REPORT

Cytoprotective activity of mulberry leaf extract against oxidative stress-induced cellular injury in rats

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Abstract: *Morus alba* Linn. (MA), mulberry leaves have been used as a beverage for prevention of various diseases including hyperlipidemia and hyperglycemia. Recently, the antioxidant activities of the MA leaf extract have been reported. The objective of this study was to investigate the effect of the MA leaf extract on free radical-induced cellular injury. In the *in vitro* models, the extract scavenged stable free radical (1, 1-diphenyl-2-picrylhydrazyl; DPPH) in a concentration-dependent manner with an IC_{50} of 20.10±0.78 μg/ml. The extract protected the erythrocytes from free radical (2, 2'-azobis (2-amidinopropane) dihydrochloride; AAPH)-induced hemolysis with an IC_{50} of 74.22±9.87 μg/ml. Additionally, the extract significantly prevented the gastric mucosal injury induced by ischemia-reperfusion (I/R) in rats when given orally at doses of 0.25 and 0.50 g/kg/day for 3 consecutive days ($p<0.05$; n=7). However, this effect was not found when the higher doses (1 and 2 g/kg/day) of the extract were tested. In conclusion, these results indicate that the MA leaf extract possesses the cytoprotective activity against free radical-induced cell injury. Therefore, when given at the appropriate dose range, the mulberry leaf may potentially be used as a food supplement in patients with certain diseases in which the oxidative stress-induced cellular injury is pathologically involved.

Keywords: *Morus alba* Linn., mulberry, cytoprotective activity, anti-oxidant activity, ischemia-reperfusion.

INTRODUCTION

Oxidative damage caused by reactive oxygen species (ROS) is harmful to the cell. Injury of cellular organelles, cellular dysfunctions and cell death are all the consequences of the oxidative damage. The condition inwhich ROS are excessively produced, known as an oxidative stress, is implicated in many diseases including gastrointestinal disorder, diabetes, cancer and cardiovascular diseases (Zimmerman and Granger, 1994; Halliwell and Gutteridge, 1999). Anti-oxidants can prevent, stabilize and terminate the reactions of ROS by defending oxidative induced cellular damage. Therefore, external supplementation of anti-oxidants is widely recommended to protect cells from the oxidative stress (Cuzzocrea *et al.*, 2001; Kwiecień *et al.*, 2003; Ha *et al.*, 2010).

Morus alba Linn. (MA), also known as white mulberry, is the important economic plant in Thailand for silk worm feeding in a silk production. Mulberry leaves possess high values in both nutrition and medicine. It has been widely promoted in Thailand as a health beverage. All parts of mulberry tree are generally used in folk medicine for the treatment of various diseases in Asia. For example, its ripe fruit is used to treat premature grey hair, constipation, diabetes, and to tonify the blood. Its bark is used to treat wheezing, edema, and to promote urination. It is also used to relieve fever, headache, red dry and sore eyes, as well as cough. Furthermore, the anti-hypertensive, anti-oxidant and neuroprotective activities of the ethanolic extract of mulberry leaves have been reported (Doi *et al.*, 2000; Kang *et al.*, 2006). It is also found that long-term administration of mulberry leaf extract at doses of 0.5-1 g/kg has anti-hyperglycemic, anti-oxidant and anti-glycation effects in chronic diabetic rats (Naowaboot *et al.*, 2009a). It is also demonstrated that MA at 0.5-1 g/kg significantly restored the vascular reactivities of chronic diabetic rats. Moreover, MA treatment significantly lessened the elevation of MDA content in tissues (liver, kidney, heart and aorta) of diabetic rats (Naowaboot *et al.*, 2009b). Mulberry leaves contain protein, carbohydrate, calcium, iron, ascorbic acid, carotene, vitamin B_1, folic acid and vitamin D (Bose, 1989; Srivastava *et al.*, 2006). Among the various nutritional components, flavonoids, morins, and moracins are known to have anti-oxidant effect or free radical scavenging activity (Kim *et al.*, 1999; Sharma *et al.*, 2001; Chung *et al.*, 2003; Yu *et al.*, 2006).

In relation to the significant role of free radical in the pathology of ischemic/reperfusion-induced tissue injury, it is thus hypothesized that the mulberry leaf extract has a cytoprotective activity against cellular injury induced by oxidative stress. Therefore, this study examined the anti-oxidant effects of MA on free radical (2, 2'-azobis (2-amidinopropane) dihydrochloride; AAPH)-induced

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hemolysis and on ischemic/reperfusion-induced gastric mucosal injury in rats. These were aimed to provide scientific evidence supporting the use of mulberry leaf as food supplement for patients with diseases related to the oxidative damage.

MATERIALS AND METHODS

Chemicals

Vitamin C (L-ascorbic acid), 2, 2'-azobis (2-amidinopropane) dihydrochloride (AAPH) and allopurinol (xanthine oxidase inhibitor) were obtained from Sigma Chemical Co., Ltd. (St. Louis, MO). 1,1-diphenyl-2-picrylhydrazyl (DPPH) were obtained from Fluka Chemika (AG, Switzerland). All other laboratory chemicals were of the analytical grade.

Preparation of mulberry leaves extract

Leaves of MA were provided by Mr. Wiroje Kaewruang, Sericultural Research Institute, Department of Agriculture, Ministry of Agriculture and Cooperation, Thailand. A voucher specimen (KKU 2513) was deposited at the Pharmaceutical Science Herbarium, Khon Kaen University, Thailand. Briefly, the MA leaves were cleaned, shade-dried, finely powdered and macerated with 50% ethanol for 3 days. The liquid extract was filtered and alcohol was removed from the filtrate using the rotary vacuum evaporator at 60°C. The concentrated filtrate was freeze-dried. By this method, the yield (calculated on the dried powder extract) was 18.70% of the dried leaves weight. The MA leaf extract was kept in air-tight containers at −20°C until used.

Determination of scavenging effect of MA extract on stable free radical (DPPH)

This *in vitro* method was modified from that described earlier by Doi *et al.* (2000). Briefly, equal volume of DPPH in ethanol and the extract of MA were mixed at final concentrations of 0.05 mM and 1-300 μg/ml, respectively. Vitamin C was used as a positive control. The mixture was left for 20 minutes at room temperature. The disappearance of DPPH radical was monitored through the absorbance (OD) at 515 nm after mixing. In the presence of radical scavenger, the OD at 515 nm will be decreased. The % scavenging activity was calculated as followed; % scavenging = $\{(OD_{\text{without test substance}} - OD_{\text{with test substance}}) / OD_{\text{without test substance}}\}*100$. IC_{50} was computed with non-linear regression method from concentration-response curve.

Preparation of rat red blood cells

The preparation of red blood cells was performed as described by He *et al* (2000). Briefly, 250-300 g male Spraque-Dawley rats were obtained from the Experimental Animal Unit, Faculty of Medicine, Khon Kaen University, Thailand. The rats were anesthetized with pentobarbital sodium (60 mg/kg). Blood was collected from the abdominal artery, and then red blood cells were isolated from plasma by centrifugation at 2500 rpm for 10 minutes at 10°C. The cells were washed three times with phosphate buffer solution (PBS; pH 7.4), and then packed red cells were suspended with four volumes of PBS.

Determination of MA effect against ROS-induced membrane damage

AAPH is an azo compound, which spontaneously gives radicals to react with oxygen and produces ROS. ROS attack membrane lipids and proteins of red blood cells resulting in cell lysis. This *in vitro* method was used as model to determine the effect of MA extract on ROS-induced membrane damage.

The effect of MA extract against ROS-induced membrane damage was determined by the method described by He *et al.* (2000). Briefly, the red blood cell suspension was treated with equal volume of various concentrations of the MA extract (as final concentrations of 25-200 μg/ml) for the treatment groups, PBS for a control group, or vitamin C for a positive control. These concentrations of added extracts were proved to be non-cytotoxic. Then, 0.25 ml of 400 mM AAPH was added to 1 ml of treated cell solution. The mixture was incubated in water bath at 37°C and gently mixed every 30 minutes for 3 hours. After that, 2 ml of PBS was added into reaction mixture, followed by centrifugation at 3000 rpm for 10 minutes at 10°C. The absorbance of the supernatant was measured at 540 nm by using spectrophotometer. The % inhibition of hemolysis was calculated as followed; % inhibition of hemolysis = $\{(OD_{\text{without test substance}} - OD_{\text{with test substance}}) / OD_{\text{without test substance}}\}*100$. IC_{50} was computed with non-linear regression method from concentration-response curve.

Determination of protective effect of MA extract on acute gastric mucosal injury induced by ischemic-reperfusion in rats

The *in vivo* procedure was performed as described by Wada *et al.* (1995) with some modification. Briefly, male Spraque-Dawley rats weighing 200-250 g were divided into 3 groups; a control group treated orally with distilled water; the treatment groups treated orally with the MA extract at the doses of 0.25, 0.50, 1 or 2 g/kg/day; a positive control group treated orally with allopurinol at the dose of 100 mg/kg/day. All the treatments were given for 3 consecutive days. Six rats were used per treatment. The given doses of the extracts were already proved to be non-acute toxic in rats.

After three days of treatments, the animals were fasted for 16-18 hours, but with freely access to water. The rats were anesthetized with pentobarbital sodium (60 mg/kg). The abdomen was opened and the ischemia of stomach was performed by clamping the celiac artery for 35 minutes, and was then allowed to reflow by releasing the

clamp for 45 minutes. The rat was euthanized by cervical dislocation and the whole stomach was removed and opened for taking the photograph of the injured area. The area of mucosal injury was computed by image analyzer (Image Pro Plus version 1.3) and was expressed as percentage of total glandular area of stomach. This study adhered to the Guide for the Care and Use of Laboratory Animals developed by the National Research Council of Thailand, and approved by the Institutional Animal Care and Use Committee of Khon Kaen University, Thailand.

STATISTICAL ANALYSIS

The data values are presented as mean±S.D. (n=number of replicate experiment). The effect of MA extract on I/R-induced gastric mucosal injury was analyzed using analysis of variance (ANOVA) followed by Student-Newmann-Keuls post hoc testing to test the difference between groups. A $P<0.05$ is considered as statistical significance.

RESULTS

Scavenging effect of MA extract on stable free radical (DPPH)

The MA extract showed the scavenging activity on DPPH in a concentration-dependent manner with an IC_{50} of 20.10±0.78 µg/ml and a maximum response of 78.41±0.31% (fig. 1). Vitamin C (a positive control) showed a prominent scavenging activity with the IC_{50} of 1.00±0.07 µg/ml and a maximum response of 95.44±0.13%. This value is quite close to the results from the study of Khammuang and Sarnthima (2011) (IC_{50} of 8.85 µg/ml). The anti-oxidant potency of the MA extract was lower than that of vitamin C with approximate potency ratio of 1:20.

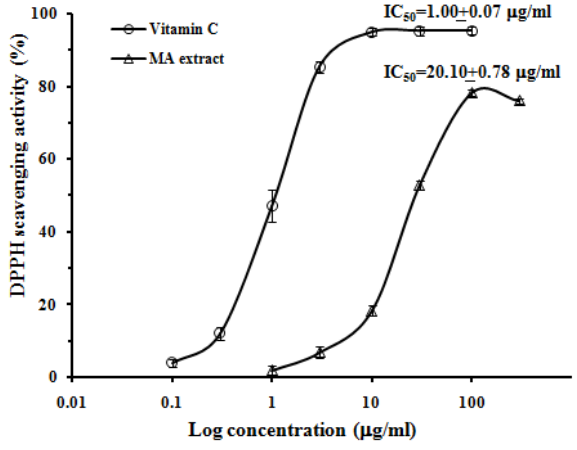

Fig. 1: The radical scavenging activity of MA extract. The log concentration-response curve represents the stable free radical (DPPH) scavenging activities of the MA extract (1-300 µg/ml) and vitamin C. IC_{50} was computed with non-linear regression method from the curve. Each point shows the mean±S.D. (n=4).

Protective effect of MA extract against ROS-induced membrane damage

Erythrocytes possess high content of membrane lipids and are also rich of oxygen supply and transition metal ions. Thus, the AAPH-induced hemolysis was used as a model of membrane damage induced by ROS. The MA extract showed hemolysis inhibitory activity with an IC_{50} of 74.22 ± 9.87 µg/ml and a maximum response of 91.60 ±0.31%. Vitamin C also showed hemolysis inhibitory activity with an IC_{50} of 8.88±0.94 µg/ml and a maximum response of 94.52±3.18% (fig. 2). In the present investigation, the IC_{50} of vitamin C is comparable to that from the study of Chepelev *et al.* (2009) (IC_{50} of 6.52 µg/ml). The inhibitory activity potency of MA was approximately 0.12 times of vitamin C. The results indicated that the MA extract could protect the ROS-induced cellular damage presumably by preventing the oxidation of lipids and proteins at the cellular membrane.

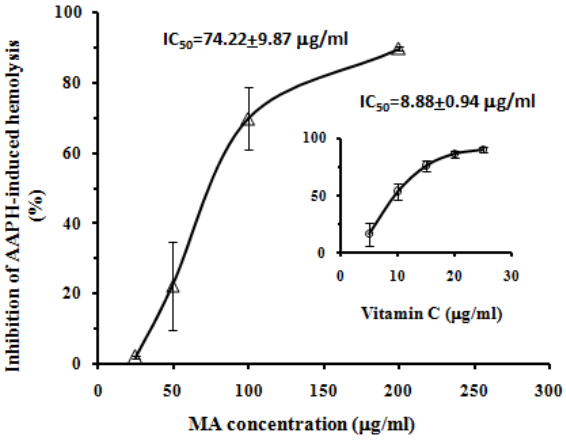

Fig. 2: Protective effects of MA extract against ROS-induced membrane damage in red blood cells. The concentration-response curve represents the inhibitory effect of the MA extract (25-200 µg/ml) and vitamin C on AAPH-induced hemolysis of rat red blood cells. IC_{50} was computed with non-linear regression method from the curve. Each point shows the mean±S.D. (n=6).

Protective effect of MA extract on acute gastric mucosal injury induced by ischemia-reperfusion (I/R)

I/R induced gastric mucosal injury in the glandular part of stomach by 11.06±1.30% of the total glandular area (table). After administration of MA extracts at doses of 0.25 and 0.50 g/kg/day for 3 days, the area of injury induced by I/R was significantly decreased ($P<0.05$) as compared to control (fig. 3 and table). On the other hand, the protective effect was apparently diminished at the higher doses, 1 and 2 g/kg/day. Allopurinol (100 mg/kg/day), a positive control agent (Wada *et al.*, 1995),

showed a significant gastric mucosal protection (P<0.05) with the injury area of 6.27±1.16% (fig. 3 and table).

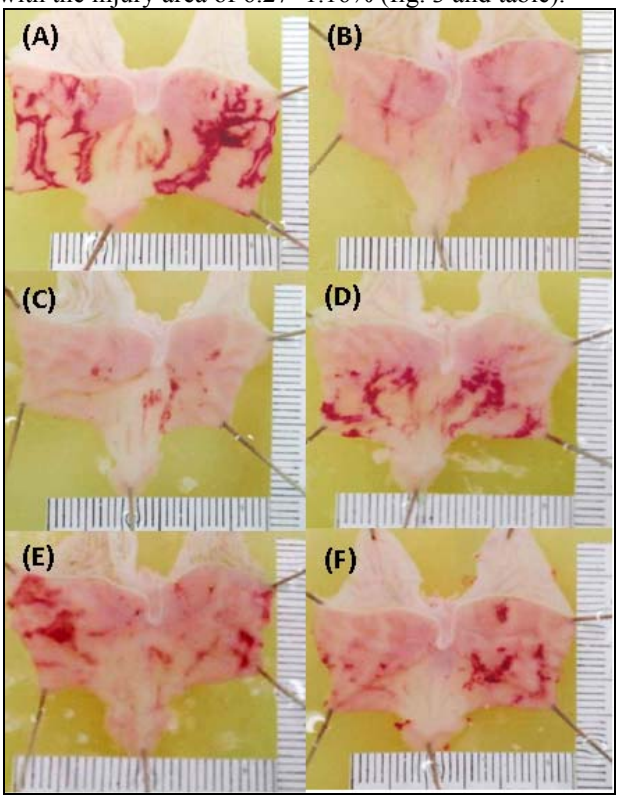

Fig. 3: Protective effect of MA extract on acute gastric mucosal injury induced by ischemia-reperfusion (I/R) in rats. The figure shows injury area of rat stomach in the control group orally administered with distilled water for 3 days (A), and treatment groups orally administered with the MA extract at the doses of 0.25 g/kg/day (B), 0.50 g/kg/day (C), 1 g/kg/day (D), and 2 g/kg/day (E), for 3 days. The positive control group was orally administered with allopurinol at the dose of 100 mg/kg/day for 3 days (F).

Table: The effect of MA extract and allopurinol on I/R-induced gastric mucosal injury in rats

Groups	Gastric mucosal injury area (% of glandular part)[a]
Control (fig. 3A)	11.06±1.30
MA 0.25 g/kg/day (fig. 3B)	5.29±2.07*
MA 0.50 g/kg/day (fig. 3C)	4.83±1.42*
MA 1 g/kg/day (fig. 3D)	8.02±1.60
MA 2 g/kg/day (fig. 3E)	7.50±2.70
Allopurinol 100 mg/kg/day (fig. 3F)	6.27±1.16*

[a] The area was computed by image analyzer and expressed as percentage of glandular part of stomach. Values are mean±S.D. (number of animals in each group=7).
*: P<0.05 as compared to control group

DISCUSSION

In the present investigation, the extract of MA showed anti-oxidant and cytoprotective activities by scavenging DPPH and protection of red cells lysis induced by AAPH. These activities were further investigated in *in vivo* model to explore the possibility of the application of MA extract in clinical use for certain pathological conditions caused by free radicals, particularly gastric mucosal injury induced by I/R. We found that the MA extract at low doses (0.25 and 0.50 g/kg/day for 3 days) decreased the gastric mucosal lesions induced by I/R, which indicating that MA has a gastroprotective effect against acute gastric mucosal lesions induced by I/R. This gastroprotective effect may be related to its anti-oxidant activity. Therefore, MA may be useful in the treatment of diseases, in which free radicals play important role in the pathological cause such as hypertension, ischemic heart disease and stress induced-gastric ulcer.

ROS contribute to I/R-induced injury in the stomach. During the reperfusion phase with oxygenated blood to ischemic tissue, xanthine oxidase can utilize hypoxanthine and oxygen to form superoxide radicals and hydrogen peroxide. They may then interact to produce the highly reactive hydroxyl radical. The cell membrane contains much lipid, especially unsaturated fatty acid. Lipid peroxidation mediated by free radicals is one of the important causes of cell membrane destruction and cell damage which was evidenced by significant increase in free radicals and MDA (Kurose and Granger, 1994; Zimmerman and Granger, 1994; Kwiecień *et al.*, 2002). It is well known that the gastric erosions due to free radicals have been prevented by free radical scavengers or antioxidants (Cuzzocrea *et al.*, 2001; Kwiecień *et al.*, 2003). According to our results in *in vitro* experiments, the MA extract possessed free radicals scavenging activity and prevented of free radicals induced membrane damage. These actions might be related to the cytoprotective activity of the extract at dose of 0.25 and 0.50 g/kg/day on I/R-induced stomach injury in rats.

In the present study, allopurinol (xanthine oxidase inhibitor) showed a protective activity and significantly decreased the injury area of gastric mucosa which was similar to that reported by Wada *et al.* (1995). Thus, the protective effect of MA extract might be correlated with the restrictive activity on this enzyme as well. The enzyme inhibitory activities of phytochemical compounds found in the MA extract have also been reported; morin (3,5,7,2',4'-pentahydroxyflavone) was found to have a potent inhibitory action on xanthine oxidase in rats (Yu *et al.*, 2006). Nitric oxide (NO) showed an important role for I/R-induced injury (Iwata *et al.*, 1998; Ishii *et al.*, 2000). Cyclo-oxygenase-2 (COX-2) contributes to the ability of stomach to resist this damage (Maricic *et al.*, 1999; Wallace *et al.*, 2004). Oxyresveratrol, obtaining from MA

extract, significantly inhibited the iNOS expression through down-regulation of NF-κB binding activity and significantly inhibited COX-2 activity (Chung *et al.*, 2003). In addition, activation of gastric PPARγ can lead to protection against I/R-related injury of stomach (Wada *et al.*, 2004; Naito *et al.*, 2011). Since, the modulation of PPAR activity of this plant has been reported (Huang *et al.*, 2009). Thus, the PPAR modulating activity might also be a mechanism of cytoprotective action of MA.

Interestingly, the gastroprotective effect of MA became diminished with higher doses of the extract (1 and 2 g/kg/day for 3 days). There is no previous study reported on this kind of effect of MA. However, the toxicity of extract would not be the cause since our acute toxicity test showed that the MA extract at single dose up to 4 g/kg/day had no acute toxicity in mice of both sexes (data not shown). The flavonoids have been reported to be the major component of the mulberry extract (Kim *et al.*, 2000; Dugo *et al*., 2009). The dietary flavonoids and other polyphenolic compounds propose the anti-oxidant activity and also pro-oxidant properties (Galati *et al*., 2002). Their pro-oxidant activity is thought to be directly proportional to the number of hydroxyl groups which significantly produce hydroxyl radicals (Hanasaki *et al.*, 1994; Cao *et al.*, 1997). Moreover, the peroxidase could catalyze phenols to form phenoxyl radical which is followed by a production of superoxide anion (Galati *et al.*, 2002). Thus, the MA extract at the higher dose may contain high amount of flavonoids and other polyphenolic compounds which act as the pro-oxidant, resulting in unexpected effects. The results suggest that the extract of MA leaves at appropriate doses have a cytoprotective activity.

In conclusion, the MA extract has an antioxidant activity in both cell-free and biological systems and possesses *in vivo* cytoprotective activity. Consumption of MA as a food supplement or drinking the mulberry tea, would be healthful when taking at appropriate doses. However, the mechanism of its action and the active ingredient in the MA extract should be further investigated. Moreover, the other altered effects which may be dependent on dose of MA, should also be further evaluated.

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