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RESEARCH

Potential Value of Bioactive and Enzymatic Antioxidant Compounds in Grapefruit (Citrus × Paradisi Macf.) Varieties

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Abstract

Growing concern about the safety of commonly used synthetic antioxidants has increased the attention toward natural antioxidants that occur as secondary metabolites in plants. The aim of this study was to investigate the bioactive composition from the fruit pulp and juice of nine varieties of grapefruit (*Citrus* × *paradise* Macf.). Total antioxidant activity performed using DPPH (2,2-diphenyl-1-picrylhydrazyl) and FRAP (ferric reducing antioxidant power) assay revealed 'Ray Ruby', 'Rio Red', and 'Ruby Red' as promising varieties with strong antioxidant activity. Pearson's correlation analysis showed that the total phenols had a significant effect on the antioxidant activity of the grapefruit, as indicated by a positive correlation with the DPPH assay ($r = 0.494^{**}$). The TPC (total phenolic content) showed significant differences (77.66-100.32 mg GAE/100 ml) between the grapefruit varieties ($p<0.5$). The two grapefruit varieties 'Rio Red' and 'Ray Ruby' had relatively higher TPC (100.32/100 ml) and 97.68/100 ml, respectively. Naringin, which is responsible for the bitter taste in grapefruit, is found in lower concentrations in deeply red grapefruit varieties like 'Ruby Red', 'Rio Red', and 'Star Ruby', making them more effective in increasing the acceptability of grapefruit among consumers. Heat map analysis based on bioactive composition clustered the genotypes representing higher antioxidant potential into a single cluster 'A' (Star Ruby, Rio Red, Ray Ruby). The genotypes 'Rio Red' and 'Ray Ruby' exhibited higher enzymatic activity (catalase, peroxidase and ascorbate peroxidase), which was effective in reducing the hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) content. The results showed that grapefruit pulp contain phenolic compounds and flavonoids, as well as antioxidant enzymatic activity. 'Ray Ruby', 'Rio Red', and 'Ruby Red' identified as promising genotypes with the optimum level of both enzymatic and non-enzymatic antioxidant compounds.

Highlights

- The study indicated that lower level of Naringin (a predominant flavonoid responsible for the bitterness) in deeply red pigmented grapefruit varieties such as 'Ruby Red', 'Rio Red' and 'Star Ruby' makes them more beneficial in increasing the grapefruit acceptability among consumers.
- 'Ray Ruby', 'Rio Red', 'Ruby Red' and 'Flame' are promising varieties due to their strong antioxidant activity based on DPPH and FRAP assay.
- Pearson's correlation analysis suggested that the total phenols contributed majorly to the antioxidant activity of the grapefruit as indicated by a positive correlation with the DPPH assay ($r = 0.494^{**}$).
- Heat map analysis based on bioactive composition clustered the genotypes representing higher antioxidant potential into a single cluster 'A' (Star Ruby, Rio Red, Ray Ruby). The genotypes 'Rio Red' and 'Ray Ruby' exhibited higher enzymatic activity (catalase, peroxidase and ascorbate peroxidase), which was effective in reducing the hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) content.

Introduction

Citrus fruit is regarded as the most valuable fruit crop globally, which not only has delicious flavours but also numerous health benefits (Wang et al. 2021). The health benefits of

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- Our results also indicated that grapefruit pulp and juice are rich source of antioxidant compounds, with 'Ray Ruby', 'Rio Red', and 'Ruby Red' identified as promising genotypes with the optimum level of both enzymatic and non-enzymatic antioxidant compounds.

Keywords Grapefruit · Antioxidant Activity · Bioactive Compounds · Phenols · Enzymes

this wonderful fruit are mainly linked to the presence of key bioactive constituents such as phenolic acids, flavanoids, vitamin C (ascorbic acid), and carotenoids, which play a crucial role in scavenging free radicals, reducing oxidative stress levels, and preventing the oxidation of biomolecules (Chen et al. 2020; Zhu et al. 2020; Khalil et al. 2022). Moreover, these phytochemicals have anti-inflammatory, anti-tumor, anti-clotting, anti-carcinogenic, anti-aging, and antioxidant properties, as well as chemo-preventive effects against chronic diseases like cancer, heart disease, and diabetes along with boosting immunity (Ke et al. 2015; Zhang et al. 2015). Grapefruit (*Citrus paradisi* Macf.), a member of the citrus family (Rutaceae) is rich in phytochemical constituents such as phenolics, flavonoids and carotenoids, and vitamin C (ascorbic acid) that can contribute to promoting good health (Cristobal et al. 2018, Lu et al. 2022). However, despite its impressive phytochemical composition, the consumption of grapefruit is often overlooked, compared to other citrus species like mandarins, due to factors such as its astringent taste, difficulty in peeling, limited availability in the market, and lack of awareness about its health benefits.

Although there have been several studies on the variation of phytochemicals and antioxidant activity in different citrus species (Canan et al. 2016; Assefa et al. 2017; Wang et al. 2021). However, information on ascorbic acid, antioxidant activity, total phenolic content, flavonoid content and antioxidant enzyme activity of grapefruit in India is difficult to obtain. Therefore, a comprehensive study on the bioactive components of different grapefruit varieties is necessary to increase consumption and provide alternatives to farmers. The amounts and types of bioactive compounds and their antioxidant capacity vary significantly between different fruit varieties, tissue types, cultivars grown in the same species or within the same cultivar in different climates and cultivation practices (Cano et al. 2008; Zhu et al. 2020). This may be due to differences in genetics, climate, soil type and other conditions (Cano et al. 2008). Variable variants offer an excellent opportunity to increase the level of potential biologically active compounds in existing cultivars. Thus, quantification of total antioxidant activity, phenolic concentrations, flavonoids, ascorbic acid content and other potentially health-related compounds is useful.

Therefore, the present study was conducted with the main objectives to understand the antioxidant and bioactive constituent levels in different grapefruit varieties so as to enhance the acceptability of grapefruit among consumers and to identify germplasm rich in bioactive composition which may be valuable for both the citrus industry and citrus crop improvement programme.

Materials and Methods

Plant Material

Nine varieties of grapefruit viz. 'Flame', 'Foster', 'Marsh Seedless', 'Oroblanco', 'Ray Ruby', 'Red Blush', 'Rio Red', 'Ruby Red' and 'Star Ruby' grafted on rough lemon (*C. jambhiri* Lush) rootstock were used for the present study conducted during 2020-22 (Fig. 1). The trees were maintained at a spacing of 6 × 3 m and were cultivated under recommended package of practices in the experimental orchard under sub–tropical conditions at Punjab Agricultural University, Ludhiana, Punjab, India (Latitude 30°54′N, Longitude 75°47′E).

Reagents

Rutin, pyrocatechol, L-ascorbic acid, Gallic acid, Naringin, were obtained from Sigma (Sigma-Aldrich Co., Bangalore, India). The 2,4,6-tripyridyl-s-triazine (TPTZ), and 2,2-diphenyl-1-picrylhydrazyl (DPPH) from Sigma (St. Louis, Missouri, United States). Folin–Ciocalteureagents,n-hexane, peroxidase, hydrogen peroxide solution (30%), EDTA, toulene, from SRL, Mumbai, India. Ninhydrin, Folin–Ciocalteu reagents, 2,2-Bipyridyl, Sodium carbonate anhydrous were obtained from HPLC, Gujrat India. All other chemicals and solvents were of analytical grade.

Hydrogen Peroxide (H_2O_2) and Malondialdehyde (MDA) Content

Hydrogen peroxide (H_2O_2) was determined according to the method given by Sinha (1971). Briefly, 500 mg of fresh fruit pulp tissue was homogenized in 2 ml of 0.1 M potassium phosphate buffer (pH 7.0) and centrifuged at 10,000 g for 25 min at 4°Cto obtain the supernatant. Then, 1 ml of supernatant was mixed with 1 ml of 0.1 M potassium phosphate buffer (pH 7.0) and 2 ml of a reaction mixture comprising 5% potassium dichromate & glacial acetic acid in the ratio of 1:3 (v/v). After filtration, the absorbance sample was read at 570 nm against blank. A standard curve of hydrogen

Fig. 1 Variation in the fruit pulp characteristics among grapefruit genotypes **(A)** Flame **(B)** Foster, **(C)** Marsh Seedless, **(D)** Oroblanco **(E)** Ray Ruby, **(F)** Red Blush, **(G)** Rio Red, **(H)**, Ruby Red and **(I)** Star Ruby

peroxide (50–200 nmole) was prepared for estimation of hydrogen peroxide content.

For malondialdehyde content estimation (Heath and Packer 1968), homogenized 0.2 g of fruit pulp tissue in 2 ml of 5% ice-cold trichloroacetic acid (TCA). After centrifugation at 10,000 rpm for 15 min at 4ºC, 1 ml of the supernatant was mixed with a solution containing 0.5% thiobarbituric acid (TBA) and incubated at 100ºC for 30 min. After cooling to room temperature, centrifuged the contents at 10,000 rpm for 10 min and read the absorbance at 532 and 600 nm against a blank. The concentration of malondialdehyde was expressed as nmol MDA per gram fresh weight of the tissues using following formula:

$$nmol\ MDA/g\ fresh\ weight = \frac{Abs@532nm - Abs@600nm\ \times Vol.\ Of\ reaction\ mixture \times 1000}{Extinction\ coefficient \times Weight\ of\ sample\ (g)}$$

Antioxidant Potential of Grapefruit Varieties Based on Various Bioactive Compounds

For phenolic compound analysis, 2 ml of fresh juice was mixed with 14 ml of 80% methanol at room temperature. After 30 min, the sample was centrifuged at 2000 rpm for 20 min, and the supernatant was collected for phenolic compounds analysis. The total phenolic content was determined using a modified Folin-Ciocalteu method (Singleton et al. 1999). Initially, 1 ml of supernatant was evaporated to dryness followed by the addition of 6.5 ml of distilled water and 0.5 ml of Folin-Ciocalteu reagent, and incubated for 5 min. Afterward, 1 ml of a saturated sodium carbonate solution was added, and the absorbance at 750 nm was measured after 60 min of room temperature incubation. Gallic acid standards (10 to 100 µg) were used for calibration. The total flavonoid content was estimated by evaporating 2 ml methanolic sample extract to dryness, then reconstituted with 1 ml of distilled water and mixed with 5 ml of 0.1 M methanolic aluminum chloride solution (Zhishen et al. 1999). After 15-minute room temperature incubation, the appearance of yellow color was measured at 420 nm. Rutin standards (40 to 200 µg) were used to create a standard curve.

The estimation of O-dihydroxy phenols was done according to Nair and Vaidyanathan (1964). After evaporating 2 ml of methanolic extract to dryness, the resulting residue was dissolved in 1 ml of double distilled water. Then, 0.3 ml of 10% TCA, 1 ml of 10% sodium tungstate, 0.5 ml of 0.5 N HCl, and 1 ml of 0.5% freshly prepared sodium nitrite were added, resulting in the development of a yellow colouration. After 5 min, 2 ml of 0.5 N NaOH was added and the light cherry colour was read after 15 min at 540 nm against the reagent blank. The standard curve using catechol in the range of 5–40 µg was prepared. Naringin was measured following method outlined by Davis (1947), which involved addition of 20 µl of grapefruit juice with 20 µl of 4 M NaOH and 1 ml of 90% diethylene glycol. The resulting mixture was thoroughly nixed and left to incubate for

15 min to develop the yellow colour. The optical density was recorded using a spectrophotometer at 420 nm against a blank without juice. The concentration of naringin (µg/ml) was then determined by referencing a standard graph of naringin created using the same procedure.

Ascorbic acid content was determined according to Law et al. (1983) in which juice vesicles were crushed in 5% meta-phosphoric acid with the help of a mortar and pestle. Following centrifugation at 1000 rpm for 20 min, the supernatant was collected for ascorbic acid estimation. In a suitably diluted sample, 0.4 ml of 5 mM EDTA and 16 mM $FeCl_3$ were added, along with 0.8 ml each of 7.6% o-phosphoric acid and 44 mM bipyridyl. After 40 min of incubation at 40º C, the optical density of the coloured sample was read at 525 nm against the reagent blank. L-ascorbic acid in the range of 25–125 µg was used for the preparation of the standard plot.

Sample Extraction for Estimation of DPPH Radical Scavenging Activity and Ferric-reducing Antioxidant Power (FRAP)

For analysis of DPPH and FRAP activity, 1 ml of fresh juice was extracted with 14 ml of 80% methanol at room temperature. After 30 min the sample was centrifuged at 2000 rpm for 20 min and the supernatant was collected for determination of DPPH and FRAP activity.

The free radical scavenging activity (DPPH) of fruit juice was measured based on the principle of reduction of DPPH free radicals by antioxidants present in the sample (Blois 1958). An aliquot of 0.20 ml of the sample was added to 2.8 ml DPPH solution (0.1 mM) prepared in 80% methanol and placed in the dark for 30 min. 80% methanol was used to set blank and DPPH solution without a sample was used as a control. Free radical scavenging activity was calculated by using the formula:

$$Antioxidant\ activity\ (\%) = \begin{pmatrix} Absorption\ of\ control - \\ Absorption\ of\ sample \end{pmatrix} / Absorption\ of\ control$$

The ability of the sample extract to reduce Fe^{3+}TPTZ solution to Fe^{2+}TPTZ was performed according to the procedure outlined by Benzie and Strain (1996).In brief, 0.1 ml of the methanolic extract was added to 1.8 ml of freshly prepared FRAP reagent. The mixture was incubated at 37°C for 10 min and the appearance of intense blue colour was read at 593 nm. A calibration curve of freshly prepared ferrous sulphate (0-1.2 mM) was used for calculation and results were expressed as mM Fe^{2+}per ml.

Extraction Buffer and Sample Extraction for Estimation for Enzymatic Activities

To prepare the extraction buffer, a mixture of 30 ml of 0.05 M Potassium phosphate buffer (pH 7.5) and 0.25 ml of 100x Triton was combined with 1 gm of PVP and 18.6 mg of EDTA. For sample extraction, 0.5 g of pulp tissue was crushed in 2 ml of cold (4 °C) extraction buffer using a pre-chilled pestle & mortar. The mixture was centrifuged at 7500 rpm for 20 min, and the resulting supernatant was used as the enzyme extract. Superoxide dismutase activity was assessed following Marklunds and Marklunds (1974). The assay mixture contained 1.5 ml of Tris-HCl buffer (pH 8.2), 0.5 ml of 6 mM EDTA, 1 ml of 6 mM pyrogallol solution, and 0.1 ml of the enzyme extract. Absorbance at 420 nm was measured at 30-second intervals for 3 min, and results were expressed as µmol per mg protein. To estimate peroxidase activity, 3 ml of chilled 0.05 M guaiacol and 0.4 ml of enzyme extract were mixed (Shannon et al. 1966). Initiation of the reaction occurred by adding 0.1 ml of 0.8 M hydrogen peroxide, and absorbance at 470 nm was recorded at 30-second intervals for 3 min.

Catalase activity was determined as per Chance and Maehley (1955). The reaction began with 0.1 ml of enzyme extract added to 1.90 ml of chilled 0.05 M potassium phosphate buffer (pH 7.5). Then, 1 ml of hydrogen peroxide was introduced, and absorbance at 240 nm was monitored at 30-second intervals for 3 min. The ascorbate peroxidase assay was assessed as per Nakano and Asada's method (1981). The reaction mixture comprised 1 ml of 0.05 M potassium phosphate buffer (pH 7.5), 0.8 ml of 0.5 mM ascorbate, 1 ml of 39 mM hydrogen peroxide, and 0.1 ml of enzyme extract. The decrease in absorbance was monitored at 290 nm at 30-second intervals for up to 3 min.

Statistical Analysis

The experiment was laid in RBD (Randomized Block Design) as set out by Gomez and Gomez (2010) with three biological replicates. Statistical significance was determined using a 5% significance level with computer software SAS (Statistical Analysis System) 9.3. Correlation among the various biochemical traits of different grapefruit varieties was done using SPSS 25 (Statistical Package for the Soil Sciences) software. A heatmap for the clustering of genotypes was generated by using ClustVis (https://biit.cs.ut.ee/clustvis/).

Results and Discussion

Hydrogen Peroxide (µmoles/g FW) and Malondialdehyde Content (nmoles/g FW)

Hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) are the key markers of cellular oxidation. Hydrogen peroxide is the most stable form of reactive oxygen species (ROS) that can initiate and cause oxidative damage in plant cells under stress. While, malondialdehyde (MDA) is considered one of the direct indicators of membrane oxidative damage (Sharma et al. 2015). The content of the hydrogen peroxide (H_2O_2) and malondialdehyde (MDA)among the grapefruit varieties under study are shown in Fig. 2 (a, b). The Hydrogen peroxide (H_2O_2) content ranged from 73.25 to 111.87 µmoles/g FW across all the varieties (Fig. 2a). Varieties such as 'Star Ruby' and 'Rio Red' recorded the lowest hydrogen peroxide content (73.25 and 75.65 µmoles/g FW, respectively), compared to 'Oroblanco' (111.87 µmoles/g FW) and 'Foster' (108.38 µmoles/g FW). Hydrogen peroxide (H_2O_2) initiates oxidative damage and content of malondialdehyde is the direct indicator of oxidative damage. The minimum content of malondialdehyde was observed in 'Flame' (4.34 nmoles/g FW), 'Rio Red' (5.38 nmoles/g FW), 'Ray Ruby' (6.12 nmoles/g FW) and 'Star Ruby' (6.37 nmoles/g FW) (Fig. 2(b) which is in association with the lower hydrogen peroxide content observed in these varieties subsequently resulting in minimum lipid peroxidation. On the other hand, 'Oroblanco' accumulated a significantly higher (8.99 nmoles/g FW) amount of malondialdehyde which was almost two-fold higher than 'Flame' (4.34 nmoles/g FW), which is expected as it also registered the higher hydrogen peroxide content. The results are in good agreement with Oustricet al. (2015), Nie et al. (2020) and Zhu et al. (2020), who reported that an increase in MDA (malondialdehyde) levels and a loss of membrane integrity in citrus fruits experiencing cellular oxidative stress are typically associated with an excessive accumulation of hydrogen peroxide and an oxidative stress burst.

Antioxidant Potential of Grapefruit Varieties Based on Various Bioactive Compounds

Phenolic compounds in citrus fruit mainly comprise phenolic acid and flavonoids (Lado et al. 2018; Zhang et al. 2018). The phenols and flavonoids are known for their antioxidant and anti-inflammatory properties that have been extensively studied for their potential health benefits (Lin et al. 2016; Wang et al. 2021). Notable variations in the total phenolic content were observed among

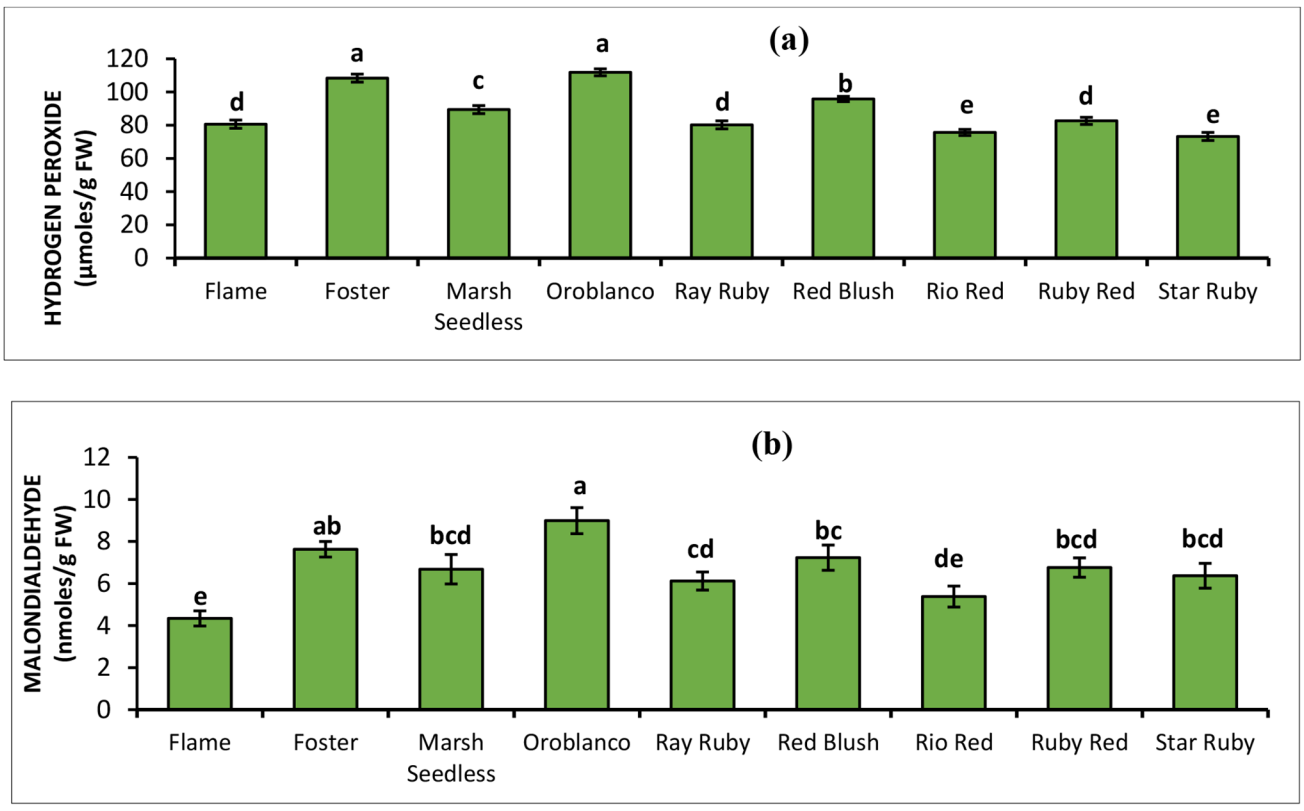

Fig. 2 Hydrogen peroxide content **(**Fig. 2**(a))** and malondialdehyde (MDA) content **(**Fig. 2**(b))** among grapefruit genotypes **(**Values with the same alphabet (s) are not significantly different at $p > 0.05$ (LSD))

the different grapefruit varieties ($p < 0.5$), ranging from 77.66 to 100.32 mg GAE/100 ml (Table 1). 'Rio Red' and 'Ray Ruby' exhibited comparatively higher levels of total phenolics, with values of 100.32 and 97.68 mg GAE/100 ml, respectively, in contrast to 'Marsh Seedless' which witnessed a significantly lower total phenolic content (77.66 mg GAE/100 ml). Similarly, the total flavonoid content ranged from 31.51 to 46.67 mg rutin equivalent (RE)/100 ml among grapefruit varieties (Table 1). 'Rio Red' which had the highest total phenolic content, also had the highest flavonoid amount (46.67 mg RE/100 ml), whereas, 'Star Ruby' observed the lowest flavonoid content of 31.51 mg RE/100 ml. Wang et al. (2021) reported that the total phenols and flavanoids are important due to their ability to scavenge free radicals. The phenolic hydroxyl groups attached to the ring structure of the flavonoids are known to be antioxidants by scavenging free radicals, inhibiting lipid oxidation, or chelating metal ions (Tripoli et al. 2007). This suggests the strong radical scavenging activity of 'Rio Red' and 'Ray Ruby' attributed to the higher content of phenols and flavonoids in them. However, the variation in bioactive compounds as observed across grapefruit varieties might be due to genetic and environmental factors which have an important role in biosynthesis of bioactive compounds, their accumulation and formation (Patil et al. 2004; Barbara et al. 2005). The present results are in line with the studies of Sicari et al. (2018); Chen et al. (2020); Morianou et al. (2021) and Singh et al. (2021).

Ortho-dihydroxyphenol content ranged from 2.02 to 2.93 mg catechol/100 ml among grapefruit varieties. Maximum content of ortho-dihydroxyphenol was witnessed in 'Marsh Seedless' (2.93 mg catechol/100 ml), while, minimum content of ortho-dihydroxyphenol was recorded in 'Ruby Red' (2.02 mg catechol/100 ml) and 'Oroblanco' (2.04 mg catechol/100 ml). Ortho dihydroxy phenol, an aromatic phenolic compound, are present in minor amount in fruit (Shahidi and Ambigaipalan 2015). However, not much study has been conducted on the ortho-dihroxyphenol in fruit crops especially citrus. Naringin is the predominant flavanoid found in grapefruit and has been recognized for its strong antioxidant and anti-inflammatory properties (Cavia-Saiz et al. 2011). However, this flavanoid is also responsible for the bitterness in grapefruit (Sudto et al. 2009). Therefore, selection of varieties with low naringin content would be beneficial in increasing the grapefruit acceptability among consumers. In the present study the varieties with low naringin content were 'Ruby Red' (23.20 mg/100 ml), 'Rio Red' (24.20 mg/100 ml), 'Oroblanco' (24.40 mg/100 ml) and 'Star Ruby' (26.20 mg/100 ml) (Table 1). It is intriguing that, the varieties with low naringin content were deeply pigmented grapefruit which is in accordance with most of the previous research studies (La Cava and Sgroppo 2015).

Citrus fruits are also renowned for their higher amount of ascorbic acid, which acts as a powerful antioxidant by neutralizing reactive oxygen species (ROS) and free radicals. According to the Food and Nutrition Board, the vitamin C dietary reference intake (DRI) is90 mg/day for adults over 19 years; from this point of view, consumption of one-half portion of grapefruit (~ 150 g) would provide atleast 69.78% of the DRI of this vitamin (Trumbo et al. 2002). Among citrus fruits, grapefruits are recognized as one of the richest sources of vitamin C, with levels ranging from 25 to 60 mg/100 mL of juice, surpassed only by oranges and pummelos, which have a range of 30 to 88 mg/100 mL of juice (Lado et al. 2018).In the current investigation, significant variability was observed in the ascorbic acid content within the studied grapefruit varieties (Table 1). The content of ascorbic acid in the fruit juice ranged from 37.60 mg/100 g to 48.72 mg/100 g on a fresh weight basis, 'Oroblanco' had the highest accumulation of ascorbic acid (48.72 mg/100 g), followed by 'Marsh Seedless' (45.94 mg/100 g), 'Rio Red' (44.88 mg/100 g), 'Ray Ruby' (42.79 mg/100 g), and

Table 1 Total antioxidant activity and content of bioactive antioxidants among grapefruit varieties

Variety	Phenolic compounds				Ascorbic acid (mg/100 g FW)
	Total Phenols (mg GAE/100 ml)	Flavanoids (mg RE /100 ml)	Naringin (mg/100 ml)	Ortho-dihroxy-phenol (mg catechol/100 ml)	
Flame	88.62 ± 1.29^{b}	34.10 ± 1.06^{d}	31.00 ± 1.50^{ab}	2.33 ± 0.42^{bcd}	41.81 ± 1.44^{cd}
Foster	87.56 ± 1.80^{b}	38.65 ± 1.36^{c}	26.80 ± 0.92^{cd}	2.59 ± 0.15^{abc}	37.00 ± 2.74^{e}
Marsh Seedless	77.66 ± 1.19^{d}	37.64 ± 0.65^{c}	34.20 ± 1.27^{a}	2.93 ± 0.16^{a}	45.94 ± 1.40^{ab}
Oroblanco	89.72 ± 2.12^{b}	44.84 ± 1.77^{a}	24.40 ± 1.39^{de}	2.04 ± 0.23^{d}	48.72 ± 2.64^{a}
Ray Ruby	97.68 ± 2.16^{a}	42.13 ± 0.94^{b}	30.40 ± 0.69^{b}	2.19 ± 0.42^{cd}	42.79 ± 2.76^{bc}
Red Blush	81.84 ± 2.16^{c}	38.82 ± 1.65^{c}	29.00 ± 0.81^{bc}	2.76 ± 0.22^{ab}	37.60 ± 2.62^{e}
Rio Red	100.32 ± 1.90^{a}	46.67 ± 0.65^{a}	24.20 ± 1.27^{de}	2.87 ± 0.12^{a}	44.88 ± 1.10^{bc}
Ruby Red	82.28 ± 1.80^{c}	35.33 ± 1.06^{d}	23.20 ± 1.15^{e}	2.02 ± 0.25^{d}	39.12 ± 1.20^{de}
Star Ruby	89.32 ± 2.16^{b}	31.51 ± 0.94^{e}	26.20 ± 1.04^{cde}	2.39 ± 0.13^{bcd}	39.00 ± 2.04^{de}
Mean	**88.33**	**38.85**	**27.71**	**2.46**	**41.87**
LSD($p \leq 0.05$)	**3.93**	**1.99**	**2.25**	**0.44**	**3.60**

*Values in the same lines that have different letters exhibit significant differences (with a significance level of $p < 0.05$)

'Flame' (41.81 mg/100 g). It is worth mentioning that the varieties with highest ascorbic acid content (Oroblanco and Marsh Seedless) were white fleshed, which is in accordance with the observation of Sicari et al. (2018) who reported that white fleshed 'Marsh Seedless' had higher vitamin C content than pink fleshed 'Star Ruby'. These findings are consistent with the results reported by Barros et al. (2012) and Morianou et al. (2021).

Total Antioxidant Activity in Terms of DPPH and FRAP Assay

Total antioxidant activity of grapefruit varieties was evaluated through two complementary assays viz. 2,2diphenylpicrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP) (Fig. 3a, b). DPPH is a stable free radical that reacts with antioxidants, and the percentage of DPPH reduction reflects the antioxidant activity of the sample (Assefa et al. 2017). The use of the DPPH free radical is more beneficial in evaluating antioxidant efficacy as it is more stable than the hydroxyl and superoxide radicals (Layina-Pathirana et al. 2006). On the other hand, the FRAP assay is typically used to measure the capacity of the sample to reduce the ferric complex (Fe III) to the ferrous form (Fe II) (Contreras et al. 2011). The highest DPPH activity of 49.40% and 48.76%, respectively was observed in 'Rio Red' and 'Ray Ruby' (Fig. 3a). In addition to this, 'Flame', 'Red Blush', and 'Ruby Red' also exhibited noteworthy DPPH activity levels, ranging from 40.19 to 44.58%. Conversely, 'Oroblanco' recorded the lowest DPPH activity of 27.64%, which was 44.04% and 43.31% lower than that of 'Rio Red' and 'Ray Ruby', respectively. Similarly, the antioxidant activity of the grapefruit samples determined by FRAP assay ranged from 1.86 to 2.87 mM Fe^{2+} equivalent/ml (Fig. 3b). 'Flame' exhibited the highest FRAP activity (2.78 mM Fe^{2+} equivalent/ml) among the varieties, which was twice as high as 'Oroblanco' followed by 'Ray Ruby', 'Rio Red', and 'Red Blush' in which the FRAP value ranged from 2.38 to 2.55 mM Fe^{2+} equivalent/ml. On the other hand, 'Oroblanco' exhibited the lowest FRAP value (1.24 mM Fe^{2+} equivalent/ml), which is consistent with the results of the DPPH assay.

Nishad et al. (2018) reported that the variation in antioxidant activity observed among various pummelo varieties may arise from the unique composition of the extract, as well as potential synergistic or antagonistic interactions among different bioactive compounds (phenolic compounds). Ogundele and Bolade (2021) observed that the free radical scavenging activity of grapefruit juice showed similar trend

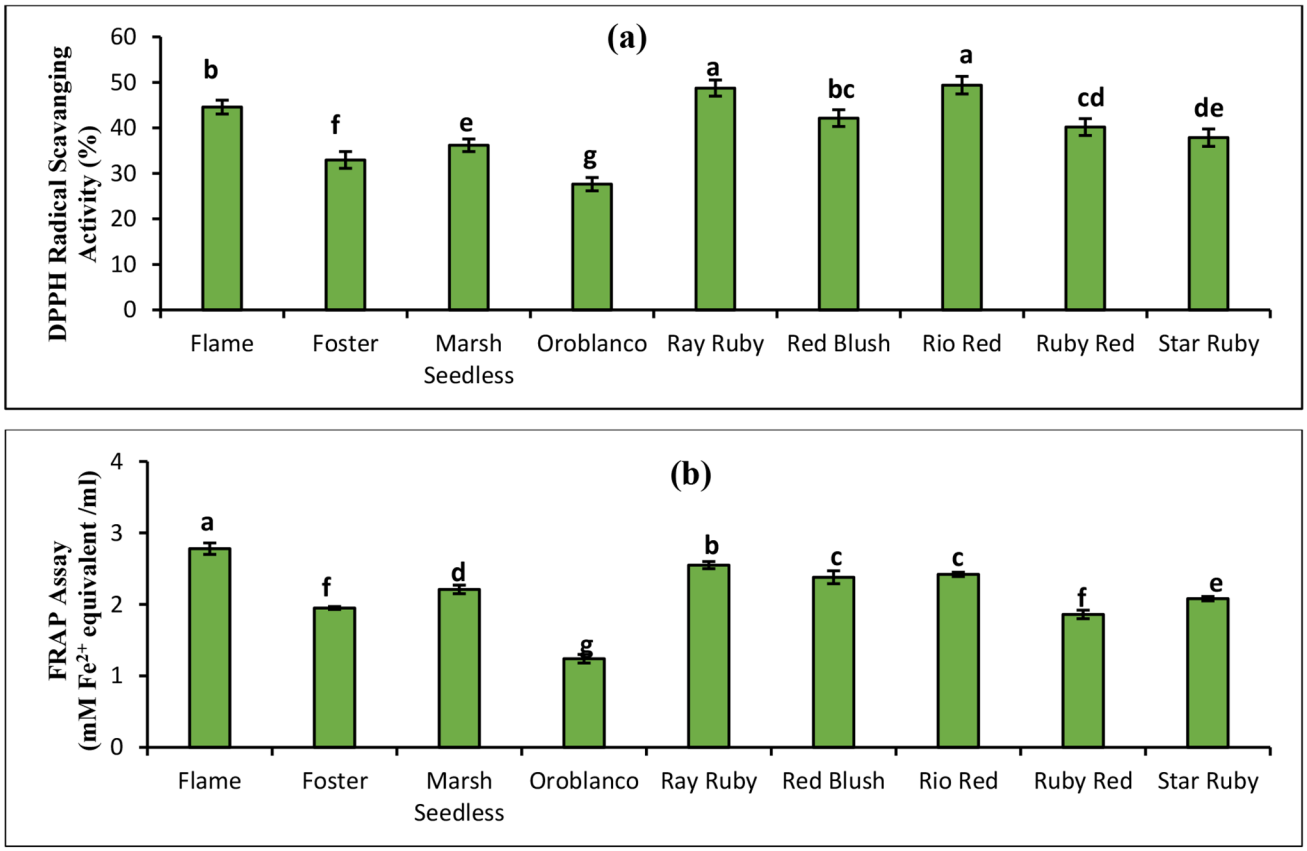

Fig. 3 Antioxidant activity of different grapefruit genotypes using DPPH **(3a)** and FRAP **(3b)** assay

as that of biochemical constitutes such as total phenols. Therefore, a higher amount of bioactive compounds i.e. total phenols and flavanoids might be the reason for higher antioxidant activity (DPPH and FRAP) reported in varieties like 'Ray Ruby' and 'Rio Red'. The results of the DPPH and FRAP assays used in this study are consistent with the findings of previous studies by Kumar et al. (2018), Ahmed et al. (2018) and Singh et al. (2021) which advocated high antioxidant activity of grapefruit juice. Overall, our present findings suggest that 'Ray Ruby', 'Rio Red', and 'Flame' are the most potent varieties in terms of their antioxidant activity, as indicated by their high DPPH radical scavenging activity and FRAP assay.

The Antioxidant Enzymes Activity among Grapefruit Varieties

The high activity of antioxidant enzymes such as superoxide dismutase (SOD), peroxidase (POX), catalase (CAT), and ascorbate peroxidase (APX) prevent the lipid peroxidation caused by active oxygen production by effectively reducing the accumulation of ROS (Reactive oxygen species) and MDA (Malondialdehyde). The enzymatic activity i.e., superoxide dismutase (SOD), peroxidase (POX), catalase (CAT), and ascorbate peroxidase (APX) activity displayed among different grapefruit varieties in Fig. 4. The varieties with the highest superoxide dismutase activity were 'Oroblanco' (9.15 µmoles/mg protein), 'Rio Red' (9.80 µmoles/mg protein), and 'Marsh Seedles's (6.86 µmoles/mg protein), in comparison to 'Foster' (2.62 µmoles/mg protein), 'Red Blush' (2.94 µmoles/mg protein), and 'Ray Ruby' (2.83 µmoles/mg protein) (Fig. 4a). Similarly, the significantly highest peroxidase activity ($p < 0.05$) was observed in 'Flame' (8.99 µmoles/mg protein) and 'Marsh Seedless' (8.07 µmoles/mg protein), than 'Foster' (4.98 µmoles/mg protein), 'Red Blush' (5.03 µmoles/mg protein), and 'Oroblanco' (3.89 µmoles/mg protein) (Fig. 4b).The grapefruit varieties also observed significant variation with respect to catalase and ascorbate peroxidase activity (Fig. 4c and d). Among varieties, 'Ray Ruby' recorded the highest catalase and ascorbate peroxidase activity (20.37 µmoles/mg protein and 222.62 µmoles/mg protein, respectively, respectively). While, 'Oroblanco' had the lowest catalase and ascorbate peroxidase activity levels with an average of 10.22 µmoles/mg protein and 113.60 µmoles/mg protein, respectively. The present results are in line with the findings of Cai et al. (2021), Khan et al. (2021) and Liaquat et al. (2023), Oustric et al. (2015) and Sharma et al. (2015).

Superoxide dismutase, a class of metalloproteins, is the first barrier against oxidative damage, which catalyzes the dismutation of superoxide (O^{2-}) radicals into molecular oxygen (O^2) and hydrogen peroxide H_2O_2. The hydrogen peroxide formed is then decomposed by catalase and peroxidases (Racchi 2013). Whereas, ascorbate peroxidase is a key enzyme of the ascorbate-glutathione cycle responsible for removing hydrogen peroxide in chloroplasts and the main enzyme for ascorbate metabolism (Cai et al. 2021). Therefore, higher activity of catalase (CAT), peroxidase (POX) and ascorbate peroxidase (APX) observedin 'Ray Ruby' and 'Rio Red' appear to be responsible for lower content of hydrogen peroxide and MDA detected in them. Liaquat et al. (2023) reported catalase, peroxidaseand superoxide dismutase activity of 30.83 U mg/protein, 11.40 U mg/protein, and 114.51 U mg/protein, respectively in the juice sample of Kinnow mandarin. While, Khan et al. (2021) reported peroxidase activity of 3.97 U/mg protein in pulp of grapefruit cultivar 'Shamber'. In another study, Soheila et al. (2021) reported that catalase activity ranged from 1.5 to 2.5 U/mg protein in pulp of grapefruit genotype 'Red Blush'. Haider et al. (2021) found catalase activity 11.80 U mg/protein in fruits of Kinnow mandarin and observed that fruit treatment with 4 mM salicyclic acid increased the catalase activity by 1.5 times (17.71 U/mg protein).

It is noteworthy that though catalase, peroxidase and ascorbate peroxidase activity were lower in 'Oroblanco', it recorded higher superoxide dismutase activity. This might be the reason that lower activity of catalase and ascorbate peroxidase along with higher superoxide dismutase activity observed in 'Oroblanco' led to higher hydrogen peroxide levels and concomitantly higher MDA levels observed in this variety. The present results are in accordance with those of Oustric et al. (2015) who suggested that the accumulation of hydrogen peroxide during fruit growth could be due to either significant superoxide dismutase activity that converts the oxygen molecule into hydrogen peroxide or inefficient activity of catalase and ascorbate peroxidase.

Heat-map Based Clustering

Heat-map analysis categorized different genotypes into two main clusters ('A' and 'B'). Cluster 'A' comprised 'Star Ruby', 'Rio Red', 'Ray Ruby', and 'Flame', while cluster 'B' included 'Ruby Red', 'Marsh Seedless', 'Oroblanco', 'Foster', and 'Red Blush' (Fig. 5). Within cluster 'A', subcluster 'A1'included only 'Star Ruby', while subcluster 'A2' comprised 'Rio Red', 'Ray Ruby', and 'Flame'. Genotypes in subcluster 'A1' demonstrated higher antioxidant potential, whereas those in subcluster 'B2' (Oroblanco and Foster) had lower antioxidant potential and reported higher hydrogen peroxide and MDA levels. Higher hydrogen peroxide levels and concomitantly higher MDA levels in 'Oroblanco' and 'Foster' genotypes might be due to inefficient enzymatic antioxidant system as depicted by lower catalase, peroxidase, and ascorbate peroxidase activity observed in

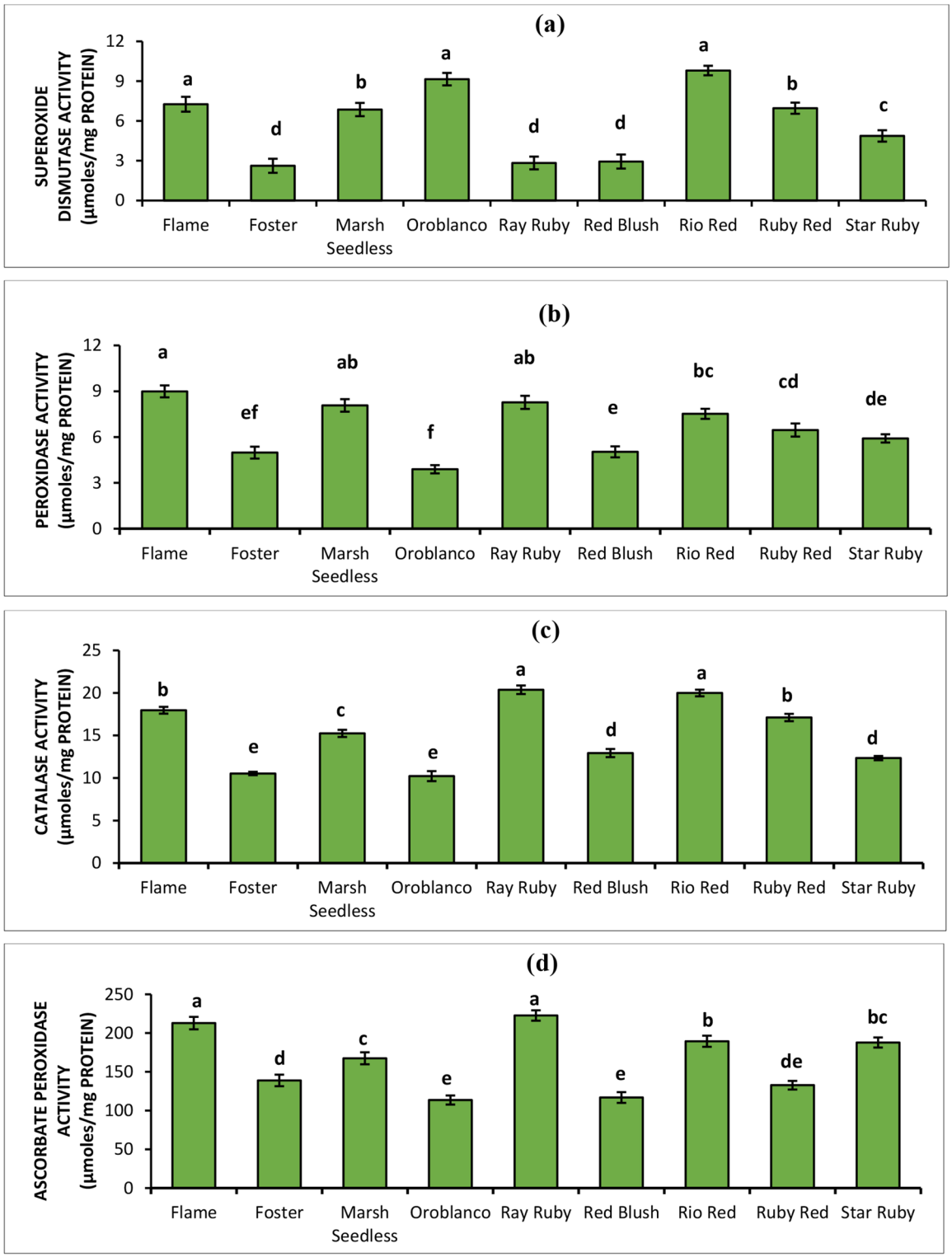

Fig. 4 Antioxidant enzymes i.e., superoxide dismutase **(4a)**, peroxidase **(4b)**, catalase **(4c)**, and ascorbate peroxidase activity **(4d)** among different grapefruit genotypes. Values with the same alphabet (s) are not significantly different at $p \leq 0.05$ (LSD)

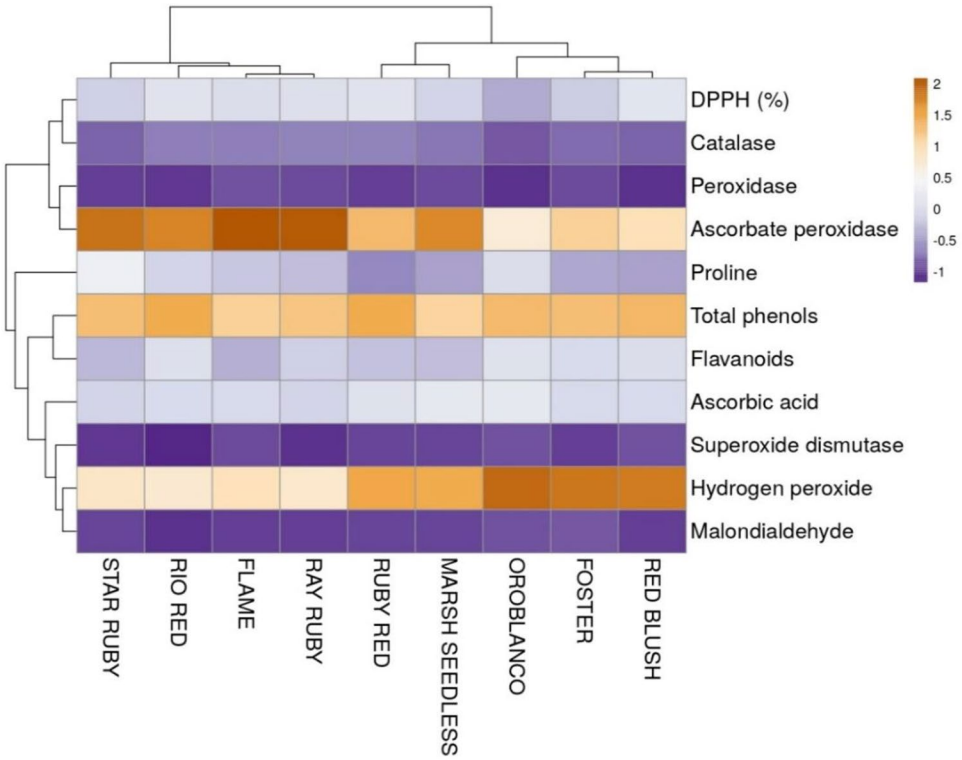

Fig. 5 Heat-map representation of grapefruit genotypes based on components of antioxidant defense mechanism

these genotypes. Further, superoxide dismutase activity was recorded higher in 'Oroblanco' which might have contributed to higher hydrogen peroxide production registered in this genotype. Oustric et al. (2015) proposed that increased hydrogen peroxide during fruit growth could result from either significant superoxide dismutase activity or inefficient catalase and ascorbate peroxidase activity. Our findings suggest that the combination of lower catalase and ascorbate peroxidase activities along with higher superoxide dismutase activity in 'Oroblanco' might have contributed to the elevated hydrogen peroxide levels observed in this genotype. Further, our results showed that 'Ray Ruby' and 'Rio Red' possess sufficient catalase (CAT), peroxidase (POX), and ascorbate peroxidase (APX) activity to effectively reduce hydrogen peroxide levels, as evidenced by their lower hydrogen peroxide and MDA content. This aligns with the findings of Oustric et al. (2015), who noted that higher catalase and ascorbate peroxidase activity led to reduced hydrogen peroxide levels in sweet lime pulp. Additionally, various studies have shown that increased activities of SOD, CAT, and APX correspond to lower levels of H2O2 and MDA (Oustric et al. 2015; Nie et al. 2020; Ma et al. 2021). Thus, from the above discussion, it can be inferred that grapefruit varieties such as 'Ray Ruby', 'Ruby Red' and 'Rio Red' possess good antioxidant potential.

Pearson's Correlation Analysis between Enzymatic and non-antioxidant Compounds

A correlation matrix, based on enzymatic and non-enzymatic antioxidant parameters of nine grapefruit varieties, was generated using Pearson's correlation coefficient (Table 2). A statistically significant association was identified between antioxidant activity, as measured by the DDPH assay with enzymes such as catalase ($r = 0.863^{**}$), and peroxidase ($r = 0.686^{**}$). Similarly, total phenolic content noticed a positive correlation with the DPPH assay ($r = 0.494^{**}$) for the antioxidant potential. The results are in agreement with those of Chen et al. (2021) and Singh et al. (2021) suggesting that polyphenols can serve as indicators of antioxidant effectiveness (Singh et al. 2021). However, no correlation was witnessed between ascorbic acid content and DPPH activity. Franke et al. (2004) reported similar findings and suggested that in citrus, polyphenols might play a dominant role in antioxidant capacity. Moreover, Abuzar et al. (2013) found a weak correlation between vitamin-C content and antioxidant activity (DPPH activity). Conversely, most studies have shown a strong correlation between ascorbic acid content and antioxidant activity measured by DPPH (La Cava and Sgroppo 2015; Rey et al. 2020). Hydrogen peroxide and MDA showed strong negative correlations with antioxidant enzymes, such as peroxidase (MDA: $r = -0.779^{**}$, H_2O_2: $r = -0.724^{**}$), ascorbate peroxidase (MDA: $r = -0.741^{**}$, H_2O_2: $r = -0.715^{**}$), and catalase (MDA: r

Table 2 Pearson's correlation coefficient between bioactive compounds and enzymatic antioxidant compounds

	H_2O_2	MDA	DPPH	FRAP	PHNL	ASC	SOD	CAT	POX	APX
H_2O_2	1.00									
MDA	0.736**	1.00								
DPPH	-0.747**	-0.737**	1.00							
FRAP	-0.625**	-0.736**	0.824**	1.00						
PHNL	-0.294	-0.290	0.494**	0.206	1.00					
FL	0.318	0.300	0.097	-0.188	0.655**					
ASC	0.137	0.132	0.106	0.223	0.257	1.00				
PRLN	-0.349	-0.144	0.088	0.003	0.622**	0.288				
SOD	-0.118	-0.114	-0.050	-0.241	0.130	0.595**	1.00			
CAT	-0.724**	-0.713**	0.863**	0.664**	0.437*	0.134	0.196	1.00		
POX	-0.672**	-0.779**	0.686**	0.740**	0.188	0.148	0.102	0.808**	1.0	
APX	-0.715**	-0.741**	0.678**	0.706**	0.515**	0.113	-0.017	0.681**	0.773**	1.0

** Correlation is significant at the 0.01 level (2-tailed).

* Correlation is significant at the 0.05 level (2-tailed).

H_2O_2-Hydrogen peroxide, MDA- malondialdehyde, DPPH- 2,2diphenyl1picrylhydrazyl radical scavenging activity, FRAP- Ferric reducing antioxidant power, PHNL- Total phenol, ASC-Ascorbic acid, SOD-Superoxide, CAT-Catalase, POX-peroxidase, APX-Ascorbate peroxidase

= -0.713**, H_2O_2: r = -0.672**). This negative correlation between reactive oxygen species and antioxidant enzymes has been consistently reported by various researchers (Cai et al. 2021; Ma et al. 2021; Nie et al. 2020).

Conclusion

The present study indicated 'Ray Ruby', 'Rio Red', 'Ruby Red' and 'Flame' as promising varieties due to their strong antioxidant activity based on DPPH (2,2-diphenyl-1-picrylhydrazyl) and FRAP (ferric reducing antioxidant power) assay. Among the bioactive compounds studied, the total phenols was found to be the main contributor to the antioxidant activity of these varieties (DPPH: $r = 0.494^{**}$). Moreover, 'Rio Red' and 'Ray Ruby' exhibited higher enzymatic activity such as catalase (CAT), peroxidase (POX) and ascorbate peroxidase (APX), which appeared to be effective in reducing hydrogen peroxide levels since lower content of hydrogen peroxide (75.65 µmoles/g FW and 80.24 µmoles/g FW, respectively) and MDA (5.38nmoles/g FWand 6.12 nmoles/g FW, respectively) were detected in them. Naringin, a predominant flavonoid responsible for the bitterness in grapefruit, was observed in lower concentration in deeply pigmented grapefruit varieties such as 'Ruby Red' (23.20 mg/100 ml), 'Rio Red' (24.20 mg/100 ml), and 'Star Ruby '(26.20 mg/100 ml), makes them more beneficial in increasing the grapefruit acceptability among consumers. Our results indicate that grapefruit pulp is rich in phenolic compounds, flavonoids, and antioxidant enzymatic activity. 'Ray Ruby', 'Rio Red', and 'Ruby Red' identified as promising genotypes with the optimum level of both enzymatic and non-enzymatic antioxidant compounds.

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Declarations

Competing Interests The authors declare that they have no conflict of interest.

Ethical Approval This rresearch did not involve Human Participants and/or Animals, so the Ethical approval is not required.

Informed Consent All authors have read the manuscript and agreed to submit it to the journal.

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