Kasetsart J. (Nat. Sci.) 44 : 816 - 823 (2010)

Antimutagenicity and Anti-HSV-2 Activity of Mulberry Tea (*Morus rotunbiloba* Koidz)

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ABSTRACT

Hot water extract from mulberry leaves, *Morus rotunbiloba* Koidz was extracted with diethyl ether, and its components were analyzed using high-pressure liquid chromatography (HPLC). Polyphenolic compounds constituted the major component (79.8%), consisting of mainly tannic acid (37.9%), epigallocatechin-3-gallate (21.1%) and caffeic acid (11.2%). The genotoxicity of the extract was evaluated by the Ames mutagenicity test, using *Salmonella typhimurium* strain TA 98 induced by a mutagen Trp-P-1. It was found that the number of revertant colonies was significantly decreased with an IC_{50} value of 4.5 mg/mL. The extract of *Morus rotunbiloba* Koidz also exhibited marked antiviral activity against herpes simplex virus type 2 (HSV-2) with an IC_{50} of 0.52 μg/mL. The results suggested the benefit of consumption of mulberry tea for prevention of cancer and HSV-2 infection.

Keywords: mulberry tea, *Morus rotunbiloba*, antimutagenicity, anti-HSV-2

INTRODUCTION

M. rotunbiloba and the other *Morus* species are widely cultivated in many Asian countries. In Thailand, besides being used mainly for feeding silkworms (*Bombyx mori* L.), the dried leaves of *M. rotunbiloba* have been consumed as a mulberry tea beverage and in food supplements. Among all herbal health teas consumed in the world, tea derived from the dried leaves of *Camellia sinensis* is the best for chemoprevention of degenerative diseases, such as cardiovascular diseases, arthritis, and diabetes (Sharangi, 2009). It has been suggested that the polyphenolic content, found in high levels, is an active ingredient providing clinical merit (Khan and Mukhtar, 2007).

Leaves of the mulberry have been reported to be a rich source of flavonoids and other polyphenolic compounds (Doi *et al*., 2001). Nine flavonoids isolated from *Morus alba* L. leaves were identified and some contained free radical scavenging properties (Asano *et al*., 2001). The antioxidant and antihyperglycemic roles of mulberry leaves have been reported in black mulberry, *Morus indica* L. (Andallu and Varadacharyulu, 2003). Ingestion of mulberry leaves was reported to reduce blood glucose concentration and total serum lipids in patients with type 2 diabetes (Andallu *et al*., 2001).

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Received date : 20/01/10 Accepted date : 26/05/10

Recently, hot-water extract from mulberry (*M. rotunbiloba* Koidz) was shown to exhibit potent scavenging effects on the $ABTS^+$ (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) free radical, as well as having antibacterial activities against some human-pathogenic bacteria (Patharakorn *et al*., 2006). Prenylated flavonoids obtained from the root bark of *Morus mongolica* and *M. alba* L. were found to exhibit strong antibacterial and antifungal activities (Shon *et al*., 2004). Two prenylated flavonoids (leachinone G and mulberroside C), from the root bark of *M. alba* L., were shown to inhibit the activities of the herpes simplex virus type 1 (Du *et al*., 2003). Recently, tea polyphenols, particularly catechin derivatives, have been shown to have antiherpetic activity (Savi *et al*., 2006).

Herpes simplex virus type 2 (HSV-2) causes neonatal infections and fatal infections in humans, with symptoms that are similar to those of meningitis and cervical cancer types (Corey and Spear, 1986). Nowadays, acyclovir and other antiviral drugs are used for the treatment of genital herpes infection. However, HSV-2 has been reported to acquire resistance to these drugs (Wagstaff *et al*., 1994). Therefore, research on seeking new anti-HSV-2 drugs from medicinal plants that provide effective treatment with the lowest cytotoxicity and low cost is of high interest.

The objectives of this study were to identify the chemical composition of the diethyl ether extract of tea derived from leaves of a mulberry, *M. rotunbiloba* Koidz (Mon-Noi) and to investigate its antimutagenicity and antiviral activity against HSV-2.

MATERIALS AND METHODS

Plant materials

Tea leaves of the mulberry, *M. rotunbiloba* Koidz (Mon-Noi) were provided by the Udon Thani Sericultural Research Center, Thailand.

Preparation of mulberry tea-leaf extract

Tea leaves of the mulberry were ground, then 150g were brewed with hot water (1.5 L) for 20 min and the resulting tea extract was decanted. After the mixture was centrifuged at 8,000 × g for 15 min, pool supernatant was recovered, freeze-dried to reduce the volume to 120 mL and stored in airtight containers at 4°C until use. Diethyl ether (3 mL) was added into the tea-leaf extract. After mixing and standing for 2 h, the upper diethyl ether soluble fraction was separated, concentrated to dryness *in vacuo*, redissolved in absolute methanol, and filtered, prior to furnishing a stock solution for HPLC analysis.

Chemical analysis by high-pressure liquid chromatography

Stock solution (2 μL) was analyzed using an analytical HPLC unit (Perkin Elmer system, comprising auto-sampler and quaternary pump coupled to a diode array) and a reverse phase Micro-Bonapak™ C_{18} silica column (300 × 3.9 mm) from Waters, which was pre-equilibrated with acetonitrile. The solvent system used a gradient of acetonitrile and 0.1% trifluoroacetic acid, increasing from 0-100% acetonitrile over 30 min at 35°C, with a solvent flow rate of 1 mL/min. The chromatogram was recorded at 255 nm. Quantification was achieved by the absorbance recorded in chromatograms relative to the reference compounds, epigallocatechin-3-gallate (EGCG), tannic acid, caffeic acid, chlorogenic acid, quercetin, quercitrin and rutin.

Antimutagenicity assay

Antimutagenicity of the mulberry tea extract against a mutagen, Trp-P-1, was assessed using the standard plate incorporation assay, as described by Maron and Ames (1983). *Salmonella typhimurium* tester strain (TA 98) and a quantity of S9 mixture were obtained from the Institute of Food Research and Product Development (IFRD), Kasetsart University. For the plate incorporation

inhibition assay, 0.05 mL of Trp-P-1 (1μg/mL in DMSO), 0.05 mL of the mulberry tea extract of various concentrations, 0.1 mL of the S9 mixture and 0.1 mL of a bacterial culture (grown overnight in Oxoid nutrient broth No. 2 at 37°C, with shaking) were mixed carefully with 2 mL molten top agar, containing 0.05 mM biotin-histidine and dispersed onto minimal glucose agar plates. The mutagenicity of Trp-P-1 was monitored against TA 98 in the presence of the S9 mixture. A series of control plates containing only the tea extracts and the bacteria in the absence and presence of the S9 mixture were also included to screen the different tea preparations for mutagenic effect. Control plates containing only DMSO, which was used as the solvent vehicle, were also included to obtain the background or spontaneous revertant count. All plates were incubated at 37°C for 48 h. Thereafter, the histidine revertants were counted. There were three replicates of each treatment. The percentage of mutagenicity was calculated according to the formula in Equation 1:

$$\text{percentage of antimutagenicity} = [1- (T / M)] \times 100 \quad (1)$$

where: T = the number of revertants per plate in the presence of the mutagen and test sample and

M = the number of revertants per plate in positive controls.

The number of spontaneous revertants was subtracted from the numerator and denominator.

Anti-HSV-2 assays

Herpes simplex virus type 2 (HSV-2) (LB-strain) was propagated in BHK-21 (baby hamster kidney) cells. The cells were cultured in Eagle's minimal essential medium (MEM) (Nissui Pharmaceutical Co., Tokyo) containing 1.2 g/L of $NaHCO_3$ and supplemented with 100 U/mL of penicillin, 50 μg/mL of streptomycin, and 10% of fetal bovine serum (FBS) (Biowhittaker, Walkersville, Maryland, USA), and incubated in a humidified 5% carbon dioxide atmosphere at 37°C. Virus stock was obtained by freezing and thawing at -70°C and 37°C, respectively, in order to break the infected cells, followed by centrifugation at 1,200 × g for 10 min. The supernatant was collected as stock seed and kept at -70°C until use.

For the determination of virus titers, the BHK-21 cells in 6-well tissue culture dishes were incubated with serially diluted infected cell culture supernatants for 1 h at 37°C and, after removal of the inoculum, the dishes were overlaid with culture medium containing 0.5% agar for visualization of induced plaque formation. Plaques were counted by neutral red assay and titers were calculated as plaque forming unit (PFU) mL^{-1}.

The antiviral activity of the mulberry tea extracts was studied using a plaque reduction assay (Wetprasit *et al*., 2000), under two conditions.

(1) Inactivation assay: Mulberry leaf hot-water extracts and the diethyl ether soluble-fractions were diluted to 1:300 in MEM medium and passed through a 0.25-μm pore diameter filter. Each extract was twofold serially diluted in MEM and then each was mixed with 100 PFU of virus. After incubation at 37°C for 30 min, 0.2 mL was inoculated into BHK-21 monolayer cells. After 1 h adsorption, the unadsorbed virus was discarded, and the cells were washed with the MEM medium. The cells were further cultured in MEM medium and inspected periodically until 80% virus-induced cytopathic effects (CPE) were observed in the control cell cultures containing no mulberry extracts. Absence of CPE indicated complete inactivation of the virus. The infected cells were stained and the number of plaques was counted. The 50% inhibition concentration (IC_{50}) was determined from the curve constructed using different sets of values of plaque number and the concentration of the extract.

(2) Post-treatment assay: BHK-21 monolayer cells were added to 0.2 mL virus corresponding to 100 PFU. After 1 h of adsorption, the unadsorbed virus was discarded and the cells were washed with MEM medium. The monolayer

cells were replaced by the medium containing mulberry extracts using a concentration range of 0.07-33.3 μg/mL. The cell cultures were inspected periodically until 80% CPE was observed in the control without any treatment. The infected cells were stained and the number of plaques was counted.

RESULTS AND DISCUSSION

Six phenolic compounds were identified by HPLC analysis, representing 79.8% of the total constituents in the diethyl ether extract of tea prepared from *M. rotunbiloba* Koidz. Table 1 shows the phenolic compounds in the tea extract, with the major ones being tannic acid (37.9%), epigallocatechin-3-gallate (21.1%) and caffeic acid (11.2%). Other authors have already identified various phenolic compounds from the mulberry leaves of *M. alba* L., including astragalin, quercetin, isoquercitrin, scopolin, skimmin, roseoside II, kaempferol-3-*O*-(6-*O*-acetyl)-β-D-glucopyranoside, quercetin-3-*O*-(6-*O*-acetyl)-β-D-glucopyranoside, rutin, kaempferol-3-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside, quercetin-3-*O*-β-D-glucopyranosyl-(1→6)-β-D-glucopyranoside, quercetin-3,7-di-*O*-β-D-glucopyranoside and benzyl D-glucopyranoside (Kim *et al*., 1999; Doi *et al*., 2001). Moreover, the flavonoid content in the leaves of some *Morus* species has been shown to be different among plant varieties and to vary by season (Zhishen *et al*., 1999).

The antimutagenicity of the diethyl ether extract from mulberry leaves of *M. rotunbiloba* Koidz was determined by measuring the extent of decrease in the frame-shift mutation of *S. typhimurium* TA 98 induced by Trp-P-1. The tea extract was found to exert a potent protective effect against the mutation, leading to a reduction in the number of revertants (Table 2). The antimutagenicity increased as the concentration of the tea extract was increased (Figure 1). Surprisingly, at the maximum concentration of the assay, the tea extract did not show any evidence of cytotoxicity compared with normal growth of the control. The

Table 1 Phenolic compounds in the diethyl ether extract of mulberry tea evaluated by HPLC.

Phenolic compounds and polyphenols	Content[a] (μg/100 μg extract)
Tannic acid	37.90
Epigallocatechin-3-gallate	21.10
Caffeic acid	11.20
Chlorogenic acid	3.85
Quercetin	3.20
Rutin	2.55
Others (unidentified)	20.20

[a] = Values are presented as the mean of three independent experiments.

Table 2 Antimutagenicity activity of the diethyl ether extract of mulberry tea against Trp-P-1 (50 ng per plate) on *Salmonella typhimurium* TA98.

Plate	Content of extract (mg per plate)	Revertants per plate [a]
Control	0	959.5 ± 47.5
Background	0	36 ± 5
Sample background	15	32 ± 0
Sample inhibition	15	52 ± 17

[a] = Each value represents mean ± standard deviation, n = 5.

lack of cytoxicity was found in both the presence and absence of the mutagen, Trp-P-1. Moreover, the tea extract showed a potent antimutagenicity, inhibiting the mutagenicity of Trp-P-1 for 95% at a dose of 20 mg/mL (0.1 mg/plate) with the 50%-inhibition concentration (IC_{50}) being 4.5 mg/mL (22.5 ng/plate).

To evaluate the inhibitory effect of the mulberry tea extract on HSV-2, the plaque formation of the virus was determined under two experimental conditions (inactivation and post-treatment). The tea extract was capable of reducing the plaque formation by HSV-2 with an IC_{50} value of 0.52 μg/mL (Table 2 and Figure 2). At a

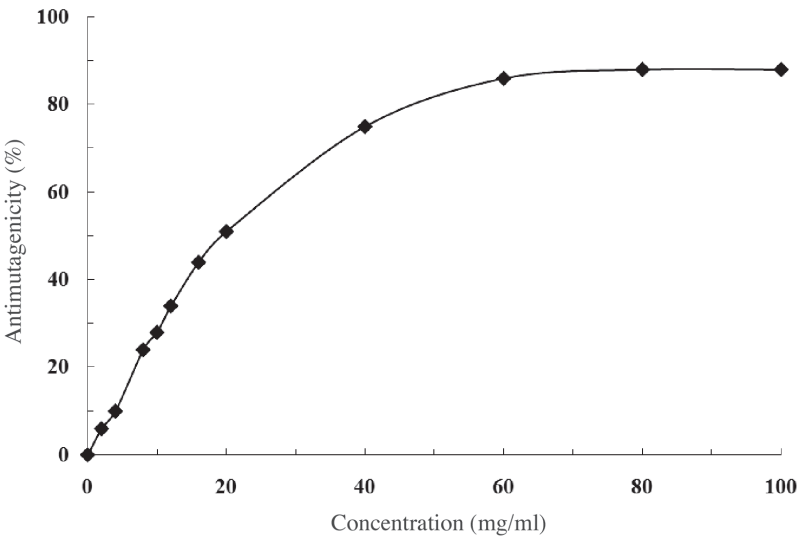

Figure 1 Antimuagenicity of the diethyl ether extract of mulberry tea on *Samonella typhimurium* TA 98 (His^-) in the presence of a mutagen, Trp-P-1 (50 ng).

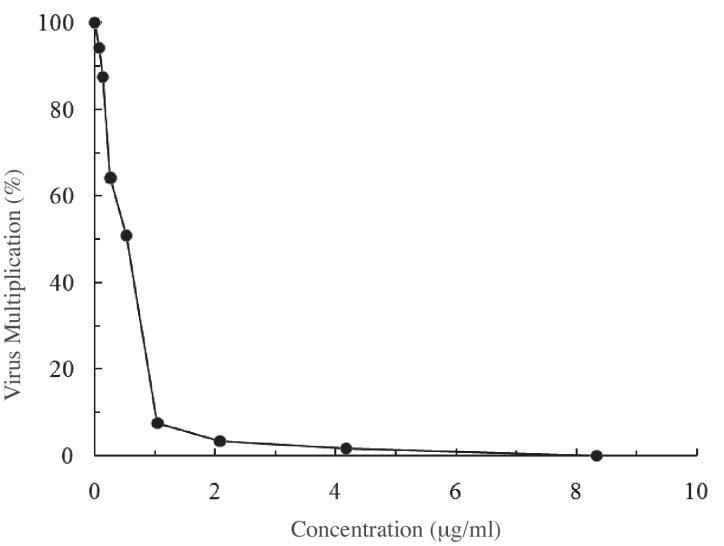

Figure 2 Dose response curve of HSV-2 multiplications of the diethyl ether extract of mulberry tea at various concentrations in the inactivation of plaque reduction assay.

concentration of 8.33 μg/mL, the tea extract exhibited complete inactivation of virus infectivity, resulting in 100% plaque reduction (Table 2). The results suggested that some components in the tea extract might bind to the virus and/or alter the viral envelope structure, thereby inhibiting the binding of the virus to the host cells at the sites for viral infection or by penetration into the cells. The effect of the mulberry tea extract on HSV-2 replication was tested by post-treatment of BHK-21 cells with the virus, followed by incubation with the tea extract. It was found that the tea extract did not affect the production of infectious virus agents (data not shown). This suggested that the tea extract did not impair productive replication of HSV-2. This could have been due to the binding of a phytochemical phenolic compound with the protein coat of the virus and arrestment absorption of the extracts into the BHK-21 cells. A number of medicinal plants had been reported to contain compounds possessing strong antiviral activity (Jassim and Naji, 2003). Methanolic extracts of some Thai medicinal plants have been reported to exhibit anti-HSV type 1 (Lipipun *et al*., 2003) and anti-HSV type 2 (Wetprasit, 2002) activities by inactivating the viral infections on the plaque reduction assay. The inhibitory activity to herpes virus of hot water and water extracts has been reported and a significant reduction in the viral infection was shown (Garcia *et al*., 1999). It had been demonstrated that two prenylated flavonoids, namely leachinone G (IC_{50} = 1.6 μg/mL) and mulberroside C (IC_{50} = 75.4 μg/mL), from seven known compounds isolated from the rootbark of *M. alba* L., were active principles for anti-HSV-1 activity (Du *et al*., 2003). It is believed that there are no reports of the antiviral activity to HSV-2 by the tea extract of the mulberry, *M. rotunbiloba* Koidz and of the other *Morus* species. Thus, the current study is the first to report that mulberry tea is a potentially effective antiviral candidate for the treatment of HSV-2. Further studies to identify active viral inhibitory molecules and their mode of actions to HSV-2 are clearly necessary for therapeutic application.

Various kinds of herbal tea have been reported to contain antimutagens and their inhibitory potency might be related to the relative levels of their constituents (Bunkova *et al*., 2005). Antimutagenic and anticarcinogenic activities of Japanese and Chinese tea-leaves were found to be associated with the antioxidant activity of their

Table 3 Antiviral activity of diethyl ether extract of mulberry tea on plaque formation in the inactivation assay.

Extract concentration (μg/mL) [b]	Percent virus multiplication [a]
0.07	94.17
0.13	87.50
0.26	64.17
0.52	50.80
1.04	7.50
2.08	3.33
4.17	1.67
8.33	0
16.67	0
33.33	0

[a] = Percent virus multiplication is percent plaque formation compared with the control in the absence of extract. Values are means of duplicate determination.

[b] = Minimal non-cytotoxic concentration of the extract = 125 μg/mL

polyphenol components (Yang *et al*., 2006). Patharakorn *et al*. (2006) reported the antioxidant and antibacterial activities of tea-leaves and root extracts of mulberry. Chemical constituents found in the mulberry tea extract included tannic acid, epigallocatechin-3-gallate, caffeic acid, chlorogenic acid, quercetin and rutin, which are probably associated with its antimutagenicity and anti-HSV-2 activity. Tannin and epigallocatechin-3-gallate have been shown to exhibit remarkable biological and pharmacological activities of various potencies, including radical scavenging activity, antimutagenic activity, antiviral activity against HSV and human immunodeficiency (Okuda, 2005), anti-inflammatory activity and anticancer activity (De Mejia *et al*., 2009). Large amounts of tannin and epigallocatechin-3-gallate accumulate in the leaves of *M. rotunbiloba* Koidz. Therefore, additional studies on their health effects are required to evaluate fully the potential of this medicinal plant in therapeutical applications.

CONCLUSION

The present study provides the first evidence showing that the diethyl ether extract of tea obtained from the leaves of *M. rotunbiloba* Koidz (Mon-Noi) has promising antimutagenic and anti-HSV-2 activities. The removal of polar substances in the mulberry tea, by hot-water extraction with diethyl ether, produced an active fraction with potent antimutagenicity and anti-HSV-2 activity. Its bioactivities may be due to the major phenolic components. Such activities of the mulberry tea extract may open a new way for chemoprevention and therapy of HSV-2 infection. This depends on further demonstration of its efficacy and safety.

ACKNOWLEDGEMENTS

This work was supported by the Kasetsart University Research and Development Institute (KURDI). The authors are grateful to Mr Viroj Kaewruang, the Queen Sirikit Institute of Sericulture, for providing the mulberry tea and to Dr Gassinee Trakoontivakorn, the Institute of Food Research and Product Development (IFRD), for her advice and the provision of bacterial cells for the antimutagenic test. Special thanks go to Dr Aree Thattiyaphong, for her comments in the preparation of the manuscript.

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