly increased both during precancerous stage and in the liver cell carcinoma (Fiala et al., 1972; Fiala et al., 1973; Tanigushi et al., 1974; Kalengayi et al., 1975) as well as in the sera of animals with liver cancer (Rojanapo et al., 1993). We therefore measured the activity of this enzyme in the sera of our animals in order to compare the biochemical changes with the histopathological findings. Results in Table 5 revealed that in the control group, the serum GGT activity in rats with liver cancer was much higher than those without cancer. Interestingly, the enzyme activity was significantly

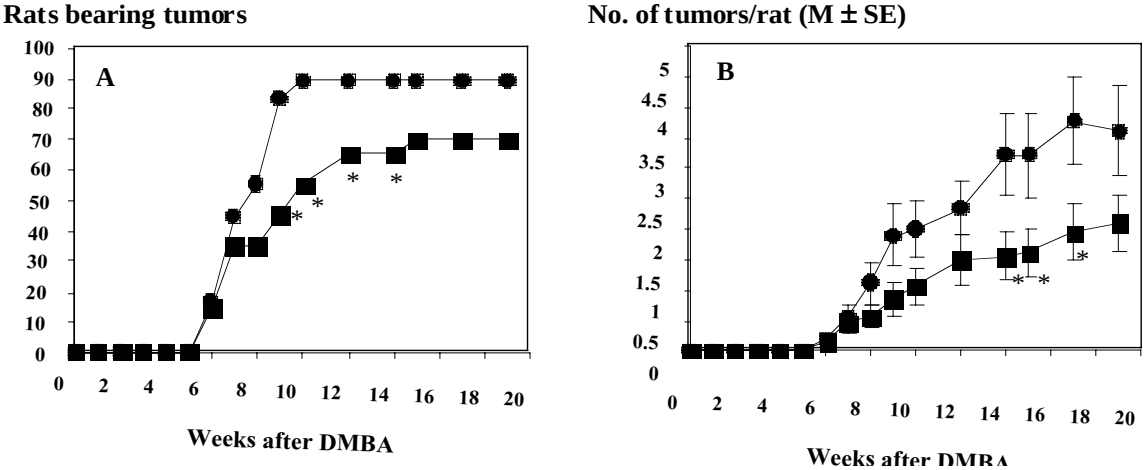

Figure 1. Effects of Dietary Neem Flowers on DMBA-Induced Mammary Gland Carcinogenesis in Female Sprague Dawley Rats.

The Anticarcinogenic Activities were Evaluated by both the Percent Tumor Bearing Rats (A) and the Numbers of Tumors per Rat (B). Animals were fed control diet (●) and diet containing 10% neem flowers (■) 1 week prior to and also 1 week after DMBA administration. *Significantly different from corresponding control at $P< 0.05$

Table 3. Effect of Dietary Neem Flowers on Survival, Body and Liver Weights of Male Wistar Rats Treated with AFB_1.

Group	Survival[a]	Body weight (g)		Liver weight (g)	Relative liver weight (g/100 g body wt.)
		Initial	Terminal		
Control	23/29	49.5 ± 1.4	676.7 ± 76.1	29.3 ± 21.7	4.3 ± 2.6
Neem flowers-12.5%	21/29	49.3 ± 0.6	633.7 ± 59.0	19.6 ± 3.4	3.1 ± 0.6

Results are expressed as means ± SD [a]Number of rats surviving at the 7th week after AFB_1 treatment

lower in neem flowers fed animals comparing to that in the control animals, both with and without cancer (some rats bearing benign liver tumors). Some rats in the control group especially those bearing large liver cancer or many cancers had very high GGT activity which then leading to large SD value. However, the statistical analysis between control and neem flowers fed groups could not be done since the blood from only one animal in that latter group was obtained.

Discussion

Dietary neem flowers, at the level of 10% in the diet, reduced both the incidence and the multiplicity (number of tumors per rat) of mammary gland tumors in female Sprague Dawley rats induced by DMBA when given 1 week prior to carcinogen administration. The average tumor onset time was also lengthened, although not significantly, by dietary neem flowers. Interestingly, dietary neem flowers, at the level of 12.5% in the diet, could also suppress the development of liver tumor in male Wistar rats induced by AFB_1. The incidence of both benign and malignant liver tumors as well as the number of tumors per rat were markedly and significantly lower in neem flowers fed group. The size of tumors in neem flowers fed group was generally much smaller than that in the control animals, in which the tumors were also more aggressive as revealed by lung metastasis. Results in this study therefore can be concluded that neem flowers possessed chemopreventive potential against DMBA-induced mammary gland and AFB_1-induced liver carcinogenesis when given prior to carcinogen administration. These results also suggested that neem flowers inhibited carcinogenesis at least at the initiation stage. The data in this study were parallel with our previous results that neem flowers were able to prevent micronucleus formation in mouse peripheral reticulocytes induced by DMBA (Kupradinun et al., 1997). Interestingly, our results were in good agreement with those of Balasenthil and colleagues (Balasenthil et al., 1999) who reported that leaf extract of Indian neem tree, which is the same species as our Thai neem tree, effectively suppressed buccal pouch carcinogenesis in male Syrian hamster initiated by DMBA. On the other hand, however, the flowers of Indian neem tree have been shown to be unable to suppress the *in vivo* chromosome aberrations caused by B(a)P in mouse bone marrow cells (Aruna and Sivaramakrishnan, 1990).

According to the hypothesis that a marked elevation of phase II enzymes may be a critical and probably sufficient condition for achieving chemoprotection (Talalay, 1992). Furthermore, chemoprotective potential may be even much greater in the condition where phase II enzymes are elevated while phase I enzymes are inhibited (lower or suppressed), which resulting in a lower yield of the electrophilic metabolites capable of covalently binding to DNA. Thus, monofunctional inducers, agents that induce only phase II enzymes, and dual-acting agents, those that induce phase II enzymes but inhibit the expression of phase I enzymes

Table 4. Effect of Dietary Neem Flowers on the Incidences of Benign and Malignant Tumors in Livers of Rats Treated with AFB_1

Group	No. of rats	No. of rats (%) with						No. of tumors per rat		
		All tumors	Benign tumors	Malignant tumors				Malignant	Benign	Total
				All types	HCC	HCC & CCC	Others[a]			
Control	23	21 (91.3)	20 (87.0)	11 (47.8)	7[b]	1[c]	3[d]	0.9 ± 1.5	3.3 ± 2.3	4.2 ± 3.0
Neem flowers-12.5%	21	7*** (33.3)	7*** (33.3)	2** (9.5)	0	0	2	0.1± 0.3*	0.4 ± 0.7***	0.5± 0.9***

[a]Include haemangiosarcoma, spindle cell sarcoma and clear cell sarcoma
[b]One rat was found to develop adenocarcinoma of kidney and another developed haemangiosarcoma of the mesentery
[c]With lung metastasis [d]Two rats had lung metastasis
*, **. *** Significantly different from control group at $P < 0.05$, $P < 0.005$ and $P < 0.00005$, respectively

Table 5. The Serum GGT Activity in Male Rats with and without Liver Carcinoma.

Group	Gamma-glutamyl transpeptidase activity (IU/L)		
	Rats with and without cancer	Rats with cancer	Rats without cancer
Control	8.76 ± 8.77 (17)	14.71 ± 13.08 (6)	5.51 ± 2.16 (11)
Neem flowers-12.5%	3.62 ± 1.01* (16)	3.59** (1)	3.62 ± 1.05* (15)

Results are expressed as means ± SD Numbers in the parentheses are numbers of rats
*Significantly different from control group at $P < 0.05$ **The significant difference could not be analyzed

(Henderson et al., 2000 and references therein), may be regarded as the potential chemopreventive agents. As already mentioned in the 'Introduction' that now there are many phase II enzyme inducers and those induce phase II enzymes as well as inhibit phase I enzymes that possess chemopreventive property (Kensler et al., 1987; Wattenberg and Bueding, 1986; Zhang et al., 1992; Zhang et al., 1994; Sparnins et al., 1988; DeLong et al., 1986; Fiala et al., 1977; Wattenberg, 1979; Hong et al., 1991; Wargovich, 1988; Wargovich et al., 1988; Tadi et al., 1991; Barcelo, 1996). We have previously demonstrated that neem flowers, when fed to the animals in the diet, increased GST activity in the liver of rats (Kusamran et al., 1998a) and mice (Chewasantikan, B., 2001, personal communication) and also increased QR activity in Hepa 1c1c7 mouse hepatoma cell line (Tepsuwan et al., 2001), but decreased the levels of cytochrome 1A1, 1A2 as well as the capacity to metabolically activate the mutagenicity of AFB_1 and B(a)P in rat liver (Kusamran et al., 1998a; Chewasantikan et al., 2001). Thus, it may be suggested that the mechanism by which neem flowers inhibit carcinogenesis in rats may be through the induction of phase II detoxification enzymes and/or the inhibition of the expression of phase I activating enzymes especially the cytochrome P450 isozymes specific for the activation of AFB_1 and polycyclic aromatic hydrocarbons.

In this study, the serum GGT level was high in control rats especially those with liver cancers. The GGT level in neem flowers fed group (with and without cancers), in which the tumors were much smaller, was significantly lower than that in the control group. Although, the statistical analysis between groups with cancers could not be analyzed due to the low number of rats in neem flowers fed group, the GGT level in neem flowers fed rat was much lower than that in control group. Thus, it seems that the level of GGT was well correlated with the size and number of large tumors in the livers, which agreed well with the previous finding (Rojanapo et al., 1993).

The chemopreventive effect as well as the biochemical activities of neem flowers may be attributable to one or more phytochemicals. At present, however, there is no report on the chemical constituents of Thai neem flowers. Indian neem flowers have been shown to contain some sterols and flavonoid glycosides such as myricetin-3-arabinoside, quercetin-3-galactoside and kaempferol-3-glucoside (Subramanian and Nair, 1972). These glycosides exhibit antioxidant activity, whereas the corresponding flavonoids possess many activities in chemopreventive assay, including inhibition of cytochrome P450-dependent activities such as B(a)P hydroxylase, ethoxyresorufin-*O*-deethylase and ethoxycoumarin-*O*-deethylase (Beecher, 1995; Moon et al., 1998; Siess et al., 1995). We are on the way to isolate and characterize the active constituents that posses strong QR inducing potency as well as inhibitory effects on some cytochrome P450 isozymes from neem flowers and our preliminary results demonstrated there were at least 2 compounds possessing strong QR inducing activity (manuscript in preparation).

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