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Influence of side chain structure changes on antioxidant potency of the [6]-gingerol related compounds

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ABSTRACT

[6]-Gingerol and [6]-shogaol are the major pungent components in ginger with a variety of biological activities including antioxidant activity. To explore their structure determinants for antioxidant activity, we synthesized eight compounds differentiated by their side chains which are characteristic of the C_1–C_2 double bond, the C_4–C_5 double bond or the 5-OH, and the six- or twelve-carbon unbranched alkyl chain. Our results show that their antioxidant activity depends significantly on the side chain structure, the reaction mediums and substrates. Noticeably, existence of the 5-OH decreases their formal hydrogen-transfer and electron-donating abilities, but increases their DNA damage- and lipid peroxidation-protecting abilities. Additionally, despite significantly reducing their DNA strand breakage-inhibiting activity, extension of the chain length from six to twelve carbons enhances their anti-haemolysis activity.

1. Introduction

Ginger (*Zingiber officinale* Roscoe), one of the most popular spices, is widely used in various foods and beverages, and has a long history of medicinal usages especially in China and India (Baliga et al., 2011; Shukla & Singh, 2007). Numerous studies have shown that ginger and its extracts possess a variety of biological and pharmacological activities, including cancer preventive and antioxidative effects (Baliga et al., 2011; Gundala et al., 2014; Shukla & Singh, 2007). The active and major pungent components of ginger consist of gingerols and shogaols, which are a series of chemical homologs with varying alkyl side chain lengths (Baliga et al., 2011; Jolad, Lantz, Chen, Bates, & Timmermann, 2005). Generally, gingerols are thermally labile owing to the presence of β-hydroxy keto moiety and readily undergo dehydration to yield the corresponding shogaols, resulting in the increased concentrations of shogaols in dry ginger compared with fresh ginger (Baliga et al., 2011; Jolad et al., 2005).

Of all the gingerols and shogaols, [6]-gingerol and [6]-shogaol (Fig. 1) bearing an unbranched alkyl chain of six carbon atoms are the most representative (Baliga et al., 2011; Jolad et al., 2005). [6]-Gingerol and [6]-shogaol share the same vanillyl moiety and have very similar chemical structures. Their structural differences exist only in the side chains, the former contains β-hydroxy keto moiety, and the latter possesses α,β-unsaturated carbonyl moiety. Such a slight difference significantly distinguishes their biological activities, as reported by numerous studies showing that [6]-shogaol is more effective than [6]-gingerol in antioxidant (Dugasani et al., 2010), anti-inflammatory (Dugasani et al., 2010; Pan, Hsieh, Hsu et al., 2008; Sang et al., 2009) and anti-tumorigenic assays (Wu et al., 2010) as well as in inhibiting proliferation (Gan et al., 2011; Pan, Hsieh, Kuo et al., 2008; Sang et al., 2009), inducing apopotosis (Pan, Hsieh, Kuo et al., 2008) and restraining invasion of cancer cells (Weng, Chou, Ho, & Yen, 2012; Weng, Wu, Huang, Ho, & Yen, 2010). Additionally, [6]-shogaol can act as a Michael acceptor to react with sulphydryl groups of cysteine residues in tubulin, and hence impair tubulin polymerisation, whereas [6]-gingerol is inactive (Ishiguro, Ando, Watanabe, & Goto, 2008). The above observations highlight the importance of the side chain structure for biological activities of the gingerol related compounds.

Because oxidative stress imposed by reactive oxygen species (ROS) or free radicals plays an important role in the development of many chronic diseases including cancer (Hussain, Hofseth, & Harris, 2003; Reuter, Gupta, Chaturvedi, & Aggarwal, 2010), antioxidant activity of the gingerol related compounds has also attracted attention (Dugasani et al., 2010; Kikuzaki & Nakatani, 1993; Li et al., 2012; Masuda, Kikuzaki, Hisamoto, & Nakatani, 2004; Yeh et al., 2014). Interestingly, Halvorsen et al. (2006) observed that ginger presented the highest antioxidant activity among the foods tested based on the ferric reducing antioxidant power assay. Antioxidant activity of gingerol related compounds should be mediated

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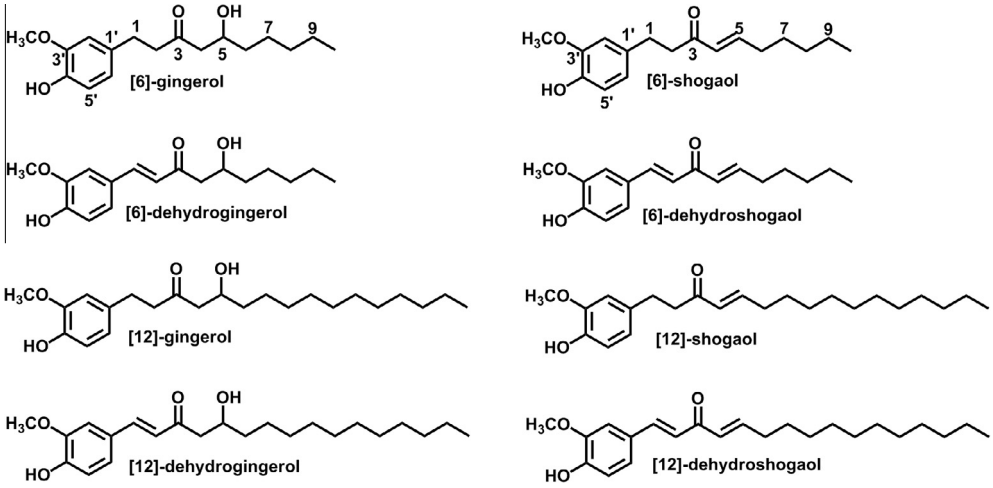


Fig. 1. Molecular structures of [6]-gingerol, [6]-shogaol and their derivatives.

by their *o*-methoxyphenolic moiety. It has been proved that hydrogen atom abstraction is surprisingly easy from intramolecularly hydrogen bonded methoxyphenols, in contrast to intermolecularly hydrogen bonded molecules (de Heer, Mulder, Korth, Ingold, & Lusztyk, 2000). Besides, the small solvent kinetic effect of *o*-methoxyphenols renders them good antioxidants, even in a polar environment (de Heer et al., 2000). It has been firmly established that in addition to its innately chemical reactivity towards radicals, the effectiveness of an antioxidant in biological system is also affected by its localisation, concentration, and mobility at the microenvironment, because the environment of biological system is quite heterogeneous (Niki & Noguchi, 2004). Therefore, we believe that antioxidant potency of the gingerol related compounds depends on not only their *o*-methoxyphenolic moiety but also functional groups, and the number of carbons in their side chains. Although a few studies has been conducted to investigate antioxidant activity of gingerol related compounds (Dugasani et al., 2010; Kikuzaki et al., 1993; Li et al., 2012; Masuda et al., 2004; Peng et al., 2012; Yeh et al., 2014), the literature regarding the influence of side chain structure on antioxidant potency of them are still limited. Thus, as a part of our ongoing research project on bioantioxidants (Cao et al., 2012; Li et al., 2014; Qian et al., 2011; Shang et al., 2009, 2010; Tang et al., 2011; Zhou, Miao, Yang, & Liu 2005), we synthesized eight gingerol related compounds (Fig. 1) including [6]- and [12]-gingerol, shogaol, dehydrogingerol and dehydroshogaol, and used them to probe how the side chain structure, such as the C_1–C_2 double bond, the C_4–C_5 double bond or the 5-OH, and the unbranched alkyl chain length, affect their antioxidant potency. Their antioxidant potency were characterised by four experimental models including the 2,2-diphenyl-1-picrylhydrazyl free radical (DPPH·)-scavenging, ferric reducing antioxidant power (FRAP), DNA strand breakage-inhibiting and human red blood cell haemolysis-protecting assays which are indicative of their formal hydrogen-transfer, electron-donating, DNA damage and lipid peroxidation-protecting abilities, respectively.

2. Materials and methods

2.1. General experimental procedures

^{1}H NMR spectra and ^{13}C NMR were recorded by a Bruker AV 400 (400 MHz) spectrometer with $CDCl_3$ or CD_3COCD_3 as a solvent. Chemical shifts (δ) are reported in parts per million (ppm) using the solvent peak. Mass spectra were recorded on a Bruker Daltonics Esquire 6000 spectrometer (ESI-MS) or a VG ZAB-HS spectrometer (EI-MS).

2.2. Materials

2,2-Diphenyl-1-picrylhydrazyl free radical (DPPH·), pBR322 DNA and 2,2′-azobis (2-amidinopropane hydrochloride) (AAPH) were purchased from Sigma-Aldrich. 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ) was from Alfa Aesar. Other chemicals used were of analytical grade. All anhydrous solvents were dried and purified by standard techniques.

2.3. Synthesis of (E)-4-(4′-hydroxy-3′-methoxyphenyl)-3-buten-2-one

(E)-4-(4′-Hydroxy-3′-methoxyphenyl)-3-buten-2-one was synthesized by following previous published procedures (Ramachandra & Subbaraju, 2006). 25 ml 20% aq. sodium hydroxide was added to the acetone (30 ml) solution of vanillin (5.0 g, 32.89 mmol) and then this was stirred overnight at room temperature. The reaction mixture was diluted with ice water and 35 ml conc. HCl was added, a brown precipitate formed which was filtered, washed with cold water and dried to obtain (E)-4-(4′-hydroxy-3′-methoxyphenyl)-3-buten-2-one.

2.4. General procedure for the synthesis of dehydroshogaols and dehydrogingerols

Dehydroshogaols and dehydrogingerols were synthesized based on the published procedures with some modifications (Fleming, Dyer, & Eggington, 1999). 1.0 equivalent of (E)-4-(4′-hydroxy-3′-methoxyphenyl)-3-buten-2-one in dry THF (0.7 M) was added to a flame-dried flask under argon, and cooled to −78 °C. 1.0 equivalent of n-BuLi in hexane (1.60 M) was then added. After the mixture was stirred for 30 min, LDA prepared with 1.3 equivalent of diisopropyl amine and 1.0 equivalent of n-BuLi in 0.7 M THF at −78 °C was sequentially added dropwise. The reaction was stirred at −78 °C for 3 h, 1.1 equivalent of the appropriate aldehyde (hexanal or dodecanal) in THF (0.7 M) was then introduced into the reaction system. After stirring for another 3 h at the same temperature, the mixture was warmed to 0 °C for 1 h, then quenched with 10% HCl and extracted using ether. The combined organic layers were dried over sodium sulphate and concentrated. Silica gel column purification of the crude product provided dehydrogingerols or dehydroshogaols, respectively.

2.4.1. (E)-5-Hydroxy-1-(4′-hydroxy-3′-methoxyphenyl)-1-decen-3-one ([6]-dehydrogingerol)

Yield: 44.5%. ^{1}H NMR (400 MHz, $(CD_3)_2CO$) δ 8.18 (s, 1H), 7.57 (d, *J* = 16.0 Hz, 1H), 7.34 (d, *J* = 1.6 Hz, 1H), 7.18 (dd, *J* = 8.0, 1.6 Hz, 1H), 6.88 (d, *J* = 8.0 Hz, 1H), 6.74 (d, *J* = 16.0 Hz, 1H), 4.11–4.04 (m, 1H), 3.91 (s, 3H), 3.72 (d, *J* = 4.4 Hz, 1H), 2.78–2.76 (m, 2H), 1.48–1.44 (m, 3H), 1.32–1.30 (m, 5H) 0.90–0.87 (m, 3H); ^{13}C NMR (100 MHz, $(CD_3)_2CO$) δ 200.2, 150.3, 148.9, 143.9, 127.8, 125.3, 124.3, 116.3, 111.6, 68.7, 56.4, 48.5, 38.2, 32.7, 26.1, 23.4, 14.4; HRMS (ESI): *m*/*z* calcd. for $C_{17}H_{24}O_4$ $(M+H)^+$: 293.1747; found: 293.1744, error = 1.0 ppm.

2.4.2. (E)-5-Hydroxy-1-(4′-hydroxy-3′-methoxyphenyl)-1-hexadecen-3-one ([12]-dehydrogingerol)

Yield: 37.5%. ^{1}H NMR (400 MHz, $(CD_3)_2CO$) δ 8.20 (s, 1H), 7.57 (d, *J* = 16.0 Hz, 1H), 7.34 (s, 1H), 7.17 (d, *J* = 8.4 Hz, 1H), 6.88 (d, *J* = 8.4 Hz, 1H), 6.73 (d, *J* = 16.0 Hz, 1H), 4.12–4.05 (m, 1H), 3.91 (s, 3H), 3.75 (d, *J* = 4.8 Hz, 1H), 2.78-2.76 (d, 2H), 1.48-1.45 (m, 2H), 1.28 (brs, 18H), 0.88 (t, *J* = 6.8 Hz, 3H); ^{13}C NMR (100 MHz, $(CD_3)_2CO$) δ 200.3, 150.3, 148.8, 144.0, 127.8, 125.2, 124.3, 116.2, 111.6, 68.7, 56.4, 48.5, 38.2, 32.7, 30.5 (3C), 30.2, 29.4, 26.4, 23.4, 14.5; HRMS (ESI): *m*/*z* calcd. for $C_{23}H_{36}O_4$ $(M+H)^+$: 377.2686; found: 377.2684, error = 1.1 ppm.

2.4.3. (1E,4E)-1-(4′-Hydroxy-3′-methoxyphenyl)-1,4-decadien-3-one ([6]-dehydroshogaol)

Yield: 17.5%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.59 (d, *J* = 16.0 Hz, 1H), 7.14 (dd, *J* = 8.4, 2.0 Hz, 1H), 7.08 (d, *J* = 2.0 Hz, 1H), 7.00 (dt, *J* = 15.6, 7.2 Hz, 1H), 6.93 (d, *J* = 8.4 Hz, 1H), 6.82 (d, *J* = 16.0 Hz, 1H), 6.44 (dt, *J* = 15.6, 1.2 Hz, 1H), 5.98 (s, 1H), 3.94 (s, 3H), 2.27 (dq, *J* = 7.2, 1.2 Hz, 2H), 1.55–1.48 (m, 2H), 1.35–1.31 (m, 4H), 0.92–0.89 (m, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 189.4, 148.3, 148.1, 146.9, 143.4, 129.2, 127.5, 123.4, 123.0, 114.9, 109.8, 56.1, 32.8, 31.5, 28.0, 22.5, 14.1; MS (EI) *m*/*z*: 275 (M^+).

2.4.4. (1E,4E)-1-(4′-Hydroxy-3′-methoxyphenyl)-1,4-hexadecadien-3-one ([12]-dehydroshogaol)

Yield: 19.9%. ^{1}H NMR (400 MHz, $(CD_3)_2CO$) δ 8.18 (s, 1H), 7.60 (d, *J* = 16.0 Hz, 1H), 7.36 (d, *J* = 1.6 Hz, 1H), 7.20 (dd, *J* = 1.6, 8.4 Hz, 1H), 7.04 (d, *J* = 16.0 Hz, 1H), 6.99 (dt, *J* = 15.6, 7.2 Hz, 1H), 6.88 (d, *J* = 8.0 Hz, 1H), 6.48 (d, *J* = 15.6 Hz, 1H), 3.91 (s, 3H), 2.27 (q, *J* = 7.2 Hz, 2H), 1.52–1.47 (m, 2H), 1.33–1.28 (m, 16H), 0.87 (t, *J* = 6.8 Hz, 3H); ^{13}C NMR (100 MHz, $(CD_3)_2CO$) δ188.9, 150.2, 148.8, 147.6, 143.7, 130.5, 128.1, 124.2, 123.3, 116.2, 111.7, 56.4, 33.3, 32.7, 30.5, 30.4 (2C), 30.2 (2C), 30.1, 29.1, 23.4, 14.5; HRMS (ESI): *m*/*z* calcd. for $C_{23}H_{34}O_3$ $(M+H)^+$: 359.2581; found: 359.2588, error = 1.9 ppm.

2.5. General procedure for the synthesis of gingerols

Gingerols were synthesized by following previous published procedures (Ramachandra & Subbaraju, 2006). A solutions of [6]-dehydrogingerol ([12]-dehydrogingerol) (1 mmol) in anhydrous ethyl acetate (10 ml) was hydrogenated over 10% Pd/C at room temperature. The resulting mixture was allowed to stir at room temperature for 4 h and then monitored by TLC. After filtering off the catalyst, the filtrate was evaporated in vacuo and then purified by column chromatography on silica gel to afford gingerols.

2.5.1. 5-Hydroxy-1-(4′-hydroxy-3-methoxyphenyl)decan-3-one ([6]-gingerol)

Yield: 80.4%. ^{1}H NMR (400 MHz, $(CD_3)_2CO$) δ 7.33 (m, 1H), 6.81 (d, *J* = 2.0 Hz, 1H), 6.71 (d, *J* = 8.0 Hz, 1H), 6.64 (dd, *J* = 8.0, 2.0 Hz, 1H), 4.03–3.99 (m, 1H), 3.81 (s, 3H), 3.72-3.68 (m, 1H), 2.77–2.76 (m, 4H), 2.54–2.52 (m, 2H), 1.42–1.25 (m, 8H), 0.89-0.86 (t, *J* = 6.8 Hz, 3H); ^{13}C NMR (100 MHz, $(CD_3)_2CO$) δ 210.2, 148.3, 145.7, 133.7, 121.5, 115.7, 112.8, 68.4, 56.3, 51.0, 46.0, 38.2, 32.7, 26.0, 23.4, 14.4; HRMS (ESI): *m*/*z* calcd. for $C_{17}H_{26}O_4$ $(M-H)^-$: 293.1747; found: 293.1751, error = 1.4 ppm.

2.5.2. 5-Hydroxy-1-(4′-hydroxy-3′-methoxyphenyl)hexadecan-3-one ([12]-gingerol)

Yield: 64.0%. ^{1}H NMR (400 MHz, $(CD_3)_2CO$) δ 7.29 (s, 1H), 6.82 (d, *J* = 2.0 Hz, 1H), 6.71 (d, *J* = 8.0 Hz, 1H), 6.64 (dd, *J* = 8.0, 2.0 Hz, 1H), 4.04–3.99 (m, 1H), 3.81 (s, 3H), 3.65 (d, *J* = 4.8 Hz, 1H), 2.79–2.74 (m, 4H), 2.53–2.52 (m, 2H), 1.42–1.38 (m, 3H), 1.29 (s, 17H), 0.89-0.86 (t, *J* = 6.8 Hz, 3H); ^{13}C NMR (100 MHz, $(CD_3)_2CO$) δ 210.2, 148.3, 145.7, 133.8, 121.5, 115.7, 112.9, 68.4, 56.3, 51.0, 46.0, 38.3, 32.7, 30.5, 30.2, 26.4, 23.4, 14.4; HRMS (ESI): *m*/*z* calcd. for $C_{23}H_{38}O_4$ $(M+NH_4)^+$: 396.3108; found: 396.3112, error = 1.0 ppm.

2.6. General procedure for the synthesis of shogaols

Shogaols were synthesized by following previously published procedures (Fleming et al., 1999). An equal volume of 10% HCl was added to the THF solution (20 ml) of [6]-gingerol ([12]-gingerol) and the mixture was heated for 4 h. The cooled mixture was extracted with ether and washed with water. The combined organic parts were washed with brine, dried over anhydrous Na_2SO_4, and concentrated in vacuo to give a crude oil. Purification with column chromatography on silica gel gave shogaols.

2.6.1. (E)-1-(4′-Hydroxy-3′-methoxyphenyl)-4-decen-3-one ([6]-shogaol)

Yield: 70.8%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.86–6.78 (m, 2H), 6.71 (d, *J* = 2.0 Hz, 1H), 6.68 (dd, *J* = 8.0, 2.0 Hz, 1H), 6.09 (dd, *J* = 16.0, 1.6 Hz, 1H), 5.55 (s, 1H), 3.87 (s, 3H), 2.86–2.84 (m, 4H), 2.20 (dq, *J* = 7.2, 1.6 Hz, 2H), 1.44 (quint, *J* = 6.8 Hz, 2H), 1.32–1.25 (m, 4H), 0.89 (t, *J* = 6.8 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 199.8, 147.9, 146.4, 143.8, 133.2, 130.3, 120.7, 114.3, 111.1, 55.8, 41.9, 32.4, 31.3, 29.8, 27.7, 22.4, 13.9; MS (EI) *m*/*z*: 277 (M^+).

2.6.2. (E)-1-(4′-Hydroxy-3′-methoxyphenyl)-4-hexadecen-3-one ([12]-shogaol)

Yield: 45.5%. ^{1}H NMR (400 MHz, $(CD_3)_2CO$) δ7.29(s, 1H), 6.90-6.81 (m, 2H), 6.71 (d, *J* = 8.0 Hz, 1H), 6.65 (dd, *J* = 8.0, 2.0 Hz, 1H), 6.10 (dt, *J* = 16.0, 1.2 Hz, 1H), 3.81 (s, 3H), 2.87–2.83 (m, 2H), 2.80-2.74 (m, 2H), 2.21 (dq, *J* = 7.2, 1.2 Hz, 2H), 1.48–1.43 (m, 2H), 1.30-1.28 (bs, 16H), 0.89-0.86 (m, 3H); ^{13}C NMR (100 MHz, $(CD_3)_2CO$) δ 199.5, 148.2, 147.8, 145.7, 133.8, 131.3, 121.6, 115.7, 112.9, 56.3, 42.5, 33.1, 32.7, 30.5 (2C), 30.4, 30.2, 30.1, 30.0, 29.0, 23.4, 14.4; HRMS (ESI): *m*/*z* calcd. for $C_{23}H_{36}O_3$ $(M+H)^+$: 361.2737; found: 361.2730, error = 1.9 ppm.

2.7. DPPH·-scavenging assay

The EC_{50} values of [6]-gingerol, [6]-shogaol and their derivatives for DPPH·-scavenging were determined by monitoring the absorbance of this radical (60 μM) at 517 nm in methanol according to the previously published method (de Gaulejac, Provost, & Vivas, 1999) with minor changes, and using a Beijing purkinje TU-1901 UV/Vis spectrometer after the solution was allowed to stand 60 min in the dark.

The reaction rates of [6]-gingerol, [6]-shogaol and their derivatives with DPPH· in methanol were determined by monitoring the absorbance change at 517 nm, using a Hitachi 557 spectrophotometer equipped with a quartz cell (optical path length, 1 cm), and using the second-order kinetics with the ratio of [compounds]/[DPPH·] being 1/1. The temperature in the cell was kept at 25 °C by means of a thermostated bath.

2.8. Assay for ferric reducing/antioxidant power (FRAP)

FRAP assay was used to evaluate the reducing capacity of [6]-gingerol, [6]-shogaol and their derivatives according to the procedure of Benzie and Strain (1996) and the related details were described in our previous work (Tang et al., 2011).

2.9. Protective effects against AAPH-induced DNA damage

The experiment was carried out by incubation of plasmid pBR322 DNA (100 ng) with AAPH (5 mM) and [6]-gingerol, [6]-shogaol or their derivatives (40 μM) in 10 mM phosphate-buffered saline (PBS, pH 7.4) at 37 °C for 1 h with the final volume of 25 μl, and the related details were described in our previous work (Qian et al., 2011).

2.10. Assay for anti-haemolysis activity

Human red blood cells (RBCs) were provided by the Red Cross Center for Blood (Gansu, China). Anti-haemolysis activity of [6]-gingerol, [6]-shogaol or their derivatives was determined as previously described (Qian et al., 2011).

2.11. Statistical analysis

The IBM SPSS Statistics 19 was used for statistical analysis of data. Data were subjected to analysis of variance (ANOVA) and multiple comparisons of means were carried out using Duncan's multiple range test. Data were expressed as means ± SD of three independent analyses. Significance was accepted at $P < 0.05$.

3. Results and discussion

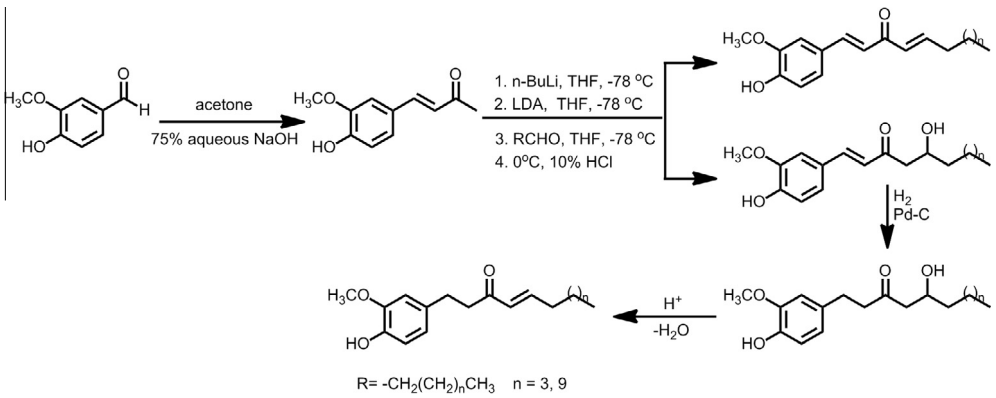
3.1. Synthesis of [6]-gingerol, [6]-shogaol and their derivatives

Synthesis of dehydrogingerols and dehydroshogaols was well-established by an Aldol condensation reaction of vanillin acetone with the different alkyl aldehyde (Ramachandra & Subbaraju, 2006). Gingerols were prepared by hydrogenation of dehydrogingerols over Pd/C catalysts, and shogaols were then obtained by dehydration of gingerols under acid conditions (Scheme 1).

3.2. DPPH·-scavenging activity of [6]-gingerol, [6]-shogaol and their derivatives

DPPH· is a stable N-centered radical with a characteristic absorbance at 517 nm and is widely used as the prototypic model for peroxyl radical to evaluate the formal hydrogen atom transfer ability of a phenolic antioxidant by monitoring the decrease in this absorbance. It has been pointed out that the radical-scavenging antioxidant activity should be assessed by the reactivity toward radicals and stoichiometric factor (n), that is, the radical-scavenging rate and number of radicals that can be trapped by each antioxidant molecule (Niki & Noguchi, 2004). Consequently, we first determined the EC_{50} values (defined as the amount of antioxidant required to eliminate the initial DPPH· concentration by 50%) of [6]-gingerol, [6]-shogaol and their derivatives by plotting the remaining DPPH· concentrations at the end of this reaction versus the concentrations of the compounds tested in methanol. This plotting gave excellent linear correlations for all the compounds and their stoichiometric factors ($n_{DPPH\cdot}$) were obtained from the gradients of the straight lines (Fig. 2). According to the EC_{50} or $n_{DPPH\cdot}$ values listed in Table 1, we can conclude that in the two series with different chain lengths, shogaols are the most active followed by gingerols, whereas dehydroshogaols and dehydrogingerols are relatively less active than shogaols and gingerols. Introduction of the C_1-C_2 double bond in the chain structure of shogaols and gingerols, to generate dehydroshogaols and dehydrogingerols, respectively, results in a reduction of the DPPH·-scavenging activity. This is line with the previous finding that [6]-shogaol is more active than [6]-dehydroshogaol in the DPPH·-scavenging assay (Li et al., 2012). A comparison of shogaols with gingerols, or dehydroshogaols with dehydrogingerols clearly indicates that the C_4–C_5 double bond is superior to the 5-OH in increasing the activity, in agreement with the previous results that [6]-shogaol can more effectively scavenge the DPPH· than [6]-gingerol (Dugasani et al., 2010). Additionally, extension of the unbranched alkyl chain length from six to twelve carbons had no significant influence on the activity of gingerols and dehydrogingerols as suggested by statistical analysis (Table 1). Previously, Masuda et al. (2004) had also found that there is no significant difference in the activity among the gingerol related compounds. In contrast, Dugasani et al. (2010) suggested that the activity increases as extension of the chain length ([10]-gingerol > [8]-gingerol > [6]-gingerol). The discrepancy in the influence of the chain length on the activity probably comes from the different incubation time of the radical with the test compounds. Taken together, our results highlight that the influence of functional group change is greater than that of the chain length change on the DPPH·-scavenging activity.

We further measured the rate constants (k_2) for hydrogen atom abstraction from the compounds by DPPH· in methanol at 25 °C by following the second order decay of the absorbance at 517 nm due to the radical. It can be judged from the k_2 values (Table 1) that similarly to that determined by the $n_{DPPH\cdot}$ values, introduction of the C_1-C_2 double bond extends the conjugated link between the aromatic region and side chain, but reduces the formal hydrogen



Scheme 1. Synthesis of [6]-gingerol, [6]-shogaol and their derivatives.

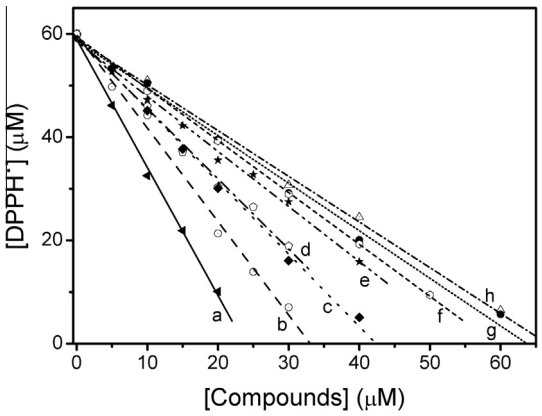


Fig. 2. Dependency of the remaining DPPH· concentrations on the concentrations of [6]-gingerol, [6]-shogaol and their derivatives in methanol after 60 min of reaction. (a) [12]-shogaol; (b) [6]-shogaol; (c) [12]-gingerol; (d) [6]-gingerol; (e) [12]-dehydroshogaol; (f) [6]-dehydroshogaol; (g) [12]-dehydrogingerol; and (h) [6]-dehydrogingerol.

Table 1
Antioxidant activity of [6]-gingerol, [6]-shogaol and their derivatives.

Compounds	DPPH·-Scavenging activity			FRAP	t_{eff}
	EC_{50} (μM)	$n_{DPPH\cdot}$	k (M^{-1} s^{-1})	n_e	(min)
[6]-Gingerol	21.4 ± 0.9[a]	1.29	243 ± 12[ab]	2.39 ± 0.04	46[af]
[6]-Shogaol	16.3 ± 0.4[b]	1.81	260 ± 5[b]	2.81 ± 0.01	33[b]
[6]-Dehydrogingerol	32.6 ± 0.7[c]	0.88	173 ± 15[c]	1.88 ± 0.02	52[c]
[6]-Dehydroshogaol	29.2 ± 0.6[d]	1.00	230 ± 11[a]	2.08 ± 0.05	41[a]
[12]-Gingerol	20.8 ± 0.6[a]	1.40	254 ± 8[b]	1.90 ± 0.02	76[d]
[12]-Shogaol	11.6 ± 0.8[e]	2.29	292 ± 11[d]	2.15 ± 0.06	44[af]
[12]-Dehydrogingerol	31.1 ± 2.0[c]	0.93	168 ± 15[c]	1.19 ± 0.02	69[e]
[12]-Dehydroshogaol	26.6 ± 0.9[f]	1.06	226 ± 16[a]	1.37 ± 0.03	48[cf]

For the DPPH·-Scavenging activity and FRAP assays, data are expressed as the mean ± SD for three determinations. For the anti-haemolysis assay, data are the averages of three determinations which were reproducible with deviation less than ± 10%. In the same column, mean values bearing different superscripts are significantly different ($P < 0.05$).

atom transfer ability of the compounds. Moreover, extension of the chain length has no significant influence on the reaction rate constant of gingerols, dehydrogingerols and dehydroshogaols with the DPPH·.

3.3. FRAP of [6]-gingerol, [6]-shogaol and their derivatives

A phenolic antioxidant can neutralize free radicals not only by transferring a hydrogen atom but also by donating an electron. Therefore, we next tested the electron-donating ability of [6]-gingerol, [6]-shogaol and their derivatives by the FRAP method (Benzie & Strain, 1996) based on the reduction of the Fe^{3+} complex of tripyridyltriazine $Fe(TPTZ)_2^{3+}$ to the intensely blue coloured Fe^{2+} complex $Fe(TPTZ)_2^{2+}$ in acidic medium. The results are expressed as the number (n_e) of donated electrons per molecule and summarised in Table 1. On the basis of the n_e values, the electron-donating ability decreases in the order of shogaols > gingerols > dehydroshogaols > dehydrogingerols for two series compounds, in agreement with the result obtained form the DPPH·-scavenging assay. Installation of the C_1–C_2 double bond attenuates the electron-donating ability as exemplified by the n_e values of shogaols versus dehydroshogaols, and gingerols versus dehydrogingerols. Replacement of the C_4–C_5 double bond by the 5-OH also reduces the ability as suggested by comparing the n_e values of shogaols with gingerols, and dehydroshogaols with dehydrogingerols. In addition, extension of the unbranched alkyl chain length from six to twelve carbons mildly decreased the ability, a result different from that of the DPPH·-scavenging assay.

3.4. Inhibitory activity of [6]-gingerol, [6]-shogaol and their derivatives against AAPH-induced DNA strand breakages

DNA is one of the most biologically important targets of free radicals, and oxidative damage of DNA including strand breakages and base and nucleotide modifications could result in gene mutation. For example, 8-hydroxy-2′-deoxyguanosine (8-OHdG), an oxidised nucleoside of DNA, induces transversion of G to T, which is also strong evidence that oxidative stress is intimately associated with carcinogenesis (Wu, Chiou, Chang, & Wu, 2004). In view of implication of DNA damage and the above two models being substrate-free, thus, we further selected the AAPH-induced DNA strand breakage model to probe inhibitory activity of [6]-gingerol, [6]-shogaol and their derivatives against the damage by using agarose gel electrophoresis analysis.

As shown in lane 2 of Fig. 3A, supercoiled pBR322 plasmid DNA was completely broken down to the open circular and linear forms upon addition of 5 mM 2,2′-azobis (2-amidinopropane hydrochloride) (AAPH), a water-soluble azo compound which is extensively used as a free radical initiator for biologic studies. The open circular and linear forms are indicative of single- and double-strand breakages, respectively. The compounds bearing a six-carbon unbranched alkyl chain significantly inhibits the DNA strand breakages with the activity sequence of [6]-dehydrogingerol > [6]-gingerol > [6]-dehydroshogaol > [6]-shogaol (lanes 3–6). The activity sequence is completely different from that obtained by the DPPH·-scavenging and FRAP assays, highlighting the importance of the 5-OH in inhibiting the DNA strand breakages. A possible reason is that this group would facilitate the binding affinity of [6]-dehydrogingerol and [6]-gingerol with plasmid DNA, resulting in the activity enhancement. Furthermore, the C_1–C_2 double bond could be responsible for their interaction with DNA. The extension from six- to twelve-carbon unbranched alkyl chains almost completely abolishes their inhibitory activity. Even in the case of [12]-dehydrogingerol bearing the 5-OH, it only retains the weak activity as evidenced by lane 4 of Fig. 3B where the linear form was decreased compared to that in the APPH control lane, but no the supercoiled form appeared. This indicates that the chain extension significantly disrupts and intervenes with their interaction with DNA.

3.5. Inhibitory activity of [6]-gingerol, [6]-shogaol and their derivatives against AAPH-induced haemolysis of human erythrocytes

Biological membranes are the heterogeneous system where antioxidant efficacy could be significantly different from that determined in the homogeneous solutions as used in the DPPH·-scavenging, FRAP and DNA strand breakage-inhibiting experiments

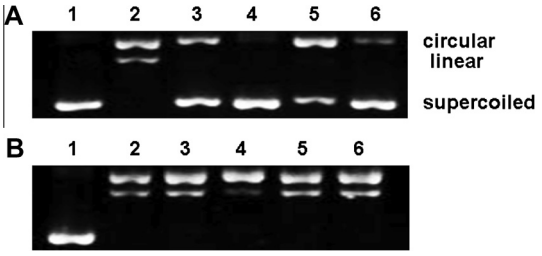


Fig. 3. Agarose gel electrophoresis pattern showing protection of [6]-gingerol, [6]-shogaol and their derivatives (40 μM) against the AAPH (5 mM)-induced strand breakages of plasmid pBR322 DNA (100 ng/25 μl) in PBS (pH 7.4) at 37 °C for 60 min. (A) Lane 1: control; Lane 2: AAPH alone; Lanes 3-6: AAPH + [6]-dehydroshogaol, AAPH + [6]-dehydrogingerol, AAPH + [6]-shogaol and AAPH + [6]-gingerol, respectively. (B) Lane 1: control; Lane 2: AAPH alone; Lanes 3-6: AAPH + [12]-dehydroshogaol, AAPH + [12]-dehydrogingerol, AAPH + [12]-shogaol and AAPH + [12]-gingerol, respectively.

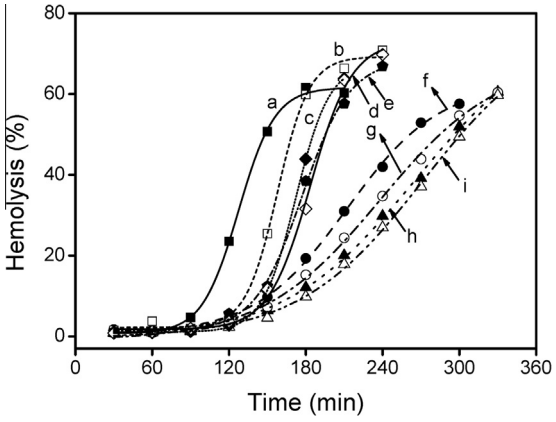


Fig. 4. Inhibitory effect of [6]-gingerol, [6]-shogaol and their derivatives (40 μM) against 50 mM AAPH-induced haemolysis of 5% human RBCs in 0.10 mM PBS (pH 7.4) under an aerobic atmosphere at 37 °C. (a) AAPH alone; (b) AAPH + [6]-shogaol; (c) AAPH + [6]-gingerol; (d) AAPH + [6]-dehydrogingerol; (e) AAPH + [6]-dehydroshogaol; (f) AAPH + [12]-shogaol; (g) AAPH + [12]-dehydroshogaol; (h) AAPH + [12]-dehydrogingerol, and (i) AAPH + [12]-gingerol.

(Niki & Noguchi, 2004; Niki, 2010). Human red blood cell membranes are rich in polyunsaturated fatty acids and are very susceptible to free radical-mediated lipid peroxidation, which induces membrane disturbance and haemoglobin leakage, ultimately leading to occurrences of haemolysis (Niki et al., 1988). Consequently, we finally employed the AAPH-induced RBC haemolysis model to assess inhibitory activity of [6]-gingerol, [6]-shogaol and their derivatives against lipid peroxidation in heterogeneous media.

The haemolysis extent can be detected by measuring absorbance of the haemolysates at 540 nm (Niki et al., 1988). When human RBCs were incubated in air at 37 °C as a 5% suspension in buffered saline solution, they were stable and little haemolysis was observed during 5 h (data not shown). When 50 mM AAPH was added to the RBC suspension, it induced fast haemolysis after an inhibition time of 88 min (t_{inh}) due to the presence of the endogenous antioxidants in the RBC membranes (line a of Fig. 4) (Esterbauer & Ramos, 1996). However, the onset of oxidative haemolysis was significantly inhibited by adding the compounds tested, as indicated by prolongation of the t_{inh} derived from the endogenous antioxidants (Fig. 4). Their antioxidant efficacy can be assessed by the prolongation portion called "an effective inhibition time (t_{eff})".

Of interest, according to the t_{eff} values (Table 1), the best results regarding the anti-haemolysis activity are again appeared in dehydrogingerols and gingerols bearing the 5-OH, a similar result to that obtained from the DNA strand breakage-inhibiting experiment. This is probably because the binding of the compounds to RBC membranes could be mediated by hydrogen bonds between the 5-OH and the membrane component, hence facilitating their reaction with the propagating lipid peroxyl radicals (LOO·) within membranes. A previous study has also proven that the hydroxyl groups at carbons 1, 3 and 4 of D-glucose play important roles for its binding to isolated human erythrocyte membranes by hydrogen bond interaction (Kahlenberg & Dolansky, 1972). On the other hand, the extension from six- to twelve-carbon unbranched alkyl chains strengthened their anti-haemolysis activity. Notably, this result is entirely opposite to that of the DNA strand breakage-inhibiting experiment and the FRAP assay, suggesting that the microenvironment change significantly influences antioxidant efficacy. This is probably due to the fact that the modest extension in the chain length facilitates their uptake into the membranes or assists in their appropriate localisation in the membranes to increase the LOO·-scavenging efficiency.

4. Conclusion

In summary, we synthesized eight gingerol related compounds and investigated influence of the side chain structure on their antioxidant potency by four different assays. Their antioxidant activity depends significantly on the side chain structures including the functional groups and chain length, the reaction mediums and substrates. Specifically, introduction of the C_1-C_2 double bond decreases their formal hydrogen-transfer and electron-donating abilities, but increases their DNA damage- protecting ability; Despite lowering their formal hydrogen-transfer and electron-donating abilities, existence of the 5-OH strengthens their DNA damage- and lipid peroxidation-protecting abilities; Extension of the chain length from six to twelve carbons significantly reduces the DNA strand breakage-inhibiting activity, however, enhances their anti-haemolysis activity. The above results provide useful information for designing [6]-gingerol and [6]-shogaol-directed antioxidants, and understanding the behaviour of antioxidant in different media and models.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2014.05.077.

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