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REGULAR ARTICLE

RP-HPLC ANALYSIS OF 6-GINGEROL AND ASSESSMENT OF ANTIOXIDANT ACTIVITIES IN EMS TREATED GINGER

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SUMMARY

Present investigation deals with RP-HPLC analysis of 6- gingerol from rhizome of ginger treated with EMS at different concentrations. The concentration levels were 0.10, 0.15, 0.20 and 0.25% for EMS 8 hour treatment and 0.30, 0.40, 0.50 and 0.60% for 4 hour treatment. The antioxidant activity of extracts were assessed by DPPH radical scavenging method and FRAP. Total phenolic content was determined using the Folin–Ciocalteu (F–C) method. The results showed that the 6- gingerol content increased with an increase in EMS dose initially (0.30%) and decreased at the higher dose (0.60%) in EMS 4 hrs treatments while there was no definite trend observed in EMS 8 hrs treatments. Antioxidant activity and the total phenolic content showed significant correlation with 6-gingerol content.

Key words: Ginger; EMS; HPLC, 6-gingerol; Antioxidant activity; Total phenolic content.

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1. Introduction

The rhizome of ginger (*Zingiber officinale*, Rosc.) Zingiberaceae has long served culinary and medicinal uses (Afjal et al., 2001). It has been used as a spice for over 2000 years (Bartley and Jacobs, 2000). Its roots and the obtained extracts contain polyphenol compounds (6-gingerol and its derivatives), which have a high antioxidant activity (Chen et al., 1986; Herrmann, 1994). Two major groups of compounds including gingerol-related compounds and diarylheptanoids have been reported as bioactive components from this plant (Koo et al., 2001; Masuda et al., 2004). The pungent bioactive compound, which have long been recognized 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 (He et al., 1998; Bhattarai, et al., 2001; Harvey, 1981). According to Leverington, (1975); Connell and Sutherland, (1969) 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. Limited reports found on the influence of EMS procedures on the antioxidant activity of spices. Hence, in the present investigation attempts have been made to shows the effect of EMS on the 6-gingerol content and antioxidant potential.

2. Material and Methods

Treatments

The rhizomes were washed free of soil and other adhering materials and then air dried overnight at room temperature. The explants used were the shoot tips of sized 1-2cm long taken from rhizomes. The explants were presoaked and treated with EMS 8hrs treatments (0.10, 0.15, 0.20 and 0.25%) and EMS 4 hrs treatments (0.30, 0.40, 0.50, and 0.60%).

Extract Preparation

EMS treated shoot tips of Ginger were grown in field. Rhizomes of all the above treatments after VM2 generation were removed from field, washed and blotted to dry with tissue towel and dried under oven for 60°C. After drying, rhizomes were

pulverized and passed through a 40 mesh sieve before extraction. The one gram powder from each treatment was dissolved in 25 ml methanol and sonicated for 30 minutes. The mixtures were centrifuged at 10,000 rpm for 10 minutes and the supernatant was filtered through Whatmans filter paper no.1. All these extracts were kept at 4°c and for assay diluted extracts were used.

Chemicals, Glasswares and Apparatus

Extracts of ginger were filtered through a 0.45 mm nylon filter into an Agilent wide opening amber vial before injection. 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.

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 µ, and 3.9×150 mm, 300 A◦. 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 1.0mL/min, the column temperature was maintained at 30^{0}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 20µl.

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 analyzed 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.

Calibration Curves 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^{0}C. The calibration curves for the 6-gingerol with the RRHT column were established by the peak areas and concentrations of working solutions.

Quantification of Total Phenolic Content (TPC)

Total phenolic content was quantified using modified Folin – Ciocalteu method described by Wolfe et al., (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 ^{0}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 equivalent per gram dry powder of the samples.

Quantitative determination of total flavonoid contents

Total flavonoid contents in all the above extracts were determined by using a method given by (Luximon-Ramma et al., 2002). 1% 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 minutes 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.

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 et al., 1995). Three ml of a methanolic solution of DPPH (25ppm) was mixed with 20 µl of different concentration of standard Ascorbic acid and the mixture was incubated for 30 min in dark. The absorbance at 515 nm was measured using methanol as blank. 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 et al., (2000).

Antioxidant activity: Ferric Reducing Antioxidant Power (FRAP)

The ferric reducing/antioxidant power (FRAP) assay was used to measure the total antioxidant power 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).

Statistical analyses

Statistical analyses was 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. The confidence limits used in this study were based on 95% ($P < 0.05$).

3. Results and Discussion

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 were y = 3.65e+004 X +3.44e+004. The result by linear regression analysis showed a very good linear relationship between peak area and concentration. The 6-gingerol contents, calculated using the standard calibration curve ($R2$ = 0.9975), were varied from 0.16% to 0.18 % (Table 1) in EMS 8 hrs treatments and 0.09% to 0.18% (Table 2) in EMS 4 hrs treatments. The concentration of 6-gingerol initially increased than control (Fig. 1B) but later decreased to the doses increased. The highest 6-gingerol content (0.18%) was detected in EMS 4 hrs 0.30% dose (Fig. 1C) while lowest (0.09%) was in EMS 4 hrs 0.60% dose (Fig. 1D) in all EMS treatments.

Table 1. Total phenolic, flavonoid and 6- gingerol content in % and antioxidant activity in mM Ascorbic acid eq. of different doses of EMS 8 hrs.

EMS 8 hrs in %	6-gingerol %	Flavonoid	Phenolic	FRAP	DPPH
Control	0.16	0.334±0.000	1.240±0.002	2.731±0.003	1.493±0.003
0.10	0.18	0.440±0.001	1.407±0.000	4.345±0.000	2.045±0.000
0.15	0.16	0.291±0.000	1.168±0.000	3.344±0.004	1.861±0.003
0.20	0.17	0.297±0.000	1.330±0.003	3.747±0.004	2.013±0.005
0.25	0.16	0.255±0.000	1.106±0.002	1.573±0.000	1.372±0.000

Biochemical analysis Calibration curves

Calibration curves were plotted for determination of the total phenolic content and flavonoid content from ginger, while DPPH and FRAP were evaluated by analyzing four concentration levels of

standard solution. The calibration curves of the standards were constructed with the correlation coefficients and regression equations (Table 3).

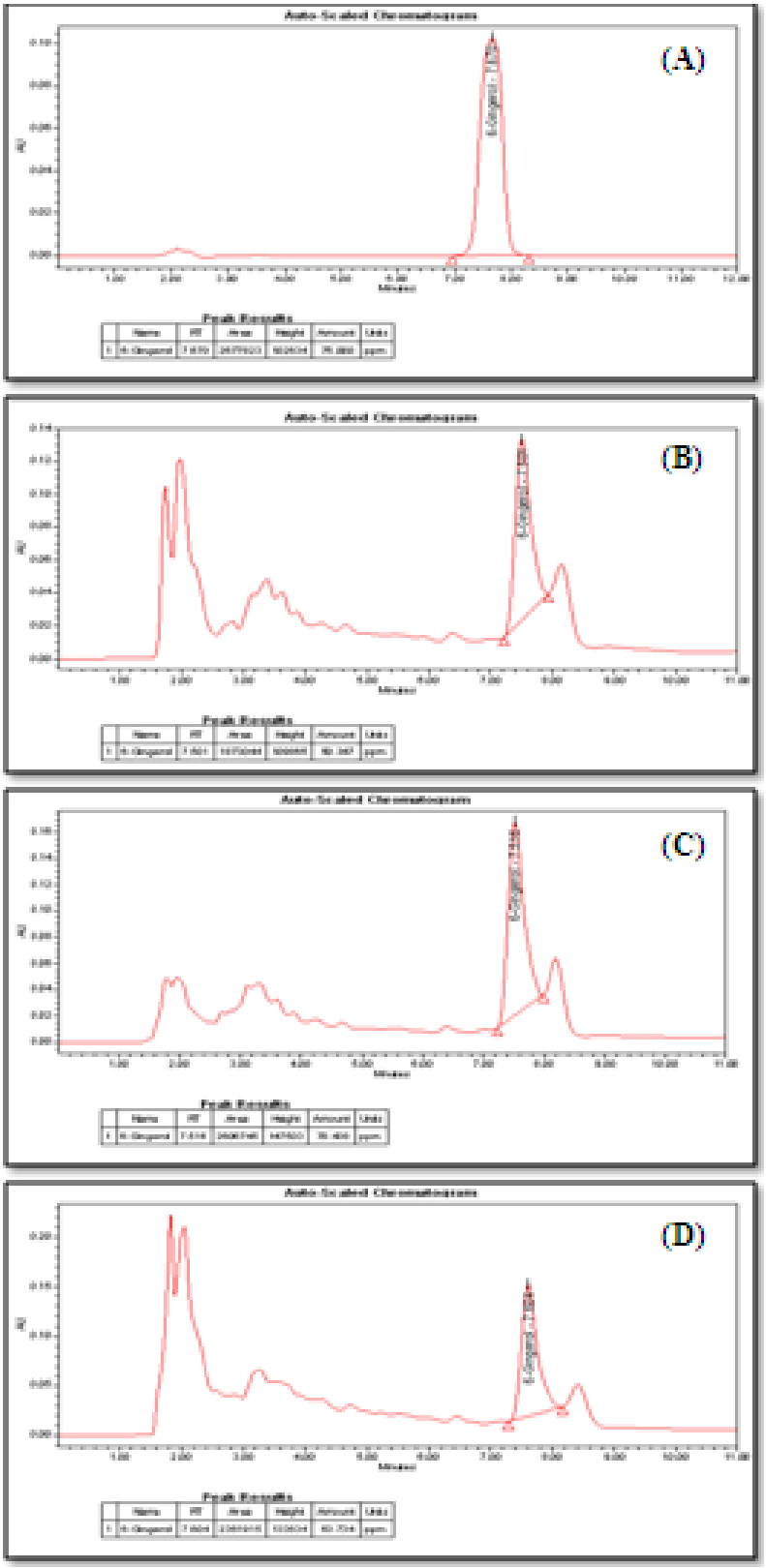

Figure 1: A: Chromatogram of Standard 6-Gingerol; B: Chromatogram of control; C: Chromatogram of highest 6-Gingerol content in EMS treatments (0.30%); D: Chromatogram of lowest 6-Gingerol content in EMS treatments (0.60%).

Table 2. Total phenolic, flavonoid and 6- gingerol content in % and antioxidant activity in mM Ascorbic acid eq. of different doses of EMS 4 hrs.

EMS 4 hrs in %	6-gingerol	Flavonoid	Phenolic	FRAP	DPPH
Control	0.16	0.334±0.000	1.240±0.002	2.731±0.003	1.493±0.003
0.30	0.18	0.319±0.001	1.266±0.002	3.072±0.002	1.513±0.003
0.40	0.15	0.318±0.000	0.918±0.001	2.401±0.002	1.333±0.016
0.50	0.14	0.239±0.000	0.957±0.000	1.646±0.002	1.242±0.003
0.60	0.09	0.197±0.000	0.639±0.003	1.113±0.000	0.838±0.000

Table 3. Values of standards calibration graphs.

Name of Standard	Name of quantified compound	Y Value	R^2
Tannic acid	Total phenolic content	0.003x + 0.080	0.999
Quercetin	Flavonoid	0.011x - 0.046	0.998
Ascorbic acid	DPPH	0.000 x + 0.023	0.978
Ascorbic acid	FRAP	0.000 x + 0.057	0.976

Table 4. ANOVA and Correlation between biochemical parameters of EMS 8 hrs treatments.

Correlations	P value	Remark	R^2 value
Flavonoid vs Phenolics	>0.05	Not significant	0.880
Flavonoid vs FRAP	<0.001	Extremely significant	0.301
Flavonoid vs DPPH	<0.001	Extremely significant	0.589
Phenolics vs DPPH	<0.001	Extremely significant	0.599
DPPH vs FRAP	<0.01	Significant	0.901
6-gingerol vs Flavonoid	>0.05	Not significant	0.982
6-gingerol vs FRAP	<0.001	Extremely significant	0.731
6-gingerol vs Phenolics	>0.05	Not significant	0.755
6-gingerol vs DPPH	<0.001	Extremely significant	0.457
Phenolics vs FRAP	<0.001	Extremely significant	0.689

Table 5. ANOVA and Correlation between biochemical parameters of EMS 4 hrs treatments.

Correlations	P value	Remark	R^2 value
Flavonoid vs Phenolics	>0.05	Not significant	0.880
Flavonoid vs FRAP	<0.001	Extremely significant	0.301
Flavonoid vs DPPH	<0.001	Extremely significant	0.589
Phenolics vs DPPH	<0.001	Extremely significant	0.910
DPPH vs FRAP	<0.01	Significant	0.859
6-gingerol vs Flavonoid	>0.05	Not significant	0.773
6-gingerol vs FRAP	<0.001	Extremely significant	0.856
6-gingerol vs Phenolics	>0.05	Not significant	0.911
6-gingerol vs DPPH	<0.001	Extremely significant	0.985
Phenolics vs FRAP	<0.001	Extremely significant	0.433

Total phenolic and flavonoid content

The phenolic content of the control sample was found to be 1.240 gm/100gm of dry weight (Table 1). EMS 4 hrs treated rhizome showed an increase in phenolic content at dose levels of 0.30% while started decrease at dose levels of 0.40% and above. The highest total phenolic content (1.407 gm/100gm of dry weight) found in 0.10% dose while lowest (0.639 gm/100gm of dry weight) was in 60% in all EMS treatments.

Flavonoid content was expressed in Quercetin equivalent which was 0.334gm/100gm dry weight equivalent in control. The range of flavonoid content of EMS doses varies from 0.197 to 0.440 gm/100gm of dry weight of ginger. The content of flavonoid decreases as dose increases for both the treatments. There is limited information available in the literature on the effect of chemical mutagen on the phenolic content of plant. However, for radiation mutagen, diverse effects on the phenolic content have been reported. Variyar et al., (1998) found increased amounts of phenolic acids in irradiated cloves and nutmeg. Harrison and Were, (2007) also reported increases in total phenolic content of gamma-irradiated almond skin extract, as compared to the control samples. Similarly, (Huang and Mau, 2006) reported a higher content of tocopherols in irradiated than in non-irradiated lyophilised mushrooms.

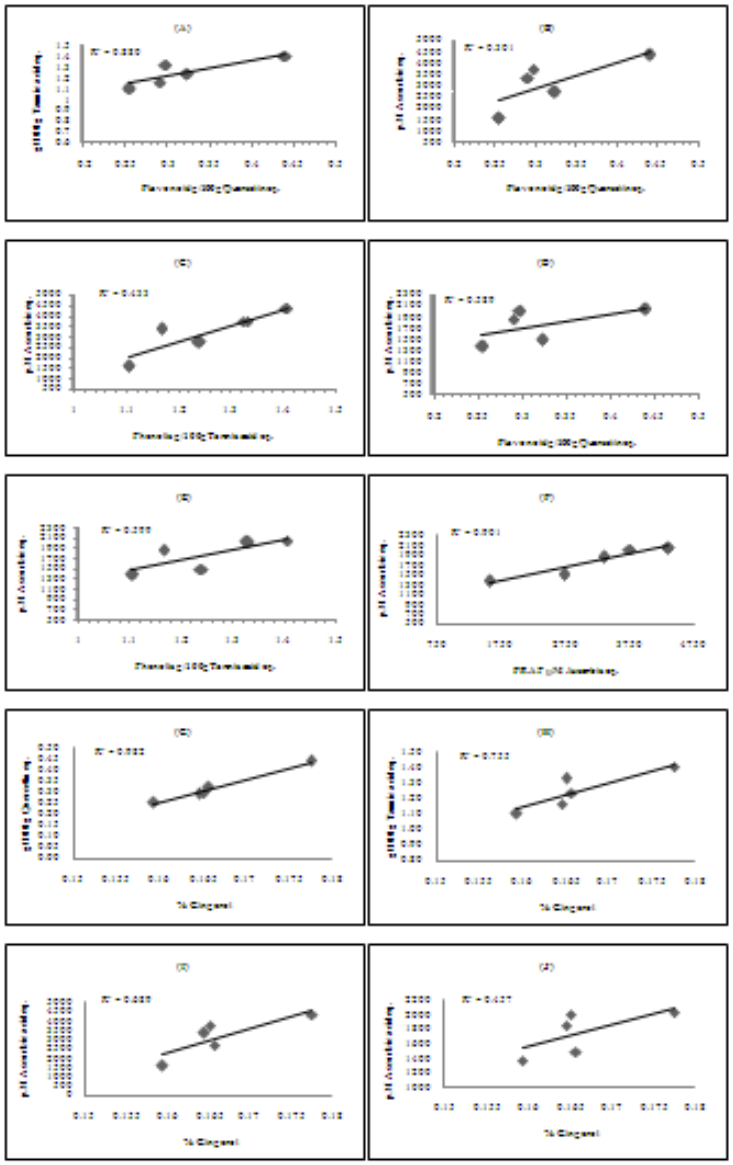

Figure 2. Correlation between biochemical parameters of EMS 8 hrs treatments:- A: Correlation between Flavonoid and Phenolics; B: Correlation between Flavonoid and Antioxidant (FRAP); C: Correlation between Phenolics and Antioxidant (FRAP); D: Correlation between Flavonoid and Antioxidant (DPPH); E: Correlation between Phenolics and Antioxidant (DPPH); F: Correlation between Antioxidant tests: FRAP and DPPH; G: Correlation between 6-gingerol and Flavonoid content; H: Correlation between 6-gingerol and Phenolic content; I: Correlation between 6-gingerol and Antioxidant activity (FRAP); J: Correlation between 6-gingerol and Antioxidant activity (DPPH).

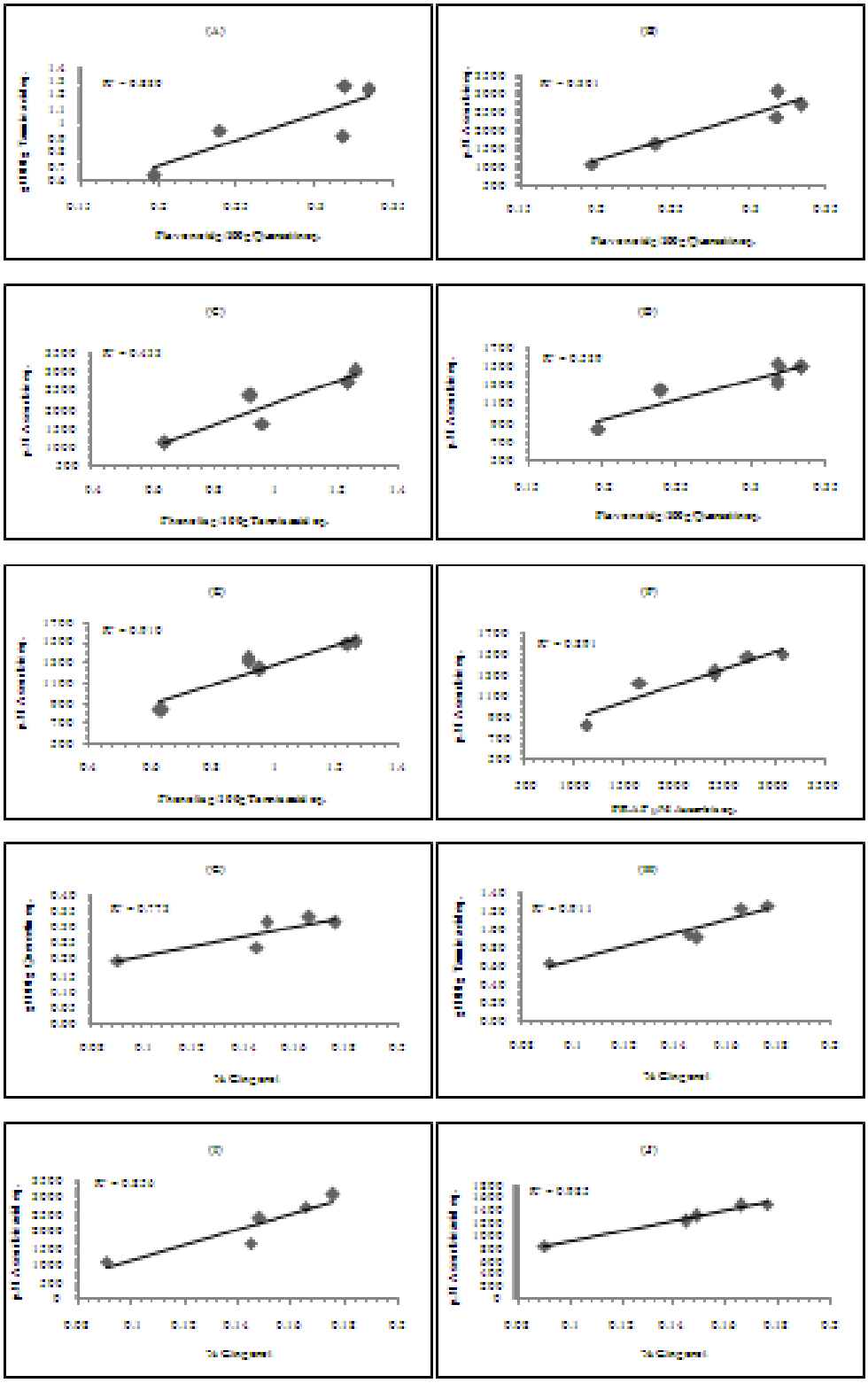

Figure 3: Correlation between biochemical parameters of EMS 4 hrs treatments:- A: Correlation between Flavonoid and Phenolics; B: Correlation between Flavonoid and Antioxidant (FRAP); C: Correlation between Phenolics and Antioxidant (FRAP); D: Correlation between Flavonoid and Antioxidant (DPPH); E: Correlation between Phenolics and Antioxidant (DPPH); F: Correlation between Antioxidant tests: FRAP and DPPH; G: Correlation between 6-gingerol and Flavonoid content; H: Correlation between 6-gingerol and Phenolic content; I: Correlation between 6-gingerol and Antioxidant activity (FRAP); J: Correlation between 6-gingerol and Antioxidant activity (DPPH).

Antioxidant activity

The radical-scavenging activity of the treated and control rhizome samples were analyzed in methanol extract, using 1, 1-diphenyl-2-picrylhydrazyl radical (DPPH) and FRAP. The highest activity was recorded in 0.10% of EMS 8 hrs dose for both DPPH and FRAP while lowest in 0.60% of EMS 4 hrs 60% dose.

Correlation between biochemical parameters in gamma rays treatments

Various authors have reported a correlation between phenolic content and antioxidant activity. (Velioglu et al., 1998) reported a significant correlation coefficient between total phenolic content and antioxidant activity in selected fruits, vegetables and grains. Statistical analysis of EMS 8 hrs treatments was depicted in Table 4 and Fig. 2 A-J while EMS 4 hrs treatments in Table 5 and Fig. 3 A-J. The three doses that ranked highest for antioxidant activity also ranked within the top four for phenolic content. Because phenolic compounds are some of the most 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 but p value is not significant. Flavonoid is the type of phenolic so the correlation will be always strong. In previous report, Ivanova et al., (2005), showed strong correlation between antioxidant activity and phenolic compounds in Bulgarian medicinal plants, Zheng and Wang, (2001) in Chinese medicinal plants. The phenolic hydroxyl groups present in plant antioxidants have redox properties (Shahidi and Wanasundara, 1992) allowing them to act as a reducing agent and a hydrogen donator in the two assays.

4. Conclusion

EMS treatments at different doses resulted in a maintenance of the natural antioxidants and increase in 6 – gingerol content for certain doses, which is necessary for the quality of spices. There was a good maintenance or slight increase in the DPPH scavenging activity and total phenolic content in all EMS doses. To conclude the EMS gives rise to rhizome with improved 6 – gingerol content and significantly protected the natural antioxidants in the ginger which could provide opportunities for the plant scientists to identify and develop special ginger genotypes for maximizing the nutraceutical value.

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