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The role of anthocyanins in activating antioxidant enzymes during postharvest degradation of (*Hibiscus sabdariffa* L.) Roselle calyx

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Abstract

The perishable roselle, calyx, and anthocyanin can swiftly degrade and become brown. The pH, temperature, light, and postharvest enzymes affect anthocyanin stability. The calyx is damaged when the seed was removed, causing stress and microbial decay. Despite this, anthocyanins benefit plants under stress, including drought, high salinity, excessive light, and damage. This study evaluates the role of anthocyanins in activating antioxidant enzymes during deterioration. Total anthocyanin content, Total phenolic content, and Total flavonoids content were assessed, as well as H_2O_2, antioxidant enzymes, polyphenol oxidase (PPO), and phenylalanine ammonia lyase (PAL). The findings revealed that the TAC, TPC, and TFC were significantly higher at ($p \leq 0.05$), from 0 hours to 4days. Both Catalase (CAT), Guaiacol Peroxidase (POD), and Ascorbate Peroxidase (APX) were expressed at significantly higher concentrations after 1 hour and 1 day, leading to a reduction in H_2O_2 concentration. Still, at 3-5-days post deterioration induction, the enzyme activity significantly decreased drastically or depleted. This indicates that anthocyanins play a role in activating antioxidant enzymes in response to stress during roselle calyx deterioration. We have also reported the biochemical changes during deterioration of roselle calyx for the first time. Therefore, anthocyanins might be a potential deterioration biomarker in roselle.

Keywords: Antioxidant enzymes; anthocyanin; calyx; postharvest; degradation; and roselle.
Abbreviations: APX-Ascorbate peroxidase; TPC-Total anthocyanin content; CAT-Catalase; H_2O_2-Hydrogen peroxide; POD-Peroxidase; PAL-Phenylalanine ammonia-lyase; PPO-Polyphenol oxidase; and TPC-Total Phenolic content; TFC- Total flavonoids content; PD-postharvest deterioration.

Introduction

Hibiscus sabdariffa (roselle) is a member of the Malvaceae family that has spread worldwide, including Malaysia (Ahmad A, 2012). Roselle calyces have been widely employed in pharmaceuticals and foods, including syrups, refreshing beverages, wines, jams, and natural food colorants (Ifie, I *et al*., 2017; Da-Costa-Rocha *et al.,* 2014). Furthermore, Roselle calyx is used to treat cardiovascular disease and hypertension and avoid pyrexia and liver issues. Red Roselle calyx variants have antioxidant and cyclooxygenase inhibitory properties. Furthermore, roselle calyx is used in the medicinal and cosmetic industries (Duy *et al.,* 2019). The leaves or calyces are rich in anthocyanins, antioxidants, and aid diuretic and sedative therapies. (Lema *et al.,* 2021). Roselle contains a high concentration of phenolic compounds, primarily anthocyanins, giving its crimson hue. When exposed to temperature, pH, metal ions, oxygen, light, or enzymatic activity, phenolic and anthocyanin molecules become unstable (Bakowska *et al.,* 2003).

Anthocyanidin is a naturally occurring water-soluble pigment that is one of the flavonoid derivatives found in phenolic compounds (Da-Costa-Rocha *et al.,* 2014). The potent roselle extract contains anthocyanins in several forms, including delphinidin-3-sambubioside, which is used in folk medicine to treat numerous diseases (Sablikova L. *et al*., 2015) Fig.1. "Delphinidin, cyanidin, malvidin, pelargonidin, petunidin, and peonidin" are anthocyanin pigments present in different antioxidant-rich plants and fruits (Aurelio *et al.,* 2006; Kahkonen MP, and Heinonen M, 2003). ROS levels can be reduced by manipulating the expression of anthocyanin biosynthesis-related genes (e.g., MYBs; F3H; DFR; ANS) through molecular techniques (Zhang *et al.* 2013; Orzaez *et al.,* 2009). During stress, plants release reactive oxygen species (ROS), subsequently employed as signalling molecules to stimulate the stress tolerance process. During high stress levels, however, overproduced ROS, causing plant oxidative damage (Naing and Kim, 2021). Poor postharvest regulation and stress responses, including

drought, increased salt, light, and wound degradation, are connected with activated anthocyanin plants (Hernández et al., 2009; Sytar et al., 2013). Similarly, plants produce anthocyanins in response to reactive oxygen species (ROS) signals by transcribing the biosynthetic genes of anthocyanins. Anthocyanins are then exploited in antioxidant activities to ensure long-term viability by scavenging excess reactive oxygen species (ROS) (Naing and Kim, 2021; Rao et al. 2022).

Deterioration begins when a crop is removed from its parent plant (Mahmud Tengku, 2017). However, deterioration can increase the production of superoxide, hydrogen peroxide, and hydroxylated radicals. Under these conditions, certain plants' genes become involved and modulate flavonoids' biosynthesis, including anthocyanins, increasing their levels during stress exposure. Additionally, anthocyanins have been shown to protect plants from oxidative stress by donating hydrogen atoms due to their ability to balance unpaired electrons in free radicals. Furthermore, they are believed to have a more potent antioxidant capacity than vitamins C and E. (Jahan et al., 2020). As a means of self-protection from oxidative damage, plants developed an antioxidant defense system that includes enzymes and non-enzymatic components present in their cells, such as CAT, SOD, POD, and APX as reported by Sairam et al., 2011. Antioxidant have the potential to remove, neutralize, and scavenge ROS from various cellular sites, therefore reducing lipid peroxidation and maintaining the integrity of the membrane (Foyer et al., 1994; Li et al., 2013; Scandalios, 1993). Postharvest degradation of cassava results in the expression of catalase, ascorbate peroxide genes, and clandestine peroxides that reduce H2O2 exploiting ascorbate or a range of inorganic and organic substrates as an electron donor (Andersen et al., 2000).

Timing and conventional postharvest processing processes influence the quality of Roselle products. Furthermore, postharvest management affects roselle products' storage time and marketability (Osei Kwarteng and Mahunu, 2021). Similarly, Roselle calyx is perishable, thus making a dry extract from the calyx is challenging because anthocyanin, which is highly reactive, soon degrades and acquires undesirable brown hues. pH, temperature, light, and postharvest-related enzymes are the most critical factors impacting the stability of anthocyanins (Lema et al., 2022). When the seed was removed from the calyx, the calyx was damaged, resulting in stress and microbial deterioration. Today, global agriculture faces an ever-increasing challenge to produce 70 percent more food crops to feed an additional 2.3 billion population by the year 2050, but this tremendous abiotic stress hinders agricultural output worldwide (Munns & Tester 2008: Polash et al. 2019). According to Cardoso et al. (2012), degradation is a natural process that may be slowed down depending on the storage circumstances (Da-Silva et al., 2021). As such, the purpose of this study is to assess the role of anthocyanins in activating antioxidant enzymes and related enzymes that are expressed or respond to stress generated during the deterioration of roselle calyx, which may lead to its degeneration; this could be a potential deterioration biomarker, and this could help in achieving food security and reduce food loss due to postharvest deterioration.

Results

Roselle calyx deterioration

The induced postharvest deterioration of roselle calyx in this study revealed that the calyx started deteriorating at 3 days and wholly degraded or depleted between 6-7days of post-deterioration induction Fig. 2. Compared to the fresh calyx, the deteriorated calyx showed a brown coloration change and loss of biological activity.

Total phenolic content (TPC)

The TPC determined in this study has shown to significantly decrease in the deteriorated calyx (60.9 mg GAE/100g) at 5-days post deterioration than in fresh calyx with a higher significant difference and concentration of 487mg (GAE/100g) Fig.3 There is also considerable difference at $p \leq 0.05$ throughout the deterioration period even though later significantly decreased. Leaving the calyx for long after ripening makes it susceptible to sores, sun cracking, and general deterioration in quality.

Total flavonoids content (TFC)

The total flavonoids content concentration during the deterioration of roselle calyx was detected in this research; the results revealed a significant difference of TFC concentration fresh, 1 hour, 1 day, and 3 days, but there is no significant difference between the 3-days and 5-days post deterioration. The concentration of TFC were higher in 1hour 213mg/ml/QE/g, followed by 1-day, 3 days (143, 125,125 and 43mg/ml/QE/g) but the least TFC concentration was observed in 5-days post deterioration 15 mg/ml/QE/g as seen in Fig.4.

Total anthocyanin content (TAC)

Anthocyanin content of roselle calyx was determined at various time intervals throughout the deterioration process in this investigation. Fresh calyx had significant anthocyanin content in both the delphinidin-3-sambubioside (129 mg/L), cyanidin-3-sambubioside (126 mg/L), and cyanidin-3-glucoside (91 mg/L) tables.1, followed by 1hour (111 mg/L), cyanidin-3-sambubioside (108 mg/L), and cyanidin-3-glucoside (78 mg/L), 3-days (37 mg/L), cyanidin-3-sambubioside (36 mg/L), and cyanidin-3-glucoside (22 mg/L); however, the anthocyanin content was entirely depleted at 5-days. Delphinidin and cyanidin were found to be the most abundant anthocyanins in this study Fig.5. There is a significant difference in Delphinidine sambubioside concentrations across fresh 1hour to 3-days. However, in cyanidin-3-sambubioside and Cyanidine 3-glucoside, the significant difference was only observed in fresh. The schematic presentation of the role anthocyanins in response to PD was explained in Fig.6.

% DPPH Radical Scavenging Assay (RSA)

In the last two decades, the "Antioxidant Bandwagon" has grown substantially and scope, with a focus on finding the best natural sources of antioxidants, adding antioxidants to beverages and other foods, medical applications, and hyper marketing (Schaich et al., 2015). In the present study, the % RSA of DPHH is higher in fresh roselle calyx 48.85; however, 1 hour, one day, 3-days, and 5-days PD of roselle has a % RSA of 37.02, 31.95, 27. 43. While 5-days shown to have the least % RSA of 14%, as indicated in Fig.7. The calculated IC50 values of the roselle, calyx, and Quercetin in the DPPH assay were 0.88, 1.72, 3.42, 5.57, and 7.73 mg/mL, respectively. The 5-days PD shows to have higher IC50, which correspond to the low % RSA observed in this study.

Hydrogen peroxide H2O2 activity

This study found H2O2 activity during PD of roselle calyx. The highest H2O2 concentration was found in the freshly harvested calyx (71.9 µmol-L), followed by 1 day (55.99

µmol/L), three days (29.44 µmol/L), and 5 days induction of PD (Fig.8).
The higher quantities detected in freshly harvested calyx might be attributed to an injury or wound sustained during the removal of the calyx from the seed, a process known as decouring.

Catalase activity (CAT)
The determination of catalase enzyme activity in this research revealed that there is a high catalase enzymatic activity in 1hour (9.8 mmol-protein-min-1), 1-day, (7.3 mmol-protein-min-1), and fresh (7 mmol mg-protein-min-1); however, the activity decreases drastically during 3-days (5.2 µmol-protein-min-1), while in 5-days no activity was detected after 5-days PD as shown in Fig. 9. The catalase activity is due to the expression of Hydrogen peroxide due to wound stress or other biotic and abiotic factors during the deterioration of roselle calyx.

Peroxidase s activity (APX and POD, EC 1.11.1.7)
As mentioned earlier, Peroxidase and Catalase oxidize hydrogen peroxide to O2 and H2O in response to stress and deterioration of roselle calyx. This finding indicates that Ascorbate Peroxidase activity is higher in 1h of deterioration (32.61 µmol-protein-min). Fresh and 1 day had the APX (activity of 16.19 and 11.9 µmol mg-protein-min-1), followed by (3 days 0.95 µmol-protein-min), as shown in Fig.10. Still, there is no activity of APX activity was observed in 5 days post deterioration. However, the Peroxidase activity observed during the PD of roselle calyx indicates that there is significant activity in 1hour and 1-day at 18 and 8.8 µmol-protein-min than 3-days and fresh 6.8 and 5.4 µmol-protein-min, the most minor POD activity was observed in 5-days 4 µmol mg-protein-min as shown in Fig.11.

Polyphenol oxidase (PPO; EC 1.10.3.1)
The result of the polyphenol oxidase activity in this study revealed that fresh calyx has the highest PPO activity (3.5 mmol-protein-min-1), followed by 1h (2.5 mmol-protein-min-1), 1(1.8 mmol-protein-min-1), day and a minor activity was observed 5-days of deterioration (0.14 mmol-protein-min-1) Fig 12. The high activity might be attributed to the wound in the calyx while harvesting and handling, i.e., removal of the calyx from the seed; this might lead to the infestation of microbes and subsequent deterioration. Furthermore, the PPO activity did not follow the pattern of antioxidant enzymes in this study, where they attain the highest activity after hours and later decline.

Phenylalanine ammonia lyase (PAL)
They were the first enzymes in the cinnamate cycle. They remove the pro-S hydrogen in position 3 from l-phenylalanine through trans elimination of ammonia (Martens, S & Forkmann, 1999). After fungal infection, the production of phenylpropanoid phytoalexins requires an extremely fast induction of PAL. The findings of the activity of PAL in this study indicated that there is higher PAL activity in 1hour post deterioration at 29 µmoL-protein-min and 1-day 28 µmoL-protein-min-L, however fresh, 3-days and 5-days showed the PAL activity at 23, 17 and the lowest activity was observed in 5
-days at 5 µmoL PAL mg-protein-min-L as shown in Fig.13.

Discussions

Research into the molecular and biochemical mechanisms of postharvest physiological deterioration (PPD) has revealed that ROS formation is among the first steps in the reaction, and several PPD related genes and their expression have been discovered and documented (Buschmann et al. 2000; Reilly et al. 2003; Reilly et al. 2007; Timothy 2009). Polyphenols are a well-known antioxidant. They provide an electron to free radicals, therefore stabilizing them. This protects cells and tissues from the deleterious consequences of oxidative stress (Tsuda et al., 2003).
Significant decreased concentration of TPC at 5 days could be ascribed to anthocyanin depletion at 5 days after PD and its instability Fig.3. When exposed to a series of biotic and abiotic aspects, roselle calyx rapidly degrades, most notably during the harvesting and manufacturing of certain products (Gweyi-Onyango et al., 2021). TPC, TAC, and radical scavenging activity of DPPH Figs.3, 4, and 7, have shown that there is a strong relationship in their activities during the PD of the roselle calyx is consistent with Turkmen et al., findings' that there is a positive correlation between the total content of phenolic compounds and antioxidant activity, and that extracts possess over 95% of their antioxidant capacity due to their phenolic components (Turkmen et al., 2007; Abdulrahman et al.2019). The concentration required for 50% radical scavenging, known as IC50, is often used to evaluate ROS activity. The greater the antioxidant activity, the lower the IC50 value. The lower IC50 0.88 mg/mL/100g observed in our findings indicates high antioxidant activity, even though as the calyx deteriorates, the IC50 7.73 mg/mL/100g increases while the radical scavenging activity decreases Fig.7; similar findings from strawberry, blackberry, raspberry, and bilberry extracts with the most potent IC50 1.7, 3.8, and 4.0 mg/100 mL, respectively (Vulic et al., 2001).
Reactive oxygen species (ROS) are a family of biological components that includes hydrogen peroxide (H2O2), a naturally occurring molecule in plants (Orozco-Cardenas, Narvaez-Vasquez and Ryan 2001). An increase in genes that produce anti-inflammatory phenolic compounds, such as phenylpropanoid (P.P.) genes, provides a local signal for hypersensitive cell death and cell wall stiffness in wound pathways (Orozco-Cardenas, Narvaez-Vasquez, and Ryan, 2001; Lawton et al. 1991; George, 2014). The antioxidant enzymes generated in response to signals that break down H2O2, such as Catalase and peroxidase, may be responsible for the significant decrease of H2O2 in 5 days DP Fig.8. This current finding is supported by Decros et al., 2019; Liu et al., 2021; Meng et al., 2013, who discovered that hydrogen peroxide regulates senescence and postharvest fruit quality deterioration.
The presence of high anthocyanin and phenolic content during the early stages of PD may be attributed to antioxidant enzymes responding to the stress caused by ROS during degeneration, as antioxidant enzymes play a role in activating antioxidant enzymes and protecting plants against both biotic and abiotic stress table 1 and Fig.3. These findings are in line with those reported by Jia et al., The postharvest performance of vegetables may be enhanced by the presence of anthocyanins. For example, anthocyanins subside lipid peroxidation and maintain the integrity of the membrane to decline cell senescence, for which they are regarded as antioxidants (Jiao et al., 2012). Another benefit of anthocyanins is that they inhibit ROS activity and signalling function. This means that fruits with higher antioxidant capacity may delay over-ripening processes. Postharvest senescence is heavily dependent on ROS, which has recently garnered attention (Decros et al., 2019; Meng et al., 2013).

Table. 1 Total Anthocyanin Content during deterioration roselle calyx at a different time interval.

Sample	Delphinidine-3-sambubioside mg/L	Cyanidine-3- sambubioside mg/L	Cyanidine -3-glucoside mg/L
Fresh	129^{a}±0.09	126^{a} ±0.14	91^{a}±0.12
1 hours	111^{a}±0.30	108^{b} ±0.38	78^{b}±0.40
3 days	37^{a}±0.24	36^{b} ±0.24	22^{b}±0.23
5 days	ND	ND	ND

Key: ND=Not detected, ± = Mean ± standard deviation.

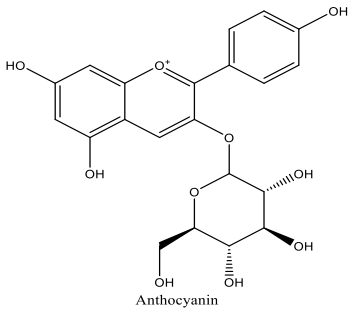

Figure 1. The structure of anthocyanin.

Figure 2. Fresh and deteriorated roselle calyx.

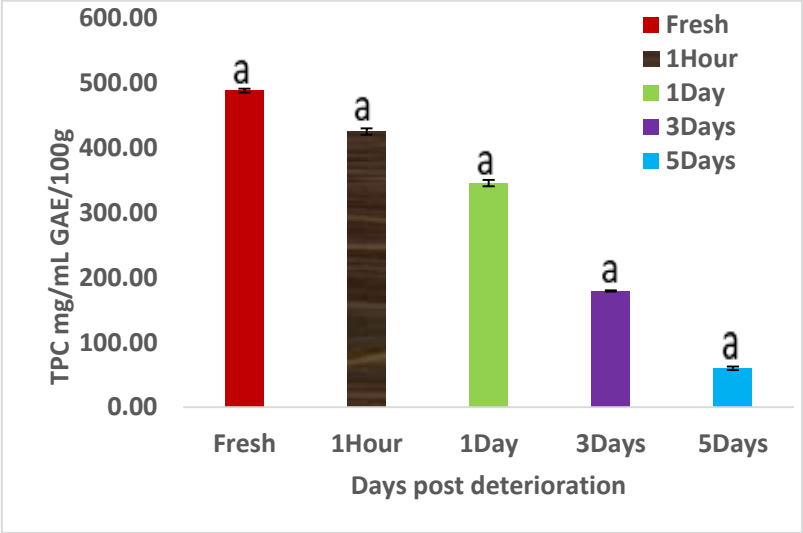

Figure 3. Total phenolic content calyx mg/mL GAE/100g of fresh and 5 days post deteriorated roselle calyx.

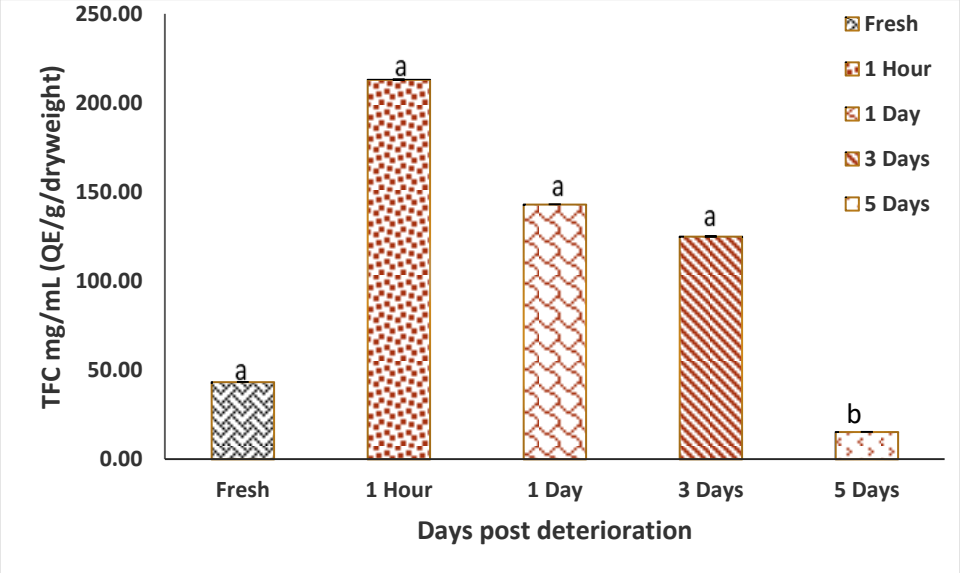

Figure 4. Total Flavonoids content calyx mg/mL QE/g/dry weight during five days post deteriorated roselle calyx.

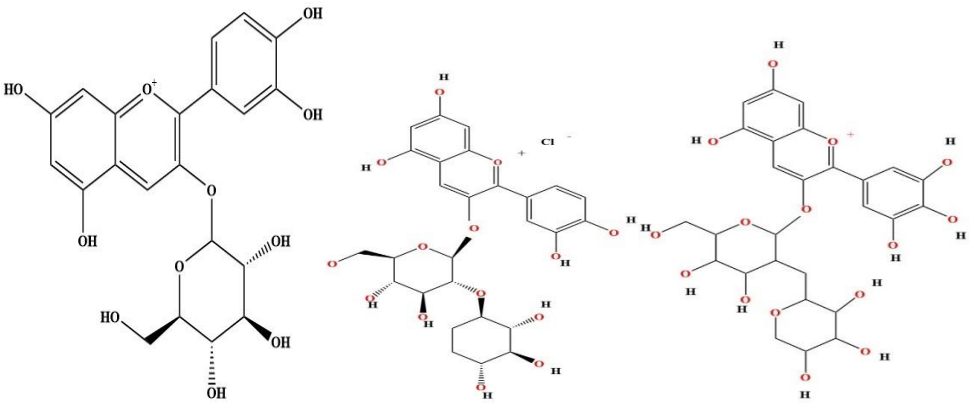

Figure 5. Structure of cyanidine glucoside, cyanidin sambubioside and delphinidin sambubioside. The most prevalent anthocyanidin revealed in this study.

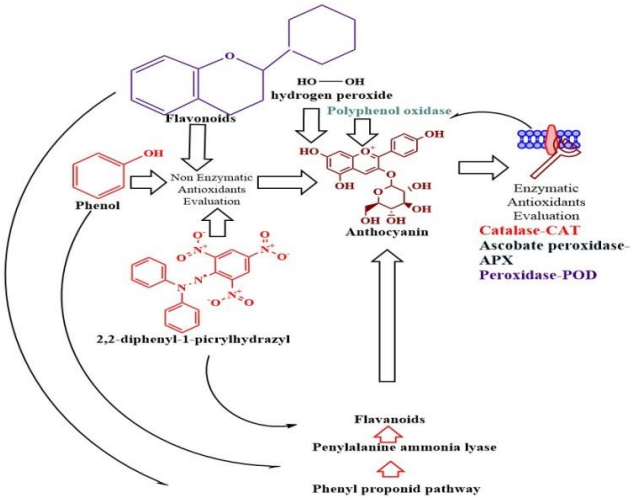

Figure 6. schematic presentation of role of anthocyanin in response to stress during postharvest deterioration of roselle calyx. Anthocyanin has shown coordinated response to stress during PD. As a result, signal by hydrogen peroxide the anthocyanin content expressed in this study lead to the activation of enzymatic antioxidant such as CAT, POD and APX which scavenge or reduced the hydrogen peroxide to water and oxygen, also non enzymatic antioxidant was also expressed such as phenolic, DPPH radical scavenging activity as well as flavonoids that are all part of the anthocyanin. Furthermore, the expression of PPO and PAL in this study indicates roselle of anthocyanin because PAL is the key enzyme in the phenylpropanoid pathway for the synthesis of anthocyanin and flavonoids. PAL its self-posse's antioxidant activity and also express in response to stress.

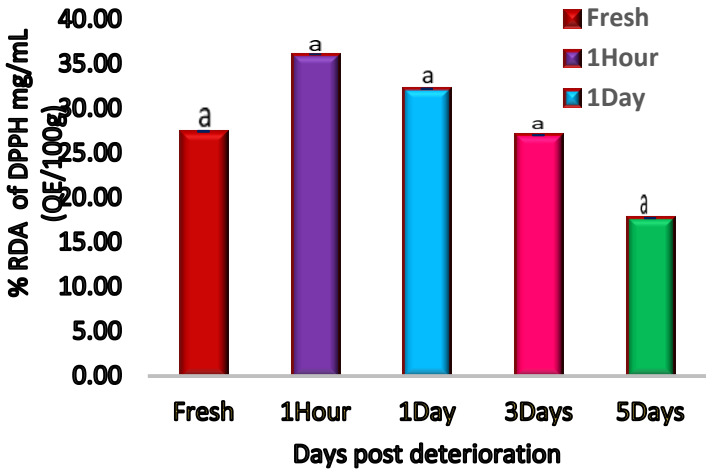

Figure 7. % RSA of DPPH Quercetin equivalent (mg-QE-100g) in deteriorated calyx five days post deterioration of roselle calyx in comparison with fresh calyx.

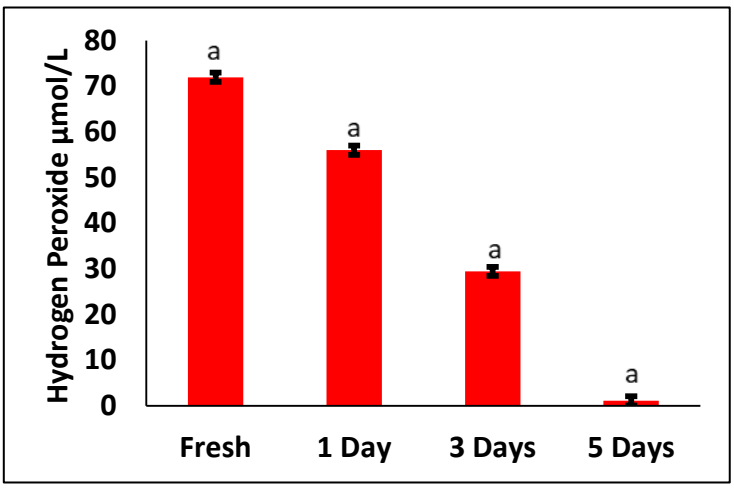

Figure 8. Hydrogen peroxide H_2O_2 concentrations µmol^{-}L in deteriorated calyx five days post deterioration of roselle calyx in comparison with fresh calyx.

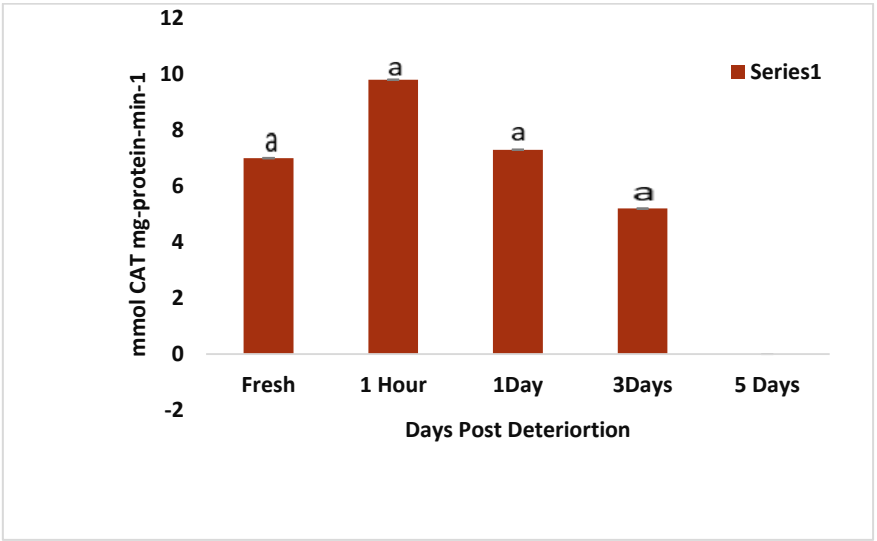

Figure 9. Catalase activity mmol CAT mg^{-}protein^{-}min^{-1} roselle calyx days post degradation was measured, with fresh calyx serving as a control.

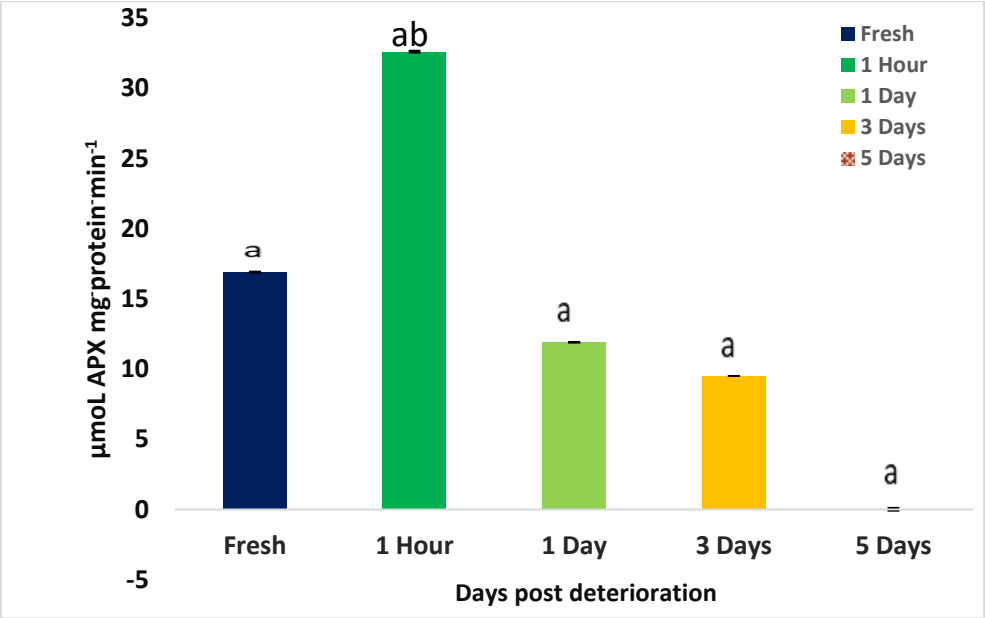

Figure 10. The activity of Ascorbate Peroxidase µmol mg-protein-min-1 roselle calyx days post degradation was measured, with fresh calyx serving as a control.

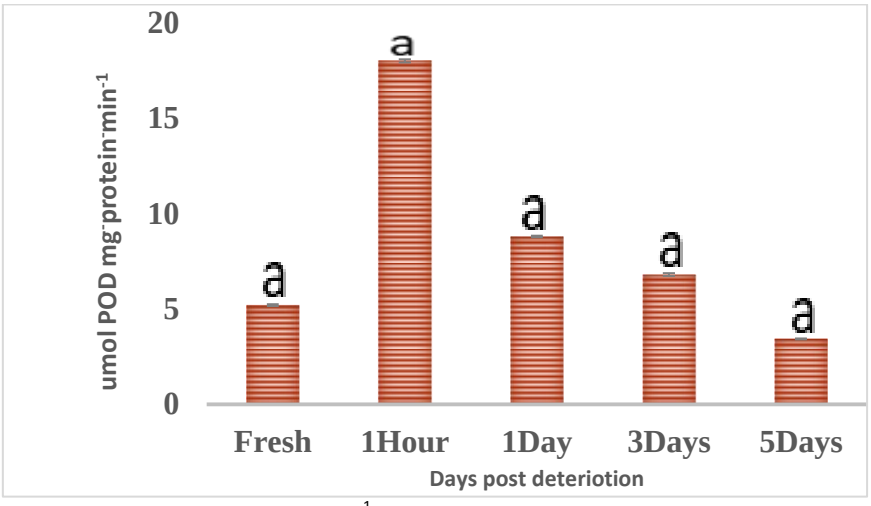

Figure 11. The activity of Peroxidase µmol mg-protein-min-1 roselle calyx days post degradation was measured, with fresh calyx serving as a control.

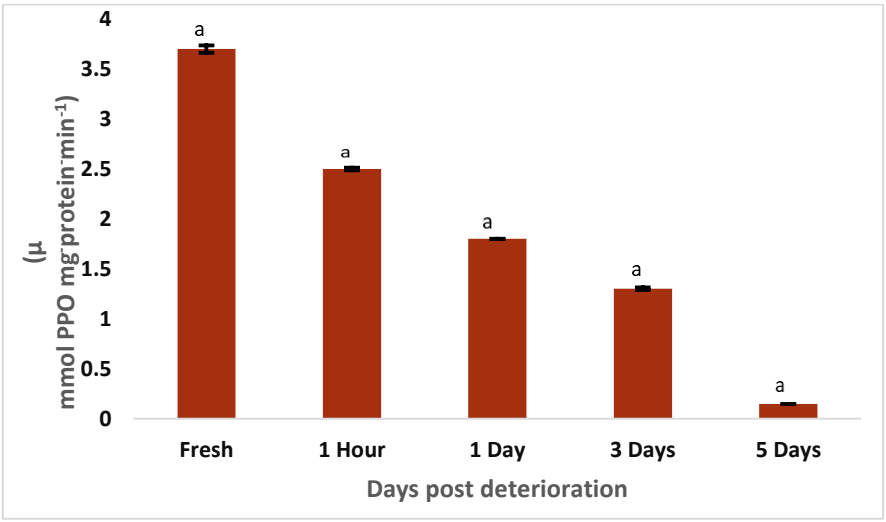

Figure 12. Polyphenol oxidase activity (mmol mg -protein-min-1) of roselle calyx days post degradation was measured, with fresh calyx serving as a control.

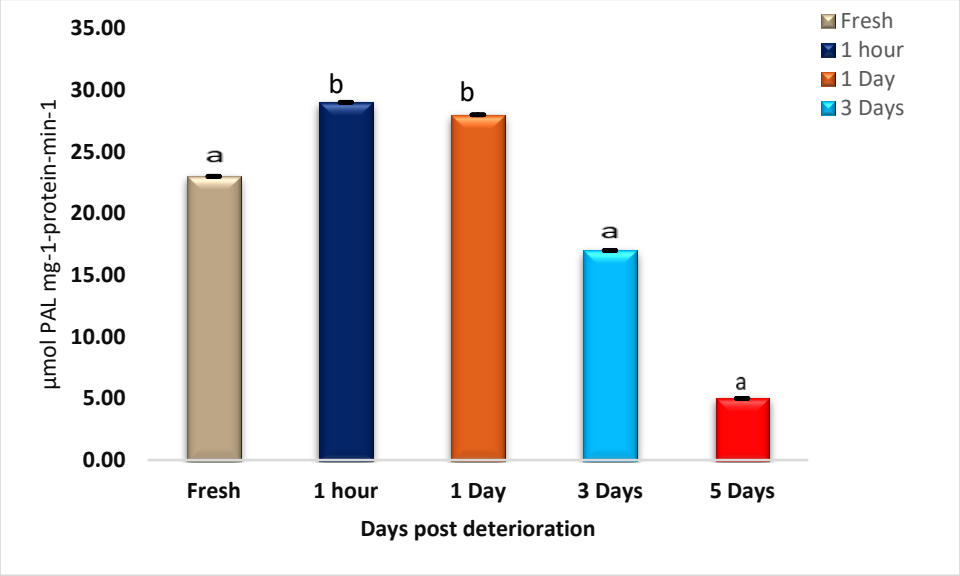

Figure 13. PAL activity in (µmol PAL mg-1-protein-min-1) of roselle calyx days post degradation was measured, with fresh calyx serving as a control

H_2O_2 may be scavenged from red leaves more quickly than green leaves, and anthocyanins have contributed further to this process than other phenols (Bi *et al.,* 2014; Gould *et al.,* 2002; Zhang *et al.,* 2012; Landi *et al.,* 2014). Anthocyanins can impact the developmental process because of their affinity for several proteins involved in signalling cascades (Grotewold, 2005; Taylor and Peer Murphy, 2006).

Catalase is found in almost all living things. Damage to the cells is prevented by a sophisticated antioxidant defense mechanism that is linked to the increased production and activity of enzymes that convert hydrogen peroxide (H_2O_2) into water (H_2O) (Scandalios, 2005). Catalase experiments Fig.9, add to the findings of Santos et al. that enzymes can be used to look at physiological and biochemical changes in plant products that have been preserved (Santos *et al.,* 2004). The considerable decline in CAT activity from 3dyas following PD (Fig.9) is consistent with Marcos-resulted Filho's enzyme inactivation or reduction, as well as a stop in enzyme production, resulted in a fall in enzyme activity during PD (Marcos-Filho 2005). The peroxidase activity data in Figs. 10 and 11 confirm Adyanthaya's earlier observations that peroxidase activity reduced dramatically during the late stages of storage, implying PD (Adyanthaya. I, 2007). Additionally, Marques *et al.* observed that catalase and ascorbate peroxidase activity increased during storage (Marques *et al.,* 2014; Ismah, 2004). However, Well-preserved fruits initially showed higher superoxide dismutase activity, but this activity declined as the apples deteriorated. This current finding indicates that higher phenolic content and antioxidant activity were connected with increased SOD and preservation (Adyanthaya, 2007). Similarly, this finding agrees with the conclusion of Uarrota *et al.,* 2015, that; during the PD of cassava, both antioxidant enzymes were found to be expressed, including CAT, POD, and APX enzymatic activity was observed. Rohman *et al.* also reported higher antioxidant enzyme activity such as SOD, POD, and APX (Rohman *et al.,* 2016; Akter *et al.* 2021). The activation of antioxidant enzymes showed decreased hydrogen peroxide, although it was not wholly scavenged due to temperature and biotic and abiotic stress.

When handling, storing, and preparing fruits and vegetables the enzymatic browning response occurs, causing the formation of brown pigment in the damaged tissues. PPO has been linked to the synthesis of pigments, oxygen scavenging, and plant defence mechanisms against infections and other diseases (Lopes *et al.,* 2015). As revealed in Fig.12, the statistically significant activity might be attributed to the wound in the calyx while harvesting and handling, i.e., removal of the calyx from the seed; this might lead to the infestation of the microbe's subsequent deterioration. Furthermore, the PPO activity did not follow the pattern of antioxidant enzymes in this study, where they attained the highest significant activity after 1hour and later significantly decline. The result shows PPO is significantly expressed even in fresh samples due to the injury or wound stress generated during harvesting or removing the calyx from the seed. The significant decrease in PPO activity at the late stage agrees with the findings of Uarrota *et al.* in cassava PPD. PAL is a key enzyme in the production of secondary compounds like lignin and phenols in higher plants as revealed by Hemm *et al.,* 2004. A plant's ability to fight infection depends on the presence of phenolic compounds and the synthesis of these compounds in response to infection. PAL is one of the most intensively studied enzymes in plants' secondary metabolism due to its crucial role in the biosynthesis of phenylpropanoid metabolites. The development of PAL and PPO during roselle calyx PD in this research, as shown in Figs12 and 13, is confirmed by Vanttha *et al.,* 2015, which function as a resistance mechanism in tomatoes in response to infection. Furthermore, numerous studies have shown that the activities of PAL and PPO increases when plants are infected with pathogens (Zeier *et al.,* 2004; Niranjanraj et al., 2006; Zheng *et al.,* 2012; Sticher, Mauch-Mani, & Métraux, 1997). Finally, this study has shown a strong connection amongst the total phenolic, antioxidant, TAC, antioxidant enzymes, and PAL in response to PD and many other studies on plant deterioration and storage shelf life.

Materials and methods

Plant materials

Induction of roselle calyx deterioration

Roselle calyces were freshly collected from the University of Sultan Zainal Abidin farm on the Besut Campus, cleaned, and dried using tissue paper. They were then kept at room

temperature 15-20^{0}C in a clean plastic bag. The rate of deterioration was tracked over time.

Total phenolic content

Using the Folin-Ciocalteu technique, the total phenolic content was determined. Simply, distilled water was used to dilute 200 L of the crude extract (1 mg/mL), thoroughly combined with 0.5 mL of Folin–Ciocalteu reagent for 3 minutes before being added to 2 mL of 20 percent (w/v) sodium carbonate. After 60 minutes in the dark, the absorbance was measured at 650 nm. The TPC was determined using a standard curve and represented as mg gallic acid equivalent /gram /dry weight. The entire experiment was conducted in three replicates (C. Kaur and H.C. Kapoor, 2002).

$$\text{Phenolic content (\% w/w)} = \frac{\text{GAE} \times \text{QE} \times \text{V} \times \text{D} \times 10^{-}6 \times 100}{W}$$

GAE – Garlic acid equivalent (µg/ml), V - total volume of sample (ml), D - dilution factor, W - sample weight (g)".

Total flavonoid content

The total "flavonoid content of the crude extract was determined by the aluminium chloride colorimetric method (Chang C and Yang M, 2002). Briefly, 50 µL of crude extract (1 mg/mL ethanol) were made up to 1 mL with methanol, mixed with 4 mL of distilled water and then 0.3 mL of 5% $NaNO_2$ solution; 0.3 mL of 10% AlCl3 solution was added after 5 min of incubation, and the mixture was allowed to stand for 6 min. Then, 2 mL of 1 mol/L NaOH solution were added, and the final volume of the mix was brought to 10 mL with double-distilled water. The mixture was allowed to stand for 15 min, and the absorbance was measured at 510 nm. The TFC was calculated from a calibration curve, and the result was being expressed as mg quercetin equivalent per g dry weight" (Kassim et al., 2011).

$$\text{Flavonoid's content (\% w/w)} = \frac{\text{QE} \times \text{V} \times \text{D} \times 10^{-}6 \times 100}{W}$$

QE – Quercetin equivalent (µg/ml), V - total volume of sample (ml), D - dilution factor, W - sample weight (g)".

% Radical scavenging assay of DPPH

The DPPH assay was conducted according to the prior method with modifications (Wu et al., 2018). Briefly, "DPPH solutions were prepared daily, and the concentrations of 0.1 mM of diluted sample were mixed with 1 mL of DPPH. The mixture was kept in the dark at room temperature for 10 minutes after vertexing. The measured absorbance was then taken spectrophotometrically at 517 nm (Abdulrahman, M. 2021). Quercetin was used as a reference standard. The formulation calculated the percent of reduction of DPPH":

$$\text{\% "DPPH radical scavenging} = \frac{AS-AC}{AC} \times 100$$

where Ac = absorbance of the control, As = absorbance of the sample".

Total anthocyanin content (TAC)

The TAC was determined using the pH-differential technique (Giusti and Wrolstad, 2001). To make two dilutions of the sample, add 1 mL of the extracted solution in a 10 mL volumetric flask. CH3COONa buffer, pH 4.5, and KCL buffer, pH 1.0, dilute the extract. For 15 minutes, the dilutions were brought to an equilibrium state. To account for haze, the absorbance of each sample was compared to that of a control sample in distilled water at 510 and 700 nm. All measurements were taken within 15 minutes and 1 hour after sample preparation (Sutharut and Sudarat, 2012) to get the best results. The maximum absorption wavelength (Aλ vismax) was used to calculate their corresponding TAC values. Each TAC value was expressed as mg cyanidin-3-glucoside (C3G) equivalents per liter of concentrate according to the following equations:

Absorbance "(A) = (Aλ vis-max x A700) pH 1.0 0 (Aλ vis-max x A700) pH 4.5

$$\text{TAC (mg/L)} = \frac{(A \times \text{MW} \times \text{DF} \times 1000)}{(\varepsilon \times \text{l})}$$

M.W.: the molecular weight, calculated as cyanidin-3-glucoside (449.2); DF: the dilution factor."

Determination of H_2O_2 concentrations

The modified technique of (Velikova *et al.,* 2000). In a cold-water bath, calyx tissue (20 mg) was homogenised in 2 ml 0.1 % TCA. 12,000 g for 15 minutes to obtain the supernatant from the homogenate was added 0.5 ml of the supernatant and 1 ml of potassium iodide. The absorption was 390 nm spectrophotometrically. Calibrating with 20 – 100 Mol H_2O_2 yielded the H_2O_2 concentration in the samples.

Enzyme's extraction

To extract the antioxidant enzymes from 200 mg of roselle calyx, 800 µL extraction buffer (pH 7.8) was used, including 100 mmol L^{-}1 EDTA, 100 mmol L^{-}1 potassium phosphate buffer (pH 7.8); and 1 mmol $^{-}$1 L^{-}ascorbic acid. The Bradford (1976) technique was used to measure the protein content in each sample.

Catalase (CAT; EC 1.11.1.6.)

The activity of Catalase was determined by the breakdown of H_2O_2 into H_2O and O_2. About 1 mM Na-EDTA, 50 mM potassium phosphate buffer (pH 7.0) were added to the reaction mixture. About 0.05 % w/v H_2O_2 and 500 µL protein extract in a total volume of 3 ml. The addition of H_2O_2 initiated the reaction, and the constant reduction in absorbance at 240 nm was measured (for 5 minutes) using quartz cuvettes.

Ascorbate peroxidase assay (APX; E.C.1.11.1.11)

The activity of APX was estimated using the Nakano and Asada (1981) technique, which included determining the rate of ascorbate oxidation. The reaction mixture of 100 mmol L^{-}1 potassium phosphate buffer (pH 7.0), 0.1 mmol L^{-}1 hydrogen peroxide (H_2O_2), 0.5 mmol L^{-}1 L^{-}ascorbic acid, distilled water, and 30 mL protein extract was incubated. The rate of ascorbate oxidation was measured at 290 nm for 3 minutes at 15-second intervals, and the enzyme activity was reported as µmol ascorbate oxidized per minute per mg protein using (extinction coefficient of 2.8 mmol L^{-}1 cm^{-}1).

Guaiacol peroxidase assay (GPX; E.C. 1.11.1.7)

The Souza and MacAdam (1998) method was used to determine the activity of GPX by observing the tetra guaiacol formation at 28°C. With 2 mL of the final volume, the mixture of the reaction contained H_2O_2, 50 mmol L^{-}1 potassium phosphate buffer (pH 7.0), guaiacol 3.5 mmol L^{-}1, distilled water, and finally 10 µL of enzyme extract. The increase in absorbance through guaiacol oxidation was measured at 470 nm for 1 min at 10-s intervals. The GPX activity was expressed as µmol of guaiacol decomposed /

minute / mg of protein using (extinction coefficient of 26.6 mmol L^{-1} cm^{-1}).

Polyphenol Oxidase (PPO; EC 1.10.3.2)

Potassium phosphate buffer (pH 6.8), 5mM (+)-catechin (dissolved in assay buffer, 0.25 ml enzyme extract) were added to the reaction mixture. A 30-minute incubation period at 30^{o}C was used. The reaction was stopped by the addition of 0.4 ml of 2 N HClO4. The response was then centrifuged at 5000 rpm for 10 minutes to finish the process. At 395 nm, the oxidized catechin absorbance was measured.

Phenylalanine ammonia lyase (PAL, EC 4.3.1.5)

The activity was expressed as the content of trans-cinnamic acid generated from L-phenylalanine by PAL. About 0.1 ml enzyme extract, 50 mM Tris-HCl pH8.5, and 1 ml 10-mM L-phenylalanine were added to the mixture. The reaction was incubated at 40 °C, whereas similar condition was performed as a control reaction but using 10-mM D-phenylalanine as substrate. The production of cinnamic acid was tracked by absorbance at 30 min intervals in quartz cuvettes at 290 nm wavelength for 2 hours (Ma *et al.* 2018).

Statistical analysis

The data obtained were subjected to one-way ANOVA and the level of significance was set at 95% confidence using Statical package for social sciences (SPSS Version 23.0).

Conclusions

Based on the biochemical results reported, roselle calyx's metabolic changes might be associated with PD. Our data show that anthocyanins, antioxidant enzymes, phenolic s-polyphenol oxidase, and Phenyl ammonia lyase are all increased in the earliest stages of deterioration, up to 3-5-days, and, as such, may be associated with PD. The anthocyanin production increases at the beginning of the degradation phase; however, the underlying mechanism in roselle calyx PD remains elucidated further more as the previously reported anthocyanin content of plants is a prominent focus of research (Rao *et al.* 2022; Roa *et al.* 2019; Ni *et al.* 2021; Jiu *et al.* 2021). We have also reported the biochemical changes during PD of roselle calyx for the first time. Therefore, Anthocyanin, PAL, and PPO might be a potential biomarker in roselle calyx PD.

Authors' contributions

The Laboratory analysis and manuscript written by AAL. NHM brought the research idea and supervised the research. MMK reviewed the manuscript and made corrections while MAD proofread the manuscript.

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