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Antioxidant Activities and Phenolic Compounds Composition of Extracts from Mulberry (*Morus alba* L.) Fruit

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ABSTRACT : The objective of this research was to evaluate the ability of water and ethanol extracts from mulberry fruit (*Morus alba* L.) to influence the inhibitory activity of angiotensin converting enzyme (ACE) and xanthine oxidase (XOase). The total phenol contents and sixteen phenolic compounds were investigated in water and ethanol extracts. In order to understand the factors responsible for the potent antioxidant and antihypertensive ability of mulberry, it has been evaluated for anti-oxidative activity using Fenton's reagent/ethyl linoleate system and for free radical scavenging activity using the 1,1-diphenyl-2-picryl hydrazyl free radical generating system. The total phenol contents and total of phenolic compounds in ethanol extract showed higher levels than water extract in mulberry fruit six phenolic compounds (chlorogenic acid, narigin, syringic acid, quercetin, naringenin, kampferol) has a higher individual phenolic compound content in the 60% ethanol extraction than 80% ethanol extract. The inhibitory activity on angiotensin converting enzyme (ACE) were highest in 80% ethanol extract (9.0%). Also, activity of xanthine oxidase (XOase) inhibition appeared highest in 80% ethanol extracts and correlated well with the total phenolic content, which was modulated by the concentration of individual phenolic compounds. This result revealed, that strong biological activity was caused by specific phenol compound contents. Utilization of water and ethanol extracts from mulberry fruit are expected to be good candidate for development into source of free radical scavengers and antihypertentive activity

Key words **:** Mulberry, Xanthine oxidase, Phenolic compounds, Angiotensin converting enzyme

INTRODUCTION

The larch tree is classified in the family Moraceae of genus *Morus*. The fruit of this tree, called mulberry, is picked when a its colour turn into black or dark red. The dried fruit has been used as both food and medicinal ingredient (Kim *et al.*, 1991) and in oriental medicine, it is used to treat dizziness, tinnitus and thirst (Kim *et al.*, 2003; Hong *et al.*, 2003). Beyond silkworm feeding, depending on regions, mulberry is also appreciated for its fruit (in juice making, processed food such as jam and liquors), for its medicinal properties (mulberry leaf tea), for landscaping, as a vegetable (leaves and young stems) and as a feed for ruminants and others animals (Zepeda, 1991).

This fruit from the *Moraceae* family is safely used with no toxic effects to prevent hypertension due to its effectiveness in removing unwanted hotness from the body (Hwang & Song, 2003). Particularly, the crude drug "Sangsimja", the fructus of *Morus alba* (Moraceae), has been used traditionally to cure and prevent diabetes, anemia, hypertension and arthritis in Chinese medicine. Recently, mulberry fruits have been reported to have several biological actions, such as antidiabetic (Cho *et al.*, 2006), antioxidant, antiinflammatory (Kim *et al.*, 1998) and antihyperlipidemic activities (Choi *et al.*, 2006). Therefore, consumer demand for the mulberry fruit has been recently increased because of its dessert and functional properties.

Many of these properties have been proved by clinical studies and various compounds present in mulberry (flavonoids, alkaloids, steroids), responsible of such therapeutic benefits, have also been recognized (Asano *et al.*, 2001; Cheon *et al.*, 2000; Doi *et al.*, 2001; Kim *et al.*, 1999; Nomura, 1999). Nevertheless, just these substances can limit the use of mulberry leaves as a food or food ingredient, especially for those peo-

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ple affected by those diseases which mulberry extracts can help to control due to interactions with other medicines or to physiological intolerance. However, few systematic analyses of antihyperglycemic, antioxidant and antihypertensive phytochemicals in mulberry fruits are still available.

The polyphenolic compounds include flavaonols, flavones, catechins, anthocyanins and ferulic acid. On a daily basis, human intake of total flavonoids is approximately in the hundreds of milligrams (Hollman & Katan 1999). Tea and red wine contain concentrated levels of these compounds, thus possibly accounting for the potential health benefits associated with the intake of these beverages (Bourne *et al.*, 2000). However, *in vivo* antioxidant effectiveness of plant-derived phenolic compounds may depend on their bioavailability (Duthie & Crozier, 2000). As antioxidants, phenolic compounds may act to break free radical chain reactions, and quench reactive oxygen and nitrogen species, thereby inhibiting oxidation of lipids and other biological molecules (Morton *et al.*, 2000).

The hydrogen-donating abilities as well as the metal-chelating properties of polyphenols may helps to explain their antioxidant potentials (Pannala *et al.*, 1998). Moreover, similar to other scavenging antioxidants, oxidized polyphenols are relatively stable and thus less likely to initiate a chain reaction. Nevertheless, under certain circumstances, such as the presence of excess phenolic compounds, transition metals, the potential for polyphenols to act as prooxidants is possible (Bravo, 1998). Although many *in vitro* studies indicate that plant phenolic compounds act as antioxidants, *in vivo* studies are still limited. This is especially the case with hydroxycinnamates (simple phenolic compounds such as ferulic acid) which are usually present in plant foods at much higher concentrations, versus other flavonoids. Nevertheless, it has been reported that ferulic acid prevents LDL peroxidation suggesting possible peroxyl radical scavenging effects (Pannala *et al.*, 1998). Ferulic acid may also possess stronger abilities to quench peroxynitrite radicals as opposed to other hydroxycinnamates, possibly indicating greater antioxidant properties. Indeed, *in vivo* studies are required to further explore the relative importance of phenolic compounds and their reducing properties, and their overall contribution to antioxidant defences.

Recently, researchers have investigated alcohol extraction utilizing domestics fruits. Mulberry fruit extract has been used for the production of foods with anti-radical activity, but the phenolic compound in the extract differed during various extraction processes. Therefore, the quantification of phenolic compounds at various ethanol concentration may influence the antihypertensive and antiradical capacity of polyphenol. This study was conducted to investigate the optimal extraction process using various ethanol concentration.

MATERIALS AND METHODS

Preparation of mulberry fruit extract

Mulberry fruits (*Morus alba* L.) at a commercially mature stage were obtained from the HyeJeon College, Hongsung, Korea. Fruits were selected according to uniformity of shape and color. The fruits were freeze dried (PVTF 200K : Ilshin Lab Co, Korea) then stored at −20 ℃ for further studies. The composition of the mulberry fruits was 83.2% moisture, 2.2% crude protein, 0.9% fat, 13.0% carbohydrate, and 0.7% ash.

Extraction

For water extraction, each mulberry fruit (50 g) was powdered in a mill and was mixed with 100 ㎖ boiling distilled water by a magnetic stirrer for 15 min. The water extract was lyophilized. The ethanol extract solution of mulberry fruit was prepared according to Lee & Wicker (1991) method, by homogenizing the mulberry fruit (50 g) with 100 ㎖ of 20%, 40%, 60% and 90% ethanol. The sample was extracted in a soxhlet apparatus until the extraction solvent became colourless. After refrigerating overnight and filtering through Whatman No. 1 paper, the extract was concentrated for further experiments by removing ethanol with a rotary evaporator at 40℃.

Measurement of Total Phenolics

TP (total phenolic) concentrations were measured using the Folin-Ciocalteu assay (Singleton & Rossi, 1965). Briefly, 5 ㎖ of Nanopure water, 1.0 ㎖ of sample, and 1.0 ㎖ of Folin-Ciocalteu reagent were added to a 25 ㎖ volumetric flask. The contents were mixed and allowed to stand for 5-8 min at room temperature. To this mixture, 10 ㎖ of a 7% sodium carbonate solution was added, followed by the addition of Nanopure water filled Solutions were mixed and allowed to stand at room temperature for 1 hour. Sample aliquots were filtered through a Whatman 0.45 ㎛ poly (tetrafluoroethylene) prior to the determination of total phenols concentration using a Beckman DU 7400 spectrophotometer, monitoring at 725 ㎚.

TP content was standardized against gallic acid and expressed as milligrams per liter of gallic acid equivalents. The linearity range for this assay was determined as 0.5-5.0 ㎎/L GAE giving an absorbance range of 0.050-0.555 AU.

Qualification of 16 phenolic compounds in various extraction type

Instrumentation for HPLC analysis was applied by Lim *et al.* (2004). The HPLC system consisted of a Young-Lin M930 liquid chromatograph pump and an M720 detector (Young-Lin Instruments, Korea, Anyang). The column for quantitative

analysis was a YMC-Pack ODS-AM-303 (250 × 4.6 ㎜ I.D.), and the UV absorption was measured at 280 ㎚. A linear HPLC gradient was employed as in Table 1.

Making the calibration curves of the 16 phenolic compounds, the 16 phenolic standards were purchased from Sigma-Aldrich (USA) in 2005. The purity of each standard was determined by HPLC chromatography, and the plotting standard concentration was obtained at five concentrations (1, 25, 50, 75 and 100 ppm). A high linearity ($r^2 > 0.998$) was obtained for each curve. Gentisic acid, cathechin, hydroxybenzoic acid, chlorogenic acid, caffeic acid, syringic acid, coumaric acid, ferulic acid, hesperidin, narigin, salicylic acid, hyricetin, quercetin, t-cinnamic acid and naringenin were identified by comparing their retention times with authentic standards. Their concentrations were calculated by comparing peak areas of samples with those of the standards (Table 2) and total phenolic compounds were obtained by adding a each of the 16 phenolic compound analyzed by HPLC.

Table 1. Analysis condition of phenolic compound using HPLC

Column	YMC AM303 (4.6 × 250 ㎜)		
Mobile phase	Gradient		
	Solvent A	Solvent B	
	98% H_2O + 2% glacial acetic acid in 0.018 M ammonium acetate	70% solvent A + 30% organic solvent (82% MeOH, 16% n-butanol and 2% 0.018 M ammonium acetate)	
Flow rate	1.0 ㎖ · min^{-1}		
Detector	SPD-10A spectrophotometer (UV), 280 ㎚		
Injection volume	20 ㎕		
Time Program	Time	Solvent A	Solvent B
	0.0	90	10
	1.0	90	10
	21.0	75	25
	36.0	55	45
	56.0	0	100
	82.0	90	10
	92.0	90	10

Measurement of angiotensin I-converting enzyme (ACE) inhibitory activity of various extraction type

Angiotensin I-converting enzyme inhibitory activity was measured by the method of Saito *et al.* (1992). with some modifications. Aliquots of 100 ㎕ of the sample solution with 100 ㎕ of 0.125U/㎖ ACE (from rabbit lung; Sigma, St. Louis, MO, USA) were preheated at 37 ℃ for 3 min. To this mixture, 150 ㎕ of substrate solution containing 2.5 mM hippuryl-L-histidyl-L-leucine (HHL; Nacalai, Kyoto, Japan), 300 mM NaCl and 100 mM borate buffer was added and pH8.3 was adjusted and the mixture was incubated at 37 ℃ for 30 min. The reaction was terminated by adding 350 ㎕ of 1 N HCl and then hippuric acid liberated from HHL by ACE was extracted by 3 ㎖ of ethyl acetate with stirring. Only aliquot of the ethyl acetate layer was transferred to another test tube.

After removal of ethyl acetate by drying in evaporator, the residue containing the hippuric acid was redissolved in 2 ㎖ of distilled water and the optical density (OD) was measured at 228 ㎚ (UV-1200; Shimadzu, Kyoto, Japan).

The ACE inhibition rate was calculated as follows:

$$\text{ACE inhibition rate } (\%) = (C - S)/(C - B) \times 100$$

where S is the OD value when the sample solution was added to the assay media, C is the control OD value when distilled water was added to the media as a substitute for the extract, and B is the blank OD value when 350 ㎕ of 1 M HCl was added to the assay media beforehand to inactivate ACE. The ACE inhibitory activity of the extract was expressed as the IC_{50} value.

Table 2. Calibration curves of 16 phenolic compounds

Standard Chemicals	Equation	Standard Chemicals	Equation
Gentisic acid	Y = 1.05X − 3.96	Ferulic acid	Y = 47.41X − 14.45
p-Hydroxy benzoic acid	Y = 32.83X − 3.42	Salicylic acid	Y = 6.96X − 5.78
Chlorogenic acid	Y = 21.38X − 7.85	*trans*-Cinnamic acid	Y = 123.80X − 19.08
Caffeic acid	Y = 43.12X − 7.43	*Kampferol*	Y = 22.97X − 32.75
Syringic acid	Y = 40.60X − 0.03	Naringenin	Y = 38.95X − 18.70
p-Coumaric acid	Y = 67.50X − 5.55	Naringin	Y = 24.92X − 3.50
Cathechin	Y = 1.876X − 0.18	Quercetin	Y = 15.99X − 36.52
Hyricetin	Y = 15.99X − 36.52	Hesperidin	Y = 20.46X − 4.47

Analysis of anti-oxidant and free-radical scavenging activities

At first, we examined anti-oxidant activity against lipid peroxidation in Fenton's reagent with ethyl linoleate. Then we examined scavenging activity against 1,1-diphenyl-2-picryl hydrazyl (DPPH) free radical generation.

A lipid peroxidation system was induced by Fenton's reagent. Each test sample (0.1 mℓ) and ethyl linoleate (10 μM) were added to incubation medium (4.98 mℓ) containing 2% sodium dodecyl sulphate (SDS), 1 μM ferrous chloride and 0.5 mM hydrogen peroxide. The known synthetic anti-oxidants, dibutylated hydroxy toluene (BHT), α-tocopherol and ascorbic acid were used as reference compounds. The incubation medium was kept at 55℃ for 16 hour. Each reaction mixture (0.2 mℓ) was transferred to a test tube, followed by addition of 4% BHT (50 μℓ) to prevent further oxidation. The anti-oxidative activity of the sample was measured using the thiobarbituric acid (TBA) assay (Ohkawa *et al.*, 1979). Absorbance was measured at 535 nm.

Scavenging effects against free radical generation was measured by the procedure of Fugita *et al.* (1988). The sample solution (2 mℓ) was added to 2 mℓ of 60 μM DPPH ethanol solution and kept at room temperature for 30 min. Absorbance was measured at 514 nm.

The Analysis of superoxide radical scavenging activity by inhibitory activity of Xanthine oxidase

The superoxide radicals were generated *in vitro* by the xanthine oxidase. The scavenging activity of the extract was determined by the nitro-blue tetrazolium (NBT) reduction method. In this method, O_2 reduces the yellow dye ($NBT2^+$) to produce the blue formazan, which is measured spectrophotometrically at 560 nm. The capacity of the extracts to scavenge the superoxide radical was assayed by mixing of 0.5 mℓ of 0.8 mM xanthine in 0.1 mM phosphate buffer (pH 8.0), 0.48 mM NBT in 0.1 mM phosphate buffer (pH 8.0) and 0.1 mℓ of extract solution. After heating to 37℃ for 5 min, the reaction was started by adding 1.0 mℓ of XOD (Xanthine oxidase, 0.049 U/mℓ) and carried out at 37℃ for 20 min. The reaction was stopped by adding 2.0 mℓ of 69 mM SDS. The absorbance of the reaction mixture was measured at 560 nm.

The results were expressed as the percentage inhibition of the NBT reduction with respect to the reaction mixture without any sample.

% inhibition = [{(C − CB) − (S − SB)}/(C − CB)], where S, SB, C and CB are absorbance of the sample, the blank sample, the control and the blank control, respectively.

Statistical analysis

All data are expressed as the mean ± the standard error. Results were assessed by ANOVA and Tukey's Honestly Significant Difference test. Differences were considered significant at $p < 0.05$

RESULTS AND DISCUSSION

Total phenol contents of mulberry extracts

The presence of phenolic compounds in plant species is very important in term of their medicinal and nutritional evaluation. TP (total phenol) were measured in various concentration of ethanol extract of mulberry. The impact of water soluble and organic solvent on TP content was also evaluated and compared to the TP content in samples that were extracted in various ethanol concentration.

Concentrations of phenolic compounds were measured separately by HPLC because phenolic compounds produces an oxidative-reduction reaction that contributes to the absorbance measurement in the Folin-Ciocalteu assay. Reported TP values were adjusted for the contributory effect of phenolic compounds in the analysis of TP activity. Levels of phenolic compounds in ethanol extract of mulberry samples were consistently higher than the levels for the water extract of mulberry (Table 3).

In the present study, phenolic compounds were evaluated in different levels with varying extraction types. Thus, it may be concluded that the occurrence of total phenol in mulberry extracted by various ethanol concentration has an important pharmacological value especially for their medicinal evaluation.

The average TP content of 20, 40, 60, 80% ethanol extract of mulberry sample were 95, 137, 153 and 170 mg/g of extract dry weight, respectively (Fig. 1). Whereas, on water extract basis, the average of TPs contents was 116 mg/g. In contrast, the levels of TPs we report for 80% ethanol extract was two times higher than 20% ethanol concentration.

HPLC profile of 16 phenolic compounds of mulberry extracts

This study was conducted to examine differences in 16 phenolic compounds with both water and various ethanol concentration extract. Generally, the low ethanol concentration resulted in lower total phenolic compounds than the high ethanol concentration extract. The 20% ethanol extract of mulberry had a higher total phenolic compounds content (1885 $\mu g \cdot g^{-1}$) than the water extract of mulberry (1796 $\mu g \cdot g^{-1}$). These results were statistically significant. The 80% ethanol extract had highest total phenolic compounds (2606 $\mu g \cdot g^{-1}$) among of the

Table 3. Distribution of 16 phenolic compounds different ethanol concentration and water extract from fruit of *Morus alba* L

Morus alba L. (fruit)	Gentisic acid	Cathechin	p-hydroxy benzoic acid	Chlorogenic acid	Caffeic acid	Syringic acid	p-coumaric acid	Ferulic acid	Hesperidin	Narigin	Salicylic acid	Hyricetin	Quercetin	t-Cinamic acid	Naringenin	Kampferol	Total
EtOH Conc. (%)	----------μg/g----------																
0	1253.1	1.1	24.43	56.78	14.45	53.43	54.56	14.32	3.21	4.3	185	43.2	10.23	0.76	1.45	75.7	1796
20	1327.3	2.3	17.77	45.34	21.32	55.64	32.43	18.43	3.76	12.4	184	56.7	26.73	0.96	1.23	79.2	1885
40	1432.2	2.3	12.34	67.84	29.35	54.34	34.92	21.42	3.42	24.5	195	78.3	32.11	0.89	5.34	114.1	2108
60	1761.4	9.2	17.38	71.18	34.73	87.42	54.94	17.45	3.71	36.0	200	34.0	68.04	1.00	6.56	121.0	2524
80	1865.1	10.5	29.63	56.71	56.92	73.32	57.32	18.96	3.43	35.7	210	34.8	65.45	1.12	2.34	85.4	2606

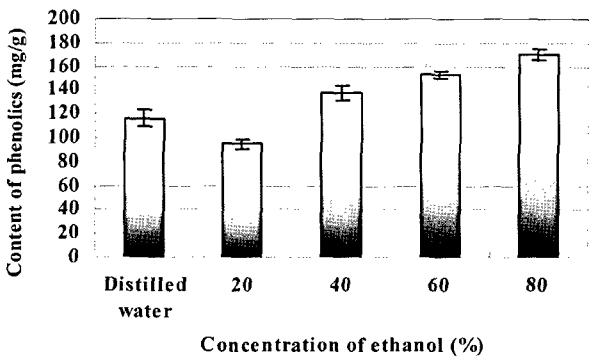

Fig. 1. Effect of ethanol concentration on extraction of total phenolic compound from fruits of *Morus alba* L.

other extraction types. Among the 16 phenolic compounds, trans-cinnamic acid were detected at trace level in all extraction type and 6 phenolic compounds (chlorogenic acid, narigin, syringic acid, quercetin, naringenin, kampferol) has a higher individual phenolic compound content in the 60% ethanol extraction than 80% ethanol extract (Table 3). Enhanced levels of TPs and phenolic compounds in cereal extracted by 60% ethanol extract as compared to other extraction methods were recently reported by Zielinski & Kozlowska (2000), which is in agreement with our result, where 60% ethanol extraction was found to have higher contents of quercetin and kampferol among all four treatments. Flavonols such as quercetin and kampferol are antimicrobial compounds synthesized by plants in response to pathogen attack (Macheix *et al.*, 1990). As organically and sustainably grown products were produced by cultural methods utilizing no or very little pesticides, the pathogenic pressures may explain the higher TP levels found in the organically and sustainably grown samples. Genstinic acid in 80% ethanol extract (1865 $\mu g \cdot g^{-1}$) had the highest content of the 16 individual phenolic compounds in the all extraction type. We found that genstinic, caffeic and salicylic acids were significantly proportional with increasing concentrations of ethanol, but hesperidin acid showed no significant differences in all extraction type. Ferulic acids and hyricetin, were increased in the 40% ethanol extract but, naringin increased 5.34 and 6.56 $\mu g \cdot g^{-1}$ in the 40% and 60% ethanol concentration respectively (Table 3). However, that the amount of p-hydroxy bezoic acid and coumaric acid increased in water extract at similar level as compared to 80% ethanol extract.

Angiotensin I-Converting Enzyme inhibitory activity of mulberry extracts

One of the long-term complications of diabetes is hypertension Angiotensin I-Converting Enzyme (ACE) is an important enzyme involved in maintaining vascular tension. ACE activates a histidyl-leucine dipeptide called angiotensin I into a potent vasoconstrictor called angiotensin II (Skeggs *et al.*, 1956). Angiotensin II also stimulates the synthesis and release of aldosterone, which increase blood pressure by promoting sodium retention in the distal tubules (Lieberman, 1975). Hypertension is a risk factor for many cardiovascular diseases and is also associated with long term diabetes. Control of hypertension via modulation of ACE by dietary anti-hypertensive agents is an important strategy to manage this risk factor.

A method for estimating the ACE inhibitory activity was developed by using hippuryl-L-histidyl-L-leucine as a substrate, based on the fact that it corresponds to the carboxy terminus of angiotensin I. The hydrolysis product (hippuric acid) was determined by a spectrophotometeric assay. In this study, we investigated the anti-hypertensive potential of various type of extracts of mulberry enriched for phenolic content by ethanol concentration. Therefore, the phenolic rich extracts of mulberry may have potential as a source of anti-ACE agents for control of hypertension, a known complication of long-term diabetes and/or hyperglycemia. Here, aqueous phenolic-optimized extracts of mulberry were assayed. The results were analyzed with respect to the antioxidant activities of the extracts.

Table 4. Effect of inhibition on angiotensin converting enzyme by fruit water and ethanol extract from fruit of *Morus alba* L

	ACE inhibitory activity (%)			
Water extract	Etanol extracts (%)			
	20	40	60	80
8.4 ± 0.2	6.9 ± 0.1	7.4 ± 0.5	8.2 ± 0.3	9.0 ± 0.7

*Mean ± S.E obtained from six experiments

Here, we investigated the ability of the various extracts and total phenolics on inhibiting the activity of rabbit lung ACE. The results indicated that 80% ethanol extract had the highest ACE inhibitory activity (9.0%) followed by water extract (8.4%) and 60% ethanol extract (8.2%) (Table 4).

ACE inhibitory activity of the extracts did not correlate well with the total phenolic content, antioxidant activity or the concentration of individual phenolics in the extracts such as hydroxy benzoic acid and coumaric acid (Table 3). However the ACE-inhibition activity of the ethanol extracts increased with the increase of total phenolic contents.

Anti-hypertensive drugs have been isolated from a number of plant species (Actis-Goretta *et al.*, 2003). It is now believed that screening plant extracts for inhibition of ACE will be an effective method to search for new anti-hypertensive agents (Wagner *et al.*, 1991).

Anti-oxidative and free radical scavenging activity of mulberry extracts

Table 5 and Fig. 2 shows the initial screening of the anti-oxidative and free radical scavenging activity of mulberry extracts by various ethanol concentration. In the lipid peroxidation assay using the TBA method, all extract showed more than 40% inhibition at a concentration of 100 $\mu g \cdot m\ell^{-1}$ or 80% inhibition at 100 $\mu g \cdot m\ell^{-1}$ in water extract. Fig. 2 shows the anti-oxidative activity of water mulberry extracts and reference compounds such as a-tocopherol and BHT showed good dose-response relationships (Fig. 2).

In the DPPH free radical assay, water mulberry extracts showed more than 50% inhibition at a concentration of 50 $\mu g \cdot m\ell^{-1}$. To investigate IC_{50} values of plant extracts showing high biological activities, the dose response relationship was studied. BHT was the most potent inhibitor of TBA-reactive material formation. IC_{50} value of BHT was 1.5 $\mu g \cdot m\ell^{-1}$, whereas, compound such as a-tocopherol showed 33.6 $\mu g \cdot m\ell^{-1}$. IC_{50} values of the water extracts selected were 28.3 $\mu g \cdot m\ell^{-1}$, which showed more potent activity than α-tocopherol.

IC_{50} values of other ethanol extracts were 48.2 $\mu g \cdot m\ell^{-1}$ for 20% ethanol extract, 50.7 $\mu g \cdot m\ell^{-1}$ for 40% ethanol extract, 38.5 $\mu g \cdot m\ell^{-1}$ for 60% ethanol extract, 42.2 $\mu g \cdot m\ell^{-1}$ for 80% ethanol extract.

Table 5. DPPH free radical scavenging activity of various concentration ethanol extract in fruit of *Morus alba* L

Extracts and reference Compound	$IC_{50}^{\dagger}$ ($\mu g/m\ell$)
water extract	28.3
20% ethanol extract	48.2
40% ethanol extract	50.7
60% ethanol extract	38.5
80% ethanol extract	42.2
α-tocopherol	34.1
BHT	1.5

†Amount required for 50% reduction of DPPH after 30 min

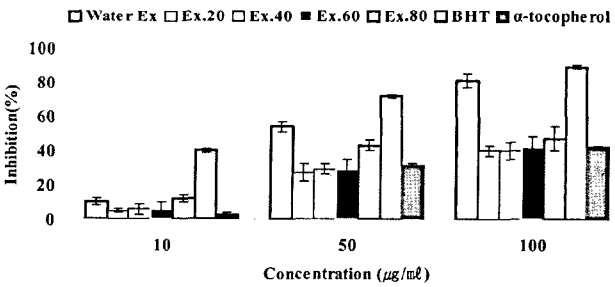

Fig. 2. Antioxdative activity of water and various ethanol concentration extract determined by Feton's reagent/ ethyl linoleate system in fruit of *Morus alba* L.

Natural anti-oxidants are usually phenolic or polyphenolic compounds and include tocopherol, flavonoid and cinnamic acid derivatives (Ames, 1983). It is known that there are two types of anti-oxidant. The first type of anti-oxidant inhibits the formation of free radicals which may initiate oxidation. In most cases, they are chelators of metal ions such as flavonoids. The second type of anti-oxidant inhibits the free-radical chain propagation reactions. Therefore, some plant extracts may act at the initiation stage of peroxidation, interfering with Fenton's reaction, thus breaking the chain reaction.

Free radical damage of biosystems is one of the major processes that contributes to degenerative diseases like cancer and ageing (Davies, 1991).

The ability of phenolics to inhibit the DPPH radical formation was measured both in water and ethanol extracts. The water extracts of mulberry had the highest DPPH radical inhibition activity followed by 60% and 80% ethanol extract (Table 5). The results indicated that the DPPH scavenging activity of 60% and 80% ethanol extracts was directly proportional to the total soluble phenolic content in them (Table 5). However, 20% and 40% ethanol extract showed low DPPH scavenging activity with higher total phenolic content than

Table 6. Effect of inhibition on xanthine oxidase by water and ethanol extracts from fruit of *Morus alba* L

Xanthine oxidase inhibition activity (%)				
Water extract	Ethanol extracts (%)			
	20	40	60	80
23 ± 0.8	46 ± 0.7	53 ± 1.4	72 ± 1.9	85 ± 0.7

water extracts. This could be due to high content of hydroxy benzoic acid, chlorogenic acid, cinnamic acid (Table 3). This suggests that the profile of individual phenolics in the extracts may be more important in contributing to the antioxidant activity than the total phenolics content.

Therefore, higher antioxidant activity of the phenolic antioxidants from the water and 60% ethanol extract in two assays suggest a possible biological functionality in preventing the oxidative degradation of membrane lipids.

In this study, it has been demonstrated that several water and ethanol extracts have potent antioxidative activity and/or free radical scavenging activity. However, we do not know clearly what components in the extracts show these biological activities and isolating the active compounds from each extract type is now underway.

Superoxide-scavenging activities of mulberry extracts

Superoxide radicals are one of the most important reactive oxygen free radicals constantly produced in living cells (Halliwell & Gutteridge, 1993; Winterbourn & Kettle, 2003). The radicals may also play an important role during the peroxidation of unsaturated fatty acids and possibly other susceptible substances (Nice & Robinson, 1992). One of the methods of generating superoxide radicals is by xanthine-xanthine oxidase (XO) assay, where XO enzyme catalyses the oxidation of xanthine to uric acid. Therefore, the study of the scavenging effects of mulberry extracts on superoxide is one of the most important ways of illustrating the mechanism of antioxidant activity.

Superoxide-scavenging activities of water extract and various concentration of ethanol extract of mulberry were measured using the xanthine-xanthine oxidase system and the results indicate the high inhibition rate of superoxide activity. As shown in Table 6, each mulberry extract exhibited superoxide-scavenging activity and these activities showed ethanol concentration dependence. Regarding to mulberry extracts, ethanol extracts exhibited higher superoxide scavenging activity than water extract. These results show that mulberry extracts have strong scavenging effects on superoxide radicals. Xanthine oxidase inhibitory activity of the extracts correlated well with the total phenolic content, which was modulated by the concentration of individual phenolics harboring hydroxyl residue in the extracts such as *p*-hydroxy benzoic acid, caffeic acid, *p*-coumaric acid (Table 3).

It can be concluded that mulberry fruit could receive adequate attention as food ingredient even for human nutrition and that the extraction with water and ethanol could be a possible way for obtaining more suitable food source by the increased content of bioactive phenolic compounds, which, on the other hand, could be recovered for potential industrial or medical utilizations. Also, this result suggests that mulberry fruit may be a safe and effective functional health prompting supplements for the prevention and treatment of various diseases.

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