JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Antioxidant and Iron-Binding Properties of Curcumin, Capsaicin, and S-Allylcysteine Reduce Oxidative Stress in Rat Brain Homogenate

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Research demonstrates that antioxidants and metal chelators may be of beneficial use in the treatment of neurodegenerative diseases, such as Alzheimer's disease (AD). This study investigated the antioxidant and metal-binding properties of curcumin, capsaicin, and *S*-allylcysteine, which are major components found in commonly used dietary spice ingredients turmeric, chilli, and garlic, respectively. The DPPH assay demonstrates that these compounds readily scavenge free radicals. These compounds significantly curtail iron- (Fe^{2+}) and quinolinic acid (QA)-induced lipid peroxidation and potently scavenge the superoxide anion generated by 1 mM cyanide in rat brain homogenate. The ferrozine assay was used to measure the extent of Fe^{2+} chelation, and electrochemistry was employed to measure the Fe^{3+} binding activity of curcumin, capsaicin, and *S*-allylcysteine. Both assays demonstrate that these compounds bind Fe^{2+} and Fe^{3+} and prevent the redox cycling of iron, suggesting that this may be an additional method through which these agents reduce Fe^{2+}-induced lipid peroxidation. This study demonstrates the antioxidant and metal-binding properties of these spice ingredients, and it is hereby postulate that these compounds have important implications in the prevention or treatment of neurodegenerative diseases such as AD.

KEYWORDS: Neurodegeneration; Alzheimer's disease; quinolinic acid; superoxide; lipid peroxidation

INTRODUCTION

Alzheimer's (AD) is the most common neurodegenerative disorder, affecting at least 5% of the population above the age of 65 years (*1*). Although the etiology of these diseases is not well understood, accumulating evidence suggests that oxidative stress is a major contributor to the initiation and progression of AD (*2*). Compared to other organs, the brain is particularly vulnerable to oxidative damage due to its high content of easily peroxidizable unsaturated fatty acids. Although the brain possesses antioxidant defense mechanisms, composed largely of glutathione, vitamin E, melatonin, and antioxidant enzymes, these cannot meet the extent of free radical generation during neurological insults. This vulnerability is exacerbated by the brain's requirement for large amounts of oxygen per unit weight; it has a high content of iron and ascorbate, which are the key ingredients required for lipid peroxidation, and it is not as highly enriched with antioxidant defense mechanisms compared to other organs (*3*).

Transition metals such as copper and iron are known to aggravate oxidative stress. Transition metals have been found in high concentrations in the brains of AD patients, and it has also been suggested that these metals are involved in the etiopathology of the changes found in these patients (*4*). These metals react with hydrogen peroxide (H_2O_2), which is a product formed by the dismutation of the superoxide anion ($O_2^{\bullet-}$) by superoxide dismutases, to produce the highly reactive hydroxyl radical ($^{\bullet}OH$) (*5*). This reaction, shown below, is known as the Fenton reaction:

$$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^- + {}^{\bullet}OH$$

In the presence of reducing agents such as ascorbic acid, the oxidized metal is reduced, allowing it to react with another molecule of H_2O_2 to generate another $^{\bullet}OH$ radical. This potent free radical directly targets nucleic acids, causing DNA damage and lipid peroxidation (*6*).

Quinolinic acid (QA), a neurotoxic metabolite of the tryptophan–kynurenine pathway, has also been implicated in many neurodegenerative disorders (*7*). This excitatory amino acid has been shown to induce lipid peroxidation and cause neurodegeneration (*8*). It has been demonstrated that QA induces lipid peroxidation in rat brain homogenate through the chelation of ferrous ions, and it is the QA–iron complex that stimulates lipid peroxidation (*9*).

Neurodegenerative disorders, such as AD, pose severe economic and social stress with great emotional and physical drain on families and caregivers. The current treatment for these diseases provides symptomatic improvements or delays the progression of the

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10.1021/jf0734931 CCC: $40.75 © 2008 American Chemical Society
Published on Web 04/19/2008

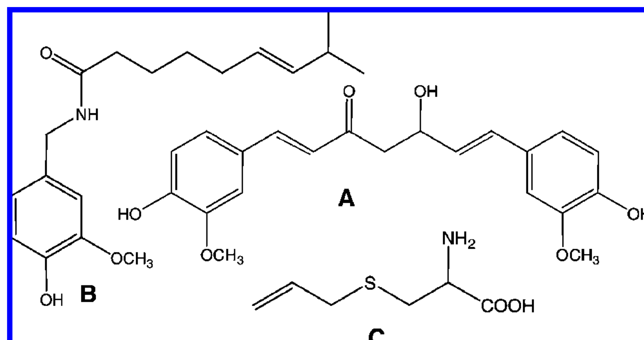

Figure 1. Chemical structures of the antioxidants investigated in this study: **A**, curcumin (CURC); **B**, capsaicin (CAP); and **C**, *S*-allylcystein (SAC).

cognitive, behavioral, and functional deficits. However, not only does the current treatment fail to arrest or reverse the progression of the diseases, but long-term treatment has many adverse side effects. Therefore, there is a need for more efficient and potent drugs that will manage these diseases without the severe side effects currently associated with treatment.

Research has demonstrated that antioxidants (*10*) and metal chelators (*11*) may be of beneficial use in the treatment of these neurodegenerative disorders. Statistics confirm that there is a reduced incidence of AD among the inhabitants of Indian villagers compared to the Western world (*12, 13*). It has been thought that this is due to the various antioxidant and neuroprotective components in the spices commonly consumed in the diet of this population group (*14, 15*). Therefore, to examine the potential neuroprotective benefits that these dietary components may confer, in this study we investigated the antioxidant and iron-binding properties of curcumin (CURC), capsaicin (CAP), and *S*-allylcysteine (SAC), which are the most active agents present in the Indian spices turmeric, chilli, and garlic, respectively. Curcumin, which also plays an important role in aryuvedic medicine and as a coloring agent, and its demethoxylated curcuminoids are known antioxidants (*16, 17*) with several biological roles. Likewise, capsaicin has also been shown to possess antiperoxidative properties (*18*). The structures of the investigated compounds are depicted in **Figure 1**.

MATERIALS AND METHODS

Chemicals and Reagents. Curcumin, capsaicin, butylated hydroxytoluene (BHT), 2-thiobarbituric acid (TBA), 1,1,3,3-tetramethoxypropane (99%), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine (ferrozine), nitroblue tetrazolium (NBT), nitroblue diformazan (NBD), ferrous chloride ($FeCl_2$), quinolinc acid, L-cysteine, allylbromide, and glutathione were purchased from Sigma Chemical Corp., St. Louis, MO. Potassium cyanide (KCN), trichloroacetic acid (TCA), ethanol, ferrous sulfate ($FeSO_4$), ascorbic acid, and butanol were purchased from Saarchem, Johannesburg, South Africa. Ethylenediaminetetraacetic acid (EDTA) was obtained from HOLPRO Analytics, (Pty.) Ltd., Johannesburg, South Africa. Hydrogen peroxide and anhydrous ferric chloride ($FeCl_3$) were purchased from BDH Laboratory Supplies, Poole, U.K. SAC was synthesized by the reaction of L-cysteine and allylbromide and purified by recrystallization from ethanol/water as previously described by Maldonado et al. (*19*). The structure and purity were confirmed by ^1H and ^{13}C nuclear magnetic resonance spectroscopy. The ^1H (400 MHz), ^{13}C (100 MHz), DEPT135, COSY, HMQC, and HMBC NMR spectra were recorded on an Avance Bruker 400 MHz spectrometer using standard pulse sequences. All other reagents used were of the highest quality available.

BHT was dissolved in absolute ethanol. TBA, TCA, ascorbic acid, EDTA, and H_2O_2 were prepared in degassed Milli-Q water. CURC, CAP, and SAC were dissolved in ethanol to reach a final concentration of ethanol of no greater than 0.1% in water. $FeSO_4$ was dissolved in Milli-Q water immediately before use.

DPPH Radical Scavenging Activity. The free radical scavenging activity of CURC, CAP, and SAC was measured by the scavenging of the DPPH radical using a modified method of Brandt-Williams et al. (*20*). DPPH (1.2 mL, 0.1 mM) dissolved in methanol was incubated for 20 min with 0.3 mL of CURC, CAP, or SAC at final concentrations of 0.625−60 μg/mL. The absorbance was read after 20 min at 517 nm. The DPPH radical scavenging activity was calculated using the equation

$$\text{scavenging activity (\%)} = \{(A_0 - A_1)/A_0\} \times 100$$

where A_0 is the absorbance of the control reactions and A_1 is the absorbance in the presence of the test compound. For comparative purposes, these experiments were also conducted using endogenous occurring antioxidants such as glutathione, melatonin (*21*), and the water-soluble antioxidant ascorbic acid at the same final concentrations.

Determination of Oxidation Potentials (OPs) by Cyclic Voltammetry. Cyclic voltammetry (CV) is an electroanalytical technique that is used to characterize the redox processes of electroactive species in solution. The total antioxidant capacity of a compound in solution is a function of combining two sets of parameters. The first is the biological oxidation potential (E_p) (OP), which indicates the specific reducing power of the compound in solution, and the second is the intensity of the anodic peak current produced. The intensity of the current produced by the compound is a reflection of the concentration of the compound in solution. However, it has been proposed that the area under the curve (AUC) response is a better parameter to measure and report the total antioxidant capacity of the compound. This method has been used extensively to measure total antioxidant capacity (*22*).

This technique was used to determine the OPs of CURC, CAP, and SAC. The OPs of the compounds in this study were also compared to those of glutathione, melatonin, and ascorbic acid. Cyclic voltammograms were recorded on the Autolab PGSTAT 30 voltammeter equipped with a Metrohm VGA cell stand. A 3 mm diameter glassy carbon electrode (GCE) was employed as a working electrode for voltammetric experiments and scanned at 100 mV/s. Silver/silver chloride (KCl = 3 M) and platinum wires were employed as reference and auxiliary electrodes, respectively, for all electrochemical work. The analyte used for the experiments was 0.2 M Tris-HCl, pH 7.4. Prior to use and between scans, the GCE was cleaned by polishing with alumina on a Buehler pad, followed by washing in nitric acid and rinsing in ethanol followed by Milli-Q water. All solutions were degassed with nitrogen.

Animals. Adult male rats of the Wistar strain, weighing between 250 and 300 g, were purchased from the South African Institute for Medical Research (Johannesburg, South Africa). The animals were housed in a controlled environment with a 12 h light/dark cycle and were given access to food and water ad libitum. The Rhodes University animal ethics committee approved protocols for the experiments.

Homogenate Preparation. On the day of the experiment, rats were killed by cervical dislocation and decapitation. The brains were rapidly removed and homogenized whole (10% w/v) in 0.1 M phosphate-buffered saline (PBS) at pH 7.4 and used immediately for assay.

Fe^{2+}-Induced Lipid Peroxidation Assay. Lipid peroxidation was determined according to a modified method of Placer et al. (*23*). To 0.8 mL of brain homogenate were added final concentrations of 1 mM ascorbate, 100 μM EDTA, 0.1 mM $FeSO_4$, and 0.2 mM H_2O_2. This was performed in either the absence or presence of CURC, CAP, or SAC at final concentrations of 25, 50, or 100 μM. The homogenate solutions were subsequently incubated in a shaking water bath for 1 h at 37 °C. At the end of the incubation period 0.5 mL of BHT (0.5 mg/mL in ethanol) and 1 mL of 25% TCA were added. The tubes were sealed and heated for 10 min in a boiling water bath to release protein-bound malondialdehyde (MDA), the product of lipid peroxidation. To avoid adsorption of MDA to insoluble proteins, the samples were cooled to 4 °C and centrifuged at 2000*g* for 20 min. Following centrifugation, 2 mL of the protein-free supernatant was removed from each tube, and 0.5 mL aliquots of 0.33% TBA were added to this fraction. All tubes were heated for 1 h at 95 °C in a water bath. After cooling, the TBA−MDA complexes were extracted with 2 mL of

butanol. The absorbance was read at 532 nm, and MDA levels were determined from a standard curve generated from 1,1,3,3-tetramethoxypropane. Final results are represented as nanomoles of MDA per milligram of tissue.

QA-Induced Lipid Peroxidation. These experiments were conducted as described above; however, lipid peroxidation was induced with 1 mM QA with and without CURC, CAP, or SAC at final concentrations of 25, 50, or 100 μM.

Superoxide Anion Assay. The assay procedure was a modification of that of Das et al. (*24*). Homogenate (1 mL) containing 1 mM KCN alone or in combination with CURC, CAP, or SAC at final concentrations of 25, 50, or 100 μM was incubated with 0.4 mL of 0.1% NBT in a shaking water bath for 1 h at 37 °C. Termination of the assay and extraction of the reduced NBT were carried out by centrifuging the samples for 10 min at 2000*g* followed by resuspension of the pellets with 2 mL of glacial acetic acid. The absorbance was measured at 560 nm and converted to micromoles of diformazan using a standard curve generated from NBD. Final results are expressed as micromoles of diformazan per milligram of tissue.

Chelating Activity of CURC, CAP, and SAC of Fe^{2+} Ions. The chelating activity of the investigated antioxidants on Fe^{2+} ions was measured using the method of Decker and Welch (*25*). Briefly, 1 mL of solution containing increasing concentrations of CURC, CAP, or SAC was added to 3.7 mL of H_2O. This mixture was then reacted with 0.1 mL of 2 mM $FeCl_2$ and 0.2 mL of 5 mM ferrozine. The absorbance was read after 20 min at 562 nm. The lower absorbance of the mixture indicated higher chelating activity. The percentage chelation activity on the Fe^{2+}−ferrozine complex was calculated using the equation

$$\text{chelating activity } (\%) = \{(A_0 - A_1)/A_0\} \times 100$$

where A_0 is the absorbance of the control reactions and A_1 is the absorbance in the presence of the test compounds at final concentrations of 1−30 μg/mL. EDTA was used as the positive control.

Determination of Fe^{3+} Ion Chelation by Adsorptive Stripping Voltammetry (ASV). Instrumentation and electrodes used for these experiments were as reported above for CV experiments. For all experiments 10 μM Fe^{3+} and increasing micromolar concentrations CURC, CAP, or SAC were introduced into an electrochemical cell. The electrolyte used was 0.2 M Tris-HCl buffer, pH 7.4. For ASV, the optimum deposition potential for Fe^{3+} was identified (0.37 V) and applied for 60 s to effect the formation and adsorption of the metal and ligand species onto the GCE. The voltammograms were then scanned in the negative direction from the deposition potential to at least 0.50 V beyond the reduction of the metal at the scan rate of 0.10 $V \cdot s^{-1}$ to strip the adsorbed metal–ligand species from the electrode. During the stripping step, current responses due to the reduction of the metal–ligand species were measured as a function of potential. All potential values quoted are referenced against the silver/silver chloride reference electrode. Blanks were performed under the same conditions in pure electrolyte. The technique that was employed to measure the extent of Fe^{3+} binding has been previously used to measure metal binding (*26, 27*).

Statistical Analysis. The lipid peroxidation assay and superoxide anion assay results were analyzed using a one-way analysis of the variance (ANOVA) followed by the Student–Newman–Keuls multiple range test. The level of significance was accepted at $p < 0.05$. The DPPH and ferrozine assay results were analyzed using two-way ANOVA followed by the Scheffe test as post hoc analysis.

RESULTS AND DISCUSSION

DPPH Radical Scavenging Activity. Figure 2 shows that all compounds potently scavenge the DPPH free radicals. At a final concentration of 60 μg/mL ascorbic acid scavenges 96.6% of the free radicals, whereas CURC, CAP, SAC, glutathione, and melatonin scavenge 92.9, 91.9, 76.2, 65.5, and 57.3% of the DPPH free radicals at the same concentration, respectively. Statistical analysis shows that all compounds significantly differ compared to each other in scavenging DPPH free radicals over the entire concentration range tested. Research has demonstrated that CURC (*28*) and CAP (*29*) are more potent free radical scavengers than vitamin E. This study shows that both of these compounds reduced the DPPH radical more potently than SAC and the well-known antioxidants glutathione and melatonin. It has been suggested that the C7-benzyl carbon in CAP (*29*) and the methoxy group on the phenol ring (*29*) as well as the β-diketone moiety (*30*) in CURC are the important functional groups on these compounds responsible for the potent antioxidant and free radical scavenging properties.

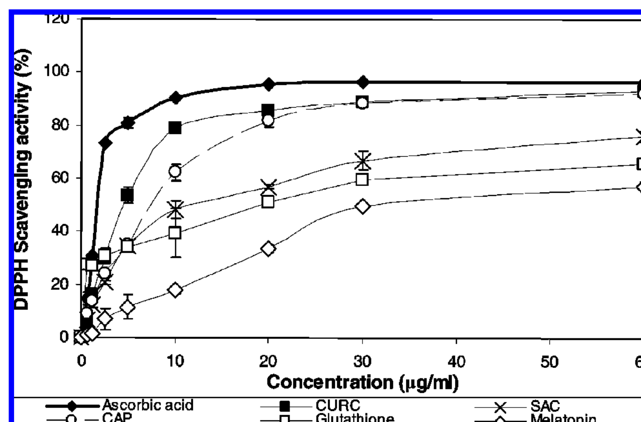

Figure 2. DPPH radical scavenging activity of CURC, CAP, SAC, ascorbic acid, glutathione, and melatonin. Each point represents the mean ± SD of five separate determinations.

Table 1. Oxidation Potentials and Areas under Curve of the Anodic Peaks of CURC, CAP, SAC, Ascorbic Acid, Glutathione, and Melatonin, Determined Using Cyclic Voltammetry[a]

antioxidant	OP (V)	AUC (μC)
ascorbic acid	0.012	0.45
capsaicin	0.37	2.27
curcumin	0.41	0.19
glutathione[b]	0.60	1.02
melatonin	0.64	0.36
S-allylcysteine	none	none

[a] All OPs and AUCs were determined using a bare GCE in 0.2 M Tris-HCl buffer, pH 7.4. Data were obtained from samples that had a final concentration of 5 μg/mL in the electrochemical cell. [b] OP of GSH obtained using a final concentration of 30 μg/mL.

Oxidation Potentials of CURC, CAP, SAC, Glutathione, and the Oxidized Species. Oxidation potentials and the area under the curve for the compounds examined are shown in **Table 1**. CURC, CAP, ascorbic acid, and melatonin produce oxidation potentials at 0.41, 0.37, 0.012, and 0.64 V, respectively. A more potent antioxidant donates its electrons at a lower OP (*22*). Ascorbic acid has the lowest OP and most potently scavenges the DPPH free radicals. CURC and CAP have lower OPs than glutathione and melatonin and also scavenge the DPPH free radicals more effectively than these endogenously occurring antioxidants. The AUC of CAP is more than that shown by ascorbic acid and melatonin. Although ascorbic acid has a smaller AUC than CAP, it has a much lower OP than these compounds and scavenges more of the DPPH radicals. Therefore, the total antioxidant capacity must be evaluated as a function of both parameters, the OP and the AUC. SAC and GSH also scavenged the DPPH free radicals but did not show any OP at this concentration, indicating that these compounds are not electroactive or, as has been reported for thiols, possess sluggish electron transfer kinetics. However, at much higher concentrations (30 μg/mL final concentration), GSH produces an OP of 0.60 V with an AUC of 1.02 μC. The OP for GSH is

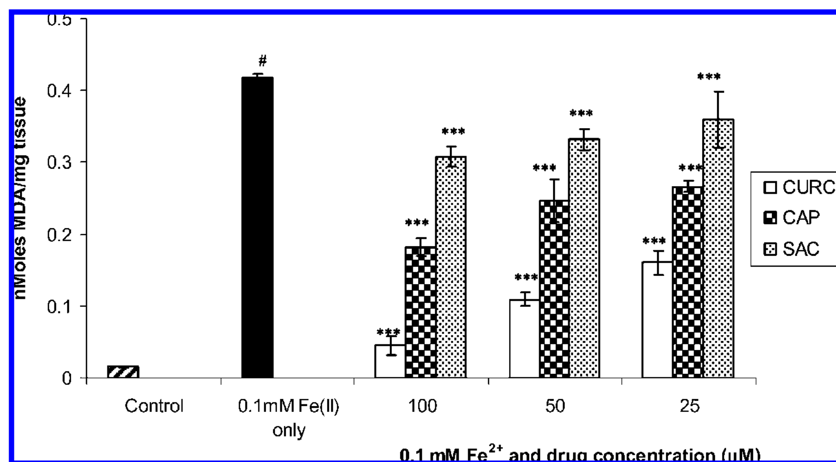

Figure 3. Effect of CURC, CAP, and SAC on Fe^{2+}-induced lipid peroxidation in rat brain homogenate. Each bar represents the mean ± SD ($n = 6$). #, $p < 0.001$, in comparison to control; ∗∗∗, $p < 0.001$, in comparison to 0.1 mM Fe^{2+}-induced lipid peroxidation.

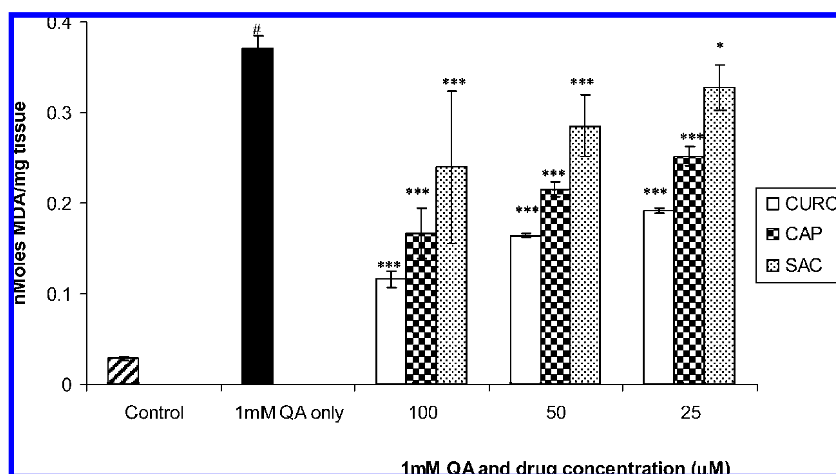

Figure 4. Effect of CURC, CAP, and SAC on QA (1 mM)-induced lipid peroxidation in rat brain homogenate. Each bar represents the mean ± SD ($n = 6$). #,$p < 0.001$, in comparison to control. ∗, $p < 0.05$, and ∗∗∗, $p < 0.001$, in comparison to 0.1 mM QA-induced lipid peroxidation.

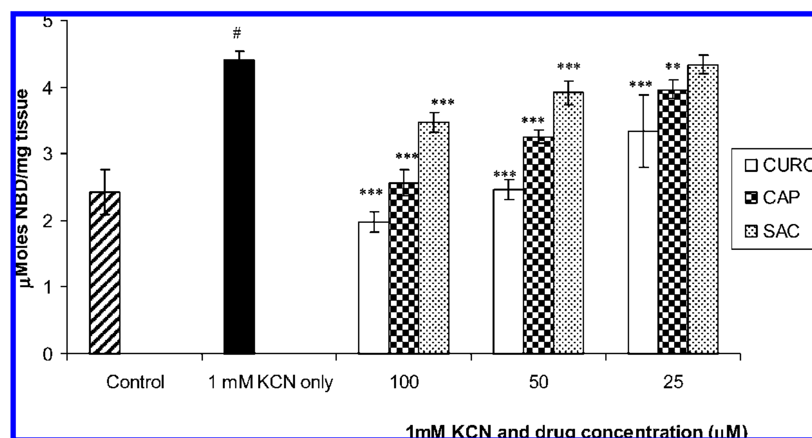

Figure 5. Effect of CURC, CAP, and SAC on KCN (1 mM)-induced superoxide anion generation in rat brain homogenate. Each bar represents the mean ± SD ($n = 6$). #, $p < 0.001$, in comparison to control. ns, $p > 0.05$, ∗∗, $p < 0.01$, and ∗∗∗, $p < 0.001$, in comparison to 1 mM KCN group.

slightly lower than that observed for melatonin, concurring with the DPPH assay results in which GSH shows greater antioxidant properties than melatonin.

It has been suggested that the use of CV may be a valuable tool in the evaluation of total antioxidant capacity in plasma, plant extracts, and tissue homogenates (*22*). It has also been stated that CV may be used for the rapid screening of antioxidant activity (*22*). The data from this study indicate that there is a strong correlation between the data obtained from the CV and the DPPH assays. However, the method is limited by the electroactivity of the screened molecules.

Fe^{2+}- and QA-Induced Lipid Peroxidation. Fe^{2+} significantly ($p < 0.001$) induces lipid peroxidation in rat brain homogenate. However, the co-incubation of the homogenates with CURC, CAP, or SAC significantly ($p < 0.001$) curtails lipid peroxidation in a concentration-dependent manner (**Figure**

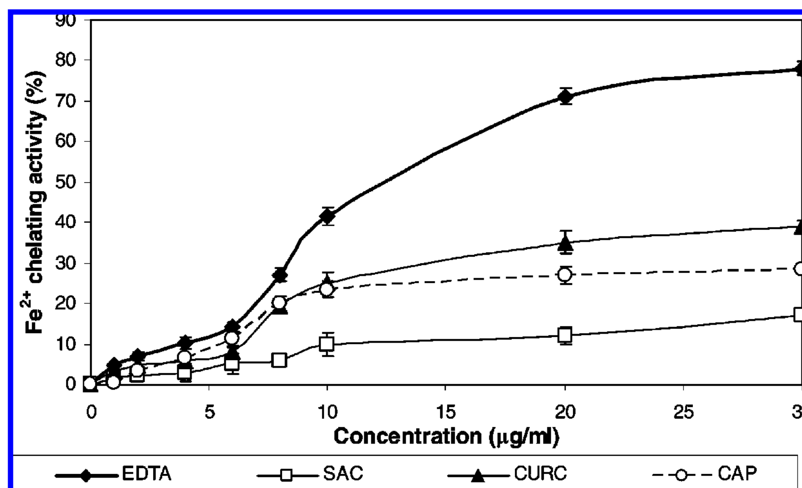

Figure 6. Fe^{2+} ion chelating activity of CURC, CAP, SAC, and EDTA. Each point represents the mean ± SD of five separate determinations.

3). **Figure 4** shows that the excitatory amino acid, QA, also induces lipid peroxidation in rat brain homogenate, but the co-incubation of the homogenates reduces this phenomenon. CURC reduces the Fe^{2+}- and QA-induced lipid peroxidation more than CAP and SAC, in that order. It is well-known that the Fenton reaction is responsible for the generation of the potent hydroxyl radical (*5*), which targets nucleic acids (*31*), causes DNA damage, and induces lipid peroxidation (*6*). QA has been shown to modulate lipid peroxidation through the chelation of ferrous ions (*9*). CURC has previously been demonstrated to reduce the Fe^{2+}-induced lipid peroxidation by its potent antioxidant (*32*) and metal-binding properties (*27*). It is possible that the antioxidant properties of CAP and SAC may have been responsible for the reduction in the lipid peroxidation because it has been previously reported that CAP (*18*) and SAC (*33*) have potent antioxidant properties. These spice ingredients also bind Fe^{2+} as demonstrated by the Fe^{2+} chelation assay and prevent the formation of the QA−ferrous ion complex and could be another mechanism through which these agents reduce lipid peroxidation.

Superoxide Anion Scavenging. One millimolar KCN significantly induces the generation of the superoxide anion in rat brain homogenate (**Figure 5**). KCN inhibits complex IV of the mitochondrial electron transport chain, and inhibition of this distal end of the chain augments reactive oxygen species generation (*34*). KCN inhibits cytochrome oxidase a_1a_3 (*35*), causing electrons to leak out of the mitochondria, and results in the reduction of oxygen to the superoxide anion. Studies have shown that CURC is a powerful scavenger of the superoxide anion (*32*). This study shows that the co-incubation of the homogenates with the spice ingredients CURC, CAP, and SAC reduces the production of NBD. SAC does not scavenge the superoxide anion at 25 µM. It is possible that these agents significantly scavenge the superoxide anion, with CURC and CAP being more potent than SAC.

Fe^{2+} and Fe^{3+} Ion Chelating Activity. Ferrozine forms complexes with Fe^{2+} in a quantitative manner, and in the presence of chelating agents this complex is inhibited or disrupted. **Figure 6** shows the Fe^{2+} ion chelating activity of EDTA, CURC, CAP, and SAC. These compounds chelate approximately 78.1, 38.9, 28.5, and 16.9% of the Fe^{2+} ions at 30 µg/mL (final concentration), respectively. The Fe^{2+} ion chelating activity of these spice ingredients diminishes the available Fe^{2+} that reduces H_2O_2 to the potent hydroxyl radical, which subsequently results in the decrease of Fe^{2+}- and QA-induced lipid peroxidation.

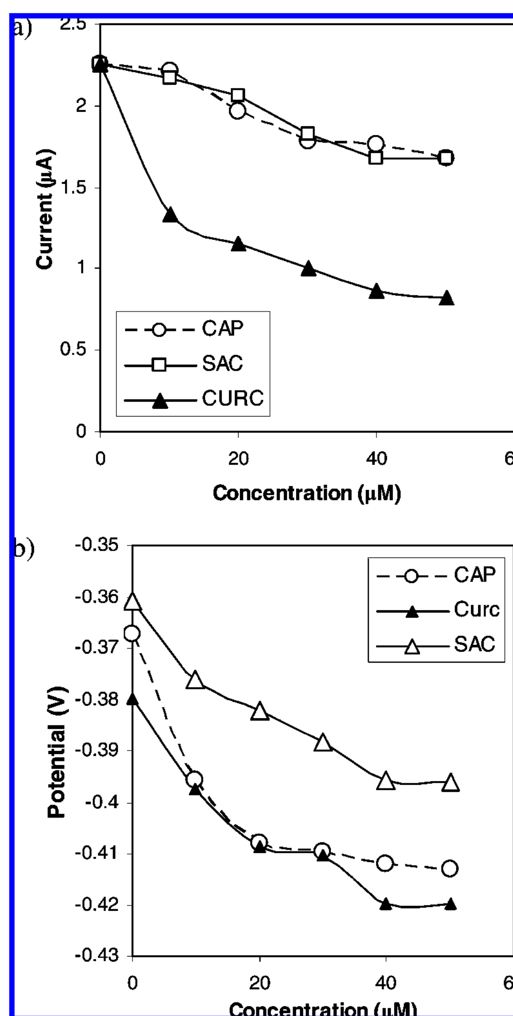

Figure 7. Effect of increasing concentrations of CURC, CAP, or SAC on (**a**) the cathodic peak current and (**b**) the cathodic peak potential of 10 µM Fe^{3+}.

Upon the addition of increasing concentrations of CURC, CAP, and SAC to the electrochemical cell containing 10 µM Fe^{3+}, there is a decrease in the current response (**Figure 7a**) and a shift of the Fe^{3+} peak to more negative potentials (**Figure 7b**). CURC causes the greatest decrease in current response and shifts the Fe^{3+} peak the most, to more negative potentials than CAP or SAC. It has been demonstrated that a decrease in the current response and a shift of the metal peak to more negative

potentials is an indication of a relatively strong metal–ligand interaction (*26, 36*). With supporting data from the ferrozine assay, these results indicates that CURC, CAP, and SAC bind Fe^{3+}, indicating that these agents may prevent the redox cycling of iron, and this may also be a mechanism through which these agents may reduce lipid peroxidation. Whereas the systemic bioavailability of CURC has been shown to be low (*37*) compared to the high bioavailability of SAC (*38*), in vivo studies may provide further conclusive evidence to support the key observations in this study.

In conclusion, this study also supports the use of CV for the rapid screening of antioxidants possessing electroactive properties. The potent antioxidant and iron-binding properties of CURC, CAP, and SAC may be mechanisms through which these commonly used spice ingredients offer neuroprotection and may explain the low incidence of neurodegenerative diseases in certain villages in India.

ABBREVIATIONS USED

AD, Alzheimer's disease; ASV, adsorptive stripping voltammetry; AUC, area under the curve; BHT, butylated hydroxytoluene; CAP, capsaicin; CURC, curcumin; CV, cyclic voltammetry; DPPH, 1,1-diphenyl-2-picrylhydrazyl; GCE, glassy carbon electrode; MDA, malondialdehyde; NBT, nitroblue tetrazolium; NBD, nitroblue diformazan; OP, oxidation potential; QA, quinolinic acid; SAC, *S*-allylcysteine; TBA, 2-thiobarbituric acid.

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Received for review November 29, 2007. Revised manuscript received February 8, 2008. Accepted March 7, 2008. This research was supported by a grant from the National Research Foundation (NRF, South Africa).

JF0734931