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Analytical Methods

RP-HPLC analysis of phenolic antioxidant compound 6-gingerol from different ginger cultivars

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ABSTRACT

The aim of this work was to assess the antioxidant capacity and phenolic content from the rhizomes of 12 ginger cultivars from different agro climatic zones of India. The quantities of phenolic compound 6-gingerol was determined with reverse phase high performance liquid chromatography (RP-HPLC) which was ranging from 0.1% to 0.2%. The antioxidant capacity was determined using DPPH (1,1-diphenyl-2-picrylhydrazyl) and FRAP (ferric-reducing antioxidant power) assays. The rhizome of Rajasthan and Rio De Janero cultivars were good sources for these compounds among the cultivars examined. The cultivars with high 6-gingerol content had strongest free radical scavenging activities and this was also supported by statistically high significant correlation between the two ($R^2 = 0.850$, $P < 0.001$).

1. Introduction

Ginger (*Zingiber officinale* Roscoe) has been used as a spice for over 2000 years (Bartley & Jacobs, 2000). It is cultivated in many tropical and subtropical countries including China, India, Nigeria, Australia, Jamaica and Haiti. Among which, China and India are the world's leading producers of ginger (Blumenthal, Goldberg, & Brinckmann, 2000). Several cultivars of ginger are grown in different ginger growing areas in India which were named after the localities from where they are cultivated or collected. The ginger has been increasingly used recently because of its low toxicity and its broad spectrum of biological and pharmacological applications, *viz.* antitumor, antioxidant, anti-inflammatory, anti-apoptotic, cytotoxic, anti-proliferative and anti-platelet activities (Sekiwa, Kubota, & Kobayashi, 2000; Shukla & Singh, 2007; Wei, Ma, Cai, Yang, & Liu, 2005; Young et al., 2005). Ginger rhizome contains rich source of pungent bioactive principles of great importance, which has been recognised long before. These substances, which are phenolic ketones include the gingerols as well as the shogaols, which exist as a series of homologues ([4], [6], [8], and [10] gingerols and shogaols) with a range of unbranched alkyl chain lengths (Harvey, 1981; He, Bernart, Lian, & Lin, 1998). For direct analysis of either gingerols or shogaols, HPLC conducted on a reversed-phase column (RP-18) turned out to be the best method (Baranowski, 1985; Steinegger & Stucki, 1982).

According to Connell and Sutherland (1969) and Leverington (1975) the main pungent principles extracted from the rhizomes were 6-gingerol, 8-gingerol, and 10-gingerol, and in terms of pungency 6-gingerol was the most pungent compounds (Govindarajan, 1982). The quantity and quality of the polyphenols present in plant foods can vary significantly due to different factors, such as plant genetics and cultivar, soil composition and growing conditions, maturity state, and post harvest conditions, among others (Jaffery et al., 2003). The aim of present study was to quantify the 6-gingerol among 12 different ginger cultivars, by using RP-HPLC, and to evaluate the antioxidant activity of each cultivar by the DPPH (2,2-diphenyl-1-picrylhydrazyl) and FRAP (ferric-reducing antioxidant power) method, in order to compare and differentiate cultivars as valuable sources of antioxidant compounds.

2. Materials and methods

2.1. Extract preparation

Rhizomes were freshly procured from field, washed and blotted to dry with tissue towel and dried under oven for 60 ± 5 °C. After drying, rhizomes were pulverized and passed through a 40 mesh sieve before extraction. One gram of powder from each treatment was dissolved in 25 ml methanol and sonicated for 30 min. The mixtures were centrifuged at 10,000 rpm for 10 min and the

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supernatant was filtered through Whatman filter paper (No. 1). All the extracts were kept at 4 °C and extracts were diluted prior to use for different assays. Extracts of ginger were filtered through a 0.45 mm nylon filter into an Agilent wide opening amber vial for HPLC analysis.

2.2. Procurement of samples and chemicals

Rhizomes of gingers were obtained from different agro-climatic regions of India. 6-Gingerol (above 99.0% purity) was obtained from Sigma–Aldrich. Acetonitrile and methanol (Fisher, USA) were of HPLC grade. Water for HPLC analysis was purified with a Milli-Q water system (Millipore Corp., Bedford, MA, USA). Calibrated analytical balance accurate to ±0.01 mg flask, volumetric, class A, assorted sizes vials, chromatography with cap. Filters: 0.45 μm, PVDF or Nylon Sonicator.

2.3. Instrumentation and chromatographic conditions

Waters HPLC system (Waters/Millipore, Milsford, MA, USA) consisting of a model 515 pump, a model 2487 dual wavelength absorbance detector was used. The separation of the TC extract was conducted in a C18 column (Delta Pak), 5 μm, and 3.9 × 150 mm, 300 Å. A mobile phase consisting of A (water) and B (acetonitrile) was used for separation, and the gradient range varied linearly from 50% to 90% B in 4 min with injection volume 2 ml for the RRHT column. The flow rate was 1.0 ml/min, the column temperature was maintained at 30 °C, and the detection wavelength of the diode array detector (DAD) was set at 280 nm. In addition, the analysis time of the conventional analytical column was 11 min with the same rate of gradient, and the injection volume was 20 μl.

For calibration and linearity, 6-gingerol was accurately weighed and dissolved in methanol to produce stock standard solutions. The stock solutions were serially diluted to prepare working solutions for the calibration curves at five concentration levels. All the solutions were stored in amber glass bottles at 4 °C. The calibration curves for the gingerol with the RRHT column were established by the peak areas and concentrations of working solutions.

2.4. System suitability

The system suitability test was assessed by six replicate injections of the standard solutions at a certain concentration. The peak area of the 6-gingerol was used to evaluate repeatability of the proposed method, and their peaks were analysed for resolution and tailing factors. According to the Chinese Pharmacopoeia, the relative standard deviations (RSD) of the peak areas were used as the indicators of repeatability, and the acceptance criterion were within 2.0%. The resolutions between peaks of interest and their adjacent peaks were greater than 1.5 and the tailing factors of peaks were between 0.95 and 1.05.

2.5. Quantification of total phenolic content (TPC)

Total phenolic content was quantified using modified Folin–Ciocalteu method described by Wolfe, Wu, and Liu (2003). The assay mixture was prepared using 0.125 ml different concentrations of standard Tannic acid with 0.250 ml of Folin–Ciocalteu reagent, 1.25 ml of distilled water and incubated for 10 min in dark. After 10 min 1 ml 7% aq. sodium carbonate and 1 ml of distilled water was added and the reaction mixture was incubated in dark for 90 min at 37 °C. The absorbance of blue colour was read at 760 nm using distilled water instead of std. tannic acid in the reaction mixture as blank on double beam spectrophotometer. Similarly, extracts prepared were also quantified and the results were compared to the standard curve of above standards and expressed as mg/tannic or equivalent per gram dry powder for the samples under study.

2.6. Quantitative determination of total flavonoid content

Total flavonoid contents in all the above extracts were determined using method given by Luximon-Ramma, Bahorum, Soobrattee, and Aruoma (2002). One percentage of plant extract (1.5 ml) was taken for the determination of total flavonoids. To this, 1.5 ml of 2% aluminium chloride in methanol was added. The reaction mixture was incubated for 10 min at room temperature. The OD was measured at 368 nm against 2% $AlCl_3$, as blank. The OD measurements were compared to standard curve of quercetin (a standard flavonoid) concentrations and expressed as milligrams of quercetin equivalent per gram dry weight of ginger.

2.7. Antioxidant activity: DPPH (2,2-diphenyl-1-picrylhydrazyl) assay

The antioxidant activities were determined as the measure of radical scavenging using DPPH assay as determined by Brand-Williams, Cuvelier, and Berset (1995). Three millilitres of methanolic solution of DPPH (25 ppm) was mixed with 20 μl of plant extract and the mixture was incubated for 30 min in dark. The absorbance at 515 nm was measured using methanol as blank. Similarly, different concentration of ascorbic acid was used instead of plant extract as reference standard during the experiment. The inhibition percentage of DPPH (% DPPH) was calculated and the results were expressed as ascorbic acid equivalent antioxidant capacity (AEAC) as per method described by Gil, Tomas-Barberan, Hess-Pierce, Holcroft, and Kader (2000).

2.8. Antioxidant activity: ferric-reducing antioxidant power (FRAP)

The ferric reducing/antioxidant power (FRAP) assay was used to measure the total antioxidant power of ginger extracts. In the FRAP assay, reductants (antioxidants) in the sample reduce Fe^{3+}/tripyridyltriazine complex, present in stoichiometric excess, to the blue colored ferrous form, with an increase in absorbance at 593 nm. The ΔA is proportional to the combined (total) ferric reducing/antioxidant power (FRAP value) of the antioxidants in the sample. Antioxidant activity assays were performed by the method described by Benzie and Strain (1996). The results were expressed as ascorbic acid equivalent antioxidant capacity (AEAC).

2.9. Statistical analyses

Statistical analyses were conducted using Graphpad. Analysis of variance (ANOVA) was done by repeated measures analysis of variance (Tukey–Kramer multiple comparisons tests). Pearson's correlation coefficients were performed to compare the data. All determinations were done at least in triplicate and all were averaged. The confidence limits used in this study were based on 95% ($P < 0.05$).

3. Results and discussion

3.1. RP-HPLC analysis of 6-gingerol

The calibration curve of the standard chromatogram (Fig. 1a) was constructed with the correlation coefficients (R^2) above 0.9975. The results of the regression equations was $y = 3.65e + 004X + 3.44e + 004$. The result by linear regression analysis showed a very good linear relationship between peak area and concentration. HPLC method has been employed for the estimation of

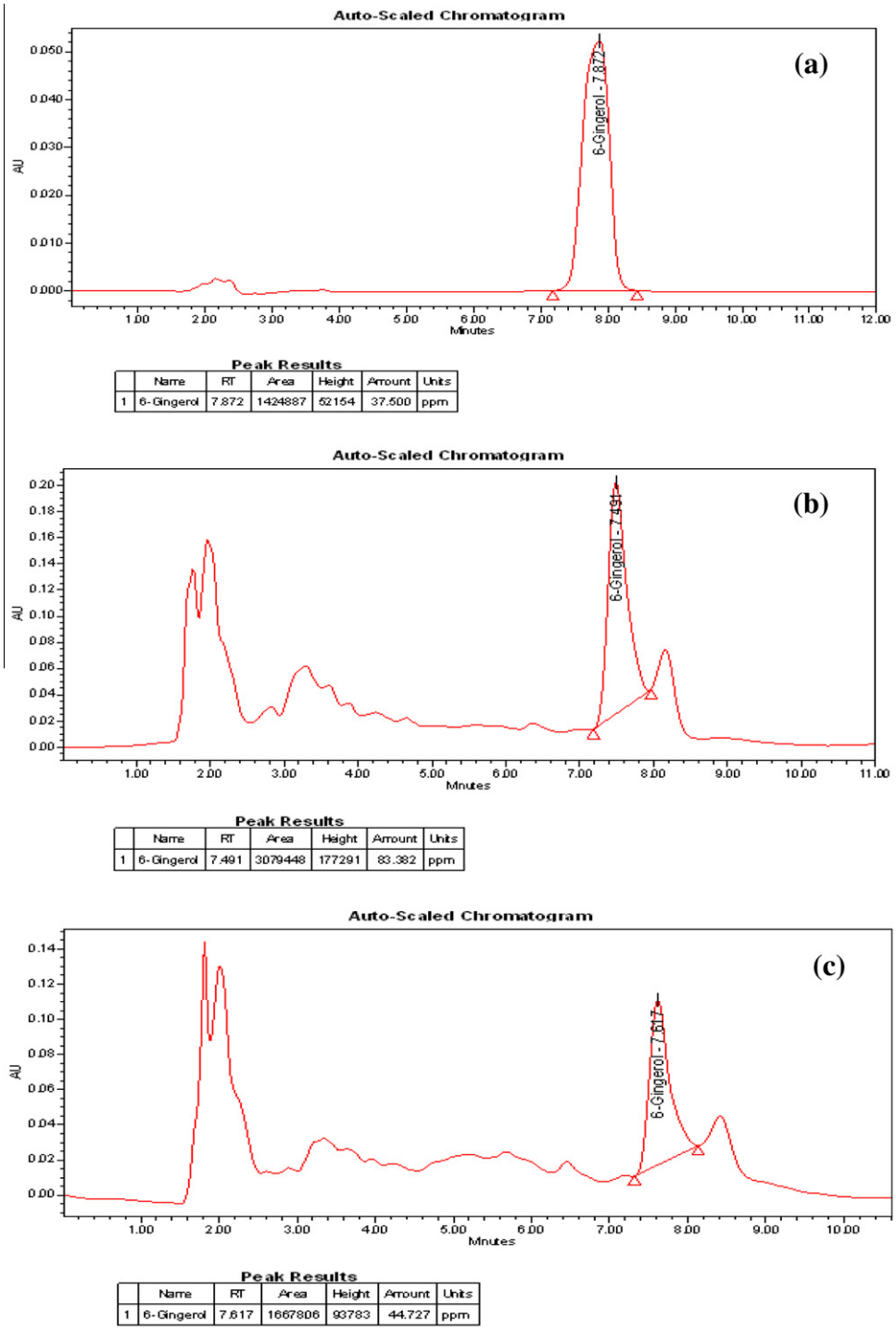

Fig. 1. (a) RP-HPLC profile of standard 6-gingerol (37.50 ppm); (b) profile of Rajasthan cultivar of ginger (83.38 ppm = 0.208%, highest 6-gingerol content cultivar); and (c) profile of Udaipur cultivar of ginger (44.73 ppm = 0.117%, lowest 6-gingerol content cultivar).

6-gingerol from different cultivars of ginger. The 6-gingerol content, was calculated by using the standard calibration curve (R^2 = 0.9975). The content of 6-gingerol for the ginger cultivars ranged from 0.117% in Udaipur to 0.208% in Rajasthan (Fig. 1b and c and Table 1). The results were in accordance with work carried out by Xiang et al. (2008), wherein they determined 0.39% 6-gingerol in dried ginger, 0.10% in baked ginger and 0.19% in fresh ginger using HPLC method. Hawlader, Conrad, and Tian (2006) determined 6-gingerol content (0.6%) by using normal air drying method. Determination of gingerol by LC–MS from raw herb and dried aqueous extract was done by Samiuela, Cheang, Clyton, Thuy, and Alan (2007). During determination of 6-gingerol, sonication extraction and methanol solvent showed maximum yield. 6-Gingerol content in dried aqueous extract was 0.18% while raw herb extract had 0.93%. Zachariah, Sasikumar, and Ravindran (1993) evaluated germplasm for oleoresin content and found range for 6-gingerol content in between 0.3% and 0.7%.

3.2. Total phenolic and flavonoid content

The antioxidant activity and total phenolic contents vary considerably among cultivars. Total phenolic contents were determined

Table 1
Total phenolic, flavonoid and 6-gingerol content in % and antioxidant activity in mM ascorbic acid equivalent of ginger cultivars.

Varieties	6-Gingerol	Phenolic	Flavonoid	DPPH	FRAP
Rio	0.208	1.344 ± 0.004	0.384 ± 0.000	1.827 ± 0.021	4.489 ± 0.001
Rajasthan	0.208	1.588 ± 0.007	0.382 ± 0.000	1.788 ± 0.000	4.817 ± 0.000
Uttar Pradesh	0.174	1.151 ± 0.001	0.333 ± 0.000	1.489 ± 0.012	3.729 ± 0.002
Maran	0.173	1.199 ± 0.002	0.309 ± 0.001	1.390 ± 0.014	3.730 ± 0.000
Chhattisgarh	0.174	1.215 ± 0.000	0.342 ± 0.000	1.453 ± 0.000	3.906 ± 0.002
Assam	0.184	1.249 ± 0.003	0.340 ± 0.001	1.526 ± 0.058	4.212 ± 0.002
Sagar	0.197	1.264 ± 0.004	0.350 ± 0.001	1.503 ± 0.003	4.281 ± 0.000
Wynad	0.142	1.021 ± 0.001	0.219 ± 0.000	1.426 ± 0.023	3.090 ± 0.004
Nadia	0.155	1.078 ± 0.002	0.294 ± 0.000	1.404 ± 0.021	3.217 ± 0.003
Udaipur	0.117	0.886 ± 0.001	0.202 ± 0.000	1.193 ± 0.029	3.161 ± 0.003
Himachal Pradesh	0.125	1.056 ± 0.001	0.261 ± 0.000	1.137 ± 0.000	3.236 ± 0.000
Satara	0.165	1.240 ± 0.002	0.334 ± 0.000	1.493 ± 0.003	2.731 ± 0.003

Table 2
Similarity matrix of ginger cultivars.

Cultivars	Satara	Sagar	HP	Rajasthan	Chattisgarh	Udaipur	Maran	Rio	Assam	UP	Nadia	Wynad
Satara	100.00	87.96	90.17	80.88	90.36	89.03	90.79	83.89	88.51	91.43	93.49	93.39
Sagar	87.96	100.00	86.73	92.75	96.55	84.52	94.48	95.86	99.14	95.03	89.47	87.42
HP	90.17	86.73	100.00	**79.68**	90.13	96.76	92.19	82.68	87.28	91.64	96.91	95.49
Rajasthan	80.88	92.75	**79.68**	100.00	89.33	**77.52**	87.29	96.41	92.19	87.83	82.36	80.35
Chattisgarh	90.36	96.55	90.13	89.33	100.00	87.90	97.93	92.42	97.09	97.94	92.89	90.82
Udaipur	89.03	84.52	96.76	**77.52**	87.90	100.00	89.95	80.50	85.07	89.41	94.97	95.81
Maran	90.79	94.48	92.19	87.29	97.93	89.95	100.00	90.36	95.05	98.73	94.75	92.33
Rio	83.89	95.86	82.68	96.41	92.42	80.50	90.36	100.00	95.29	90.91	85.39	83.36
Assam	88.51	99.14	87.28	92.19	97.09	85.07	95.05	95.29	100.00	95.59	90.02	87.97
UP	91.43	95 03	91.64	87.83	97.94	89.41	98.73	90.91	95.59	100.00	94.41	92.34
Nadia	93.49	89.47	96.91	82.36	92.89	94.97	94.75	85.39	90.02	94.41	100.00	97.56
Wynad	93.39	87.42	95.49	80.35	90.82	95.81	92.33	83.36	87.97	92.34	97.56	100.00
Colour index												
Range		100		<100–95		<95–90		<90–85		<85–80		<80

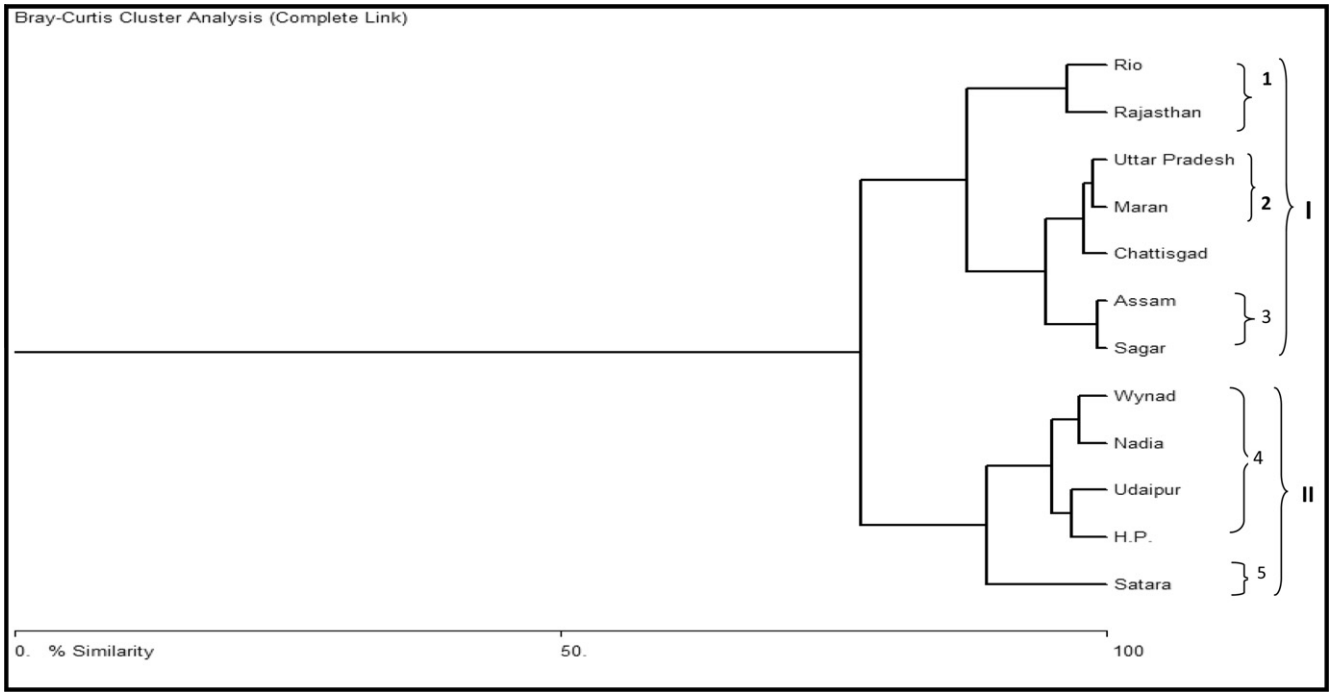

Fig. 2. Dendrogram of ginger cultivars.

using Folin–Ciocalteu reagent and were expressed as tannic acid equivalent per gram. The total phenolic contents, calculated using the standard curve of tannic acid (R^2 = 0.999), ranged from 0.7 to 1.58 gm tannic acid eq./100 g of dry weight. Total phenolic content for the ginger cultivars were highest in Rajasthan 1.58 g while 0.886 g tannic acid eq./100 g of dry weight in Udaipur was the lowest (Table 1). Shamina, Zachariah, Sasikumar, and George (1997) determined the variability in total phenols in ginger using 25 cultivars.

The total flavonoid contents of the samples derived from 12 different ginger varieties were expressed as quercetin equivalent per

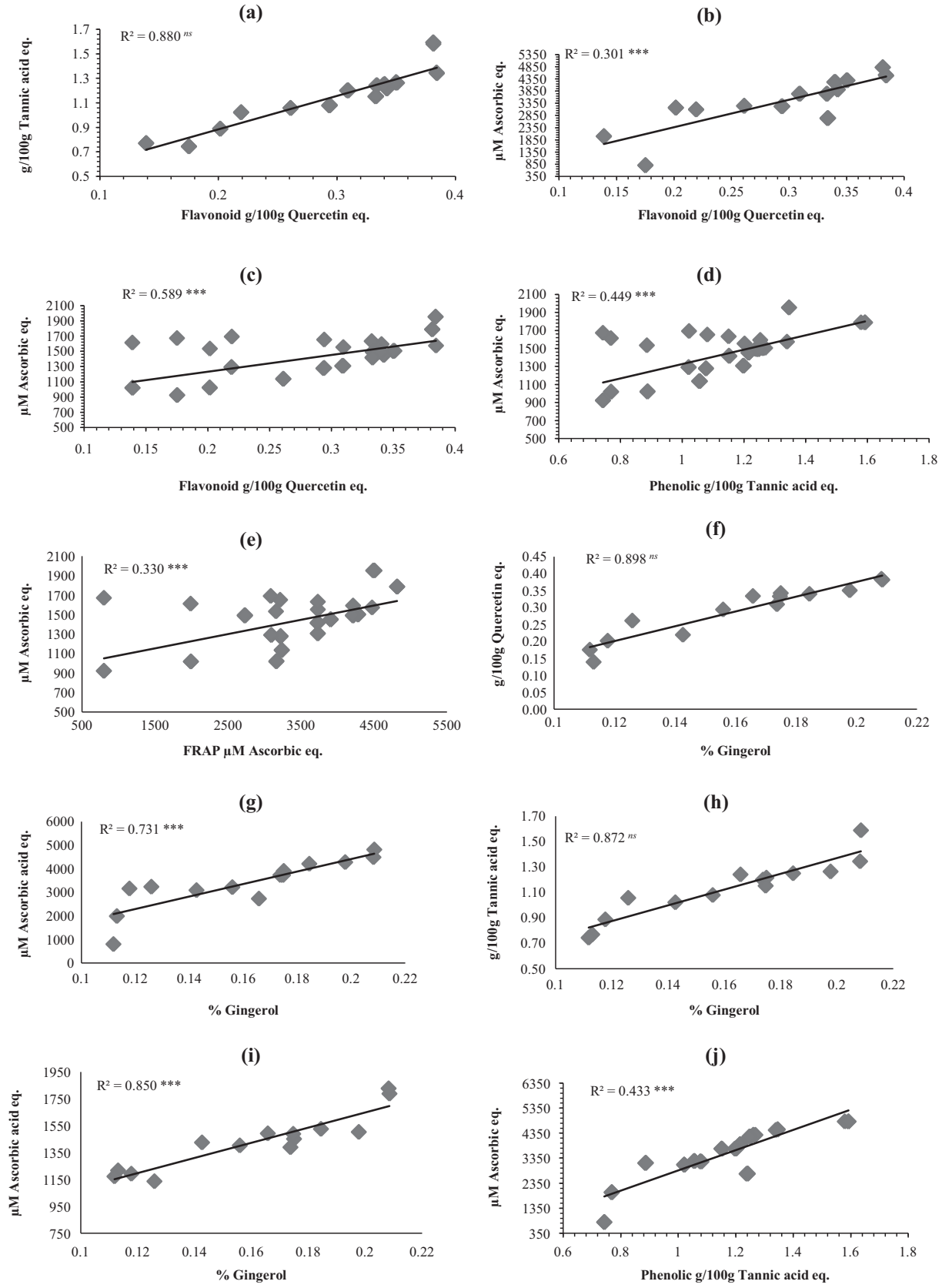

Fig. 3. Correlation between biochemical parameters of different ginger cultivars (>0.05; not significant^{ns}, <0.001: extremely significant***): (a) correlation between flavonoid and phenolics; (b) correlation between flavonoid and antioxidant (FRAP); (c) correlation between flavonoid and antioxidant (DPPH); (d) correlation between phenolics and antioxidant (DPPH); (e) correlation between antioxidant tests: FRAP and DPPH; (f) correlation between gingerol and flavonoid content; (g) correlation between gingerol and antioxidant activity (FRAP); (h) correlation between gingerol and phenolic content; (i) correlation between gingerol and antioxidant activity (DPPH); and (j) correlation between phenolics and antioxidant (FRAP).

gram. The total flavonoid contents ranged from 0.13 to 0.38 g quercetin eq./100 g of dry weight. The highest content of flavonoids was observed in cv. Rajasthan (0.38 g) while cv. Udaipur showed lowest, i.e. 0.202 g quercetin eq./100 g of dry weight (Table 1).

3.3. Total antioxidant activity

The antioxidant activity of extracts were investigated using DPPH and FRAP assays. The reduction of DPPH by antioxidants in the ginger extracts was expressed in terms of μM ascorbic acid equivalent. Total DPPH activity of the ginger cultivars ranged from 1137 μM to 1827 μM ascorbic acid equivalent (Table 1). The highest antioxidant activity was observed in cv. Rio De Janero, while the lowest activity was in cv. Assam. The group of cultivars which had higher phenolic content had highest antioxidant activity. It has also been noted that the antioxidant activity, total phenolic, flavonoid, and 6-gingerol content varied considerably from cultivar to cultivar. These results are in accordance with previous reports on antioxidant capacities of medicinal plants which exhibit polyphenols to play important role in the activity (Kalt, Forney, Martin, & Prior, 1999). The highest ferric reducing capacity was found in cv. Rajasthan (4816.84 μM) followed by cv. Rio De Janero (4489.93 mM), while among the remaining cultivars Sagar (4281.21 μM) had significant ferric reducing capacities as compared to other ginger varieties.

3.4. Multivariate analysis

A multivariate Bray Curtis Cluster analysis (complete link) was applied to the means of different biochemical parameters to produce a similarity matrix (Table 2). Analysis showed two major clusters (Fig. 2) among which cluster I comprised of 7 cultivars (cv. Rio De Janero, Rajasthan, Uttar Pradesh, Maran, Chhattisgarh, Assam and Sagar) and cluster II had 5 cultivars (cv. Wynad, Nadia, Udaipur, H.P. and Satara). Cluster I was formed of three subclusters whereas, cluster II had two subcluster. It was observed that in cluster I: cv. Uttar Pradesh, Maran, Chhattisgarh, Assam and Sagar all formed two subclusters and the subcluster of cv. Rio De Janero, Rajasthan, formed an outgroup (Fig. 2). In cluster II Wynad, Nadia, HP and Udaipur formed one subcluster for which cultivar 'Satara' was an out group. Multivariate analysis showed that cluster I comprised of cultivars with high biochemical content than cluster II. Satara cultivar was found isolated from all other cultivars and stood biochemically intermediate.

3.5. Correlation between biochemical parameters

Statistical analysis of ginger data is graphically presented in Fig. 3a–j. The three cultivars that ranked highest for antioxidant activity also ranked within the top four for phenolic contents. As phenolic compounds are one of the important water soluble antioxidants and can be present at high concentrations in plants, the correlation between these two traits was expected. Antioxidant activity increased proportionally to the phenolic content and a linear relationship between DPPH-radical scavenging activity and total phenolic was established. In case of flavonoid and phenolic there was also strong correlation ($R^2 = 0.880$) with poor significance. However, flavonoids being grouped in phenolics, the correlation is always assumed to be strong. All the ginger cultivars which contained high phenolic compounds exhibited high antioxidant activity when determined by DPPH and FRAP assays. This supports previous findings and confirms role of phenolic compounds as antioxidants (Harborne, 1998).

Correlation between antioxidant activity and phenolic compounds were significant in Bulgarian medicinal plants (Ivanova, Gerova, Chervenkov, & Yankova, 2005), Chinese medicinal plants (Zheng & Wang, 2001), some fruits, vegetables and grain products (Velioglu, Mazza, Gao, & Oomah, 1998). The phenolic hydroxyl groups present in plant antioxidants have redox properties (Pietta, 2000; Shahidi, & Wanasundara, 1992) allowing them to act as a reducing agent and a hydrogen donator in the two assays. Thus, phenolic compounds could be the major antioxidant in these ginger cultivars.

4. Conclusion

The results of present study demonstrated that 6-gingerol content of most widely grown ginger varieties had shown large variation. It would be interesting to investigate the genotypes like Rio De Janero and Rajasthan which have high 6-gingerol content. These cultivars may be widely used in agriculture for large scale production, which are rich source of pungent compound. Significant genotypic variation observed for antioxidative properties within cultivars of ginger provides opportunities for the plant scientists to identify and develop special ginger genotypes for maximizing the nutraceutical value.

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