

# Characterization of gingerol analogues in supercritical carbon dioxide (SC CO<sub>2</sub>) extract of ginger (*Zingiber officinale*, R.)

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**Abstract** Organically grown ginger rhizome (*Zingiber officinale* Roscoe) SC CO<sub>2</sub> extract obtained at 280 bar and 40 °C and its column chromatographic fractions are characterised for its composition. The components in the extract and fractions are identified by HPLC and LC based MS and are used as standard for the estimation of gingerol analogues in the extract. HPLC and mass analysis of the extracts confirmed the various forms of gingerol constituents [4]-, [6]-, [10]-gingerols and [6]-, [8]-, [10]-shogaols in ginger extracts. SC CO<sub>2</sub> extract of organic ginger was found to show 6-gingerol around 25.97 % of total extract. The estimation of [6]-gingerol, [6]-shogaols, [4]gingerol, [10]-gingerol and 6-gingediol content of the SC CO<sub>2</sub> purified ginger extract was found to be 75.92±1.14, 1.25±0.04, 4.54±0.04, 13.15±0.30 and 0.37±0.00 % respectively. Antioxidant activity was measured by 2, 2-diphenyl-1-picryl-hydrazyl (DPPH) free radical scavenging and ferric reducing antioxidant power (FRAP) and the assay have shown 652±0.37 mg TE/g and 3.68±0.18 mg TE/100 g respectively, are significantly higher results with SC CO<sub>2</sub> organic ginger extract. Paradol analogues are not detected in this study. Small quantities of [4]-, [10]gingediol and [6]-gingediacetate are also found in ginger extract.

**Keywords** Organic ginger · SC CO<sub>2</sub> ginger extract · [6]-Gingerol · Shogaols · Total phenolic content · Antioxidant activity

## Introduction

Ginger (*Zingiber Officinale Rascoe*) rhizome has been used since ancient times for its flavouring and medicinal properties,

as an aid to digestion and as a carminative. Ginger is a household remedy for dyspepsia, flatulence, colic and diarrhoea (Yai 1991). Gingerols that occurs in the species, has been found to possess various pharmacological and physiological effects including antioxidant (Masuda et al. 2004), anti-inflammatory, analgesic (Young et al. 2005), antipyretic, gastroprotective (Suekawa et al. 1984), cardiotoxic (Kobayashi et al. 1987), antihepatotoxic (Hikino et al. 1985), antifungal (Ficker et al. 2003), antinuclear, antiemetic (Kawai et al. 1994) and antischistosomal activities (Chen and Ho 1988). In addition, [6]-gingerol has been known to suppress cytokine formation (Phan et al. 2005) and promote angiogenesis (Kim et al. 2005). The phytochemical, pharmacological and physiological properties of ginger are well reviewed (Ali et al. 2008). A study has confirmed that the spices derived from plants exert their anticancer properties through the suppression of NF-κB (Nuclear Factor-kappa B) (Aggarwal and Shishodia 2006). [6]-gingerol is a natural chemopreventive agent that has been found to be potent inhibitor of NF-κB (Kim et al. 2004).

The gingerols are responsible for the pungency of ginger; they are homologous series of phenolic ketones known as gingerols, shogaols and paradols. Shogaols and paradols are even more pungent than the gingerols is virtually absent in fresh ginger and is derived from gingerols during thermal processing or long term storage (Zhang et al. 1994). Shogaols are gingerol analogues with a 4, 5 double bond, resulting from elimination of 5-hydroxy group. Paradols are formed by hydrogenation of the corresponding shogaol. [6]-Gingerol (5-hydroxy-1-(4'-hydroxy3'-methoxyphenyl)-3-decanone) is one of the most abundant constituent in fresh ginger but it found to decrease during thermal processing and post harvest storage and shogaols even more pungent than gingerols, is virtually absent in fresh ginger and is derived from gingerol during long term post harvest storage (Zhang et al. 1994).

Ginger contains number of different pungent and active ingredients mainly gingerols (Govindarajan 1982a, b) are homologous series of phenolic ketones known as ginger

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constituents such as [4]-, [6]-, [8]-, [10]- and [12]-gingerols (Kikuzaki et al. 1994). SC CO<sub>2</sub> extraction is a best method to extract the gingerol constituents from fresh ginger without the drawbacks of the chemical solvent extraction and SC CO<sub>2</sub> extraction can be considered to result in products that are near natural for food applications (Diaz-Reinoso et al. 2006). The pressure and temperature dependent can fractionate rich compositions of non-polar components, essential oils and isoprenoids individually (Udaya Sankar 1989; Yonei et al. 1995). In addition CO<sub>2</sub> has a low surface tension, viscosity and high diffusivity (Roy et al. 1996) that help in faster mass transfer rates. Very few reports available on supercritical carbon-dioxide extraction, estimation and characterization of gingerol (Yai 1991).

In the present work, organically grown ginger is used for carbon dioxide extraction. The agriculture practice of organic production results in superior produce/products than that were grown with intervention of fertilizers and there is also a need to characterize the composition of the same (Aldrich 1981; Siderer et al. 2005). In the present study, SC CO<sub>2</sub> extracts of organic ginger are fractionated on silica gel column chromatography for the isolation, characterization of compounds; the gingerol constituents estimated by HPLC (Chen et al. 1986; Wood 1987) LC based mass chromatography has been used to analyse the ginger analogues (Hiserodt et al. 1998) and the extracts and the fractions are evaluated for DPPH radical scavenging, ferric reducing antioxidant power (FRAP) to evaluate the antioxidant activity. Total phenolic contents had been determined by the Folin–Ciocalteu method.

## Materials and methods

**Chemicals** Organically grown ginger SC CO<sub>2</sub> extract was obtained from M/s South East Agro India Limited, Mysore and SC CO<sub>2</sub> extraction of ginger (origin, Assam, India) was carried out at a pressure of 280 bar at 40 °C in a commercial SCF CO<sub>2</sub> extraction plant of capacity 2×200 lit (M/s Natex, Austria) on 90 kg of ginger powder. Analytical grade reagents hexane and diethyl ether procured from Merck Ind.Ltd. All the solvents distilled once before use. Folin–Ciocalteu reagent from Loba chemicals Ind Ltd., TPTZ (2,4,6-tripyridyl-S-triazine), DPPH (2,2'-diphenyl-1-picryl-Hydrazyl), Gallic acid and Trolox (6-Hydroxy-2,5,7,8-tetramethyl chroman-2-carboxylic acid) from Sigma USA. Sodium carbonate, ferric chloride hexa hydrate and sodium acetate are from Rankem Fine Chemicals India Ltd., glacial acetic acid from SD fine chemicals India Ltd.

**Column chromatographic fractionation of ginger extract** Supercritical fluid carbon dioxide extract (400 mg) was fractionated on silica gel column to obtain fractions by eluting with

different proportions of (successively) n-hexane: diethyl ether (80:20) 77 mg I (19.2 %), (50:50) 158 mg II (39.5 %), (40:60) 138 mg III (34.5 %) and (20:80) 20 mg (5 %) after evaporating to dryness. These fractions were characterized for their compositions by HPLC and LCMS.

**High performance liquid chromatography** The ginger fractions which were purified on silica gel column chromatography were subjected to high performance liquid chromatography in both gradient and isocratic mode of elution was used for identification and quantification of gingerols, while gradient was used for identification of components, using HPLC grade (A) water and (B) acetonitrile. The gradient elution had the following profile: 0–8 min, 45–50 % B; 8–15 min, 50–55 % B; 15–40 min, 55–90 % B; 40–45 min, 90–45 % B; 45–55 min, 45–45 % B. For isocratic (A) water and (B) acetonitrile (55:45 v/v), equipped with a reverse phase column C18 (250×4.6 mm), flow rate 1 ml/min. UV detector set at 282 nm. The compounds were identified and quantified based on retention time. HPLC measurements will be ±5 %. HPLC analyzed gingerols fractions were characterized by mass to determine the individual constituents.

**Preparative HPLC** Purified fractions from column chromatography were subjected to preparative HPLC, of stationary phase column Pursuite ERS C18 (250×21.2 mm) particle size, 10 μm, flow rate 5 ml/min UV detector 282 nm, fractions (10 %) in acetonitrile injected 500–1,000 ml, isocratic mode acetonitrile: water (55:45 v/v), the fractions eluted were collected, separately, pooled and evaporated under N<sub>2</sub> to obtain purified components.

**Mass spectroscopy** The aliquots were subjected mass analysis directly on the mass head of LCMS system using mass spectrometry (Waters 2996, PDA detector and mass by MS-Q-ToF, Ultima, Mass Lynx 4.0 software) equipped with an ESI (electrospray ionization) positive mode under the following conditions. Capillary, -3.00KV; Cone, 100KV; Source temperature, 120 °C; Dissolution temperature, 300 °C; Cone gas, 50 L/h; Dissolution, Gas flow 500 L/h; scan range, m/z 100–800 with a scan speed of 1,000 amu/s. A peak threshold of 1 % identity is applied to the mass spectra.

**Total phenolic assay** Total phenolic content was determined by the Folin–Ciocalteu method adapted from Swain Hillis (1959). The different concentration of extract was made up to 150 μl using distilled ethanol. To this, 150 μl of 0.25 N Folin–Ciocalteu reagent, 2,400 μl of distilled water was added and then mixed well using vortex. Mixture was allowed to react for 3 min then 300 μl of 1 N Na<sub>2</sub>CO<sub>3</sub> solution is added and mixed well. The solution is incubated at room temperature (23 °C) in the dark for 2 h. The

absorbance was measured at 725 nm using a spectrophotometer. And the results were expressed in gallic acid equivalents (GAE; mg/100 g fresh mass) using a standard gallic acid curve. Additional dilution was done if the observed absorbance value was over the linear range of the standard curve. Error in measurements will be  $\pm 5$  %.

**Antioxidant activity measurements** Antioxidant activity was measured by DPPH method (Blois 1958) with few modifications. Three milliliter of ethanolic solution of ginger extract (35.06  $\mu\text{g}/3$  ml) and standard trolox solutions (0.1 mM) were taken in different test tubes. To this solution 1 ml of 0.4 mM ethanolic solutions of DPPH was added. The mixture was vigorously mixed in a vortex mixer for a minute and allowed to stand at 27 °C in dark for 20 min. Reagent blank was prepared as above without addition of sample or standard. Then the absorbance was taken at 517 nm. Results are expressed in mg TE/g fresh mass using standard trolox calibration curve. Error in measurements will be  $\pm 5$  %.

The FRAP assay was done according to (Guo et al. 2003) with some modifications. The stock solutions included 300 mM acetate buffer pH 3.6, 10 mM TPTZ (2,4,6-tripyridyl-triazine) solution in 40 mM HCl and 20 mM  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  solution. The fresh FRAP working solution was prepared by mixing 10:1:1 ratio respectively and then warmed at 37 °C before using. Then sample extracts in different concentrations were allowed to react with 2.9 ml of FRAP solution for 30 min in the dark condition. Readings of the colored product (ferrous tripyridyl-triazine complex) were then taken at 593 nm. The standard trolox curve was used to express results in mg TE/100 g fresh mass. Error in measurements will be  $\pm 5$  %.

## Result and discussion

In this study, the estimation of gingerol analogues from crude SC  $\text{CO}_2$  organic ginger extract as well as purified fractions was carried out. There are several disadvantages associated with GC and GC-MS methods for analyzing 6-gingerol and its analogues as the column temperature significantly affect the results due to conversion of 6-gingerol to 6-shogaol (Zhang et al. 1994). The crude ginger extract and column chromatographic fractions were subjected to HPLC both isocratic and gradient method to identify the compounds in the samples.

Identification of compounds in ginger extract:

As the gradient HPLC method showed better separation and was used for characterization and identification of the compounds. SC  $\text{CO}_2$  crude extract of ginger showed fifteen peaks with different retention time varied from 6.2 to 45.5 (Fig. 1a). We could identify the twelve compounds and

respective mass obtained using LC-MS by UV diode array detector given in the Table 1.

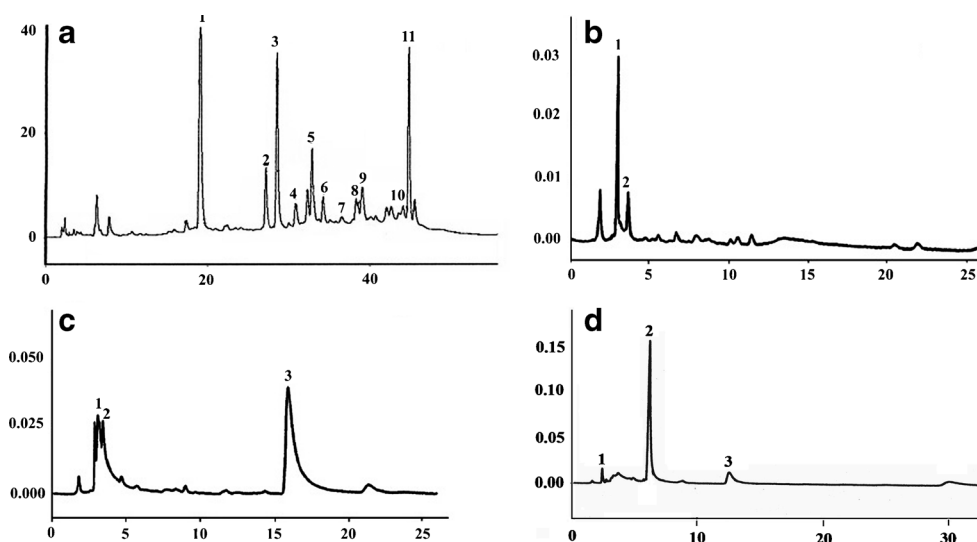
To aid the identification of the components the SC  $\text{CO}_2$  crude ginger extract was fractionated by silica gel column chromatography using n-hexane:diethyl ether with increasing polarity of 80:20 I (Fig. 1b), 50:50 II (Fig. 1c) and 40:60 III (Fig. 1d) to obtain three fractions. It has been observed that the HPLC chromatography of the fractions showed three different sets of compounds as observed from the peaks with different retention times in each of the chromatogram (Fig. 1b, c and d). It is found that the fractions eluted with n-hexane: diethyl ether (40:60 III) was found to contain only gingerols.

Both the crude SC  $\text{CO}_2$  and fractionated extract were characterized by ESI + mass spectra to identify the peaks in the HPLC. [6]-gingerol gave major fragment with 295 molecular weight. All the peaks were assigned based on the molecular mass and compounds were given Tables 1, 2 and 3 separately. Crude ginger extract was showed fewer amounts of gingerols and shogaols when compared to purified fraction. Purified fraction showed  $[\text{M} + \text{H}]^+$ ,  $[\text{M} + \text{Na}]^+$  peaks for [6]-[8]- and [10]-gingerols and  $[\text{M} + \text{H}]^+$  peaks for [10]-, [8]-shogaols with small  $[\text{M} + \text{Na}]^+$  peak for [4]-gingediol (Table 2 and Fig. 2a, b). Crude ginger extract was showed  $[\text{M} + \text{H}]^+$  peak for [6]-, [8]-shogaols and [6]-gingerol,  $[\text{M} + \text{K}]^+$  peak with the molecular weight of 391, which could be [4]gingediacetate or [10]-gingediol,  $[\text{M} + \text{Na}]^+$  peak for [6]-gingediacetate, [10]-shogaol and [4]gingediol,  $[\text{M}-\text{H}]^+$  peak for [8]-gingerol (Table 3 and Fig. 3a, b) respectively. Mass results illustrate that [8]-, [10]-shogaol exists in fresh ginger as measurable constituent. However, the occurrence of [6]-, [8]- and [10]-shogaols in the ginger extract is owing to the conversion from corresponding gingerols but are insignificant quantities as observed by HPLC. Also the mass chromatogram demonstrates that [6]-gingerol was a major constituent in purified fraction III in various forms such as  $[\text{M} + \text{H}]^+$ ,  $[\text{M} + \text{Na}]^+$  peak with low intensity of  $[\text{M} + \text{H}]^+$  peaks are also observed for [8]-, [10]-gingerols. One of the minor peaks was also identified in mass spectrum with molecular weight of 358 which could not be determined. In this study, we did not find any paradol constituents like [6]-, [8]- and [10]-paradol in both crude and purified ginger extracts.

Based on relative retention times from literature (Schwertner and Rios 2007), mass fragments by ESI-MS spectra the 4-gingerol, 6-gingediol, 6-gingerol, methyl-6-gingerol, 8-gingerol, 6-shogaol, 12-gingediol, methyl-6-shogaol, 10-gingerol, 1-gingedione and 8-gingediol were identified in the HPLC chromatogram (Table 1A).

Quantification:

[4]-gingerol, [6]-gingerol and [10]-gingerol were the major compounds found in 40:60 III fraction. These compounds were collected by preparative HPLC and confirmed by ESI-



**Fig. 1** High performance liquid chromatography of super critical carbon dioxide ginger extract and its column chromatographic fractions. **a** Ginger extract by gradient HPLC. Peak 1-[4]-gingerol, 2-[6]-gingediol, 3-[6]-gingerol, 4-methyl-6-gingerol, 5-[8]-gingerol, 6-[6]-shogaol, 7-[12]-gingediol, 8- methyl-6-shogaol, 9-[10]-gingerol, 10-[1]-gingedione and 11-[8]-gingediol. **b** HPLC (isocratic) Column

chromatographic fraction I of ginger extract. Peak 1-[4]-gingerol and 2-[6]-gingediol. **c** HPLC (isocratic) Column chromatographic fraction II of ginger extract. Peak 1-[4]-gingerol, 2-[6]-gingediol and 3-[1]-gingedione. **d** HPLC (isocratic) Column chromatographic fraction III of ginger extract. Peak 1-[4]-gingerol, 2-[6]-gingerol and 3-[10]-gingerol

MS, used for quantification by injecting different concentration in isocratic mode. The standard curve of [4]-gingerol, [6]-gingerol and [10]-gingerol was linear in the range of 500–3,000 ng. The calibration curve of [4]-gingerol, [6]-gingerol and [10]-gingerol was represented by the linear equation  $Y=13360X$  and  $R^2$  value 0.889,  $Y=22059X$  and  $R^2$  value 0.891 and  $Y=21142X$  and  $R^2$  value 0.761. This indicates [6]-gingerol content was abundant than the other constituents. The HPLC percentage area of [6]-gingerol was found to be

30–35 % in crude extract and 75–79 % in purified ginger extract. HPLC analysis also shows abundance of gingerol and shogaols in purified ginger extract. The [6]-gingerol, [6]-shogaol, [4]-gingerol, [10]-gingerol and [6]-gingediol content of the SC CO<sub>2</sub> purified ginger extract was found to be  $75.92 \pm 1.14$ ,  $1.25 \pm 0.04$ ,  $4.54 \pm 0.04$ ,  $13.15 \pm 0.30$  and  $0.37 \pm 0.00$  % ( $n=3$ ) respectively.

Estimation and biological activity measurements showed best results for purified SC CO<sub>2</sub> ginger extract than crude

**Table 1** Components of SCCO<sub>2</sub> organic ginger extract identified based on retention time and mass using gradient HPLC program (RT-Retention time, RRT-Relative Retention time with respect to 6-gingerol)

Sl.No.	RT	RRT	M+	[M + H] <sup>+</sup>	[M + Na] <sup>+</sup>	Compound name
1	6.2	0.218	–	–	–	–
2	7.737	0.272	–	–	–	–
3	17.278	0.607	–	–	–	–
4	18.933	0.665	–	266	289	4-gingerol
5	27.054	0.950	–	297	320	6-gingediol
6	28.481	1.000	–	294	317	6-gingerol
7	30.834	1.083	308	–	331	methyl-6-gingerol
8	32.257	1.133	–	–	–	–
9	32.804	1.152	–	322	–	8-gingerol
10	34.193	1.201	–	277	–	6-shogaol
11	36.475	1.281	–	403	–	12-gingediol
12	38.258	1.343	–	291	313	methyl-6-shogaol
13	39.074	1.372	–	351	373	10-gingerol
14	42.034	1.476	–	–	–	–
15	44.059	1.547	–	–	–	1-gingedione
16	44.76	1.572	–	–	347	8-gingediol
17	45.478	1.597	–	–	–	–

**Table 2** Components of the purified ginger extract as indicated in the mass spectra of Fig. 2a and b

Peak no		Mass value	Analysis	Compound	Actual molecular weight
Chromatogram in Fig. 2a	Chromatogram in Fig. 2b				
1	1	291	[M + Na] <sup>+</sup>	[4]-Gingediol	268
2	2	295	[M + H] <sup>+</sup>	[6]-Gingerol	294
3	3	305	[M + H] <sup>+</sup>	[8]-Shogaol	304
4	–	317	[M + Na] <sup>+</sup>	[6]-Gingerol	294
5	–	323	[M + H] <sup>+</sup>	[8]-Gingerol	322
6	4	333	[M + H] <sup>+</sup>	[10]-Shogaol	332
7	–	351	[M + H] <sup>+</sup>	[10]-Gingerol	350

ginger extract, confirming the [6]-Gingerol and its analogues contribute to the activity measurements. [6]-gingerol is the most abundant in the SC CO<sub>2</sub> extracts and extraction with SC CO<sub>2</sub> was the best method for obtaining bioactive compounds like gingerols without transformation to paradols. HPLC analysis of the extracts gave the various forms of gingerol constituents [4]-, [6]-, [10]-gingerols, [6]-shogaol and [6]-gingediol. Mass analysis of the extracts gave the various forms of gingerol constituents [8]-, [6]-, [10]-gingerols and [6]-, [8]-, [10]-shogaols in ginger extracts. [6]-gingerol and [6]-shogaol are found to be in maximum quantity than other gingerols and shogaols in purified ginger extract. Small quantities of [4]-, [10]-gingediol and [6]-gingediacetate were also found in ginger extract.

The carbon dioxide extraction resulted in a yield of 4.8 % of oleoresin on the basis of dried ginger rhizome with an essential oil content of 20 % in the oleoresin. The gingerol content is found to be around 34.5 % of the total extract, which is much higher than the earlier reports (11–27 %) by Govindarajan et al. (1982a, b).

Ginger extract from SC CO<sub>2</sub> as well as purified ginger fraction from column chromatography are taken into account to determine total phenolic content. Gingerol constituents showing significant amount of gallic acid equivalence

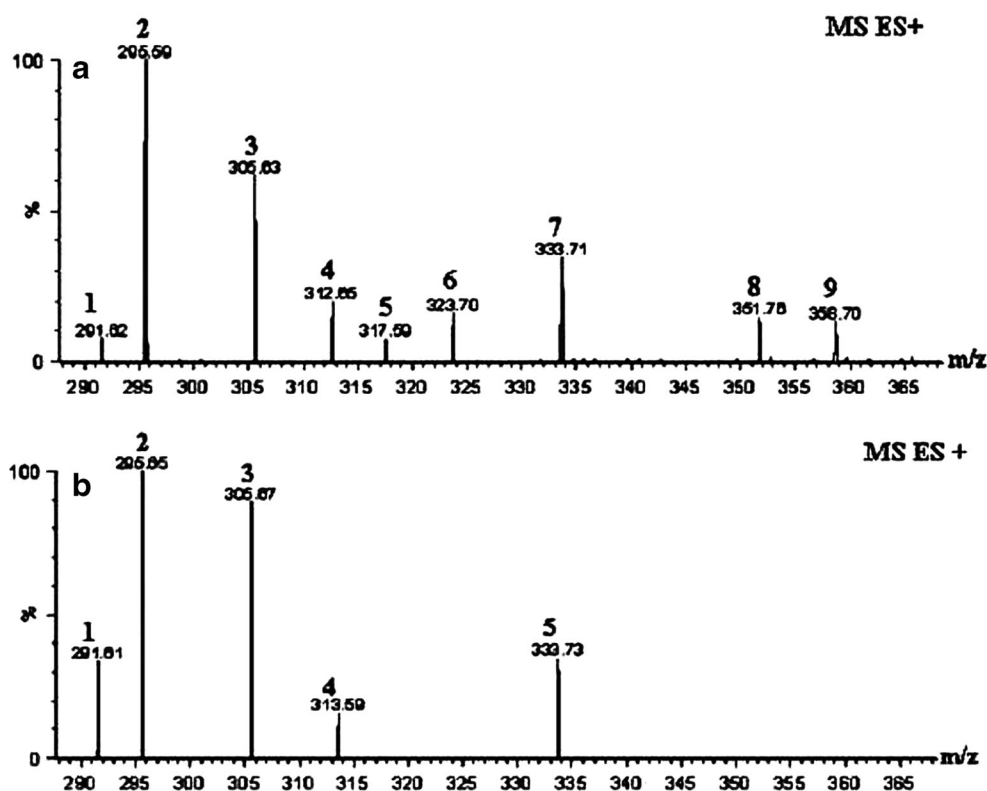
for the total phenolic activity. Total phenolic content is increased with increasing concentration of gingerol. Purified ginger extract is showing higher phenolic content due to the higher gingerol constituents. Total phenolic content of SC CO<sub>2</sub> ginger extract is found to be 46.0±2.3 g GAE/100 g and purified ginger fraction (40:60) was found to be 58.5±2.93 g GAE/100 g.

Antioxidant activity of ginger has been proposed as one of the major possible mechanisms for the protective actions of the plant and animal against toxicity and lethality of radiation (Haksar et al. 2006) and a number of toxic agents like carbon tetrachloride and cisplatin (Yemitan and Izegbu 2006) showing higher toxicity. DPPH and FRAP assays have been carried out to measure antioxidant property of ginger with various concentrations of SC CO<sub>2</sub> ginger extract and purified fraction ginger extracts. Activity results are an average of all the measurements. DPPH assay showed 415±0.3 mg TE/g for SC CO<sub>2</sub> ginger extract and 652±0.37 mg TE/g for purified fraction ginger (40:60). According to FRAP method, SC CO<sub>2</sub> ginger extract exhibits 2.67±0.13 mg TE/100 g and purified fraction ginger is 3.68±0.18 mg TE/100 g for antioxidant activity. Assay results strongly suggest that gingerol constituents have potent antioxidant power with high content of phenolic compounds. SC

**Table 3** Components of the crude ginger supercritical carbon dioxide extract as indicated in the mass spectra of the Fig. 3a and b

Peak no		Mass value	Analysis	Compound	Actual molecular weight
Chromatogram in Fig. 3a	Chromatogram in Fig. 3b				
1	–	277	[M + H] <sup>+</sup>	[6]-Shogaol	276
–	1	291	[M + Na] <sup>+</sup>	[4]-Gingediol	276
–	2	295	[M + H] <sup>+</sup>	[6]-Gingerol	294
2	3	305	[M + H] <sup>+</sup>	[8]-Shogaol	304
–	4	321	[M-H] <sup>+</sup>	[8]-Gingerol	322
3	5	333	[M + Na] <sup>+</sup>	[10]-Shogaol	332
4	–	391	[M + K] <sup>+</sup>	[4]-Gingediacetate Or [10]-Gingediol	352
5	–	403	[M + Na] <sup>+</sup>	[6]-Gingediacetate	380

**Fig. 2** Mass chromatograms of purified ginger extract showing gingerol constituents such as gingerols, shogaols and gingediols with different intensity of peaks

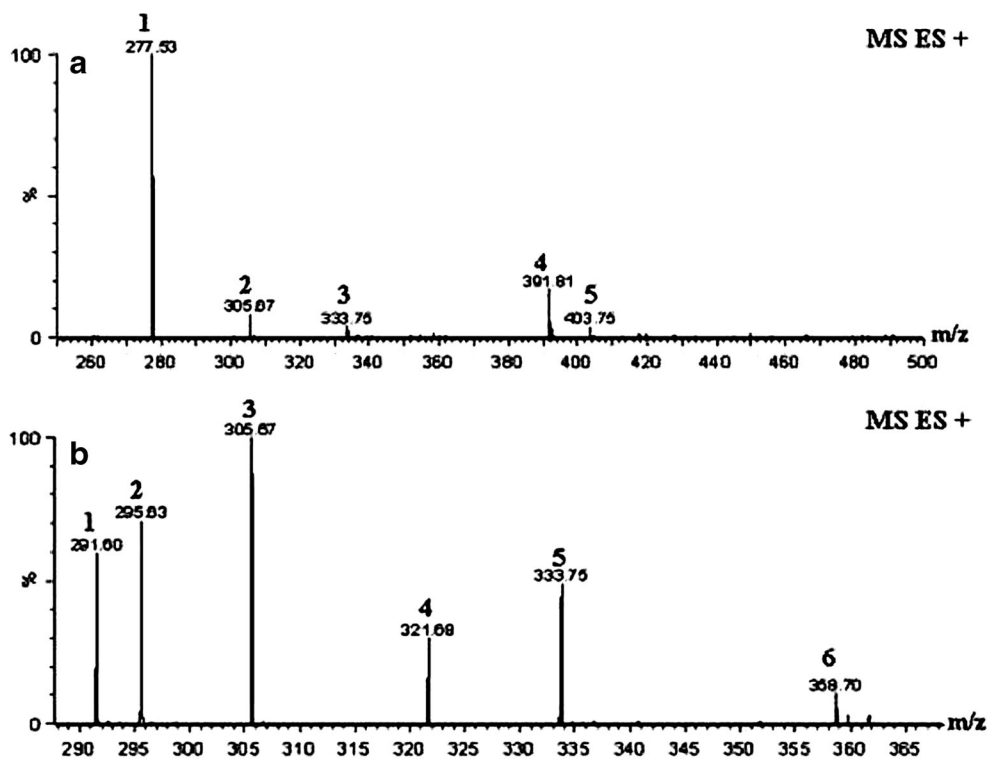


CO<sub>2</sub> fractionated ginger extract showed the antioxidant activity significantly higher than that was reported earlier (Puengphian and Sirichote 2008). Successfully we could extract high bioactive compounds using SC CO<sub>2</sub> with high [6]-gingerol content (> 30 %).

### Conclusion

HPLC determination of 6-gingerol and its analogues has confirmed no significant transformation of gingerols occur in the extracts obtained by supercritical carbon dioxide and

**Fig. 3** Mass chromatogram of the crude ginger supercritical carbon dioxide ginger extract showing gingerol constituents such as gingerols, shogaols, [4]-gingediacetate, [6]-gingediateacte and gingediols with different intensity of peaks



occurrence of higher amount of gingerols are reported as estimated by HPLC as high as 40 % in oleoresin with 6-gingerol content of 30–35 % in the extract. The 6-gingerol content in the gingerol fraction is found to be 79–82 %. Several different components of gingerols are identified and the antioxidant activity of the gingerols is quite significant as measured by DPPH and FRAP assays.

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