

Research Article

Demethylcurcumin analogs, the highly potent compounds for antioxidation in G6PD normal and deficient subjects

Duangmanee Sanmun^{1*}, Chatchawan Changtam², Sarawut Saichanma¹,
Sucha Chulsomlee¹, Pornsuri Pongsuchart¹ and Apichart Suksamrarn³

¹Division of Clinical Microscopy, Faculty of Medical Technology, Huachiew Chalermprakiet University, Samutprakarn 10540, Thailand

²Division of Physical Science, Faculty of Science and Technology, Huachiew Chalermprakiet University, Samutprakarn 10540, Thailand

³Department of Chemistry and Center of Excellence for Innovation in Chemistry, Faculty of Science, Ramkhamhaeng University, Bangkok 10240, Thailand

* E-mail: dsanmun@gmail.com

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Abstract

Glucose-6-phosphate dehydrogenase (G6PD) deficiency can cause hemolytic anemia in response to oxidative stress. This study was undertaken to study the protective effect of natural curcuminoids (curcumin, demethoxycurcumin, bisdemethoxycurcumin) with curcuminoid analogs (di-*O*-demethylcurcumin and mono-*O*-demethylcurcumin) in healthy and G6PD-deficient red blood cells. After pretreatment blood with the test compounds, 0.75 mM sodium nitrite-oxidized hemolysate was determined for methemoglobin (metHb) formation. Concurrently, the released hemoglobin from 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH)-challenged cells was measured for hemolysis. We found that most of the compounds reduced metHb formation and oxidative hemolysis in red cells. After pretreatment with curcuminoid analogs, the oxidized hemoglobins were lower than treated with natural compounds. Furthermore, this study showed that demethylcurcumin analogs certainly protect AAPH-induced red cell lysis. This study can be concluded that curcuminoid analogs with different phenol rings possibly have higher antioxidant activity in order to protect the oxidative stress condition, especially in G6PD deficiency.

Keywords: antioxidant, curcuminoids, curcumin analog, red blood cell, G6PD deficiency

Introduction

Glucose-6-phosphate dehydrogenase (G6PD) deficiency is the most common enzymatic disorders of red blood cells. Patients with G6PD deficiency are sensitive and susceptible to oxidative agents and affects to phagocytic function depending on the severity (Vives Corrons et al., 1982; Beutler, 1994). Avoiding of toxic chemical substances should be concerned in G6PD patients in order to protect hemolytic anemia (Francis et al., 2013). Factors affect clinical severity in G6PD deficiency, including the age, the sex, the genetic variant, the pharmacology of the drug and coexisting disease conditions (Pamba et al, 2012). Supplement with some herbs that compose of antioxidant compounds may possibly applied (Brahmachari, 2012). Turmeric (*Curcuma longa* L.) is popular supplemented in Asian and Arab people because abundance with curcuminoids (Mueller and Mechler, 2005; Singh et al., 2010). Curcuminoids are active ingredients that consist of 46-67% curcumin, 11-24% demethoxycurcumin and

6-14% bisdemethoxycurcumin (Pothirat & Gritsanapa, 2005). Curcumin has been extensively used as anti-inflammation, antioxidant or anticancer agent (Anand et al, 2008; Ammon & Wahl, 1991; He et al., 2015). Since synthetic drugs can cause adverse effect toward the patient. The compounds derived from natural product have benefits because such natural compound can be modified as the analog. In addition, the analog compound offers the researchers to manipulate its structure with the target molecule (Lahlou, 2013). Biological activities of curcuminoid analogs have been interested in investigating their potential therapeutic benefits. In this study, in order to determine the effect of turmeric on erythrocytes, curcuminoids and various forms of analogs were examined in healthy and G6PD-deficient blood. Therefore, aim of this study was to evaluate the antioxidant activity between natural curcuminoids and its analogs.

Materials and methods

Reagents and compound preparation

Reagents and chemicals were purchased from the following sources: dimethyl sulfoxide (DMSO); 2,2'-azobis(2-methylpropionamide) dihydrochloride (AAPH); from Sigma-Aldrich (St. Louis, MO, USA). Sodium nitrite was purchased from Merck (Darmstadt, Germany) and Dulbecco's PBS (pH 7.4) was purchased from Gibco, Life Technologies (Grand Island, NY, USA). The individual natural curcuminoids, curcumin (C1), demethoxycurcumin (C2) and bisdemethoxycurcumin (C3), were isolated from the rhizomes of *Curcuma longa* L. as previously described (Changtam et al., 2010). The curcuminoid analogs, di-*O*-demethylcurcumin (C4) and mono-*O*-demethylcurcumin (C5), were prepared from curcumin by the method of Changtam et al. (2010). The spectroscopic (¹H NMR and mass spectra) data of the synthesized compounds were consistent with a previous report (Venkateswarlu et al., 2005). All of five compounds were reconstituted in 60% DMSO, as a stock solution.

Sample collection

Male subjects (age range 20-30 years old) recruited in this study were students of faculty Medical Technology at Huachiew Chalermprakiet University, Thailand. This study was approved by the ethical committee at Huachiew Chalermprakiet University, no.082/2555 according to the Declaration of Helsinki (Samutprakarn, Thailand) and informed consent was signed from individual participating subjects. Blood samples were collected in sterile tube containing citrate phosphate dextrose anticoagulant before investigation. Diagnosis of G6PD deficiency has been performed by fluorescent spot test, using the G6PD screening kit (R&D Diagnostics Ltd, Holargos, Greece). Here, venous blood samples from four G6PD-deficient subjects (age 20.5 ± 5.1 years old) and six healthy subjects (age 21.7 ± 4.1 years old) were obtained.

Assay for methemoglobin

Once freshly isolated red blood cells from healthy blood volunteers were obtained, doses of 10-40 μM of compounds were tested in pilot studies. Experiment in control cells, whole blood was washed with PBS three times and then centrifuged. Packed red cells were separated and diluted in PBS as 40% hematocrit. Next, the suspension of red blood cells was dissolved in distilled water in the ratio 1:100 (v; v) to produce the hemolysate. Hemolysate sample was pretreated with compounds C1-C5 for 15 min and then added with 0.75 mM sodium nitrite. After 20 minute incubation, the level of methHb was measured by spectrophotometer at absorbance 630 nm.

To verify whether G6PD deficiency responded to curcuminoids similar to red blood cells, blood samples were also investigated as described above. The formation of methHb was

monitored with time interval from 5 min to 45 min (Lewis and Roper, 2006). Treatment conditions were divided into three conditions with 2 mL volume: negative control (hemolysate+ control DMSO + sodium nitrite), positive control (hemolysate + control DMSO + sodium nitrite + ascorbic acid) and test tubes (hemolysate + sodium nitrite + compound in 60%DMSO). Control DMSO can be performed by adding 3.3 μ L of 60%DMSO for 2 mL reaction to yield a final concentration at 0.1%DMSO.

Assay for oxidative hemolysis

The washed red blood cells were diluted in PBS to give 4% hematocrit. Cell suspension from healthy volunteers was pretreated with compounds C1-C5 (concentration range 5-40 μ M) at 37 °C for 30 minutes. The cells were washed once and then mixed with 25 mM AAPH at 37 °C for 3 hours. The supernatant was collected from treated samples by centrifugation (3,000 rpm, 5 minutes). The absorbance of released Hb was determined by spectrophotometer at 540 nm (Niki et al., 1988). Percent of hemolysis was computed by reading the absorbance of hemoglobin in supernatant compared with 100% hemolysis (blood mixed in distilled water containing 0.1%DMSO and 25 mM AAPH). Value of inhibition hemolysis was calculated using equation (1): where $ABS_{DMSO+AAPH}$ is the absorbance of supernatant containing control buffer plus AAPH and ABS_{C+AAPH} is the absorbance of supernatant containing individual compound plus AAPH.

$$\% \text{ Inhibition hemolysis} = \frac{(ABS_{DMSO+AAPH} - ABS_{C+AAPH})}{ABS_{AAPH+DMSO}} \times 100 \quad \text{Eq.1}$$

Data analysis

The program packages of R version 3.2.0 statistical software (Copyright 2015, The R Foundation for Statistical Computing Platform: i386-w64-mingw32/i386) was used to analyze the data. The median differences between sample control and sample treated with compound were compared using Kruskal-Wallis test, followed by Wilcoxon rank sum test for pair wise comparison. All data were expressed as median \pm IQR of three separated experiments and statistical test was considered to be significant when $P < 0.05$.

Results

The chemical structures of curcuminoids examined are shown in Figure 1. Three natural curcuminoids, curcumin (C1), demethoxycurcumin (C2) and bisdemethoxycurcumin (C3), and the synthesized curcuminoid analogs, di-*O*-demethylcurcumin (C4) and mono-*O*-demethylcurcumin (C5) had been investigated for the possible reduction of metHb formation and hemolysis in erythrocytes.

Protective effect against oxidation

In this *in vitro* experiment, curcuminoids inhibited nitrite-induced metHb formation, at a concentration range 10-40 μ M (Figure 2). Ascorbic acid is a common standard antioxidant compound, so it was used to co-treatment as a positive control as same as various curcuminoids study in this protocol. The experiment was performed in G6PD-deficient cells at dose 40 μ M in five compounds including ascorbic acid. Noticeably, hemolysate with the analogs C4 and C5 at 10 μ M concentration were able to inhibit the transformation of reduced hemoglobin to oxidized hemoglobin more than two times (2.52-3.53 fold change) as compared to the DMSO condition (negative control) and were two most potent compounds (Figure 3).

As shown in Figure 2, natural compounds (C1, C2 and C3) at 20-40 μM concentration was determined to be the most efficient. Interestingly, analog compounds (C4 and C5) at 10 μM concentration can inhibit methHb formation as compared with control DMSO. Obviously, the methHb formation in hemolysate pretreated with analogs were lower than the natural curcuminoids (Figure 3).

Figure 4A (G6PD-deficient samples) shows the change of absorption spectra of methHb produced by hemolysate containing 0.75 mM sodium nitrite. C5 was found to be the most efficient for anti-oxidation of hemoglobin induced by sodium nitrite followed by AA, C4, C2, C1 and C3, respectively. In comparison with studies conducted in healthy blood samples, figure 4B showed that C5, AA and C4 were found to be the most efficient for anti-oxidation of hemoglobin induced by sodium nitrite followed by C2, C3 and C1, respectively. All together, the potent of C4 versus C5 and C1 versus C3 is slightly different, the variation of the experiment probably due to dose optimization, low number of replicates or small sample size.

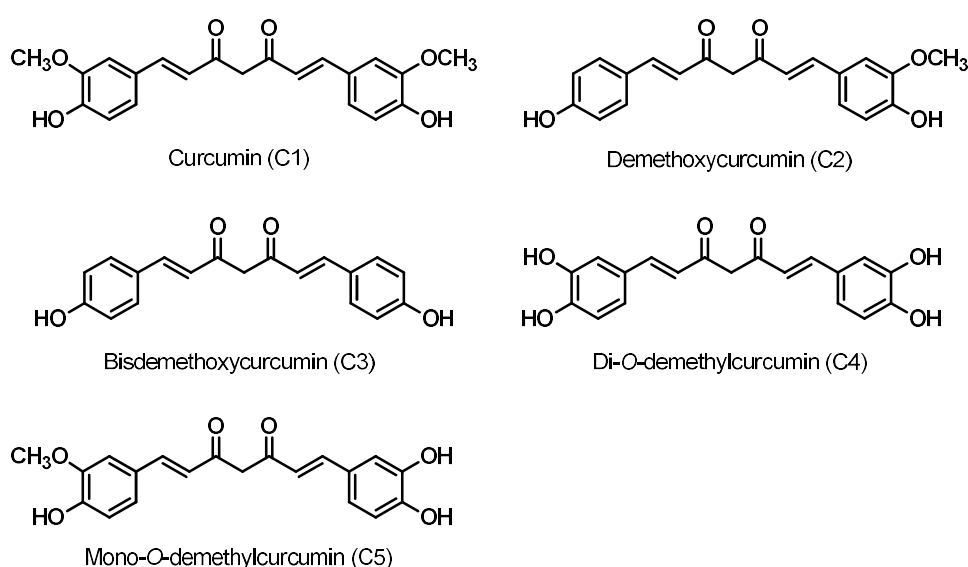


Figure 1. Structures of curcuminoids C1-C5

Effect of natural and curcuminoids analogs on hemolysis

At concentration 5-40 μM , curcuminoids and analogs suppressed hemolysis in a dose-dependent manner (Table 1). As observed, inhibitory effects of individual compound was remarkable at 20 μM concentration (all of them can suppressed hemolysis by more than 50%). To validate this finding, G6PD-deficient cells were investigated. This study showed that C5, C4, C3 and C2 significantly suppressed hemolysis but the action was less pronounced in C1 as seen in Figure 5. As shown in Table 1, 20 μM concentration was chosen to be effective dose for inhibition hemolysis. G6PD-deficient cells were further evaluated to see whether natural curcuminoids or analogs could prevent hemolysis differently (Figure 5).

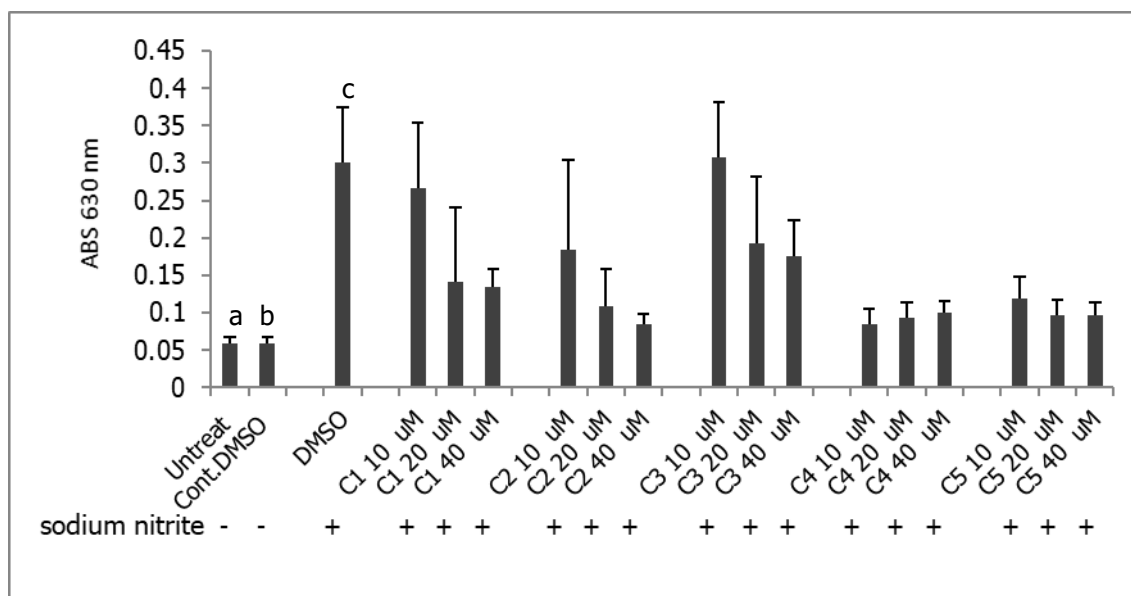


Figure 2. The absorbance of hemolysate from healthy red cells were pretreated with different conditions. The level of methHb was determined at 630 nm after incubating with sodium nitrite for 20 minutes. Data represent the median of three separated experiments (n=3). (a) hemolysate alone, (b) hemolysate containing control DMSO (c) hemolysate + DMSO + sodium nitrite, and (d) hemolysate + sodium nitrite + compounds (curcumin: C1, demethoxycurcumin: C2, bisdemethoxycurcumin: di-*O*-demethylcurcumin: C4 or mono-*O*-demethylcurcumin: C5)

Discussion

It seems that didemethylcurcumin analog is a very potent antioxidant. The ability of C4 and C5 to inhibit hemoglobin oxidation was two- to three-fold higher than that of parent compound (C1). This difference may be due to the number of hydroxyl group (-OH) in the benzene ring. We also found that curcuminoids and analogs exhibit anti-radical property as proved in the hemolytic assay. Our results are in agreement with elegant previous studies which showed that the phenolic group of curcumin analogs inhibited nitrite-oxidized hemoglobin and hemolysis in the AAPH-challenged red cells (Venkatesan et al., 2003; Zhang et al., 2014). Portes et al. (2007) also claimed that the benzylic hydrogens (not the beta-diketone moiety) are responsible for the anti-oxidation process. Recent studies pointed out that tetrahydrocurcumin, the hydrogenated analog, seems to enhance a greater biological activity than the parent compound (Okada et al., 2001; Nakmaroeng et al., 2011). Therefore, further investigation should be carried out to convince that tetrahydrocurcuminoids and curcuminoid analogs are more promising than the natural curcuminoids.

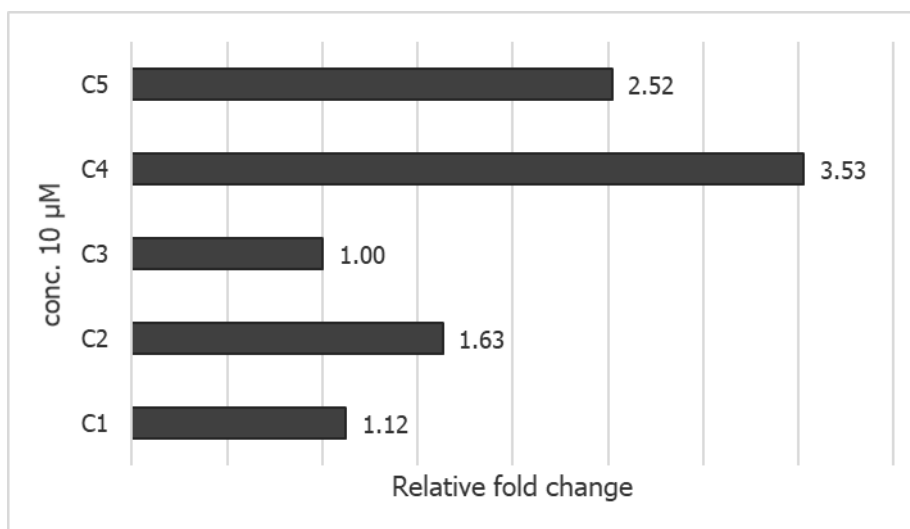


Figure 3. The fold change methHb on methHb formation after treated with different curcuminoids; curcumin: C1, demethoxycurcumin: C2, bisdemethoxycurcumin: di-*O*-demethylcurcumin: C4 or mono-*O*-demethylcurcumin: C5 and DMSO treated with nitrite (data represent the median±IQR). The fold change ratio of methHb formation calculated by setting the absorbance of DMSO control as denomination divided by the absorbance of individual compound.

Previous studies showed that the number of methoxyl group (-OCH₃) on the benzene ring involves in the radical scavenging activities, the relative potency followed by C1, C2, and C3, respectively (Somparn et al., 2007; Barzegar & Moosavi-Movahedi, 2011). Nevertheless, this investigation showed the strong effect of C2 and the weak activity of C3 and C1. There is one -OH group on demethoxycurcumin, while two on curcumin and none of -OH group on bisdemethoxycurcumin. Our study indicated that the number of -OH group on the benzene rings is not crucial for the antioxidant activity. Thus, some contrasting finding suggests that removal of methoxy group can enhance potency of natural compound (Zhang et al., 2008).

Comparing the biological effect of C5 versus C4, we usually found that didemethylcurcumin analog suppressed AAPH radicals more effectively than if we were to use monodemethylcurcumin analog. The maximal activity of the demethylated analogs C4 could be due to an extra phenolic hydroxyl groups on the phenyl rings (Tocharus et al., 2012). However, the ability of the two analogs in anti-hemolytic action did not depend on structure only but could perhaps be the result of its ability to scavenge free radicals in different microenvironment (Malik & Mukherje, 2014).

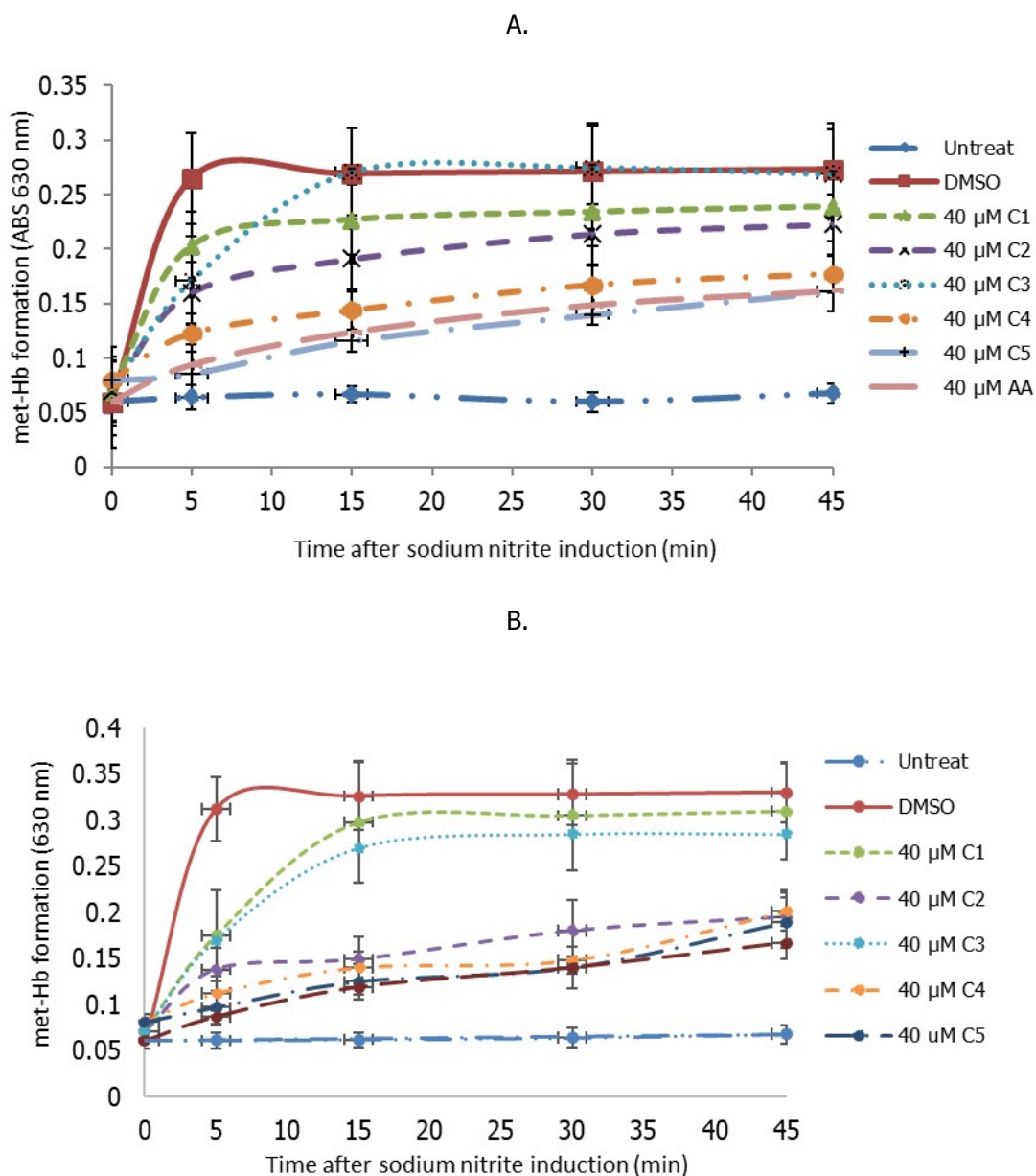


Figure 4. Changes of metHb formation in pre-treated hemolysates with different compounds during incubation with sodium nitrite for 45 min. The degree of metHb was measured at 15 minute intervals under sodium nitrite induction. Data represent the median values of hemolysate samples from G6PD deficient subjects (A; n=4) and healthy subjects (B; n=3) at different time points. Note: C1 = curcumin, C2 = demethoxycurcumin, C3 = bisdemethoxycurcumin, C4 = di-*O*-demethylcurcumin, C5 = mono-*O*-demethylcurcumin and AA = ascorbic acid

The parent compound curcumin has some concern about its poor bioavailability and rapid metabolism. In attempt to solve these problems, the researchers have developed new delivery systems and modified parent's chemical structure for improving curcumin as therapeutic agent (Vyas et al., 2013). Our results have shown that substitution of a -OCH₃ of the benzene rings in the curcumin structure leads to dramatically increase in anti-radical activity. We believe that the biochemical effect of curcuminoids and their analogs is not only due to the phenolic hydroxyl group, but is also associated with the number of free -OH groups on the benzene ring as shown in Figure 1. Collectively, there is a possibility of using the modified analogs as supplement for human diseases (Gupta et al., 2012; Bergamaschi et al., 2011). However, additional studies are needed to elucidate this finding into the realm of clinical management of G6PD-deficient patients.

Table 1. The percentage of hemolysis inhibition between compounds compared to DMSO control. Data were expressed as median values±IQR of three separated experiments (n=3).

| % Inhibition hemolysis in healthy subjects | | | | |
|--|-------------|------------|------------|-------------|
| Compound | 5µM | 10µM | 20µM | 40µM |
| DMSO .Cont | 0 | 0 | 0 | 0 |
| C1 | 0±70.15 | 95.12±91.6 | 88.55±91.6 | 76.83±78.10 |
| C2 | 28.29±44.11 | 46.69±07.2 | 53.75±07.2 | 54.77±01.4 |
| C3 | 78.46±91.13 | 42.81±18.6 | 10.71±18.6 | 82.78±91.11 |
| C4 | 63.90±11.02 | 89.42±1.77 | 94.91±1.77 | 94.21±2.49 |
| C5 | 54.05±17.02 | 88.42±0.85 | 89.84±0.85 | 91.95±3.90 |

Note: C1 = curcumin, C2 = demethoxycurcumin, C3 = bisdemethoxycurcumin, C4 = di-*O*-demethylcurcumin and C5 = mono-*O*-demethylcurcumin

Conclusion

Our study has demonstrated the antioxidant properties of natural curcuminoids and analogs in two ways: (1) The demethylcurcumin analogs (C4 and C5) have higher activity on reduction the methHb formation, and (2) Di-*O*-demethylcurcumin and mono-*O*-demethylcurcumin also have more potential effect on protect the red cell hemolysis than the natural compounds.

It was obvious that the two curcuminoid analogs which are structurally modified have been found to be the more highly potent compounds for antioxidant activity than the parent compound. Moreover, individual curcuminoids might have different effects on biological blood. It is particularly important when patients have vulnerability in red blood cells, especially G6PD deficiency. Therefore, considering pure curcuminoids to be the medicinal herbs possibly applied, but it also needed to more clinical studied in the future.

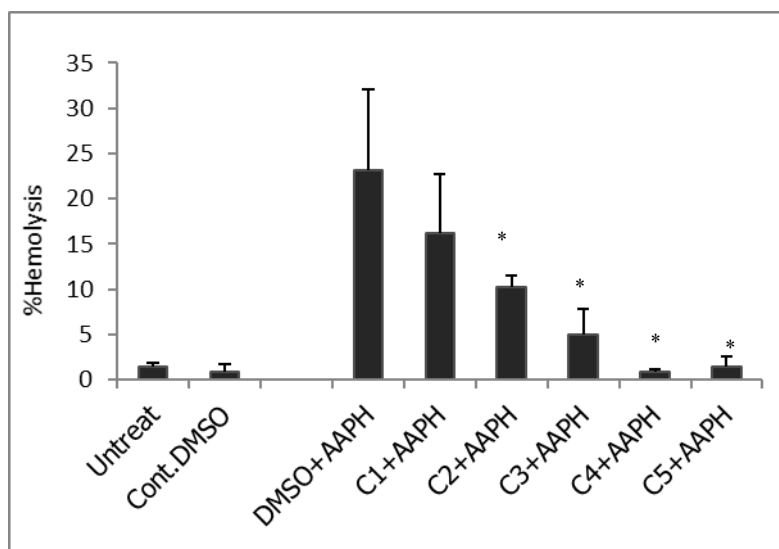


Figure 5. Percentage of red cells hemolysis when added AAPH in pre-incubation with compounds (curcumin: C1, demethoxycurcumin: C2, bisdemethoxycurcumin: C3, di-*O*-demethylcurcumin: C4 or mono-*O*-demethylcurcumin: C5) compared to untreated and DMSO treated cells. * $P < 0.05$ as compared with Control DMSO+AAPH condition.

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