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DNA Protective Effect of Mangosteen Xanthones: an in Vitro Study on Possible Mechanisms

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ABSTRACT

Purpose: The aim of this study was to evaluate antioxidant ability of mangosteen shell and explore the non-enzymatic repair reaction and possible mechanism of xanthones in mangosteen shell.

Methods: Mangosteen shell was extracted by methanol to obtain the extract of mangosteen shell. The extract was then determined by various antioxidant assays in vitro, including protection against DNA damage, •OH scavenging,DPPH• (1,1-diphenyl-2-picryl-hydrazl radical) scavenging, $ABTS^{+}$• (2,2′-azino-bis(3-ethylbenzo- thiazoline-6-sulfonic acid diammonium) scavenging, Cu^{2+}-chelating, Fe^{2+}-chelating and Fe^{3+} reducing assays.

Results: Mangosteen shell extract increased dose-dependently its percentages in all assays. Its IC_{50} values were calculated as 727.85±2.21,176.94±19.25, 453.91±6.47, 84.60±2.47, 6.81±0.28, 1.55±0.10, 3.93±0.17, and 9.52±0.53μg/mL, respectively for DNA damage assay, •OH scavenging assay, Fe^{2+}-Chelating assay, Cu^{2+}-Chelating assay, DPPH• scavenging assay, $ABTS^{+}$• scavenging assay, Fe^{3+} reducing assay and Cu^{2+} reducing assay.

Conclusion: On the mechanistic analysis, it can be concluded that mangosteen shell can effectively protect against hydroxyl-induced DNA oxidative damage. The protective effect can be attributed to the xanthones. One approach for xanthones to protect against hydroxyl-induced DNA oxidative damage may be ROS scavenging. ROS scavenging may be mediated via metal-chelating, and direct radical-scavenging which is through donating hydrogen atom (H·) and electron (*e*). However, both donating hydrogen atom (H·) and electron (*e*) can result in the oxidation of xanthone to stable quinone form.

Introduction

As we know, reactive oxygen species (ROS) are various forms of activated oxygen including free radicals and non-free-radical species. ROS, particularly hydroxyl radical (·OH) with high reactivity, can attack DNA to cause its transient damage. If the transient damage cannot be repaired in time, it may be developed to permanent damage which causes severe biological consequences including mutation, cell death, carcinogenesis, and aging.[1,2] It is well known that the transient DNA damage can be repaired via enzymatic or non-enzymatic mechanisms.[2] Although the non-enzymatic repair reaction is faster than the enzymatic one, however, it is not well-known yet.[2] In general, non-enzymatic repair is finished by phenolics from plants.

Recent study has indicated a potent antigenotoxic effect of xanthones in mangosteens shell (山竹壳 in Chinese).[3] Hence, we used mangosteen shell as a reference to explore the non-enzymatic repair reaction and its possible mechanism.

Materials and Methods

Plant materials

Mangosteen (*Garcinia mangostana* L.) was purchased from Changzhou fruit market, Guangzhou, China. It was peeled off to obtain mangosteen shell. The voucher specimens were deposited in our laboratory.

Chemicals

DPPH• (1,1-diphenyl-2-picryl-hydrazl), ABTS [2,2′-azino-bis(3-ethylbenzo- thiazoline-6-sulfonic acid diammonium salt)], neocuproine, BHA (butylated hydroxyanisole), Trolox [(±)-6- hydroxyl-2,5,7,8-tetramethlychromane-2-carboxylic acid] were purchased from Sigma Co. (Sigma-Aldrich Shanghai Trading Co., China). Other chemicals used in this study were of analytical grade.

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Preparation of methanol extract from mangosteen shell

The dried mangosteen shell was coarsely powder then extracted with methanol by Soxhlet extractor for 12 hours. The extract was concentrated under reduced pressure to a constant weight. Then the dried extract was stored at 4°C until used.

Total phenol content determination

Total phenol contents of methanol extract from mangosteen shell was determined using the Folin-Ciocalteu method[4] with slight modifications. Briefly, 0.5 mL sample methanolic solution (1 mg/mL) was mixed with 0.5 mL Folin-Ciocalteu reagent (0.25 M). The mixture was kept for 3 min, followed by the addition of 1.0 mL Na_2CO_3 aqueous solution (15 %, w/w). After incubation at ambient temperature for 30 min, the mixture was centrifuged at 3500 rpm for 3 min. The supernatant was measured using a spectrophotometer (Unico 2100, Shanghai, China) at 760 nm. The results were expressed as pyrogallol equivalents (Pyr.) in milligrams per gram extract.

Protective effect against hydroxyl-induced DNA damage

The experiment was conducted using our method.[5] Briefly, sample was dissolved in methanol to prepare the sample solution. Various amounts (50 – 250 µL) of sample solutions (4 mg/mL) were then separately taken into mini tubes. After evaporating the sample solution in tube to dryness, 300 µL phosphate buffer (0.2 M, pH 7.4) was brought to the sample residue. Then, 50 µL DNA (10.0 mg/mL), 75 µL H_2O_2 (33.6 mM), 50 µL $FeCl_3$ (0.3 mM) and 100 µL Na_2EDTA solutions (0.5 mM) were added. The reaction was initiated by mixing 75 µL ascorbic acid (1.2 mM). After incubation in a water bath at 50 °C for 20 min, the reaction was terminated by 250 µL trichloroacetic acid (0.6 M). The color was then developed by addition of 150 µL 2-thiobarbituric acid (TBA) (0.4 M, in 1.25% NaOH aqueous solution) and heated in an oven at 105 °C for 15 min. The mixture was cooled and absorbance was measured at 530 nm against the buffer (as blank). The percent of protection of DNA is expressed as follows:

$$\text{Protective effect \%} = (1\text{-}A/A_0)\times 100\%$$

Where A is the absorbance with samples, while A_0 is the absorbance without samples.

Hydroxyl (•OH) radical-scavenging assay

The hydroxyl radical-scavenging activity was investigated by the deoxyribose method improved by our laboratory.[6] In brief, the sample was dissolved in methanol, and then the sample solution was aliquoted into mini tubes. After evaporating the sample solutions in the tubes to dryness (64-192 µg), 300 µL of phosphate buffer (0.2 M, pH 7.4) was added to the sample residue. Subsequently, 50 µL deoxyribose (2.8 mM), 50 µL H_2O_2 (2.8 mM), 50 µL $FeCl_3$ (25 µM), and 100 µL Na_2EDTA (0.8 mM) were added. The reaction was initiated by mixing 50 µL ascorbic acid (1.2 mM) and the total volume of the reaction mixture was adjusted to 600 µL with buffer. After incubation in a water bath at 50 °C for 20 min, the reaction was terminated by addition of 500 µL trichloroacetic acid (5%, w/w). The color was then developed by addition of 500 µL TBA (1g/100 mL, in 1.25% NaOH aqueous solution) and heated in an oven at 105 °C for 15 min. The mixture was cooled and the absorbance was measured at 532 nm against the buffer (as a blank control). The inhibition percentage on ·OH was expressed as follows:

$$\text{Inhibition \%} = (1\text{-}A/A_0)\times 100\%$$

Where A is the absorbance containing samples, while A_0 is the absorbance without samples.

Fe^{2+}-chelating activity

The Fe^{2+} chelating activity of methanol extract from mangosteen shell was estimated by the method as described by Li.[7] Briefly, 200 µL samples (200, 400, 600, 800, 1000 and 1200 µg/mL in methanol) were added to 100 µL $FeCl_2$ aqueous solutions (250 µM). The reaction was initiated by the addition of 150 µL ferrozine aqueous solutions (1 mM) and total volume of the system was adjusted to 1000 µL with methanol. Then, the mixture was shaken vigorously and stood at room temperature for 10 min. Absorbance of the solution was measured spectrophotometrically at 562 nm. The percentage of chelating effect was calculated by the following formula:

$$\text{Chelating effect \%} = (1\text{-}A/A_0)\times 100\%$$

Where A_0 is the absorbance without sample, and A is the absorbance with sample.

Cu^{2+}-chelating activity

The Cu^{2+}-chelating activity of methanol extract from mangosteen shell measured by a complexometric method using murexide.[7] Briefly, 60 µL $CuSO_4$ aqueous solution (20 mM) was added to hexamine HCl buffer (pH 5.0, 30 mM) containing 30 mM KCl and 0.20 mM murexide. After incubation for 1 min at room temperature, 20-120 µL sample solutions (2 mg/mL in methanol) were added. The final volume was adjusted to 1500 µL with methanol. Then, the mixture was shaken vigorously and left at room temperature for 10 min. Absorbance of the solution was then measured spectrophotometrically at 485 nm and 520 nm. The absorbance ratio (A485/A520) reflected the free Cu^{2+} content. Therefore, the percentage of cupric chelating effect was calculated by the following formula:

$$\text{Relative chelating effect \%} = [(A_{485}/A_{520})_{max}\text{-}(A_{485}/A_{520})]/[(A_{485}/A_{520})_{max}\text{-}(A_{485}/A_{520})_{min}]\times 100\%$$

Where (A_{485}/A_{520}) is the absorbance ratio in the presence of the samples, while $(A_{485}/A_{520})_{max}$ is the maximum absorbance ratio and $(A_{485}/A_{520})_{min}$ is the minimum absorbance ratio in the test.

DPPH• radical-scavenging assay

DPPH• radical-scavenging activity was determined as described.[8] Briefly, 1 mL DPPH• ethanolic solutions (0.1 mM) were mixed with 10 mg/mL sample methanolic solutions (2-12 μL). The mixtures were kept at room temperature for 30 min, and then measured with a spectrophotometer (Unico 2100, Shanghai, China) at 519 nm. The DPPH• inhibition percentages were calculated:

$$\text{Inhibition \%} = (1\text{-}A/A_0)\times 100\%$$

Where A is the absorbance with samples, while A_0 is the absorbance without samples. Trolox and BHA were used as the positive controls.

$ABTS^+$• radical-scavenging assay

The $ABTS^+$• scavenging activity was measured as described,[9] with some modifications. The $ABTS^+$• was produced by mixing 0.35 mL ABTS diammonium salt aqueous solution (7.4 mM) with 0.35 mL $K_2S_2O_8$ aqueous solution (2.6 mM). The mixture was kept in the dark at room temperature for 12 h to allow completion of $ABTS^+$• generation. Before usage, it was diluted with 95% ethanol (about 1:50) so that its absorbance at 734 nm was 0.70 ± 0.02. Then, 1.2 mL diluted $ABTS^+$• reagent was mixed with 0.3 mL sample ethanolic solution. After incubation for 6 min, the absorbance at 734 nm was read on a spectrophotometer (Unico 2100, Shanghai, China). The percentage inhibition was calculated as:

$$\text{Inhibition \%} = (1\text{-}A/A_0)\times 100\%$$

Where A_0 is the absorbance of the mixture without sample, A is the absorbance of the mixture with sample (or positive control).

Reducing power (Fe^{3+}) assay

Ferric (Fe^{3+}) reducing power was determined by the method of Oyaizu.[10] In brief, x μL sample methanolic solution (1 mg/mL) was mixed with (350-x) μL Na_2HPO_4/KH_2PO_4 buffer (0.2 M, pH 6.6) and 250 μL $K_3Fe(CN)_6$ aqueous solution (1 g/100 mL). After the mixture was incubated at 50 ℃ for 20 min, 250 μL trichloroacetic acid (10 g/100 mL in distilled water) was added. The mixture was then centrifuged at 3500 rpm for 10 min. As soon as 400 μL supernatant was mixed with 400 μL $FeCl_3$ (0.1 g/100 mL in distilled water), the timer was started. At 90 s, absorbance of the mixture was read at 700 nm. Samples were analyzed in groups of three, and when the analysis of one group has finished, the next group of three samples were mixed with $FeCl_3$ to avoid oxidization by air. The relative reducing ability of the sample was calculated by using the formula:

$$\text{Relative reducing power\%} = [(A\text{-}A_{min})/(A_{max}\text{-}A_{min})]\times 100\%$$

Here, A_{max} is the maximum absorbance in the test and A_{min} is the minimum absorbance in the test. A is the absorbance of sample. BHA and Trolox were used as the positive controls.

Reducing power (Cu^{2+}) assay

The cupric ions (Cu^{2+}) reducing power capacity was determined by the method,[11] with a slight modification. Briefly, 125 μL $CuSO_4$ aqueous solution (10 mM), 125 μL neocuproine ethanolic solution (7.5 mM) and 500 μL CH_3COONH_4 buffer solution (100 mM, pH 7.0) were brought to test tubes with different volumes of samples (1 mg/mL, 2-12 μL). Then, the total volume was adjusted to 1000 μL with the buffer and mixed vigorously. Absorbance against a buffer blank was measured at 450 nm after 30 min. The relative reducing power of the sample as compared with the maximum absorbance, was calculated by using the formula:

$$\text{Relative reducing power\%} = [(A\text{-}A_{min})/(A_{max}\text{-}A_{min})]\times 100\%$$

Here, A_{max} is the maximum absorbance in the test and A_{min} is the minimum absorbancein the test. A is the absorbance of sample.

Results and Discussion

It has been demonstrated that there are many xanthones in the mangosteen shell[1,12,13](Figure 1).

As seen in Figure 1, all xanthones bear a phenolic –OH and they can beconsidered as the phenolics. The result in the present study suggested a high level of total phenolics content (317.14±5.16 mg Pyr./g) in mangosteen shell extract. Obviously, these phenolic xanthones can be responsible for the antioxidant ability of mangosteen shell. Here we use a typical xanthone, γ-mangostin, as a reference compound for the following discussion.

As we know, hydroxyl radical (·OH) can be generated via Fenton reaction (Eq. **1**):

$$Fe^{2+} + H_2O_2 \rightarrow HO\cdot + OH^- + Fe^{3+} \qquad \text{Eq. } \mathbf{1}$$

As the most reactive ROS, hydroxyl radical can easily attack DNA to bring about various classes of oxidative lesions from base, nucleoside, nucleotide, oligonucleotide and DNA fragment. In addition, malondialdehyde (MDA) was also yielded. As discussed in our previous report,[5,14] MDA could reflect the protective percentages well. In the study, the protective percentages of mangosteen shell increased in a dose-dependent manner (Figure 2A). As listed in Table 1, the IC_{50} values of mangosteen shell, BHA and Trolox were respectively 727.85±2.21, 979.29±54.05, 285.27±56.33 μg/mL. It means that mangosteen shell can more effectively protect against hydroxyl-induced DNA oxidative damage than a standard antioxidant BHA.

Previous works have demonstrated that there are two approaches for natural phenolic antioxidant to protect DNA oxidative damage: one is to fast repair the deoxynucleotide radical cations damaged by free radicals,[15,16] one is to scavenge ROS (especially ·OH radicals) prior to DNA damage. To explore whether the protective effect of mangosteen shell is associated to ROS scavenging, we further determined its ·OH radical-scavenging ability by deoxyribose degradation assay.

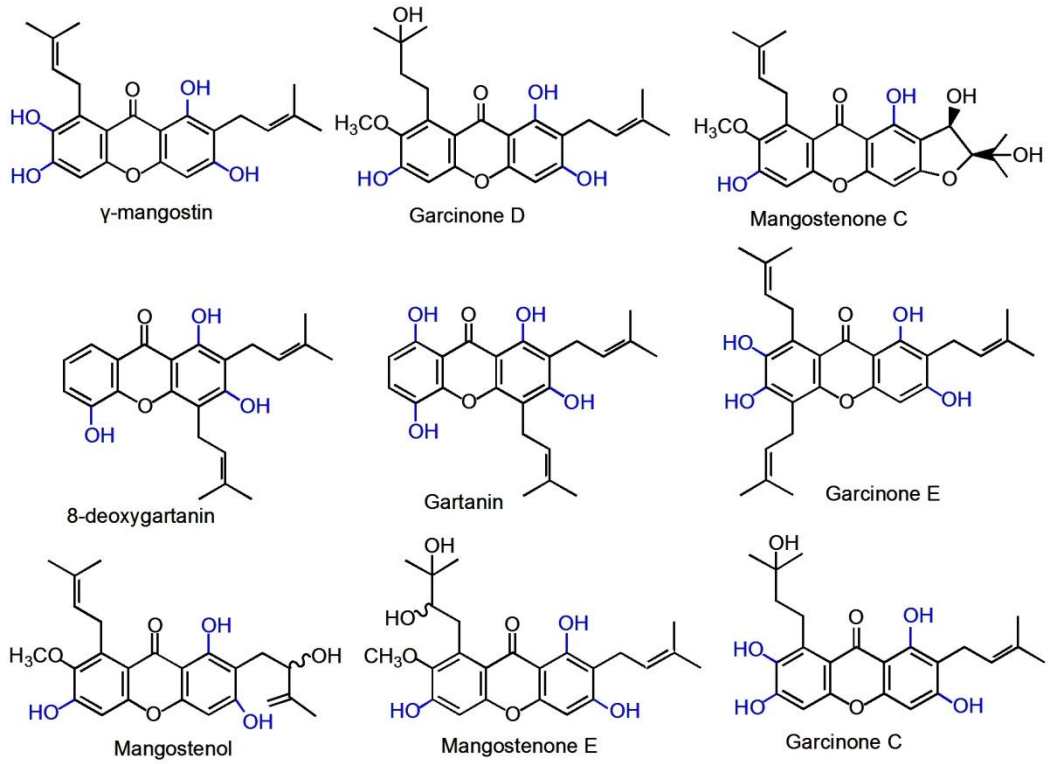

Figure 1. The structures of main xanthones in mangosteen shell.

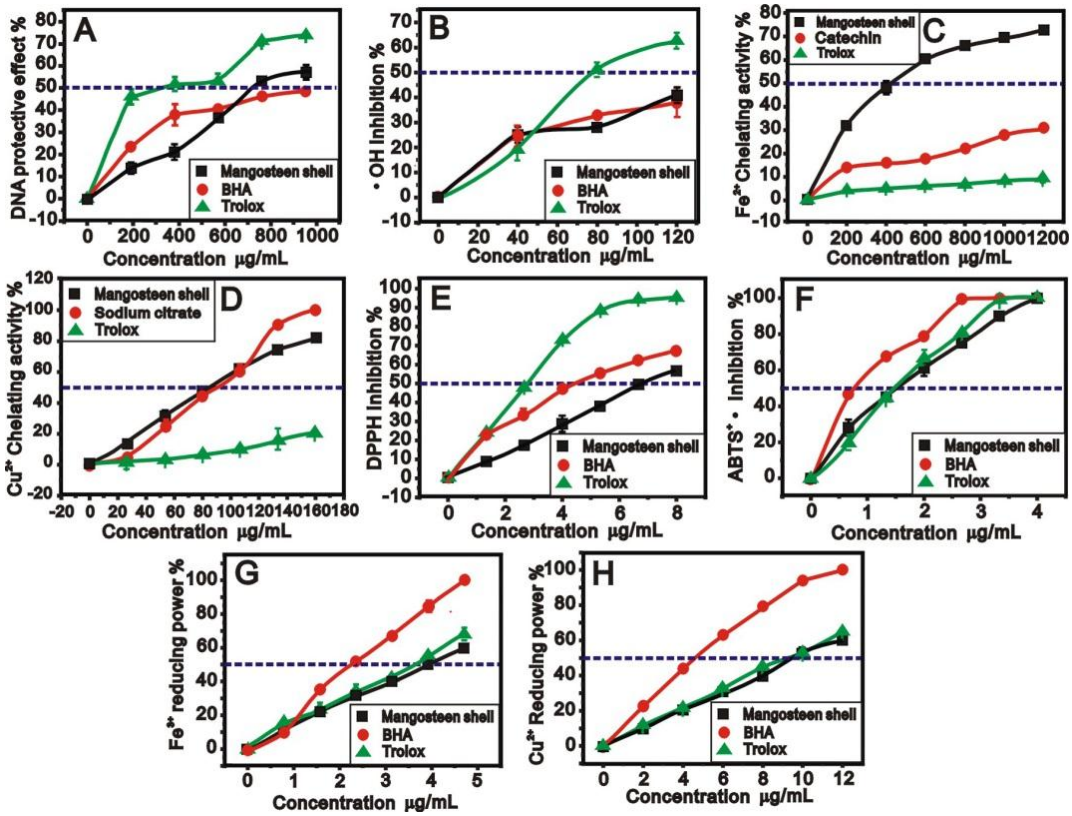

Figure 2. The dose response curves of mangosteen shell in various assays. A: DNA damage assay, B: hydroxyl radical (·OH) scavenging assay, C: Fe^{2+}-chelating assay, D: Cu^{2+}-chelating assay, E: DPPH• scavenging assay, F: $ABTS^{+}$• scavenging assay, G: Fe^{2+} reducing power assay, H: Cu^{2+} reducing power assay. Values are means ± SD (n = 3).

Since there is a strong solvent interference in ·OH scavenging assay, we have improved the experimental procedure.[6] Using our method, mangosteen shell was analyzed and the dose response curves are shown in Figure 2B. In terms of IC_{50} values (Table 1), mangosteen shell exhibited a similar ·OH radical-scavenging ability to BHA. It suggests that a possible approach for mangosteen shell to protect against oxidative DNA damage is ROS scavenging.

Table 1. The IC_{50}values of mangosteen shell extract and the positive controls (μg/mL)

Assays	Mangosteen shell	Positive controls	
		BHA	Trolox
DNA damage assay	727.85±2.21 [b]	979.29±54.05 [c]	285.27±56.33 [a]
•OH scavenging	176.94±19.25 [b]	172.97±33.04 [b]	79.08±3.54 [a]
Fe^{2+}-Chelating	453.91±6.47 [a]	1878.71±35.83 [b] *	5896.73±1574.22 [c]
Cu^{2+}-Chelating	84.60±2.47 [a]	89.96±0.48 [a] **	308.38±10.60 [b]
DPPH• scavenging	6.81±0.28 [c]	4.39±0.03 [b]	2.76±0.03 [a]
$ABTS^{+}$•scavenging	1.55±0.10 [b]	0.74±0.00 [a]	1.51±0.09 [b]
Fe^{3+} reducing	3.93±0.17 [b]	2.51±0.09 [a]	3.58±0.06 [b]
Cu^{2+} reducing	9.52±0.53 [b]	4.65±0.03 [a]	9.23±0.10 [b]

IC_{50} value is defined as the concentration of 50% effect percentage and calculated by linear regression analysis and expressed as mean ± SD (*n* = 3). The linear regression was analyzed by Origin 6.0 professional software. Means values with different superscripts in the same row are significantly different ($p < 0.05$), while with same superscripts are not significantly different ($p < 0.05$). * The positive control was Catechin, instead of BHA. ** The positive control was Sodium citrate, instead of BHA.

As illustrated in Eq. 1, the generation of •OH radical relies on the catalysis of transition metals (especially Fe and Cu). We then explored the metal-chelating ability of mangosteen shell. The dose-response curves in Figure 2C&D indicated an effective metal-chelating ability of mangosteen shell. The IC_{50} values in Table 1 suggest that mangosteen shell had the stronger metal chelating ability than positive controls Trolox and BHA. Now it is clear that metal-chelating may be one approach for mangosteen shell to scavenge ·OH radical. For example, γ-mangostin naturally occurring in mangosteen shell, may bind metal ions via the following proposed mechanism (Figure 3).

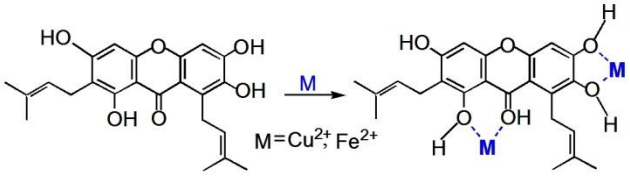

Figure 3. The proposed reaction for γ- mangostin to bind Cu^{2+} and Fe^{2+}.

To verify whether mangosteen shell can directly scavenge free radicals, we further investigated the radical-scavenging effects on DPPH· and $ABTS^{+}$· which don't require metal catalysis.

The DPPH assay confirmed that mangosteen shell could efficiently eliminate DPPH· radical (Figure 2E) and its IC_{50} was 6.81±0.28 μg/mL (Table 1). The previous studies suggested that DPPH· may be scavenged by an antioxidant through donation of hydrogen atom (H·) to form a stable DPPH-H molecule.[17] On the basis of previous reports,[18,19] γ-mangostin, for example, may scavenge DPPH• via the following proposed mechanism (Figure 4).

It has been demonstrated that *ortho*-dihydroxyl groups in benzenze ring play a critical role in the antioxidant ability of phenolic antioxidants.[20] Hence, in γ-mangostin molecule, *ortho*-dihydroxyl groups were thought to homolysis to produce H· and γ-mangostin· radical (Ⅰ). H· then combined DPPH· to generate DPPH-H molecule and the γ-mangostin· radical might transform into (Ⅱ), which could be further extracted H· by excess DPPH·to form the stable quinone form (Ⅲ) (Figure 4).

Figure 2F showed that mangosteen shell could also scavenge $ABTS^{+}$· in a dose-dependent manner and the IC_{50} value was 1.55±0.10μg/mL (Table 1). Hence, mangosteen shell was an effective radical scavenger on $ABTS^{+}$· as well. Unlike DPPH· radical, $ABTS^{+}$· radical cation however needs only an electron (***e***) to neutralize the positive charge. Therefore, $ABTS^{+}$· scavenging is an electron (***e***) transfer process.[21] For example, γ-mangostin scavenged $ABTS^{+}$· possiblyvia the following mechanism. At first, γ-mangostin produced electron (***e***) and H^{+} cation. The electron (***e***) was then donated to $ABTS^{+}$· to form stable ABTS molecule. Meanwhile, γ-mangostin molecule was changed to γ-mangostin· radical (Ⅰ), which could also be further converted to (Ⅱ), even (Ⅲ) in excess $ABTS^{+}$· (Figure 5).

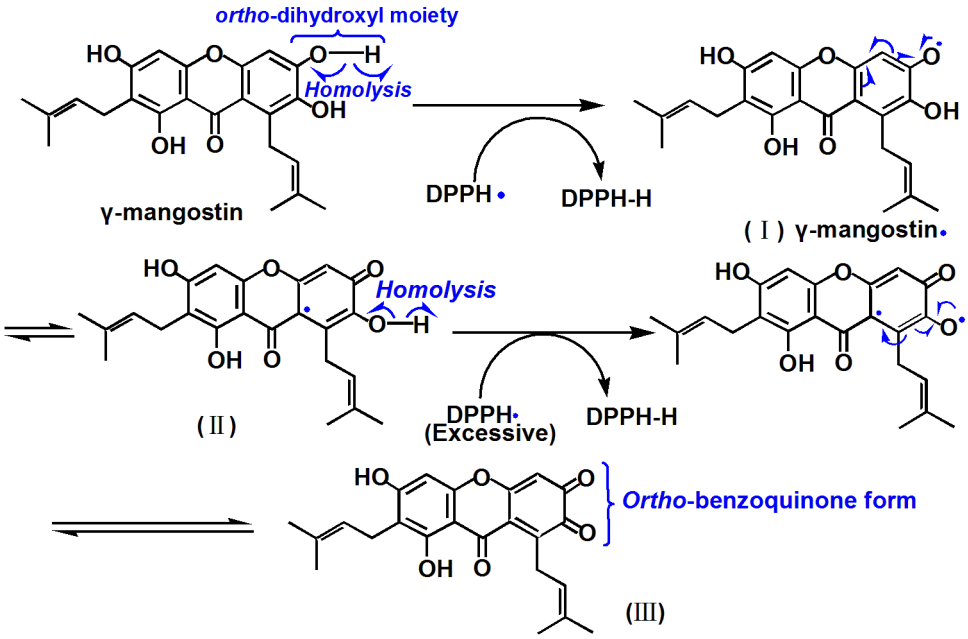

Figure 4. The proposed reaction for γ- mangostin to scavenge DPPH• radical

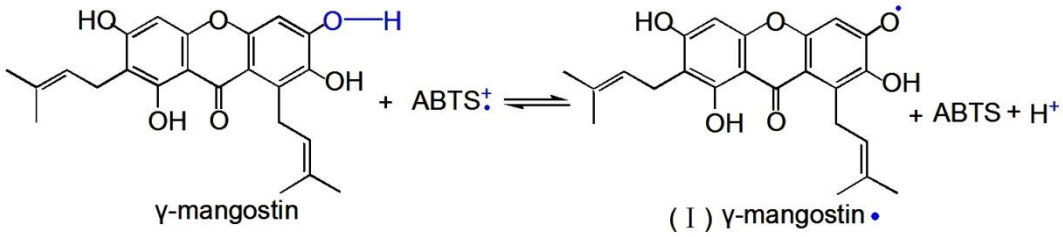

Figure 5. The proposed reaction for γ- mangostin to scavenge $ABTS^{+}$• radical cation.

The electron (***e***) transfer mechanism of ABTS assay was also supported by the Cu & Fe-reducing power assays, in which mangosteen shell exhibited a good dose response. The IC_{50} values (3.93±0.17 and 9.52±0.53 μg/mL, Table 1) suggest that mangosteen shell could successfully reduce Cu^{2+} to Cu^{+}, and Fe^{3+} to Fe^{2+}. As we know, reductive reaction is actually accepting electron (***e***) process, so it agrees with the findings of ABTS assay above.

Conclusion

In conclusion, mangosteen shell can effectively protect against hydroxyl-induced DNA oxidative damage. The protective effect can be attributed to the xanthones. One approach for xanthones to protect against hydroxyl-induced DNA oxidative damage may be ROS scavenging. ROS scavenging may be mediated via metal-chelating, and direct radical-scavenging which is through donating hydrogen atom (H·) and electron (***e***). However, both donating hydrogen atom (H·) and electron (***e***) can result in the oxidation of xanthone to stable quinone form.

Conflict of interest

The authors report no conflicts of interest.

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