ELSEVIER

Food Chemistry 64 (1999) 555–559

Food Chemistry

Analytical, Nutritional and Clinical Methods Section

The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals

Jia Zhishen,* Tang Mengcheng, Wu Jianming

Chemistry Laboratory, Department of Basic Course, Zhejiang Agricultural University, 310029 Hangzhou, People's Republic of China

Received 29 August 1997; received in revised form 30 April 1998; accepted 30 April 1998

Abstract

Flavonoid content of mulberry leaves of 19 varieties of species, determined spectrophotometrically in terms of rutin equivalent, varied from 11.7 to 26.6 mg g^{-1} in spring leaves and 9.84 to 29.6 mg g^{-1} in autumn leaves. Fresh leaves gave more extract than air-dried or oven-dried ones. HPLC showed that mulberry leaves contain at least four flavonoids, two of which are rutin and quercetin. The percentage superoxide ion scavenged by extracts of mulberry leaves, mulberry tender leaves, mulberry branches and mulberry bark were 46.5, 55.5, 67.5 and 85·5%, respectively, at a concentration of 5 μg ml^{-1}. The scavenging effects of most mulberry extracts were greater than those of rutin (52.0%).

1. Introduction

For some time it has been recognized that several classes of flavonoids show antioxidant activity towards a variety of readily-oxidizable compounds. Flavonoids exist widely in the plant kingdom and are especially common in leaves, flowering tissues and pollens (Larson, 1988). Plant flavonoids are an important part of the diet because of their effects on human nutrition (Frankel, 1995). These phytochemicals can modulate lipid peroxidation involved in atherogenesis, thrombosis and carcinogenesis. Known properties of the flavonoids include: free radical scavenging, strong antioxidant activity, inhibition of hydrolytic and oxidative enzymes (phospholipase A_2, cyclooxygenase, lipoxygenase), and antiinflammatory action (Frankel, 1995). Some evidence suggests that the pharmacological effects of flavonoids are correlated with their antioxidant activities (Gryglewski et al., 1987). Superoxide radicals have been observed to kill cells, inactivate enzymes and degrade DNA, cell membranes and polysaccharide (Fridovich, 1978). Moreover, it is suggested that the overall antioxidant effect of flavonoids on lipid peroxidation may be related to their ·OH and $O_2^{-}\cdot$ scavenging properties and their reaction with peroxy radicals (Husain et al., 1987). Thus, $O_2^{-}\cdot$ may play an important role during the peroxidation of unsaturated fatty acids and possibly other susceptible substances (Nice and Robinson, 1992). Therefore, the study of the scavenging effects of antioxidants on $O_2^{-}\cdot$ is one of the most important ways of making clear the mechanism of antioxidant activity and has therefore caused growing interest among researchers.

Flavonoids can be used directly to scavenge $O_2^{-}\cdot$ and ·OH by single electron transfer. The scavenging process can generally be followed by means of electron spin resonance (ESR) (Chen et al., 1989; Dan et al., 1989), but the expense of such instruments hinders their use by the average laboratory. The photochemical reduction of riboflavin was first used to determine the dismutation of $O_2^{-}\cdot$ by superoxide dismutase (SOD) (Beauchamp and Fridovich, 1971) and has been adapted for analysis of the dismutation of $O_2^{-}\cdot$ by a model compound of superoxide dismutase and other natural compounds (Luo et al., 1990).

Although the mulberry mainly supplies leaves to raise silkworms, mulberry leaves, mulberry bark and mulberry branches have long been used in Chinese medicine to treat fever, protect the liver, improve eyesight, strengthen the joints, facilitate discharge of urine and lower blood pressure. It is only recently that the mechanism of their action has been related to their antioxidant activity. The chemical composition of mulberry leaves includes rutin, quercetin, isoquercitrin and other flavonoids.

* Corresponding author.

0308-8146/99/$—see front matter
PII: S0308-8146(98)00102-2

This article is mainly concerned with the method of extracting flavonoids from mulberry leaves and the determination of the content of flavonoids in the extracts. In parallel, the scavenging effects of mulberry extracts on the superoxide radical was analysed using the photochemical reduction of riboflavin. The confirmation of the antioxidant potential of flavonoids has made possible the exploitation of a natural antioxidant from superfluous mulberry leaves.

2. Materials and methods

2.1. Materials

Mulberry leaves were obtained from the sample sections of Zhejiang Agricultural University. Spring mulberry leaves were picked on 5 May, 1996 and autumn mulberry leaves on 11 November, 1996. The mulberry leaves were washed with distilled water and air-dried. The dry leaves were ground in a mortar, sieved through a 40-mesh sieve and stored in a glass bottle.

2.2. Determination of percentage dry weight

Between 30 and 50 g of fresh leaves were accurately weighed and dried to constant weight in an oven at 100–105°C for 4 hours.

2.3. Extraction of flavonoids

Powdered air-dried or oven-dried leaves (1 g) were extracted in a Soxhlet extractor with 100 ml distilled water or ethanol for 1 hour and the extract filtered. Fresh leaves (1.4 g) were cut into small pieces before extraction.

2.4. Determination of flavonoids

A known volume of extract was placed in a 10 ml volumetric flask. Distilled water was added to make 5 ml, and 0.3 ml $NaNO_2$ (1:20) were added. 3 ml $AlCl_3$ (1:10) were added 5 min later. After 6 min, 2 ml 1 mol litre^{-1} NaOH was added and the total was made up to 10 ml with distilled water. The solution was mixed well again and the absorbance was measured against a blank at 510 nm with a M8500 UV-VISIBLE spectrophotometer (Taizhou Radio Factory) (Zhuang et al., 1992). Since the rutin content is high in mulberry leaves, it was used as the standard for a calibration curve. The flavonoid content was calculated using the following linear equation based on the calibration curve:

$$A = 0.01069C - 0.001163,\ r = 0.9998$$

where A is the absorbance

C is the flavonoid content in μg g^{-1}.

2.5. Separation of flavonoids in mulberry by HPLC

Reversed-phase high-performance liquid chromatography was used for the separation and determination of rutin and quercetin in the extracts of mulberry leaves. The extract (3 ml) was filtered through a 0.45 μm membrane, and 10 μl filtrate was injected into the HPLC. The instruments used were a P100HPLC system and DL-80 chromatographic work station (National Chromatography Research Centre). The column used was YWG-C_{18}, 10 μm, 250 mm×4.6 mm i.d. (National Chromatography Research Centre) and the eluent was MeOH–H_2O–H_3PO_4 (60:40:0·4 v/v). The flow rate was 1.0 ml min^{-1}. The column temperature was 25°C.

2.6. Determination of the scavenging of superoxide radical

Superoxide radicals were generated by a modified method based on that of Beauchamp and Fridovich (1971). All solutions were 0.05 mol litre^{-1} in phosphate buffer (pH 7.8). The photo-induced reactions were performed in an aluminum foil-lined box with two 20 W fluorescent lamps. The distance between reactant and lamp was adjusted until the intensity of illumination reached 4000 lx. The total volume of reactant was 5 ml and the concentrations of riboflavin, methionine and nitro blue tetrazolium (NBT) were 3×10^{-6} mol litre^{-1}, 1×10^{-2} mol litre^{-1} and 1×10^{-4} mol litre^{-1}, respectively. The reactant was illuminated at 25°C for 25 min. The photochemically-reduced riboflavins generated $O_2^{-\cdot}$ which reduced NBT to form blue formazan. The unilluminated reaction mixture was used as a blank. Absorbance (A) was measured at 560 nm. Mulberry extracts or other flavonoids were added to the reaction mixture, in which $O_2^{-\cdot}$ was scavenged,thereby inhibiting the NBT reduction. Absorbance (A_1) was measured and the decrease in $O_2^{-\cdot}$ was represented by $A-A_1$. The degree of scavenging was calculated by the following equation:

$$\text{Scavenging}\ (\%) = (A - A_1/A) \times 100\%$$

3. Results and discussion

3.1. Effects of different pretreatments on the flavonoid content extracted from mulberry leaves

The amount of flavonoids extracted from fresh leaves, oven-dried and air-dried leaves was determined as above (Table 1).

The results indicate that fresh leaves allow the largest amount to be extracted, air-dried leaves rank second and oven-dried leaves come last. The order may be explained by the decomposition of flavonoids after a

Table 1
Flavonoids extracted after different pretreatments

Mulberry variety	Flavonoids extracted (mg g^{-1} dry wt)		
	Fresh leaves	Oven-dried	Air-dried
Xin Yi Yuan Lai (*Morus alba* L.)	15.3	11.5	14.8
Hei You Sang (*Morus alba* L.)	29.6	25.0	29.5
Jing Bai Sang (*Morus alba* L.)	23.4	19.3	20.9

long period of storage or under high temperature. However, there is a possibility that the matrix has changed to make the flavonoids less extractable.

3.2. Qualitative analysis of flavonoids in the mulberry

Fig. 1 shows the HPLC of flavonoid extracts and of standard rutin and quercetin. Comparing the two chromatograms shows that the flavonoids extracted from the mulberry contained both rutin and quercetin, the content of rutin being the higher. Some other flavonoids were not well separated and have not been identified so far, further research being needed.

3.3. Flavonoid contents in different mulberry varieties and species

The respective flavonoid contents in six varietal species of spring mulberry leaves and 13 varietal species of autumn leaves were determined (Tables 2 and 3).

Tables 2 and 3 show that the flavonoid content differs with variety and species. The flavonoid content in spring leaves lies between 11.7 and 26.6 mg g^{-1} and that in autumn leaves between 9.84 and 23.4 mg g^{-1}. The flavonoid content in spring leaves is higher than that in autumn leaves of the same mulberry variety species, spring leaves being mature whereas autumn leaves are senescent.

In summary, leaves of all species of mulberry contain flavonoids. It is worth pointing out that autumn mulberry leaves, even though possessing less flavonoids, provide useful material for extraction, as such leaves are generally not used to feed silkworms.

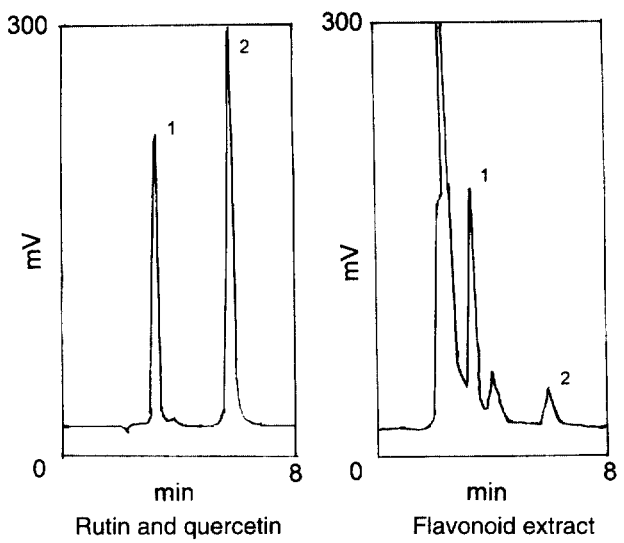

Fig. 1. Chromatograms of standard rutin and quercetin and of a flavonoid extract. 1. rutin, 2. quercetin.

Table 2
Flavonoid content in spring mulberry leaves

Mulberry variety and species	Original place	Flavonoid content (mg g^{-1} dry wt)
He Ye Bai (*Morus multicaulis* Perr.)	Zhejiang	18.7
Xin Yi Yuan Lai (*Morus alba* L.)	Japan	26.6
TieYe Huang Lu (*Morus multicaulis* Perr.)	Shandong	24.3
Nan Chong Zao Sheng (*Morus alba* L.)	Sichuan	20.7
Tong Xiang Qing (*Morus multicaulis* Perr.)	Zhejiang	26.6
Lun Jiao (*Morus atropurpurea* Roxb.)	Guangdong	11.7

Table 3
Flavonoid content in autumn mulberry leaves

Mulberry variety and species	Original place	Flavonoid content (mg g^{-1} dry wt)
Xin Jiang Bai Sang (*Morus alba* L.)	Xin Jiang	9.84
Gai Liang Shu Fan (*Morus alba* L.)	Japan	13.8
Xin Yi Yuan Lai (*Morus alba* L.)	Japan	15.3
Huang Lu Sang (*Morus multicaulis* Perr.)	Shandong	14.3
Lun Jiao (*Morus atropurpurea* Roxb.)	Guangdong	17.2
Qun Ma Chi Mu (*Morus bornbycis* Koidz.)	Japan	12.4
Da Ji Guan (*Morus multicaulis* Perr.)	Shandong	18.3
Jing Bai Sang (*Morus alba* L.)	Heilongjiang	23.4
Tong Xiang Qing (*Morus multicaulis* Perr.)	Zhejiang	14.9
He Ye Bai (*Morus multicaulis* Perr.)	Zhejiang	22.5
Hu Sang (*Morus multicaulis* Perr.)	Zhejiang	12.5
Huang Sang (*Morus multicaulis* Perr.)	Zhejiang	14.3
Huo Sang (*Morus bornbycis* Koidz.)	Zhejiang	10.8

3.4. The scavenging effects of flavonoids extracted from mulberry leaves on superoxide radical

The superoxide radicals were generated by illuminating a solution. The relative scavenging effects of mulberry flavonoids were assessed. The results are illustrated in Fig. 2.

All the flavonoid extracts had a scavenging action on superoxide radicals, the highest scavenging ability being exhibited by the flavonoids extracted from mulberry branches. The effect of flavonoids extracted from mulberry tender leaves is greater than that of flavonoids extracted from mulberry leaves. When the scavenging effect is 50%, the concentrations of mulberry leaves, mulberry bark, mulberry tender leaves and mulberry branches are 5.3, 4.3, 3.1 and 1.5 μg mlitre^{-1}, respectively.

The scavenging effects of rutin and quercetin on superoxide radical have already been established by the ESR method (Chen et al., 1989). The differeces in content of these two flavonoids in the mulberry samples are likely to be responsible for their varied scavenging effects.

3.5. The comparison of scavenging abilities of flavonoids extracted from different parts of mulberry trees with those of rutin and ascorbic acid

The scavenging effects of flavonoids extracted from mulberry leaves, rutin and ascorbic acid, on $O_2^{-\cdot}$ at a concentration of 5 μg mlitre^{-1}, were determined by the NBT method. The result (Table 4) indicates that the scavenging effects of rutin and ascorbic acid agree with the preceding report (Chen et al., 1989), which confirms the reliability of the NBT method. The flavonoids extracted from mulberry tender leaves, mulberry branches and mulberry bark proved to be better at scavenging $O_2^{-\cdot}$ than rutin and ascorbic acid. This may be explained by the interaction of the different flavonoids in these extracts. The flavonoids extracted from mulberry leaves are stronger scavengers than ascorbic acid.

Table 4
The comparison of scavenging abilities of flavonoids extracted from different parts of mulberry trees with that of rutin and ascorbic acid

	Leaves	Tender leaves	Branches	Bark	Rutin	Ascorbic acid
Scavenging (%)	46.5	67.5	85.5	55.5	52.0	38.2

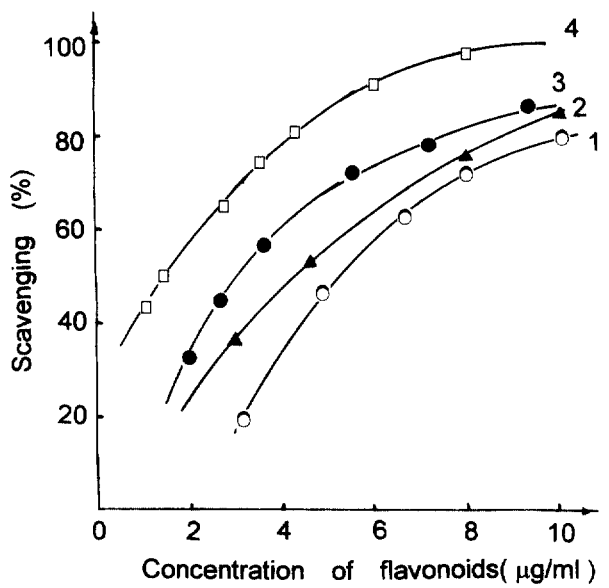

Fig. 2. The scavenging effects of flavonoids extracted from different parts of mulberry trees on superoxide radicals: 1. mulberry leaf; 2. mulberry bark; 3. mulberry tender leaves; 4. mulberry branches.

It has been reported that the flavonoid molecules with polyhydroxylated substitutions on rings A and B, a 2–3 double bond, a free 3-hydroxyl substitution and a 4-keto moiety, would confer potent antiperoxidative properties upon the compounds (Younes, 1981; Das and Pereira, 1990). The main flavonoids in mulberry leaves, rutin and quercetin, both contain a 3′,4′ ortho-position hydroxyl which provide active hydrogen to take part in the following reaction to scavenge $O_2^{-\cdot}$.

$$2H^+ + 2O_2^{-\cdot} = H_2O_2 + O_2$$

The superoxide dismutase participates the above reaction by catalysis, and as an antioxidant through the supply of hydrogen. Tajima used ESR to determine the scavenging effect of V_E on P_{450} and proved that the mechanism requires the supply of active hydrogen (Tajima et al., 1983). It is assumed that flavonoids from mulberry trees (e.g. rutin and quercetin) scavenge $O_2^{-\cdot}$ in the same way.

The results presented above demonstrate that mulberry leaves, mulberry tender leaves, mulberry branches and mulberry bark contain at least four flavonoids, including rutin and quercetin. The flavonoids extracted from mulberry parts have strong scavenging effects on superoxide radicals, which provide new evidence of their antioxidant effect. The extraction of antioxidants from waste mulberry leaves and branches makes comprehensive exploitation of the mulberry a possibility.

Acknowledgements

The authors wish to thank the Department of Sericulture for providing mulberry samples and Professor Lou Chenfu for identifying the mulberry species.

References

Beauchamp, C., & Fridovich, I. (1971). Superoxide dismutase: Improved assays and an assay applicable to acrylamide gels. *Analytical Biochemistry, 44*, 276–287.

Chen, Y. T., Li, X. J., Zao, B. L., Zheng, R. L., & Xin, W. J. (1989). ESR study of the scavenging effect of natural products of rutin etc superoxide and hydroxyl radical. *Acta Biophysica Sinica, 5*, 235–240.

Dan, N., Li, X. J., Zhao, B. L., Zhang, T. M., & Xin, W. J. (1989). Scavenging effects of probimane on active oxygen free radicals by electron spin resonance. *Acta Pharmacologica Sinica, 10*, 443–447.

Das, N. P., & Pereira, T. A. (1990). Effects of flaononids on thermal autoxidation of Plam oil: structure–activity relationship. *Journal of the American Oil Chemists' Society, 67*, 255–258.

Frankel, E., (1995) Nutritional benefits of flavonoids. *International conference on food factors: Chemistry and cancer prevention*, Hamamatsu, Japan. Abstracts, C6-2.

Fridovich, I. (1978). The biology of oxygen radicals. *Science, 201*, 875–880.

Gryglewski, R. J., Korbut, R., & Robak, J. (1987). On the mechanism of antithrombotic action of flavonoids. *Biochemical Pharmacology, 36*, 317–321.

Husain, S. R., Cillard, J., & Cillard, P. (1987). Hydroxyl radical scavenging activity of flavonoids. *Phytochemistry, 26*, 2489–2491.

Larson, R. A. (1988). The antioxidants of higher plants. *Phytochemistry, 27*, 969–978.

Luo, Q. Q., Sen, M. C., Gao, W., & Peng, Q. (1990). A study on reaction kinetics of superoxide dimutase and its model compounds with superoxide ion by illumination. *Chemical Journal of Chinese Universities, 11*, 928–932.

Nice, D. J., & Robinson, D. S. (1992). Inhibition of lipid autoxidation by bovine superoxide dismutase. *Food Chemistry, 45*, 99–103.

Tajima, K., Sakamoto, M., & Okada, K. (1983). Reaction of biological phenoic antioxidants with superoxide generated by Cytochrome P_{450} model system. *Biochemical and Biophysical Research Communications, 115*, 1002–1008.

Younes, M. (1981). Inhibitory action of some flavonoids on enhanced spontaneous lipid peroxidation following glutathione depletion. *Planta Medica, 43*, 240–245.

Zhuang, X. P., Lu, Y. Y., & Yang, G. S. (1992). Extraction and determination of flavonoid in ginkgo. *Chinese Herbal Medicine, 23*, 122–124.