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Metabolomic comparison and antioxidant activities of the of Broccoli and Cauliflower residuals

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ABSTRACT

Our body defends itself from oxidative stress via endogenous antioxidants. However, in case of deficiency, antioxidant supplements from plant and animal sources may help in maintaining overall health. The current research was conducted to evaluate the activity of enzymatic antioxidants like Superoxide dismutase, Ascorbate Peroxidase, Catalase, Glutathione Peroxidase, Glutathione Reductase, and non-enzymatic antioxidant Ascorbic acid in floret and stem of cauliflower and broccoli cultivar. Native PAGE was performed for SOD, CAT, and Glutathione Peroxidase. Antioxidant activity significantly varied among broccoli and cauliflower and their different parts. The activity of APX, GPX, and Protein was found maximum in Broccoli floret. Whereas SOD and CAT activity was higher in broccoli stems. Cauliflower floret had maximum ascorbic acid followed by broccoli floret. GR activity was highest in the cauliflower floret. Except for CAT & GR , all other enzymatic antioxidant activity like SOD, APX, GPX, was 2.5%, 47.59% and 63.59%, higher in broccoli floret as compared to cauliflower floret. CAT activity was 29.64% higher in cauliflower floret compared to broccoli floret. Our findings support future efforts to utilize broccoli and cauliflower agro-waste as sources of compounds for the nutraceutical and pharmaceutical industries.

Keywords: Oxidative stress, Antioxidant supplements, Enzymatic antioxidants, Broccoli and cauliflower cultivar

INTRODUCTION

Oxidative stress and free radicals are widely recognized as harmful to human health. When there is an imbalance between pro-oxidants and antioxidant mechanisms, it leads to oxidative stress. This condition can be caused by various environmental factors, including exposure to pollutants, alcohol consumption, certain medications, infections, a poor diet, toxins, radiation exposure, and more (Pizzino *et al.,* 2017).

Numerous studies have provided substantial evidence that oxidative damage to DNA, proteins, and other macromolecules significantly contributes to the development and advancement of various illnesses. These include cardiovascular diseases (CVD), cancer (Tousoulis *et al*., 2017; Spickett & Pitt, 2018, Sullivan *et al.,* 2018; Trachootham *et al.,* 2019) as well as neurological disorders such as amyotrophic lateral sclerosis (Nowsheen et al., 2020), multiple sclerosis, Parkinson's disease (Reddy *et al.,* 2018), and Alzheimer's disease (Zhao & Zhao, 2020).

ROS, which stands for reactive oxygen species, encompasses highly reactive oxygen-containing molecules, including free radicals. Various types of ROS include hydroxyl radical, hydrogen peroxide, superoxide anion radical, nitric oxide radical, singlet oxygen, hypochlorite radical, and various lipid peroxides (Singh, 2022).

Antioxidants are molecules capable of inhibiting the oxidation of other molecules (Norma *et al.,* 2019). In simpler terms, antioxidants are compounds that prevent oxidation. They delay autoxidation by hindering the formation of free radicals and counteract oxidation by donating electrons from their hydroxyl (-OH) group. Additionally, they may indirectly reduce the production of free radicals by inhibiting the effectiveness or expression of enzymes that create free radicals or by enhancing the activities and expressions of other antioxidant enzymes (Hasanuzzaman *et al.,* 2020).

Our body employs endogenous antioxidants to defend against oxidative damage. However, when the endogenous antioxidants become insufficient or imbalanced in countering oxidants, exogenous antioxidant supplements can help restore the balance (Kurukutas, 2016). Exogenous antioxidants of animal or plant origin are primarily introduced through diet or nutritional supplementation. Plants contain phytochemicals or "plant chemicals," many of which exhibit antioxidant properties (Forni *et al.,* 2019). Brassica vegetables, such as cauliflower and broccoli, serve as excellent sources of natural antioxidants due to their high levels of carotenoids, tocopherols, and ascorbic acid. These compounds have demonstrated the ability to inhibit tumor growth, induce

apoptosis, and prevent the formation of cancerous cells (Sun *et al.,* 2018; Wang *et al.,* 2019). For instance, sulforaphane, a well-known glucosinolate found in broccoli, has shown the potential to suppress cancer cell proliferation and reduce tumor formation in preclinical models (Traka & Mithen, 2019). Apart from antioxidant vitamins, carotenoids, and polyphenols, Brassica vegetables also contain glucosinolates, which possess relatively low antioxidant activity, but their hydrolysis products can protect against cancer (Anna, Podsędek, 2005; Ramirez *et al.,* 2020). The antioxidant content of Brassica vegetables varies due to factors such as variety, harvest maturity, growing conditions, soil quality, and post-harvest storage conditions (Alfredo, 2015). The present study aimed to assess the activity of enzymatic antioxidants, such as superoxide dismutase, ascorbate peroxidase, catalase, glutathione peroxidase, and glutathione reductase, as well as the non-enzymatic antioxidant ascorbic acid in the florets and stems of cauliflower and broccoli cultivars.

MATERIAL AND METHODS

Material: *Brassica oleracea var. italica* and Brassica oleracea var. botrytis stem and florets were selected for the current study as these vegetables are good sources of antioxidant enzymes.

Collection of Materials:

Cauliflower and Broccoli samples were collected near Nandan Sankar school, Trimurti Nagar, Jabalpur, Madhya Pradesh in sterilized polyethene bags. These were brought to the lab in an ice box within two hours of collection. The samples were then washed with abundant tap water (to remove any soil residues). Florets and stems of broccoli and cauliflower were sorted and selected for uniform size, color and without evidence of insect, disease, or mechanical damage, for use in the experiments.

Enzymatic Antioxidant Extraction:

The extraction of enzymes followed the protocol recommended by Wang *et al.* (2008). Each gram of cauliflower and broccoli stem and floret was collected, finely crushed, and homogenized in 1 ml of cold potassium phosphate buffer (100 mM, pH 7.0) containing 1% polyvinylpyrrolidone (PVP) and 1 mM EDTA. The mixture was then centrifuged at 4°C for 20 minutes at 10,000 xg. The resulting supernatant was separated and stored at -14 degrees Celsius until enzyme activity assessment. These extracts were utilized for both spectrophotometer assays and native PAGE assays of all enzymes, except for APX, where the extraction buffer included 2 mM L-ascorbate.

Protein Quantification:

Protein content was determined following the Lowry *et al.* (1951) method, using bovine serum albumin (BSA) fraction V (Sigma) as a standard.

Catalase Assay:

The catalase (CAT) activity was evaluated according to the procedure outlined by Goth (1991). The reaction utilized reagents such as 65 µM H_2O_2, 6.0 mM sodium phosphate buffer, and 32.4 mM ammonium molybdate. For the assay, a mixture containing 100 µg protein of enzyme extract and 360 µl of sodium phosphate buffer with 1 ml of H_2O_2 was prepared. The reaction was initiated by adding 1 ml of ammonium molybdate at 60-second intervals, and the reduction in absorbance at 405 nm was monitored. The enzyme activity was expressed as (Ku)/liter.

Ascorbate Peroxidase Assay:

The activity of ascorbate peroxidase (APX) was determined based on Nakano and Asada's (1981) method. The reaction mixture consisted of 50 mM potassium phosphate buffer with 0.5 mM L-ascorbic acid and 1 mM H_2O_2. To start the reaction, 100 µg protein of enzyme extract and 1 ml of potassium phosphate buffer with ascorbic acid were combined. Then, 0.1 ml of 1 mM H_2O_2 was added, and the reduction in absorbance at 290 nm was measured at 15-second intervals over a 2-minute period. Enzyme activity was presented as units per milligram of protein per minute, where a 0.1 absorbance decrease corresponded to one unit of enzyme activity.

Superoxide Dismutase Assay:

The superoxide dismutase (SOD) activity assay followed the Beyer and Fridovich (1987) method. The reaction mixture included 27 ml of 50 mM potassium phosphate buffer (pH 7.8), 1.5 ml of L-methionine (300 mg/10 ml), 1 ml of nitroblue tetrazolium salt (NBT) (14.4 mg/10 ml), and 0.75 ml of Triton X-100. Aliquots (1 ml) of this mixture, along with 100 µg protein of enzyme extract and 10 ml of riboflavin (4.4 mg/100 ml), were added to glass tubes. The tubes were exposed to light, and the increase in absorbance at 560 nm was recorded.

Guaiacol Peroxidase Assay:

The guaiacol peroxidase activity was measured at 25°C using the method described by Tatiana *et al.* (1999). The reaction mixture contained 50 mM potassium phosphate (pH 7.0), 2 mM H_2O_2, and 2.7 mM guaiacol. Enzyme extract equivalent to 5 g protein was added to initiate the reaction, and the formation of tetra guaiacol was

measured at 470 nm. Enzyme activity was quantified as the amount of enzyme oxidizing 1 µ mol of substrate per minute.

Glutathione Reductase Assay:

The glutathione reductase (GR) activity was determined as per the Hasanuzzaman et al. (2011) method. The reaction mixture consisted of 0.1 M potassium-phosphate buffer (pH 7.0), 1 mM EDTA, 1.0 mM oxidized glutathione (GSSG), 0.2 mM NADPH, and the enzyme solution. The decrease in absorbance at 340 nm was recorded over 1 minute, and the enzyme activity was calculated as nanomoles of NADPH oxidized per minute per milligram of protein.

Non-enzymatic Antioxidant Extraction:

Non-enzymatic antioxidants were extracted by homogenizing samples in 3 ml of ice-cold acidic extraction buffer (6% metaphosphoric acid containing 1 mM EDTA). The resulting mixture was centrifuged at 4°C for 15 minutes at 11,500 xg. For NPT, 5% sulfosalicylic acid (SSA) was used in a 1:6 ratio instead of an acidic extraction buffer, and the supernatant was stored at -20°C.

Ascorbic Acid Assay:

Ascorbic acid content was estimated following the Mukherjee & Choudhuri (1983) method. An extract aliquot (4 ml) was mixed with 2 ml of 2% dinitrophenyl hydrazine in an acidic medium, followed by the addition of thiourea in 70% ethanol. After heating and cooling, 5 ml of 80% H_2SO4 was added, and the absorbance was measured at 530 nm. An ascorbic acid standard curve was used to estimate the unknown ASA content.

In-Gel Assay for Enzymatic Antioxidants:

The native PAGE method according to Laemmli (1970) was employed to assess changes in proteins with ROS scavenging enzyme isozyme activity. Equal volumes of enzyme extract (100 µg protein) and loading buffer were loaded into each gel lane. PAGE was carried out for SOD, CAT, and APX.

Native Gel SOD Assay:

The native gel activity of SOD was visualized following the Yen *et al.* (1996) procedure. The gel was exposed to light, and the appearance of achromatic bands against the background indicated SOD activity.

In-Gel CAT Assay:

CAT activity in native PAGE gels was visualized using the method by Woodbury *et al.* (1971). After electrophoresis, the gel was incubated with H_2O_2 and then with potassium ferricyanide and ferric chloride solution to visualize catalase isozymes.

In-Gel APX Assay:

The in-gel activity of APX was determined as per the protocol of Mittler & Zilinskas (1993). The gel was equilibrated with a solution containing ascorbate, followed by incubation with a mixture of ascorbate and H_2O_2. The presence of APX was indicated by the appearance of achromatic bands against a purple background.

Statistical Analysis:

Data analysis was performed using MS Excel. ANOVA was used to compare significant differences in antioxidant activity. Duncan's Multiple Range test identified significant variations, with a heatmap generated using XLSTAT 2020 to visualize differences in antioxidant activity among samples ($P < 0.05$).

RESULTS

The present study was conducted to study the antioxidants in the stem and floret of cauliflower and broccoli. Salient points of the results obtained are reported below and further discussed in light of available literature.

In the present work total protein was estimated in the extracts of broccoli and cauliflower stem and floret. Maximum protein was present in the Broccoli floret and minimum in the Broccoli stem. Cauliflower floret had less protein compared to broccoli floret (Fig.1)

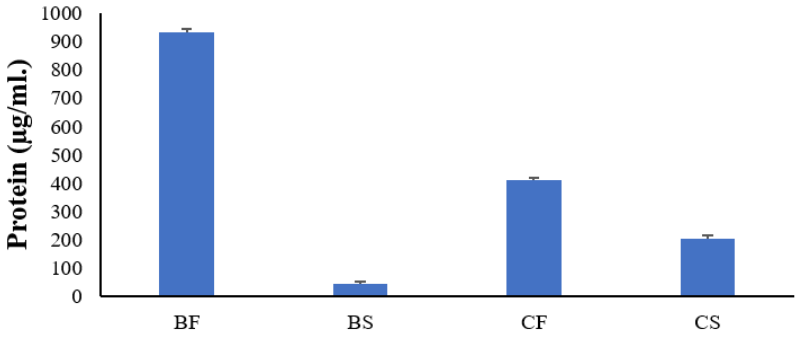

Fig. 1- Protein content in different samples of BF=Broccoli floret, BS= Broccoli stem, CF=Cauliflower floret and CS= Cauliflower stem

SOD activity in broccoli and cauliflower stem and floret was studied. SOD activity was found in all four extracts viz broccoli floret (1.614 units/100µg protein), cauliflower floret (1.574 units/100µg protein), broccoli stem (2.182units/100µg protein), and cauliflower stem (1.998units/100µg protein). The maximum activity of SOD was found in broccoli stem followed by cauliflower stem, broccoli floret, and cauliflower floret. SOD activity in broccoli floret was 2.5 % high compared to cauliflower floret. In broccoli stem, it was 8.5 % higher compared to cauliflower stem. In the present study, we noticed a higher activity of SOD was found in broccoli than in cauliflower (Fig.2).

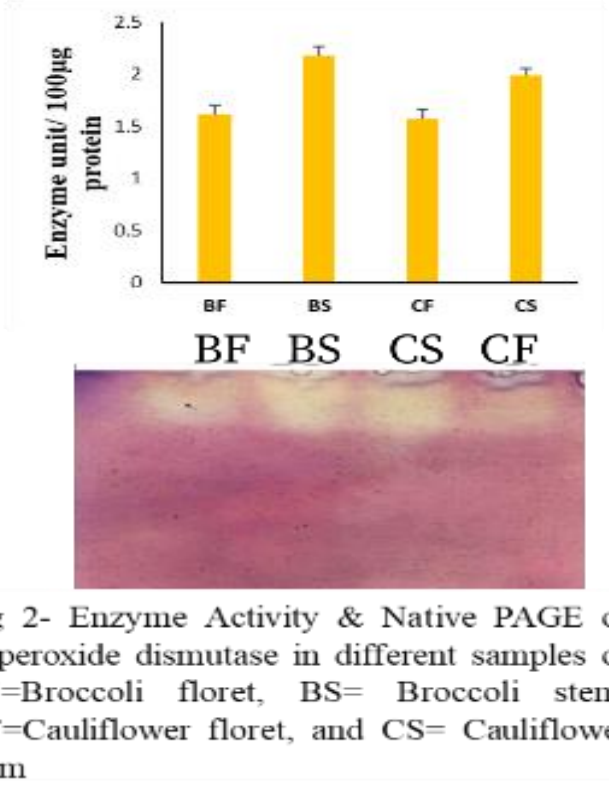

Fig 2- Enzyme Activity & Native PAGE of Superoxide dismutase in different samples of BF=Broccoli floret, BS= Broccoli stem, CF=Cauliflower floret, and CS= Cauliflower stem

CAT activity in broccoli and cauliflower stem and floret was studied. CAT was found in all extracts viz broccoli stem (211.23 units/l), cauliflower floret (112.06 units/l), cauliflower stem (105.95 units/l), and broccoli floret (78.78 units/l) (Fig 3). Maximum activity of CAT was found in broccoli stem followed by cauliflower floret, cauliflower stem, and broccoli floret. In broccoli stem, it was 49.81% higher compared to cauliflower stem and cauliflower floret had 29.64% higher activity compared to broccoli floret. In the present study, we noticed that maximum activity of CAT was found in broccoli stem and minimum activity was found in broccoli floret (Fig 3).

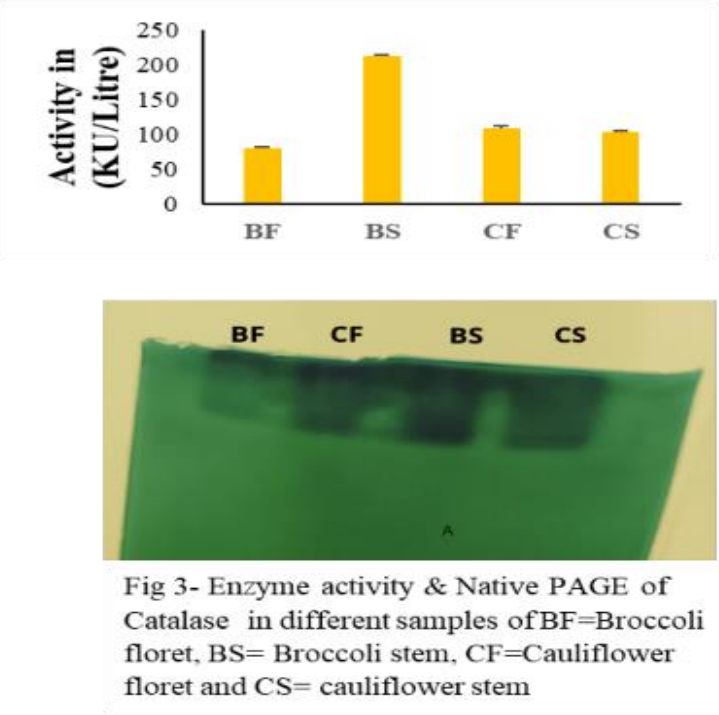

Fig 3- Enzyme activity & Native PAGE of Catalase in different samples of BF=Broccoli floret, BS= Broccoli stem, CF=Cauliflower floret and CS= cauliflower stem

APX activity in broccoli and cauliflower stem and floret was studied. APX activity was found in all four extracts viz broccoli floret (0.61728 units/l), cauliflower floret (0.2976 units/l), broccoli stem (0.28467units/l), and cauliflower stem (0.14880units/l) (Fig.4). Maximum activity of APX was found in broccoli floret followed by cauliflower floret, broccoli stem, and cauliflower stem. APX activity in broccoli floret was 47.59 % high compared to cauliflower floret. In broccoli stem, it was 51.98% higher compared to cauliflower stem. In the present study, we noticed the higher activity of APX was found in broccoli than in cauliflower (Fig 4).

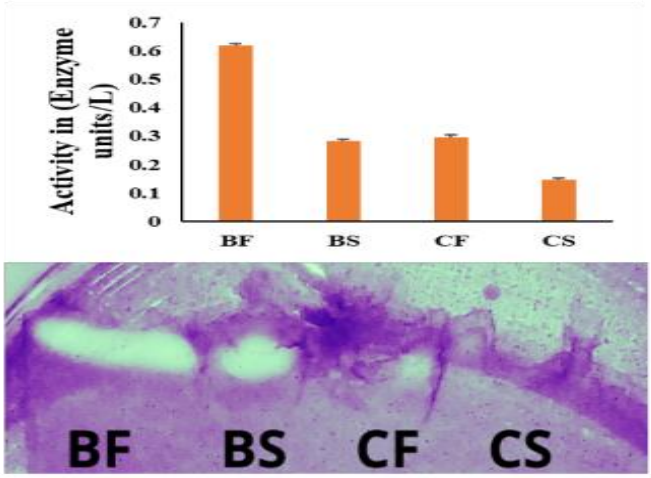

Fig 4 – Enzyme activity & Native PAGE of Ascorbate Peroxidase enzyme in different samples of BF=Broccoli floret, BS= Broccoli stem, CF=Cauliflower floret and CS= Cauliflower stem

GPX was found in all extracts viz broccoli floret (2.45 units/100µg), broccoli stem (0.135 units/100µg), cauliflower floret (0.160 units/100µg), cauliflower stem (0.892 units/100µg). Maximum activity of GPX was found in broccoli floret followed by cauliflower stem, cauliflower floret, and broccoli stem. In cauliflower stem, it was 56.07 % higher compared to broccoli stem and broccoli floret had 63.59% higher activity compared to cauliflower floret. In the present study, we noticed that maximum activity of GPX was found in broccoli floret and minimum activity was found in broccoli stem (Fig. 5).

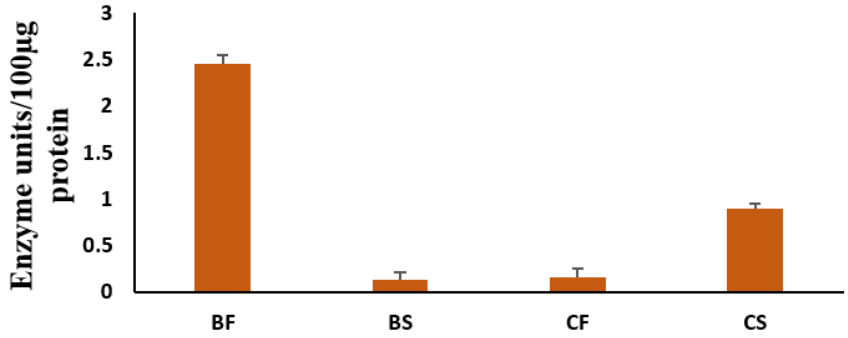

Fig. 5 – GPX activity in different samples of BF=Broccoli floret, BS= Broccoli stem, CF=Cauliflower floret and CS= Cauliflower stem

GR activity was found in all four extracts viz broccoli floret (1.614 units/100µg protein), broccoli stem (1.574 units/100µg protein), cauliflower floret (2.182 units/100µg protein), and cauliflower stem (1.99 units/100µg protein). Maximum activity of GR was found in cauliflower floret followed by, cauliflower stem, broccoli floret and broccoli stem. GR activity in cauliflower floret was higher compared to broccoli floret. In cauliflower stem, it was 21 % higher compared to a broccoli stem (Fig.6)

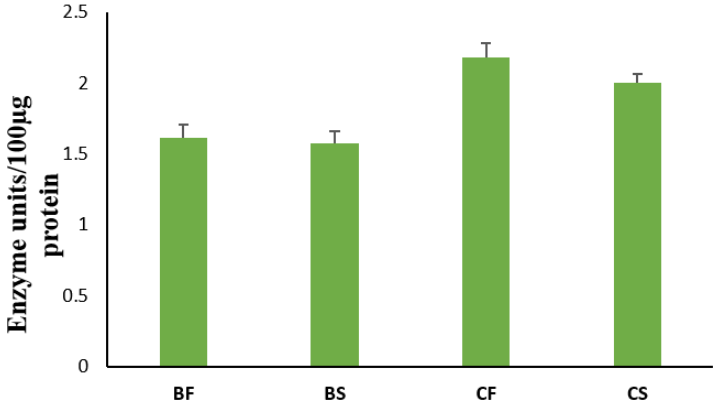

Fig. 6- GR activity in different samples of BF=Broccoli floret, BS= Broccoli stem, CF=Cauliflower floret and CS= Cauliflower stem

Non-enzymatic oxidant, AA's activity in broccoli and cauliflower stem and floret was studied. Ascorbic acid was found in all extracts viz. broccoli floret (3.9 mg./ml) broccoli stem (3.7 mg/ml.), cauliflower floret (4.1 mg/ml), cauliflower stem (3.3 mg/ml). Maximum activity of AA was found in cauliflower floret followed by broccoli floret, broccoli stem, and cauliflower stem. In broccoli stem, it was 11.29% higher compared to cauliflower stem and cauliflower floret had 6.21% higher activity compared to broccoli floret. In the present study, we noticed that the maximum activity of AA was found in cauliflower floret and minimum activity was found in cauliflower stem (Fig.7).

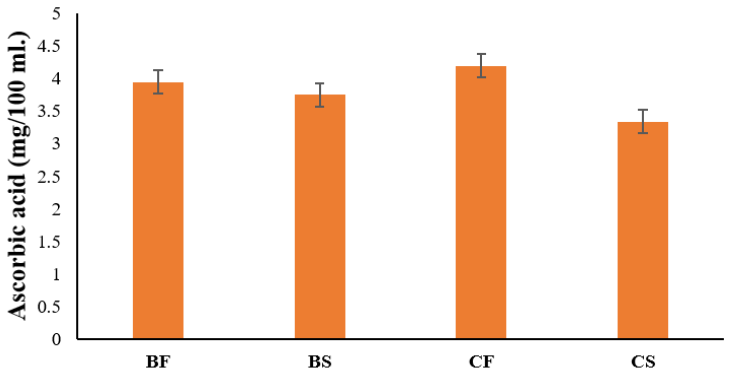

Fig. 7- Ascorbic Acid content in different samples of BF=Broccoli floret, BS= Broccoli stem, CF=Cauliflower floret and CS= Cauliflower stem

In-gel assay- Antioxidant isozyme patterns were also investigated by means of activity staining of the native-PAGE (Fig 8,9,10). In the case of SOD, there was a high molecular weight band that appeared most significantly in the Broccoli stem extract followed by cauliflower stem, broccoli floret, and cauliflower floret. The result is in accordance with spectrophotometric assay results (Fig.1). In order to analyze CAT isozyme profiles, the extract was subjected to a native page and stained for CAT activity (Fig 2). Bands for CAT activity was present in all extracts though the pattern was diffused. In the case of APX, there is one high molecular weight band that appeared most significantly in the Broccoli floret extract. However, the intensity of APX bands decreased in cauliflower floret, broccoli stem, and cauliflower stem (Fig. 3)

The antioxidant profile of broccoli and cauliflower floret tissue was significantly different from that of the stem. The activity of most antioxidants in the tissue of broccoli was higher than those in cauliflower except for Glutathione reductase and ascorbic acid which are higher in cauliflower tissues. The heat map presented in Fig.11, showed two clusters were identified. The dendrogram similarities in antioxidant activity between broccoli floret and cauliflower floret and between broccoli stem and cauliflower stem. The dendrograms of individual antioxidant activity show that SOD and CAT, GR, and AA are in a positive correlation.

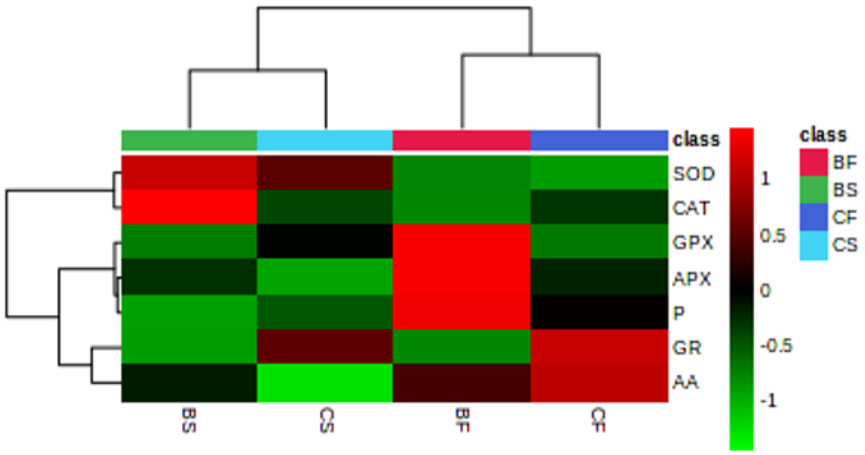

Fig 11. Heat map based on Pearson and Ward for determining distance and clustering of variables (SOD, CAT, APX, GPX, GR, AA and Protein) (row) and cauliflower, broccoli florets and stem (columns). Color intensity is proportional to correlation coefficient.

DISCUSSION

Our research reveals notable variations in the distribution of antioxidant properties within different parts of broccoli and cauliflower plants. Both the florets and stems of these vegetables were found to be abundant sources of antioxidants, both enzymatic and non-enzymatic, although their levels differed significantly between individual organs. These findings align with previous studies conducted on broccoli, cauliflower, kale, turnips, and Arabidopsis, which have reported similar results (Chang *et al.,* 2019). Thus, it suggests that, apart from the edible portions, other organs of cauliflower, particularly the stem, could serve as valuable food products or potential ingredients for the development of new functional foods.

According to the Optimal Defense Theory (ODT), younger leaves, flowers, and fruits are crucial for growth and reproduction, making them more vulnerable and important. Therefore, these plant parts may exhibit higher antioxidant activity as the distribution of phytochemicals in different plant organs depends on their significance (McCall and Fordyce, 2010). Liu et al. (2018) found higher concentrations of amino acids, glucoraphanin, and neoglucobrassicin in broccoli florets compared to other tissues, which supports our current result of higher protein content in broccoli florets followed by cauliflower florets. Chang *et al.* (2019) reported that inflorescences and leaves contained the highest levels of antioxidants and antioxidant capacity. Our study's results, which revealed the highest antioxidant activity in broccoli florets, are consistent with findings from Kumar *et al.* (2019) and Mishra *et al.* (2021). Peroxidase activity in plants varies depending on anatomical location, tissue/plant age, and freshness. In our investigation, we observed higher peroxidase activity in the florets of broccoli and cauliflower. However, Deva *et al.* (2014) reported higher peroxidase activity in the stems of cauliflower compared to the leaves. Rodica *et al.* (2017) suggested that broccoli exhibited the highest catalase activity, followed by kale, white cabbage, and cauliflower. Our study also found higher catalase activity in the stem of broccoli, which is consistent with Renata et al. (2013) who observed lower values in the generative organs of plants from the same family. In their research, CAT activity in the heads was several times lower than in the leaves, ranging between 146.4 and 168.2 µmol H2O2 g^−1 min^−1. Native PAGE analysis supported the spectrophotometric analysis of these enzyme activities (Figure 5). Recent studies have recognized antioxidant enzymes like glutathione reductase (GR) as primary defense mechanisms against degenerative and chronic diseases (Boddupalli *et al.,* 2012). Sharma and Kaur (2017) reported that the antioxidative capacity of aqueous broccoli extract induces the phase II enzyme system, including GR, which reduces lipid peroxidation, restores cell membranes, and improves tissue homeostasis. Ole *et al.* (1995) found that increased glutathione peroxidase (GSH-Px) activity can decrease the potential toxic effects of peroxides. They also observed increased glutathione concentration in different segments of the intestinal mucosa when dietary cruciferous vegetables, including dried broccoli, were consumed. Some broccoli samples also increased renal GSSG-Red activity.

Superoxide dismutase (SOD) is a group of metalloenzymes that protect cells from oxidative damage by dismutation of superoxide radicals into molecular oxygen and hydrogen peroxide (Singh, 2022). SOD and catalase (CAT) activities have been documented in various plant products, including green peppers, grapes, and cucumbers (Jia *et al.,* 2018; Huang *et al.,* 2019; Zhang *et al.,* 2020). Ascorbic acid (AA) sensitivity makes it a useful parameter for evaluating the effects of food processing on vitamin content. Bureau *et al.* (2015) reported that raw broccoli contains a significant amount of vitamin C compared to other raw vegetables. Ahmed and Ali (2013) found the highest AA content in fresh cauliflower florets compared to blanched and water-boiled florets. Our results are consistent with Bhandari and Shiva (2015), who found higher AA activity in cauliflower compared to broccoli. Although the leaves of the broccoli cultivar exhibited higher AA content, cauliflower florets showed higher AA content in all three cultivars.

Our findings strongly corroborate previous studies highlighting the remarkable antioxidant effects and significant reduction in oxidative damage observed in extracts derived from Brassica vegetables (Larocca *et al.,* 2017; Zhang *et al.,* 2021). Moreover, the groundbreaking research by Gudiño *et al.* (2022) unequivocally establishes that even the traditionally undervalued parts of broccoli hold tremendous promise as abundant sources of bioactive compounds, including the highly sought-after glucosinolates. This pivotal discovery opens up new avenues for harnessing the untapped potential of these plant components and revolutionizing the field of functional foods and nutraceuticals

CONCLUSION

In summary, our study unequivocally demonstrates that both *Brassica oleracea* var. *botrytis* and *Brassica oleracea L*. var. *italica* extracts possess a remarkable abundance of potent antioxidant agents. This remarkable

discovery strongly suggests that the entire plant holds immense potential as a valuable source of natural antioxidants. Moreover, our findings provide compelling evidence for the future exploration and extraction of novel antioxidant and bioactive compounds from these plants. Particularly, the extracts derived from the stem and floret of cauliflower and broccoli exhibit profound capabilities in preventing various degenerative diseases by effectively inhibiting the production of reactive oxygen species (ROS). This crucial mechanism reduces the associated risks and ensures the optimal functioning of vital organs. With these groundbreaking insights, our findings lay a solid foundation for future endeavors aimed at harnessing broccoli agro-waste directly for human consumption and as a rich source of compounds for the nutraceutical and pharmaceutical industries.

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