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Effect of Brining on Biological Activity of Leaves of *Vitis vinifera* L. (Cv. Sultani Çekirdeksiz) from Turkey

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Leaves of *Vitis vinifera* (Fam. Vitaceae) cv. 'Sultani Çekirdeksiz' cultivated in Manisa-Alaşehir in western Turkey, were processed with or without brine. Fresh, brined, and nonbrined leaves (after being subjected to 3 months of fermentation) were sampled and extracted with distilled water under reflux. Antioxidant, anti-inflammatory, and anti-nociceptive activities of the water extracts were investigated using in vitro and in vivo methods. Free radical scavenging activity (1,1-diphenyl-2-picrylhydrazyl, DPPH• assay), iron(III) reductive activity (reducing power activity assay), capacity of inhibition of linoleic acid peroxidation (ferric thiocyanate and thiobarbituric acid method), anti-nociceptive activity (*p*-benzoquinone-induced abdominal constriction test), and anti-inflammatory activity (carrageenan-induced hind paw edema model) were used to determine biological activities of the extracts. In addition, the contents of total phenolics, flavonoids, and flavonols in the extracts were determined by spectrophotometrical methods. Results were compared with those of ascorbic acid, butylated hydroxytoluene, and gallic acid as reference antioxidants. The extracts of fresh, brined, and nonbrined leaves showed almost the same activity in all antioxidant assays. These extracts inhibited the oxidation of linoleic acid to the same extent as BHT. Compositions of the extracts were analyzed by a reverse phase HPLC-PDA method. The occurrence of hydroxycinnamic acids (e.g., caffeic acid) and flavonoids (e.g., quercetin) was verified in the extracts. The content of total flavonoids as well as quercetin was increased by fermentation.

KEYWORDS: ***Vitis vinifera*; HPLC; 1,1-diphenyl-2-picrylhydrazyl (DPPH•); linoleic acid peroxidation; anti-inflammatory activity; anti-nociceptive activity; antioxidant activity**

INTRODUCTION

Turkey has a vast diversity of different types of foods thanks to its rich culture and history dating back several millennia. Fermentation has been used for the production of several ethnic products. These products contain organic and phenolic acids, flavonoids, anthocyanins, etc. They are used by the people of Turkey for flavoring as well as for their nutritious properties.

The leaves of *Vitis vinifera* (vine leaf) have been traditionally used as a food in both fresh and brined forms in Turkey. Fresh vine leaves tend to decompose in a very short time during storage. Therefore, people generally prepare different kinds of brines to preserve vine leaves for future use. Generally, high-salt brines are used in Turkey. However, in some parts of Turkey, vine leaves are packed into jars without salt or any brining agent to protect the leaves from rotting during storage. The advantage of this method is that such products can be used by people with hypertension (*1*−*3*). Moreover, the leaves, due to their astringent and hemostatic properties, are used in the treatment of diarrhea, hemorrhage, and varicose veins, and the juice of leaves has been used as an eye bath. Flavonoids, terpenes, organic acids, vitamins, carbohydrates, lipids, and enzymes have been isolated from fruits and leaves of *V. vinifera* (*4*).

The phenolic compositions of vine leaves have been studied, and the occurrence of chlorogenic acid, quercetin, quercetin-3-*O*-glucoside, quercetin-3-*O*-glucoside-7-*O*-glucronide, isorhamnetin-3-*O*-glucoside, and kaempherol-3,7-*O*-diglycoside were reported in the literature (*4*, *5*). These compounds are well-known antioxidants (*4*, *5*). Many flavonoids have strong free radical scavenging and lipid peroxidation activities. Food-derived flavonoids such as flavonols quercetin, kaempferol, and

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myricetin have been reported to reduce the risk of cancer. The average daily intake of flavonoids in the Western diet is estimated to be 1 g (*6−9*). The effects of flavonoids on inflammation were reported (*10*). They affect the enzymes regarding inflammation and show anti-inflammatory activity (*10*, *11*).

The aim of this work was to compare the composition and antioxidant, anti-inflammatory, and anti-nociceptive activities of fresh, brined, and nonbrined vine leaves. It was also intended to prove the safety and nutritious features of the products,which have been used as foodstuffs for millennia.

MATERIALS AND METHODS

Plant Material and Reagents. *V. vinifera* L. cv. 'Sultani Çekirdeksiz' grafted on *V. vinifera* 41B was grown in Alaşehir in Manisa province in western Turkey. Fourth and fifth leaves of cv. 'Sultani Çekirdeksiz' from the tip of the branch were used as study material in the experiments. Fresh leaves were kept at −20 °C until the extraction. For brining, 200 g of leaves was placed in a 720 mL screw-cap jar with 400 mL of 5% salt-brine solution. After the cap was closed, the jar was left to ferment at room temperature (22 ± 2 °C) for 120 days (*1*). For unbrined material, 350 g of leaves was placed well in a 720 mL screw-cap jar with no air space left. After the cap had been kept in boiling water for 5 min, the jar was closed (*2*).

Preparation of Extracts. Frozen fresh leaves and brined and unbrined materials (100 g for each) were extracted with water under reflux for 8 h. The water phase was filtered and freeze-dried. All of the extracts were stored at −20 °C. Prior to analysis, an aliquot of each extract was dissolved and filtered through a 0.45 μm membrane (Whatman, Maidstone, U.K.).

Total Phenolics, Flavonoids, and Flavonols. Total phenols were estimated as gallic acid equivalents (GAE), expressed as $mg_{gallic\ acid}/g_{extract}$ (*12*). To ca. 6.0 mL of H_2O was transferred 100 μL of sample in a 10.0 mL volumetric flask, to which was subsequently added 500 μL of undiluted Folin−Ciocalteu reagent. After 1 min, 1.5 mL of 20% (w/v) Na_2CO_3 was added, and the volume was made up to 10.0 mL with H_2O. After 2 h of incubation at 25 °C, the absorbance was measured at 760 nm and compared to a gallic acid calibration curve. The data are presented as the average of triplicate analyses.

Total flavonoids were estimated as rutin equivalents (RE), expressed as $mg_{rutin}/g_{extract}$ (*13*). One milliliter of plant extract in methanol (10 g/L) was mixed with 1 mL of aluminum trichloride in ethanol (20 g/L) and diluted with ethanol to 25 mL. The absorption at 415 nm was read after 40 min at 20 °C. Blank samples were prepared from 1 mL of plant extract and 1 drop of acetic acid and diluted to 25 mL. The rutin calibration curve was prepared in ethanolic solutions with the same procedure. All determinations were carried out in quadruplicate, and the mean values were used.

Total flavonols were estimated as rutin equivalents (RE), expressed as $mg_{rutin}/g_{extract}$ (*13*). The rutin calibration curve was prepared by mixing 2 mL of 0.5−0.015 mg/mL rutin ethanolic solutions with 2 mL (20 g/L) of aluminum trichloride and 6 mL (50 g/L) of sodium acetate. The absorption at 440 nm was read after 2.5 h at 20 °C. The same procedure was carried out with 2 mL of plant extract (10 g/L) instead of rutin solution. All determinations were carried out in quadruplicate, and the mean values were used.

Qualitative−Quantitative Chromatographic Analysis. The liquid chromatographic apparatus (Shimadzu LC 10A*vp*, Ant Ltd., Istanbul, Turkey) consisted of an in-line degasser, pump, and controller coupled to an SPD-M10A*vp* photodiode array detector equipped with an automatic injector interfaced to Class VP chromatography manager software (Shimadzu, Ant Ltd., Istanbul, Turkey). Separations were performed on a 250 × 4.6 mm i.d., 5 μm particle size, reverse-phase Discovery-C18 analytical column (Supelco, Bellefonte, PA) operating at room temperature (22 °C) at a flow rate of 1 mL/min. Detection was carried out with a sensitivity of 0.1 aufs between the wavelengths of 200 and 550 nm. Elution was effected using a nonlinear gradient of the solvent mixtures aqueous 5% HCOOH (solvent A) and acetonitrile (solvent B). The composition of B was held in 5% for 5 min, increased to 15% in 15 min, increased to 85% in 10 min and held for 5 min, then returned to initial conditions in 3 min and held for 7 min. A 10 min equilibrium time was allowed between injections. Components were identified by comparison of their retention times to those of authentic standards under analysis conditions and UV spectra with an in-house PDA library.

All extracts and standards were dissolved in 70% aqueous acetonitrile at concentrations of 1 and 10 mg/mL, respectively. The concentration used for the calibration of reference compounds was 0.00−0.10 mg/mL. All of the standard and sample solutions were injected in triplicate.

Iron(III) to Iron(II) Reduction Activity. The ability of the extracts to reduce iron(III) was assessed according to the method of Oyaizu (*14*). One milliliter of each extract dissolved in H_2O was mixed with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of a 1% (w/v) potassium hexacyanoferrate solution. After 30 min of incubation at 50 °C, 2.5 mL of 10% (w/v) trichloroacetic acid (TCA) was added and the mixture was centrifuged for 10 min. Finally, 2.5 mL of the upper layer was mixed with 2.5 mL of H_2O and 0.5 mL of 0.1% (w/v) $FeCl_3$, and the absorbance was recorded at 700 nm. The reductive activities of the extracts are expressed as ascorbic acid equivalents (AscAE) in $mmol_{ascorbic\ acid}/g_{sample}$ (*15*). The bigger the AscAE value, the greater the reducing power of the sample. The data are presented as the average value of quadruplicate analyses.

1,1-Diphenyl-2-picrylhydrazyl (DPPH•) Radical Scavenging Activity. The ability of the extracts to scavenge DPPH• radicals was determined according to the method of Gyamfi et al. (*16*). A 50 μL aliquot of each extract, in 50 mM Tris-HCl buffer (pH 7.4), was mixed with 450 μL of Tris-HCl buffer and 1.0 mL of 0.1 mM DPPH• in MeOH. After 30 min of incubation in darkness and at ambient temperature, the resultant absorbance was recorded at 517 nm. The percentage inhibition was calculated using eq 1. Estimated IC_{50} values are presented as the average of quadruplicate analyses.

$$\%\ \text{inhibition} = \left[\frac{(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}})}{\text{Abs}_{\text{control}}}\right] \times 100 \qquad (1)$$

Determination of Inhibition of Linoleic Acid Peroxidation. *Iron(II) Thiocyanate Method.* The ability of the extracts to inhibit the linoleic acid peroxidation was determined according to the method of Llorach et al. (*17*) and Mohd Zin et al. (*18*). All reagents were prepared fresh. Reaction mixture (2.525 mL) in a screw-cap bottle included linoleic acid (2.55) (0.25 mL) in ethanol, 50 mM sodium phosphate buffer, pH 7 (1 mL), ethanol (0.25 mL), distilled water (0.9 mL), sample solution (0.1 mL), and 1.8 mM AAPH (25 mL) for acceleration. This mixture was mixed vigorously and placed in an oven at 50 °C for 10 h of incubation. Thirty microliters of reaction mixture was taken in every 2 h into 2910 μL of ethanol and mixed with 30 mL of ammonium thiocyanate (3.86 M) solution. Thirty microliters of iron(II) solution was added and mixed vigorously, and then the absorbance at 500 nm was read after 3 min. Blank solution included all reagents except the sample. Ascorbic acid, BHT, and gallic acid were used as positive controls. The average of quadruplicate analyses was given as result.

Measurement of Malondialdehuyde (MDA) Value (TBA Method). The amount of MDA formed in the reaction mixture using the above method was determined by thiobarbituric acid (TBA) reagent (*18*). One milliliter of reaction mixture, 1 mL of TCA (2.8%), and 1 mL of TBA (1%) were mixed vigorously and then placed in a water bath at 90 °C for 20 min. After incubation, reaction was stopped in an ice bath for 10 min. Two milliliters of *n*-butanol was added into the reaction mixture and mixed vigorously. The butanol phase was separated after centrifugation at 3000 rpm at 5 min. The absorbance of the *n*-butanol phase was measured at 532 nm using *n*-butanol as blank. The average of quadruplicate analyses was given as the result.

Pharmacological Procedures. *Animals.* Male Swiss albino mice (20−25 g) were purchased from the animal breeding laboratories of Refik Saydam Central Institute of Health (Ankara, Turkey). The animals left for 2 days for acclimatization to animal room conditions were maintained on standard pellet diet and water ad libitum. The food was withdrawn 1 day before the experiment, but free access to water was allowed. A minimum of six animals was used in each group.

Table 1. Fraction Yield, Total Phenols, Flavonoids, Flavonols, and HPLC Qualitative and Quantitative Data for Vine Leaf Extracts

		spectrophotometric results			HPLC results[a]			
					identified		unidentified	
sample[b]	yield[c]	total phenols[d]	total flavonoids[e]	total flavonols[f]	caffeic acid	quercetin	hydroxycinnamates[g]	flavonoids[h]
A	59.87	152.06 ± 0.68	65.10 ± 0.48	2.32 ± 0.05	3.60 ± 0.08	3.16 ± 0.01	52.39 ± 0.08	66.24 ± 0.58
B	55.30	160.17 ± 0.21	75.85 ± 0.43	3.10 ± 0.01	2.40 ± 0.01	5.34 ± 0.02	47.06 ± 0.09	67.24 ± 0.17
C	69.06	178.99 ± 0.47	81.99 ± 0.43	0.62 ± 0.01	3.07 ± 0.00	11.63 ± 0.02	62.63 ± 0.16	54.13 ± 0.13

[a] Values (mg/g) are expressed as means ± standard error ($p < 0.05$, $n = 6$). [b] A, fresh leaves extract; B, brined leaves extract; C, unbrined fermented leaves extract. [c] Extract yields expressed as milligrams of extract per gram (dry weight) of aerial material. [d] Total phenols expressed as gallic acid equivalent milligrams of gallic acid per gram (dry weight) of extract. [e] Total flavonoids expressed as rutin equivalent milligrams of rutin per gram (dry weight) of extract. [f] Total flavonols expressed as rutin equivalent milligrams of rutin per gram (dry weight) of extract. [g] Quantified using caffeic acid. [h] Quantified using quercetin.

Throughout the experiments, animals were processed according to the suggested ethical guidelines for the care of laboratory animals.

Preparation of Test Samples for Bioassay. All of the materials were given orally to test animals in 200 mg/kg doses after suspension in a mixture of distilled H_2O and 0.5% sodium carboxymethyl cellulose (CMC). The control group animals received the same experimental handling as those of the test groups except that the drug treatment was replaced with appropriate volumes of the dosing vehicle. Either indomethacin (10 mg/kg) or acetylsalicylic acid (ASA) (200 mg/kg) in 0.5% CMC was used as reference drug.

Anti-nociceptive Activity. A *p*-benzoquinone-induced abdominal constriction test was performed on mice for determination of anti-nociceptive activity (*19*). According to the method, 60 min after the oral administration of test samples, the mice were intraperitonally injected with 0.1 mL/10 g of body weight of 2.5% (w/v) *p*-benzoquinone (PBQ; Merck) solution in distilled H_2O. Control animals received an appropriate volume of dosing vehicle. The mice were then kept individually for observation, and the total number of abdominal contractions (writhing movements) was counted for the next 15 min, starting at the fifth minute after the PBQ injection. The data represent the average of the total number of writhings observed. The anti-nociceptive activity was expressed as percentage change from writhing controls. Aspirin (ASA) at 100 and 200 mg/kg doses was used as the reference drug in this test.

Anti-inflammatory Activity. Carrageenan-induced hind paw edema model was used with modifications in measuring periods for the determination of anti-inflammatory activity (*20*). The difference in footpad thickness between the right and left foot was measured with a pair of dial thickness gauge calipers (Ozaki Co., Tokyo, Japan). Mean values of treated groups were compared with mean values of a control group and analyzed using statistical methods. Sixty minutes after the oral administration of test sample or dosing vehicle, each mouse was injected with freshly prepared (0.5 mg/25 μL) suspension of carrageenan (Sigma, St. Louis, MO) in physiological saline (154 nM NaCl) into the subplantar tissue of the right hind paw. As the control, 25 μL saline solutions were injected into that of the left hind paw. Paw edema was measured every 90 min during 6 h after induction of inflammation. The difference in footpad thickness was measured by a gauge calipers (Ozaki Co., Tokyo, Japan). Mean values of treated groups were compared with mean values of a control group and analyzed using statistical methods. Indomethacin (10 mg/kg) was used as the reference drug.

Acute Toxicity. Animals employed in the carrageenan-induced paw edema experiment were observed during 48 h, and morbidity or mortality was recorded, if necessary, for each group at the end of the observation period.

Gastric Ulcerogenic Effect. After the anti-nociceptive activity experiment, mice were killed under deep ether anesthesia, and stomachs were removed. Then the abdomen of each mouse was opened through the greater curvature and examined under dissecting microscope for lesions or bleedings.

Statistical Analysis. Data are presented as mean values ± standard error. All statistical analyses were carried out using SPSS 10.0.1. (SPSS Inc., Chicago, IL). Analysis of variance (ANOVA) was performed by ANOVA procedures. Significant differences between means were determined by Tukey's pairwise comparison test at a level of $p < 0.05$. IC_{50} values were estimated using a nonlinear regression algorithm. Data obtained from animal experiments were expressed as mean standard error (±SEM). Statistical differences between the treatments and the control were evaluated by ANOVA and Student−Newman−Keuls post-hoc tests. $p < 0.05$ was considered to be significant (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

RESULTS AND DISCUSSION

In Turkish folk medicine, vine leaves are known to have a diuretic effect. In addition, the fresh and brined or fermented leaves are used as food throughout the year, especially in winter months. Mixtures of rice and spices with or without meat are wrapped with vine leaves and served with or without garlic-yogurt dressing after cooking. It is a well-known dish of the Turkish cuisine served both at home and in restaurants.

Fruits, seeds, and leaves of *V. vinifera* have been used not only in the diet but also in medicine since ancient times for their astringent and hemostatic properties. *V. vinifera* is also used for skin diseases and for eye inflammations in traditional European medicine. It contains different types of phenolic compounds such as tannins, flavonoids, procyanidins, anthocyanins, and phenolic acids within the leaves and berries. *V. vinifera* is reported to show antioxidant (free radical scavenging and lipid peroxidation activities) and anti-inflammatory activities because of these phenolic compounds (*21*).

Extract Yields, Total Phenol, Total Flavonoids, Total Flavonols, and Compositional Analysis. Both fresh and brined/unbrined vine leaves were extracted with water under reflux. The results of extract yields, total phenols, total flavonoids, total flavonols, and compositional analysis of samples are presented in **Table 1**. According to their results, yields and total phenol and flavonoid amounts of all the samples were slightly different, whereas the amounts of total flavonols were found to vary ($p < 0.05$). The unbrined fermented leaves (sample C) contained the highest amount of total flavonoid content, whereas the same extract had the lowest amount of flavonols.

The results of qualitative−quantitative analyses of the vine leaf extracts, carried out using an HPLC apparatus coupled to a PDA detector, are presented in **Table 1**, with associated chromatograms given in **Figure 1**. Phenolic compounds were identified and quantified at 330 and 360 nm as hydroxycinnamates and flavonoids, respectively. Caffeic acid and quercetin (**Figure 2**) were identified by comparison to the retention times and UV spectra of authentic standards, whereas quantitative data were calculated from their calibration curves. The extract from unbrined fermented leaves was found to be the richest in total phenolics and total flavonoids by both UV spectrophotometry and HPLC. Hydroxycinnamic acids and flavonoids were found as the main compounds in all of the extracts. The amounts of caffeic acid were variable. The amount of quercetin was observed to increase with fermentation. The occurrence of

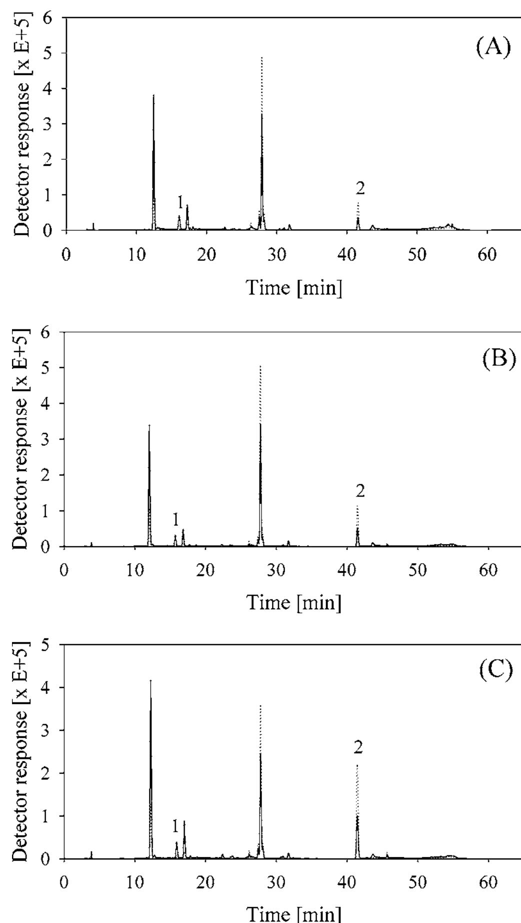

Figure 1. HPLC-PDA analysis of the water extracts of vine leaves with responses at 320 and 360 nm overlaid: (**A**) fresh leaves; (**B**) brined leaves; (**C**) unbrined leaves; **1**, caffeic acid; **2**, quercetin.

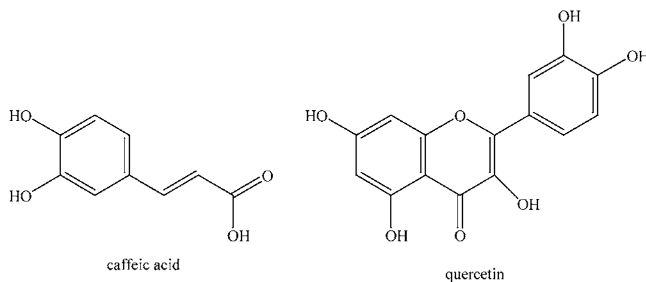

Figure 2. Structural formulas of identified compounds.

quercetin, caffeic acid, and their derivatives in vine leaves had previously been reported (*22*).

Iron(III) to Iron(II) Reduction Activity. The ability of a fraction to reduce iron(III) represents the extract's ability to donate electrons (*23*). Reducing activity is very important in terminating radical chain reactions (*23*). As the literature survey suggests, there is a high correlation between iron(III) to iron(II) reduction activity of aqueous extracts and antioxidant activity (*15*, *24*, *25*); however, this may not always be the case (*25*). The ability of all the fractions to reduce ferric iron to ferrous iron was investigated, and the results are shown in **Figure 3**. As seen in **Figure 3**, all of the extracts reduced iron(III) to iron(II). None of the extracts was found to have activity as the positive controls ascorbic acid, BHT, and gallic acid (5.7 ± 0.2, 5.2 ± 0.0, and 13.7 ± 0.3 mmol/g AscAE, respectively), whereas extracts A and C were shown to have 3 times less activity than BHT. Therefore, these two extracts can be used at greatermore concentration than BHT as a natural antioxidant.

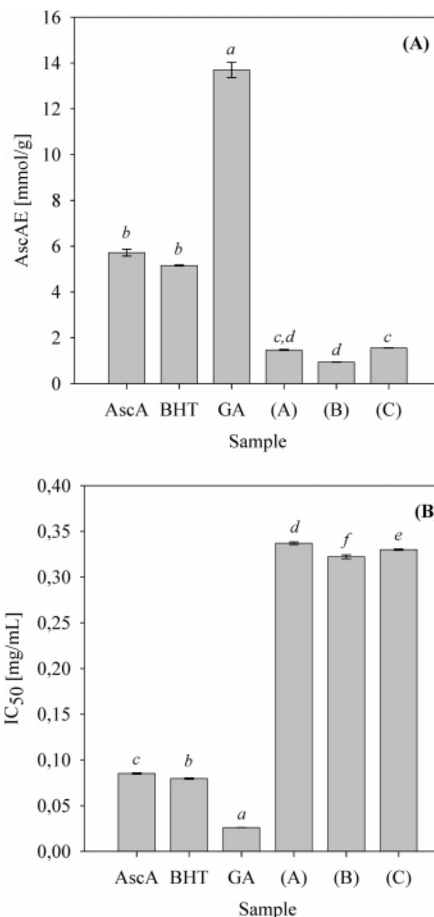

Figure 3. Effect of extracts of vine leaves and positive controls upon (**A**) iron(III) reduction and (**B**) DPPH• radical scavenging. AscA, ascorbic acid; BHT, butylated hydroxytoluene; GA, gallic acid; A, fresh leaves; B, brined leaves; C, unbrined leaves. Values are presented as means ± 95% confidence interval. Bars with the same lowercase letter (a–f) are not significantly ($p < 0.05$) different.

A significant ($p < 0.05$) correlation was found between the reductive capacity and DPPH• scavenging activity ($r^2 = 0.7340$; $p = 0.0293$) and between the reductive capacity and the performance of the fractions against linoleic acid peroxidation ($r^2 = 0.6943$; $p = 0.0394$). Phenolic acids and flavonoids are well-known as natural antioxidants. Phenolic acids, especially hydroxycinnamates, show antioxidant activity by hydrogen-donating mechanisms (*6*, *26*). In this study, the extracts contained hydroxycinnamic acids as the main phenolic acids and some flavonoids such as quercetin and its glycosides. Both phenolic acids and flavonoids are soluble in polar solvents and show strong activity in the polar test systems. Both iron(III) reduction and DPPH• radical scavenging activities are performed in polar media. Therefore, a correlation was found between the reducing power and DPPH• radical scavenging activity of vine extracts.

DPPH• Radical Scavenging Activity. The DPPH• radical is a stable radical with an absorption maximum at 517 nm. When reduced to the hydrazine derivative by an antioxidant via electron or hydrogen atom transfer reactions, this absorption maximum decreases (*27*). All of the vine leaf extracts were able to scavenge DPPH• radicals at physiological pH and did so in a concentration-dependent fashion (data not shown). IC_{50} values, defined as the concentration required to scavenge 50% of the available free radicals, estimated by nonlinear regression for all extracts are presented in **Figure 3**. None of the extracts was as active as the positive controls, ascorbic acid, BHT, and gallic

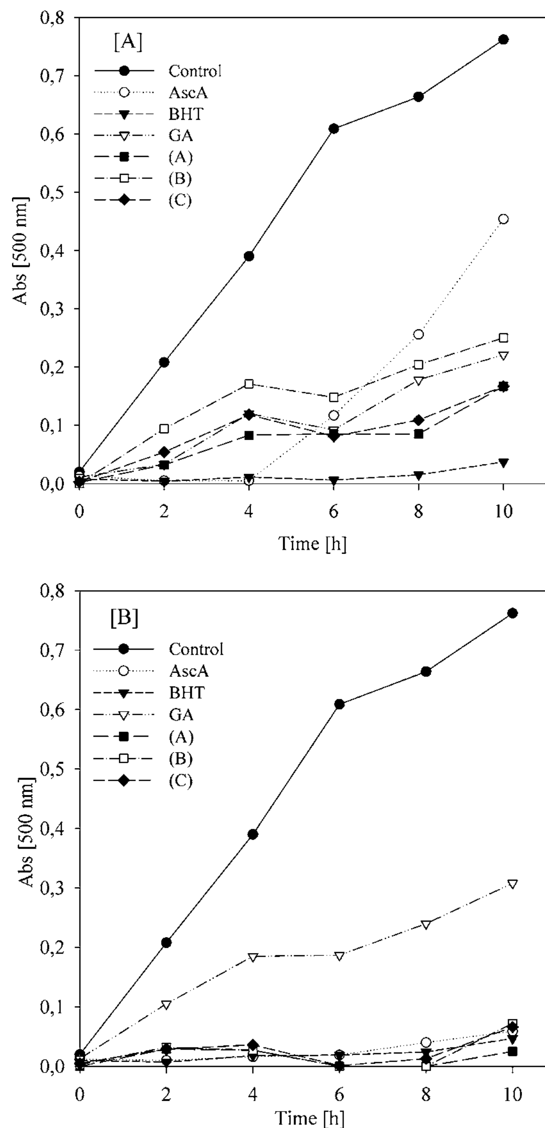

Figure 4. Effect of extracts of vine leaves and positive controls on the inhibition of the linoleic acid peroxidation: (**A**) 0.25% extract; (**B**) 1% extract. AscA, ascorbic acid; BHT, butylated hydroxytoluene; GA, gallic acid; A, fresh leaves; (B) brined leaves; (C) unbrined leaves.

acid, but all extracts showed 3 times less activity than BHT. The vine leaf extracts can be used more concentrated as natural free radical scavengers in both fresh and brined/unbrined form. All extracts showed the same IC_{50} values on DPPH• radicals (0.3 ± 0.0 mg/mL). The berries of *V. vinifera* are well-known as a natural source of antioxidants and widely used for purposes related to this property. Berries contain resveratrol, hydroxycinnamic acid derivatives, anthocyanins, and procyanidins. These groups of phenolics are natural antioxidants. They are principally responsible for the fruit's activity as they are very strong radical scavengers (*4*, *27*, *28*). Vine leaves contain hydroxycinnamic acids and flavonoids, especially quercetin and its derivatives. Reports on antioxidant activities of vine leaves are very scarce (*29*), but the effects of hydroxycinnamic acids and quercetin derivatives are well-known. As seen in **Table 1**, all of the extracts contain hydroxycinnamic acids and flavonoids such as quercetin. Unidentified components were determined using the HPLC-PDA data as hydroxycinnamates and flavonoids. Flavonoids exhibit relatively weak DPPH• radical scavenging activity, and glycosylation has been reported to decrease radical scavenging activity (*27*, *30*). Chlorogenic acid

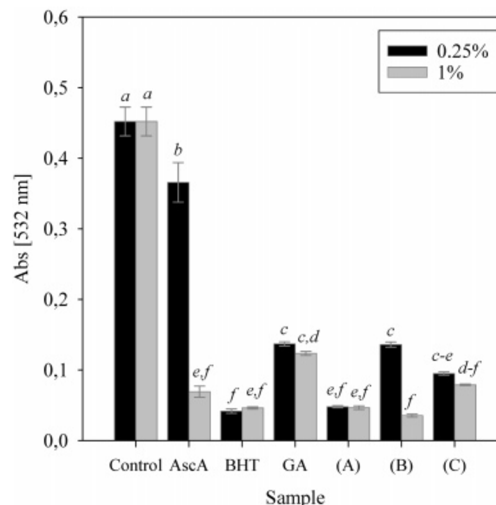

Figure 5. Effect of extracts of vine leaves and positive controls on the formation of MDA: AscA, ascorbic acid; BHT, butylated hydroxytoluene; GA, gallic acid; A, fresh leaves; B, brined leaves; C, unbrined leaves. Values are presented as means ± 95% confidence interval. Bars with the same lowercase letter (a–f) are not significantly ($p < 0.05$) different.

and its derivatives, hydroxycinnamates, are reported to be excellent DPPH• radical scavengers (*27*). In this study, the extracts contained hydroxycinnamic acids as the main phenolic acids and some flavonoids such as quercetin and its glycosides. Both phenolic acids and flavonoids are soluble in polar solvents and show strong activity in the polar test systems. Both iron(III) reduction and DPPH• radical scavenging activities are performed in polar media. Therefore, a correlation was found between the reducing power and DPPH• radical scavenging activity of vine extracts.

Determination of Inhibition of Linoleic Acid Peroxidation. *Iron(II) Thiocyanate Method (FTC).* Peroxidation of lipids is a principal cause of the oxidative deterioration of susceptible foodstuffs and the loss of physiological function in cellular organelles within the human body (*31*). Thus, phospholipids are a useful model substrate for the in vitro assessment of dietary components and membrane phospholipids as potential antioxidants. Furthermore, synthetic free radicals used to assess an antioxidant's reactivity to free radicals are physiologically irrelevant. The hydroxyl radical is physiologically relevant: these extremely reactive oxygen-derived species are capable of initiating deleterious in vivo chain reactions and are considered to play a role in the pathogenesis of numerous diseases (*32*). Thus, it is important to characterize the effectiveness of the extracts at inhibiting Fenton chemistry catalyzed hydroxyl radical mediated degradation of bovine brain derived phospholipid liposomes and to compare it to that of selected control substances (*32*).

In this method, the concentrations of 0.25 and 1% of each extract were used. These two concentrations are generally used for antioxidants within the foods in the food industry. Therefore, these two concentrations were chosen to test the activity of the extracts on linoleic acid peroxidation assays. The FTC method was used to measure the peroxide level during the initial stage of lipid oxidation. Low absorbance values would indicate high levels of antioxidative activity. The effects of the extracts on the peroxidation of linoleic acid were determined using an accelerated oxidation method, and the results are shown in **Figure 4**. As seen in **Figure 4**, all of the extracts were found to be more active in the lipid system at both concentrations. Such extracts were also found to be as active as positive controls

Table 2. Effects of Vine Leaves and Their Ethnic Products against Carrageenan-Induced Paw Edema in Mice

sample[b]	swelling thickness[a] ($\times 10^{-2}$ mm) [inhibition %]			
	90 min	180 min	270 min	360 min
control	44.8 ± 3.37	50.0 ± 3.37	56.0 ± 3.65	62.5 ± 4.68
indomethacin (10 mg/kg)	34.5 ± 2.99 [**22.9**]*	31.0 ± 2.02 [**38.0**]**	33.2 ± 2.15 [**40.7**]***	34.6 ± 2.38 [**44.6**]***

	dose											
	100 mg/kg	200 mg/kg	400 mg/kg	100 mg/kg	200 mg/kg	400 mg/kg	100 mg/kg	200 mg/kg	400 mg/kg	100 mg/kg	200 mg/kg	400 mg/kg
A	37.9 ± 2.98 [15.4]	42.0 ± 3.12 [6.3]	43.3 ± 3.01 [3.3]	39.7 ± 2.93 [20.6]	47.0 ± 3.11 [6.0]	46.6 ± 3.21 [6.8]	42.5 ± 2.88 [**24.1**]*	53.2 ± 3.66 [5.0]	51.8 ± 3.94 [7.5]	45.7 ± 3.10 [**26.9**]*	58.5 ± 4.05 [6.4]	57.6 ± 3.87 [7.8]
B	43.7 ± 2.15 [2.5]	44.7 ± 4.48	45.3 ± 3.01	49.8 ± 2.72	49.2 ± 4.67 [1.6]	51.2 ± 3.45	53.4 ± 3.15 [4.6]	54.0 ± 4.49 [3.6]	57.3 ± 3.92	59.7 ± 3.26 [4.5]	57.8 ± 4.38 [7.5]	63.3 ± 4.03
C	41.8 ± 2.92 [6.7]	53.2 ± 5.57	45.2 ± 3.94	45.7 ± 2.16 [8.6]	57.2 ± 5.44	50.4 ± 3.70	51.4 ± 2.93 [8.2]	61.5 ± 5.52	57.8 ± 4.12	54.9 ± 3.01 [12.2]	65.8 ± 5.40	63.1 ± 4.92

[a] Mean ± SEM; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ ($n = 8$). [b] A, fresh leaves extract; B, brined leaves extract; C, unbrined fermented leaves extract.

Table 3. Effects of Vine Leaves and Their Ethnic Products against *p*-Benzoquinone-Induced Writhings in Mice

test sample[a]	no. of writhings	inhibitory ratio (%)	ratio of ulceration
control	50.5 ± 4.15		0/6
ASA (200 mg/kg)	21.8 ± 1.74	**57.0***	5/6

	dose								
	100 mg/kg	200 mg/kg	400 mg/kg	100 mg/kg	200 mg/kg	400 mg/kg	100 mg/kg	200 mg/kg	400 mg/kg
A	38.4 ± 1.97[b]	47.8 ± 4.84	43.8 ± 2.93	23.9*	5.3	13.3	0/6	0/6	0/6
B	43.3 ± 2.61	45.3 ± 5.52	46.7 ± 4.15	14.3	10.3	7.5	0/6	0/6	0/6
C	42.2 ± 2.95	48.5 ± 3.67	49.8 ± 2.17	16.4	3.9	1.4	0/6	0/6	0/6

[a] A, fresh leaves extract; B, brined leaves extract; C, unbrined fermented leaves extract. [b] Mean ± SEM; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ ($n = 8$).

ascorbic acid, BHT, and gallic acid. The order of the inhibition activity of extracts was as follows: unbrined fermented leaves extract (78.8 ± 0.5%) > fresh leaves extract (77.3 ± 0.6%) > brined fermented leaves extract (66.7 ± 0.4%) at the concentration of 0.25%; all of the extracts showed the same significant ($p < 0.05$) activity at the concentration of 1% (99.0 ± 0.2, 91.3 ± 0.1, and 90.4 ± 0.6%, respectively).

Measurement of MDA Value (TBA Method). During the oxidation process, peroxides are gradually decomposed to lower molecular weight compounds. One such compound is malonaldehyde, which is measured by the TBA method on the final day of the incubation period. After the oxidation of lipids, MDA appears in the medium, and it is reacted with TBA (*18*). The MDA formed after oxidizing linoleic acid was measured using the TBA assay, and the results are given in **Figure 5**. Oxidized linoleic acid solutions used above were used in this assay, and the same results were obtained with the thiocyanate assay. The extract of unbrined fermented leaves was found to be as effective as BHT to inhibit lipid oxidation at both concentrations (**Figure 5**). One percent of the extract of brined leaves showed the same significant ($p < 0.05$) activity as with BHT. All of the extracts at the concentration of 1% showed significant ($p < 0.05$) activity similar to those of BHT and ascorbic acid.

Extracts contained almost similar amounts of hydroxycinnamic acid derivatives (caffeic acid) and flavonoids (quercetin) (**Table 1**). Hydroxycinnamic acid derivatives and flavonoids in free form are dissolved in the lipoid medium. It is well-known that lipophilic compounds are more active than hydrophilic compounds in the assay of lipid peroxidation (*28*, *33*, *34*). All extracts showed strong activity against the oxidation of linoleic acid. Antioxidative activities observed in the plants could be the synergistic effect of compounds that may be present in the plant. It has been reported that most natural antioxidative compounds often work synergistically with each other to produce a broad spectrum of antioxidative activities that creates an effective defense system against free radical attack (*18*).

Anti-inflammatory and Anti-nociceptive Activities. Water extracts of vine leaves were tested for their anti-inflammatory effects using a carrageenan-induced hind paw edema model in mice as a common in vivo activity screening model. The dose-dependent swelling thickness and inhibition effects of aqueous vine leaf extracts are presented in **Table 2**. Indomethacin (10 mg/kg) was used as positive control in this assay. As shown in **Table 2**, none of the extracts was found to be as effective as the positive control. The aqueous extract prepared from fresh leaves showed moderate inhibition ($p < 0.05$) at the 100 mg/kg dose after 270 and 360 min (24.1 and 26.9%, respectively). This extract can be used as a potential anti-inflammatory agent after further research.

The analgesic activities of the materials were studied by using a *p*-benzoquinone-induced writhing model in mice. The number of writhings, inhibition ratio, and the ratio of ulceration by the extracts are given in **Table 3**. According to **Table 3**, none of the extracts was found to be as effective as ASA used as positive control. The extract of fresh leaves showed moderate anti-nociceptive activity ($p < 0.05$) at the 100 mg/kg dose. None of the extracts caused any gastric damage, whereas ASA was found to have a high ulceration ratio (5/6). Therefore, this extract can be used for its anti-nociceptive properties. Vine leaves are rich in hydroxycinnamic acids and flavonoids. Flavonoids are known to have important physiological effects on various biological systems. The therapeutic applications of flavonoids on inflammation have previously been reported (*10*, *11*). Inflammation is important in many serious diseases, including cancer, Alzhe-

imer's, and AIDS. Therefore, intake of flavonoids is very important in the management of these diseases. In addition, flavonoids are known to prevent the synthesis of prostaglandins. Biochemical investigations on the mechanism of action of flavonoids have shown that these compounds can inhibit a wide variety of enzymes. Linoleic acid and arachidonic acid are indigenous compounds of the cell membrane with a task to protect the cell. The release of arachidonic acid is closely related to the cyclooxygenase (CO) and 5-lipoxygenase (LO) enzyme systems. The ability of flavonoids to inhibit both CO and LO pathways of the arachidonate metabolism has been suggested to contribute to anti-inflammatory action (*11*). Therefore, the protection of membrane lipids is important in inducing both anti-inflammatory and antioxidant activities. Quercetin derivatives are also known to possess many beneficial health effects such as cardiovascular protection, anticancer activity, and antiulcer activity. However, quercetin was found to be inactive in acetic acid induced writhing (*33*), with a negative response reported when it was given at a higher concentration (1 g/kg, ip). The anti-nociceptive effect of quercetin was reported to act through a central mechanism. Anti-inflammatory activity of quercetin against carrageen-induced paw edema model was observed at a dose of 20 mg/kg ip (*33*). In this study, hydroxycinnamates and flavonoids were found in vine leaves as the main phenolic groups. Biochemical investigations of the flavonoid mechanism of action have shown that these compounds inhibit a wide variety of enzymatic systems. The ability of flavonoids to inhibit both cyclooxygenase and 5-lipoxygenase pathways of the arachidonate metabolism may contribute to the anti-inflammatory properties. The antioxidant activities of flavonoids such as free radical scavenging and lipid peroxidation activities are also well-known. These activities seem to be directly related to the number of hydroxyl groups at ring B (*34*). Water extracts possessing moderate antioxidant activity can play an important role in anti-nociception. In this study, extract A was found to be active in anti-inflammatory and anti-nociceptive activity assays, whereas it was more active in the antioxidant assays, particularly in the linoleic acid peroxidation system (*35*−*37*).

In conclusion, vine leaves contain hydroxycinnamic acid derivatives (via caffeic and chlorogenic acids) and flavonoids (via quercetin and its derivatives) as the main compounds responsible for antioxidant activities as previously reported (*4*, *5*). Extracts were found to have strong activity in inhibiting linoleic acid peroxidation in in vitro tests. Further studies on the identification and purification of unidentified components responsible for the antioxidative activities in vine leaf are now in progress.

NOTE ADDED AFTER ASAP PUBLICATION

Reference citations in the original posting of May 9, 2007, were incomplete. This has been corrected in the posting of May 10, 2007.

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