LC–MS/MS analysis, antioxidant and anticholinergic properties of galanga (*Alpinia officinarum* Hance) rhizomes

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ABSTRACT

Galanga (*Alpinia officinarum* Hance) is a pungent and aromatic rhizome, which is a member of the ginger family (*Zingiberaceae*). The rhizomes of this plant have been used as a traditional medicine in far east countries for relieving stomach ache, invigorating the circulatory system, treating colds, and reducing swelling. The aim of this study was to investigate antioxidant and anticholinergic properties of galanga. For this purpose, we elucidated the antioxidant activity of water (WEG), ethanol (EEG) and water/ethanol (50:50 v/v WEEG) extracts of galanga by different *in vitro* antioxidant assays. Radical scavenging activities of galanga extracts were performed by 2,2-diphenyl-1-picrylhydrazyl (DPPH$^\bullet$) and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS$^{\bullet+}$) radical scavenging assays. Also, reducing power galanga extracts were evaluated by Cu^{2+}–Cu^+ reducing (CUPRAC) and Fe^{3+}–Fe^{2+} reducing and $[Fe^{3+} - (TPTZ)_2]^{3+}$–$[Fe^{2+}–(TPTZ)_2]^{2+}$ (FRAP) abilities. Finally, Fe^{2+} chelating activity by pipyrdyl reagent and anticholinergic activities of galanga extracts were realised. All galanga extracts demonstrated antioxidant activity. α-Tocopherol ((2R)-2,5,7,8-tetramethyl-2-[(4R,8R)-(4,8,12-trimethyltridecyl)]-6-chromanol), trolox (3,4-dihydro-6-hydroxy-2,5,7,8-tetramethyl- 2H-1-benzopyran-2-carboxylic acid), butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) were used as positive controls. On the other hand, IC_{50} values were calculated for DPPH$^\bullet$, ABTS$^{\bullet+}$ scavenging Fe^{2+} chelating, and anticholinergic effects of galanga extracts. This study clearly showed that galanga had marked antioxidant, anticholinergic effect, reducing ability, radical scavenging and metal binding activities.

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1. Introduction

Oxidation is the transfer of electrons between two atoms and stands for a required part of aerobic life and our metabolism. It is essential to many living organisms for the production of energy to fuel biological processes. However, problems may occur when the electron flow becomes disconnected, generating free radicals and ROS (Gülçin, 2012; Bursal et al., 2013). A free radical has one or more uncoupled electrons situated outermost orbital of molecular or atomic orbitals. ROS includes non-free radical kinds like singlet oxygen ($1O_2$), ozone (O_3), hydrogen peroxide (H_2O_2) and free radical species like superoxide anion radicals ($O_2^{\bullet-}$), peroxyl (ROO$^\bullet$), hydroxyl radicals (OH$^\bullet$) and hydroperoxyl radicals (HOO$^\bullet$) (Gülçin, 2006a; Bursal and Gülçin, 2011). Free radicals may be occurring by living cells during pathophysiological and biochemical processes as well as due to environmental pollutants, radiation, chemicals and toxins (Elmastaş et al., 2006a; Gülçin, 2011). They are produced naturally in mammalian systems as a result of oxidative metabolism (Bursal et al., 2013).

A normal cell has convenient prooxidant-antioxidant equilibrium. Yet this equilibrium can be changed towards the prooxidants when production of ROS is gone up enormously or when ranges of antioxidants are decreased. This stage is entitled as oxidative

stress (Elmastas et al., 2006; Gülçin, 2012). In recent years, interest has remarkably increased in identifying alternate safe and innate source materials of food antioxidants, and the search for natural antioxidants, particularly of plants (Gülçin, 2006b; Gülçin et al., 2011a), vegetables (Gorinstein et al., 2011b) and fruits (Park et al., 2014, 2015; Gorinstein et al., 2011a,c). Also, restricting the use of synthetic antioxidants leaded to an incremental interest of natural antioxidant sources. Therefore, there is an increasing way in consumer priorities based on natural antioxidants, which have given a driving force to the initiatives to detect many new sources of antioxidants (Dymerski et al., 2015; Gorinstein et al., 2013; Gülçin, 2008a, 2009; Namiesnik et al., 2014).

Galanga (*Alpiniae officinarum*) Hance (*Zingiberaceae*) is an annual plant and cultivated in far east countries including India, Vietnam, Southern China and Thailand because of its use as a spice and as a traditional medicine. There are about 46 species in this genus in China (Zhang and Shen, 1996). Medicinal properties of this plant were well documented. It has been used as a traditional medicine for several purposes such as relieving stomach ache, treating colds, invigorating the circulatory system and reducing swelling (Liu et al., 2014). Galangal had anti-inflammatory (Kiuchi et al., 1982a,b), antioxidant (Shen et al., 1998), antibacterial (Subramanian et al., 2009), antiparasitic (Alves et al., 2003), anti-proliferative (Ali et al., 2001), anticancer (Heo et al., 2001), antitumor (Matsuda et al., 2009), anti-arthritic (Matsuda et al., 2006), antiemetic (Shin et al., 2002), anti-nociceptive, (Lee et al., 2009), anti-hepatotoxic (Hikino et al., 1985), anti-genotoxic (Heo et al., 2001), anti-phlogistic (Matsuda et al., 2006), anti-psychiatric (Lee et al., 2009), analgesic (Matsuda et al., 2006), antalgic (Zhang et al., 2010), cholagogue (Zhang et al., 1996), carminative (Matsuda et al., 2006), immunomodulatory (Subramanian et al., 2009) and anti-spasmodic (Jung and Shin, 1990) effects in oriental medicine. In addition, inhibitions of prostaglandin (Kiuchi et al., 1982a,b) and leukotriene (Kiuchi et al., 1992) biosynthesis, pancreatic lipase (Shin et al., 2004), and α-reductase (Kim et al., 2003) have been reported. Also, pharmacological studies indicated that galangal exhibited anti-ulcer, anti-diarrhoea, antithrombotic effects (Zhang et al., 1996; Ling et al., 2010 Ling et al., 2010). It is generally used for treatment of pyogenic diseases, ringworm, venereal diseases, carminative, abdominal discomfort (Athamaprasangsa et al., 1994).

Recently, some studies related to the chemical and pharmacological properties of the galanga rhizomes showed that it has three groups of important chemical constituents, flavonoids, glycosides and diarylheptanoids (Eumkeb et al., 2010). Realizing the importance of medicinal plants, different plant parts extracts are extensively explored and studied for different bioactivities including antioxidants (Boo et al., 2012; Heo et al., 2013, 2014). Some bioactive chemical constituents in galanga include volatile oil (Jirovetz et al., 2003), phenylpropanoids (Ly et al., 2002), and diarylheptanoids. Flavonoids (Lu and Jiang, 2006) such as galangin (Eumkeb et al., 2010), 3-O-methyl galangin (Tao et al., 2006), officinin A (Zhao et al., 2010), kaempferide and kaempferide-3-O-β-d-glucoside (Eumkeb et al., 2010), alpinin (Liu et al., 2012), and cadinane sesquiterpene (Sheng-Mei et al., 2012) have been reported so far. Diarylheptanoids are among the characteristic compounds and categorized into linear diarylheptanoids, cyclic diarylheptanoids, dimeric diarylheptanoid, special diarylheptanoids or diarylheptanoid bearing flavonol moiety (Shin et al., 2002; Sun et al., 2008; Liu et al., 2012). However, galangin, a member of the flavonol class of flavonoids, was present in high concentrations in galanga (Heo et al., 2001). 3-O-methyl galangin had hypolipidemic effect (Shin et al., 2003). Thus, galanga has increasing and important applications in food and in medicinal herb industry.

Alzheimer's disease (AD) is characterized by memory loss, dementia, and cognitive impairment. AD is one of the most common diseases in elderly people (Förstl and Kurz, 1999). At the present time, the treatment of AD focuses on acetylcholinesterase (AChE, E.C. 3.1.1.7) inhibitors, such as tacrine, donepezil, rivastigmine and galantamine. AChE inhibitors are used in the treatment of several neuromuscular diseases, and were studied for treatment of AD (Meng et al., 2012). However, the potential effectiveness of these inhibitors in clinical use is often complicated by their associated side effects. For example, clinical studies have shown that tacrine, which putative AChE inhibitor, causes hepatotoxicity (Göçer et al., 2013). Since AD is a multi-pathogenic illness, a current drug-discovery strategy is to develop novel anti cholinergic agents with multiple potencies including inhibition of AChE (Akıncıoğlu et al., 2014). AChE exists in high concentrations in red blood cells and brains. This enzyme catalyses the hydrolysis and involves the regulation of acetylcholine. The use of agents with enhanced selectivity for AChE indicated potential therapeutic benefit of inhibiting AChE in AD and related dementias. Therefore, AChE might be considered as an important target for novel and natural antioxidant drug development for treatment of AD (Göçer et al., 2013; Akıncıoğlu et al., 2014).

In this study, we determined the antioxidant, antiradical and anticholinergic activities of galanga extracts used by different bioanalytical methods. Also, another intention of the current study was to explain the antioxidant, anticholinergic, antiradical and chelating ability with metal of galanga extracts.

2. Materials and methods

2.1. Chemicals

Ellman's reagent [DTNB, 5,5′-dithio-bis(2-nitro-benzoic acid)], acetylthiocholine iodide (AChI), N,N-dimethyl-p-phenylenediamine, 2,9-dimethyl-1,10-phenanthroline, BHA (butylated hydroxyanisole), nitrobluetetrazolium, BHT (butylated hydroxytoluene), DPPH• (2,2-diphenyl-1-picrylhydrazyl), ABTS•+ (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), linoleic acid, trichloroacetic acid and α-tocopherol were purchased commercially from Sigma–Aldrich GmbH, Sternheim, Germany. Ammonium thiocyanate was obtained from Merck. All other chemicals were used analytical grade and attained from either Sigma–Aldrich or Merck.

Following compounds were used as standards in LC–MS/MS analysis: *p*-hydroxybenzoic acid (99%, Merck), *p*-coumaric acid (98 %, Sigma–Aldrich), quercetin (98%, Sigma–Aldrich), pyrogallol (98%, Sigma–Aldrich), apigenin (95%, Sigma–Aldrich), kaempferol (90 %, Sigma–Aldrich), luteolin (98%, Sigma–Aldrich) and isorhamnetin (98%, ExtraSynthese, Genay-France). Stock solutions were prepared as 10 mg/L in methanol, which were prepared as 0.1 mg/L and 5 mg/L, respectively, in the same solvent. HPLC grade methanol was purchased from Merck (Darmstadt, Germany). Calibration solutions were prepared in methanol in a linear range (Table 1). A portion (0.1 g/L) of curcumin solution was freshly prepared for using as an internal standard (IS) in all experiments.

2.2. Preparation of test solution

50 mg of the plant material were dissolved in 5 mL of ethanol-water (50:50 v/v) in a volumetric flask were refluxed in ethanol–water (50:50 v/v) for 1 h, from which 1 mL was transferred into a 5 mL of volumetric flask. Then, 50 μL of curcumin was added and diluted to the volume with methanol. From the final solution, 1 mL was transferred into capped autosampler vial and 10 μL of sample was injected to LC. The samples in autosampler were kept at 15 °C during the experiment.

Table 1

LC–MS/MS parameters and of selected compounds of galanga (*Alpinia officinarum* Hance) rhizome with different extracts: WEG, water; EEG, ethanol; and WEEG, water/ethanol (50:50 v/v).

	Compounds	Parent ion	Daughter ion	Collision energy (V)
1	Kaempferol	287.0	152.3	30
2	Pyrogallol	125.0	80.0	16
3	*p*-Hydroxibenzoic acid	136.7	92.6	12
4	*p*-Coumaric acid	163.2	118.7	14
5	Apigenin	269.0	151.0	22
6	Luteolin	285.0	132.0	34
7	Quercetin	301.0	178.5	16
8	Isorhamnetin	315.0	300.0	20
9	Curcumin[a]	369.3	176.9	20

[a] Used as internal standard.

2.3. Instruments and chromatographic conditions

Experiments were performed by a Zivak® HPLC and Zivak® Tandem Gold Triple quadrupole (Istanbul, Turkey) mass spectrometry equipped with a Synergy Max C18 column (250 × 2 mm i.d., 5 μm particle size). The mobile phase was composed of water (A, 0.1% formic acid) in methanol (B, 0.1% formic acid), the gradient program of which was 0–1.00 minute 55% A and 45% B, 1.01–20.00 min 100% B and finally 20.01–23.00 55% A and 45% B. The flow rate of the mobile phase was adjusted to 0.25 mL/min, and the column temperature was set to 30 °C. The injection volume was taken as 10 μL.

2.4. Optimization of HPLC methods and LC/MS/MS procedure

The best mobile phase solution was determined to be a gradient of acidified of methanol and water system. Such a mobile phase was determined to be satisfactory for the ionization abundance and separation of the compounds. The good ionization of small and relatively polar antioxidants was obtained by the ESI source. Ionization technique and collision energies of the experiments are the most important parameters in quantitative mass spectrometry analyses. Triple quadrupole mass spectrometry system is commonly used due to its fragmented ion stability (Gören et al., 2009). Therefore, triple quadrupole mass spectrometry was used. The optimum ESI parameters were determined as 2.40 mTorr CID gas pressure, 5000 V ESI needle voltage, 600 V ESI shield voltage, 300 °C drying gas temperature, 50.00 °C API housing temperature, 55 psi Nebulizer gas pressure and 40.00 psi drying gas pressure. Detailed information on experiment parameters is given in Table 1.

2.5. Validation of experiments and uncertainty evaluation

In validation experiments of all compounds, curcumin was used as an internal standard. The validation parameters were determined for linearity, repeatability, LOD (limit of detection) and LOQ (limit of quantification) experiments.

The linearity for each compound for the reported method was determined by analysing standard solution. The linearity ranges of each compound are given in Table 2A and B. The correlation coefficients (r^2) were found to be ≥0.99. Linear regression equations of the reported compounds are also presented in Table 2, where y is the peak area and x is the concentration.

Precision of the method was evaluated by repeating the measurements of three concentrations for each compound. A good precision was determined, and the results were implemented to the uncertainty budget.

LOD and LOQ of the LC–MS/MS methods for the above compounds were calculated to be 0.5–50 mg/L. The LODs were determined to be 3 times bigger than standard deviation while LOQs were determined to be 10 times bigger (Table 2). The

concentration of each analyte within the linear range and concentration of the reported method was obtained from the calibration curve. Then, concentrations were converted to mg/kg of crude sample by the equation given below.

$$A = \frac{C_a x V_f}{m x V_i} \times 1000$$

where A is a quantity amount of compounds (μg/mg), C_a is the analyte concentration obtained by calibration curve (mg/L), V_f is the final diluted volume before the analysis, m is the amount of extract as gram, V_i is the initial sample volume. The EURACHEM/CITAC guide was used for evaluation of sources and quantification of uncertainty of LC–MS/MS method. The maximum contribution comes from the calibration curve. Detailed procedures of uncertainty evaluation are reported previously (Gören et al., 2007).

2.6. Determination of antioxidant activities

1 Fe^{3+} reducing assay. For determination of Fe^{3+} reducing ability of galanga, Fe^{3+} $(CN^-)_6$ to Fe^{2+} $(CN^-)_6$ reduction method was used (Gülçin, 2007, 2010). In brief, varied concentrations of galanga (10–30 μg/mL) in 0.75 mL of deionized H_2O were added with 1.25 mL of phosphate buffer (0.2 M, pH 6.6) and 1.25 mL of potassium ferricyanide $[K_3Fe(CN)_6]$ (1%). Then, the solution was incubated at 50 °C during 20 min. After incubation period, trichloroacetic acid (TCA) was added (1.25 mL, 10%). Lastly, a portion of $FeCl_3$ (0.5 mL, 0.1%) was transferred to this mixture and the absorbance value was enrolled at 700 nm in a spectrophotometer. According to the obtained results, when reduction capability increases, absorbance indicates greater value (Elmastaş et al., 2006b; Bursal et al., 2013 Bursal et al., 2013).

2 CUPRAC assay. Cu^{2+} reducing power was used as a second reducing ability method for galanga extracts. Cu^{2+} reducing capability was performed according to the method of Apak et al. (2006) with slight modification (Gülçin, 2008b) For this purpose, aliquots of $CuCl_2$ solution (0.25 mL, 0.01 M), ethanolic neocuproine solution (0.25 mL, 7.5 × 10⁻³ M) and NH_4Ac buffer solution (0.25 mL, 1.0 M) were transferred to a test tube, which contains galanga extracts at different concentrations (10–30 μg/mL). Total volume was completed with distilled H_2O to 2 mL and shaken vigorously. Absorbance of samples was recorded at 450 nm after 30 min (Gülçin, 2008).

3 FRAP assay. FRAP assay is based upon reduction of Fe^{3+}-TPTZ complex under acidic medium and conditions. Increased absorbance of blue-coloured ferrous form (Fe^{2+}-TPTZ complex) is recorded at 593 nm. TPTZ solution (2.25 mL, 10 mM TPTZ in 40 mM HCl) was freshly prepared, then transferred to acetate buffer (25 mL, 0.3 M, pH 3.6), and $FeCl_3$ solution (2.25 mL, 20 mM) in water. Then, different concentrations of galanga extracts (10–30 μg/mL) dissolved in 5 mL of appropriate buffer solvent, stirred and incubated at 37 °C for 30 min. Finally the absorbance of mixture was measured at 593 nm (Göçer and Gülçin, 2011).

4 Fe^{2+} chelating assay. Metal chelating ability of galanga extracts was predicted according to Dinis et al. (1994) with slight modification (Gülçin and Daştan, 2007). Fe^{2+}-binding capacity of galanga extracts was spectrophotometrically recorded at 562 nm. In brief, to a mixture of $FeCl_2$ (0.1 mL, 0.6 mM) galanga extracts were added at three different concentrations (10–20 μg/mL) in methanol (0.4 mL). The reactions were started by pipyrdyl solution addition (0.1 mL, 5 mM). After that, the solution was mixed and incubated at room temperature for ten minutes. Finally, absorbance value of the mixture was quantified spectrophotometrically at 562 nm versus blank sample.

5 DPPH• scavenging assay. The solution of DPPH• was daily prepared, stored in a flask coated with aluminium foil and kept in the dark at 4 °C. In brief, fresh solution of DPPH•

Table 2
(A) Validation and uncertainty parameters; (B) Amount (mg/kg) of secondary metabolites in extracts of galanga (*Alpinia officinarum* Hance).

(A)

Compounds	Linear regression equation	R^2	LOD (mg/L)	LOQ (mg/L)	RSD (%)
Kaempferol	$y = (8.11E+15)x + (4.07E+14)$	0.9942	0.002	0.008	5.47
Pyrogallol	$y = (1.33E+15)x + (5.24E+15)$	0.9813	0.001	0.002	5.47
p-OH benzoic acid	$y = (4.35E+16)x + (9.83E+15)$	0.9939	0.002	0.007	4.78
p-Coumaric acid	$y = (1.02E+17)x + (5.48E+16)$	0.9972	0.006	0.021	6.39
Apigenin	$y = (5.54E+16)x + (3.79E+16)$	0.9904	0.150	0.501	4.01
Luteolin	$y = (7.75E+16)x + (5.18E+16)$	0.9932	0.062	0.207	15.9
Quercetin	$y = (4.08E+16)x + (1.90E+16)$	0.9946	0.001	0.002	11.4
Isorhamnetin	$y = (7.39E+16)x + (5.10E+16)$	0.9908	0.088	0.294	3.67

(B)

Compounds	WEEG	EEG	WEG
Kaempferol	44.84 ± 4.35	87.02 ± 10.40	7.38 ± 0.52
Pyrogallol	135.45 ± 9.32	6.36 ± 0.42	–
p-OH benzoic acid	–	10.98 ± 0.73	–
p-Coumaric acid	8.46 ± 0.59	12.08 ± 0.83	17.17 ± 1.28
Apigenin	30.53 ± 4.32	49.37 ± 8.27	25.72 ± 1.69
Luteolin	1.88 ± 0.12	3.85 ± 0.29	–
Quercetin	4.36 ± 0.28	7.18 ± 0.55	–
Isorhamnetin	15.65 ± 1.62	34.37 ± 9.33	1.84 ± 0.12

(0.1 mM) was prepared in ethanol. Then, 1.5 mL of each galanga extracts in ethanol was added an aliquot of this solution (0.5 mL) (10–30 μg/mL). These mixtures were mixed vigorously and incubated in the dark for 30 min. Finally the absorbance value was recorded at 517 nm in a spectrophotometer (Gülçin, 2006b).

6 ABTS•+ scavenging assay. ABTS radical scavenging activity of galanga extracts was performed using the spectroscopic method described by Re et al. (1999). The ABTS radical cation (ABTS•+) was acquired by reacting 7 mM solution of ABTS with 2.45 mM $K_2S_2O_8$. Prior to assay, the ABTS radical cation solution was diluted with ethyl alcohol to an absorbance of 0.750 ± 0.05 at 734 nm. Then, 1 mL of ABTS•+ solution was supplement to 3 mL of each galanga extract and control solutions. The extent of decolorization is calculated as percentage reduction of absorbance.

Percentage of metal chelating, and radicals scavenging were computed using the following equation:

$$SE(\%) = [1 - (\frac{As}{Ac})] \times 100$$

where SE is scavenging effects, Ac is the absorbance value of control and As is absorbance value of sample (Gülçin et al., 2006a,b).

• Anticholinergic assay. Inhibition effects of galanga extracts on AChE activities were measured by Ellman et al. (1961). AChI, and DTNB (Product no: D8130-1G, Sigma–Aldrich) were used for the determination of the AChE activity. Namely, 100 mL of buffer (Tris/HCl, 1 M, pH 8.0), 10 mL of sample solution dissolved in deionised water at different concentrations. Then, 50 mL AChE (5.3210^{-3} EU) solution was added and incubated for 10 min at 25 °C. After incubation, a portion of DTNB (50 mL 0.5 mM) was added. Finally, the reaction was started by the addition of 50 mL of AChI (10 mM, Product no: 1,480-1G, Sigma–Aldrich). The enzymatic hydrolysis of these substrates was determined spectrophotometrically by the formation of yellow 5-thio-2-nitrobenzoate anion as the result of the reaction of DTNB with thiocholine at a wavelength of 412 nm. For determination of the effect of galanga extracts on AChE, different galanga extracts concentrations were added to the reaction mixture. Then, AChE activity was measured. IC_{50} values were obtained from activity (%) versus compounds plots (Aksu et al., 2013; Innocenti et al., 2010a,b; Çetinkaya et al., 2014).

2.7. Statistical analysis

Each experiment was implemented 3 times. The acquired data were enrolled as mean ± standard deflection and analysed by SPSS (version 11.5 for Windows 2000, SPSS Inc.). One-way analysis of type ANOVA was implemented by procedures. Considerable distinctions between means were identified by Duncan's multiple range tests, and $p < 0.05$ was seen as important and $p < 0.01$ as very important.

3. Results and discussion

Galanga (*Alpinia officinarum*) belongs to the *Zingiberaceae* family and is a medicinal plant that has been traditionally used for treating inflammatory and gastrointestinal disorders (Subramanian et al., 2009). Galanga is one of the most commonly medicinal plant consumed sources of phenols. Standard chromatogram of antioxidant phenolic acids by LC–MS/MS (mg/mL) is presented in Fig. 1A. Diluted sample chromatogram is also given in Fig. 1B–D. According to LC–MS/MS experiment, as a main phenolic compound, 87.02 ± 10.40 mg/kg of kaempferol, 135.45 ± 9.32 mg/kg pyrogallol and 25.72 ± 1.69 mg/kg Apigenin were detected in 1 mg of EEG, WEEG and WEG, respectively (Table 2B). On the other hand, antioxidant compounds from natural sources are the only alternative to synthetic antioxidants counteracting the ROS associated diseases. For this purpose, majority of naturally taking place substances have been noticed to own antioxidant abilities. Also, various *in vitro* methods have been used to assess antioxidant activity (Gülçin, 2012; Gülçin and Beydemir, 2013; Çakmakçı et al., 2015). In the present study, several different antioxidant activity assays based on different reaction mechanisms are used to detect the potent antioxidant activity of galanga extracts. The first of these methods is Fe^{3+} reduction method. Generally, reducing properties depend on the presence of reductones, which have been shown to exert antioxidant activity and radical scavenging ability by donating a hydrogen atom (Duh, 1998; Sehitoglu et al., 2015). The Fe^{3+} $(CN^-)_6$ reduction method detects the antioxidant effect of any molecule as reducing capability in the reaction. Galanga extracts had the most influential reducing capacity using Fe^{3+} $(CN^-)_6$ reduction and Cu^{2+} ions reducing ability when classed with the standards (trolox, α-tocopherol, BHT, and BHA). As was seen in Fig. 2A and Table 3, WEG (r^2: 0.9761), EEG (r^2: 0.9787) and WEEG (r^2: 0.9837) demonstrated potent Fe^{3+} reducing capability and these diversities were statistically
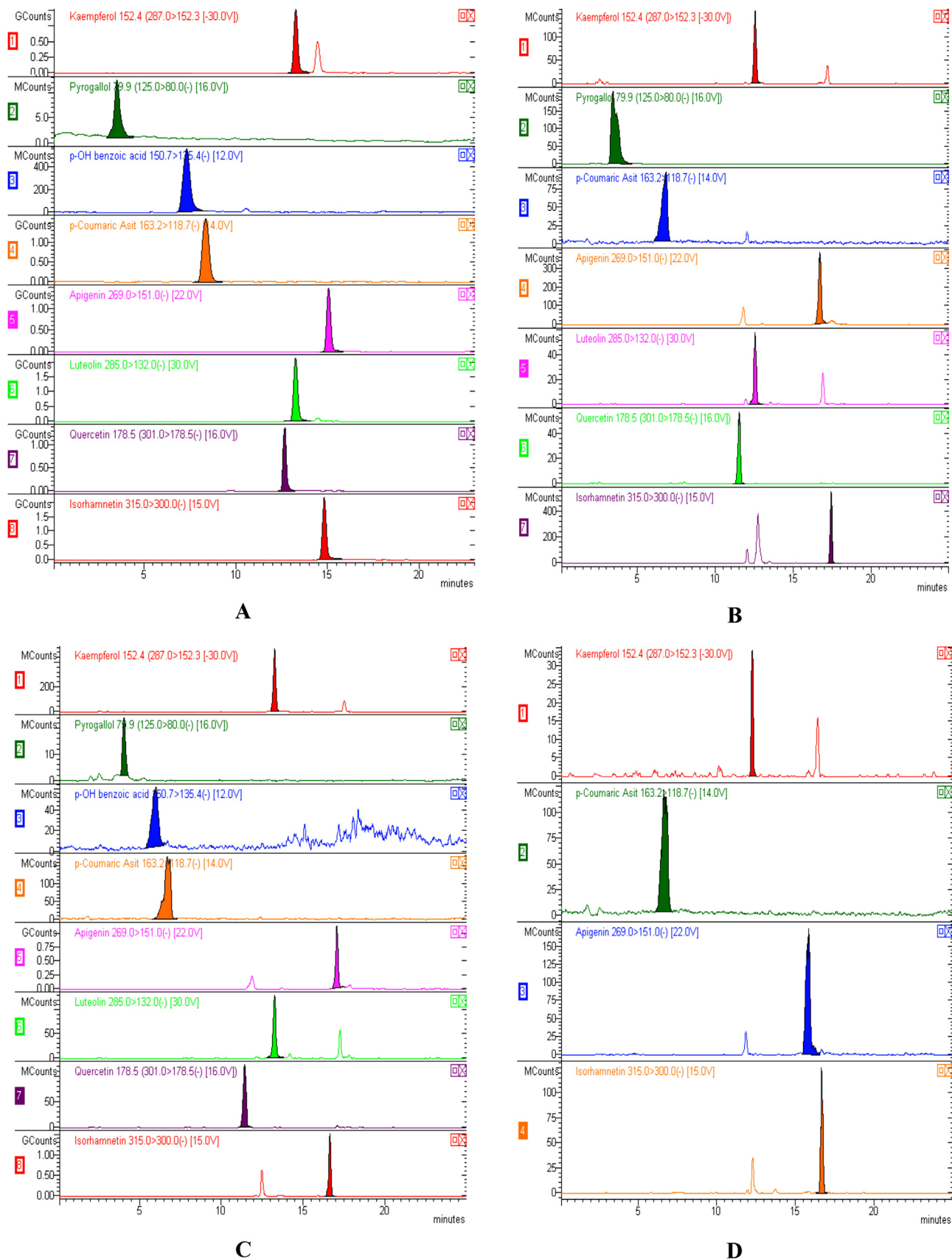
Fig. 1. (A) Standards chromatograms of secondary metabolites by LC-MS/MS (2.5 mg/L); (B–D), Chromatograms of secondary metabolites of galanga (*Alpinia officinarum* Hance) rhizome] in WEEG, EEG, WEG [WEG, water, EEG, ethanol, and WEEG, water/ethanol (50:50 v/v)] extracts.

Table 3
Determination of reducing power by potassium ferricyanide reduction, FRAP and FRAP methods of galanga (*Alpinia officinarum* Hance) rhizome [WEG, water, EEG, ethanol and WEEG, water/ethanol (50:50 v/v)] extracts.

Antioxidants	Fe^{3+}-Fe^{3+} reducing[a]		Cu^{2+}-Cu^+ reducing[a]		Fe^{3+}-TPTZ reducing[a]	
	IC_{50} (µg/mL)	R^2	IC_{50} (µg/mL)	R^2	IC_{50} (µg/mL)	R^2
BHA	2.170 ± 0.005	0.9616	2.396 ± 0.018	0.9107	2.853 ± 0.003	0.8282
BHT	1.490 ± 0.002	0.9950	2.020 ± 0.004	0.9206	2.026 ± 0.002	0.8870
Trolox	1.170 ± 0.001	0.9955	1.452 ± 0.050	0.9970	2.102 ± 0.003	0.9201
α-Tocopherol	1.101 ± 0.006	0.9631	1.262 ± 0.018	0.9920	1.855 ± 0.001	0.9175
WEEG	1.276 ± 0.004	0.9837	1.164 ± 0.002	0.9900	2.020 ± 0.003	0.9133
EEG	1.030 ± 0.002	0.9787	0.959 ± 0.006	0.9948	1.976 ± 0.002	0.9187
WEG	0.712 ± 0.003	0.9761	0.649 ± 0.002	0.9935	1.332 ± 0.002	0.8838

[a] Expressed as absorbance values.

seen to be considerably important ($p < 0.01$). Reducing capacity of 30 µg/mL concentration of WEG (r^2: 0.9761), EEG (r^2: 0.9787) and WEEG (r^2: 0.9837) and standard reducing agents were given as the following order: BHA (2.170; r^2: 0.9616) > BHT (1.490; r^2: 0.9950) > trolox (1.170; r^2: 0.9955) > WEEG (1.276; r^2: 0.9837) > α-tocopherol (1.101; r^2: 0.96631) ≈ EEG (1.030; r^2: 0.9787) > WEG (0.712; r^2: 0.9761). Results proved that galanga extracts had marked ferric ions (Fe^{3+}) reducing capability and also had electron-releasing features to neutralize free radicals by creating steady products. *In vivo*, as a result of reduction reactions, radical chain reactions finish and it may be very destructive.

Reducing capability of a bioactive compound and plant extracts can be evaluated by reduction of Fe $[(CN)_6]_3$ to Fe $[(CN)_6]_2$. In this technique, the presence of reductants or plant extracts would result in the reduction of Fe^{3+} to Fe^{2+} (Gülçin et al., 2011b,c). Addition of free Fe^{3+} to the reduced product brings about the formation of intensive Perl's Prussian blue complex, Fe_4 [Fe $(CN^-)_6]_3$, which has a strong absorbance at 700 nm. Fe^{3+} reducing assay gets advantage of an electron chain reaction where a ferric salt is utilized as an oxidant (Inatani et al., 1983). In addition, the yellow colour of the tested mixture changes into diverse tons of green and blue by ability of galanga extracts.

Cu^{2+} reducing power of galanga extracts and positive controls is shown in Fig. 2B. A positive relationship was found between Cu^{2+} reducing power and different concentration of galanga extracts. It was detected that Cu^{2+} reducing capacity of galanga extracts was addicted to different concentration (10–30 µg/mL, Table 3). Cu^{2+} reducing capability of galanga extracts and positive controls at the same concentration (30 µg/mL) showed the following order: BHA (2.396; r^2: 0.9107) > BHT (2.020; r^2: 0.9206) > trolox (1.452; r^2: 0.9970) > α-tocopherol (1.262; r^2: 0.9920) ≈ WEEG (1.164; r^2: 0.9900) > EEG (0.959; r^2: 0.9948) > WEG (0.649; r^2: 0.9935). There was a positive control between $Fe^{3+} - Fe^{2+}$ and $Cu^{2+} - Cu^+$ reducing power orders. The most powerful reducing power was observed in BHA and relatively the lowest powerful reducing power was observed in WEG for both reducing power methods.

The CUPRAC method is a simple, rapid, selective, cost-effective, steady and versatile antioxidant assay useful for a wide variety of polyphenols, as well as for thiols, synthetic antioxidants, and vitamins C and E. Also, this chromogenic redox reaction is conducted at a neutral pH (7.0) and the method allows measuring antioxidants including thiol like glutathione and non-protein thiols. CUPRAC reactions are essentially complete within 30 min (Apak et al., 2006).

The FRAP assay can be used to measure the total reducing capability of pure antioxidant compounds or plant extracts. Fe^{2+} can be detected spectrophotometrically due to its coloured complex with TPTZ, which has a maximum absorbance at 593 nm (Benzie and Strain, 1996). As can be seen in Fig. 2C and Table 3, Fe^{3+} reducing capability of galanga extracts and positive controls at the same concentration (30 µg/mL) showed the following order: BHA (2.853; r^2: 0.8282) > trolox (2.102; r^2: 0.9201) > BHT (2.026; r^2: 0.870) ≈ WEEG (2.020; r^2: 0.9133) ≥ EEG (1.976; r^2: 0.9187) > α-tocopherol (1.855;
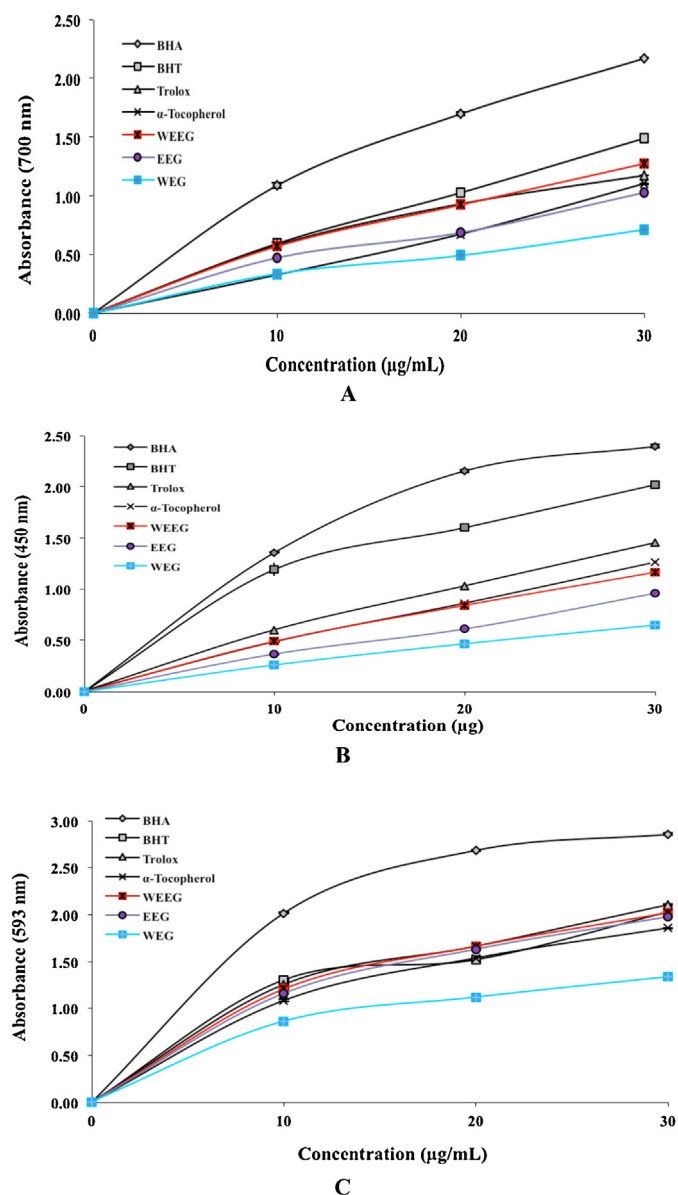

Fig. 2. (A) $Fe^{3+} \rightarrow Fe^{2+}$ reductive potential of different concentrations (10–30 µg/mL) of galanga extracts and reference antioxidants. (B) Cu^{2+} reducing ability of different concentrations (10–30 µg/mL) of galanga extracts and reference antioxidants. (C) $Fe^{3+} - TPTZ - Fe^{2+} - TPTZ$ reducing ability of different concentrations (10–30 µg/mL) of galanga extracts and reference antioxidants [BHA, butylated hydroxyanisole, BHT, butylated hydroxytoluene, WEG, water, EEG, ethanol and WEEG, water/ethanol (50:50 v/v)].

r^2: 0.9175) > WEG (1.332; r^2: 0.8838). FRAP assay was selected for evaluation of the reducing ability of galanga extracts for following reasons. Firstly, the FRAP assay treats the antioxidants in the samples as reductant in a redox-linked colorimetric and it is relatively simple and easy to be standardized. Also, this reductive method has been frequently used for a rapid determination of the total antioxidant capacity of various food and medicinal and pharmaceutical plants. Additionally, it has been applied for measurement of antioxidant activity of phenolic compounds, polyphenols and flavonoids in vitro (Cavalli et al., 2008).

DPPH test is generally used as the substrate to gauge free radical scavenging effectiveness of antioxidants. This methodology is based on the reduction of a DPPH solution in alcohol in the source of a hydrogen donating antioxidant, owing to the formation of non-radical form DPPH-H by the reaction (Şerbetçi Tohma and Gülçin, 2010; Gülçin et al., 2012). Galanga extracts have the ability to reduce steady radical DPPH to yellow-coloured DPPH-H. Fig. 3A and Table 3 define a crucial decrement ($p < 0.01$) in the concentration of DPPH radical owing to the scavenging capability of galanga extracts and reference radical scavenging agents like trolox, α-tocopherol, BHT and BHA. IC$_{50}$ values were found as 14.75 μg/mL (0.9948) for WEEG, 31.51 μg/mL (0.9973) for EEG, 49.51 μg/mL (0.973) for WEG, 14.16 μg/mL (0.9787) for trolox, 9.62 μg/mL (0.8966) for α-tocopherol, 24.76 μg/mL (0.9879) for BHT and 9.37 μg/mL (0.9466) for BHA. DPPH radical scavenging of samples increased in the order of trolox > BHA $\approx$ α-tocopherol > WEEG > BHT > $\approx$ EEG > WEG. A lower EC$_{50}$ value demonstrates a higher DPPH radical scavenging activity. DPPH exposed an absorbance at 517 nm, which vanished after acceptation of an electron or hydrogen radical from an antioxidant compound to become a steadier diamagnetic molecule (Gülçin et al., 2005).

As shown in Fig. 3B and Table 3, galanga extracts had effective ABTS$^{\bullet+}$ radical scavenging in a concentration-dependent manner (10–30 μg/mL). The EC$_{50}$ values for WEEG, EEG and WEG in this analysis were 12.38 μg/mL for WEEG (r^2: 0.9878), 16.12 μg/mL for EEG (r^2: 0.9870) and 33.01 μg/mL for WEG (r^2: 0.9955). It is seen that concentration of ABTS$^{\bullet+}$ ($p < 0.01$) declines substantially owing to the scavenging capability at all galanga extracts concentrations. Moreover, EC$_{50}$ values for trolox, α-tocopherol, BHT and BHA were found to be 9.00 μg/mL (r^2: 0.9453), 13.33 μg/mL (r^2: 0.9946), 4.02 μg/mL (r^2: 0.9344) and 3.75 μg/mL (r^2: 0.9404), respectively. The scavenging efficacy of galanga extracts and standards on the ABTS$^{\bullet+}$ increased in the following order: BHA $\approx$ BHT > trolox > WEEG > α-tocopherol > EEG > WEG. As well as in DPPH free radical scavenging activity, a lower EC$_{50}$ value indicates a higher ABTS$^{\bullet+}$ scavenging activity galanga extracts and positive controls.

Another improved technique for the determination of radical scavenging is ABTS$^{\bullet+}$ scavenging activity (Gülçin et al., 2010a). Generation of ABTS$^{\bullet+}$ defined here includes direct production of blue/green ABTS$^{\bullet+}$ chromophore as a result of the reaction between ABTS and K$_2$S$_2$O$_8$. ABTS$^{\bullet-}$, the oxidant, was produced by the agency of K$_2$S$_2$O$_8$ oxidation of ABTS^{2-} and radical cation is calculated spectrophotometrically (Gülçin et al., 2010b). This is a direct production of a steady form of radical to create a blue-green ABTS$^{\bullet+}$ chromophore before the reaction with antioxidants (Gülçin et al., 2009). ABTS$^{\bullet+}$ cation can be prepared by running distinct oxidants like permanganate (MnO$_4^-$), chromate (CrO$_4^{2-}$) and perchlorate (ClO$_4^-$). In this sense, the oxidizing agent can be called an oxygenation reagent or oxygen-atom transfer agent. Results acquired using K$_2$S$_2$O$_8$ as oxidant show that the occurrence of K$_2$S$_2$O$_8$ increases the ratio of ABTS$^{\bullet+}$. These radicals were generated in ABTS/K$_2$S$_2$O$_8$ system (Ak and Gülçin, 2008).

On the other hand, galanga extracts had also effective Fe^{2+} ions chelating effect. The distinction between different concentrations
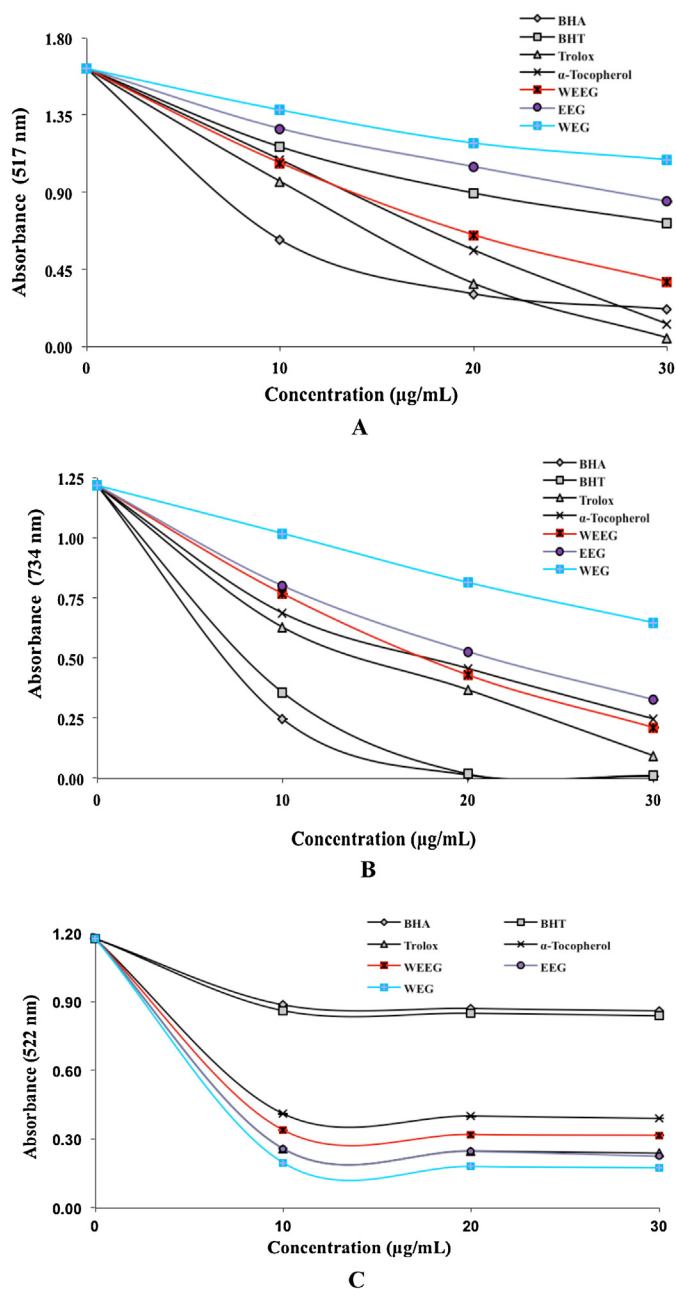

Fig. 3. (A) DPPH free radical scavenging activity of different concentrations (10–30 μg/mL) of galanga (*Alpinia officinarum* Hance) rhizome extracts and reference antioxidants. (B) ABTS radical scavenging activity of different concentrations (10–20 μg/mL) of galanga extracts and reference antioxidants. (C) Fe^{2+} chelating activity of galanga extracts and standard antioxidant compounds [BHA, Butylated hydroxyanisole, BHT, Butylated hydroxytoluene; DPPH$^{\bullet}$, 1, 1-diphenyl-2-picrylhydrazyl free radical, ABTS$^{\bullet+}$, 2, 2′-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid); WEG, water; EEG, ethanol and WEEG, water/ethanol (50:50 v/v) extracts].

of galanga extracts (10–30 μg/mL) and the control value was fixed to be statistically important ($p < 0.01$). Furthermore, it is found that IC$_{50}$ values for WEEG, EEG and WEG were found as 12.38, 10.05 and 8.56 μg/mL, respectively (Fig. 3C and Table 4). Whereas, IC$_{50}$ values belonging to Fe^{2+} ions chelating capacity of positive controls like trolox, α-tocopherol, BHT and BHA was found to be 10.35, 14.75, 49.51 and 53.32 μg/mL, respectively. A lower EC$_{50}$ value reflects a higher Fe^{2+} ions binding activity. These results clearly introduce that Fe^{2+} ions chelating effect of galanga extracts was parallel to trolox, α-tocopherol, BHA and BHT ($p > 0.05$). Fe^{2+} ions are the most efficient pro-oxidants in pharmacology systems and

Table 4
Determination of half maximal concentrations (IC$_{50}$) of taxifolin and standard radical scavenger belonging to Fe^{2+}chelating, DPPH$^•$, ABTS$^•$, scavenging and AChE inhibition assays in WEG, water; EEG, ethanol; and WEEG, water/ethanol (50:50 v/v) extracts of galanga (*Alpinia officinarum* Hance) rhizome].

Antioxidants	Fe^{2+} chelating		DPPH$^•$ scavenging		ABTS$^{•+}$ scavenging		AChE inhibition	
	IC$_{50}$	R^2	IC$_{50}$	R^2	IC$_{50}$	R^2	IC$_{50}$	R^2
BHA	53.319	0.5412	9.36685	0.9466	3.74674	0.9404	–	–
BHT	49.5105	0.5194	24.7553	0.9879	4.02993	0.9344	–	–
α-Tocopherol	14.7478	0.4828	9.62704	0.8966	13.3298	0.9946	–	–
Trolox	10.3455	0.4862	7.00149	0.8975	9.00191	0.9453	–	–
WEEG	12.3776	0.4901	14.7478	0.9948	12.3776	0.9878	0.25521	0.9955
EEG	10.0456	0.5239	31.5067	0.9973	16.1197	0.9870	0.273971	0.9985
WEG	8.55737	0.5007	49.5105	0.9787	33.007	0.9955	2.100446	0.9902
Tacrine	–	–	–	–	–	–	1.03765	0.9922

food (Balaydın et al., 2010). Ferrozine can create complexes with Fe^{2+}. In the presence of Fe^{2+} chelating compounds, Ferrozine-Fe^{2+} complex formation is a broken down, resulting in a decrease in the red colour of Ferrozine-Fe^{2+} complex (Wood et al., 2006; Gülçin, 2012).

Folin–Ciocalteu reagent was used for determination of total phenolic contents in galanga (*Alpinia officinarum* Hance) rhizome extracts. The standard graph of gallic acid was drawn (r^2: 0.994) described as previously (Gülçin et al., 2008). The amount of total phenolics in galanga (*Alpinia officinarum* Hance) rhizome extracts was determined from the standard graph equation as gallic acid equivalents per 1 mg of extract (GAE/mg extract). As can be seen in Fig. 4A, 0.507, 0.531 and 0.234 μg of GAE of phenolic content were calculated from 1 mg of EEG, WEEG and WEG, respectively. On the other hand, a standard graph of quercetin was drawn for determination of total flavonoid content as quercetin equivalent (QE). The amount of total flavonoid as QE was determined by the equation obtained from this standard graph. The results obtained in Fig. 4B showed that 2.758, 0.675 and 0.437 μg of QE of flavonoid content were calculated from 1 mg of EEG, WEEG and WEG, respectively.

Polyphenols are common constituents of the human diet, with fruits and vegetables being the major dietary source of these bioactive compounds. The possible health benefits of polyphenol consumption have been suggested to derive from their antioxidant properties (Gülçin et al., 2006a,b). Polyphenols display strong antioxidant activity and various beneficial physiological functions *in vivo* and *in vitro*. Plant polyphenols have received much attention over the past decades for their diverse roles in human health issues (Köksal et al., 2009).

Different types of AChE inhibitors have been studied for the treatment of Alzheimer's disease (AD). As AChE inhibitors Rivastigmine and Galantamine are frequently used as drugs for the treatment of AD. Besides, Rivastigmine was shown to be effective of opposite from the scopolamine of consciousness disorders (Raschetti et al., 2007). It rapidly causes inhibition on AChE and BChE alone (Akıncıoğlu et al., 2014). Results obtained from these studies showed that an investigation into the mechanism of action of AChE might cause the design of inhibitors, which can be used as a therapeutic in the future. Additionally some studies showed that AChE inhibitors have different properties in terms of action mechanism, metabolism, and brain selectivity.

In the current study, we focused on the influence of galanga extracts on the inhibition kinetics of AChE. In our study, galanga extracts were investigated for their ability to inhibit AChE. According to our data, inhibitory effect of galanga extracts revealed significant elevation in the case of AChE. All galanga extracts had significantly higher AChE inhibition activity than that of putative standard AChE inhibitor like Tacrine (Imramovsky et al., 2012). Also, as can be seen in Table 4, IC$_{50}$ values were found as 0.255 μg/mL (r^2: 0.996) for WEEG, 0.274 μg/mL (r^2: 0.9985) for EEG and 2.101 μg/mL (r^2: 0.992) for WEG. On the other hand, it was reported that Tacrine as a standard compound demonstrated IC$_{50}$ values of 1.038 μg/mL.
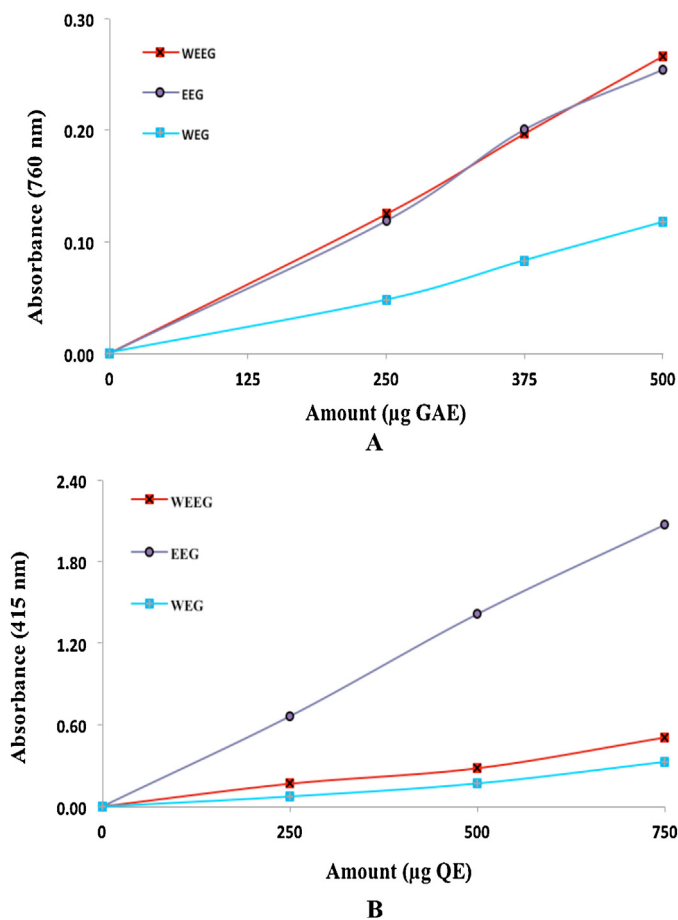

Fig. 4. (A) Total phenolic contents in galanga (*Alpinia officinarum* Hance) rhizome extracts. (B) Total flavonoid contents in EEG, ethanol, WEEG, water/ethanol (50:50 v/v) and WEG, water extracts.

As can be seen in Table 3, the results clearly showed that all galanga extracts possessed strong AChE inhibition effects.

4. Conclusion

Galanga extracts were found to be powerful antioxidant, antiradical and anticholinergic effects in different bioassays when compared to standard antioxidant and anticholinergic compounds. As discussed above, galanga extracts can be used for minimizing or preventing lipid oxidation in food or pharmaceutical products, delaying the formation of toxic oxidation products, maintaining nutritional quality, and prolonging the shelf life of food or pharmaceutical materials. On the other hand, acetylcholine is one of the key neurotransmitter for peripheral nervous system and therefore, inhibition of AChE has been proposed as a drug for

neurotoxicity. Galanga extracts had effective AChE inhibition properties and can be used for the treatment of mild-to-moderate AD and various other memory diseases.

Conflict of interests

The authors declare that they have no any conflict of interests.

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