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Cucurbitacin glucosides: Antioxidant and free-radical scavenging activities

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Abstract

The cucurbitacins are of great interest because of the wide range of biological activities they exhibit in plants and animals. We studied the antioxidant properties of cucurbitacin B + E glucosides (cucurbitacin glucoside combination, CGC) and their direct free-radical scavenging properties, using ESR spectroscopy. Antioxidant activity was measured by the ability of the CGC to reduce preformed $ABTS^{\bullet+}$ into its native form and to inhibit MDA formation during the oxidation of linoleic acid. In both methods, the CGC exhibited antioxidant activity in a dose-dependent manner as expected from antioxidants. Using ESR spectroscopy, we found that the CGC inhibited $^{\bullet}OH$-dependent DEPMPO–OH adduct formation, $O_2^{\bullet-}$-dependent DEPMPO–OOH adduct formation, and the $^1O_2$-dependent TEMPO adduct generated in the photoradiation–porphin system. The IC_{50} values were 0.38, 8, and 11 mM, respectively. Together, these data demonstrate that the CGC exhibits antioxidant properties, probably through the involvement of a direct scavenging effect on several free radicals.

Keywords: Antioxidant; Cucurbitacin glucoside combination (CGC); ESR; Hydroxyl radical; Singlet oxygen; Superoxide anion

Oxidative stress induced by reactive oxygen species (ROS) such as hydrogen peroxide, superoxide anions, and singlet oxygen is believed to be a primary factor in various diseases as well as in the normal process of aging [1,2]. Reactive oxygen species can initiate, for example, the peroxidation of membrane lipids, leading to the accumulation of lipid peroxides. The elimination or inactivation of reactive oxygen species, or the prevention of their cellular formation by antioxidants and free-radical scavengers, is considered to be a practical approach to reducing the risk of these diseases. The importance of antioxidants in human health has become increasingly clear due to spectacular advances in understanding the mechanisms of their reaction with oxidants.

Plants contain a wide variety of chemicals that have potent biological effects [3]. As a result, there has been a growing interest in the use of herbs as a source of therapeutic drugs. Our group has succeeded in isolating and characterizing a water-soluble antioxidant from spinach [4], and we presented its antioxidant and broad free-radical scavenging properties [5,6]. Recently, our group has also described the isolation and chemical identification of cucurbitacin B and E glucosides isolated from *Citrullus colocynthis* (L.) Shrad [7].

Citrullus colocynthis (L.) Shrad (Cucurbitaceae), locally known as Sherry or Handal, is used in folk medicine in rural areas as a purgative, antirheumatic, and a remedy for skin infections. This plant contains cucurbitacins A, B, C, and D, α-elaterin, and probably other constituents [8].

The cucurbitacins (highly oxygenated tetracyclic triterpens) are of great interest because of the wide range of biological activities they exhibit in plants and animals. They are predominantly found in the Cucurbitaceae family, but are also present in several other families of the plant kingdom. Species of the plants containing cucurbitacins have been used for centuries in various pharmacopoeia. A number of compounds of this group have been investigated for their cytotoxic, hepatoprotective, cardiovascular, and antidiabetic effects [9]. Cucurbitacins B and I exhibit antioxidant activity [9]. Cucurbitacin B was also shown to exhibit anti-inflammatory activity [10,11] and cucurbitacins B, D, E, and I inhibit the COX-2 enzyme, but not COX-1

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[9]. Additionally, several studies indicated that different cucurbitacin species inhibit the proliferation of cancer cells through different mechanisms [12–15].

Recently, we isolated cucurbitacin B and E glucosides from *C. colocynthis*, and explored the mechanism by which their combination (1:1) reduces the proliferation of human breast cancer cells [7].

The aim of the present study was to examine the effect of a cucurbitacin glucoside combination (CGC) (1:1) as an antioxidant and free-radical scavenger. We report here that this CGC exhibits both antioxidant and free-radical scavenging activities.

Materials and methods

Materials

TEMP (2,2,6,6-tetramethyl-4-piperidone hydrochloride), xanthine, xanthine oxidase, PBS, $FeSO_4$, AAPH (2,2′-azobis(2-amidinopropane)dehydrochloride), and ABTS (2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)) were obtained from Sigma Chemicals, USA. DEPMPO (5-diethoxyphosphoryl-5-methyl-1-pyrroline-N-oxide) was purchased from Calbiochem, CA, USA. $TPPS_4$ (meso-tetra(4-sulfonatophenyl) porphine) was purchased from Porphyrin Products, UT, USA. Trolox was obtained from Acros Organics, NJ, USA.

Determination of total antioxidant activity by measuring the reducing power of cucurbitacin glucosides

The total antioxidant capacity of CGC (compared to trolox, resveratrol, and ascorbic acid) was measured by the (2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)) $ABTS^{\bullet+}$ decolorization assay involving preformed $ABTS^{\bullet+}$ radical cation, as described previously by Kerem and others [16]. ABTS radical cation ($ABTS^{\bullet+}$) was generated by mixing 5 ml of an aqueous ABTS solution (7 mM) and 88 μl of a potassium persulfate solution (140 mM) followed by incubation for 12 h at room temperature in the dark [17]. The resulting $ABTS^{\bullet+}$ radical cation was diluted with ethanol to give an absorbance of $\sim$0.7 OD $\pm$ 0.02 at 734 nm. Test samples (0.02–0.2 mM CGC) and PBS were transferred to a 96-well plate and the assay was started by the addition of 200 μl of the preformed $ABTS^{\bullet+}$ solution. Absorbance at 734 nm was measured, and the percentage of reduction of $ABTS^{\bullet+}$ was calculated.

Determination of antioxidant activity by the ability of CGC to inhibit linoleic acid oxidation

Antioxidant activity was determined by measuring the inhibition of linoleic acid oxidation initiated by AAPH. Linoleic acid was prepared in Tween 20, as described [18]. In brief, a stock solution of 3×10^{-2} M linoleic acid was prepared by addition of 3 nmol of linoleic acid to 50 ml of distilled water containing 1 ml of Tween 20. To clarify the resulting emulsion, 3–5 ml of 1 N NaOH was added, and the volume was adjusted to 100 ml with distilled water. This stock solution was diluted with 100 mM phosphate buffer, pH 7.0, to 7.5×10^{-3} M. The solution was stored at 4 °C for up to 2 weeks. For the oxidation assay, 80 μl of this solution was incubated with 25 mM AAPH in the presence or absence of CGC at 39 °C for 2 h. From this solution, 40 μl was taken for MDA assay.

Oxidation level was determined by measuring MDA (malonedialdehyde) and 4-HNE (4-hydroxynonenal), a linoleic acid chain-cleavage product. This determination was based on the reaction of the chromogenic reagent, *N*-methyl-2-phenylindole, with MDA and 4-hydroxyalkenals at 45 °C. The chromophore absorbs at 586 nm [19].

For the assay, an aliquot of 40 μl of the tested sample was added to each well in a 96-well plate, followed by 130 μl of R-1 (7.725 mM *N*-methyl-2-phenylindole diluted in methanol 3:1) and 30 μl of R-2 (15.4 M methanesulfonic acid). Three replicates were prepared for both controls and samples. The microplate was incubated for 1 h at 45 °C. The microplate was read at 586 nm using a spectrofluorometer (Tecan).

ESR measurements

Recording of ESR signals. The ESR spectra were recorded on a Bruker ER 100d X-band spectrophotometer. The measurements were repeated three times for each sample. After acquisition, the spectra were processed using Bruker WIN-EPR software (version 2.11) for baseline correction, noise filtration, and integration of the signals (in all the figures, the intensity was expressed in arbitrary units). The ESR parameters were set at microwave frequency—9.74 GHz; modulation frequency—100 kHz; microwave power—20 mW; modulation amplitude—1 G; time constant—163.840 ms; sweep time—41.9 s; and resolution—1024 points.

Hydroxyl free-radical assay

In our experiments, DEPMPO was used as a spin trap, as it was reported to be one of the most suitable spin traps for various types of radicals, including superoxide and hydroxyl radical, peroxy radical, and sulfur radical. Additionally, DEPMPO represents an improvement over the widely used DMPO in terms of higher stability of the spin adducts [20].

The Fenton reaction ($Fe^{+2} + H_2O_2 \rightarrow Fe^{+3} + {}^{\bullet}OH + OH^{-}$), a well-known and defined generator of $^{\bullet}OH$, was utilized to examine whether cucurbitacin glucosides scavenge $^{\bullet}OH$ [21]. The reaction mixture contained DEPMPO (30 mM), $FeSO_4$ (100 μM), and H_2O_2 (10 mM), in the absence or presence of CGC at a final volume of 100 μl. Hydroxyl radical detection was based on the specific reaction between $^{\bullet}OH$ and DEPMPO, which forms a stable and ESR-detectable DEPMPO–OH adduct.

Superoxide anion assay

The reaction of xanthine and xanthine oxidase was used as a source of superoxide radical anion $O_2^{\bullet-}$ [22]. Prior to performing ESR studies, we examined whether CGC inhibits xanthine oxidase activity by monitoring uric acid formation spectrophotometrically at 290 nm. There was no significant change in urate formation, indicating that CGC did not inhibit this enzyme. The typical reaction mixture consisted of 30 mM DEPMPO, 20 μM xanthine, 0.0115 U xanthine oxidase, and CGC. The reaction was initiated by adding xanthine oxidase from a stock solution prepared in PBS, pH 7.4. Superoxide anion radical detection was based on the specific reaction between $O_2^{\bullet-}$ and DEPMPO, which forms a stable and ESR-detectable DEPMPO–OOH adduct.

Determination of singlet oxygen

Singlet oxygen detection was based on the specific reaction between $^1O_2$ and TEMP, which forms a stable and ESR-detectable TEMPO adduct. Singlet oxygen was generated in the photoradiation–porphin system. The reaction mixture contained 0.054 mM TPPS4, 50 mM TEMP, and various concentrations of CGC. The mixture was irradiated by a visible light projector at 400–800 nm (40 mW/cm^2) at room temperature for 85 s, and ESR spectra were recorded.

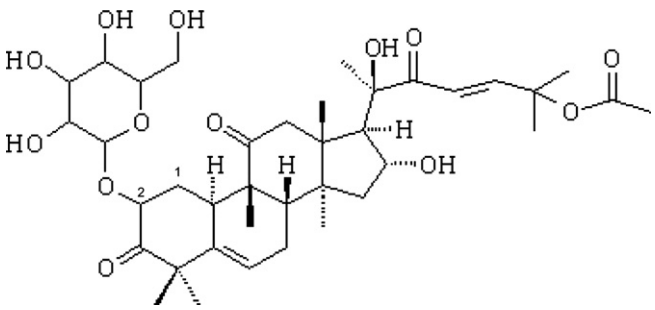
Statistical analysis. All experiments were performed at least three times. Where appropriate, the data are expressed as means $\pm$ standard error of the mean (SEM). Probability (P) was calculated using a Student's t test. P values lower than 0.05 were considered significant.

Results

Antioxidant activity of cucurbitacin glucoside combination

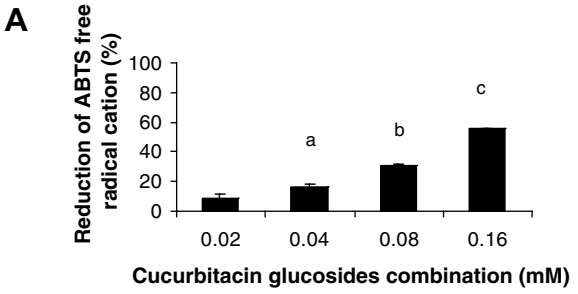
The main components isolated and characterized from *C. colocynthis* are cucurbitacin B glucoside and cucurbitacin E glucoside [7]. In the following scheme, we present

the structure of cucurbitacin B glucoside. Cucurbitacin E glucoside has the same structure, except for a double bond between carbons 1 and 2.



The total antioxidant activity was measured by the ability of CGC to reduce preformed $ABTS^{\bullet+}$ into its native form. As shown in Fig. 1A, CGC reduced $ABTS^{\bullet+}$ in a dose-dependent fashion. At a concentration of 0.04 mM, 16% reduction in $ABTS^{\bullet+}$ was detected, and at a concentration of 0.16 mM, 55% reduction was detected. The IC_{50} for CGC was 145 μM. The antioxidant capacity of CGC was compared to those of known antioxidants such as resveratrol, ascorbic acid, and trolox, and is expressed as IC_{50} values (Fig. 1B). The IC_{50} of resveratrol was 9 μM, ascorbic acid—15 μM, and trolox—26 μM.

To further assess the antioxidant potential of CGC, its ability to inhibit linoleic acid oxidation and, hence, MDA and 4-HNE formation, was measured. Using 0.2 mM CGC, 24% inhibition of linoleic acid oxidation was obtained, and using 2 mM CGC, 55% inhibition was obtained (Fig. 1C).



B

Antioxidant	IC_{50} (μM)
Resveratrol	9
Ascorbic acid	15
Trolox	26
CGC	145

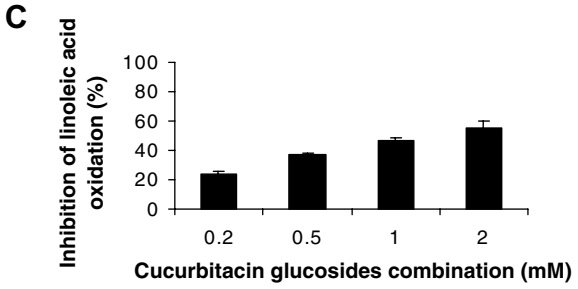


Fig. 1. Antioxidant activity of CGC. (A) Total antioxidant capacity of CGC as measured by the ABTS method. $ABTS^{\bullet+}$ was generated by mixing ABTS solution with potassium persulfate solution followed by incubation for 12 h at room temperature in the dark. The ability of CGC to reduce $ABTS^{\bullet+}$ into its native form was measured at 734 nm, and the percentage of reduction of $ABTS^{\bullet+}$ was calculated. Trolox served as a standard. Different letters indicate significant differences ($P < 0.05$). (B) Comparison between IC_{50}s of different antioxidants as measured using the ABTS assay. (C) Inhibition of linoleic acid oxidation by CGC. Linoleic acid was exposed to oxidation (by AAPH) at 39 °C, and the level of MDA and 4-HNE formation (an index of lipid peroxidation) was determined by the reaction with the chromogenic reagent *N*-methyl-2-phenylindole. The chromophore was measured at 586 nm. $P < 0.05$ vs control.

Effect of CGC on hydroxyl radicals

Hydroxyl radicals ($^{\bullet}OH$) were generated in a Fenton-type system. The ability of cucurbitacin glucosides to scavenge this free radical was tested. The typical ESR spectra of DEPMPO–OH adducts observed in the Fenton reaction are shown in Fig. 2 (as a control). Addition of CGC inhib-

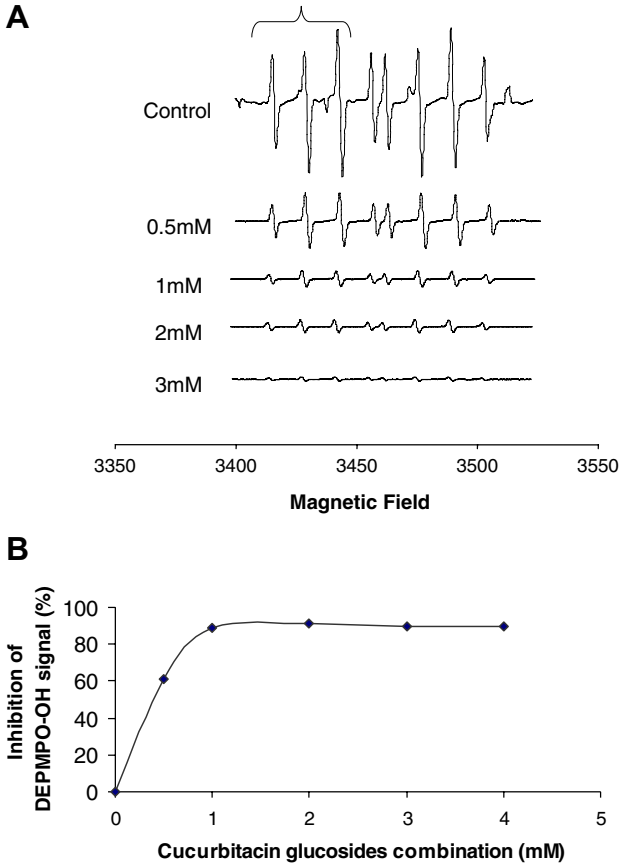


Fig. 2. Effect of CGC on DEPMPO–OH adduct formation. (A) Effect of different concentrations of CGC on the formation of DEPMPO–OH adduct. Hydroxyl radicals were generated and detected as described in Materials and methods. The reaction mixture contained 10 mM H_2O_2 and 30 mM DEPMPO in the presence of 100 μM $FeSO_4$, and the indicated concentrations of CGC. (B) The percentage of inhibition was calculated from the intensity of ESR signal of the three left peaks, as indicated. The length of the ESR signal is plotted against concentrations of CGC as a percentage of control.

ited the signal intensity in a dose-dependent manner. Addition of 0.5 mM CGC decreased the DEPMPO–OH adduct formation by 62%, and addition of 1 mM decreased the DEPMPO–OH adduct formation by 90% (Fig. 2). At a concentration of 0.38 mM, CGC inhibited the ESR signal intensity of DEPMPO–OH adduct by ~50%.

Effect of cucurbitacin glucoside on superoxide anion radical

Superoxide anion ($O_2^{\cdot -}$) is known to be produced when xanthine oxidase acts on xanthine in the presence of molecular oxygen [23]. The ability of CGC to scavenge the superoxide free radical was monitored. The spectrum of DEPMPO–OOH adducts generated using the xanthine–xanthine oxidase system is presented in Fig. 3. CGC reduced the typical ESR spectrum of DEPMPO–OOH adducts: 2 mM CGC decreased the DEPMPO–OOH adduct formation by 17% while 5 mM CGC decreased the DEPMPO–OOH adduct formation by 29%. IC_{50} for superoxide anion was 8 mM.

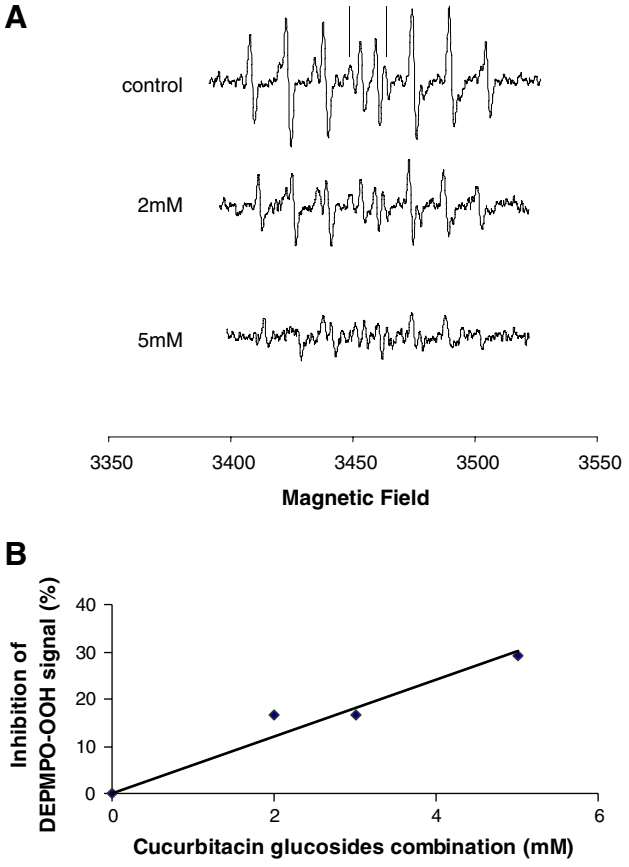


Fig. 3. Effect of CGC on DEPMPO–OOH adduct formation. (A) Effect of different concentrations of CGC on the formation of the DEPMPO–OOH adduct. Superoxide anions were generated in a xanthine–xanthine oxidase system as described in Materials and methods. The reaction mixture contained xanthine (20 μM), xanthine oxidase (0.0115 U), DEPMPO (30 mM), and the indicated concentrations of CGC. (B) The percentage of inhibition was calculated from the intensity of ESR signal of the peaks, indicated by arrows. The length of the ESR signal is plotted against concentrations of CGC as a percentage of control.

Effect of cucurbitacin glucosides on singlet oxygen

Singlet oxygen was detected as TEMP–$^1O_2$ adduct (TEMPO) using TEMP as a singlet oxygen trap, as described previously [24]. A typical ESR spectrum of TEMPO adducts is shown in Fig. 4 (as a control). The TEMPO adduct formation decreased by 27% in the presence of 6 mM CGC, and the TEMPO adduct formation decreased by 74% in the presence of 16 mM. IC_{50} for singlet oxygen was 11 mM.

Discussion

In the present study, we examined the antioxidant and free-radical scavenging properties of CGC (cucurbitacin B + E glucosides) isolated from *C. colocynthis*. In a previous paper, cucurbitacin B and cucurbitacin I were shown to exhibit antioxidant activity by inhibition of lipid perox-

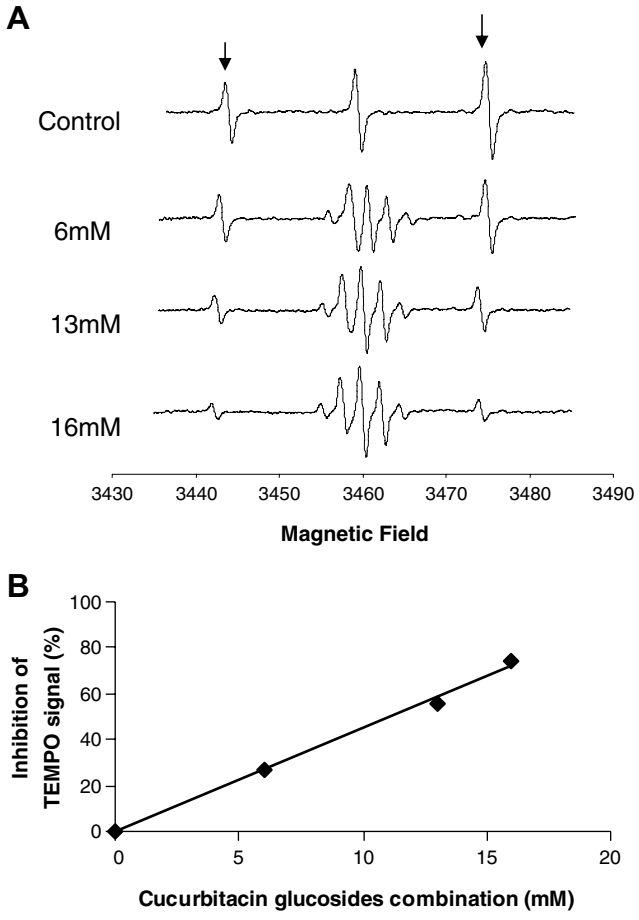


Fig. 4. Effect of CGC on TEMPO adduct formation. (A) Effect of different concentrations of CGC on the formation of TEMPO adduct. The experimental conditions are described under Materials and methods. Singlet oxygen was generated in the photoradiation–porphine system. The reaction mixture contained 0.054 mM TPPS4, 30 mM TEMP, PBS (pH 7.4), and the indicated concentrations of CGC. TEMPO adduct was recorded after irradiating the reaction mixture for 85 s with visible light. (B) The percentage of inhibition was calculated from the intensity of the ESR signal of the peaks indicated by arrows.

idation [9], but the antioxidant properties of cucurbitacin glucosides were not yet studied.

Using two different systems, we found that the CGC exhibits antioxidant activity. The activity was found to be dose-dependent, as is to be expected from an antioxidant.

First, we examined the total antioxidant activity of CGC by measuring its ability to reduce preformed $ABTS^{\bullet+}$ into its native form through electron donation. CGC indeed exhibited the ability to reduce $ABTS^{\bullet+}$ (Fig. 1A). The IC_{50} obtained for CGC was 145 μM. The IC_{50} obtained for trolox (which served as a standard) was 26 μM (Fig. 1B), indicating that the antioxidant activity of trolox was ~5.5-fold higher than that of CGC.

The antioxidant activity of CGC was further assessed by its ability to inhibit linoleic acid oxidation as measured by the level of MDA and 4-HNE (chain-cleavage products produced in lipid oxidation) formed. As shown in Fig. 1C, CGC inhibited the formation of MDA and 4-HNE in a dose-dependent manner. Lipid-oxidation products represent a health risk [19]. These molecules are more stable than free radicals, and, hence, are more harmful to cell systems. 4-HNE, for example, disturbs gap-junction communications in cultured endothelial cells and induces several genotoxic effects in hepatocytes and lymphocytes. It was suggested that *in-vivo* modification of low-density lipoproteins by certain lipid-peroxidation products (e.g., 4-hydroxynonenal and malonaldehyde) renders this lipoprotein more atherogenic and causes foam-cell formation. In addition, proteins modified by 4-hydroxynonenal and malonaldehyde were detected by immunological techniques in atherosclerotic lesions [19].

As mentioned by Noguchi et al. [25], antioxidants can be classified into four categories: preventive antioxidants, radical scavenging antioxidants, repair and de novo antioxidants, and adaptation antioxidants. It was suggested that radical scavenging antioxidants have the greatest benefits and their chemical structure is of key importance. Therefore, we used the ESR spin-trapping technique to measure the ability of CGC to scavenge reactive oxygen species (ROS). ESR spin trapping is a reliable technique for detecting activated species of oxygen in aqueous solutions. Using ESR spectroscopy, we studied the ability of CGC to scavenge hydroxyl radical ($^{\bullet}OH$), superoxide anion ($O_2^{\bullet-}$), and singlet oxygen ($^1O_2$).

In living systems, oxygen species, such as hydroxyl radicals, superoxide anion radicals, and singlet oxygen, may attack proteins, polyunsaturated fatty acids in cell membranes (giving rise to lipid peroxidation), or DNA, causing the alteration of gene expression and cellular metabolism. Although the amount of free radicals can be minimized by cellular defense systems, endogenous antioxidant defenses are inadequate in scavenging them completely [26]. Ongoing oxidative damage to lipids, proteins, DNA, and other molecules may contribute to the development of cancer, cardiovascular disease, and, possibly, to neurodegenerative diseases [27].

In the present report, we showed that CGC is able to scavenge hydroxyl radical, superoxide anion, and singlet oxygen (Figs. 2–4). This broad spectrum of radical scavenging activity is not characteristic of all natural antioxidants that have been studied. For example, Yamaguchi et al. [28] examined the scavenging effects of grape-seed extract using ESR, and reported that it showed strong scavenging activity on superoxide radicals but weak activity against hydroxyl radicals.

Boveris and Pontarulo [29] tested the potential of wheat, alfalfa, and ginkgo biloba extracts on scavenging free radicals resulting from lipid peroxidation in rat liver microsomes. While ginkgo biloba extracts were efficient in scavenging superoxide radical anions formed in microsomes, both wheat and afalfa extracts were not able to inhibit the superoxide free radicals. Comparison between the IC_{50} values of CGC revealed that hydroxyl radicals are the most effectively scavenged by CGC. The IC_{50} obtained was 0.38 mM for hydroxyl radical, 8 mM for superoxide anion, and 11 mM for singlet oxygen. It should be mentioned that the IC_{50} values obtained are within the range reported in the literature. For example, the IC_{50} for chlorogenic acid scavenging of $^{\bullet}OH$ is 0.11 mM [27], for trolox—0.2 mM [30], and in the presence of 2 mM glucurinated flavonoid, the intensity of DMPO–OH signal decreases by 60% [5]. In another study, 5 mM of sodium disuccinate derivative of lutein inhibited the superoxide radical signal by approximately 75%. No significant scavenging (0% inhibition) was observed at 0.1 mM [31]. Additionally, 10 mM glucurinated flavonoid inhibited the intensity of DMPO–OOH signal by about 90% [5], the IC_{50} for trolox scavenging of superoxide radical was 0.05 mM [30], and for resveratrol—about 3 mM [32]. The IC_{50} for histidine and sodium azide, known singlet oxygen scavengers, was 0.6 mM and 6 mM, respectively [33], and 4 mM glucurinated flavonoid inhibited the intensity of TEMPO signal by about 70% [5].

Taken together, our results demonstrate the antioxidant capacities and free-radical scavenging activities of cucurbitacin B+E glucosides. Although the mechanism by which cucurbitacin glucosides act as antioxidants is not well understood, it is generally believed that the beneficial properties of cucurbitacin glucosides are due to their ability to directly interact with reactive oxygen species. These results suggest that there is promising potential for cucurbitacin glucosides in treating human diseases that involve free radical and oxidative damage.

Acknowledgment

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