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ORIGINAL ARTICLE

In vitro and *In vivo* Antioxidant Activity of Flavonoid Extracted from Mulberry Fruit (*Morus alba* L.)

Sivakumar Thasma Raman, Ajay Krishna Palani Gounder Ganeshan, Cheng Chen, Chao Jin, Shao-Hui Li, Hui-Juan Chen, Zhongzheng Gui

School of Biotechnology, Jiangsu University of Science and Technology, Zhenjiang 212018, Jiangsu Province, P.R. China

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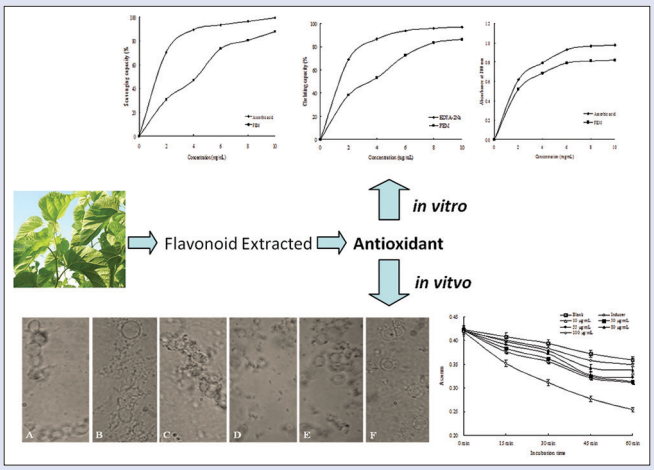
ABSTRACT

Background: Many plants possess antioxidants that exhibit additive or synergistic activities. **Objective:** In this study, an ethanol-extracted flavonoid extracted from mulberry fruit (FEM) was evaluated for the antioxidant activity *in vitro* and the hemolysis in red blood cell (RBC) and lipid peroxidation in liver *in vivo*. **Materials and Methods:** Antioxidant activities *in vitro* were measured by quantifying its 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity, reducing power, and Fe^{2+}-chelating ability. FEM inhibits hemolysis in RBCs and effects of lipid peroxidation in the liver were estimated. **Results:** The total content of flavonoid compounds was 187.23 mg of quercetin equivalents per grams dried material. In the *in vitro* assays, FEM demonstrated a strong antioxidant effect, especially in DPPH scavenging activity and reducing power. Mouse RBC hemolysis induced by H_2O_2 was significantly inhibited by FEM in a dose- and time-dependent manner. The effects of FEM on lipid peroxidation in liver, mitochondria, and microsome were investigated. The percentage of inhibition at high concentration (100 μg/mL) of FEM was 45.51%, 39.36%, and 42.78% for liver, mitochondria, and microsomes, respectively. These results suggest that the FEM possesses a strong antioxidant activity both *in vivo* and *in vitro*.

Key words: Antioxidant activity, flavonoid, hemolysis, lipid peroxidation, mulberry fruit

SUMMARY

- The total content of flavonoid compounds in mulberry fruit was 187.23 mg/g dried material
- FEM showed a strong antioxidant effect, especially in 2,2-diphenyl-1-picrylhydrazyl scavenging activity and reducing power
- Mouse red blood cell hemolysis induced by H_2O_2 was significantly inhibited by FEM in a dose- and time-dependent manner
- The inhibition percentage at high concentration of FEM was 45.51%, 39.36%, and 42.78% for mouse's liver, mitochondrial, and microsomes, respectively.



Abbreviations used: FEM: Flavonoid Extracted from Mulberry fruit, H_2O_2: Hydrogen peroxide, DPPH: 2,2-diphenyl-1-picrylhydrazyl, EDTA: Ethylene diamine tetraacetic acid, MDA: malondialdehyde, TBA: 2-thiobarbituric acid, RBC: Red blood cells, DNJ: 1-deoxynojirimycin, LDL: low density lipoprotein, ROS: reactive oxygen species, EDTA2Na: Ethylenediaminetetraacetic acid disodium salt.

Correspondence:

Prof. Zhongzheng Gui,
School of Biotechnology, Jiangsu University of Science and Technology, Sibaidu, Zhenjiang 212018, Jiangsu Province, P.R. China.
E-mail: srizzgui@hotmail.com

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INTRODUCTION

Mulberry (*Morus alba* L.) is not only a plant source for feeding *Bombyx mori*, but also a long-used medicinal plant in Eastern Asia countries. All parts of the mulberry, including roots, bark, and leaves are reported to have antioxidants,[1,2] antidiabetic,[3] antihyperlipidemic properties.[4] It may also function to prevent from cardiovascular diseases,[5] and inhibit cancer, as well as a diuretic.[6]

Mulberry fruit is used as an herbal medicine and is widely regarded as a nutritious food in China. Mulberry fruit may protect against liver and kidney damage, strengthen the joints, improve eyesight, have anti-aging effects, and be a treatment for sore throats, fever, hypertension, and anemia.[7] The constituents of mulberry fruit include flavonoids, polyphenols, anthocyanins, polysaccharides, fatty acids, vitamins, and trace elements.[7-9] Numerous studies have demonstrated that mulberry fruits and leaves exhibited significant scavenging effects of free radicals and protected low-density lipoprotein (LDL) against oxidative damage. Fresh fruit extracts are excellent sources of polyphenolic compounds that exhibit antioxidant activity.[10] These medicinal capabilities are

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attributable to the presence of active ingredients with notable therapeutic functions.[11] Anthocyanins and water extracts from mulberry fruit can scavenge free radicals, inhibit LDL oxidation, and have beneficial effects on blood lipid levels and atherosclerosis.[6,12,13]

Antioxidants are molecules that can neutralize free radicals by accepting or donating electrons to eliminate the unpaired form of the radical, breaking the chain of oxidation reactions.[14] Many studies have shown that natural antioxidants not only play a major role against reactive oxygen species (ROS), but also trigger lipid peroxidation.[15-17] Oxidative damage caused by ROS, such as the superoxide and hydroxyl radicals to lipids, proteins, and nucleic acids may trigger various diseases, including cardiovascular disease. Epidemiological studies have shown that the administration of antioxidants may decrease the probability of cardiovascular diseases.[18]

However, few studies have evaluated the ethanol flavonoid extract from mulberry fruit (FEM) and its effect on *in vitro* and *in vivo* antioxidant activity. In the present study, our goal was to extract flavonoids from mulberry fruit using ethanol and to evaluate the antioxidant activity *in vitro* and the hemolysis in red blood cell (RBC) and lipid peroxidation in liver *in vivo*.

MATERIALS AND METHODS

Materials

Mulberry fruit was harvested from the plantation of the National Mulberry Orchard (Zhenjiang, PR China). Mice of clean grade were provided by the Experimental Animal Center of Jiangsu University (Zhenjiang, China). 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferrozine, potassium ferricyanide, ferrous chloride ($FeCl_2$), and ferric chloride were purchased from Bio Basic Inc., (Toronto, Canada). Other reagents were obtained from the Sinopharm Chemical Reagent Co., Ltd., (Beijing, China). All reagents used in this study were of analytical grade.

Extraction of flavonoid from mulberry fruit

The fresh mulberry fruit (500 g) was milled using a blender and extracted with 1000 mL of ethanol (70%) at 80°C for 2 h using a Soxhlet apparatus. The extract was filtered and extracted again under the same conditions with fresh solvent. The extract was centrifuged at 1000 g for 10 min. The supernatant (which constitutes the FEM) was concentrated to 100 mL under reduced pressure in a rotary evaporator.

Estimation of total flavonoid content

Total flavonoid content was determined using the aluminum chloride method.[19] From the extractions, different concentrations of the quercetin standard solution were diluted appropriately and mixed with 0.3 mL 10% $NaNO_2$. After standing for 5 min at room temperature, 0.3 mL of 10% $AlCl_3$ and 2 mL of 1 M NaOH were added. The mixture was allowed to rest for 15 min at room temperature. The absorbance was measured at 510 nm, with distilled water used as a blank control. All determinations were performed in triplicate.

Flavonoid content was calculated as the quercetin concentration (mg/g) using the following equation based on the calibration curve.

$Y = 0.0056x - 0.0013$, $R^2 = 0.998$

Where, Y is the absorbance and x is the quercetin equivalent (μg/g).

Antioxidant activity assay *in vitro*

2,2-diphenyl-1-picrylhydrazyl radical-scavenging activity

The DPPH radical scavenging capacity was determined according to the method used by Wu *et al.*[20] A sample of the different concentrations (2, 4, 6, 8, and 10 mg/mL) was mixed with 2 mL of 0.04 mg/mL DPPH in ethanol. The mixture was shaken vigorously and allowed to stand at 25°C for 30 min. Then, it was centrifuged at 1500 g for 10 min, after which, the absorbance of the supernatant was measured at 517 nm. Ascorbic acid was used as a control. The DPPH radical scavenging capacity was calculated using the following formula:

$$\text{Scavenging capacity } (\%) = \frac{1-(A_1 - A_2)}{A_0} \times 100$$

where A_0 is the absorbance of the control (without extract), A_1 is the absorbance in the presence of the extract, and A_2 is the absorbance without DPPH.

Ferrous ion chelating capacity

The ferrous ion chelating capacity was determined following the method of Wu *et al.*[20] The reaction mixture, containing 3 mL of sample at different concentrations (2, 4, 6, 8, and 10 mg/mL), 0.05 ml of 2 mmol/L $FeCl_2$ solution, and 0.2 mL of 5 mmol/L ferrozine solution, was shaken vigorously and incubated at 25°C for 10 min. The absorbance of the mixture was then measured at 562 nm. Ethylenediaminetetraacetic acid disodium salt (EDTA-2Na) was used as a positive control. The ferrous ion chelating capacity of the sample was calculated as follows:

$$\text{Chelating capacity } (\%) = \frac{A_0 - (A_1 - A_2)}{A_0} \times 100$$

where A_0 is the absorbance of the control (without extract), A_1 is the absorbance in the presence of the extract, and A_2 is the absorbance without EDTA.

Reducing power

Reducing power was determined based on the method of Wu *et al.*[20] Briefly, 1 mL of the sample at different concentrations (2, 4, 6, 8, and 10 mg/mL) in phosphate buffer (0.2 mol/L, pH 6.6) was mixed with 2 mL potassium ferricyanide (1%) and incubated at 50°C for 20 min. Next, 2 mL of 10% trichloroacetic acid (TCA) was added to the mixture to stop the reaction. After centrifugation at 3000 g for 10 min, 2.5 mL of supernatant was mixed with 2.5 mL distilled water and 0.5 mL 0.1% ferric chloride. The mixture was allowed to rest for 10 min and absorbance was measured at 700 nm. Ascorbic acid was used as a control.

Antioxidant activity assay *in vivo*

Red blood cell preparation

RBCs were collected in heparinized tubes from the eyes of mice via the cardiopuncture method of Žabar *et al.*[21] Whole blood was centrifuged at 1000 g for 10 min at 4°C. The plasma and buffy coat layers were discarded, and an equal volume of phosphate-buffered saline (PBS: pH 7.4) was added. This procedure was repeated 3 times and the erythrocytes were diluted with PBS to obtain a 4% suspension.

Erythrocyte hemolysis

Erythrocytes were hemolyzed using H_2O_2 and the modified method of Lalitha and Selvam.[22] One milliliter of erythrocyte suspension (4%) was mixed with 1 mL of FEM at the concentrations (10, 30, 55, 80, and 100 μg/mL) and then added 1 mL of H_2O_2 (100 mmol/L in PBS). The blank control consisted of 2 mL of PBS and 1 mL RBC suspension and the induced control consisted of 1 mL of PBS, 1 mL RBC suspension, and 1 mL of H_2O_2. The mixture was incubated in a shaking water bath at 37°C for 30 min, 60 min, 90 min, 120 min, and 150 min, and then centrifuged at 1000 g for 10 min at 4°C. The RBC-free supernatant solution from each tube was transferred to cuvettes. Absorbance was measured at 415 nm in a spectrophotometer (Shimadzu UV-VIS 1650, Tokyo, Japan).

Each sample was measured in triplicate. The percentage of hemolysis was calculated using the equation:

Hemolysis percentage (%) = $[Ab_{(sample)}/Ab_{(induced\ control)}] \times 100\%$

Percent of hemolysis inhibition

$$= [(Ab_{(induced\ control)} - Ab_{(sample)})/Ab_{(induced\ control)} - Ab_{(blank\ control)}] \times 100\%$$

Preparation of mitochondrial and microsomal suspension

Mice livers were collected and homogenized in buffer (25 mmol sucrose, 0.5% protease inhibitor cocktail, and 10 mmol HEPES, pH 7.4) using a hand homogenizer. The crude mitochondria were prepared by differential centrifugation at 1000 g for 30 min and at 10,000 g for 20 min at 4°C. The mitochondrial suspension was obtained from the precipitate using a 10 mmol/L Tris-HCl buffer solution. The supernatant was centrifuged at 10,000 g for 30 min at 4°C. The extracted microsome was collected from the precipitate and dissolved in a 10 mmol/L Tris-HCl buffer solution for future use.[23]

Determination of mitochondria swelling

The swelling of liver mitochondria was achieved using the method of Dutra and Bechara.[24] Briefly, 1 mL of liver mitochondrial suspension (1%) was mixed with different concentrations of FEM (10, 30, 55, 80, and 100 μg/mL) and 2 mL of the inducer was added (5 μmol/L $FeSO_4$ and 0.1 mmol/L ascorbic acid) to stimulate mitochondrial swelling. The mixture was incubated in a shaking water bath at 37°C for 0 min, 15 min, 30 min, 45 min, and 60 min, and centrifuged at 1000 g for 10 min at 4°C. Absorbance was measured at 520 nm.

Inhibition of lipid peroxidation by thiobarbituric acid reactive substance method

Lipid peroxidation was determined using the thiobarbituric acid (TBA) reactive substance method.[25] 1 mL of liver mitochondria suspension (1%) was thoroughly mixed with the different concentrations of FEM (10, 30, 55, 80, and 100 μg/mL). Then, 100 μL of 15 mM $FeSO_4$ and 50 μL of 0.1 mmol/L ascorbic acid were added, and the mixture was incubated at 37°C for 1 h. Next, 1 mL of 15% TCA and 1 mL of 0.67% TBA were added to stop the reaction. The mixture was incubated in boiling water for 15 min. After it had cooled down, the absorbance was measured spectrophotometrically at 532 nm.

Inhibition ratio

$$= [(Ab_{(induced\ control)} - Ab_{(sample)})/Ab_{(induced\ control)} - Ab_{(blank\ control)}] \times 100\%.$$

Statistical analysis

All tests were carried out in triplicate for the three separate experiments. Statistical significance was calculated by one-way analysis of variance. Values are represented as mean ± standard deviation.

RESULTS

Extract yield and total flavonoids content in mulberry fruit

The dried FEM was obtained by freeze-drying (EYELA FDU-2100, Japan) the extract after concentrating in a rotary evaporator. The yield of ethanol extract was 53 mg/g from fresh mulberry fruit. Flavonoids content was 20.4 mg/g in fresh mulberry fruit and 187.23 mg/g in dried fruit.

Antioxidant activity *in vitro*

The antioxidant properties *in vitro* were largely DPPH radical scavenging, ferrous ion chelating capacity, and reducing power. The ethanol extract of mulberry fruit exhibited all these properties in a concentration-dependent manner [Figures 1-3]. The decrease in absorbance of the DPPH radical was caused by antioxidant scavenging of the radical and donating hydrogen. Ascorbic acid exhibited an excellent scavenging activity (IC_{50} = 0.186 mg/mL). FEM also showed strong scavenging activity with an IC_{50} value of 0.518 mg/mL [Figure 1]. Similarly, EDTA-2Na exhibited strong Fe^{2+}-chelating activity, and even at the lowest concentration of 2 mg/mL, the chelating rate was 69.12%. However, while FEM showed little Fe^{2+}-chelating activity at low concentrations, activity increased rapidly with increasing concentration. At 6 mg/mL, FEM reached a level of 72.6% [Figure 2]. As shown in Figure 3, the reducing power of FEM was 0.522 at 2.0 mg/mL and 0.685 at 4.0 mg/mL. Ascorbic acid exhibited only slightly higher activity, with a reducing power of 0.617 and 0.794 at 2.0 mg/mL and 4.0 mg/mL, respectively.

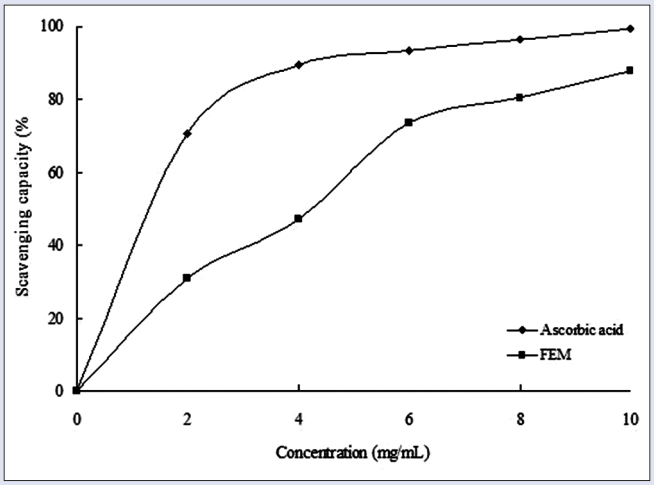

Figure 1: The 2,2-diphenyl-1-picrylhydrazyl radical scavenging capacity of FEM. The absorbance values were converted to scavenging capacity (%) and data were plotted as the mean of replicate scavenging capacity (%) ± standard deviation (n = 3) against extract concentration in mg extract per ml reaction volume. Linear regression analysis was used to calculate IC_{50} value

Red blood cell hemolysis by H_2O_2-induced oxidant stress

To determine the effects of FEM on hemolysis of RBCs, both hemolytic and antihemolytic (i.e., hemolytic inhibition) tests were conducted. The ethanol extract of mulberry fruit exhibited both hemolytic and antihemolytic properties in a dose- and time-dependent manner [Table 1]. In both experiments, the lowest dose of FEM exhibited slightly beneficial effects on RBC membranes. The percentage of hemolysis was the highest for 10 μg/mL and it gradually declined, as incubation time was increased from 30 min to 150 min. In addition to the incubation time, higher concentrations resulted in a decline in RBC hemolysis. Hemolysis was the lowest for the 100 μg/mL concentration with incubation time of 150 min. Conversely, the percentage of hemolytic inhibition increased with increasing concentration and incubation time [Table 1]. The morphology of RBCs treated with FEM during H_2O_2-induced oxidant stress is shown in Figure 4.

Lipid peroxidation of mice liver

Lipid peroxidation of mice liver induced by $FeSO_4$-ascorbic acid created membrane damage. Figure 5 shows that the absorbance at 520 nm decreased with the incubation time in all groups; however, it decreased rapidly in the induced group. These results show that mitochondrial

swelling is increasing. They also indicate that FEM retards liver mitochondrial swelling in a dose-dependent manner. The effect of FEM on lipid peroxidation in the mitochondria and microsome from liver cells is shown in Table 2. The absorbance at 520 nm corresponded to the amount of malondialdehyde (MDA). The content of MDA was high in all treatment groups. FEM decreased the amount of MDA in a dose-dependent manner. The percentage of inhibition at the highest concentration (100 μg/mL) of FEM was 45.51%, 39.36%, and 42.78% for liver, mitochondria, and microsomes, respectively.

DISCUSSION

Flavonoids are major secondary metabolites and play an important role in mulberry plants. Flavonoids were detected in all investigated parts of the plant. Overall, the flavonoid content was 28.7 mg/g in mulberry leaves,[4] 88 mg/g in mulberry twigs, and 141 mg/g in mulberry root bark.[2] The present study showed that the flavonoid content in mulberry fruit was 187.23 mg/g in dried material. Mulberry fruit contained the highest levels of flavonoid in all the parts of the plant, and flavonoid content was much higher than in *Choerospondias axillaris* fruit (36.5 mg/g).[26]

The role of an antioxidant is to remove free radicals. *In vitro* antioxidant activities of DPPH radicals, Fe^{2+}-chelating capacity, and reducing power were used to test the antioxidant power of mulberry extract. The DPPH assay is a popular radical scavenging test for natural products.[20] The decrease in absorbance of the DPPH radical due to the antioxidant was caused by scavenging of the radical by hydrogen-donating entities.[27] Ferrous ions (Fe^{2+}) can catalyze and induce superoxide anions to form the more harmful hydroxyl radicals.[28] Reducing power is widely used to evaluate the antioxidant activity of plant extracts. Bae and Suh reported that an ethanol extract of mulberry fruit effectively scavenged free radicals, including the DPPH radical, hydroxyl and superoxide anions, and exhibited a moderate ability to inhibit linoleic acid oxidation induced by hemoglobin *in vitro*.[29]

High-performance liquid chromatography analysis illustrated that the main flavonols in mulberry fruit were rutin, morin, quercetin, and myricetin. These four flavonols are reported to be effective antioxidants.[17,29,30] In this study, FEM exhibited DPPH radical scavenging activity in a concentration-dependent manner with an IC_{50} value of 0.518 mg/mL [Figure 1], Fe^{2+}-chelating activity reached a level of 72.6% at high concentrations (6 mg/mL) [Figure 2], and the reducing power of FEM showed similar activity to ascorbic acid [Figure 3]. Chang *et al.* reported that flavonols from mulberry twigs and root bark have shown significant antioxidant effects, including superoxide inhibition and reducing activity.[2] The antioxidant mechanism of flavonols is reflected in its scavenging potential and metal chelating ability, and these factors are dependent upon their structure and the number and position of the hydroxyl groups.[31]

The RBC membrane is well adapted to the formation of OH^- and O_2 from H_2O_2. Oxidant damage of the cell membrane, induced by H_2O_2 can result in increased erythrocyte hemolysis and inhibition rate.[21] Our result indicated that RBC hemolysis was inhibited by FEM with varying degrees of inhibition. An increase in the concentration of FEM at the

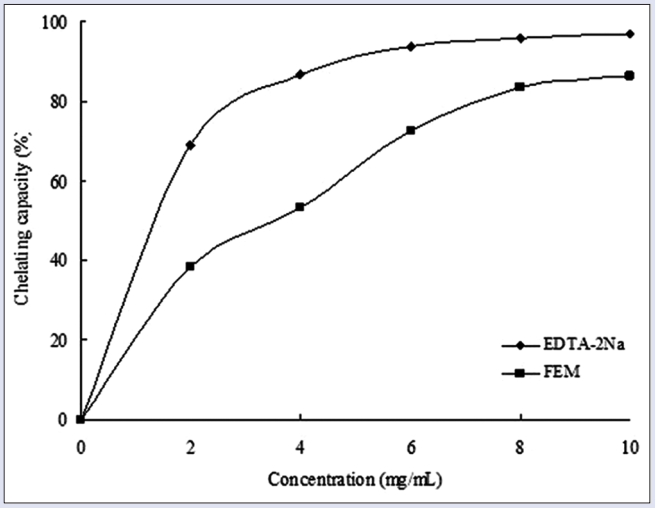

Figure 2: Fe^{2+}-chelating activities of FEM. The absorbance values were converted to chelating effects (%) and data were plotted as the mean of replicate chelating effects (%) ± standard deviation (n = 3) against extract concentration in mg extract per ml reaction volume

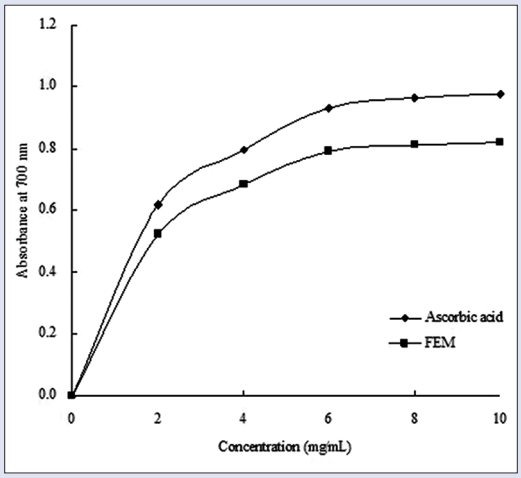

Figure 3: Reducing power of FEM. The absorbance values were converted directly plotted as the mean of replicate absorbance values ± standard deviation (n = 3) against extract concentration in mg extract per ml reaction volume

Table 1: Effect of flavonoid extract from mulberry fruit on hemolysis and inhibition of red blood cells from mice induced by H_2O_2

Group	Hemolysis (%)					Inhibition (%)				
	30 min	60 min	90 min	120 min	150 min	30 min	60 min	90 min	120 min	150 min
RBC	16.12	15.83	16.31	16.54	16.65					
RBC+H_2O_2	100	100	100	100	100					
FEM 10 μg/mL	76.14	74.48	73.24	72.19	71.37	29.41	31.46	32.99	34.27	35.29
FEM 30 μg/mL	73.65	71.78	70.75	69.92	69.5	32.48	34.78	36.06	37.08	37.60
FEM 55 μg/mL	66.18	64.73	63.07	62.03	61.61	41.69	43.48	45.52	46.80	47.32
FEM 80 μg/mL	59.75	58.51	57.46	56.85	56.02	49.62	51.15	52.43	53.20	54.22
FEM 100 μg/mL	54.56	52.70	51.45	50.20	49.58	56.01	58.31	59.85	61.38	62.15

FEM: Flavonoid extract from mulberry fruit

Table 2: Effect of flavonoid extract from mulberry fruit on malondialdehyde of mitochondria and microsome from mice liver

Group	Liver		Liver mitochondria		Liver microsome	
	A_{532} nm	Inhibition (%)	A_{532} nm	Inhibition (%)	A_{532} nm	Inhibition (%)
Normal control	0.061±0.01	-	0.054±0.01	-	0.036±0.01	-
Induced control	0.239±0.02	-	0.651±0.07	-	0.625±0.06	-
FEM 10 μg/mL	0.211±0.03	15.73	0.579±0.09	12.06	0.569±0.08	9.51
FEM 30 μg/mL	0.197±0.02	23.59	0.538±0.10	18.93	0.515±0.05	18.68
FEM 55 μg/mL	0.184±0.05	37.67	0.509±0.08	23.79	0.497±0.07	21.73
FEM 80 μg/mL	0.171±0.02	38.20	0.471±0.08	30.15	0.426±0.09	33.79
FEM 100 μg/mL	0.158±0.04	45.51	0.416±0.07	39.36	0.373±0.07	42.78

FEM: Flavonoid extract from mulberry fruit

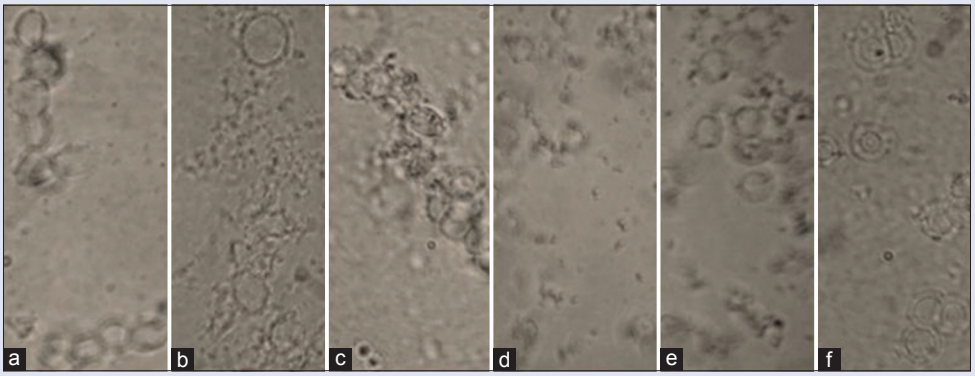

Figure 4: Morphology of red blood cells during H_2O_2-induced oxidant stress. (a) normal control; (b) H_2O_2-induced control; (c) FEM 10 μg/mL; (d) FEM 55 μg/mL; (e) FEM 80 μg/mL; (f) FEM 100 μg/mL

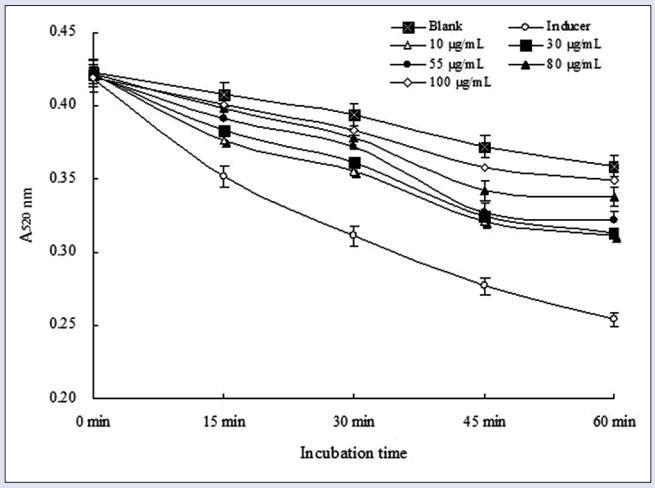

Figure 5: Effects of FEM on mitochondrial swelling in mice liver

outer membrane leads to diffuse to the internal membrane until it gets to the specific concentration that promotes membrane disruption and induces hemolysis.[32] The flavonoid extract from mulberry fruit can accept electrons and scavenge OH that was induced by H_2O_2. Certain flavonoids may interact with the membrane, leading to a decrease in its fluidity and the diffusion of free radicals into the RBC.[33]

Lipid peroxidation is a complex process involving the oxidation of polyunsaturated fatty acids that are responsible for long-term damage to cells.[2,34] Chen *et al.* obtained similar results and found that hypobaric storage could reduce MDA accumulation of ROS.[35] MDA, a lipid peroxidation product, is an indicator of ROS generation in the tissue.[36] The inhibition of lipid peroxidation is of great importance in disease processes that involves free radicals.[37] The inhibition of lipid peroxide formation by FEM showed the maximum inhibition of peroxide formation with a high concentration for liver, mitochondria, and microsome. The levels of MDA were significantly higher in the liver of $FeSO_4$-Vc-induced mice than in those of normal mice [Table 2]. Similar findings have been reported in tissues of D-gal-treated mice.[38,39] The effect of flavonoids from mulberry fruit accelerates the repair of mitochondrial membrane injury induced by Fe^{2+}-Vc.[40]

Yang *et al.* showed that a freeze-dried powder of *Forsythia suspense* Leaves inhibited the formation of a lipid peroxidation product, increased antioxidant enzyme activity, and repressed the development of atherosclerosis in hyperlipidemic rats.[31] In general, mulberry fruit is a natural and healthy food with hypolipidemic and antioxidant effects, and these beneficial effects may be attributed to phytochemical constituents. It contains flavonoids, anthocyanins, phenolics, fiber, fatty acids, vitamins, and trace elements.

CONCLUSION

The flavonoid ethanol extracted from mulberry fruit (FEM) was 53 mg/g from fresh mulberry fruit. FEM exhibited a strong antioxidant effect, especially in DPPH scavenging activity and reducing power. RBC hemolysis induced by H_2O_2 was significantly inhibited by FEM in a dose- and time-dependent manner. FEM exhibited the lipid peroxidation in liver, mitochondria, and microsomes.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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Zhongzheng Gui

ABOUT AUTHOR

Zhongzheng Gui, is a Professor at Jiangsu University of Science and Technology, China. He is the chief of Functional Bioactive Products Laboratory since 2003. His experience in the area of Functional Natural Products, working mainly in: Silkworm, mulberry, and their biomedical applications. His research program covers various subject areas, including insect biochemistry and molecular biology, microbiology and biotransformation, and biomedical.