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ANTIOXIDANT ACTIVITY OF THE POLYPHENOL MANGIFERIN

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ABSTRACT

The antioxidant properties of alcohol solutions of mangiferin were studied. The antioxidant activity in linoleic acid/water emulsion system, determined by the thiobarbituric acid reactive substances (TBARS), was higher at 37 ^{0}C – 72.0 %, and by inhibiting conjugated dienes formation – 70.4 %. At 80 ^{0}C the antioxidant activity at the highest concentration was less efficient: 67.2 % for conjugated dienes and 69.1 % for TBARS. Mangiferin inhibited the hydroxyl radicals by 84.3 % at 37 ^{0} C and 79.8 % at 80 ^{0}C, as it showed a higher antioxidant activity than quercetine. The IC_{50} concentration for the inhibition of OH· at 37 ^{0}C was lower than that at 80 ^{0}C – 0.88 μg/ml and 1.81 μg/ml, respectively. Mangiferin chelated Fe^{3+} in the solution. The inhibiting effect of mangiferin with regard to the xanthine oxidase activity was weaker – 71.4 %, as IC_{50} concentration for the reduction of nitroblue tetrazolium was 14.17 μg/ml.

KEYWORDS

mangiferin; lipid peroxidation; OH·; chelating capacity; superoxide radical

INTRODUCTION

The consumption of foods containing considerable amounts of polyunsaturated fatty acids makes the use of antioxidants to prevent their oxidation more significant. Adding antioxidants is a way of prolonging the storage of lipids and foods containing lipids. Besides, the addition of antioxidants to foods would help the human body reduce the losses from oxidation disturbances related to ageing and diseases, such as atherosclerosis, cancer, and cirrhosis. Although almost all organisms have an antioxidant protection and systems for correction and prevention of oxidation damages, these systems are insufficient to wholly prevent the damages.

Synthetic antioxidants, such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) are included in various foods, but their safety, however,can be doubted (Imadia et al., 1983). For this reason, he importance and demand for natural antioxidants has grown over the recent years. These antioxidants occur in all plants and in all parts of the plants (Kim et al., 1997). It is already well known that the antioxidant activity shown by plants is mainly due to the presence of polyphenol components, which are secondary products from the plant metabolism (Helle and Grete, 1995; Yen and Chen, 1995; Yen et al., 2003). The initiation of lipid peroxidation is induced by the superoxide radical or by hydroxyl radicals. Therefore, antioxidation is an extremely significant action, which can be used as a preventive agent against a number of diseases (Aruoma et al., 1989; Aruoma, 1994; Basaga, 1990; Basaga et al., 1997; Halliwell and Chirico, 1993). Polyphenols capture the free radicals by giving hydrogen atoms or electrons. Furthermore, their bioactivity may be related to the ability to chelate metals and inhibit lipooxygenases (Decter, 1995). Consequently, polyphenols can be used as natural preservatives, inhibiting the onset of lipid peroxidation and hydroperoxidation in foodstuffs and in living tissues.

Mango leaves (*Mangifera indica* L.) have been used by traditional medicine of a number of peoples for centuries. The key biological substance in them is a polyphenol called mangiferin. It has been established that it has antiviral and antitumor (Guha et al., 1996; Ngo, 2001; Zhu et al., 1993), spasmolytic (Tona et al., 2000), antidiabetic (Ichiki et al., 1998; Miura et al., 2001) and immunostimulating properties (Garcia et al., 2003). The study of its antioxidant properties would expand the scope of its application to the food industry as an inhibitor of lipid autooxidation.

The aim of this study is to assess the antioxidant properties of mangiferin, isolated from mango leaves with regard to the lipid peroxidation and hydroxyl radicals at body temperature – 37 ^{0}C and at 80 ^{0}C, as well as the inhibition of the superoxide radical.

MATERIAL AND METHODS

Materials. The mangiferin used in the study was provided by "VIMEDIMEX- II" Inc. from Republic of Vietnam. Mangiferin is a yellow odorless powder with a degree of purity of 97 %. Alcohol solutions of mangiferin were prepared with concentrations from 0.1 μg/ml to 30 μg/ml and from 0.005 % to 0.1 %.

Evaluation of antioxidant activity in linoleic acid model system. Linoleic acid emulsions were prepared by mixing 0.285 g of linoleic acid, 0.289 g of Tween 20 as emulsifier and 50 ml phosphate buffer (pH 7.2). The mixture was homogenized for 5 min (Yen et al., 2003). The antioxidant was added at the final concentrations of 0 %, 0.005 %, 0.01 %, 0.02 % and 0.05 % wt/vol of dry extract, BHT 0.01 % was used as control. The mixture was incubated in an oven at 37 ^{0}C and 80 ^{0}C for 12 d and 10 h, respectively. The course of oxidation was monitored by measuring conjugated dienes formation (CD) and thiobarbituric acid reactive substances (TBARS).

The antioxidative activity at the end of assay time was expressed for each indicator as reduction percent of peroxidation (RP %) with a control containing no antioxidant being 0 %.

RP %=[(peroxidation indicator value without antioxidant) – (peroxidation indicator value with antioxidant)/ peroxidation indicator value without antioxidant)]x100. A higher percentage indicates a higher antioxidant activity.

Determination of conjugated dienes formation. Aliquots of 0.02 ml were taken at different intervals during incubation. After incubation, 2 ml of methanol in deionised water (60 %) were added, and the absorbance of the mixture was measured at 233 nm. The conjugated dienes concentration was expressed in ml/mg in each sample. The results were calculated as CD = B x vol/wt; where B is the absorbance reading, vol denotes the volume (ml) of the sample and wt is the mass (mg) of emulsion measured (Zainol.et al.,2003).

Determination of thiobarbituric acid reactive substances. A modified thiobarbituric acid reactive substances (TBARS) method was used to measure the antioxidant activity of dry extract in terms of inhibition on lipid peroxidation. 0.1 ml of sample was taken every day, hour, respectively from the emulsion, the following were sequentially added: 2 ml of the TBA-TCA solution (20 mm TBA in 15% trichloroacetic acid (TCA). The mixture was heated in a 100 ^{0}C water bath for 15 min and cooled at room temperature. After 2 ml of chloroform were added, the mixture was mixed and centrifuged at 2000 rpm for 15 min. The chloroform layer was separated and the absorbance of the supernatant was measured at 532 nm against a blank containing 2 ml TBA-TCA solution. Malonaldehyde standard curves were prepared by 1,1,3,3-tetramethoxypropane and TBARS were expressed as mg of malonaldehyde/kg dry matter.

Detection of hydroxyl radicals by deoxyribose assay. The assay was performed as described by Halliwell and Chirico, 1993 with minor changes. All solutions were freshly prepared. 1.0 ml of the reaction mixture contained 100 µl of 28 mM 2-deoxy-D-ribose (dissolved in KH_2PO_4 K_2HPO_4 buffer pH 7.4), 500 µl solution of various concentrations of the ginger extract, 200 µl 0f 200 µM $FeCl_3$ and 1.04 mM EDTA (1:1 v/v), 100 µl H_2O_2 (1.0 mM) and 100 µl ascorbic acid (1.0 mM). After an incubation period of 1 h at 37 ^{0}C and at 80 ^{0}C the extent of deoxyribose degradation was measured by the TBA reaction. 1.0 ml of TBA (1 % in 50 mM NaOH) and 1.0 ml of TCA were added to the reaction mixture and the tubes

were heated at 100 ^{0}C for 20 min. After cooling the absorbance was read at 532 nm against a blank (containing only buffer and deoxyribose). The percentage inhibition was calculated by the formula:

$$I(\%)=100 - (Abs_{sample}/Abs_{control})x100$$

The IC_{50} value represented the concentration of the compounds that caused 50 % inhibition. Quercetin was used as a positive control.

The Assay of the superoxide radical anion. Superoxide anions were generated in an enzymatic system (xanthine-xanthine oxidase) and assayed by the reduction of nitroblue tetrazolium. The former comprised a solution of 100 μM xanthine, 60 μM nitroblue tetrazolium in 0.1 M phosphate buffer at pH 7.4 and 0.07 U ml^{-1} xanthine oxidase in a total volume of 1 ml. This mixture was incubated at 25 ^{0}C for 10 min and the optical density was read at 560 nm against a blank which did not contain the enzyme (Robak and Gryglewski, 1988). In order to check the inhibitory effect of mangiferin on xanthine oxidase activity, the enzyme was assayed by measuring the formation of uric acid from xanthine (Robak and Gryglewski, 1988; Yuting et al., 1990). Various concentrations of mangiferin were added to the samples before the enzyme was added.

The data obtained at each point were the average of three measurements.

Statistical analysis. The sstatistical processing of the data obtained from all studies was implemented by means of dispersion analysis with the Sigma Plot 7.0 software. Data are expressed as means ± standard deviation (SD). Statistical analysis was performed with Student's *t*-test. A difference was considered statistically significant when $p \leq 0.01$.

RESULTS AND DISCUSSION

Evaluation of antioxidant activity in linoleic acid model system. The antioxidant activity of mangiferin was assessed using an emulsion of linoleic acid as a model system. It was tested both for its capacity to inhibit the formation of hydroxiperoxides at an earlier stage of oxidation of linoleic acid, and in the cases of occurrences of secondary oxidation products, such as aldehydes, ketones, and hydrocarbons. To identify these properties of mangiferin, 2 indicators were used, corresponding to the different degree of lipid peroxidation – the use of coupled double bonds and TBARS. In order to conduct a quality assessment of the effect of mangiferin on the lipid peroxidation, storage took place at two temperatures: the body temperature of 37 ^{0}C and 80 ^{0}C.

Determination of conjugated dienes formation. The observation of the inhibition of the lipid peroxidation at both temperatures was performed until almost the same levels of peroxidation was reached. The experiments showed an antioxidant activity of mangiferin at both temperatures manifested at the three concentrations under study, as a stronger effect was exhibited at 37 ^{0}C (Fig.1).

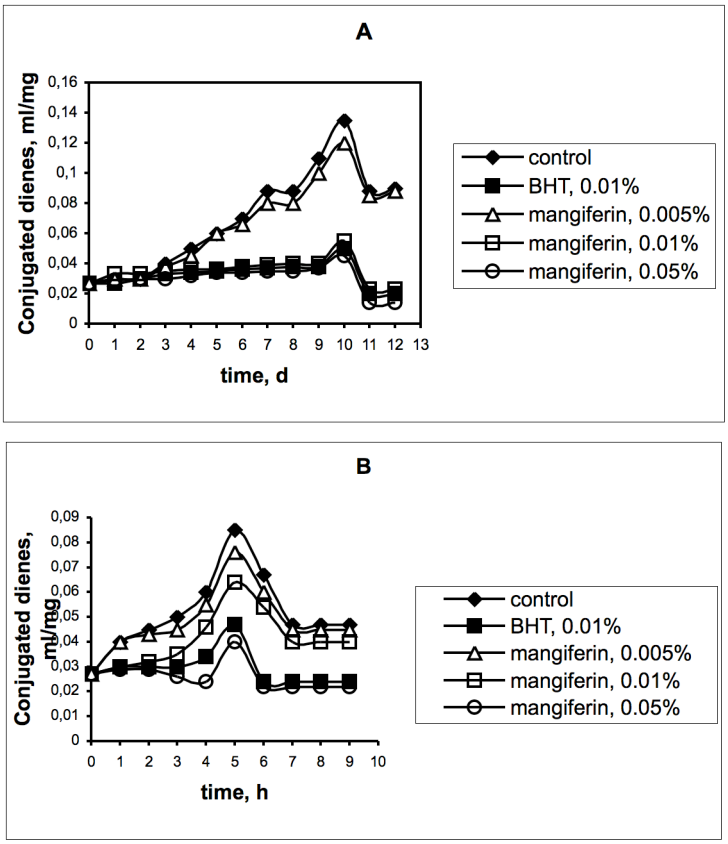

Fig.1. Effect of mangiferin on conjugated dienes in a linoleic acid/water emulsion system at 37 ^{0}C (A) and 80 ^{0}C (B)

During this study it was established that there was an increase in the formation of coupled double bonds from 4d of incubation of the linoleic acid at 37 ^{0}C, as the maximum was reached on 10 d. With the increase of the concentration of mangiferin from 0.005 to 0.05 %, its antioxidant activity kept rising and was highest at 0.05 %, as the degree of inhibition was 66.7 % compared with 62.9 % of BHT on the 10th day. An effect weaker than that of BHT was shown by the lower concentrations of mangiferin – 0.005 and 0.01 %.

At 80 ^{0}C a maximum in conjugated dienes formation was observed on 5 h of storage, as the level of conjugated dienes was lower than that at 37 ^{0}C. Most effective antioxidant activity was performed by 0.05 % mangiferin, as it exceeded that of the control – 52.9 % compared with 44.7 % of BHT and reaching 67.2% on the 6th h.

With the increase of the storage time at both temperatures the trend was an increase in the antioxidant activity of mangiferin at the highest concentration under study, similar to that of BHT.

Determination of thiobarbituric acid reactive substances. When the other indicator was applied – TBARS, the maximum formation of malonaldehyde was found on 10 d at 37 ^{0}C and on 4 hat 80 ^{0}C (compare Fig. 1 with Fig.2), i.e. the process developed almost similarly to that of the formation of coupled double bonds. It is worth mentioning that in order to

obtain a degree of inhibition of the process comparable with that of BHT, it a higher concentration of mangiferin was needed – 0.1 %, i.e. 2 times higher than the one necessary for the inhibition of conjugated dienes formation. Such a relation was not mentioned in the publication by Romero et al., 2004. This was probably due to a difference in the activation energy of the 2 processes and is a further evidence that conjugated dienes did not precede the formation of degrading products.

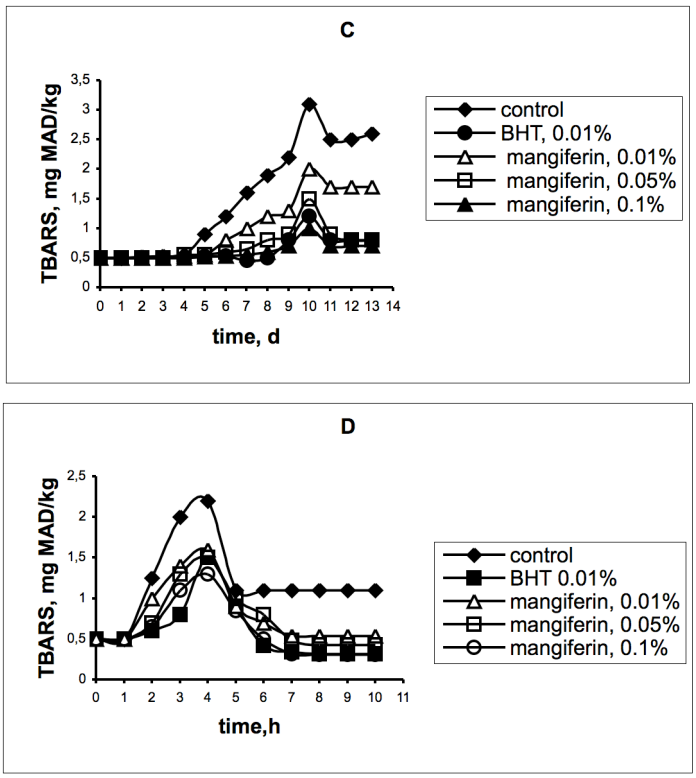

Fig.2. Effect of mangiferin on TBARS in a linoleic acid/water emulsion system at 37 ^{0}C (C) and 80 ^{0}C (D)

As for TBARS, the antioxidant activity of 0.1 % mangiferin was higher than that of the control – 67.7 % and 61.3 % on 10 d at 37 ^{0}C and 40.9 %, respectively, and 31.8 % at 80 ^{0}C on 4 h of the study, respectively.

At both stages of lipid peroxidation – conjugated dienes formation and TBARS at 80 ^{0}C, the results showed a lower antioxidant effectiveness than that at 37 ^{0}C, but nevertheless a good antioxidant potential comparable with that of BHT was found. In emulsion systems, Wettasinghe and Shihidi, 1999 found that the hydrophobic antioxidants oriented to the fatty-aqueous border, preventing the fatty phase from oxidation. It was evident that there were also other factors affecting the effectiveness of the process running in dispersion systems.

Detection of hydroxyl radicals by deoxyribose assay. Ferric-EDTA was incubated with H_2O_2 and ascorbic acid at pH 7.4. Hydroxyl radicals were formed in free solution and were detected by their ability to degrade 2-deoxy-D-ribose into fragments that on heating with TBA at low pH from a pink chromogen (Aruoma et al., 1989; Halliwell et al., 1987). The mangiferin added to the reaction mixture removed the hydroxyl radicals from the sugar and

protected it from degradation. These results are illustrated in Fig. 3. The effect from the inhibition of the hydroxyl radicals was stronger at 37 ^{0}C than at 80 ^{0}C – IC_{50} at 37 ^{0}C it was 0.88 μg/ml, while at 80 ^{0}C a higher concentration of mangiferin was needed - IC_{50} e 1.81 μg/ml. IC_{50} values were with statistical significance $p \leq 0.01$ and a high regression coefficients – $R^2 = 0.975$ and $R^2 = 0.983$, respectively. This trend to a higher concentration of antioxidant for capturing the OH· at the higher temperature of incubation was also observed in the control. The quercetin had an IC_{50} 4.61 μg/ml ($R^2 = 0.985$) at 37 ^{0}C and 7.41 μg/ml ($R^2 = 0{,}987$) at 80 ^{0}C.

If the model system of Fe^{3+} and H_2O_2 was used in the absence of ascorbat, a weak level of generation of hydroxyl radicals was observed. The addition of ascorbic acid considerably increased the level of generation of OH· by means of converting the Fe^{3+} into Fe^{2+}(Table 1). The results showed that mangiferin is a powerful scavenger of OH· rivaled by 2-deoxy-D-ribose. This analysis can also be used to assess the capacity of mangiferin to inhibit the metal-ion dependent generation of OH·, and not only the capacity to capture the released radicals. When Fe^{3+} ions were added to the reaction mixture, some of them formed a complex with deoxycarbose. Only these molecules which were able to chelate Fe ions and render them inactive, could inhibit the degradation of deoxyribose. The results obtained clearly showed that when Fe^{3+} was complexly bound to EDTA, deoxyribose degradation was weakly changed, which was evident in terms of the degree of inhibition, manifesting the metalchelating properties of mangiferin. Mangiferin's manifested property was similar to other plant polyphenols (Basaga, 1990; Martinez et al., 2000; Burits and Bucar, 2000).

Table 1. Effect of mangiferin on deoxyribose degradation by OH·

Additions	A_{532} ± CD	% inhibition
Fe^{3+} +H_2O_2 +DR	0.816±0.007	
Fe^{3+}+ mangiferin+H_2O_2 +DR		
0.5 μg/ml	0.592±0.002	17.5
1.0 μg/ml	0.586±0.003	28.2
2.0 μg/ml	0.580±0.004	28.8
2.5 μg/ml	0.569±0.004	30.0
Fe^{3+}+ ascorbat +H_2O_2 +DR	0.919±0.008	
Fe^{3+}+ ascorbat + mangiferin +H_2O_2 +DR		
0.5 μg/ml	0.675±0.005	26.5
1.0 μg/ml	0.667±0.006	27.4
2.0 μg/ml	0.664±0.005	27.7
2.5 μg/ml	0.617±0.001	32.8
Fe^3 + EDTA*+ ascorbat +H_2O_2 +DR	0.848±0.007	
Fe^3 + EDTA*+ mangiferin + ascorbat +H_2O_2 +DR		
2.0 μg/ml	0.591±0.004	30.3

2.5 μg/ml	0.533±0.003	0.533
Fe^{3+} + ascorbat +H_2O_2 +DR	0.919±0.008	
∗Fe^{3} + mangiferin + ascorbat +H_2O_2 +DR		
2.0 μg/ml	0.547±0.004	40.5
2.5 μg/ml	0.521±0.003	43.3
∗∗Fe^{3+}-EDTA+ ascorbat +H_2O_2 +DR	1.51±0.008	
Fe^{3+}-EDTA+ mangiferin + ascorbat +H_2O_2 +DR		
2.0 μg/ml	0.545±0.004	63.9
2.5 μg/ml	0.458±0.003	69.7

EDTA* was present in the reaction mixture before Fe^{3+} was added

∗Fe^{3} -5 min after the mixing of Fe^{3+} and mangiferin, the rest of the components were added

∗∗Fe^{3+} and EDTA were previously mixed

The Assay of the superoxide radical anion. Superoxide anions indirectly initiate lipid oxidation as a result of superoxide and hydrogen peroxide serving as precursors of singlet oxygen and hydroxyl radicals (Robak and Gryglewski, 1988). Xanthine-xanthine oxidase is the system which is often used as a generator of superoxide radicals. Our results showed that the polyphenol mangiferin is an inhibitor of the enzyme xanthine oxidase - Fig. 4. The maximum inhibiting effect – 71.4 % occurred at mangiferin concentrations of 30 μg/ml, as IC_{50} was 14.17 μg/ml (R^2 = 0.972). Superoxydedismutase 100 U/ml with inhibiting effect 77.8 % was used as a standard. It is possible, however, for polyphenols to have more than one ways of affecting xanthine-xanthioxidase action, viz. as inhibitors of xanthine, viz. As inhibitors of xanthine oxidase and as scavengers of the superoxide, but most probably in both ways (Jubert et al., 2004).

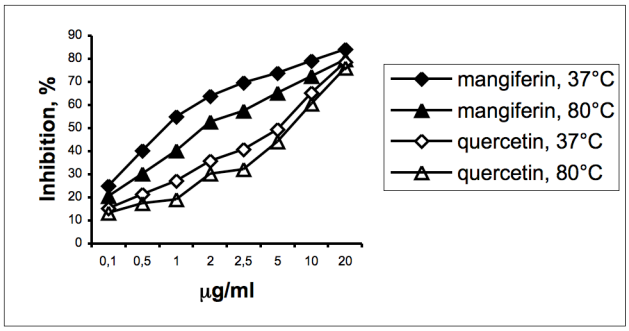

Fig. 3. Inhibition of the radical degradation of 2-deoxy-D-ribosa of mangiferin and quercetin (control)

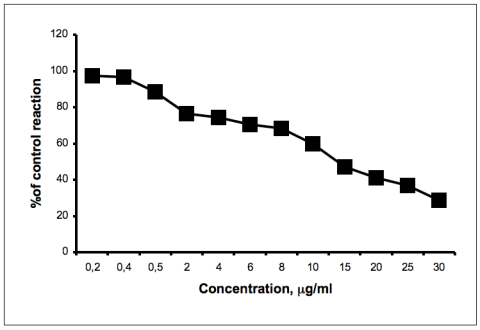

Fig. 4. Effect of mangiferin on xanthine oxidase activity

CONCLUSION

It is well known that the antioxidant activity of polyphenols is basically attributed to their ability to release H atoms, through which they neutralize the free radicals. The results obtained in the course of this study., however, showed that the alcohol solution of mangiferin can effectively inhibit conjugated dienes formation and the yield of secondary products form the lipid peroxidation both at 37 ^{0}C, and at high temperature – 80 ^{0}C. I was found that there was stronger inhibition by the stage of formation of secondary products from the autooxidation of fats. This behavior can be attributed to mangiferin's property to capture the superoxide radical.

The alcohol solutions of mangiferin also showed inhibiting capacity with regard to hydroxyl radicals, which was better than that of quercetine at both temperatures applied. Under the conditions of the conducted experiments mangiferin demonstrated not only a good capturing capacity of already existing hydroxyl radicals, but also a significant effect on the reduction of the formation of Fe dependent hydroxyl radicals form H_2O_2, through the chelatoforming capacity.

Since mangiferin is used in both traditional and formal medicine to treat a number of conditions, possibly its effectiveness is partly due to its capacity to neutralize free radicals – superoxides and hydroxyl radicals. These properties make mangiferin applicable to both food and pharmaceutical industries.

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