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Antioxidative activity of three herbal water extracts

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The antioxidative activity of water extracts of three herbs, including the flower of *Chrysanthemum morifolium* Ramat (FCMR), the calyx of *Hibiscus sabdariffa* L. (CHSL) and roasted seed of *Hordeum vulgare* L. (RSHVL), which are commonly called Hang Chu, Lo Shen and Chao Mai in Taiwan, respectively, were investigated. FCMR, CHSL and RSHVL showed marked antioxidative activity, not only in linoleic acid but also in liposome model systems, indicating that the three herbal water extracts may protect the cell from damage by lipid peroxidation. FCMR, CHSL and RSHVL possessed high contents of phenolic compounds and exhibited reducing power, revealing that these herbal extracts may contain reductones. The water extracts of the three herbs also showed good hydrogen-donating abilities, indicating that they had effective activities as radical scavengers. No mutagenicity in the water extracts of the three herbs was found in *Salmonella typhimurium* TA98 and TA100, either with or without S9 mix.

INTRODUCTION

Autoxidation of fats and oils not only lowers the nutritional value of food, but is also associated with aging, membrane damage, heart disease, stroke, emphysema and cancer in living organisms (Marx, 1987; Addis & Warner, 1991). The addition of antioxidants to food is effective in retarding the oxidation of fats. It is impressive that many substances have been identified which prevent lipid peroxidation. Some of these compounds are synthetic antioxidants, and others occur as natural dietary constituents.

Much attention has been focused on the antioxidative compounds present in edible plants, because of some safety concerns in synthetic antioxidants (Imaida *et al.*, 1983). In Asia, some herbs have long been used to prevent and/or cure certain diseases. For example, tea has been used as a daily beverage and a crude medicine in China for thousands of years. Some literature reported that the pharmacological effects of tea include antioxidative activity (Satoshi & Hara, 1990), an antimutagenic effect (Kada *et al.*, 1985; Jain *et al.*, 1989; Yen & Chen, 1995) and anticancer effects (Isao, 1990). Other herbs, in Taiwan, such as the flower of *Chrysanthemum morifolium* Ramat (FCMR), the calyx of *Hibiscus sabdariffa* L. (CHSL) and roasted seed of *Hordeum vulgare* L. (RSHVL), are usually processed as drinks, which are commonly called Hang Chu, Lo Shen and Chao Mai, respectively. However, whether these three herbal drinks possess pharmacological effects such as antioxidative activity or toxic side effects is still unclear. Thus, the aims of this work were to evaluate the antioxidative activity and mutagenicity of water extracts of these three herbs.

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MATERIALS AND METHODS

Materials

Three different kinds of dried herbs, including the flower of CMR, and the calyx of HSL were dried by sunlight, while the seeds of *Hordeu vulgare* L. were roasted and named RSHVL. These dried herbs were purchased from local oriental herbal stores in Tainan, Taiwan.

Chemicals

Linoleic acid and catechin were purchased from Sigma Co. (St. Louis, MO). 1,1-Dipheny-2-picrylhydrazyl (DPPH) was obtained from Fluka Chemie AG (Switzerland). DL-α-Tocopherol was purchased from E. Merck (Darmstadt, Germany).

Extraction

Each herb (20 g) was extracted with boiling water (600 ml) for 10 min, and the filtrate was evaporated in a vacuum below 70°C on a rotary evaporator. The yields of crude extracts for FCMR, CHSL and RSHVL were 5.59, 9.28 and 1.03 g. respectively. These crude extracts were dissolved in distilled water and used for the assessment of antioxidant activity and mutagenicity.

Determination of antioxidant activity in linoleic acid system

Antioxidant activity was carried out by using the linoleic acid system (Osawa & Namiki, 1981). Herbal extracts (5 mg) of each sample were added to a solution mixture of linoleic acid (0.13 ml), 99.8% ethanol (10 ml), and 0.2 M phosphate buffer (pH 7.0, 10 ml). The total volume was adjusted to 25 ml with distilled water. The solution was incubated at 40°C and the degree of oxidation was measured according to the thiocyanate method (Mitsuda *et al.*, 1966), with 10 ml of ethanol (75%), 0.2 ml of an aqueous solution of ammonium thiocyanate (30%), a 0.2 ml sample solution and a 0.2 ml ferrous chloride solution (20 mM in 3.5% HCl) being added sequentially. After stirring for 3 min, the absorption values of the mixtures measured at 500 nm were taken as the peroxide contents. The percent inhibition of linoleic acid peroxidation, 100-[(Abs increase of sample/Abs increase of control) × 100] was calculated to express antioxidative activity. All test data are the averages of triplicate analyses.

Determination of antioxidative action in a liposome model system

Egg lecithin (300 mg) was sonicated in an ultrasonic cleaner (Branson 8210, Branson Ultrasonic Corporation, USA) with 30 ml phosphate buffer (10 mM, pH 7.4) for 2 h. The water extracts (1.0 mg) of each sample were added to a solution mixture of sonicated solution (0.5 ml, 10 mg/ml), $FeCl_3$, (0.5 ml, 400 mM), ascorbic acid (0.5 ml, 400 mM). The antioxidative action was measured by the method of Buege and Aust (1978) after incubation for 1 h at 37°C. The absorbance of the sample was determined at 535 nm. The results were expressed in nmoles of malondialdehyde (MDA) per mg lipid and were calculated by using an extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}. All test data are the averages of triplicate analyses.

Determination of reducing power

The reducing power of water extracts was determined according to the method of Oyaizu (1986). Water extracts (2.5–15.0 mg) were mixed with phosphate buffer (5.0 ml, 2.0 M, pH 6.6) and potassium ferricyanide (5.0 ml, 1.0%); the mixture was incubated at 50°C for 20 min. A portion (5.0 ml) of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 650 × *g* for 10 min. The upper layer of the solution (5 ml) was mixed with distilled water (5 ml) and ferric chloride (1 ml, 0.1%), and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power.

Determination of total phenolic compounds

The total phenolic compounds present in herbs were determined spectrophotometrically using Folin–Denis reagent (AOAC, 1984). The water extracts (0.1 ml) in a volumetric flask were diluted with glass-distilled water (75 ml). Folin–Denis reagent (5 ml) was added and the contents of the flask mixed thoroughly. After 3 min, Na_2CO_3 solution (10 ml, 10%, w/v) was added. The contents were mixed and diluted to volume with water. The blue colour produced was measured spectrophotometrically at 760 nm. The concentration of the total phenolic compounds in herbal water extracts was determined by comparison with the absorbance of standard, catechin, at different concentrations.

Scavenging effect on DPPH radical

The effect of herbal water extracts on DPPH radical was estimated according to the method of Hatano *et al.* (1988). The herbal water extracts were passed through the cartridge (Sep-Pak C_{18}, Waters) with a syringe; the eluates were repeated twice under the same condition. The extracts decolorized were added to a methanolic solution (1 ml) of DPPH radical (final concentration of DPPH was 0.2 mM). The mixture was shaken vigorously and left to stand at room temperature for 30 min; the absorbance of the resulting solution was measured spectrophotometrically at 517 nm.

Mutagenicity assay

The mutagenicity assays of herbal water extracts were performed according to the Ames test, with 20 min pre-incubation at 37°C (Maron & Ames, 1983). The histidine-requiring strains of *Salmonella typhimurium* TA98 and TA100 were kindly supplied by Dr B. N. Ames (UC, Berkeley). The S9 mix (Organ Teknika Co., Switzerland), was prepared from Sprague-Dawley male rats treated with Aroclor 1254, according to Ames *et al.* (1975). Diluted herbal water extracts (0.05–5.0 mg $plate^{-1}$) were added to the overnight-cultured *Salmonella typhimurium* TA98 or TA100 (0.1 ml) and S9 mix (0.5 ml) or phosphate buffer (0.1 ml) in place of the S9 mix. The entire mixture was pre-incubated at 37°C for 20 min before molten top agar (2 ml) was added; the mixture was poured on a minimum agar plate. The his^+ revertant colonies were counted after incubating at 37°C for 48 h. Assay of each sample was determined in triplicate plates

per run and data presented are means ± SD of three determinations. Two runs of a single experiment were performed to validate the reproducibility. The mutagenicity is expressed as the number of revertants per plate, at a given concentration of each sample. In this mutagenicity testing, the result was recognized as positive when the number exceeds twice the number of spontaneous revertants (Ames *et al.*, 1975).

Statistical analysis

Statistical analysis involved use of the Statistical Analysis Systems (SAS, 1985) software package. Analysis of variance was performed by ANOVA procedures. Significant differences between means were determined by Duncan's multiple-range tests.

RESULTS AND DISCUSSION

The data of linoleic acid peroxidation, determined by the thiocyanate method, at 40°C after the addition of extracts of FCMR, CHSL and RSHVL, respectively, are plotted in Fig. 1. In the early stages, the autoxidation of linoleic acid without added herbal water extracts was accompanied by a rapid increase of peroxide value at 3 days of testing. Significant differences ($P < 0.5$) were found between the control and the linoleic acid containing herbal water extracts, which slowed the rate of peroxide formation. The antioxidative activities of herbal water extracts were compared with commercial antioxidants such as DL-α-tocopherol (Toc) and butylated hydroxyanisole (BHA). Among the three samples, 5.0 mg water extracts of FCMR, and CHSL showed stronger antioxidative activity than 200 ppm of Toc and BHA at twelve and a half days of testing; however, no significant difference ($P > 0.05$) was found between BHA and RSHVL, which was stronger than Toc. The main purpose of using antioxidants is to prolong the induction period of autoxidation, and thus to improve the oil stability (Wanasundara *et al.*, 1994). It is evident that addition of all the extracts significantly prolongs the induction period of linoleic acid, as shown by the low rate of accumulation of oxidative products as compared with commercial antioxidants.

To elucidate the formation and character of lipid peroxides in biological systems, the liposome model system was used to evaluate antioxidative activity (Tsuda, 1993). The antioxidative actions of herbal water extracts in the liposome system, induced by $FeCl_3$ plus ascorbic acid and determined by the thiobarbituric acid method, are shown in Fig. 2. Significant differences ($P < 0.05$) were found between the control and the liposome-containing herbal water extracts. All the water extracts at 1.0 mg show 1.05, 1.15 and 1.68 nmole MDA/mg lipid for FCMR, CHSL and RSHVL, respectively. However, the formation of the MDA for the control is 3.09 nmol MDA/mg lipid, indicating that the herbal water extracts of three samples show 66.0, 62.8 and 45.6% inhibition of peroxidation of lecithin. Moreover, each herbal water extract at 1.0 mg had a significantly lower nmol MDA/mg lipid than did the liposome containing 200 ppm of Toc. These results show that the herbal water extracts used have a strong antioxidative action both in the

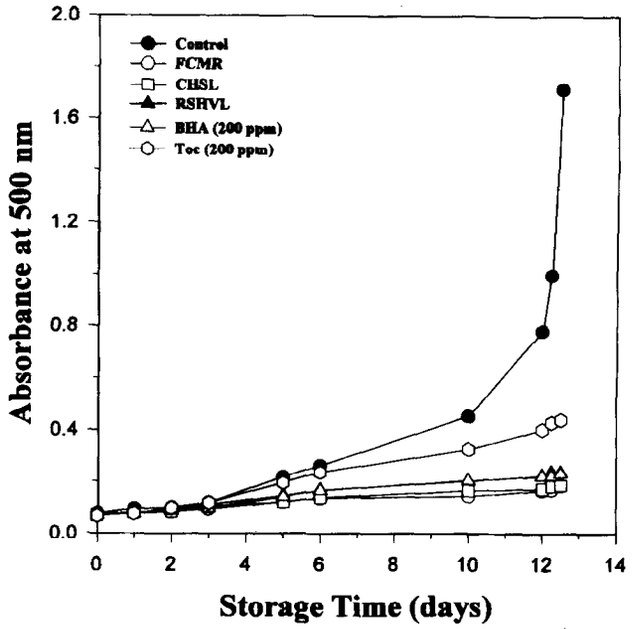

Fig. 1. Antioxidative activity of water extracts of herbs (5 mg), as measured by the thiocyanate method. FCMR, flower of *Chrysanthemum morifolium* Ramat; CHSL; calyx of *Hibiscus sabdariffa* Linn; RSHVL; roasted seed of *Hordeum vulgare* L; BHA, butylated hydroxyanisole; Toc: DL-α-tocopherol.

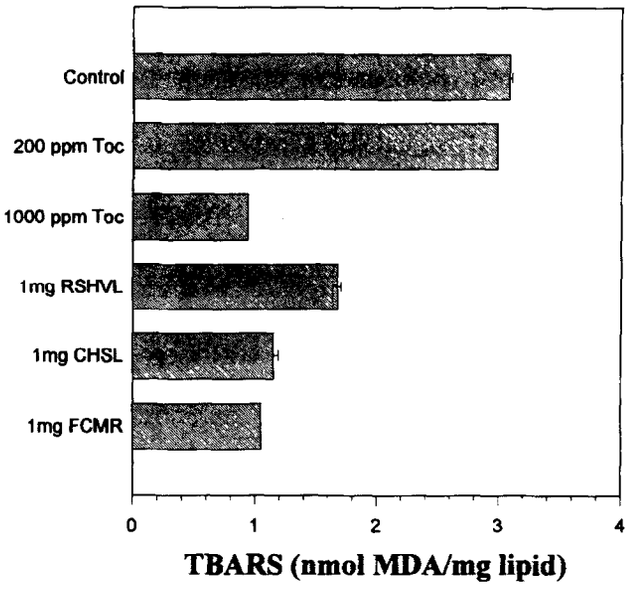

Fig. 2. Antioxidative action of water extracts of herbs in the liposome model system, as determined by thiobarbituric acid method. Lipid peroxidation was induced by $FeCl_3$ and ascorbic acid, compared with Toc. TBARS, thiobarbituric acid reactive substance; MDA, malondialdehyde; FCMR, flower of *Chrysanthemum morifolium* Ramat; CHSL, calyx of *Hibiscus sabdariffa* Linn; RSHVL, roasted seed of *Hordeum vulgare* L.

linoleic acid and in the liposome model systems. Okuda *et al.* (1983) reported that tannins and related compounds may prevent the destructive effects of lipid peroxide in liver cells by lowering the level of lipid peroxide in liver cells. Apparently, the three herbal water extracts used in this work may play an important role in protecting against damage to cell membrane function, as a result of lowering the level of lipid peroxide (Osawa *et al.*, 1985).

The antioxidative effectiveness in natural sources was reported to be mostly due to phenolic compounds (Hayase & Kato, 1984). Gutfinger (1981) and Ramarathnam *et al.* (1986) discovered that phenolic compounds play an important role in inhibiting autoxidation of the oils; Yen *et al.* (1993) reported that peanut hulls exhibited marked antioxidative activity as a result of their containing many phenolic compounds. Yen and Chen (1995) noted that polyphenols are the most abundant group of compounds in tea leaf and seem to be responsible for antioxidative activity. These investigations implied that the total phenolic compounds are closely related to antioxidative activity. From Table 1, the amounts of total phenolic compounds of water extracts of three herbs are in the order FCMR > CHSL > RSHVL. Although a significant difference ($P < 0.05$) in total phenolic compounds was found between FCMR and CHSL, the amount of total phenolic compounds of FCMR and CHSL are 11.9 and 9.9 times more than RHVL, respectively. Furthermore, significant differences ($P < 0.05$) in antioxidant activity were found between RSHVL and both FCMR and CHSL. Apparently, the contents of phenolic compounds seem to relate to the antioxidant activity. Yen *et al.* (1993) reported that peanut hulls with total phenolic compounds greater than 0.1671 mg g^{-1} of hulls displayed strong antioxidative activity. The amounts of total phenolic compound in the samples tested were greater than 0.1671 mg g^{-1} of hulls. Consequently, FCMR, CHSL and RSHVL exhibited strong antioxidative activity ($\geq$ 90%) during the oxidation of linoleic acid in an aqueous dispersion (Table 1). This result indicates that three herbal extracts containing high levels of total phenolic compounds may contribute to inhibition of lipid peroxidation.

Table 1. Total phenolic compounds of water extracts of three herbs and their antioxidant effects on inhibition of linoleic acid peroxidation

Herbs[a]	Total phenolics (mg g^{-1} of herbs)[b]	Antioxidant activity[e] (%)
FCMR	17.2 ± 0.160A[c,d]	93.3 ± 0.200A[b,c]
CHSL	14.4 ± 0.139B	93.0 ± 1.151A
RSHVL	1.45 ± 0.000C	90.0 ± 0.255B

[a]FCMR: flower of *Chrysanthemum morifolium* Ramat; CHSL: calyx of *Hibiscus sabdariffa* Linn; RSHVL: roasted seed of *Hordeum vulgare* L.
[b]Based on the dry weight of herb.
[c]Values are mean ± standard deviation of three replicate analyses.
[d]Means within a column with the same upper case letters are not significantly different ($P > 0.05$).
[e]The activity was determined by the thiocyanate method.

Okuda *et al.* (1983) elucidated that tannins in medicinal plants and drugs are effective against liver injury by inhibiting the formation of lipid peroxide owing to their reducing effect on coexisting substances, or by preventing their oxidation. Yen and Duh (1993) reported that the reducing power of methanolic extracts of peanut hulls containing high levels of polyphenols was significantly correlated to the extent of antioxidative activity. It appears that antioxidative activity may have a mutual correlation with the reducing effect. The reducing powers of various amounts of herbal water extracts are summarized in Table 2. The reducing power decreased in the order FCMR > CHSL > RSHVL. The water extracts of FCMR at 5.0 mg, as well as CHSL and RSHVL at 15.0 mg, exhibited a greater reducing power than 0.3 mg of ascorbic acid, which is a reducing agent as well as a reductone (Shimada *et al.* 1992). Therefore the water extracts of three samples tested were electron donors and can react with free radicals to convert them to more stable products and terminate radical chain reactions. Gordon (1990) reported that the antioxidative activity of reductones is believed to break radical chains by donation of a hydrogen atom. Tanaka *et al.* (1988) noted that the antioxidative effect exponentially increased as a function of the development of the

Table 2. Reducing powers of various amounts of water extracts of three herbs

Amounts (mg)	Reducing power[a]		
	FCMR[b]	CHSL	RSHVL
0.0	0.035 ± 0.000E[d,e]	0.035 ± 0.000E	0.035 ± 0.000E
2.5	0.554 ± 0.036D	0.388 ± 0.002D	0.278 ± 0.003D
5.0	0.936 ± 0.011C	0.648 ± 0.004C	0.449 ± 0.004C
7.5	1.321 ± 0.033B	0.792 ± 0.043B	0.567 ± 0.058B
15.0	3.032 ± 0.003A	1.146 ± 0.026A	0.900 ± 0.020A
Ascorbic acid[c]		0.871 ± 0.035	

[a]The reducing power of extracts was determined by absorbance at 700 nm with a spectrophotometer.
[b]Abbreviation as in Table 1.
[c]The amount of ascorbic acid was 0.3 mg.
[d]Values are mean ± standard deviation of three replicate analyses.
[e]Means within a column with the same upper case letters are not significantly different ($P > 0.05$).

reducing power, indicating that the antioxidative properties are concomitant with the development of the reducing power. Therefore, the marked antioxidative activities of the three herbal extracts may be concerned with their reducing power.

It is well known that free radicals play an important role in autoxidation of unsaturated lipids in foodstuffs (Kaur & Perkins, 1991). For example, oxidation of muscle cholesterol may be initiated by free radicals generated during the oxidation of polyunsaturated fatty acids (Hoelscher *et al.*, 1988). 1,1-Diphenyl-2-picrylhydrazyl (DPPH) was used as a free radical to evaluate antioxidative activity of some natural sources (Shimada *et al.*, 1992; Yen & Duh, 1995; Yen & Chen 1995). On the other hand, antioxidants are believed to intercept the free-radical chain of oxidations, and to contribute hydrogen from the phenolic hydroxyl groups themselves, thereby forming stable free radicals which do not initiate or propagate further oxidation of lipids (Sherwin, 1978; Dziezak, 1986). In the present work, the scavenging effects of the three herbal water extracts on the DPPH radical are summarized in Table 3. The scavenging activity of the herbal water extracts, on inhibition of the DPPH radical, was related to the amounts of the extracts added. The scavenging effects of the three herbal water extracts decreased in the order FCMR > CHSL > RSHVL. All the water extracts at 25.0 mg ml^{-1} show 91.9, 88.6 and 87.0% inhibition of the DPPH radical for FCMR, CHSL and RSVL, respectively, and significant differences ($P < 0.05$) were found among the samples, indicating that the three samples exhibited a potent scavenging effect on free radicals, although the data presented are less than 0.02 mg ml^{-1} of BHA. These results demonstrated that the three water extracts have effective activities as hydrogen donors and as primary antioxidants by reacting with the lipid radical. This may be responsible for the main cause of suppression of autoxidation, both in linoleic acid and the liposome model systems.

The three herbal water extracts used in this work are well-known prepared beverages in Asia. However, as far as health is concerned, a large amount of data and careful considerations on their safety are needed. The mutagenicity of the three herbal water extracts toward *Salmonella typhimurium* TA98 and TA100 were evaluated. From Tables 4 and 5, for testing doses at 0 to 5.0 mg per plate, no mutagenicity in *Salmonella typhimurium* TA98 or TA100, either with or without S9 mix, in the three herbal water extracts, was observed, indicating that all the samples tested do not induce base-pair substitution and frame-shift mutation toward *Salmonella typhimurium* TA98 and TA100.

In conclusion, we have demonstrated that water extracts of FCMR, CHSL and RHVL contain high

Table 3. Scavenging effects of water extracts of three herbs on 1,1 diphenyl-2-picrylhydrazyl (DPPH) radical

Concentration (mg ml^{-1})	Inhibition (%)[a]		
	FCMR[b]	CHSL	RSHVL
0.0	0.0 ± 0.00D[c,d]	0.0 ± 0.00D	0.0 ± 0.00D
5.0	68.6 ± 1.30C	49.9 ± 1.22C	47.7 ± 1.10B
15.0	81.4 ± 0.30B	77.1 ± 0.00B	72.9 ± 0.45B
25.0	91.9 ± 0.35A	88.6 ± 0.35A	87.0 ± 0.68A
BHA		94.4 ± 0.35	

[a]The concentration of BHA was 0.02 mg ml^{-1}.
[b]Abbreviation as in Table 1.
[c]Values are mean ± standard deviation of three replicate analyses.
[d]Means within a column with the same upper case letters are not significantly different ($P > 0.05$).

Table 4. Mutagenicity of water extracts of three herbs toward *S. typhimurium* TA98 either with or without S9 mix

Sample (mg $plate^{-1}$)	His^+ revertants/plate					
	FCMR[a]		CHSL		RSHVL	
	−S9	+S9	−S9	+S9	−S9	+S9
0.05	37 ± 1B[b,c]	56 ± 2A	39 ± 1A	55 ± 1A	38 ± 1B	55 ± 2A
0.25	38 ± 3B	56 ± 2A	38 ± 4A	57 ± 3A	38 ± 1B	55 ± 4A
0.50	38 ± 3B	57 ± 2A	37 ± 3A	57 ± 1A	37 ± 2B	56 ± 2A
1.00	37 ± 3B	57 ± 3A	38 ± 3A	58 ± 4A	38 ± 2B	56 ± 4A
2.00	38 ± 3B	58 ± 4A	38 ± 2A	57 ± 3A	38 ± 1B	55 ± 2A
5.00	37 ± 2B	57 ± 2A	38 ± 3A	57 ± 2A	37 ± 2B	56 ± 2A
SR[d]	40 ± 1A	57 ± 1A	40 ± 1A	57 ± 1A	40 ± 1A	57 ± 1A

[a]Abbreviation as in Table 1.
[b]Values are mean ± standard deviation of three replicate analyses.
[c]Means within a column with the same upper case letters are not significantly different ($P > 0.05$).
[d]Spontaneous revertants (SR) were obtained without water extracts of the three herbs.

Table 5. Mutagenicity of water extracts of three herbs toward *S. typhimurium* TA100 either with or without S9 mix

Sample (mg plate^{-1})	His$^+$ revertants/plate					
	FCMR[a]		CHSL		RSHVL	
	−S9	+S9	−S9	+S9	−S9	+S9
0.05	142 ± 7A[b,c]	160 ± 12A	141 ± 7A	158 ± 11B	140 ± 9A	161 ± 10A
0.25	141 ± 11A	159 ± 10AB	140 ± 8A	159 ± 9AB	138 ± 8A	160 ± 8A
0.50	139 ± 9A	157 ± 13B	140 ± 4A	157 ± 4B	140 ± 10A	158 ± 5A
1.00	140 ± 8A	160 ± 5A	139 ± 3A	159 ± 7AB	140 ± 7A	159 ± 4A
2.00	139 ± 10A	158 ± 7B	139 ± 5A	158 ± 10B	139 ± 8A	158 ± 11A
5.00	140 ± 11A	158 ± 14B	140 ± 9A	159 ± 10AB	140 ± 9A	157 ± 6A
SR[d]	141 ± 3A	160 ± 8A	141 ± 3A	160 ± 8A	141 ± 3A	160 ± 8A

[a]Abbreviations as in Table 1.
[b]Values are mean ± standard deviation of three replicate analyses.
[c]Means within a column with the same upper case letters are not significantly different ($P > 0.05$).
[d]Spontaneous revertants (SR) were obtained without water extracts of the three herbs.

levels of total phenolic compounds and show reducing power and scavenging effects on free radicals. These properties could, in part, explain the antioxidative activity of three herbal water extracts. In addition, according to the above, the water extracts of FCMR, CHSL and RSHVL not only do not induce mutagenicity, but also may play a major role as dietary antioxidants after ingestion, in the chemical protection against oxidative damage of cell membranes. However, further investigations are required to assay the antioxidant effect *in vivo* and to evaluate its relevance to human health.

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