ORIGINAL

Antimutagenicity and DT-diaphorase inducing activity of *Gynostemma pentaphyllum* Makino extract

Chollada Kulwat[1], Nirush Lertprasertsuke[2], Pranee Leechanachai[3], Prachya Kongtawelert[1], and Usanee Vinitketkumnuen[1]

[1]*Department of Biochemistry,* [2]*Department of Pathology, Faculty of Medicine, and* [3]*Faculty of Associated Medical Science, Chiang Mai University, Chiang Mai, Thailand*

Abstract : The hot water extract of the herbal tea, *Gynostemma pentaphyllum* Makino, was not found to be mutagenic in *Salmonella* mutation assay with or without metabolic activation. However, the extract had both DT-diaphorase inducing activity in the murine hepatoma (Hepa1c1c7) cell line and antimutagenic properties towards chemical-induced mutation in *Salmonella typhimurium* strains TA98 and TA100. Mutagenicity of aflatoxin B1 (AFB_1), 2-amino-6-methyldipyrido [1, 2-*a* : 3', 2', 3-*d*] imidazole (Glu-P-1), 2-aminodipyrido [1, 2-*a* : 3', 2', 3-*d*] imidazole (Glu-P-2), 2-amino-1, 4-dimethyl-5*H*-pyrido [4, 3-*b*] indole (Trp-P-1), 3-amino-1-methyl-5*H*-pyrido [4, 3-*b*] indole (Trp-P-2), 2-amino-3-methylimidazo [4, 5-*f*] quinoline (IQ) and Benzo [*a*] pyrene (B[*a*]P) was inhibited by the extract of *Gynostemma pentaphyl*lum Makino in a dose-dependent manner, but no effect was found on the mutagenic activity of 2-(2-Furyl)-3-(5-nitro-2-furyl) acrylamide (AF-2). However, the extract enhanced the mutagenicity induced by 2-aminoanthracene (2AA), and *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (MNNG). J. Med. Invest. 52 : 145-150, August, 2005

Keywords **:** *antimutagen, DT-diaphorase inducer,* **Gynostemma pentaphyllum**

INTRODUCTION

Herbal medicines are increasingly utilized to treat a wide variety of disease processes. *Gynostemma pentaphyllum* Makino, from the cucumber family, Cucurbitaceae, is a Chinese medicinal herb currently being promoted and sold as a herbal tea that is advantageous to one's health and beauty. It contains a large quantity of saponins known as gypenosides. They have a very powerful regulatory effect on a number of body systems such as blood pressure, reproductive function, digestive function, the immune response, mental function, physical performance and anticarcinogenicity in many kinds of cancer (1). Previous investigations have proved that *Gynostemma pentaphyllum* Makino has a positive anticarcinogenic function (2, 3). The use of antimutagen and anticarcinogen has been suggested as the most effective method of preventing human cancer. An increase of anticarcinogenic enzymes such as DT-diaphorase was reported to protect animals from toxic or carcinogenic substances (4). This led us to attempt a study on the antimutagenic and DT-diaphorase inducing activity of *Gynostemma pentaphyllum* Makino extract. The antimutagenic activity was determined by the modified Ames test, and the induction of DT-diaphorase activity was examined in murine hepatoma cell lines (Hepa1c1c7).

Received for publication February 23, 2005 ; accepted April 2, 2005.

Address correspondence and reprint requests to Usanee Vinitketkumnuen, Department of Biochemistry, Faculty of Medicine, Chiang Mai University, Chiang Mai 50200, Thailand and Fax : +66-53-217144.

MATERIALS AND METHODS

Gynostemma pentaphyllum Makino was obtained from a cultivated garden at Chom Thong district, Chiang Mai, Thailand. Glucose-6-phosphate, D-biotin, dimeth-

ylsulfoxide, Benzo [*a*] pyrene (B[*a*]P), *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (MNNG), and aflatoxin B1 (AFB_1) (A6636, Lot 38F4061), $NADP^+$, Flavin adenine dinucleotide, menadione, bovine serum albumin, Tris-base, glucose-6-phosphate dehydrogenase, and Tween-20 were obtained from the Sigma Chemical Company, St.Louis, USA. NADPH and NADH were obtained from the Oriental Yeast Company, Japan ; MTT from Fluka, Switzerland ; and 2-amino-1, 4-dimethyl-5*H*-pyrido [4, 3-*b*] indole (Trp-P-1), 3-amino-1-methyl-5*H*-pyrido [4, 3-*b*] indole (Trp-P-2), 2-amino-6-methyldipyrido [1, 2-*a* : 3', 2', 3-*d*] imidazole (Glu-P-1), 2-aminodipyrido [1, 2-*a* : 3', 2', 3-*d*] imidazole (Glu-P-2) and 2-amino-3-methylimidazo [4, 5-*f*] quinoline (IQ) from the Nard Institute, Osaka, Japan. Microtiter wells were acquired from Maxisorp, Nunc ; alpha-minimal essential medium and 0.25% trypsin-EDTA from GIBCO (Grand Island, NY) ; fetal calf serum from STARRATE PTY. LTD., Australia ; pennicillin G sodium and streptomycin sulfate from M & H Manufacturing CO., LTD., Thailand ; and Hepa 1c1c7 was purchased from ATCC, No.CRL-2026, USA.

Preparation of Gynostemma pentaphyllum *Makino extract*

The aerial part of *Gynostemma pentaphyllum* Makino was washed with tap water, dried in an oven with the air flow at 45°C overnight, and subsequently ground to a fine powder. The 250 mg of powder was extracted for 10 minutes in 200 ml of hot water. The extract was filtered through filter paper and the filtrates were evaporated to dryness by a vacuum rotatory evaporator at 60°C. The residue was dissolved in distilled water, and sterilized by Millipore filter membrane (0.45 μm) for use in antimutagenicity and DT-diaphorase induction assay.

Antimutagenicity assay

The tester strains, *Salmonella typhimurium* TA 98 and TA100, were kindly provided by Prof. Taijiro Matsushima, Director, Japan Bioassay Research Center, Kanagawa, Japan. The bacteria were kept and regularly checked for their genetic markers as previously described (5). The assay was performed essentially as a preincubation technique (6). 50 μl of known mutagens, 0-50 μl of plant extract, 500 μl of S9 mix (or 500 μl of 0.2 M phosphate buffer, pH 7.4) and 100μl of stationary phase bacteria ($2\text{-}3 \times 10^9$ cells/ml) were preincubated at 30°C for 30 min. The tubes were shaken at a moderate speed in a shaking water bath. After the preincubation period, 2 ml of molten top agar supplemented with L-histidine and D-biotin at 45°C was added to the mixture, gently mixed and poured onto a minimal glucose agar plate. The plates were inverted and incubated at 37°C for 48 h. His^+ revertant colonies were counted. The experiments were repeated, and 3 plates were used for each concentration of plant extract. Results were expressed as the mean of 6 plates per point minus the mean of spontaneous revertants.

Metabolic activation

The rat liver, S9 fraction, was used as the metabolic activation system in this assay. Phenobarbital and 5,6-benzoflavone were used to induce liver microsomal enzymes (7). S9 mix was freshly prepared each day and kept in ice. 1 ml of S9 mix contained 0.1 mmol of sodium phosphate buffer, pH 7.4, 8 μmol of $MgCl_2$-KCl, 5 μmol of Glucose-6-phosphate, 4 μmol of NADPH and NADH and 0.1 ml of S9 fraction.

Assay of DT-diaphorase inducer potency

The induction of DT-diaphorase was examined as previously described (8). Hepa1c1c7 murine hepatoma were grown in alpha minimal essential medium, minus nucleosides but supplemented with 10% FCS, 100 U/ml of penicillin G and 100 mg/ml of streptomycin at 37°C in a humidified incubator with an atmosphere of 5% CO_2. A total of 10,000 cells in 200 μl of medium were plated on each well of a 96 well microtiter plate. After the cells had grown in the incubator for 24 h, the medium was decanted and each well was replaced with 200 μl of fresh medium containing the extract dissolved in distilled water. The plates were subsequently incubated for an additional 24 h. DT-diaphorase activity (based on the formation of the blue-brown reduced tetrazolium dye) was measured with an optical microtiter plate scanner in cell lysates prepared in one plate. Duplicated plates were prepared and the cell density was determined in the second plate by staining with crystal violet . The control wells contained the media only. Specificity activity of DT-diaphorase could be calculated from the following equation :

$$\text{Specific activity} = \frac{\text{absorbance change of MTT/min} \times 3345\ \text{nmol/mg}}{\text{absorbance of crystal violet}}$$

Where 3345 nmol/mg is the ratio of the proportionality constant determined for crystal violet and the extinction coefficient of MTT (9).

DT-diaphorase activity assay

The media was decanted after the cells were exposed to the extracts for 24 h and lysed by incubation at 37°C for 10 min with 50 μl of a solution containing 0.8% digi-

tonin and 2mM EDTA pH 7.8 in each well. The plates were agitated on an orbital shaker for 10 min and then 200 μl of complete reaction mixture was added to each well (the following complete reaction was prepared for each assay : 7.5 ml of 0.5 M Tris-Cl, pH 7.4, 100 mg of bovine serum albumin, 1 ml of 0.5% Tween-20, 0.1 ml of 7.5 mM FAD, 1 ml of 150 mM glucose-6-phosphate, 90 μl of 50 mM NADP, 300 U of yeast glucose-6-phosphate dehydrogenase, 45 mg of MTT, and distilled water to a final volume of 150 ml ; and 1 μl T of 50 mM menadione dissolved in acetonitrile was added to the reaction mixture just before it was dispensed onto the microtiter plates). After 5 min, the reaction was stopped by the addition of 50 μl of a solution containing 0.3 mM of dicoumarol in 0.5% DMSO and 5 M potassium phosphate pH 7.4. The plates were scanned at 610 nm. The average absorbance value of the non-enzymatic blank wells containing only the reaction mixture and digitonin solution was substracted from all other absorbance values.

Crystal violet staining

After the cells were exposed to the extracts for 24 h, the media was decanted, and the plates were immersed in a vat of 0.2% crystal violet in 2% ethanol for 10 min and rinsed with tap water for about 2 min. After rinsing, 200 μl of 0.5% sodium dodecyl sulfate in 50% ethanol was added to each well. The plates were scanned at 610 nm after incubating at 37°C for 1 h.

RESULTS

1) Antimutagenicity of **Gynostemma pentaphyllum**

Investigation on the mutagenicity of the hot water extract of *Gynostemma pentaphyllum* Makino to the *Salmonella typhimurium* strain, TA 98 and TA 100, indicated that the extract had no mutagenic effects on both strains with and without metabolic activation (data not shown). Antimutagenic activity of *Gynostemma pentaphyllum* Makino extract against various known mutagens in *S. typhimurium* TA 98 was shown in Table 1. A decrease in mutant colonies of *S. typhimurium* strain TA98 induced by AFB_1, Glu-P-1, Glu-P-2, Trp-P-1, and Trp-P-2 in a dose-dependent manner by different amounts of the extract was demonstrated. The extract also decreased the mutagenicities of IQ- and B[a]P-induced mutant colonies of *S. typhimurium* strain TA100 with dose-dependency, but had no effect on the mutagenicity of AF-2 (Table 2). The extract enhanced the mutagenicity induced by 2-AA in *S. typhimurium* strain TA 98 in a dose-depentdent manner. The extract also enhanced MNNG-induced mutation in *S. typhimurium* strain TA100, but this enhancing was decreased as the concentration of the extract increase.

2) Anticarcinogenic enzyme inducer activity of **Gynostemma pentaphyllum**

The optimal time for measuring DT-diaphorase activity was 24 h after the cells were exposed to the extract. The induction of enzyme raised with the concentration of the extract, and 200 μg/well of extract induced more than a 2- fold increase of enzyme specific activity, when compared with the sample having no extract. At this level of extract no cytotoxicity was observed (Table 3). The extract at higher concentration (4000 μg/well) showed approximately 41 % cytotoxic effects on the cell line.

Table 1. Effect of *Gynostemma pentaphyllum* Makino extract on mutagenesis induced by various mutagens in *Salmonella typhimurium* strain TA98

Amount of Extract	Number of His^+ revertants induced by mutagens/ plate					
	AFB_1	2 AA	Glu-P-1	Glu-P-2	Trp-P-1	Trp-P-2
(μg)	(0.05 μg)	(0.5 μg)	(0.001 μg)	(0.5 μg)	(0.05 μg)	(0.001 μg)
0	856±55	785±18	418±17	291±12	508±24	708±23
340	724±59(-15)	1007±23(+28)	469±10(+12)	542±125(+86)	600±35(+18)	762±59(+8)
680	549±27(-36)	1449±40(+85)	426±10(+2)	204±83(-30)	511±13(+0.6)	742±69(+5)
1360	207±12(-76)	2115±42(+169)	288±29(-31)	190±76(-35)	410±21(-19)	566±65(-20)
3400	26±3(-97)	2760±87(+252)	69±6(-84)	25±7(-91)	222±45(-56)	192±30(-73)

Spontaneous revertants have been substracted already. The results are mean+SEM of 6 plates from 2 independent experiments. Zero concentration indicates mutagens alone without any extract. Number in parentheses is percentage of inhibition, -indicates % antimutagenicity, + indicates % enhancement.

Table 2. Effect of *Gynostemma pentaphyllum* Makino extract on mutagenesis induced by various mutagens in *Salmonella typhimurium* strain TA100

Amount of Extract (μg)	Number of His+ revertants induced by mutagens/plate			
	IQ (0.1 μg)	B(a)P (5.0 μg)	AF-2 (0.01 μg)	MNNG (0.4 μg)
0	1096±83	350±15	464±14	883±138
340	884±72(-19)	332±22(-5)	465±16(+0.2)	1518±189(+72)
680	472±18(-57)	289±10(-17)	524±13(+13)	1301±238(+47)
1360	214±16(-81)	311±19(-11)	523±33(+13)	1088±155(+23)
3400	31±6(-97)	189±11(-46)	476±25(+3)	1037±183(+17)

Spontaneous revertants have been substracted already. The results are mean + SEM of 6 plates from 2 independent experiments. Zero concentration indicates mutagens alone without any extract. Number in parentheses is percentage of inhibition, -indicates % antimutagenicity, + indicates % enhancement.

Table 3. Induction of DT-diaphorase specific activity in Hepa 1c1c7 by *Gynostemma pentaphyllum* Makino extract

Extract[2] (μg/well)	DT-diaphorase activity[1]	
	Specific (nmol/min/mg protein)	Relative (treated/control)
0	520±100	-
1	638±40	1.26±0.33
10	557±29	1.09±0.16
100	914±140	1.77±0.07
200	1230±23	2.42±0.51
400	1977±136	3.91±1.01
800	2632±35	5.16±0.93
1000	2924±867	5.57±0.59
2000	2513±361	4.86±0.24
4000	650±590	1.17±0.91

[1]Mean±SD from 2 replicates of 8 wells each/treatment. Approximately 41% of the cytotoxicity was observed at extract concentrations of ≥4,000 μg/well.
[2]The 0 concentration treatment consisted of H_2O and served as the control.

DISCUSSION

The hot water extract of *Gynostemma pentaphyllum* Makino may contain active compounds which exhibit antimutagenicity in *Salmonella typhimurium* strains TA98 and TA100, which are induced by various known mutagens such as AFB_1, Glu-P-1, Glu-P-2, Tryp-P-1, Tryp-P-2, IQ and B(a)P and exhibit DT-diaphorase inducer activity. The extract also contains the enhancing effect on 2-AA- and MNNG-induced mutagenesis.

Many evidences suggested that the induction of chemoprotective enzymes such as glutathione-S-transferase, UDP-glucuronyl transferase and DT-diaphorase by any compounds is the predominant mechanism by which these compounds are able to be used as chemopreventive agent (8-11). Much evidence has indicated that protection by these compounds depends on altering the metabolism of carcinogens. DT-diaphorase was selected as a marker enzyme for assessing the detoxificating activity because this enzyme protects against toxicities of quinones and their metabolic precursors. It is well established that quinones play an important part in many types of pathological conditions including cancer (12). The toxicity of quinones was produced by a number of mechanisms including redox cycling, induction of DNA strand breaks, generation of free radicals and interference with mitochondria respiration. Two electron reduction of quinones by DT-diaphorase was not mutagenic in *Salmonella* test and has generally been considered to be a detoxification pathway (13). The enzyme induced co-ordination with other chemoprotective enzymes in many animal tissues in response to the administration of various anticarcinogens. DT-diaphorase was induced by many chemically dissimilar substances that have protecting activity against carcinogens. It was induced coordinately with other phase II enzymes and its regulated by enhancer elements similar to those that control glutathione-S-transferase (8). DT-diaphorase can be induced in murine Hepa 1c1c7 hepatoma cells and 3T3 embryo fibroblasts by compound that are chemoprotector *in vivo* (14). Prochaska and his associates have reported that many vegetable extracts could induce DT-diaphorase, especially *Cruciferae* which is a potent inducer. Sulforaphane is an example of an inducer, which has been isolated from broccoli. Sulforaphane was discovered as it is a major and very potent phase II enzyme inducer in broccoli (*Brassica oleracea italica*), which

correlates to the epidemiology study that a high consumption of yellow and green vegetable could reduce the risk of developing cancer in various organs (8, 15). Therefore, the induction of detoxification enzyme is a major mechanism whereby a wide variety of chemical agents protect against neoplastic, mutagenic and other toxicities of carcinogens.

Many active substances in *Gynostemma pentaphyllum* Makino have been identified. They comprise many effective saponins named gypenosides, as well as trace minerals, amino acids, proteins, and vitamins. The extract has been detected by scientific study to have at least eighty-two saponins (16, 17). The direct effects of gypenosides on cancer cells from carcinomas of human liver, lungs, skin and uterus were studied and the results showed that these saponins inhibited the proliferation and growth of the cancer cells, indicating that *Gynostemma pentaphyllum* Makino might be used to treat cancer patients (18, 19). The results showed that the extract could induce DT-diaphorase specific activity in dose-dependent manner. A previous paper has suggested that the high activity of enzymes is an evolutionary adaptation in response to the exposure of the cell line to high levels of toxic, mutagenic, and carcinogenic substance (20). This might be concerned with the cytotoxicity of extract at higher dose.

In conclusion, *Gynostemma pentaphyllum* may contain active compounds, which exhibit antimutagenicity against either direct-acting mutagen or indirect-acting mutagen, and exhibit DT-diaphorase inducer activity. The extract also contains enhancing effect on MNNG- and 2-AA-induced mutagenesis and cytotoxic effect. The antimutagenic and anticarcinogenic enzyme inducer activities of the extract may suggest its chemoprotective activity.

ACKNOWLEDGEMENT

This work was supported by the Faculty of Medicine Endowment Fund for Medical Research, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand, 50200.

REFERENCES

1. Hou J, Liu S, Ma Z, Lang X, Wang J, Wang J, Liang Z : Effects of *Gynostemma pentaphyllum* Makino on the immunological functions of cancer patients. J Tradi Chin Med 11(1) : 47-52, 1991
2. Zhou Z, Wang Y, Zhou Y, Zhong S : Effect of *Gynostemma pentaphyllum* Makino on carcinomatous conversions of golden hamster cheek pouches induced by dimethylbenzanthracene : a histological study. Chin Med J (Engl) 111(9) : 847-50, 1998
3. Wang C, Wang X, Li Y, Deng S, Jiang Y, Yue L : A preliminary observation of preventive and blocking effect of *Gynostemma pentaphyllum* (Thunb) Makino on esophageal cancer in rats. *Hau Xi Yi Ke Da Xue Xue Bao*. Chinese 26(4) : 430-2, 1995
4. Talalay P:Mechanisms of induction of enzymes that protect against chemical carcinogenesis. Adv. Enzyme Regu 28 : 149-159, 1989
5. Maron DM, Ames, BN : Revised methods for the *Salmonella* mutagenicity test. Mutation Res 113 : 173-215, 1983
6. Matsushima T, Sugimura T, Nagao T, Shirai A, Sawamura M: Factors modulating mutagenicity in microbial test, In : Norpoth K.H, and Garner R.C, eds. Short-term Test System for Detecting Mutagens and Carcinogens. Springer, Berlin, 1980, pp.273-285
7. Matsushima T, Sawamura M, Hara K, Sugimura T : A safe substitute for polychlorinated biphenyl as an inducer of metabolic activation system, In : F. de Serres *et al*., eds. *In Vitro* Metabolic Activation in Mutagenesis Testing. Elsevier/North-Holland, Amsterdam, 1976 pp.85-88
8. Prochaska H J, Santamaria A B, Talalay P : Rapid detection of inducers of enzymes that protect against carcinogens. Proc Natl Acad Sci USA 89 : 2394-2398, 1992
9. Prochaska H T, Santamaria A B : Direct measurement of NAD(P)H : quinone reductase from cells cultured in microtiter wells : a screening assay for anticarcinogenic enzyme inducers. Anal Biochem 169 : 328-336, 1988
10. Long MJD, Prochaska HJ, and Talalay P : Induction of NAD(P)H : quinone reductase in murine hepatoma cells by phenolic antioxidants, azo-dyes, and other chemoprotectors : a model system for the study of anticarcinogens. Proc Natl Acad Sci USA 83 : 787-791, 1986
11. Riley R J, Workman P : DT-diaphorase and cancer chemotherapy. Biochem Pharmacol 43 : 1657-1669, 1992
12. Ernster L : DT-diaphorase: a historical review. Chemica Scripta 27A : 1-13, 1987
13. Chesis PL, Levin DE, Smith MT, Eruster L, Ames B N : Mutagenicity of quinones : pathways of metabolic activation and detoxification. Proc Natl Acad Sci USA 81 : 1696-1700, 1984

14. **Prochska H J, Long M J D, and Talalay P: On the mechanisms of induction of cancerprotective enzymes : a unifying proposal. Proc Natl Acad Sci USA 82 : 8232-8236, 1985**
15. **Zhang M, Talalay P, Cho C, Posner G H : A major inducer of anticarcinogenic protective enzymes from broccoli : isolation and elucidation of structure. Proc Natl Acad Sci USA 89 : 2399-2403, 1992**
16. **Li H : Inhibitory effect of gypenosides on lung cancer cells *in vitro*. Xi An Yi Ke Da Xue Xue Bao Chinese 15(4) : 346, 1994**
17. **Li GY : Effect of gypenosides on synthesis of DNA and protein of liver carcinoma cells *in vitro*. Xian Yi Ke Da Xue Bao Chinese 14(2) : 14, 1993**
18. **Yoshikawa K, Arimitsu M, Kishi K, Takemoto T, Arihira S : Studies on the constituents of Cucurbitaceae plants. XVIII. On the saponin constituents of *Gynostemma pentaphyllum* Makino. Yakugaku Zasshi 107(5) (in Japanese) : 361-366, 1987**
19. **Liao D F, Lu N, Lei L S, Yu L, Chen J X : Effects of gypenosides on mouse splenic lymphocyte transformation and DNA polymerase II activity *in vitro*. Acta Pharmacologica Sinica 16(4) : 322-324, 1995**
20. **Benson A M, Hunkeler M J, Talalay P : Increase of NAD(P)H : quinone reductase by dietary antioxidants : possible role in protection against carcinogenesis and toxicity. Proc Natl Acad Sci USA 77 : 5216-5220, 1980**