

Comprehensive Assessment of Brassica juncea variety rugosa (Pahari Rai) Accessions from the Sub-Himalayan Region: Phytochemical, Antioxidant, Enzymatic, Mineral, and Fatty Acid Profiling

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Comprehensive Assessment of *Brassica juncea* variety rugosa (Pahari Rai) Accessions from the Sub-Himalayan Region: Phytochemical, Antioxidant, Enzymatic, Mineral, and Fatty Acid Profiling

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Abstract

Brassica juncea var. rugosa (Pahari rai), a broad leafy cruciferous vegetable is an essential and easy source of nutrition with numerous health-promoting factors. The phytochemical and antioxidant activity of leaf extract showed the highest phenolic, flavonoid, orthodihydroxyphenol (ODP), and tannin content observed in the EEC-25 hydro-ethanolic leaf extract. The highest protein content in EEC-25 followed by IC-524259 and IC-350800. The foremost concentration of carbohydrates was observed in germplasms IC-363758 (31.47±0.31 mg glucose g⁻¹), IC-410471 (31.12±0.18 mg glucose g⁻¹) and EEC-25 (26.45±0.19 mg glucose g⁻¹). *In-vitro* antioxidant potential viz., total antioxidant content was observed highest in EEC-25 hydro-ethanolic leaf extract (41.91±0.28 mg AAE g⁻¹), FRAP activity in EEC-25 (5.91±0.68 mg AAE g⁻¹), maximum 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity in IC-597933 (IC₅₀=21.45±3.4 µg mL⁻¹), 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), free radical scavenging activity (FRAP) in IC-524259 (94.17±0.48 µg mL⁻¹), superoxide free radical scavenging activity of hydro-ethanolic leaf extract of PR-15 (IC₅₀=124.37 µg mL⁻¹), highest metal ion chelating activity of EEC-25 hydro-ethanolic extract (IC₅₀=52.22 µg mL⁻¹), maximum reducing power activity in EEC-25 and IC-399839, all being significantly different (p≤0.05) from each other. Positive correlations have been observed among the total antioxidant, phenolic, flavonoid content, ODP, DPPH, and FRAP. Micronutrient analysis Pahari rai leaf was observed to be a rich source of essential minerals such as Mn, Fe, Zn, Ca, Cu, and Mg. Pahari rai was observed to be a rich source of monounsaturated fatty acid (MUFA) and polyunsaturated fatty acid (PUFA). An essential metric for assessing the nutritional value of various edible oils is the ω6:ω3 ratio was observed to be maximum in IC-350800 (2.53), IC-338535 (2.34), and IC-410471 (2.19). The multiutility of this leafy vegetable against numerous pathological ailments was attributed to the pharmacological activities of phytochemicals and the development of functional food for human consumption. Therefore, the current study would aid in a deeper comprehension of the chemical composition of compounds that may be further separated as well as investigations into their potential pharmacological significance.

Key Words

Brassica juncea variety rugosa, Phytochemicals, Antioxidant, Correlation, Mineral

Introduction

Leafy vegetables are a cost-effective source of nutrients that everyone can adopt. *Brassica juncea* var. rugosa, popularly known as broad leafy mustard belongs to the family Cruciferae, also known as Brassicaceae. It is a tall, slow-growing annual green leafy vegetable (Rabi season: October-March) with large leaves whose color ranges from light green to deep purple. It is native to Central and Eastern Asia. This leafy mustard is a staple crop, grown and supplied mostly in Asian nations such as India, China, Japan, Nepal, and Bhutan. In India, it is mainly cultivated in small patches in the home backyard, agricultural land, and mountainous regions including Uttarakhand, Himachal Pradesh, Arunachal Pradesh, Nagaland, Meghalaya, Mizoram, Assam, and Manipur. The Popularity of this leafy vegetable owes to its ease of cultivation, low risk of production, traditionality, and versatility of applications.

The crop is grown by direct sowing or transplantation (Parajuli 2015). The leaves of well-grown rugosa are 40-50 cm long and 20-25 cm wide having flat petioles, smooth to crumpled margins, fleshy and non-hairy (Rauniyar and Bhattarai 2017; Adhikari et al. 2022). Enriched with water content, fiber, and vitamins along with several

phytochemicals and nutrients, rugosa leaf is widely considered a wholesome food (Pant et al. 2020). Being one of the mustard varieties, *Brassica juncea* var. rugosa is rich in nutrients, such as polyphenols and glucosinolates that hold value in promoting human health and are commonly eaten in the form of pickle (Harbaum et al. 2008; Lin et al. 2011).

Phytochemicals, also known as secondary metabolites, are biologically active molecules synthesized by plants through primary or secondary metabolism that aid in the plant's defensive mechanisms against infection. Recent research suggests that consuming plants high in polyphenols can significantly reduce the development of cardiovascular disease, neurological illness, cancer, and diabetes (Chauhan et al. 2012; Sharma & Cannoo, 2016). *Brassica juncea* var. rugosa is surplus in many such phytochemical compounds that exhibit several medicinal and biological properties, along with high protein and carbohydrate content (Moirangthem and Praveen 2020). It includes considerable iron, calcium, protein, and Vitamins A, B, C, and E (Pant et al. 2020). Due to their great nutritional value and disease-fighting capabilities, green mustard leaves can be a nutritious substitute for most leafy winter vegetables. Antioxidants are important in inhibiting various diseases and pathophysiological dysfunctions (Bhardwaj et al. 2019).

It is a promising low-cost source of critical nutrients, especially beneficial for vulnerable groups such as women and developing children. In response to this potential, germplasms from diverse hilly regions in various Indian states were meticulously collected. Subsequently, a comprehensive analysis involving phytochemical, antioxidant, mineral, and fatty acid characterization was conducted to pinpoint. The ultimate goal is to leverage these findings to enhance people's nutritional status and overall well-being. Pahari rai's significance lies in its role as an edible mustard species that has been consistently exploited for its nutraceutical attributes, holding immense potential for future direct applications in healthcare products. Natural antioxidants derived from plant sources are gaining popularity since they have fewer negative effects. The detailed insights into the nutraceutical properties derived from this research have the potential to revolutionize the development of health-enhancing products, providing accessible and cost-effective solutions to address nutritional deficiencies and improve public health. If not neutralized, free radicals can damage different cells and organs in humans and plants, increasing their vulnerability to biotic and abiotic stresses and leading to various degenerative illnesses. An innate or built-in antioxidant system can scavenge these free radicals, contributing directly to the plant defense system and promoting human health by restraining degenerative ailments such as cardiovascular diseases and cancer and even reducing the effects of aging. A review of the existing literature reveals a scarcity of information and research articles on *Brassica juncea* var. rugosa. Consequently, this study was designed to assess this particular variety's phytochemical composition, antioxidant properties, mineral content, and fatty acid profile.

Material and Methods

Plant Material and Extraction

The details of the plant material that has been used for secondary metabolite profiling, enzymatic activity, mineral analysis, and fatty acid profiling of *Brassica juncea* var. rugosa are provided in Table 1. The seeds were procured from NBPGR, New Delhi, and were sown at Crop Research Center (CRC), G. B. Pant University of Agriculture and Technology, and the research article was published on morphological data (Adhikari et al., 2022). For the preparation of extracts of leaf, 1 g of dried leaf powder was dissolved in 100 mL 70% methanol and kept in an orbital shaker (UNILAB) at 37°C for 24 hours. The solution was filtered using Whatman filter paper no. 1 and then the filtrate was concentrated in rotavapor (Biogen) at 55°C (Vaijanathappa et al. 2008). The collected concentrate was re-suspended in absolute methanol to the final concentration of 1 g mL⁻¹.

Table 1: *Brassica juncea* var. rugosa germplasm from various sub-Himalayan regions of India

S.No.	Accessions	Cultivar Name	District	State	Latitude	Longitude
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1	IC-338751	Peli sarson	Champawat	Uttarakhand	29° 20' 9.9528" N	80° 5' 27.6936" E
2	IC-524259	Rai	Pauri	Uttarakhand	30° 09' 10.30" N	78° 46' 37.56" E
3	IC-338535	Lai	Almora	Uttarakhand	29° 35' 39.0804" N	79° 39' 14.0148" E
4	EEC-25	Lahi	Ranikhet	Uttarakhand	29° 38' 60.00" N	79° 25' 12.00" E
5	IC-399826	'Chhanakti' sarson	Chamba	Himachal Pradesh	32° 33' 19.12" N	76° 07' 35.29" E
6	IC-399839	Desi sarson	Kangra	Himachal Pradesh	32° 5' 59.2944" N	76° 16' 8.7744" E
7	IC-399880	Desi sarson	Kullu	Himachal Pradesh	31° 57' 28.2636" N	77° 6' 34.0524" E
8	IC-363758	Kodra	Sirmaur	Himachal Pradesh	30° 33' 46.2456" N	77° 28' 12.7092" E
9	IC-597917	Tusut	East Siang	Arunachal Pradesh	28° 4' 12" N	95° 19' 48" E
10	IC-597873	Ogiyi	West Siang	Arunachal Pradesh	28° 23' 59.99" N	94° 32' 59.99" E
11	IC-417128	Pettu (Lai patta)	Upper Siang	Arunachal Pradesh	28° 36' 37.33" N	95° 2' 51.11" E
12	IC-597933	Sarson	Lower Subansiri	Arunachal Pradesh	27° 47' 59.99" N	93° 35' 59.99" E
13	IC-298019	Antum	Serchhip	Mizoram	23° 20' 29.9688" N	92° 51' 0.8172" E
14	IC-410471	Tryso	Ri-Bhoi	Meghalaya	25° 53' 14.9676" N	91° 52' 15.0996" E
15	IC-350800	Janem	Jaintia Hill	Meghalaya	25° 27' 0" N	92° 12' 0" E
16	IC-276011	Rayo	Tinsukia	Assam	27° 29' 22.07" N	95° 21' 36.52" E
17	IC-413486	Kala sarisha	Dhalai	Tripura	23° 54' 58.82" N	91° 53' 16.15" E
18	IC-597821		Jhansi	Uttar Pradesh	25° 30' 0" N	78° 30' 0" E
19	Pusa Saag		Pantanagr	Uttarakhand	28° 58' 12" N	79° 24' 36" E
20	PR-15 (Kranti)		Pantnagar	Uttarakhand	28° 58' 12" N	79° 24' 36" E

Phytochemical estimation

Total phenolic content

The total phenolic content was determined by the Folin-Ciocalteu reagent (FCR) based method (**McDonald et al. 2001**). Briefly, 0.5 mL of hydro-ethanolic extracts were dried in the water bath. Subsequently, 1 mL of DW was added followed by 0.5 mL of 50% (v/v) FCR and 1 mL Na₂CO₃. The standard curve was established using various concentrations (10 µg-100 µg) of gallic acid and the results were expressed as gallic acid equivalents (mg GAE g⁻¹ extract).and concentration was measured by UV-Vis spectrophotometer (Thermo Scientific Genesys 10S) at 725 nm.

Total flavonoid content

The total flavonoid content was determined by the method of **Mandal et al. (2009)**. 200 µL leaf extract was diluted with methanol up to 1 mL followed by 4 mL of distilled water. Thereafter, 0.3 mL each of 5% Sodium nitrite and 10% AlCl₃ were added and the tubes were left at RT for 5 minutes. AlCl₃ forms stable complexes with C-4 keto

groups and C-3 or C-5 hydroxyl groups of flavones and flavonols. The reaction was terminated by adding 2mL of 1M NaOH in each tube and the absorbance of colored solution was recorded at 510 nm. The concentration of total flavonoids was calculated as mg quercetin equivalent g^{-1} extract (mg QE g^{-1}), from the standard curve of quercetin. The concentration of quercetin used for preparing the calibration curve ranged between 10-100 μg .

Orthodihydroxy phenol

The content of the orthodihydroxy phenol (ODP) was estimated by Arnov's reagent (**Arnow 1937**). Briefly, 0.2 mL extract was added to 1 mL 0.05N HCl. Sequentially add 1 mL of Arnov's reagent, 10 mL distilled water, and 2 mL of 10 N NaOH. The tubes were incubated for 2 minutes and absorbance was taken at 515. By using the standard curve of catechol the amount of orthodihydroxy phenol was calculated as mg catechol equivalent g^{-1} extract (mg CE g^{-1}). The concentration of ODP was calculated from the standard curve of catechol (10-100 μg).

Total tannin content

Tannin quantification was done by the Folin-Denis method given by **Bae et al. (1993)**. 0.2 mL hydro-ethanolic extract was added to 0.5 mL Folin-Denis reagent followed by 1 mL saturated Na_2CO_3 . The volume of the reaction mixture was made up of 10 mL using DW and incubated for 30 min at RT. Tannins reduce phosphotungstomolybdic acid to a blue color solution under alkaline conditions. The intensity of the blue color was read at 760 nm. The amount of tannin was estimated as mg tannic acid equivalent g^{-1} extract (mg TAE g^{-1}) from the calibration curve of the tannic acid. The concentration of tannic acid used for preparing the calibration curve ranged between 10-100 μg .

Total Soluble Protein estimation

The quantitative analysis of the protein content in fresh leaves was determined according to **Lowry et al. (1951)**. FCR gives a blue-colored complex when react with protein which is absorbed at 660 nm. A standard curve was prepared using different concentrations of BSA (100 $\mu\text{g mL}^{-1}$).

Total carbohydrate content

The phenol-sulphuric acid method was performed to quantify the total carbohydrate content in fresh leaves of *Brassica juncea* var. rugosa (**Hossain et al. 2015**). The concentration was measured spectrophotometrically at 490 nm with glucose as standard (100 $\mu\text{g mL}^{-1}$).

In-vitro Antioxidant activity

Determination of Total antioxidant activity

The total antioxidant activity was evaluated by phosphomolybdenum method. The plant extract reduces Mo (VI) to green phosphate Mo (V) complex under acidic pH. (**Prieto et al. 1999**). 1 mL of freshly prepared phosphomolybdate reagent was added to 1 mL of leaf extract and incubated at 95° C for 90 min. Once the reaction mixture cooled to RT, the absorbance was recorded at 695 nm. The total antioxidant activity of the extract was expressed as mg ascorbate equivalents g^{-1} of extract. Ascorbic acid as a standard ranging between 10-100 μg was used for preparing the calibration curve.

Ferric reducing antioxidant power (FRAP)

The FRAP assay was determined spectroscopically at 593 nm by creating a complex with Fe(III)-TPTZ (**Benzie and Strain 1996**). This assay is based on the reduction of 2,3,5-triphenyl-1,3,4-triaza-2-azoniacyclopenta-1,4-diene chloride-ferric iron (Fe(III)-TPTZ) complex to the blue ferrous form (Fe (II)-TPTZ) at low pH. Briefly, FRAP reagent was pre-warmed at 37°C then 1.8 mL was added to 100 μL extract followed by 100 μL distilled water.

The antioxidant potential of extracts was evaluated from the calibration curve of ascorbic acid prepared in methanol for which a 10-100 µg concentration range was used.

DPPH radical scavenging activity

The free radical scavenging capacity of leaf extracts was determined using DPPH according to **Yen and Duh 1993** with some modification. DPPH free radical absorbs at 517 nm i.e., purple hue and the addition of substrate causes decolorization of purple color. In short, 1 mL of hydro-ethanolic solution containing varying concentrations (100 -500 µg mL⁻¹) of leaf extracts or ascorbic acid was mixed with 0.5 mL of (0.5 mM) hydro-ethanolic solution of DPPH and incubated for 30 min in the dark at RT. The free radical scavenging activity was calculated by the equation:

$$DPPH\ Scavenging\ (\%) = \left[1 - \left(\frac{Sample_{Abs}}{Control_{Abs}} \right) \right] \times 100$$

Superoxide free radical scavenging activity

Superoxide anion radicals are one of the ROS resulting from various metabolic processes *in vivo*. The superoxide anion scavenging ability of hydro-ethanolic leaf extracts was evaluated following the method of **Nishikimi et al. (1972)**. The active compounds in plant extract quench anionic radicals inhibiting the reduction of nitro blue tetrazolium (NBT) to purple formazan. 3 mL of tris-HCl (16 mM, pH-8.0) was added to a premix containing 1 mL of 50 µM NBT and 1 mL of 78 µM NADH. The reaction was started by adding 1 mL of 10µM phenazine methosulfate. Thereafter, different concentrations of plant extract (100-500 µg mL⁻¹) were added and the reaction mixture was incubated for 5 minutes at 25°C. The absorbance was read at 560 nm, decreasing absorbance correlates to increasing superoxide anion scavenging activity of extracts. The percentage inhibition of superoxide anion was calculated using the given formula:

$$Superoxide\ radical\ Scavenging\ (\%) = \left[1 - \left(\frac{Sample_{Abs}}{Control_{Abs}} \right) \right] \times 100$$

Metal ion Chelating activity

The metal ion chelation ability of the extract was estimated according to **Decker and Welch (1990)**. Briefly, 1 mL of 0.1 mM FeSO₄ was mixed with 1 mL of extract at various concentrations. Subsequently, 1 mL of 0.25 mM ferrozine was added followed by 10 min incubation at RT. Ferrozine chelates Fe (II) to form a pink-colored ferrozine-Fe (II) complex. Plant extract competes with ferrozine, restricting the initial reaction thus resulting in a decrease in red color intensity. The absorbance was measured at 562 nm with EDTA (100-500 µg mL⁻¹) as a reference and the percentage inhibition of ferrozine-Fe II complex formation was given by the following equation:

$$Ferrous\ ion\ Chelating\ Activity\ (\%) = \left[1 - \left(\frac{Sample_{Abs}}{Control_{Abs}} \right) \right] \times 100$$

ABTS Free Radical Scavenging Activity

ABTS radical cation decolorization assay performed the free radical scavenging activity of plant samples (**Re et al. 1999**). 7 mM ABTS was mixed with 2.45 mM potassium persulfate (1:1) and allowed to react by incubating in the dark at RT for 12-16 hr, resulting in ABTS^{•+} radical production. This solution was diluted with methanol till an absorbance of 0.700 ± 0.01 units at 734 nm was obtained. 5 µL of plant extract was added to 3.995 mL of diluted ABTS^{•+} solution and allowed to react for 30 min. Ascorbic acid is used as standard (100 µg mL⁻¹). The absorbance was measured at 734 nm and percentage inhibition was calculated using the formula.

$$ABTS\ scavenging\ effect = \frac{AB - AA}{AB} * 100$$

AB=absorbance of ABTS radical + methanol

AA= absorbance of ABTS radical + sample extract/standard

Enzymatic Analysis

Peroxidase determination

Peroxidase ($\mu\text{mol guaiacol min}^{-1} \text{ g}^{-1} \text{ FW}$) acts as an antioxidant enzyme. 100 mg of fresh leaf tissue was homogenized with 1.5 ml ice-cold 100 mM sodium phosphate buffer (pH 7.5) containing 1 mM EDTA, 1% PVP and 10 mM β -mercaptoethanol. The homogenate was centrifuged at 10,000 RPM for 15 min at 4°C and supernatant was collected for enzymatic assay. A reaction mixture was prepared containing 3 mL of 50 mM guaiacol in 0.1 M phosphate buffer (pH 6.5), to this 0.1 mL of enzyme extract was added. The reaction was initiated by adding 0.1 mL of 800 mM H_2O_2 and a change in absorbance was recorded at 470 nm for 3 min at an interval of 30 sec (Shannon et al. 1966).

Superoxide dismutase determination

Superoxide dismutase ($\mu\text{mol pyrogallol min}^{-1} \text{ g}^{-1} \text{ FW}$) is also an antioxidant enzyme that catalyzes the dismutation of superoxide to H_2O_2 and O_2 . The enzyme was extracted the same as peroxidase. For SOD quantification, 1.5 mL of 100 mM 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 enzyme extract were added into the cuvette. Change in absorbance was recorded at 420 nm at an interval of 30 seconds up to 3 min (Marklund and Marklund 1974).

Mineral Composition in Leaves

The mineral analysis of Mn, Zn, Pb, Fe, Mg, Cd, Cu, Ni, and Ca in the leaf was estimated through atomic absorption spectroscopy (AAS) (SensAA GBC Scientific Equipments) after digestion of dried and powdered leaf samples (Moscow and Jothienkatachalam 2012). The elemental analysis was based on the absorbance of the species at its resonance wavelength.

Micronutrient (NPK) Analysis

For the analysis of nitrogen and potassium, dried leaf samples were ground and then digested by H_2SO_4 and H_2O_2 on an open digestion furnace at 380°C followed by extraction with 4 M HNO_3 for 4 h. The Kjeldahl method determined the nitrogen content in the digested solution according to Snell and Snell (1959) while potassium content was determined by flame photometric method (Bhargava and Raghupathi 1994). Phosphorus content was estimated by the molybdate-vanadate method. The orthophosphates obtained in triacid digestion react with ammonium molybdate, and ammonium vanadate in an HNO_3 medium producing a yellow color complex that absorbs at 470 nm. The concentration was estimated against a standard curve of phosphorous (Jackson 1973).

Fatty acid profiling in seeds of *Brassica juncea* var. *rugosa* germplasms

The fatty acid profile is an indicator of the quality and stability of the oil. Oil was extracted from seed by cold percolation method. 20 mg of oil sample were transferred into the soxhlet extraction flask. Toluene was mixed with dried methanolic H_2SO_4 in the ratio of 99:1. Nitrogen was passed for 2 min to get rid of moisture. The sample was refluxed for 12 hrs at 50°C and then cooled down. Further, 4% NaCl and 4% KCl solution and 20mL of n-Hexane were added to it. The n-hexane layer was vortexed and separated. Further 4mL Na_2CO_3 (4%)/KCl (4%) was added and vortexed again, and the n-hexane layer was separated. 1-1.5 μL of the upper layer was subjected to gas chromatography. The injection volume was 1-1.5 μL and the carrier gas was nitrogen. The flow rate was 40-50 mL mL^{-1} with split injection mode. A flame ionization detector was used to determine the analyte. The temperature of the detector was kept between 250-300°C (Christie 1990). Selfed seeds were analyzed for fatty acid profiles, viz., palmitic, stearic, oleic, ecosinic, erucic,

linoleic, and linolenic acid by gas chromatography (NUCON – 5765) through the separation of methyl esters on a diethylene glycol succinate (DEGS) column. The column was maintained at 190°C with a nitrogen flow of 40 ml/min and the flame ionization detector was maintained at 240°C. Hydrogen and air pressure rate was maintained at 2 kg/m². 10 to 12 oven-dried (45°C, 6 hours) seeds were crushed in a capped test tube, 0.5 ml of petroleum ether (BP40 – 60°C) was added, and samples were left for one hour. The supernatant was transferred to another capped test tube, and 0.5 ml of 0.05 M sodium methoxide solution was added. After 45 minutes, 0.75 ml of 8% NaCl solution was added, followed by vortexing. Layers were separated and 1 µL of the upper layer was injected for fatty acid analysis (Chauhan et al. 2002).

Statistical Analysis

One way Analysis of variance (ANOVA) and Duncan post hoc tests were carried out for the data obtained in various experiments. Statistical analysis of the data obtained was performed using the software SPSS. The graphs were plotted with the help of MS-Excel for Windows version 7. Results were expressed as mean±SD_m (n=3). The means and standard deviation (SD_m) were calculated using MS Excel. The probability (p) value<.05 was considered significant for ANOVA and the marked correlations among the different assay.

Results and Discussion

Total Phenolic, flavonoid, orthodihydroxyphenol, and tannin content

Phenolic compounds and flavonoids are key indicators of the antioxidant capacity of plant extracts, while ODP (Orthodihydroxy phenol) and tannins contribute to their characteristic astringency and medicinal properties. A significant difference (p<0.05) was observed in the total phenolic, flavonoid, ODP, and tannin contents. Figure 1 illustrates the various phenolic, flavonoid, ODP, and tannin concentrations in hydro-ethanolic extracts. Among the 20 samples, the hydro-ethanolic extracts of EEC-25 (6.45 mg g⁻¹ DW), IC-524259 (5.45 mg g⁻¹ DW), IC-338751 (4.89 mg g⁻¹ DW), and IC-597933 (4.99 mg g⁻¹ DW) showed the highest concentrations of phenolic content. In contrast, the rest of the samples had similar concentrations ranging from 2.41 to 3.40 mg g⁻¹ DW. The maximum flavonoid concentrations were found in EEC-25 (6.32 mg g⁻¹ DW) and Pusa saag

(5.11 mg g⁻¹ DW). The highest ODP contents were observed in EEC-25 (4.66 mg g⁻¹ DW), IC-399839 (3.31 mg g⁻¹ DW), and IC-597821 (3.08 mg g⁻¹ DW). The highest tannin contents were observed in EEC-25 (3.46 mg g⁻¹ DW), IC-338751 (2.22 mg g⁻¹ DW), and IC-597917 (2.13 mg g⁻¹ DW). The variability in total Phenolic, flavonoid, ODP, and tannin content in hydro-ethanolic extracts of different germplasm of *Brassica juncea* var. *rugosa* can be attributed to several factors. Genetic diversity among germplasm plays a significant role, as variations in genes governing the biosynthesis of secondary metabolites lead to differences in the production of phenolics, flavonoids, and tannins. Environmental factors such as soil composition, temperature, light intensity, and water availability during plant growth also influence the accumulation of these compounds. The developmental stage at harvest further impacts the phytochemical profile, as metabolite levels vary with plant maturity. Additionally, differences in extraction efficiency due to procedural variations and the inherent diversity of metabolite structures contribute to variability.

Phenolic compounds, characterized by a benzene ring and one or more hydroxyl groups, play crucial roles as both radical scavengers and metal chelators due to their structural chemistry. According to Moirangthem and Praveen (2020), the hydro-ethanolic extracts of *Brassica juncea* var. *rugosa* leaves contain total phenolic, flavonoid, and tannin concentrations of 6.52 mg g⁻¹, 1.25 mg g⁻¹, and 2.64 mg g⁻¹, respectively. The total phenol concentration in the study ranged from 4.68-5.47 mg g⁻¹, while ODP concentrations ranged from 0.40-0.99 mg g⁻¹. Flavonoid concentrations varied from 3.10-4.88 mg g⁻¹ as reported by Pant et al. (2020), and the total flavonoid content in *Brassica juncea* var. *gemmifera* was 4.02 mg g⁻¹ as found by Sun et al. (2018). ODP, an essential component of plant polyphenols, was reported by Parihar et al. (2012) to range from 0.36-0.48 mg g⁻¹. Mobeen et al. (2021) found tannin concentrations in *Brassica juncea* leaves to be between 0.3-0.5 mg g⁻¹, which is lower than the values observed in the present study. Tannins contribute to astringency and defend plants from insects and pests by binding to the digestive proteins of insects, creating insoluble complexes that render the enzymes inactive (Rao et al. 2017).

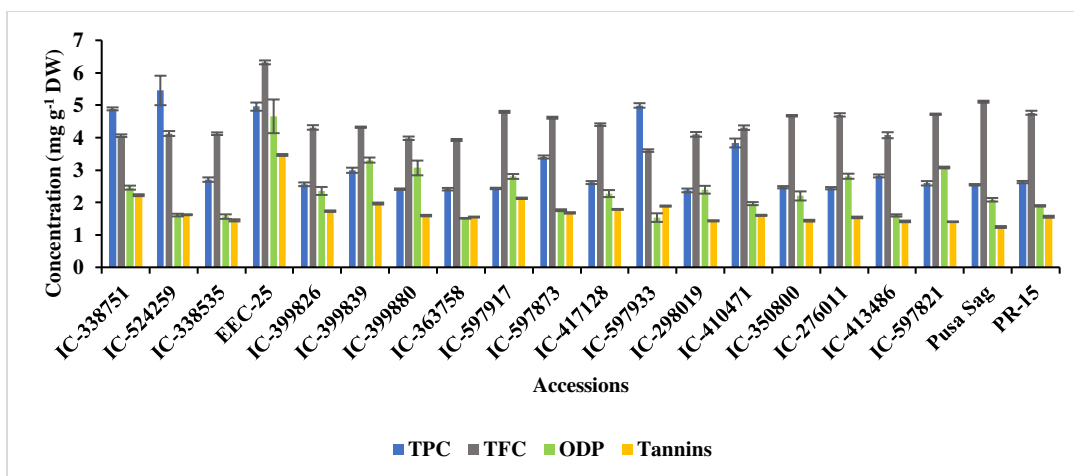


Fig 1: Total phenolic content (TPC), total flavonoid content (TFC), orthodihydroxyphenol (ODP) and tannins concentration in twenty germplasm of *Brassica juncea* var. *rugosa*

Total Soluble Protein and Total Carbohydrate Content

The protein content of *Brassica juncea* var. *rugosa* is depicted in Fig. 2. There was a significant difference ($p < 0.05$) in the total soluble protein concentration, expressed as $\mu\text{g BSA g}^{-1}$ FW, in the leaves of different accessions. The maximum protein content was observed in EEC-25 ($400.97 \pm 16.5 \mu\text{g BSA g}^{-1}$ FW), followed by IC-524259 ($380.09 \pm 17.22 \mu\text{g BSA g}^{-1}$ FW) and IC-350800 ($379.23 \pm 18.26 \mu\text{g BSA g}^{-1}$ FW). Lower concentrations of protein were noted in genotypes IC-417128 ($209.07 \pm 10.73 \mu\text{g BSA g}^{-1}$ FW), IC-597933 ($231.37 \pm 23.12 \mu\text{g BSA g}^{-1}$ FW), and IC-298019 ($237.95 \pm 19.76 \mu\text{g BSA g}^{-1}$ FW). Total soluble proteins in fresh samples of *B. juncea* var. *rugosa*, determined using the Bradford technique by **Moirangthem and Praveen (2020)**, were found to be 2.2 mg g^{-1} . The soluble protein content in the individual edible parts of *Brassica juncea* var. *gemmifera* ranged from 38.81 – 91.10 mg g^{-1} (**Sun et al., 2018**). Another study reported total protein concentrations in *B. juncea* leaves varying from 0.6 – 0.9 mg g^{-1} (**Mobeen et al., 2021**).

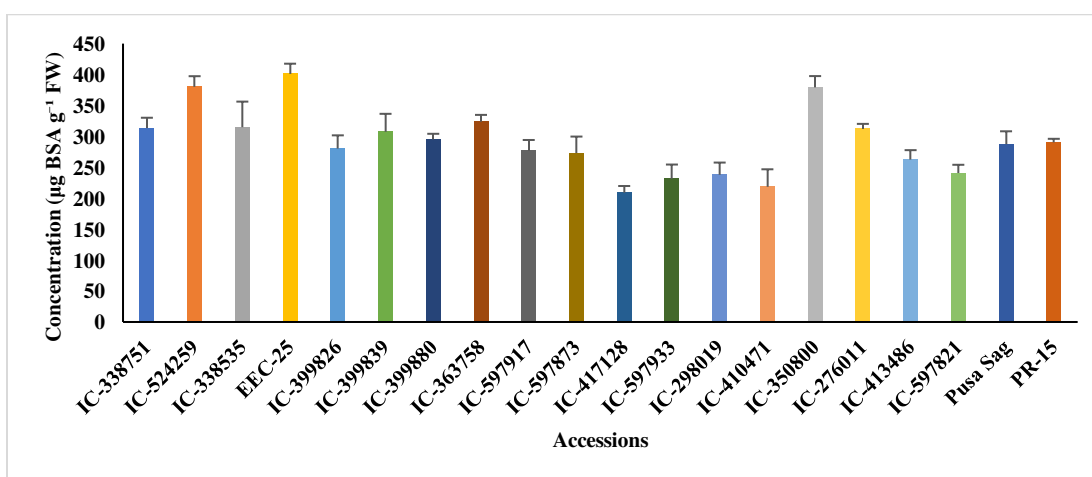


Fig 2. Total soluble protein in *B. juncea* var. *rugosa* germplasm

The total carbohydrate content of *Brassica juncea* var. *rugosa* is shown in Fig. 3. There was a significant difference ($p < 0.05$) in the total carbohydrate concentration, expressed as mg glucose g^{-1} FW, in the leaves of different accessions. The highest concentrations of carbohydrates were observed in genotypes IC-363758 ($31.47 \pm 0.31 \text{ mg}$

glucose g⁻¹ FW), IC-410471 (31.12±0.18 mg glucose g⁻¹ FW), and EEC-25 (26.45±0.19 mg glucose g⁻¹ FW). In contrast, the lowest concentrations of carbohydrates were found in IC-399839 (5.06±0.25 mg glucose g⁻¹ FW), IC-338535 (7.63±0.23 mg glucose g⁻¹ FW), and IC-597821 (7.60±0.10 mg glucose g⁻¹ FW). Carbohydrate profile of this plant demonstrated that leaves are a rich source of sugar and can act as an energy source. The carbohydrate content of fresh leaf samples of *B. juncea* var. *rugosa* was determined to be 16.66 mg g⁻¹ by **Moirangthem and Praveen (2020)**. In a study by **Mobeen et al. (2021)**, the carbohydrate content in leaves of *B. juncea* ranged from 0.47 to 0.89 mg g⁻¹. Green vegetables, when consumed alongside other carbohydrate-rich foods like cereals, are recognized as a notable source of soluble carbohydrates that the body primarily utilizes for energy provision, thereby contributing to substantial energy levels that facilitate metabolism and the assimilation of meals (**Saha et al. 2015**). The differences in total soluble protein and total carbohydrate content among various samples are due to genetic, environmental, and physiological factors. Genetic diversity among the samples results in inherent differences in the metabolic pathways that control protein and carbohydrate synthesis. Environmental influences like soil quality, nutrient availability (e.g., sources of nitrogen and carbon), temperature, and water stress can greatly affect the levels of proteins and carbohydrates since these substances are crucial in stress responses and energy storage. Additionally, the growth stage or maturity of the samples at the time of collection impacts these biomolecules, with proteins being more plentiful during periods of active metabolism and carbohydrates accumulating during storage or energy excess phases.

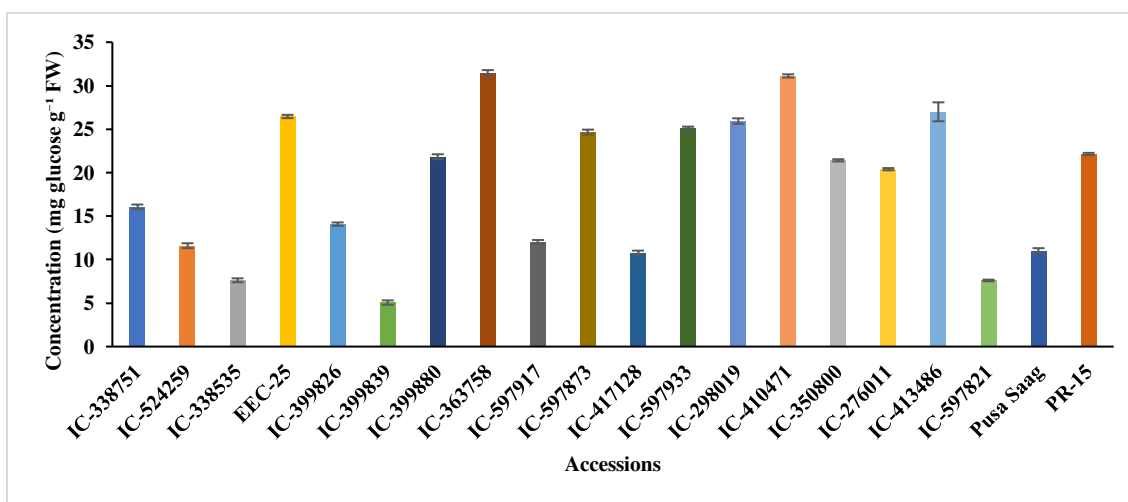


Fig 3: Total carbohydrate in twenty germplasm of *B. juncea* var. *rugosa*

In-vitro Antioxidant Activity

Total antioxidant content

The total antioxidant content of *Brassica juncea* var. *rugosa* was measured as mg ascorbic acid equivalent (AAE) per gram of dry weight (DW) extracts. The total antioxidant content of all accessions differed significantly ($p < 0.05$), with the highest values observed in EEC-25 (41.91±0.28 mg AAE g⁻¹ DW), followed by IC-399839 (39.96±0.28 mg AAE g⁻¹ DW) and IC-276011 (36.2±0.45 mg AAE g⁻¹ DW), as shown in Fig. 4. A significant amount of antioxidant activity in green vegetables is an indicator of disease prevention. A similar study by **Pant et al. (2020)** reported that the antioxidant content of *Brassica juncea* var. *rugosa* ranged from a low of 14.94 mg g⁻¹ to a high of 20.09 mg g⁻¹.

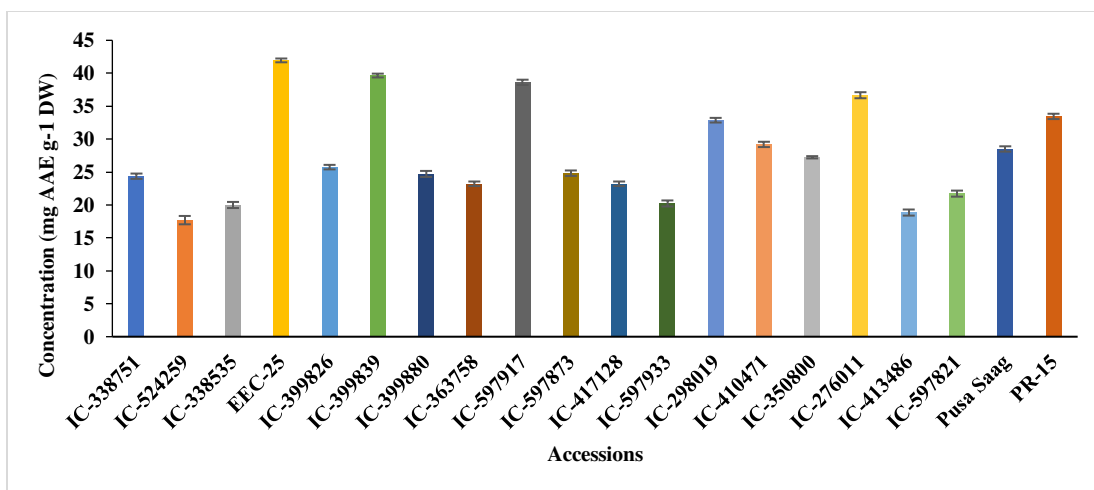


Fig 4: Total antioxidant content in leaves of *B. juncea* var. *rugosa* in hydro-ethanolic extract

Ferric ion-reducing antioxidant power (FRAP)

Ferric-reducing antioxidant power (FRAP) activity, expressed as mg ascorbic acid equivalent (AAE) per gram of dry weight (DW), varied significantly ($p < 0.05$) among all the accessions, as detailed in Fig. 5. The highest FRAP activity was observed in the EEC-25 leaf extract (5.91 ± 0.68 mg AAE g⁻¹ DW), followed by the IC-597917 extract (2.36 ± 0.06 mg AAE g⁻¹ DW). The FRAP activity reported in our study is higher than that of Deng et al. (2013) for *Brassica juncea*, which was 8.28 ± 0.58 μ mol g⁻¹.

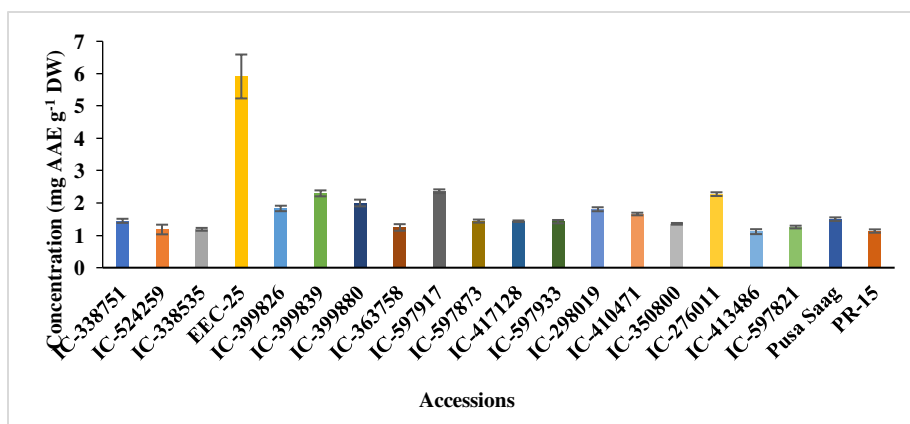


Fig 5: Ferric Reducing Antioxidant Power in leaves of *B. juncea* var. *rugosa* in hydro-ethanolic extract

ABTS Free Radical Scavenging Activity

All hydro-ethanolic extracts' free radical scavenging activity was expressed as percentage inhibition against ABTS. IC-524259 showed the highest ability to quench ABTS ($94.17 \pm 0.48\%$), followed by IC-399880 ($92.91 \pm 0.62\%$), IC-597917 ($93.83 \pm 0.62\%$), IC-597873 ($93.09 \pm 0.77\%$), and Pusa Saag ($94.01 \pm 0.62\%$) as depicted in Fig. 6. The percentage inhibition of *Brassica rapa* subsp. *Chinensis* (Pak Choi) reported by Khanam et al. (2012) was 96.97%, which corresponds well with the values observed in our study. In another study by Fernández León et al. (2014), the ABTS activity was reported to be 56.62 mg/100g in the petroleum ether extract of *Brassica oleracea* L. convar. *capitata* var. *aabauda*.

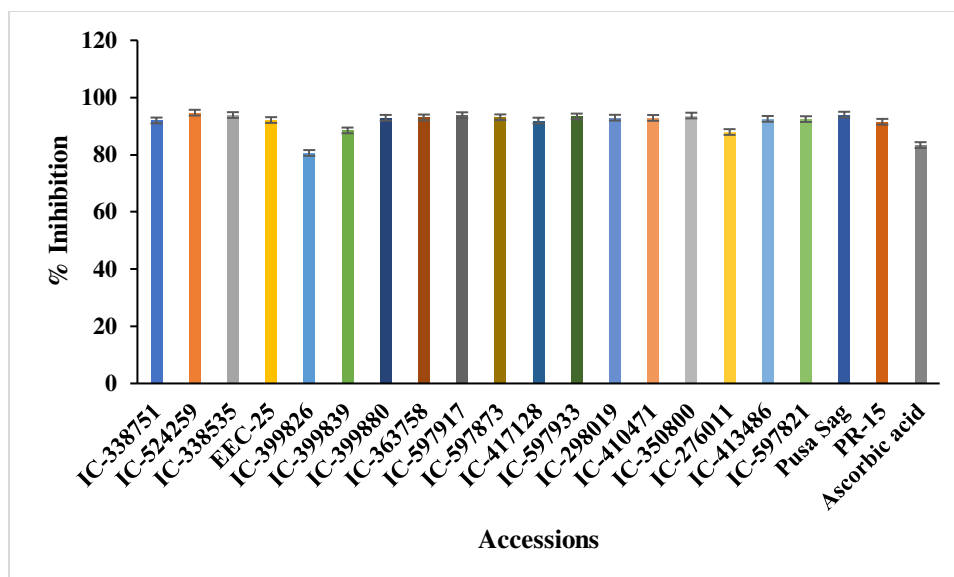


Fig 6: ABTS Free Radical Scavenging Activity in hydro-ethanolic leaf extract of *B. juncea* var. *rugosa*

DPPH, Superoxide free radical, and metal ion chelation scavenging activity

Table 2: IC₅₀ of different antioxidant assays in leaves of *B. juncea* var. *rugosa* in hydro-ethanolic extract

Accessions	DPPH scavenging ($\mu\text{g mL}^{-1}$ DW)	Superoxide Free Radical Scavenging Activity ($\mu\text{g mL}^{-1}$ DW)	Metal ion chelation ($\mu\text{g mL}^{-1}$ DW)
IC-338751	(353.05 $\pm$ 4.07) ^c	(24.38 $\pm$ 1.56) ^s	(283.91 $\pm$ 5.16) ^f
IC-524259	(837.08 $\pm$ 5.60) ^a	(1241.35 $\pm$ 3.08) ^d	(202.70 $\pm$ 5.17) ^l
IC-338535	(112.84 $\pm$ 3.66) ⁿ	(523.05 $\pm$ 2.49) ⁿ	(205.97 $\pm$ 5.01) ^{kl}
EEC-25	(676.05 $\pm$ 4.58) ^b	(732.28 $\pm$ 2.53) ⁱ	(52.22 $\pm$ 4.48) ^o
IC-399826	(503.89 $\pm$ 4.20) ^d	(1404.19 $\pm$ 3.82) ^b	(213.56 $\pm$ 3.49) ^k
IC-399839	(77.76 $\pm$ 4.04) ^o	(433 $\pm$ 3.52) ^o	(365.34 $\pm$ 4.51) ^b
IC-399880	(285.49 $\pm$ 3.52) ^f	(1696.30 $\pm$ 4.48) ^a	(222.28 $\pm$ 4.03) ^j
IC-363758	(625.31 $\pm$ 4.05) ^c	(382.98 $\pm$ 3.08) ^p	(203.64 $\pm$ 3.54) ^l
IC-597917	(124.52 $\pm$ 4.55) ^l	(1092.52 $\pm$ 2.99) ^f	(384.25 $\pm$ 4.53) ^a
IC-597873	(51.98 $\pm$ 4.08) ^p	(1024.35 $\pm$ 2.64) ^g	(306.73 $\pm$ 5.09) ^e
IC-417128	(72.85 $\pm$ 3.58) ^o	(652.12 $\pm$ 2.02) ^k	(336.92 $\pm$ 5.52) ^c
IC-597933	(21.45 $\pm$ 3.48) ^q	(1302.11 $\pm$ 3.79) ^c	(184.93 $\pm$ 3.47) ^m
IC-298019	(117.16 $\pm$ 5.25) ^{mn}	(544.58 $\pm$ 2.53) ^m	(284.04 $\pm$ 4.55) ^f
IC-410471	(156.19 $\pm$ 4.01) ^j	(326.40 $\pm$ 3.23) ^q	(327.81 $\pm$ 5.55) ^d
IC-350800	(178.70 $\pm$ 2.11) ⁱ	(15.91 $\pm$ 1.54) ^t	(264.12 $\pm$ 2.49) ^h
IC-276011	(148.18 $\pm$ 3.09) ^k	(1131.93 $\pm$ 3.04) ^e	(271.51 $\pm$ 3.63) ^g
IC-413486	(235.06 $\pm$ 4.01) ^g	(664.11 $\pm$ 2.99) ^j	(243.05 $\pm$ 3.46) ⁱ
IC-597821	(123.53 $\pm$ 2.10) ^{lm}	(590.48 $\pm$ 1.50) ^l	(97.26 $\pm$ 4.10) ⁿ
Pusa Saag	(160.75 $\pm$ 2.50) ^j	(961.95 $\pm$ 2.02) ^h	(212.98 $\pm$ 6) ^k
PR-15	(222.38 $\pm$ 2.56) ^h	(124.37 $\pm$ 2.03) ^r	(290.50 $\pm$ 4.03) ^f
Ascorbic Acid	(32.68 $\pm$ 0.76) ^r	(41.46 $\pm$ 1.41) ^u	-
EDTA	-	-	(84.63 $\pm$ 1.27) ^p

The values with the same superscript are not ($p < 0.05$) significantly different (Duncan Post Hoc analysis)

DPPH is a relatively stable organic radical widely used to determine the antioxidant activity of single compounds and various plant extracts. The DPPH free radical scavenging activity, superoxide free radical scavenging activity, and metal ion chelating activity were expressed as IC₅₀ values for the hydro-ethanolic extracts (Table 2). Significant differences ($p < 0.05$) were observed in the IC₅₀ values for all activities among the different accessions. IC-597933 exhibited the maximum DPPH free radical scavenging activity with the lowest IC₅₀ value ($21.45 \pm 3.4 \mu\text{g mL}^{-1}$), followed by IC-597873 ($51.98 \pm 4.08 \mu\text{g mL}^{-1}$) and IC-417128 ($72.85 \pm 3.58 \mu\text{g mL}^{-1}$) which is lower in comparison to the positive control (Ascorbic acid) ($32.77 \mu\text{g mL}^{-1}$). The superoxide free radical scavenging activity was highest in IC-350800 with an IC₅₀ value of $15.91 \mu\text{g mL}^{-1}$, followed by IC-338751 ($24.38 \pm 1.56 \mu\text{g mL}^{-1}$), both of which were lower than the reference standard, ascorbic acid ($41.46 \mu\text{g mL}^{-1}$). The IC₅₀ value for metal ion chelating activity was lowest in EEC-25 ($52.22 \mu\text{g mL}^{-1}$), followed by IC-597821 ($97.26 \mu\text{g mL}^{-1}$), both of which were lower than the standard EDTA ($84.63 \mu\text{g mL}^{-1}$).

At a concentration of $400 \mu\text{g mL}^{-1}$, the DPPH scavenging percentages were 62.75, 59.61, 62.95, 67.68, 69.52, 64.49, 60.06, and 68.77 for hydro-ethanolic extracts of IC-350800, IC-413486, IC-410471, IC-597873, IC-298019, Pusa saag, PR-15, and standard, respectively (Fig 7). At a concentration of $200 \mu\text{g mL}^{-1}$ superoxide anion radical scavenging percentages were 58.33, 66.01, 64.75, and 60.52 for the hydro-ethanolic extract of IC-338751, IC-410471, PR-15 and standard, respectively. At a concentration of 400 and 500 mg mL^{-1} , the scavenging percentages of hydro-ethanolic extracts of all the genotypes are nearly the same or less as compared to the standard (Fig. 8).

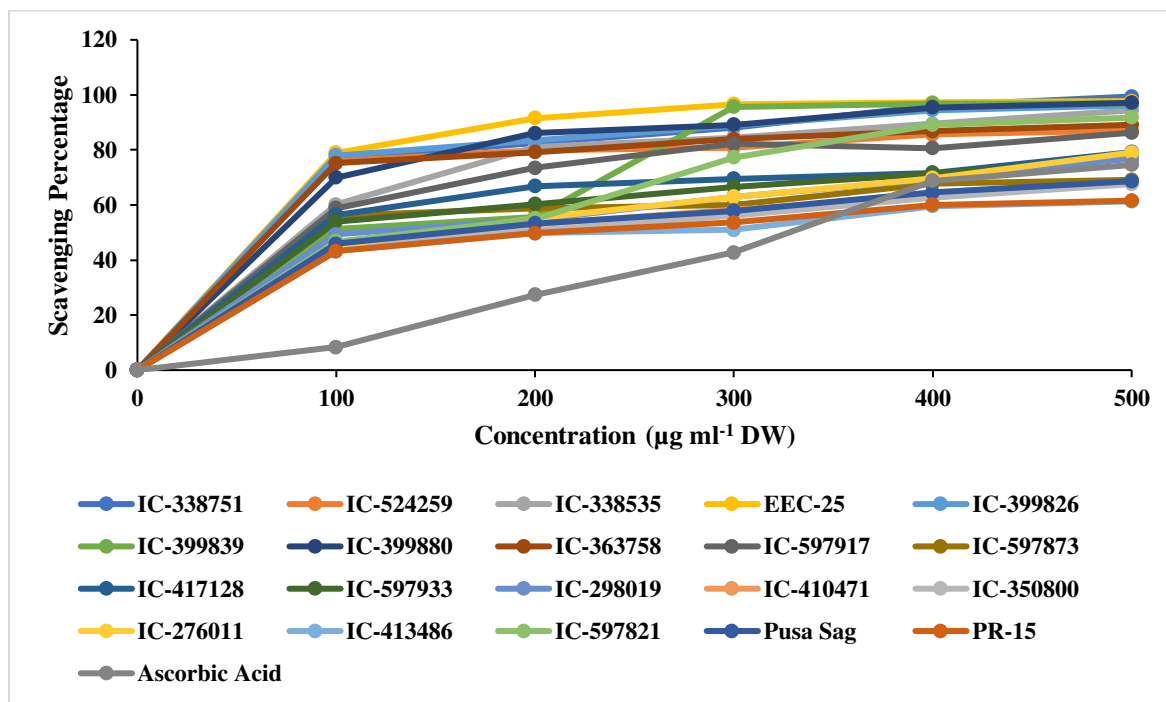


Fig 7: Dose-dependent DPPH scavenging activity in hydro-ethanolic leaf extract of *B. juncea* var. *rugosa* germplasm

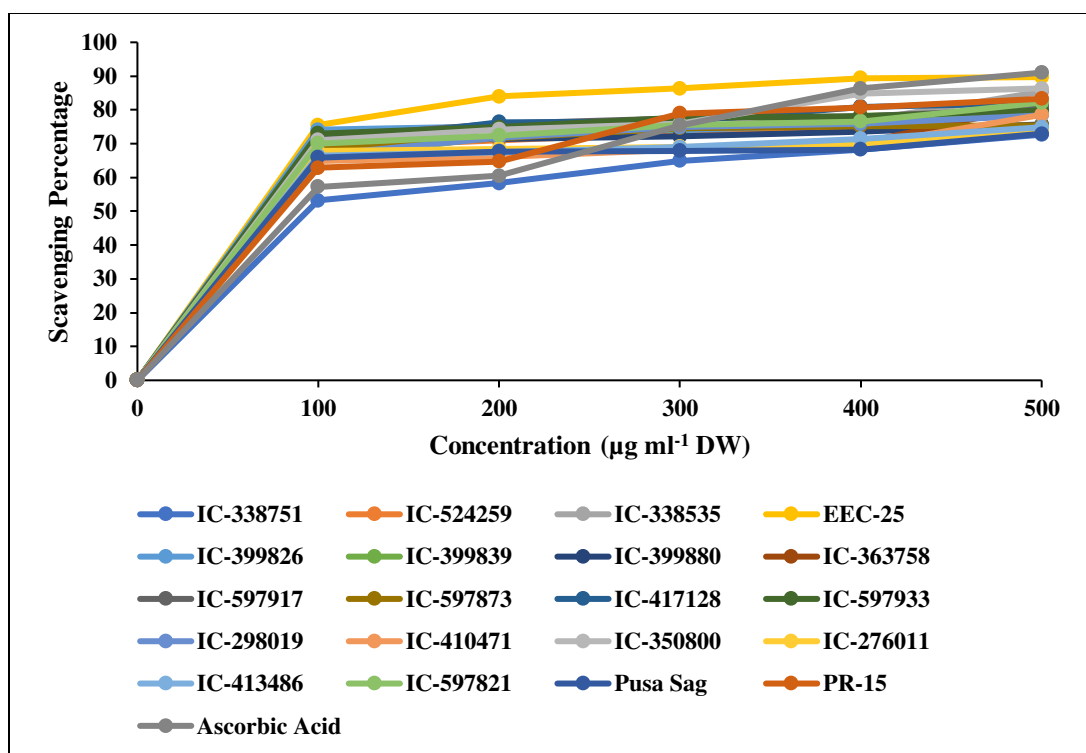


Fig 8: Dose-dependent superoxide anion radical scavenging activity hydro-ethanolic leaf extract of *B. juncea* var. *rugosa* germplasm

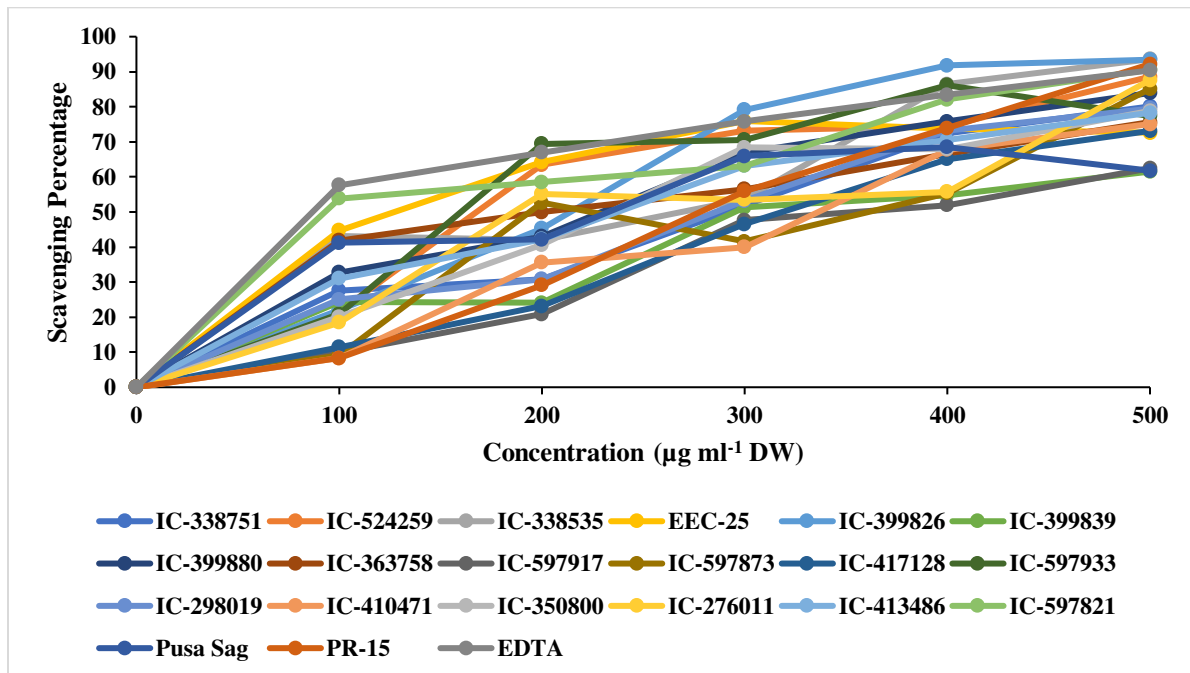


Fig 9: Dose-dependent Metal ion chelating activity hydro-ethanolic leaf extract of *B. juncea* var. *rugosa* germplasm

In the hydro-ethanolic extract of all the genotypes, at all the concentrations from 100-500 $\mu\text{g mL}^{-1}$, the metal ion chelating activity was lower and similar to the reference (Fig. 9). In a similar study by Moirangthem and Praveen

(2020) determined the DPPH activity in methanolic, ethyl acetate, petroleum ether, and chloroform extract of *B. juncea* var. *rugosa*. At 50 g mL⁻¹ concentration the ethyl acetate, petroleum ether, and chloroform extract exhibited 41.14, 40.33, and 10.33% scavenging activity respectively. The leaf of *B. juncea* showed 95.55% DPPH free radical scavenging activity (Saha et al. 2015). The DPPH percentage inhibition of *Brassica rapa* subsp. *Chinensis* (Pak choi) were found to be (72.16 ± 8.35 mg g⁻¹) (Khanam et al. 2012). Since phenolics are a significant component of overall antioxidant activity, a higher level of total phenols may be responsible for the excellent DPPH activity. No literature reports were available on the quantification of superoxide activity in *B. juncea* var. *rugosa*. But in similar studies by Jaiswal et al. (2011) among the different Brassica vegetable extracts, York cabbage showed the H₂O₂ scavenging capacity (1.15 ± 0.06 mg mL⁻¹), followed by broccoli (1.29 ± 0.10 mg mL⁻¹), Brussels sprouts (2.14 ± 0.02 mg mL⁻¹) and white cabbage (2.84 ± 0.21 mg mL⁻¹). In a study, various Brassica vegetables were studied for their ferrous ion chelating capacity. Among the vegetables studied, the Fe²⁺ chelating capacity was found in York cabbage (1.49 ± 0.03 mg mL⁻¹), followed by broccoli (1.58 ± 0.10 mg mL⁻¹), Brussels sprouts (2.14 ± 0.36 mg mL⁻¹) and white cabbage (3.45 ± 0.37 mg mL⁻¹). Plant extracts contain flavonoids, which are known to form complexes with metal ions and are hence liable for the ability to scavenge free radicals (Rice- Evans and Miller 1996).

In the hydro-ethanolic extracts of all the genotypes, at concentrations ranging from 100 to 500 µg mL⁻¹, the metal ion chelating activity was lower and similar to the reference (Fig. 9). In a similar study, Moirangthem and Praveen (2020) determined the DPPH activity in methanolic, ethyl acetate, petroleum ether, and chloroform extracts of *Brassica juncea* var. *rugosa*. At a concentration of 50 µg mL⁻¹, the ethyl acetate, petroleum ether, and chloroform extracts exhibited scavenging activities of 41.14%, 40.33%, and 10.33%, respectively. The leaves of *B. juncea* showed 95.55% DPPH free radical scavenging activity (Saha et al., 2015). The DPPH percentage inhibition of *Brassica rapa* subsp. *Chinensis* (Pak choi) was found to be 72.16 ± 8.35 mg g⁻¹ (Khanam et al., 2012). Since phenolics are a significant component of overall antioxidant activity, a higher level of total phenols may be responsible for the excellent DPPH activity.

There are no available literature reports on quantifying superoxide activity in *B. juncea* var. *rugosa*. However, in similar studies by Jaiswal et al. (2011), among different Brassica vegetable extracts, York cabbage showed H₂O₂ scavenging capacity of 1.15 ± 0.06 mg mL⁻¹, followed by broccoli (1.29 ± 0.10 mg mL⁻¹), Brussels sprouts (2.14 ± 0.02 mg mL⁻¹), and white cabbage (2.84 ± 0.21 mg mL⁻¹). Additionally, various Brassica vegetables were studied for their ferrous ion chelating capacity. Among the vegetables studied, the Fe²⁺ chelating capacity was found in York cabbage (1.49 ± 0.03 mg mL⁻¹), followed by broccoli (1.58 ± 0.10 mg mL⁻¹), Brussels sprouts (2.14 ± 0.36 mg mL⁻¹), and white cabbage (3.45 ± 0.37 mg mL⁻¹). Plant extracts contain flavonoids, which are known to form complexes with metal ions, hence contributing to their ability to scavenge free radicals (Rice-Evans and Miller, 1996).

The variation between DPPH and ABTS antioxidant assays, despite both being radical scavenging methods, arises from differences in their radical stability, solvent compatibility, spectral properties, reaction mechanisms, and sensitivity. DPPH, a stable free radical typically used in organic solvents, and ABTS, a more reactive radical often employed in aqueous solutions, interact differently with antioxidants. Additionally, DPPH and ABTS absorb light at different wavelengths (517 nm and 734 nm, respectively), and the mechanisms by which antioxidants neutralize these radicals vary, leading to different observed antioxidant activities. Moreover, ABTS is generally more sensitive than DPPH, which can result in higher antioxidant activity measurements in the ABTS assay. These factors collectively explain the discrepancies in the results of the two assays. Genetic, environmental, and biochemical factors are responsible for the difference in antioxidant potential among various germplasms. Each germplasm has a unique genetic composition, influencing the synthesis and accumulation of antioxidant compounds like phenolics, flavonoids, and glucosinolates. Environmental conditions, such as soil type, climate, and exposure to sunlight, also play a critical role in modulating these biochemical pathways. Additionally, differences in agricultural practices, including fertilization and pest control, can further contribute to the observed variability. Post-harvest handling and processing methods may also impact the stability and concentration of these antioxidants. Ultimately, the interplay of these factors results in the distinct antioxidant profiles observed in each germplasm.

Peroxidase and Superoxide Dismutase Enzymatic Analysis

Further investigations were performed to identify the levels of peroxidase and superoxide dismutase in crude extract. The highest peroxidase enzyme activity found in the IC-350800, IC-410471, IC-298019, and IC-338535, were 0.268, 0.177, 0.163, 0.142 units guaiacol min⁻¹ g⁻¹ FW, respectively. Whereas, the lowest enzymatic activity of IC-338751 (0.002±0.0035 µmol guaiacol min⁻¹ g⁻¹ FW), followed by IC-413486 (0.05±0.005 µmol guaiacol min⁻¹ g⁻¹ FW) was observed (Fig. 10). Peroxidase activity enhances the shelf life by inhibiting lipid peroxidation, yellowing and chlorophyll degradation, ethylene production, and senescence (Zhuang et al. 1995). However, our study does not attempt to identify the compounds responsible for observed antioxidant effects from *B. juncea* var. rugosa. In a study published by Singh et al. (2010), the peroxidase activity in *Brassica oleracea* var. capitata genotypes ranged from 415-5314, with a genotype mean of 1929±410.97 µmol tetraguaiacol min⁻¹ g⁻¹ FW.

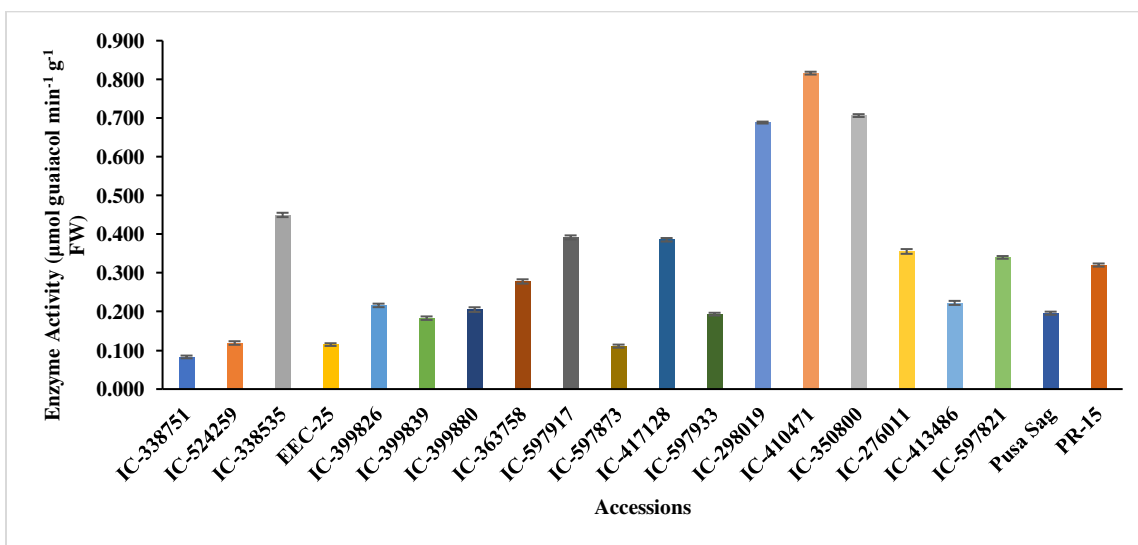


Fig 10: Peroxidase enzymatic activity in fresh leaves of *B. juncea* var. rugosa germplasm

The enzymatic activity of superoxide dismutase was found to be highest in IC-338751, followed by IC-524259 (3.65±0.04 µmol pyrogallol min⁻¹ g⁻¹ FW), and IC-338535 (3.35±0.03 µmol pyrogallol min⁻¹ g⁻¹ FW). But the rest of the genotypes had a similar activity (Fig. 11).

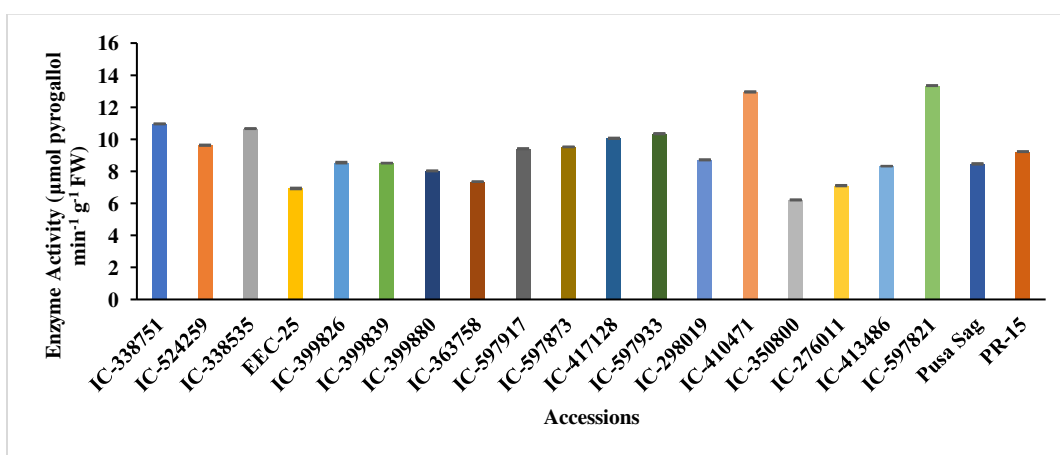


Fig 11: Superoxide dismutase enzymatic activity in fresh leaves of *B. juncea* var. rugosa germplasm

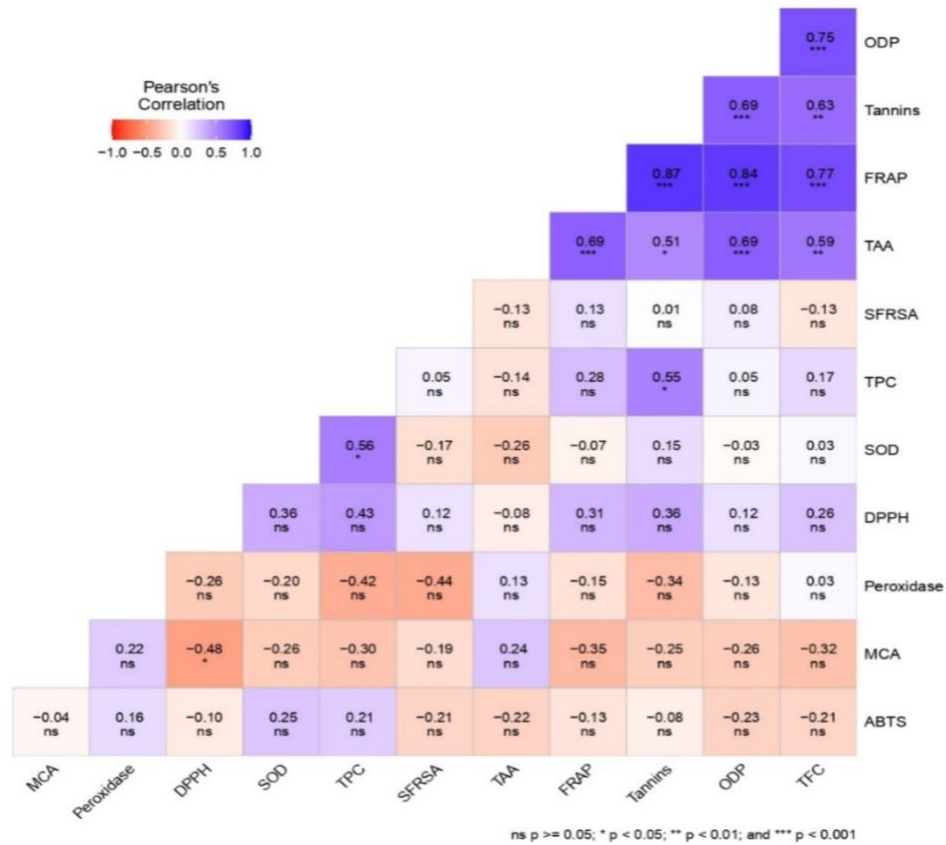
Understanding the plant's enzymatic antioxidant activity is important for a better plant defense system and longer shelf life. In a study by Singh et al. (2010) SOD activity in *Brassica oleracea* var. capitata lines ranged from

4.86-7.63 with a mean value of 6.46 ± 0.25 unit of $\text{SOD min}^{-1} \text{g}^{-1} \text{FW}$. In research by **Gul et al. (2013)**, the levels of SOD in *Brassica rapa L.* crude extract were found to be 1 mg mL^{-1} , obtained in chloroform and ethyl acetate fraction from *Brassica rapa L.* (both at 190 U mL^{-1}), while 5 mg mL^{-1} in aqueous fraction (215 U mL^{-1}) and 10 mg mL^{-1} in aqueous and ethyl acetate fraction (both at 220 U mL^{-1}).

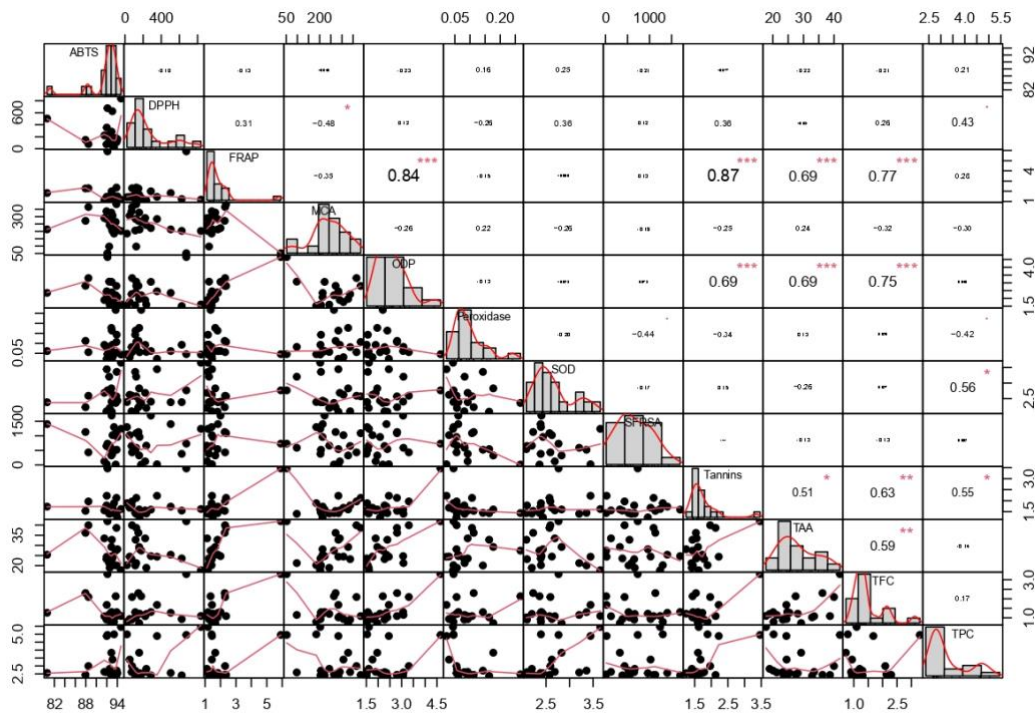
Pearson-Correlation Analysis

Figure 12 (a), (b) represents a correlation matrix. It provides an overview of the relationships between antioxidant assays (TAA, ABTS, DPPH, FRAP, MCA, SFRSA), enzymatic activities (peroxidase, SOD), and phytochemical constituents (TFC, TPC, Tannins). The upper triangular portion displays pairwise correlation coefficients, with significance levels denoted by asterisks (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Strong positive correlations are observed between ABTS and FRAP ($r = 0.84$), FRAP and SRBCA ($r = 0.87$), and FRAP and TPC ($r = 0.75$), suggesting that these antioxidant assays are closely linked to phenolic content. Moderate positive correlations between tannins, TFC, and TAA also highlight the role of flavonoids and tannins in antioxidant activity. Weak or negligible correlations are noted for parameters like DPPH and peroxidase, indicating their limited association with the others. The diagonal histograms represent the distribution of each variable, showing their spread and frequency Fig 12 (b). Scatterplots in the lower triangular matrix illustrate pairwise relationships, with trends highlighted by smoothed red lines, visually complementing the numeric correlation values. This matrix provides a comprehensive view of how antioxidant activity and phytochemical content are interrelated, with phenolic compounds emerging as key contributors to antioxidant potential.

Flavonoids reduce ferric ions, scavenge ROS, and neutralize their harmful effects, thereby reducing oxidative stress, and preventing cellular damage (**Rice-Evans et al. 1997; Middleton et al. 2000**). The strong positive correlation of total flavonoid content with FRAP attributed to the antioxidant activity of flavonoids. Since proteins are involved in key biological processes, the positive correlation between total flavonoid content and protein may indicate that flavonoids, being powerful antioxidants, help protect them from oxidative damage thereby maintaining their structural integrity and functionality (**Amarowicz et al. 2008; Ebrahimzadeh et al. 2008**). The structural similarities and antioxidant properties shared between flavonoids and ODPs explain the positive correlation between total flavonoid content and ODP levels. Tannins, one of the major classes of phenolic compounds found in plants are known for their ability to bind and precipitate proteins, contributing to their astringent taste. Total phenolic content, commonly used as an indicator of the antioxidant capacity of plant extracts, is a measurement that encompasses various phenolic compounds, including tannins. Their positive correlation with ODP can be attributed to the fact that tannins contribute significantly to the overall phenolic content and exhibit strong antioxidant activity due to their ability to scavenge free radicals and chelate metal ions (**El Gharras 2009**). Total phenolic content positively correlated with the DPPH and SOD assays as the phenolic compounds, such as flavonoids and tannins possess strong radical scavenging capabilities thus effectively neutralizing the DPPH radical. The ability of phenolic compounds to scavenge superoxide radicals and reduce oxidative stress, thereby supporting the activity of SOD (**Martins et al. 2015**). The protein content showed a moderate correlation with the DPPH assay. This positive correlation is because certain proteins like catalase and peroxidase, or peptides inherently exhibit antioxidant effects by catalyzing the breakdown of ROS, scavenging free radicals, or chelating metal ions (**Shahidi and Zhong 2008**). A positive correlation between total antioxidant activity and total phenolic content suggested that phenolics may be one of the major sources of these vegetables' antioxidant potential. A significant positive correlation of total flavonoid content with ODP, tannins, FRAP, total antioxidant activity, and protein infers that the selection of antioxidant-rich lines should be based on total flavonoid content either alone or in combination with ODP, tannins, FRAP, total antioxidant activity, and protein. Therefore, ODP, tannins, FRAP, total phenolic content, and protein could serve as a reliable biochemical marker to identify the germplasms having higher activity of other antioxidants such as total antioxidant, DPPH and SOD.



(a)



(b)

Fig 12: (a) Heatmap of Pearson's correlation analysis; (b) Correlation matrix plot combined with scatterplots and distribution histograms depicting antioxidant activities and phytochemical constituents. At 5% of the parameter. *, **, *significant at 0.05, 0.01 and 0.001 probability levels. (TCC-Total carbohydrate content; SOD-Superoxide Dismutase; TPC-Total Phenolic Content; SARSA=Superoxide Free radical scavenging activity; TAA-Total antioxidant activity, TFC-Total Flavonoid content; MCA-Metal Chelating Activity, FRAP-Ferric Reducing Antioxidant Power)**

Mineral composition in *Brassica juncea* var. *rugosa* leaves

Leafy mustard is a rich source of minerals, according to mineral profiling (Table 3). The concentration of copper was found to be highest in IC-338751 (0.069 ppm), EEC-25 (0.053 ppm), and IC-597873 (0.048). In IC-597917, PR-15, and IC-399880 the calcium concentration was found to be foremost 0.038, 0.034, and 0.026, respectively. Manganese was found to be maximum in IC-413486 (0.284 ppm), PR-15 (0.278 ppm), and Pusa saag (0.267). IC-399880, IC-399826, and IC-597873 contain the fruitful amount of Zn which is 0.157, 0.134, and 0.135, respectively. In contrast, the Fe concentration in IC-524259 (5.321 ppm), IC-413486 (4.374 ppm), and IC-338751 (4.092 ppm) was found paramount. In IC-413486 (8.006 ppm), IC-399839 (7.292 ppm), and IC-597917 (6.889 ppm) the magnesium concentration was found to be maximum. The genotypes IC-410471 (0.165 ppm), IC-597873 (0.141 ppm), and IC-597933 (0.132 ppm) contain the highest amounts of nickel.

The detection of heavy metals such as Pb, and Cd in *Brassica juncea* var. *rugosa* leaves through AAS can be related to both environmental factors and the plant's natural properties. One primary reason is the plant's natural ability to absorb and tolerate heavy metals from the soil, known as phytoremediation. This trait makes Brassica species effective in cleaning up contaminated soils, but it also results in the accumulation of toxic metals in their tissues. Environmental contamination is a secondary source of heavy metal accumulation in plants. Industries, vehicular emissions, agricultural practices, and improper waste disposal can release heavy metals into the environment, which plants absorb. Environmental pollution from industrial activities, mining, and using contaminated water for irrigation can introduce these heavy metals into the soil, which are then taken up by the plants (Mourato et al., 2015). This dual nature of *Brassica juncea* var. *rugosa*—its ability to absorb heavy metals for soil remediation and its tendency to accumulate toxic metals—highlights the complex interplay between environmental contamination and plant physiology. Understanding these dynamics is crucial for developing strategies to mitigate heavy metal accumulation in edible plants and ensure food safety.

Table 3: Mineral Composition in leaves of *Brassica juncea* var. *rugosa* (ppm)

Accessions	Cu	Ni	Ca	Mn	Zn	Fe	Mg
IC-338751	0.069	0.033	0.009	0.202	0.118	4.092	5.042
IC-524259	0.044	0.068	0.016	0.199	0.133	5.312	4.561
IC-338535	0.038	0.081	0.015	0.188	0.122	3.951	4.886
EEC-25	0.053	0.108	0.015	0.198	0.14	4.02	5.37
IC-399826	0.032	0.04	0.013	0.196	0.134	3.898	5.321
IC-399839	0.035	0.09	0.017	0.195	0.14	3.611	7.292
IC-399880	0.022	0.062	0.026	0.188	0.157	3.249	4.674
IC-363758	0.03	0.088	0.03	0.195	0.115	3.226	4.202
IC-597917	0.034	0.15	0.038	0.21	0.111	3.655	6.889
IC-597873	0.048	0.141	0.002	0.224	0.135	3.53	6.559
IC-417128	0.031	0.11	0.007	0.207	0.109	3.372	5.512
IC-597933	0.01	0.132	0.009	0.206	0.068	3.703	4.339

IC-298019	0.033	0.133	0.012	0.246	0.124	3.363	5.628
IC-410471	0.016	0.165	0.018	0.243	0.077	3.45	4.013
IC-350800	0.028	0.083	0.018	0.246	0.119	3.096	5.158
IC-276011	0.023	0.092	0.022	0.248	0.114	3.251	4.983
IC-413486	0.021	0.084	0.023	0.284	0.112	4.374	8.006
IC-597821	0.023	0.131	0.026	0.273	0.095	3.4	5.608
Pusa Saag	0.017	0.121	0.026	0.267	0.09	3.13	5.676
PR-15	0.015	0.049	0.034	0.278	0.114	3.118	6.676

Cd	0.014	0.005	0.001	0.006	0.01	0.017	0.021	0.059	0.064	0.074	0.08	0.086	0.085	0.103	0.111	0.105	0.12	0.113	0.113	0.121
Pb	0.248	0.453	0.109	0.512	0.181	0.023	0.016	0.482	0.128	0.34	0.1	0.112	0.216	0.158	0.148	0.227	0.151	0.116	0.183	0.226

For women and growing children, iron is vital, and mustard green is the greatest solution to provide them with their daily needs. The *B. juncea* var. *rugosa* accessions possessed Fe ranging from 11.81-20.23 mg/100g. Iron has significance in metabolic processes like DNA synthesis, respiration, and photosynthesis, as well as a crucial part in physiological processes and biochemical pathways, iron is an essential component of the enzyme cytochrome of the electron transport chain (Rout and Sahoo 2015). Zinc concentrations ranged from 13.6-2.73 mg/100g. Manganese levels in *B. rugosa* genotypes ranged from a high of 6.49 mg/100g to a low of 4.17 mg/100g (Pant et al. 2020). As per Saha et al. (2015) Fe, Zn, Cu, Mn, Ca, and Mg content in *Brassica juncea* were 118.50, 7.50, 2.60, 45.97, 36.6, 16.33 mg/100g, respectively. Optimum potassium levels enhance iron absorption and are advantageous for those taking diuretics to control hypertension (Archana et al. 2013). Mustard green can supply a significant amount of calcium, which is similarly crucial for bone health, especially in women and growing babies. Calcium functions as a secondary messenger in numerous physiological and developmental processes, as well as its involvement in the formation of cell walls, stability of cell membranes, and how it responds to biotic stress by concentrating when necessary (Thor 2019). The human body requires copper for the production of enzymes, and it functions as a biological electron carrier, whereas magnesium plays a pivotal role in calcium metabolism, regulating blood pressure and insulin release (Alinnor and Oze 2011).

In 2013, Babar et al. reported that zinc, a protein cofactor involved in the production of chlorophyll, growth regulation, and stem elongation, serves as a functional, structural, or regulatory cofactor of several enzymes. Magnesium plays a vital role in various biological processes, such as glycolysis, oxidative phosphorylation, nucleic acid synthesis, energy production, mitochondrial ATP synthesis, cell signaling, and activation of cAMP (Saris et al. 2000; Barbagallo and Dominguez 2007). Nickel is one of the micronutrients in plants that hydrolyze urea in plant tissues and it is part of the active site of the enzyme. The experimental results from the current study will be crucial for the creation of new Ayurvedic formulations and will be used to manage the dosage and determine the ratio of different active ingredients in each formulation.

Micronutrient (NPK) Analysis

The maximum concentration of Nitrogen (N) in EEC-25, IC-597917, and IC-410471 was 0.644%, 0.588%, and 0.504%, respectively. IC-524259 and IC-399839 showed the minimum amount of N. In the case of phosphorous (P) EEC-25 (0.751%), IC-298019 (0.772%), and IC-524259 (0.749%) had the paramount concentration. The lowest amount of P was found in IC-363758 (0.334%), and IC-399826 (0.315%). IC-413486 (0.099%), IC-597873 (0.097%), and IC-399839 (0.093%) had the maximum concentration of potassium (K). furthermore, PR-15 (0.047%), IC-597933 (0.055%), and IC-338535 (0.050%) showed the lowest concentration of K (Fig 13).

A study by Pant et al. in 2020 determined the phosphorous content ranged from 513mg/100g to high as 720 mg/100g. Only one piece of literature is available for the NPK content in *B. juncea* var. *rugosa*. However, in a study by Devkota et al. (2020), the concentration of NPK was 1.08, 1.6, and 2.83 respectively. Nitrogen is a necessary

component of plant elements such as proteins, amino acids, nucleotides, nucleic acids, and chlorophyll content, nitrogen is essential for crop development.

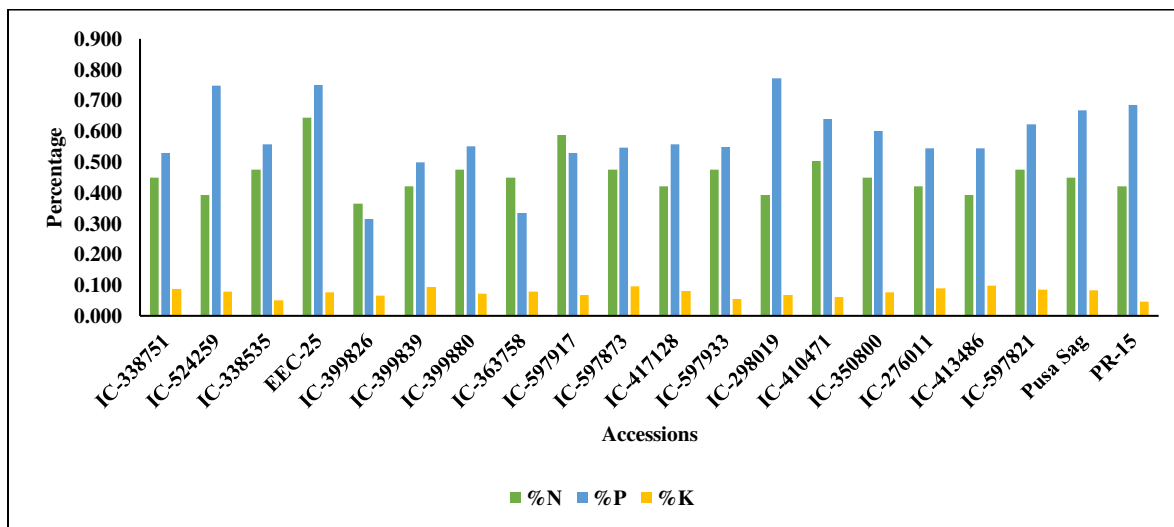


Fig 13: Nitrogen (N), Phosphorous (P), and Potassium (K) analysis of different accessions of *B. juncea* var. *rugosa*

Potassium regulates protein turnover, effect the Rubisco activity, nitrate reduction process, ribosome polypeptide syntheses, translational process, synthesis, and activity of nitrate red, affects glucose starch synthase, glucose pyrophosphorylase, and β -amylase (Berg et al. 2009). The deficiency of potassium in the plasma can cause gastrointestinal problems such as vomiting, diarrhea, increased renal loss, muscle weakness, ECG abnormalities, decreased reflex response, and cardiac arrhythmias in severe cases (Kardalas et al. 2018). Phosphorous is one of the crucial macronutrients for the plant, which is involved in nucleic acids, enzymes, coenzymes, nucleotides, phospholipids, cell organization, root development, stalk or stem growth and strength, root development, photosynthesis, respiration, macromolecular biosynthesis (Thakur et al. 2018).

Fatty acid, oil stability index, protein, and glucosinolates profiling

The fatty acid composition, oil stability index (OSI), as well as the oil content, protein, and glucosinolate (GSL) content in seeds of *Brassica juncea* var. *rugosa* accessions (Table 4). The saturated fatty acid palmitic acid (16:0) was highest in genotypes IC-338751 (3.4%) followed by EEC-25 (2.98%), IC-597821 (2.62%), and IC-413486. Stearic acid (18:0) was maximum in IC-298019 (0.94%), but IC-524259 (0.88%), and IC-597933 (0.88%) almost have the same amount of stearic acid but IC-597873 (0.11%), and PR-15 (0.12%) had the lowest amount was observed. In a study by Rai et al. (2018) on the fatty acid composition of various *Brassica* genotypes in different cultivars of *Brassica*, *Brassica juncea* genotypes contained the highest levels of palmitic acid, as a saturated fatty acid. The study also reported that the total SFA content in various *Brassic*as was less than 7%, which is regarded as appropriate for human consumption. Oleic acid one of the monounsaturated fatty acids was present foremost in IC-367658 (20.11%), IC-597917 (17.11%), IC-524259 (16.13%) whereas IC-276011 (9.9%), and PR-15 (9.3%) had the modest amount. IC-597873 (10.56%) and IC-417128 (10.65%) have almost the same amount of oleic acid observed. The oleic acid content in the *Brassica* genotypes varied from 0.80 to 48.7% in *Brassica juncea*, 16.15-37.98% in *Brassica napus*, and 6.21-16.15% in *Brassica rapa*, among other MUFAs (Rai et al. 2018). Since oleic acid improves the concentration of high-density lipoproteins and reduces the concentration of low-density lipoproteins in the blood, higher doses of oleic acid are thought to have nutritional value for human nutrition (Chang and Huang 1998). It was observed that eicosenoic acid was highest in IC-338535 (10.11%), IC-524259 (9.68%), and IC-597933 (9.58%). In a finding by Velasco et al. (1998), the eicosenoic acid concentration was 6.2% in *B. napus*. Erucic acid was paramount in PR-15

(59.55%), IC-597873 (58.47%), and IC-597821 (53.29%). The cardiac conductance in humans is hampered by higher erucic acid concentration in cooking oil, which raises blood cholesterol levels (Bozzini et al. 2007). However, numerous germplasm undertaken in the current study that have higher quantities of erucic acid will be crucial for a variety of industrial applications. Erucic acid-rich oil is utilized as a raw ingredient in the detergent, plastic, tannery, cosmetic, and polyester industries (Rakow and Raney 2003; Coonrod et al. 2008). Brassica breeding projects are currently concentrated on the establishment of zero erucic lines for nutritional purposes. In a study by Tripathi (2019), the erucic content in various Indian mustard genotypes ranged from 0.93-51.4%.

The utmost linoleic acid (omega-6) content was observed in EEC-25 (22.48%), IC-338751 (20.23%), and IC-399880 (20.22%). On the other hand, for linolenic acid (omega-3), among all twenty germplasm, IC-276011 (12.68%), IC-399826 (12.65%) and EEC-25 (12.18%) had highest amount. In a similar study in *B. juncea*, *B. napus*, and *B. rapa* genotypes, the respective linoleic acid content ranged from 11-45.3%, 18.57-26.93%, and 14.08-18.18% while, linolenic acid content ranged from, 11-26.72%, 9.99-17.23%, and 9.82-26.66% (Rai et al. 2018). In a study by El-Beltagi et al. (2010) the level of PUFAs in various cultivars of *Brassica napus* varied from 10.52-13.41 for linoleic acid and 8.83-10.32 for linolenic acid. Which is very similar to the current study. The PUFAs are recognized as the long chain fatty acid with more than one double bond, that functions as precursors for the production of molecules with crucial physiological functions like prostaglandins, leukotrienes and thromboxane. PUFAs cannot be synthesized by the human body, hence they must be supplemented through diet. High concentrations of linoleic acid in edible oils are said to lower blood cholesterol and prevent atherosclerosis (Ghafoorunissa, 1994). Despite the health benefits of linolenic acid, its inclusion in the oil elevates the risk of rancidity and bad flavor (Sharafi et al., 2015). Therefore, its concentration in the cooking oil, should be low. According to this study, *Brassica juncea* var. *rugosa* germplasm with low erucic and high linoleic acid concentrations can be employed in *Brassica* breeding programs aimed at strengthening the grade of oil for dietary and industrial applications.

An essential metric for assessing the nutritional value of various edible oils is the $\omega 6:\omega 3$ ratio. The $\omega 6:\omega 3$ ratio was observed to be maximum in IC-350800 (2.53), IC-338535 (2.34), and IC-410471 (2.19). The SFA:PUFA ratio was found highest in both germplasms IC-338751 (3:34) and EEC-25 (3:34), followed by IC-399826 (3:32), IC-524259 (3:29). The ratio of SFA:MUFA:PUFA was observed highest in IC-597873 (1:47:14), IC-597917 (1:26:11), IC-363758 (1:23:11) while lowest in germplasms IC-410471 (1:17:8), IC-399826 (1:17:9) and EEC-25 (1:17:9). The oil stability index of IC-363758 (1.047), IC-597917 (0.940), and Pusa saag (0.896) were highest in these genotypes. In all the genotypes protein percentage ranged from 22-30. But the EEC-25 (123.96), IC-298019 (116.42) and IC-417128 (115.98) have the highest content of glucosinolates.

The lack of these essential fats in our diets may contribute to the expansion of numerous chronic diseases in humans, including heart failure, cancer, diabetes, asthma, depression, accelerated aging, Alzheimer's, obesity, and arthritis (Simpoulos, 2002). Balanced diets high in MUFA and PUFA have been advised over saturated fats to lower plasma cholesterol levels. A similar finding in *B. napus* L. by El-Beltagi et al. (2010) depicted the ratio of $\omega 6:\omega 3$ ranged from 3.85-5.32. Which is very much similar to the several genotypes but in some genotypes, it was found to be much higher. The excessive concentration of PUFA in edible oils is more susceptible to oxidation, which can lower the quality of both the oil itself and the products made with it. Rancidity and the production of free radicals are mostly caused by the oxidation of the double and triple bond formation in PUFA. So, it means that those oils have maximum OSI, having a longer shelf-life. In a study by Chauhan et al. (2010), the OSI ranged from 0.40 (Kranti of Indian mustard) to 4.96 (GSC-5 of *Brassica napus*). The $\omega 6:\omega 3$ ratio ranged from 0.55-3.5, which is very low compared to the current study.

The findings presented here have been previously shared as a preprint (Adhikari et al., 2024).

Table 4: Fatty acid, oil stability index (OSI), oil, protein, and glucosinolate (GSL) content in seeds of *Brassica juncea* var. *rugosa*

	SFA		MUFA			PUFA							
Accessions	Palmitic	Stearic	Oleic	Ecosinic	Erucic	Linoleic (ω6)	Linolenic (ω3)	ω6:ω3	SFA:PUFA	SFA:MUFA:PUFA	OSI	Protein (%)	GSL
IC-338751	3.4	0.54	13.8	7.09	44.2	20.23	14.1	1.43	3:34	1:16:8	0.682	23.15	92.06
IC-524259	2.52	0.88	16.1	9.68	35.41	18.68	10.98	1.70	3:29	1:18:8	0.863	24.45	91.17
IC-338535	2.5	0.74	14.1	10.11	42.5	19.2	8.21	2.34	1:09	1:20:8	0.735	24.33	75.32
EEC-25	2.98	0.58	15.5	5.25	40.42	22.48	12.18	1.85	3:34	1:17:9	0.690	23.68	123.96
IC-399826	2.55	0.84	12.1	9.21	39.42	20.18	12.65	1.60	3:32	1:17:9	0.600	25.28	89.99
IC-399839	2.5	0.72	13.2	8.23	40.41	18.65	11.12	1.68	3:29	1:19:9	0.706	26.18	98.13
IC-399880	2.4	0.59	15.1	8.72	41.52	20.22	17.67	1.14	2:37	1:21:12	0.748	27.40	61.11
IC-363758	2.18	0.58	20.1	6.5	39.38	19.21	12.48	1.54	2:31	1:23:11	1.047	24.66	97.09
IC-597917	2.12	0.51	17.1	7.45	45.22	18.21	11.53	1.58	2:29	1:26:11	0.940	27.25	98.19
IC-597873	1.47	0.11	10.6	6.3	58.47	12.78	10.01	1.28	1:22	1:47:14	0.826	25.06	101.24
IC-417128	2.18	0.5	10.7	0.55	45.41	17.64	9.68	1.82	2:27	1:21:10	0.604	27.92	115.98
IC-597933	2.52	0.88	15.1	9.58	38.32	18.63	10.48	1.78	3:29	1:18:8	0.812	26.37	109.92
IC-298019	2.25	0.94	11.7	7.45	45.2	14.15	11.36	1.25	3:25	1:20:7	0.828	27.40	116.42
IC-410471	2.4	0.73	11.5	0.84	42.34	18.29	8.37	2.19	3:26	1:17:8	0.629	23.56	82.39
IC-350800	2.14	0.81	11.7	0.47	48.92	17.65	6.99	2.53	1:12	1:20:8	0.660	23.30	94.68
IC-276011	2.19	0.75	9.9	7.2	45.88	16.02	12.68	1.26	1:14	1:21:9	0.618	26.90	100.06
IC-413486	2.58	0.86	11.8	7.53	45.69	16.28	10.74	1.52	1:09	1:18:7	0.723	23.46	77.37
IC-597821	2.62	0.36	11.2	5.68	53.29	17.76	11.67	1.52	2:29	1:23:9	0.631	22.32	81.9
Pusa Saag	2.36	0.96	13.3	7.07	45.42	14.83	11.82	1.25	3:26	1:19:8	0.896	26.88	97.53
PR-15	2.13	0.12	9.3	5.21	59.55	15.22	6.94	2.19	1:11	1:32:9	0.611	22.72	102.24

Conclusion

In conclusion, this study provides significant insights into the phytochemical content, *in-vitro* antioxidant activity, correlation analysis, mineral composition, and fatty acid profiling of *Brassica juncea* var. *rugosa*, commonly known as Pahari rai, accessions from the Indian sub-Himalayan region. Among the 20 genotypes analyzed, the EEC-25 hydro-ethanolic leaf extract stood out due to its high levels of total phenolics, flavonoids, ODP, and tannins, alongside its remarkable antioxidant activity measured through total antioxidant activity, FRAP activity, and reducing power. This suggests its potential as a valuable candidate for functional food applications. Furthermore, the correlation analysis unveiled intriguing associations between phytochemicals, antioxidant activity, and enzymatic analyses, providing a deeper understanding of the mechanisms behind the antioxidant potential in these accessions.

The considerable variation in total soluble protein, carbohydrate content, and antioxidant activity among different germplasms of *Brassica juncea* var. *rugosa* can be attributed to the intricate interplay of genetic diversity, environmental factors, and cultivation practices. Genetic diversity influences metabolic pathways, regulating the biosynthesis and accumulation of proteins, carbohydrates, and phytochemicals such as phenolic, flavonoid, tannin, and ODP. Environmental factors, growth stages, and post-harvest processing further modulate these biochemical processes, leading to distinct nutritional and phytochemical profiles across germplasms. Variations in antioxidant activity, as determined by DPPH and ABTS assays, reflect differences in radical stability, solvent compatibility, spectral properties, reaction mechanisms, and assay sensitivity. These findings underscore the complex interactions between genetic and environmental determinants, highlighting their role in shaping the nutritional and antioxidant potential of *Brassica juncea* var. *rugosa*. A comprehensive understanding of these factors is essential for optimizing cultivation and post-harvest strategies to enhance the crop's nutritional and functional attributes.

The fatty acid profiling of seeds offered vital insights into their nutritional characteristics, with the $\omega 6:\omega 3$ ratio of IC-350800, IC-338535, and IC-410471 highlighting their superior nutritional value for edible oil production. Additionally, the oil stability index identified IC-363758, IC-597917, and Pusa saag as promising sources of vegetable oil with excellent shelf-life stability. This comprehensive investigation enhances our knowledge of the phytochemical and antioxidant capabilities of *Brassica juncea* var. *rugosa*, paving the way for future breeding and crop improvement programs. It also opens opportunities for developing functional foods and stable vegetable oils with potential health benefits, contributing to the enhancement of human health and well-being. Such advancements may lead to improved dietary options and healthier lifestyles for consumers. By integrating these findings into breeding strategies and food product development, there is potential to create health-promoting, nutritious, and sustainable food sources that could have a significant impact on public health and agricultural practices. The study's results underscore the importance of exploring and utilizing the rich biodiversity found in traditional crops like Pahari rai, which holds promise for both nutritional and economic benefits in the future. It is essential to further explore the chemical composition of the polar constituents present in these extracts. Therefore, future research should concentrate on isolating, identifying, and characterizing bioactive compounds. Additionally, conducting *in vivo* safety studies using experimental animal models remains necessary before these compounds can be practically applied.

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References

1. Adhikari A, Punetha H, Pant U (2022) Genetic diversity analysis using agro-morphological traits of *Brassica juncea* subspecies *rugosa* (Pahari rai) from North-Eastern Himalayan region. *Electronic J of Plant Breeding* 13(3):1-10. <https://doi.org/10.37992/2022.1303.106>.
2. Ankur Adhikari, Himanshu Punetha. Comprehensive Assessment of Brassica juncea variety rugosa (Pahari Rai) Accessions from the Sub-Himalayan Region: Phytochemical, Antioxidant, Enzymatic, Mineral, and Fatty Acid Profiling, 20 May 2024, PREPRINT (Version 1) available at Research Square [<https://doi.org/10.21203/rs.3.rs-4340286/v1>]
3. Alinnor IJ, Oze R (2011) Chemical evaluation of the nutritive value of *Pentaclethra macrophylla benth* (African Oil Bean) Seeds. *Pakistan J Ntr* 10(4):355-359. <https://doi.org/10.3923/pjn.2011.355.359>.
4. Amarowicz R, Dykes GA, Pegg RB (2008) Antibacterial activity of tannin constituents from *Phaseolus vulgaris*, *Fagopyrum esculentum*, *Corylus avellana* and *Juglans nigra*. *Fitoterapia* 79(3):217-219. <https://doi.org/10.1016/j.fitote.2007.11.019>.
5. Archana GN, Pradeesh S, Chinmayee MD, Mini I, Swapna TS (2013) *Diplazium esculentum*: a wild nutrient-rich leafy vegetable from Western Ghats. In *Prospects in Bioscience: Addressing the issues*. Springer, India (pp. 293-301). https://doi.org/10.1007/978-81-322-0810-5_35.
6. Arnou LE (1937) Colorimetric determination of the components of 3, 4-dihydroxyphenylalanine-tyrosine mixtures. *J Biol Chem* 118(2):531-537.
7. Babar H, Yusop MK, Saleem M (2013) Role of zinc in plant nutrition-a review. *J Exp Agri Inter* 3(2):374-391. <https://doi.org/10.9734/AJEA/2013/2746>
8. Bae HD, McAllister TA, Muir AD, Yanke LJ, Bassendowski KA, Cheng KJ (1993). Selection of a method of condensed tannin analysis for studies with rumen bacteria. *J of Agri and Food Chem* 41(8):1256-1260. <https://doi.org/10.1021/jf00032a018>.
9. Barbagallo M, Dominguez LJ (2007). Magnesium metabolism in type 2 diabetes mellitus, metabolic syndrome and insulin resistance. *Archives of Biochem and Biophy* 458(1):40-47. <https://doi.org/10.1016/j.abb.2006.05.007>
10. Benzie IF, Strain JJ (1996). The ferric reducing ability of plasma (FRAP) as a measure of “antioxidant power”: the FRAP assay. *Analytical Biochem* 239(1):70-76. <https://doi.org/10.1006/abio.1996.0292>
11. Berg WK, Cunningham SM, Brouder SM, Joern BC, Johnson KD, Volenec JJ (2009) Influence of phosphorus and potassium on alfalfa yield, taproot C and N pools, and transcript levels of key genes after defoliation. *Crop Sci* 49:974–982. <https://doi.org/10.2135/cropsci2008.07.0395>
12. Bhardwaj P, Thakur MS, Kapoor S, Bhardwaj AK, Sharma A, Saxena S, Chaurasia OP, Kumar R. Phytochemical Screening and Antioxidant Activity Study of Methanol Extract of Stems and Roots of *Codonopsis clematidea* from Trans-himalayan Region. *Pharmacog J*. 2019;11(3):536-46.
13. Bhargava BS, Raghupathi HB (1994) Analysis of plant materials for macro and micronutrients. *Methods of analysis of soils, plants, water and fertilizers* 49-82.
14. Bozzini A, Calcagno F, Soare T (2007) “SINCRO”, A NEW *Brassica carinata* Cultivar For Biodiesel Production/“Sincron”, La Nueva Variedad De *Brassica Carinata* Para La Producción De Biodiesel/“Sincron” Un Nouveau Cultivar De *Brassica Carinata* Pour La Production De Biodiesel. *Helia* 30(46):207-214. <https://doi.org/10.2298/hel0746207b>
15. Chang NW, Huang PC (1998) Effects of the ratio of polyunsaturated and monounsaturated fatty acid to saturated fatty acid on rat plasma and liver lipid concentrations. *Lipids* 33(5):481-487. <https://doi.org/10.1007/s11745-998-0231-9>
16. Chauhan JS, Kumar S, Singh KH, Meena SS, Meena ML (2010) Oil and seed meal quality indices of Indian rapeseed-mustard varieties. *J of Plant Biochem and Biotech* 19(1):83-86. <https://doi.org/10.1007/BF03323440>
17. Chauhan PK, Jaryal M, Kumari K, Singh M (2012) Phytochemical and in vitro antioxidant potential of aqueous leaf extracts of *Brassica juncea* and *Coriandrum sativum*. *Int J of Pharma Sci and Res* 3(8):2862-2865.
18. Chauhan, J. S., Tyagi, P., Tyagi, M. K., & Tyagi, P. (2002). Inheritance of erucic acid content in two crosses of Indian mustard (*Brassica juncea* L.). *SABRAO Journal of Breeding and Genetics*, 34, 19-26.

19. Christie, W. W. (1990). Preparation of methyl esters-Part 1. *Lipid technology*, 2(2), 48-49.
20. Coonrod D, Brick MA, Byrne PF, DeBonte L, Chen Z (2008) Inheritance of long chain fatty acid content in rapeseed (*Brassica napus* L.). *Euphytica* 164(2):583-592. <https://doi.org/10.1007/s10681-008-9781-7>
21. Decker EA, Welch B (1990) Role of ferritin as a lipid oxidation catalyst in muscle food. *J of Agri and Food Chem* 38(3):674-677. <https://doi.org/10.1007/s10681-008-9781-7>
22. Deng GF, Lin X, Xu XR, Gao LL, Xie JF, Li HB (2013) Antioxidant capacities and total phenolic contents of 56 vegetables. *J. of Functional Foods* 5(1):260-266. <https://doi.org/10.1016/j.jff.2012.10.015>.
23. Devkota C, Bhattarai BP, Mishra SR, Ghimire P, Chaudhari D (2020) Effect of integrated plant nutrient management on growth, yield and leaf nutrient status of broadleaf mustard (*Brassica juncea* var. *rugosa*). *Horti Int J* 4(3):78-81. <https://doi.org/10.15406/hij.2020.04.00162>
24. Ebrahimzadeh MA, Pourmorad F, Bekhradnia AR (2008). Iron chelating activity, phenol and flavonoid content of some medicinal plants from Iran. *African J of Biotech*, 7(18):3188-3192.
25. El Gharas H (2009) Polyphenols: food sources, properties and applications—a review. *Int J of Food Sci and Tech*, 44(12):251-2518. <https://doi.org/10.1111/j.1365-2621.2009.02077.x>
26. El-Beltagi HEDS, Mohamed AA (2010). Variations in fatty acid composition, glucosinolate profile and some phytochemical contents in selected oil seed rape (*Brassica napus* L.) cultivars. *Grasas Y Aceites* 61(2):143-150. <https://doi.org/10.3989/gya.087009>
27. Fernández León AM, Lozano RM, González GD, Ayuso YMC, Fernández León MF (2014) Bioactive compounds content and total antioxidant activity of two savoy cabbages. *Czech J Food Sci* 32(6):549-554.
28. Ghafoorunissa G (1994) Dietary fats/oils and heart diseases. Sustainability in oil seeds. *Indian Society of Oil Seeds Res Hyderabad*, 486-490.
29. Gul S, Ahmed S, Gul H, Shad KF, Zia-Ul-Haq M, Badiu D (2013) The antioxidant potential of *Brassica rapa* L. on glutathione peroxidase, superoxide dismutase enzymes and total antioxidant status. *Romanian Rev of Lab Med* 21:161-169. <https://doi.org/10.2478/rrlm-2013-0008>
30. Harbaum B, Hubbermann EM, Zhu Z, Schwarz K (2008) Impact of fermentation on phenolic compounds in leaves of pak choi (*Brassica campestris* L. ssp. *Chinensis* var. *communis*) and Chinese leaf mustard (*Brassica juncea* Coss). *J of Agri and Food Chem* 56(1):148-157. <https://doi.org/10.1021/jf072428o>
31. Hossain M, Ahmed K, Chowdhury F, Roksana K, Islam S, Barman A (2015) Experimental study on grain weight, moisture, ash, carbohydrates, protein, oil, total energy and minerals content of different varieties of rapeseed and mustard (*Brassica spp.*). *Int J Sci Res Publ* 5:394-400.
32. Jackson ML (1973) Soil chemical analysis. Prentice hall of India Pvt. Ltd., New Delhi, India 498: 151-154.
33. Jaiswal AK, Rajauria G, Abu-Ghannam N, Gupta S (2011) Phenolic composition, antioxidant capacity and antibacterial activity of selected Irish *Brassica* vegetables. *Nat Prod Comm* 6(9):1299-304. <https://doi.org/10.1177/1934578X11006009>
34. Kardalas E, Paschou SA, Anagnostis P, Muscogiuri G, Siasos G, Vryonidou A (2018) Hypokalemia: a clinical update. *Endocr. Connect* 7:135–146. <https://doi.org/10.1530/EC-18-0109>
35. Khanam UKS, Oba S, Yanase E, Murakami Y (2012) Phenolic acids, flavonoids and total antioxidant capacity of selected leafy vegetables. *J of Fun Foods* 4(4):979-987. <https://doi.org/10.1016/j.jff.2012.07.006>
36. Lin LZ, Sun J, Chen P, Harnly J (2011) UHPLC-PDA-ESI/HRMS/MS n analysis of anthocyanins, flavonol glycosides, and hydroxycinnamic acid derivatives in red mustard greens (*Brassica juncea* Coss variety). *J of Agri and Food Chem* 59(22):12059-12072. <https://doi.org/10.1021/jf202556p>
37. Lowry O, Rosebrough N, Farr AL, Randall R (1951) Protein measurement with the Folin phenol reagent. *J of Bio Chem* 193(1):265-275.
38. Mandal S, Hazra B, Sarkar R, Biswas S, Mandal N (2009) *Hemidesmus indicus*, an age old plant: study of its *in vitro* antioxidant and free radical scavenging potentials. *Pharmacologyonline* 1:604-617.
39. Marklund S, Marklund G (1974) Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. *European J of Biochem* 47(3):469-474.

40. Martins N, Barros L, Santos-Buelga C, Silva S, Henriques M, Ferreira IC (2015) Decoction, infusion and hydroalcoholic extract of cultivated thyme: Antioxidant and antibacterial activities, and phenolic characterisation. *Food Chem* 167:131-137. <https://doi.org/10.1016/j.foodchem.2014.06.094>
41. McDonald S, Prenzler PD, Antolovich M, Robards K (2001) Phenolic content and antioxidant activity of olive extracts. *Food Chem* 73(1):73-84. [https://doi.org/10.1016/S0308-8146\(00\)00288-0](https://doi.org/10.1016/S0308-8146(00)00288-0)
42. Middleton E, Kandaswami C, Theoharides TC (2000) The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. *Pharmacological Rev* 52(4):673-751.
43. Mobeen Wang X, Saleem MH, Parveen A, Mumtaz S, Hassan A, Yasin G (2021) Proximate composition and nutritive value of some leafy vegetables from Faisalabad, Pakistan. *Sustainability* 13(15):8444. <https://doi.org/10.3390/su13158444>
44. Moirangthem L, Praveen N (2020) Phytochemical analysis and antioxidant activity studies of leaf of *Brassica juncea* var. *rugosa* through sequential solvent extraction. *Int J of Pharm Sci and Res* 11(1):343-351
45. Moscow S, Jothivenkatachalam K (2012) Study on mineral content of some Ayurvedic Indian medicinal plants. *Int J Pharma Sci and Res* 3(2): 294-299.
46. Mourato, M. P., Moreira, I. N., Leitão, I., Pinto, F. R., Sales, J. R., & Martins, L. L. (2015). Effect of Heavy Metals in Plants of the Genus *Brassica*. *International Journal of Molecular Sciences*, 16(8), 17975-17998. <https://doi.org/10.3390/ijms160817975>.
47. Nishikimi M, Rao NA, Yagi K (1972) The occurrence of superoxide anion in the reaction of reduced phenazine methosulfate and molecular oxygen. *Biochem and Biophy Res Comm* 46(2):849-854. [https://doi.org/10.1016/S0006-291X\(72\)80218-3](https://doi.org/10.1016/S0006-291X(72)80218-3)
48. Pant U, Bhajan R, Singh A, Kulshethra K, Singh AK, Punetha H (2020) Green leafy mustard: A healthy alternative. *E J of Plant Breeding* 11(01):267-270. <https://doi.org/10.37992/2020.1101.045>
49. Parajuli A (2015) Cultivation and Management practices of leafy vegetable in Nepal. Wordpress.com. <https://parajulianish.wordpress.com/2015/02/13/cultivation-and-management-practices-of-leafy-vegetables-in-nepal/> Accessed 13 Feb 2015
50. Parihar PS, Prakash O, Punetha H (2012) Changes in metabolites of *Brassica juncea* (Indian mustard) during progressive infection of *Alternaria brassicae*. *Nat Sci* 10: 39-42.
51. Prieto P, Pineda M, Aguilar M (1999) Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: specific application to the determination of vitamin E. *Anal Biochem* 269(2):337-341. <https://doi.org/10.1006/abio.1999.4019>
52. Rai GK, Bagati S, Rai PK, Rai SK, Singh M (2018) Fatty acid profiling in rapeseed mustard (*Brassica* species). *Int J Curr Microbiol App Sci* 7(5):148-157. <https://doi.org/10.20546/ijcmas.2018.705.019>
53. Rakow G, Raney JP (2003) Present status and future perspectives of breeding for seed quality in Brassica oilseed crops. In Proc. 11th Int. Rape Seed Congress, Copenhagen, Denmark (pp. 181-185).
54. Rao DE, Divya K, Prathyusha IVSN, Krishna CR, Chaitanya KV (2017) Insect-resistant plants. *Current Dev in Biotech and Bioeng* 47-74. <https://doi.org/10.1016/B978-0-444-63661-4.00003-7>
55. Rauniyar K, Bhattarai BP (2017) Growth, yield and soil nutrient status of broad leaf mustard (*Brassica juncea* var. *rugosa*) under integrated nutrient management. *Nepalese J Agri Sci* 15:98-106.
56. Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C (1999) Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Bio and Med* 26(9-10):1231-1237. [https://doi.org/10.1016/S0891-5849\(98\)00315-3](https://doi.org/10.1016/S0891-5849(98)00315-3)
57. Rice-Evans C, Miller N, Paganga G (1997) Antioxidant properties of phenolic compounds. *Trends in plant Sci* 2(4):152-159. [https://doi.org/10.1016/S1360-1385\(97\)01018-2](https://doi.org/10.1016/S1360-1385(97)01018-2)
58. Rice-Evans CA, Miller NJ (1996) Antioxidant activities of flavonoids as bioactive components of food. *Biochemical Society Transactions*, 24(3):790-795. <https://doi.org/10.1042/bst0240790>
59. Rout GR, Sahoo S (2015) Role of iron in plant growth and metabolism. *Rev in Agri Sci* 3:1-24. <https://doi.org/10.7831/ras.3.1>
60. Saha J, Biswal AK, Deka SC (2015) Chemical composition of some underutilized green leafy vegetables of Sonitpur district of Assam, India. *Int Food Res J (Malaysia)* 22(4):1466-1473

61. Saris NEL, Mervaala E, Karppanen H, Khawaja JA, Lewenstam A (2000) Magnesium: an update on physiological, clinical and analytical aspects. *Clinica chimica acta* 294(1-2):1-26. [https://doi.org/10.1016/S0009-8981\(99\)00258-2](https://doi.org/10.1016/S0009-8981(99)00258-2)
62. Shahidi F, Zhong Y (2008) Bioactive peptides. *Journal of AOAC international* 91(4):914-931. <https://doi.org/10.1093/jaoac/91.4.914>
63. Shannon LM, Kay E, Lew JY (1966) Peroxidase isozymes from horseradish roots: I. Isolation and physical properties. *J of Bio Chem* 241(9):2166-2172. [https://doi.org/10.1016/S0021-9258\(18\)96680-9](https://doi.org/10.1016/S0021-9258(18)96680-9)
64. Sharafi Y, Majidi MM, Goli SAH, Rashidi F (2015) Oil content and fatty acids composition in Brassica species. *Inter J of Food Properties* 18(10):2145-2154. <https://doi.org/10.1080/10942912.2014.968284>
65. Sharma, A., & Cannoo, D. S. (2016). Comparative evaluation of extraction solvents/techniques for antioxidant potential and phytochemical composition from roots of *Nepeta leucophylla* and quantification of polyphenolic constituents by RP-HPLC-DAD. *Journal of Food Measurement and Characterization*, 10, 658-669.
66. Simopoulos AP (2002) The importance of the ratio of omega-6/omega-3 essential fatty acids. *Biomed. & Pharmacotherapy* 56(8):365-379. [https://doi.org/10.1016/S0753-3322\(02\)00253-6](https://doi.org/10.1016/S0753-3322(02)00253-6)
67. Singh BK, Sharma SR, Singh B (2010) Antioxidant enzymes in cabbage: variability and inheritance of superoxide dismutase, peroxidase and catalase. *Scientia Horti* 124(1):9-13. <https://doi.org/10.1016/j.scienta.2009.12.011>
68. Snell FD, Snell CT (1959) Colorimetric methods of analysis. D. van Nostrand. (Book)
69. Sun B, Tian YX, Jiang M, Yuan Q, Chen Q, Zhang Y, Tang HR (2018) Variation in the main health-promoting compounds and antioxidant activity of whole and individual edible parts of baby mustard (*Brassica juncea* var. *gemmafera*). *RSC Adv* 8(59):33845-33854. <https://doi.org/10.1039/C8RA05504A>
70. Thakur AK, Singh KH, Singh L, Nanjundan J, Khan YJ, Singh D (2018) SSR marker variations in *Brassica* species provide insight into the origin and evolution of *Brassica* amphidiploids. *Hereditas* 155(1):1-11. <https://doi.org/10.1186/s41065-017-0041-5>
71. Thor K (2019) Calcium—Nutrient, and messenger. *Frontiers in Plant Sci* 10:440. <https://doi.org/10.3389/fpls.2019.00440> (Review Article)
72. Tripathi CSM (2019) Biochemical studies in Indian mustard (*Brassica juncea* L.) Czern and Coss for fatty acid profiling. *Int J of Chem Sci* 7(4):338-343.
73. Vajjanathappa J, Badami S, Bhojraj S (2008) *In vitro* antioxidant activity of *Enicostemma axillare*. *J of Health Sci* 54(5):524-528. <https://doi.org/10.1248/jhs.54.524>
74. Velasco L, Goffman FD, Becker HC (1998) Variability for the fatty acid composition of the seed oil in a germplasm collection of the genus *Brassica*. *Genetic Res and Crop Ev* 45(4):371-382. <https://doi.org/10.1023/A:1008628624867>
75. Yen GC, Duh PD (1993) Antioxidative properties of methanolic extracts from peanut hulls. *J of the American Oil Chemists' Society* 70(4):383-386. <https://doi.org/10.1007/BF02552711>
76. Zhuang H, Hildebrand DF, Barth MM (1995) Senescence of broccoli buds is related to changes in lipid peroxidation. *J of Agri and Food Chem* 43(10):2585-2591. <https://doi.org/10.1021/jf00058a006>.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in Research Square at <https://www.researchsquare.com/article/rs-4340286/v1>.

CONFLICT OF INTEREST

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STATEMENT OF EXPERIMENTAL RESEARCH

The collected seed material was cultivated at Norma E. Borlaug, Crop Research Centre of Govind Ballabh Pant University of Agriculture and Technology, Pantnagar, Uttarakhand. The study was in accordance with relevant institutional, national, and international guidelines and legislation.

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