The Influence of Salicylic Acid and Methyl Jasmonate on Ripening and Stress Regulation Mechanisms of Tropical Fruits during Cold Storage

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DEDICATION

My family: A source of unconditional love, trust and faith

ABSTRACT

The Influence of Salicylic Acid and Methyl Jasmonate on Ripening and Stress Regulation Mechanisms of Tropical Fruits during Cold Storage

Postharvest tools have long been developed for minimising postharvest stresses and preserving fruit quality. However increasing evidence points towards the role of abiotic stress in improving the bioactivity and nutraceutical value of fruits. This study was an investigation into the effects of postharvest stresses on the bioactive content of the tropical fruits carambola (*Averrhoa carambola*), dragonfruit (*Hylocerues* sp.) and mangosteen (*Garcinia mangostana*). The fruits were subjected to stresses induced by cold storage at 6 °C along with exposure to four different levels of one of the stress hormones salicylic acid (0, 0.1, 1, 2 and 5 mM) and methyl jasmonate (0, 0.01, 0.1, 0.2 and 0.5 mM). Physicochemical responses of all three fruits to the postharvest stresses along with changes in the antioxidant activity were assessed throughout the shelf life of the fruits. The goal was to characterise physiological and biochemical associated stress responses of the tropical fruits, and to evaluate the potential of using these stress responses for enhancing fruit quality.

Assessment of weight loss, colour evolution, textural changes, soluble solids content (SSC), titratable acidity (TA), total phenolic content (TPC) and antioxidant activity (AA) of the fruits during the storage period was initially conducted. Significant increase ($p < 0.05$) in the SSC, TA and AA of carambola was observed in response to the applied stresses, along with delayed weight loss. This provided physiological evidence of a relationship between stress coping mechanisms in carambola and enhanced quality attributes. Thus, carambola was selected for further studies exploring the stress regulation mechanisms associated with bioactivity of the produce.

The aim of the second stage of the research was to evaluate the effect of the applied stresses on the bioactivity of carambola. The impact of the postharvest stresses on the synthesis and oxidation of phenolic content of carambola was examined during the established storage period by assessing the activity of phenylpropanoid ammonia lyase (PAL), peroxidase (POD) and polyphenol oxidase (PPO). Different patterns of activity were observed for the PAL and POD and PPO. The applied stresses significantly enhanced ($p < 0.05$) the activity of POD in carambola. PAL activity declined during the storage period for all of the fruits, and the decline was more rapid for the treated fruits, although an initial increase in PAL activity was observed for lower hormone doses.

The immediate effect of the stresses was evaluated by characterising changes in the specific phenolic content of the carambola fruits after 0 and 4 days of cold storage. This was followed by an assessment of the bioactive components of the fruit samples as anti-proliferative and antioxidant compounds on HepG2 cultures. Finally an attempt to identify the gene families that were associated with stress amelioration was carried out. This involved optimisation of a protocol for successful isolation of high quality RNA from carambola. Carambola has not been sequenced, thus ANS, C4H, PAL and SnrK1 genes were identified for the first time in carambola through the use of degenerate primers.

Further studies exploring the phenolic content of carambola along with transcriptomic studies on the fruit stress responses will provide a deeper understanding of the cellular and developmental processes activated. Stress amelioration systems in plants and humans rely on quenching of radical oxygen species, thus the defence strategies of plants can be exploited for benefiting human health.

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I hereby declare that the thesis is based on my original work except for the quotations and citations, which have been duly acknowledged. I also declare that it has not been previously submitted or concurrently submitted for any other degree at the University of Nottingham or other institutions.

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Date: 29/11/2015

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CHAPTER 1: REVIEW OF LITERATURE

1.1 Introduction

Consumption of fruits today is dependent on two aspects of fruit quality: taste and nutritional benefits. As the agroindustry continues to expand steadily, tropical fruits are becoming of increasing importance due to their desirability by consumers (Silva et al., 2014). Tropical fruits, such as kiwi, pineapple and papaya, are recognised for their potent bioactivity, which is associated with the prevention of degenerative diseases. Thus, these fruits are important economic tools for local producers, and have become the subject of numerous studies. However, there are a recognizable number of tropical fruits which have been overshadowed in spite of the undisputable nutraceutical value they render, such as mangosteen (*Garcinia mangostana*), dragon fruit (*Hylocereus* sp.), carambola (*Averrhoa carambola*) (Wichienchot et al. 2010; Kondo et al. 2009; Pedraza-Chaverri et al. 2008; Shui & Leong 2006, 2004; Wu et al. 2006)

The nutraceutical value of fruits is associated with the presence of secondary metabolites in plants, to which several human health-maintaining values are attributed (Patil et al. 2009). Bioactive molecules such as phenolics, carotenoids and ascorbic acid are compounds that are ubiquitously found in plants serving a variety of protective functions such as protection from damaging ultra-violet rays. These molecules have become of increasing importance in the human diet and a multitude of health benefits have been associated to their consumption. Fruits constantly experience transient changes in the phytochemical composition of plants. This has been exploited throughout the dynamic evolution of the bioactive pathway of plants (Piljac-Žegarac & Šamec 2011).

While some studies have attributed the decline of these bioactive molecules in response to stress, other studies have reported that the controlled application of stresses can improve the bioactive content of fruits and vegetables, such as heat stress increasing carotenoids and phenolic compounds, ultimately enhancing the nutraceutical value (Gonzalez-Aguilar et al. 2010). Thus, this may actually highlight a possible beneficial aspect of the impact of stress on stored fresh produce. Complex reactions are constantly in play in fruits and vegetables during postharvest storage, which could account for increases in the bioactive content while the fresh produce is experiencing overall deterioration with regards to texture, flavour and appearance (Piljac-Žegarac & Šamec 2011).

Stress is a potentially unfavourable environmental factor that a living organism may experience (Toivonen & Hodges 2011). There are numerous sources of abiotic stresses a plant may experience, the most notable stemming from environmental factors that plants are exposed to regularly such as temperature extremes (Gonzalez-Aguilar et al. 2010). The stresses may be experienced by the plant at various stages of its life cycle, thus affect the fruit at a variety of stages, both preharvest and postharvest (Toivonen & Hodges 2011).

Postharvest technologies were developed to preserve the quality of perishable food products from stresses that may occur during storage and transportation (Gonzalez-Aguilar et al. 2010; Wills et al. 2007). Tools and technologies such as modified atmosphere and cold storage are commonly used by growers and distributors to delay ripening and extend the shelf life of perishable fruits and vegetables during transportation and delivery to the consumers (Lee and Kader, 2000). These technologies are becoming of increasing importance due to the combined effects of trade globalization and climate change, which have expanded the global market for fresh

produce whilst limiting the production areas (FAO, 2010). As such, there are increasing concerns on the impact of postharvest stresses on the nutritional content of the fresh produce (Piljac-Žegarac & Šamec 2011).

Stress related increases in bioactivity are associated with the production of reactive oxygen species (ROS), which is the first observable change in plants after exposure to stress (Mittler et al. 2004). Production of ROS is an elaborate defence mechanism, involving signalling pathways that lead to the production of antioxidants (Gonzalez-Aguilar et al. 2010). Although there is a wealth of information available on the mechanisms of ROS regulation and the genes involved in this process there is a gap with regards to the specific processes and regulatory mechanisms involved (Mittler et al., 2004). Moreover, a vast range of pathways, even within the ROS mechanism, respond to stress which are all intricately interrelated (Foyer et al. 2003). Thus, it is of interest to elucidate the mechanisms of the overall response involving the various pathways and their multiple constituents and regulatory factors.

Ripening is a genetically programmed cellular response under the regulation of phytohormones such as ethylene. Ethylene is the key trigger for the ripening of climacteric fruits, but plays a less important role in non-climacteric fruits (Concha et al., 2013). In non-climacteric fruits other phytohormones function as the key ripening regulators either inhibiting or inducing ripening. Auxin is a ripening inhibitor, reducing the rate of colour change and decline in firmness by supressing anthocyanin biosynthesis and activity of cell wall degrading enzymes. Meanwhile, abscisic acid induces ripening, and interacts with ethylene stimulating colour change, softening and sugar accumulation. Another hormone, jasmonic acid (JA), plays a role in fruit ripening as well as other cellular processes such as stress tolerance (Concha et al. 2013).

Climacteric fruits respond to treatment with JA by increased ethylene production which enhances chlorophyll degradation and colour acquisition (Fan et al. 1998).

Understanding plant physiology makes it possible to predict reactions that may occur within the plants at different stages of growth. Although postharvest is usually associated with preserving fruit quality (Gonzalez-Aguilar et al. 2010), invoking abiotic stress on fruits may improve the nutraceutical value of the fruit. The enhancement of bioactivity of plants will be significant in aspects of crop production and human nutrition alike (Gould 1999). This study explores the role of natural volatiles that are stress inducers, on the bioactive compounds and antioxidant properties of selected tropical fruit. The aim is to understand the negative effects of stress on the marketing of these underutilised fruits as well as the positive effect that controlled application of stress may have on fruits, which will be studied at the physiological, biochemical and genetic level.

1.2 Postharvest life of tropical fruits

World fruit market shares are steadily increasing which has been attributed to the ever-increasing rate of population growth and a global improvement of living standards. Positive correlation between the consumption of fruits containing various phytonutrients such as carotenoids and flavonoids, with the enhanced protection from chronic diseases such as cardiovascular diseases and cancers were established in several studies (Seymour et al. 2013). However, the consumption of fruits and vegetables is generally below the recommended levels in numerous nations. It has been estimated that a mere 6 – 24% of children in Europe meet the WHO recommendations (EUFIC 2012).

Southeast Asia is bountifully endowed with an abundance of horticultural products, however hindrances arising from the shortfall of the supply chain have compromised these nations' full capacity for trade (Mohd. Adzahan & Benchamaporn 2007). Tropical fruits possess highly beneficial bioactive compounds, giving them nutritional, healing and antioxidant properties (Dembitsky et al. 2011; Naczk et al. 2011; Rufino et al. 2010; Shui & Leong 2006). Alas, these perishable food products are often sporadically cultivated in small farms, impeding their potential for commercialization (Mohd. Adzahan & Benchamaporn 2007). Consequently, pre and postharvest losses in Southeast Asia are estimated at around 20 – 50%. This has a major impact on food security as well as economic and environmental development. Sustainable use of resources through the reduction of postharvest losses could be a cost-effective and environmentally friendly approach to combat the imbalances between consumption increases and the hefty demands of increased food production (FAO 2010). Postharvest handling and storage is associated with losses that may result from deterioration of fruit quality in the time frame between harvest at the farm and distribution at the market (FAO 2010; Wills et al. 2007). Thus, postharvest technologies occupy a central role for enhancing the efficiency of the food supply chain.

The current statistics on postharvest losses in fruits and vegetables are generalized with current estimates that one third of the global production is annually wasted (Gustavsson et al. 2011; FAO 2010). These losses are incurred throughout the supply chain, starting from the initial phases to the final stages of production. In low-income countries, such as Malaysia, most of the losses are incurred at the early to middle stages of the distribution chain and this is associated with technical as well as financial limitations (FAO 2010). For example, tropical fruits encounter major losses during transportation and storage stemming from fungal decay, chilling and rapid

maturation. According to Gustavsson et al. (2011) out of an estimated 37% loss in the total volume of produced fruits and vegetables in Southeast Asia a mere 1.8% loss is attributed to consumers. Such losses can be attributed to shortages in the system that include poor infrastructure or insufficient cold storage facilities (FAO 2010). The consequence of these losses affects farmers and consumers alike, since it generates unstable pricing which can also lead to rural impoverishment through its direct effects on farmers' livelihoods.

During the distribution chain, fresh produce pass through many hands and at relatively large quantities. As a result, distributors rarely have the luxury of optimizing the storage conditions to suit each food product and consequently compromise in terms of the ideal storage conditions (Paull 1999) This results in postharvest stresses on the various fresh fruits and vegetables which are passed through the distribution chain, which may ultimately lead to deterioration of quality and shortened shelf-life (Paull 1999). Moreover, consumers increasingly demand food products with minimal chemical fungicides, forcing exporters to resort to lower storage temperatures to reduce losses resulting from decay (Tietel et al. 2012). Unfortunately, this practice has detrimental effects on the shelf-life as well as flavour life of chilling-sensitive food products (Tietel et al. 2012).

There are major data gaps in the understanding of food losses during the supply chain, and attempts to address this issue will pave the way for postharvest technologies more adapted to the distribution chain, which will result in food products of higher quantity and quality reaching the market. Understanding the different processes that make up the supply chain and the value that they add will highlight their significance in the overall operation (Whicker et al. 2009). The same concept applies for the use of postharvest tools and technologies, through the understanding of their applications and

the efficiency of these tools, their value in the supply chain will be grasped. Understanding the roles of postharvest tools requires an initial understanding of the physiology of fruits and their responses to the stresses encountered throughout the supply chain. As tropical fruits are increasingly transported over long distances, it is essential to dissect the responses of these fruits.

1.2.1 Carambola (*Averrhoa carambola* L.)

Averrhoa carambola (carambola) is a tropical oblong fruit with a characteristic star-shaped cross-section (Figure 1.1). The fruits are characterised with three to six longitudinal ribs which grant a distinct shape when cut longitudinally, hence the name 'star fruit'. The edible flesh of the fruit ranges from smooth to fibrous and its colour varies from creamy-white to orange (O'Hare 1993). It is a popular fruit in the tropics and sub-tropics, with Malaysia being a major producer. Although statistics on global market for carambola is scarce, Malaysia is identified as one of the leading countries in the production of carambola at approximately 900 ha and producing almost 24,000 million tonnes, following Taiwan and Guyana (Sepiah et al. 2003). Estimates in 2008 of Malaysian exports of carambola are at 28 million Malaysian ringgit (Zainudin et al. 2014).

Moreover, as the global demand for exotic fruits increases, the demand for carambola has witnessed an increase as well over the last two decades (Sepiah et al. 2003). This increased demand has been associated with increased cultivation of the sweet, mildly-acidic cultivars. The acidic cultivar is highly desirable and generally consumed in salads or as garnish. Germplasm improvement has been carried out, for example in Malaysia by Malaysian Agricultural Research and Development Institute

(MARDI) which have propagated the B series, in particular B-10. There are a number of varieties of carambola that are propagated globally, with different attributes associated with each variety, such as the B series which have been developed in Malaysia (Table 1.1)

Figure 1.1 Carambola (*Averrhoa carambola*) arranged longitudinally and transversely.

Carambola is non-climacteric with no accounts for autocatalytic ethylene generation, thus is usually harvested at colour break (yellowish-green) to avoid susceptibility to postharvest damage while allowing for maximal sugar accumulation (O'Hare 1993). The maturity stages as identified by Federal Agricultural Marketing Authority (FAMA), Malaysia, are stage 1: mature green; stage 2: light green; stage 3: yellowish green; stage 4: yellow; stage 5: yellowish orange; and stage 6: orange (Ali et al. 2004).

Carambola is produced sporadically throughout Malaysia, and is exported in large quantities, especially cultivars B10 and B17, to several markets throughout Europe, Middle East and the Far East (Zainudin et al, 2014). Thus, local producers usually rely on middle men to handle the exporting and marketing of the product. The exporting companies, source the carambola from local farms and assume the

responsibility of assessing the pesticide residues on the fruits and devising the most feasible postharvest technologies to be applied.

Fruits transported by sea are left to mature for longer on the tree to allow accumulation of sufficient moisture for firmness retention throughout the 6 weeks of transportation and delivery. Meanwhile, shorter maturity times are more economic for the producer and exporter, as the fruit mass will be lower, reducing transportation costs. However, the shorter the maturity time, the more fragile the fruit more susceptible it is to rib browning. In the domestic market more emphasis is placed on the flavour, thus fruits are allowed to mature on the tree for an additional 10 days to develop the desired flavour attributes. Special markets have specific requirements, such as Hong Kong where the demand is for large fruits for use in religious festivities. This requirement urges the farmer to reduce the fruit load per branch, allowing for maximum size development of fruits per branch.

The storage life of carambola is approximately 6 weeks after harvest under optimum storage conditions and maturity, which are at 7 ^{o}C and 85-95% RH. At ambient temperature, the fruits can be kept with reasonable quality up to 9 days. Containers by sea do not have humidifiers, but possess a ventilation window that allows transfer of air at 25 cbm/hr to ensure sufficient humidity. Thus, during this stage fruits are exposed to conditions that may adversely affect the quality. Although profit margins for export by sea are higher than by air, but so are the risks involved.

Various postharvest technologies have been developed for carambola, such as modified atmosphere storage and low temperature storage which effectively delay softening and water loss (Ali et al. 2004). Fruits are susceptible to mould growth, thus are commonly stored under cold environments. However, the fruit is susceptible to chilling injury, which is characterized by rib browning, surface pitting and skin

desiccation, as well as decline in membrane integrity (Pérez-Tello et al. 2001). The effect of different storage temperatures on the quality of carambola has been evaluated for different varieties (Table 1.1). The characteristic shape of carambola cannot be handled through automated machinery such as a conveyor belt. Thus, special considerations must be made for packing and storage of the fruit. Careful handling of carambola is essential as they are highly susceptible to rib browning upon impact or on exposure to extreme temperatures, which is a major quality limiting factor.

Carambola occupies a significant role in folk medicine, for example in India it is used for treating haemorrhages, fevers and liver problems (Bhat et al. 2011). The main antioxidant constituents of carambola are phenolic compounds such as gallic acid, catechin and epicatechin as well as vitamin C (Dembitsky et al. 2011; Leong & Shui 2002). Additionally, it contains an abundance of other bioactive compounds which include L-alanine, L-aspartic acid, citric acid, fumaric acid, L-glutamic acid, glycine, niacin, oxalic acid, riboflavin, succinic acid and thiamine (Dembitsky et al. 2011).

Table 1.1 Response of carambola fruits subjected to various storage conditions

Cultivar	Temp.	Duration	Observation	Reference
Golden Star	21°C	14 days	Necrotic lesions, shrivelling, bronzing	Grierson & Vines (1965)
Golden Star	16°C	21 days	Necrotic lesions, shrivelling, bronzing	Grierson & Vines (1965)
Golden Star	10°C	28 days	Maintained appearance	Grierson & Vines (1965)
Golden Star	0 & 4°C	35 days	Maintained appearance	Grierson & Vines (1965)
Golden Star & Arkin	5°C	44 days	Less necrosis, desiccation and lower decline in TSS and TA	Campbell et al., (1987, 1989)
Fwan Tung	10°C	7 days	Maintained appearance	Keeney & Hull (1986)
Fwan Tung	7°C	42 days	Maintained appearance	Keeney & Hull (1986)
Fwan Tung	5°C	42 days	Chilling injury	Keeney & Hull (1986)
B10	5°C	42 days	Chilling injury, TSS initially dropped but was then maintained	Wan & Lam (1984)
B10	10°C	42 days	Maintained firmness, colour, weight better than 5°C Chilling injury observed after 20 days	Ali et al. (2004)
B10	5°C	42 days	Maintained firmness, colour, weight Chilling injury observed after 10 days	Ali et al. (2004)
Yau	10°C	30 days	Chilling injury, sucrose content increase	Perez-Tello et al. (2001)
Yau	2°C	30 days	Chilling injury. Correlation between PPO and PAL activity and development of chilling injury	Perez-Tello et al. (2001)

1.2.2 Dragon fruit (*Hylocereus polyrhizus* L.)

Dragon fruit (pitaya) are medium-large non-climacteric fruits with large green or red scales (Figure 1.2), to which the name 'dragon fruit' is attributed (Nerd & Mizrahi 1999; Nerd et al. 1999). The plant is a cactus cultivated in tropical and subtropical areas under protection from excessive exposure to the sun as well as subfreezing temperatures (Nerd et al. 1999). The edible pulp, which is juicy and bears a multitude of tiny soft seeds, can vary in colour from white (*H. undatus*) to a distinct purplish-red colour (*H. polyrhizus* and *H. costaricensis*). The distinct colours of dragon fruit are attributed to the betalain content, a pigment characteristic of cacti (Wu et al. 2006) The white pigmentation is due to the presence of the yellow betaxanthins and the purplish-red pigment to betacyanins.

Figure 1.2 Whole piece and cross-sectional view of dragon fruit (*H. polyrhizus*)

The betacyanin content of dragon fruit have been characterised and identified as betanin phyllocactin and betacyanin hylocerenin (Wu et al. 2006). Betanin possesses antioxidant properties, inhibiting lipid peroxidation as well as heme decomposition (Wu et al. 2006). Further studies have revealed that the phenolic and ascorbic acid content of the white variety of dragon fruits are higher in comparison to the purplish-red (Dembitsky et al. 2011). Moreover, dragon fruits were also found to contain a significant amount of phytosterols such as phytol, tocopherols, and taraxasterol (Dembitsky et al. 2011). Phytosterols have been associated with cholesterol reduction in the blood, along with other health benefits (Kritchevsky & Chen 2005; Quilez et al. 2003). These include protection from colon, breast and prostate cancer as well as immune-modulatory and anti-inflammatory properties (Quilez et al. 2003). Consequently, this fruit is gaining global acknowledgment for its health-improving properties.

There have been a few studies on the postharvest quality of dragon fruits and technologies to extend its shelf-life. Optimum storage temperatures for this tropical fruit, which is susceptible to chilling injury at 6 °C, is at 14 - 20 °C (Hoa et al. 2006; Nerd et al. 1999). Harvesting the fruit after it reaches maturity minimizes the incidence of chilling injury, even after exposure to near-freezing temperatures for four weeks. Additionally, it allows the fruit to maintain market quality for almost 2 weeks of storage at 14°C (Nerd et al. 1999). Heat treatment has also been found to extend the fruit shelf-life (Hoa et al. 2006). However, there is a general lack of information on the effects of various abiotic stresses on the physiology of the fruit as well as on the antioxidant and phenolic content of the fruits.

1.2.3 Mangosteen (*Garcinia mangostana* L.)

Mangosteen is a tropical fruit characterized by a thick purplish-maroon peel covering an edible white aril (Figure 1.3), which is juicy and sweet (Dembitsky et al. 2011; Palapol et al. 2009). Although mangosteen is generally identified as climacteric, there are some contradictory reports that classify it as non-climacteric (Ketsa and Koolpuksee, 1993). Few studies have been conducted on the postharvest life of mangosteen, however, significant research has been conducted on the bioactivity of the fruit (Wittenauer et al. 2012; Zadernowski et al. 2009; Chin et al. 2008; Pedraza-Chaverri et al. 2008; Asai et al. 1995).

The purple pericarp contains an abundance of bioactive molecules such as anthocyanins, xanthones (phenolic) and proanthocyanins (Naczk et al. 2011; Palapol et al. 2009). Numerous medicinal properties have been attributed to the pericarp such as anti-inflammatory properties and the fruit has traditionally been used to treat diarrhoea, ulcers and infections (Kondo et al. 2009; Weecharangsan et al. 2006). The main pigments in the pericarp were identified as the two anthocyanins cyanidin-3-sophoroside and cyanidin-3-glucoside by analytical studies conducted by Palapol et al. (2009).

In vitro and *in vivo* studies on the polyphenol, antioxidant potential and protein profiles of the fruit have shown high antioxidant activity (Dembitsky et al. 2011). Crude ethanolic extracts of mangosteen have been used in folk medicine throughout Southeast Asia for treating abdominal pain, wound infections and chronic ulcers (Dembitsky et al. 2011). These uses can be attributed to the abundance of xanthone and benzophenone compounds such as α-mangostin, β-mangostin, γ-mangostin and epicatechin (Dembitsky et al. 2011).

Fruits are harvested at stage 3 of ripening (Table 1.2), which is characterised by irregular pink spots covering the entire surface. If harvested at an earlier stage such as stage 2 the fruit will fail to fully ripen (Palapol et al. 2009). The two anthocyanins found in mangosteen pericarp increase as ripening progresses (Dembitsky et al. 2011; Palapol et al. 2009) although a concomitant decrease in phenolic content has also been reported (Palapol et al. 2009; Ketsa & Koolpluksee 1993). A characteristic increase in firmness and lignin content occurs during storage and in response to stress, such as chilling or wounding (Ketsa & Atantee 1998; Ketsa & Koolpluksee 1993). This results in pericarp hardening which greatly reduces the fruit quality. Further studies by Ketsa and Koolpuksee (1993) have reported an increase in respiration in response to damage along with a decline in ethylene, which is not a typical response for climacteric fruits.

Table 1.2 Maturity stages of mangosteen (Palapol et al. 2009).

Maturity stage	**Description**
Stage 0	Yellowish white or yellowish light with light green
Stage 1	light greenish yellow with 5 – 50% pink spots
Stage 2	light greenish yellow with 51 – 100% pink spots
Stage 3	spots not distinct as stage 2 or reddish pink
Stage 4	red to reddish purple
Stage 5	dark purple
Stage 6	purple black

Figure 1.3 Whole piece and cross-sectional view of mangosteen (*Garcinia mangostana*)

1.3 The regulation of ripening and stress responses of fruits

Ripening of fleshy fruits is a highly coordinated developmental event which has been the subject of numerous studies (Bapat et al. 2010). The physiological and biochemical changes associated with ripening are irreversible. The ripening pathway, starting with the biosynthesis of ethylene and its perception by the target cells, is believed to be fundamentally similar amongst fleshy fruits (Seymour & Manning 2002). Although ripening in non-climacteric fruits is not dependent on ethylene to the same extent, the regulatory networks controlling ripening in fruits from different taxa seem to have many shared features (Klee & Giovannoni 2011).

Scrutinising studies into the regulation of ripening have identified several key similarities between the molecular networks regulating ripening in *Arabidopsis* and tomatoes (*Solanum lycopersicum*) (Seymour et al. 2008). Similarities in the molecular framework governing ripening have also been established between tomatoes and other fleshy fruits such as muskmelon (*Cucumis melo*), banana (*Musa* sp.) and strawberry (*Fragaria x ananassa*) (Seymour et al. 2013). Thus, although the specific ripening-

related physiological and biochemical changes observed in fruits may differ between species, these changes revolve around the same aspects (colour, texture, taste, flavour and nutritional value) and involve similar regulatory factors (Seymour et al. 2013; Valero & Serrano 2010).

Stress is characterized by the subsequent destabilization of organ functionality due to increasing demands on the system (Gould et al. 2003). It results in physiological changes within the plant organ which may affect the chemical composition of the organ in question (Wang & Frei 2011). Some of the noticeable changes include altered assimilate translocation as well as altered water homeostasis. Moreover, changes will also be experienced in gene expression and enzyme activity, which will include the synthesis of several key bioactive molecules including antioxidant compounds (Wang & Frei 2011). In the most extreme cases, these bioactive molecules may result in programmed cell death (PCD).

The various physical and chemical disorders within the environment lead to a variety of stresses that fruits or vegetables may experience during postharvest (Hernández et al. 2009). Water stress is one of the first stresses that a fruit may experience due to the dissociation of the organ from the plant, which is the sole water source. Heat shock and chilling are another type of stress that fruits may experience, both of which result from temperature imbalance. A few examples of these stages have been studied extensively and they include the control of ripening and pigment or carbohydrate metabolism (Gonzalez-Aguilar et al. 2010). Responses to heat shock involve the accumulation of heat shock proteins, antioxidants as well as enzymes such as superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) (Gonzalez-Aguilar et al. 2010). Temperature extremes, drought, salinity, wounding, and the various other environmental stresses share the common feature of triggering the

accumulation of reactive oxygen species (ROS) such as hydrogen peroxide and superoxide radicals which leads to oxidative stress (Cao, Hu, et al. 2010; Hernández et al. 2009). Consequently, this triggers a cascade resulting in the increased production and accumulation of terpenes, phenols, other antioxidants and antioxidant enzyme activity, some of which are inherently higher in stress resistant plants (Suza et al. 2010; Hernández et al. 2009).

Direct physical pressure may also injure the plant and result in abrasive wounds that alter the fruit's physiology and elicit a variety of defence responses. Meanwhile, natural chemicals, which may function as elicitors, are another source of stress endured by plants. Elicitors are generally defined as chemicals that trigger physiological and morphological responses leading to secondary metabolism (Xi et al. 2010; Dong et al. 2010). Elicitors can induce plant defence pathways, such as the phenylpropanoid pathway, in a process similar to disease infection or wounding (Gonzalez-Aguilar et al. 2010; Heredia & Cisneros-Zevallos 2009a). It is important to recognize that fruits or vegetables rarely experience a single source of stress during storage, the combination of which may be synergistic (Gould 1999).

1.3.1 Effect of cold storage on ripening process

The importance of lowering the storage temperature to preserve the postharvest life of perishable food products is a long established technique and has been adopted for fruits since the ancient times (Paull 1999). Low temperature storage maintains the quality of perishable food products primarily by reducing respiration and ethylene production as well as water loss and disease incidence (Tietel et al. 2012; Maul et al. 2011). However, tropical and subtropical fruits are susceptible to damage after prolonged exposure to low temperatures (Pérez-Tello et al. 2001).

Chilling injury occurs in tropical and subtropical fruits when exposed to temperatures below their tolerance level (typically 0 – 12 °C) for a period exceeding a certain duration (Maul et al. 2011; Zhao et al. 2009). The symptoms of chilling injury are usually not apparent during the storage period, however it manifests after the fruit is transferred to a warmer environment (Chidtragool et al. 2011; Pérez-Tello et al. 2001). The key symptoms associated with chilling injury are surface lesions, internal and external discolouration and abnormal ripening patterns (Chidtragool et al. 2011). Physiological changes associated with chilling injury include increased respiration and ethylene evolution along with disrupted membrane and cellular structures (Maul et al. 2011).

Membrane dysfunction is a characteristic result of chilling injury and manifests as water loss and external discolouration (Ali et al. 2004). This results in increased membrane water permeability as well as breakdown in the subcellular compartmentalisation, leading to the intermixing of enzymes and substrates that are usually separated (Ali et al. 2004). The intermixing of phenolic compounds and oxidative enzymes results in browning and discolouration. Moreover, chilling results in massive losses in ascorbic acid content of chilling-sensitive fruits and vegetables, and these losses may occur ahead of any visible symptoms associated with chilling (Lee & Kader 2000).

Studies exploring chilling injury and chilling tolerance have established that numerous bioactive compounds, such as heat shock proteins, antioxidants, dehydrins and reducing sugars, are in operation during chilling tolerance (Maul et al. 2011). The induction of various enzymes in response to stress includes enzymes such as oxidases, POD, polyphenoloxidases (PPO) and phenylalanine ammonia-lyase (PAL) (Pérez-Tello et al. 2001). Chilling tolerance can be induced by exposing the fruit to

temperatures slightly above the chilling range, a technique known as low temperature conditioning (Cai et al. 2006).

1.3.2 Effect of methyl jasmonate on ripening process

Abiotic stresses elicit the production of a variety of secondary metabolites, such as jasmonic acid (JA) and methyl jasmonate (MeJA) (Kim et al. 2007). JA and MeJA are phytohormones derived from the oxidation of fatty acids by lipoxygenase. They operate through the octadecanoid defence signalling pathway, enhancing the defence pathway by the perception and transduction of wound signalling (Heredia & Cisneros-Zevallos 2009a, 2009b; Kim et al. 2007; González-Aguilar et al. 2004). They are involved in a variety of stress responses, both biotic and abiotic, which commonly induce the production of ROS species. Studies have shown that plants deficient in JA signalling are more susceptible to damage resulting from oxidative stress, thus it can be predicted that JA is implicated in balancing stress related redox reactions (Suza et al. 2010).

JA and MeJA activate the phenylpropanoid pathway, resulting in the accumulation of phenolic compounds (Heredia & Cisneros-Zevallos 2009b; Kim et al. 2007). A positive correlation has been demonstrated between the exogenous application of MeJA and the induction of the phenylpropanoid pathway and consequent production of phenolic compounds (Heredia & Cisneros-Zevallos 2009a). An interaction between JA and ascorbic acid has also been reported, with exogenous application of JA increasing endogenous ascorbic acid levels (Suza et al. 2010). However, the mechanism behind this response is not yet understood.

MeJA is a non-ionized more volatile derivative of JA, thus can be easily incorporated into the plant tissue (Fan et al. 1998). The volatility allows MeJA to be applied as a vapour treatment rather than in solution as a dip. Moreover, MeJA is

capable of functioning as an organic volatile stress signal inducing plant defence responses in neighbouring plants in the absence of any stress factor (Kim et al. 2007). Studies into the exogenous application of MeJA to induce bioactive compound accumulation have proven successful (Table 1.3), such as elevated phenolic content of lettuce (Kim et al. 2007) and accumulation of anthocyanins in tulips and carotenes in apples (Cisneros-Zevallos 2003).

Previous studies by Cao et al. (2009) have demonstrated the effect of MeJA in balancing redox reactions, which was observed through enhanced activity of SOD, catalase (CAT) and ascorbate peroxidase (APX) in loquat. This phytohormone also serves a protective role for the plant cell membrane reducing membrane lipid peroxidation (Meng et al. 2009). Nonetheless, possible trade-offs in response to the application of MeJA, which can be considered as a pseudo-stress, in terms of other bioactive compounds are a likely occurrence as well.

Table 1.3 Responses of fruits to exogenous application of MeJA

Fruit	**Response observed**	**Reference**
Apple	Pre-climacteric applications increased ethylene and ester biosynthesis	Fan et al (1997)
Apple	Enhanced colour change and accumulation of anthocyanin, β-carotene content and other phenolic compounds	Rudell et al (2002)
Blackberry	Increased anthocyanin accumulation	Wang et al (2005)
Raspberry	Increased anthocyanin accumulation	Wang et al (2008)
Sweet cherry	Enhanced activity of phenylalanine ammonia lyase (PAL) and peroxidase (POD) enzymes	Yao et al (2006)

1.3.3 Effect of salicylic acid on ripening process

Salicylic acid (SA) is an ubiquitous phenolic compound in plants that acts as the key signalling component of the systemic acquired response (SAR). It functions primarily through the increased activity of POD as well as pathogenesis related (PR) proteins (Zhang et al. 2003; Pieterse & van Loon LC 1999; Raskin 1992). Application of SA induces the same spectrum of genes as those induced by disease infection. It also induces the hypersensitive response (HR) by inhibiting the activity of CAT and resulting in the accumulation of hydrogen peroxide (Raskin 1992). More general functions associated with SA are its involvement in plant growth and development and a range of activities that include seed germination, ion absorption, stomatal movement and disease resistance (Zhang et al. 2003).

As a phenolic compound, SA is produced via the phenylpropanoid pathway, which is also responsible for the production of secondary metabolites such as lignin, phytoalexins, hydroxybenzoic acids. Cao et al (2010) reported that MeJA, which is involved in activating the phenylpropanoid pathway, is capable of inducing the activity of SA. The internal levels of SA can be controlled by the moderation of SA production through the phenylpropanoid pathway (Zhang et al. 2003). Another means of moderating SA levels is through conjugation with glucose. In a typical plant system, SA is converted and stored in the conjugated form (Zhang et al. 2003). This conjugation of SA is catalysed by SA glucosyl-transferase (SA-Gtase), and it occurs in the form of a negative feedback loop, where the plant conjugates excess SA. Research has demonstrated that, similar to phenols, the conjugated form of SA is inactive (Raskin 1992). It is only after subsequent challenges by a pathogen and the resulting damage to the cell and membrane permeability that SAG is hydrolysed to release SA inducing defence responses. The inactive glucoside and its respective glucosidase are spatially

separated within the cell, which is integral for the regulation of bioactive compounds (Raskin 1992).

The role of SA in fruit ripening, senescence and chilling injury alleviation has been the subject of numerous studies (Lu et al. 2011; Luo et al. 2011; Sayyari et al. 2011; Cao et al. 2010). SA is involved in the induction of a range of resistance related enzymes such as PAL and ß1-3 glucanase (Dong et al. 2010). SA induces the accumulation of hydrogen peroxide, which is involved in activating systemic resistance, and superoxide radicals (Babalar et al. 2007). Moreover, scavenging of reactive oxygen species (ROS) is enhanced by SA due to increased activity of POD and SOD (Dong et al. 2010). Although there have been contrasting results reported on the effect of SA on PPO activity, these contradictions arise from a clear cut difference between climacteric and non-climacteric plants, where it is induced in the former (Sun et al. 2013).

SA has been demonstrated to play an important role in controlling ethylene biosynthesis, as it inhibits the activity of ACC synthase and ACC oxidase (Zhang et al. 2003). Wound induced transcription of ACC synthase was inhibited by the application of SA in a study by Zhang et al. (2003). Additionally, SA can suppress the activity of LOX which is correlated with ethylene biosynthesis and postharvest ripening through the regulation of free radical production (Zhang et al. 2003). Ascorbic acid levels are also influenced by SA, which induces the activity of APX and glutathione reductase (GR) (Cao et al. 2010).

To sum up, SA regulates cell expansion and division while also serving as a potential inducer of cell death. It plays a crucial role in plant cell homeostasis through a critical balance of growth and catabolism (Zhang et al. 2003). This is specifically done through the suppression of LOX and enhancement of antioxidative enzyme activity, to

ultimately delay superoxide production and ethylene biosynthesis (Dong et al. 2010). However, the relationship between SA, protective compounds and antioxidative enzymes is highly complex and not fully understood.

1.4 The mechanism of stress regulation

Reactive oxygen species (ROS) are a group of bioactive molecules, such as hydrogen peroxide, singlet oxygen and superoxide. These are unstable and highly reactive molecules that are implicated in causing oxidative damage to cells - one of the most damaging injuries a plant may endure (Lee et al. 2007). These short-lived radicals disrupt the membrane integrity and denature proteins and DNA through a series of reduction chain reactions (Gould et al. 2003).

ROS can be found in various locations within the plant cell, including the chloroplast, mitochondria, endoplasmic reticulum, membrane, peroxisome and glyoxysome (Gould 1999). The production of hydrogen peroxide occurs mainly in the peroxisomes by the activity of glycolate oxidase, or can occur as a side product of fatty acid oxidation during lipid catabolism (Mittler et al. 2004). Meanwhile, the production of singlet oxygen is often attributed to the over-reduction of the electron transport chain (Mittler et al. 2004).

Although ROS are clearly toxic to plants, they are also involved in the control of numerous processes that are vital for a plant's existence (Mittler et al. 2004). Enhanced production of ROS occurs in response to stresses that disrupt the intricate metabolic balance of cells. Thus, plants have evolved to utilize ROS as signalling molecules for the coordination of specialized processes. This forms a signalling pathway that leads to the accumulation of bioactive molecules with protective functions.

Plants have evolved through time to develop scavenging mechanisms to quench ROS using enzymes and secondary metabolites that possess antioxidant properties. Antioxidants are defined as substances that have the capacity to delay or inhibit the oxidation of a substrate, regardless of the relatively low levels of that substance in comparison to the abundance of the oxidizable substrate (Gould 1999). This is achieved either through chain-breaking activities or by quenching free radicals (Heredia & Cisneros-Zevallos 2009b).

Antioxidant defences in the plant can be subdivided into enzymatic and non-enzymatic. The enzymatic can further be subdivided into two types: direct such as superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione peroxidase (GPX) and catalase (CAT); and indirect which restore spent antioxidants into their reduced forms such as dehydroascorbate reductase and glutathione reductase (Lee et al. 2007). The non-enzymatic antioxidants are also referred to as low molecular weight antioxidants (LMWA), and they include chemicals such as tocopherols, ascorbate, glutathione and carotenoids (Xi et al. 2010). The first committed step in the phenylpropanoid pathway is catalysed by the enzyme PAL (Heredia & Cisneros-Zevallos 2009a). Consequently, PAL is considered the key enzyme in the synthesis of diverse classes of phenolic compounds.

1.4.1 Enzymatic antioxidant defence

The oxidative burst associated with postharvest stress is moderated and regulated, which can be observed through the associated increase in the activity of antioxidant enzymes and LMWA following oxidative bursts (Erdeva et al., 1998). APX and CAT are the main enzymatic antioxidants in oxidative damage studies. APX is located in the chloroplast and cytosol and scavenges hydrogen peroxide in the presence of ascorbic

acid, while CAT functions in glyoxysomes and peroxisomes in the presence of high levels of hydrogen peroxide (Delaplace et al., 2009). SOD is another important enzyme which scavenges superoxide radicals, producing hydrogen peroxide which is then converted by CAT and POD (Dong et al. 2010).

Oxidation in fruits usually manifests as skin browning, which is due to the oxidation and polymerization of phenol compounds by the enzymes POD and PPO (Yingsanga et al. 2008). POD and PPO are both induced by cell damage during wounding, injury or infection and catalyze the oxidation of phenols to quinines. POD is induced by the presence of hydrogen peroxide, decomposing hydrogen peroxide to oxygen and water through the oxidation of phenols, thus preventing lipid peroxidation (Dong et al. 2010). Meanwhile, PPO activity is enhanced by disruption of intracellular compartmentalization or loss of membrane integrity since PPO is located in plastids while its substrates are located in the vacuole (Chidtragool et al. 2011; Yingsanga et al. 2008). PAL, which is associated with the biosynthesis of phenol compounds, can be considered a limiting factor in the process of browning.

Membranes of various cell constituents are commonly at risk of oxidative damage during oxidative stress. Polyunsaturated fatty acids (PUFA) are membrane components of most living organisms and are susceptible to oxidative damage by ROS, which results in the production of biologically active compounds called oxilipins (Delaplace et al., 2009). Oxilipins are consequently oxidized into fatty acid hydroperoxides either enzymatically by lipoxygenases or non-enzymatically through auto-oxidation. These fatty acid hydroperoxides are the intermediates for several compounds such as the stress hormone JA (Delaplace et al., 2009). An array of bioactive molecules exists within the plant to protect membranes from oxidative stress,

the chief ones being GPX and tocopherols, which are kept in reduced form by ascorbic acid (Mittler et al. 2004).

Ascorbic acid is the major non-enzymatic antioxidant in plants, and is involved in both enzymatic and non-enzymatic quenching of ROS, particularly in the scavenging of hydrogen peroxide (Suza et al. 2010; Lee & Kader 2000). It exists in plants in two forms; ascorbic acid or dehydroascorbic acid. The latter being the oxidized form of ascorbic acid but still exhibits biological activity (Lee & Kader 2000). Ascorbate oxidase catalyses the oxidation of ascorbic acid under oxidative stress, in the presence of ROS, and it accumulates during postharvest stress (Lee & Kader 2000). Ascorbic acid and glutathione are maintained in a reduced state by (mono)dehydroascorbate reductase and glutathione reductase, which use NAD(P)H to regenerate glutathione and ascorbic acid (Mittler et al. 2004).

1.4.2 Non-enzymatic antioxidant defence

Plant responses to stress involve the production of three main groups of secondary metabolites that serve as antioxidants; terpenes, phenolics and nitrogen-containing secondary metabolites (Cisneros-Zevallos 2003). These compounds serve numerous functions within the plant such as protection from pathogens and herbivores. Controlled application of stress, both preharvest and postharvest, can be an effective tool in the manipulation of secondary metabolites. Stresses can be physical or chemical, ranging from water stress and cold storage to wounding and exogenous application of phytohormones. Studies have documented numerous responses to postharvest stresses that exhibit enhanced nutraceutical content of fruits, such as increased anthocyanin content in temperature stressed strawberries, elevated phenolic acids and anthocyanins

in wounded red lettuce, and methyl jasmonate induced carotene accumulation in apples (Cisneros-Zevallos 2003).

Phenylalanine (PA) regulates ROS activity whilst also regulating pH, cell homeostasis and the structural integrity of membranes and cell walls (Edreva et al., 1999). It is also the precursor of the phenylpropanoid pathway, which is of significant importance in the inducible defence mechanism, and can be induced by treatment with elicitors or exposure to stress (Heredia & Cisneros-Zevallos 2009b). More than 4000 compounds have been classified as 'phenolic compounds', and are produced by a variety of fruits, vegetables, fungi and bacteria (Bernal et al. 2011). Phenolic compounds regulate the activity of ROS and operate in one of two ways: chain breaking reaction activities or free radical scavenging (Heredia & Cisneros-Zevallos 2009b). Meanwhile, the pigment carotenoids offer the cells protection primarily from singlet oxygen (Mittler et al. 2004). Carotenoids and terpenes play a variety of roles that include enhancing the thermostability of membranes, scavenging ROS and reducing the incidence of lipid peroxidation (Gonzalez-Aguilar et al. 2010).

Ascorbic acid is a highly important nutritional component of fruits and vegetables, and it is susceptible to degradation in response to postharvest stresses (Lee & Kader 2000). Factors such as high temperature, chilling and wounding accelerate ascorbic acid loss. Postharvest technologies that slow down respiration, such as modified storage or edible coatings, can reduce ascorbic acid loss (Lee & Kader 2000). Although wounding can increase ascorbic acid loss, this effect is highly dependent on the specific plant organ, nature of injury and the plant species (Suza et al. 2010). However, JA stress induces the accumulation of ascorbic acid (Suza et al. 2010).

The biosynthetic pathway for ascorbic acid involves number of compounds, primarily D-mannose/L-galactose, D-galacturonate, L-glucose and myo-inositol.

Nonetheless, there is sparse information on the enzymes involved in ascorbic acid metabolism (Pignocchi et al. 2003). Ascorbic acid participates in the regulation of cell wall glycoproteins and hormones such as ethylene and gibberellic acid, as well as in the moderation of cell expansion and division (Suza et al. 2010). It is also involved in the scavenging of hydrogen peroxide, reducing it to water (Equation 1.1).

$$2 \text{ ascorbic acid} + H_2O_2 \rightarrow H_2O + 2 \text{ monodehydroascorbate}$$

Equation 1.1 Scavenging of hydrogen peroxide by ascorbic acid (Zhang et al. 2001).

Clearly, a dynamic interaction exists amongst the complex machinery involved in ROS homeostasis to minimize oxidative trauma in the plant (Hernández et al. 2009). This machinery comprises of plant secondary metabolites such as ascorbic acid, tocopherols, carotenoids and phenolic compounds. All of these compounds indiscriminately control degenerative reactions that occur in all living tissues as a result of oxidative stress (Cisneros-Zevallos 2003). Consequently, they can dually inhibit degradation of perishable fruits and vegetables during storage while serving as nutraceutical compounds.

1.4.3 Signalling pathways and networks associated with stress regulation

Plant tissues acclimate to improve stress tolerance, which involves the moderation of numerous pathways. An example of timely regulation of signalling pathways is heat shock response, which is characterized by biosynthesis of heat shock proteins, ambiguous proteins that offer protection against additional stresses (Cisneros-Zevallos 2003). Flavonoid biosynthesis genes are generally upregulated under various stresses such as wounding or drought, which increase the flavonoid levels within the plant tissue

(Hernández et al. 2009). The general signalling pathway in response to wounding involves the production of ROS and a concomitant increase in respiration levels (Heredia & Cisneros-Zevallos 2009b). Signalling pathways are complex with constant overlapping and interlinking (Heredia & Cisneros-Zevallos 2009a). Dissecting the hierarchical response to stresses will be of great interest as it can be used practically to modulate plant responses to other stresses (Cisneros-Zevallos 2003).

Transcription factors involved in regulation of cold resistance have been elucidated, such as c-Repeat/dehydration-responsive element binding factor (CBF), a transcription factor which recognizes cold regulated genes and is involved in cold acclimation (Zhao et al. 2009). CBF is linked to cold tolerance in selected plant cultivars, and a functional CBF cold response pathways has been established through the identification of genes located upstream and downstream of CBF(Zhao et al. 2009).

Lipoxygenases (LOX) are mainly involved in pathogen infection and wound stress responses by catalysing hydroperoxidation of polyunsaturated fatty acids, which is the first committed step in the synthesis of fatty acid metabolites (Gomez-Lobato et al. 2012). Subsequent catalysis of the intermediate bioactive molecules produces JA and its methylated form, which serve important functions such as signalling and stress response (Gomez-Lobato et al. 2012). Six genes encoding lipoxygenases have been identified, alas, success in describing the physiological function of the genes was only successful for one of the four genes (Gomez-Lobato et al. 2012). Moreover, the role of LOX in plant defence responses is more elaborate, involving interplay between LOX, PAL and ROS.

1.4.4 Elicitor induction of stress responses

A hypothetical pathway was developed by Gonzalez-Aguilar et al. (2010) for elicitors of plant defence mechanisms, such as MeJA and chitosan (Equation 1.2). This pathway involves the recognition by a plasma membrane localised receptor, which activates receptor-coupled effectors such as GTP-binding proteins, kinases, phosphatases. This is then followed by the mobilization of signalling molecules that include ROS and NO_2, leading to the synthesis of LMWA, increased activity of oxidative stress enzymes such as PAL and CHS as well as an overall cell reinforcement (Gonzalez-Aguilar et al. 2010).

Stress response in plants is associated with increased respiration, and a functional link between hydrogen peroxide and ethylene in stress signalling is established (Desikan et al. 2006). Ethylene signalling is linked to JA signalling pathways as well, as both pathways need to be concomitantly triggered to activate certain defence pathways (Pieterse & van Loon LC 1999). This crosstalk between JA and ethylene implicate JA in some ripening related responses, such as the promotion of chlorophyll degradation by MeJA (Fan et al. 1998).

Stress → ROS → H_2O_2 → moderate stress-induced LMWA → PAL and CHS

Equation 1.2 Proposed signalling pathway for elicitor induction of stress responses (Gonzalez-Aguilar et al. 2010).

The phenylpropanoid pathway has received considerable scientific interest, and a dynamic relation that has been extensively studied is between the phenylpropanoid pathway and SA. Whilst PAL is recognised as the rate limiting enzyme in SA biosynthesis, SA has also been demonstrated to regulate PAL activity (Dong et al.

2010). In a study conducted by Nugroho et al. (2002), overproduction of SA in tobacco resulted in the downregulation of the phenylpropanoid pathway, ultimately reducing the biosynthesis of flavonoids. SA and flavonoids are produced via the same pathway, which goes to illustrate the diversion of enzymes and precursors to SA biosynthesis limits the production of other compounds via the same pathway.

SA and MeJA operate through independent pathways, where SA primarily controls PR gene expression while MeJA moderates PDF1.2 gene expression (Pieterse & van Loon LC 1999). NPR1 is the receptor of SA signalling, activating downstream responses which primarily involve pathogenesis related (PR) gene expression; PR1, PR2 (β1-3, glucanase) and PR3 (chitinase) (Sun et al., 2013). However, initiation of lesion formation appears to be a common initial step in the signal transduction pathways of both SA and MeJA. It is generally accepted that wounding operates through MeJA while pathogen attack through SA and MeJA. However, the relationship between MeJA and SA is more complex, as in some cases SA supresses the production of MeJA as shown in mutant lines of SA biosynthesis, while in other cases a synergistic effect of applying SA and MeJA exists (Pieterse & van Loon LC 1999).

1.5 Hypothesis

The application of stress hormones SA and MeJA during cold storage will influence the ripening behaviour of the fruits and the bioactive content. Addition of SA will delay ripening and enhance nutritional quality, while MeJA will promote ripening whilst enhancing nutritional quality.

1.6 Research objectives

It is apparent that the different pathways involved in ripening and abiotic stress response are interlinked. Thus, there is a need to elucidate the interaction between these different pathways. Moreover, the activation of the antioxidant system as a result of postharvest stresses has not been well documented. The quintessence of which is the dynamics of PAL, PPO and POD under postharvest stresses, where a substantial lack of data exists. The genetic network is fundamentally redundant and flexible, thus more elaborate information linking other factors related to stress response, such as ROS, and the transcriptional information is pertinent. Finally, although the nutraceutical value of tropical fruits is generally associated with the radical quenching activity, there is surprisingly minimal amount of studies exploring the benefits of enhanced fruit bioactivity to human health.

On the basis of this literature review, an assessment of the response of three tropical fruits – carambola, mangosteen and dragon fruit – to postharvest stresses caused by chilling, MeJA and SA, was carried out. Physico-chemical changes and altered enzymatic activities were assessed and compared to fruit ripening under cold storage. Moreover, the enhanced bioactivity resulting from these responses was also evaluated in terms of the nutraceutical benefits that are associated with the bioactive compounds. This study was not aimed at developing a new technology, as salicylic and jasmonic acids have long been established to alleviate chilling injury. The aim of this study was to illustrate the role that controlled application of stress within the postharvest supply chain might play in promoting the quality of selected fruits. This could ultimately provide a holistic picture relating aesthetic quality with nutritional quality and functionality of edible fruits by understanding the stability of the fruits under stress and the defense mechanisms in operation.

The main objectives of this study were as follows:

1. To explore the effects of methyl jasmonate and saliciylic acid in combination with chilling on physico-chemical responses of carambola, dragonfruit and mangosteen.
2. To assess the effect of the postharvest stresses on bioactive content and antioxidant activity of the fruits.
3. To evaluate the impact of the postharvest stresses on selected enzymatic activities of the carambola fruit samples with enhanced bioactivity.
4. To assess the effect of enhanced bioactivity of the carambola fruit samples on Human Hepatoma (HepG2) cell cultures.
5. To identify the members of gene families associated with stress amelioration in carambola

CHAPTER 2: GENERAL MATERIALS AND METHODS

2.1 Plant material

2.1.1 Carambola

Small, disease free and uniform sized carambola fruit (cv. B10) at green-maturity stage (stage 1) were purchased from a commercial farm (Seng Chew Hup Kee Sdn Bhd) located at Kajang, Selangor State of Malaysia. The fruits were washed with 0.05% sodium hypochlorite followed by distilled water then air-dried at ambient temperature.

2.2.2 Dragonfruit

Disease free and uniform sized red dragonfruit at mature stage (28 – 32 days after fruit set) were purchased from a commercial farm located at Broga, Negeri Sembilan State of Malaysia. The fruits were washed with 0.05% sodium hypochlorite followed by distilled water then air-dried at ambient temperature.

2.2.3 Mangosteen

Small, disease free and uniform sized mangosteen at purple-red maturity (stage 5) were purchased from a commercial farrm located at Malacca State of Malaysia. The fruits were washed with 0.05% sodium hypochlorite followed by distilled water then air-dried at ambient temperature.

2.2 Treatments and storage conditions of fruits

2.2.1 Methyl jasmonate vapour treatment

Five concentrations of methyl jasmonate (Sigma-Aldrich, USA) were chosen for this experiment, which consisted 0.00 mM, 0.01mM, 0.1mM, 0.2mM and 0.5mM. Fruits

were subjected to methyl jasmonate vapours by incubating the fruits in 45L air-tight storage box with the selected methyl jasmonate treatment, for 16 hours at ambient temperature. After the incubation period, boxes were allowed to ventilate for 2 hours and the fruits were then packed in cardboard boxes and stored at 6°C for the desired storage period.

2.2.2 Salicylic acid treatment

Five treatments of salicylic acid (Sigma-Aldrich, USA) (0.00 mM, 0.1mM, 1mM, 2mM and 5mM) were used for the study. Fruits were immersed in salicylic acid solutions at the desired concentration and were then allowed to dry at room temperature for 30 minutes. Fruits were then packed in packaging cartons and stored at 6 °C for the desired storage period.

2.3 Statistical analysis

All the experiments conducted were arranged in a completely randomised design (CRD) with four replications. The data was subjected to analysis of variance (ANOVA) and means were separated by Duncan's Multiple Range Test (DMRT) ($P < 0.05$) using SAS® (9.1, SAS Institute Inc., USA). Data was presented as the means of four replications of four units for each treatment ± standard error in graphs.

CHAPTER 3: FRUIT PHYSICO-CHEMICAL RESPONSE TO POSTHARVEST INDUCED STRESSES

3.1 Introduction

Ripening is a programmed process leading to several changes in the quality attributes of the fruits, ultimately enhancing the fruit's desirability (Seymour et al. 2013). These include changes in flavour, colour, texture and palatability, which are generally independent of each other. Moreover, compositional changes also occur that involve the conversion of energy reserves in the form of complex carbohydrates or organic acids into simple molecules (Valero & Serrano 2010). Ripening behaviours of fruits are characterised as one of two: climacteric and non-climacteric. These definitions are rather arbitrary now, which has prompted further research on the biochemistry of ripening and associated changes.

Dissection of ripening begins with an understanding of respiration patterns. Respiration is the oxidative breakdown of complex matter into simple matter in the process of generation of energy and essential molecules for biosynthesis reactions (Wills et al. 2007). Thus, metabolic activity has been commonly adopted as an indicator of the storage life of fruits. Variations in the rate of respiration are often experienced during growth, maturation, ripening and senescence of fruits. In climacteric fruits, a respiratory peak is observed which is triggered by ethylene perception. This respiratory peak is not observed in non-climacteric fruits.

All fruits produce minute quantities of ethylene, although climacteric fruits produce ethylene at a much higher rate than non-climacteric fruits. The effect of ethylene on respiration has been a priority of previous studies into fruit ripening (Concha et al. 2013). This has yielded substantial information on the mechanism behind

the interaction of ethylene and respiration. Ethylene enhances ripening of climacteric fruits, although the magnitude of enhancement in ripening is independent of the ethylene concentration applied (Wills et al. 2007). Meanwhile, in non-climacteric fruits a transient dose-dependent increase in ripening is observed in response to ethylene. Moreover, non-climacteric fruits are distinct from climacteric fruits in that several peaks can be observed, whilst for climacteric fruits a singular ripening peak is observed. Stress and wounding have been reported to increase respiration, similarly to ethylene.

Although ripening enhances the desirability and marketability of fresh produce, over-ripening is a potential risk that ultimately decreases the storage life and marketability of the product (Valero & Serrano 2010). The shelf life of fresh produce varies, depending on the produce and its sensitivity to the storage conditions. Nonetheless, percentage weight loss of 5 % is sufficient to render most types of fruits undesirable. A 5 % loss causes extensive wilting resulting in a shrivelled fruit with reduced quality in terms of firmness, colour, nutritional and sensorial quality (Wills et al. 2007)). Thus, weight loss exceeding 3 % was considered as a determining factor for selecting the end of the storage period for the fruits in this experiment.

Cold storage can effectively slow down respiration and undesirable changes in the fruit. However, tropical fruits are intolerant of chilling conditions and may suffer from further undesirable changes as a result of chilling injury (Vyas et al., 2015). The effect of cold storage on carambola, dragonfruit and mangosteen was initially assessed. Storage at 10 °C was found to be optimal for storage of the fruits with minimal symptoms of chilling injury observed (results not shown), while storage at 6 °C exhibited sufficient stress on the produce. Thus, 6 °C was selected as the temperature for inducing chilling stress, and the stress hormones were selected at concentrations that equal to or exceed doses reported to alleviate chilling injury.

The postharvest shelf life of the selected fruits was characteristically short. Cold storage increases the storage life of mangosteen, however prolonged storage results in pericarp hardening of the fruit (Dangcham et al. 2008). Extensive increase in firmness of the fruit was observed after 12 days of storage at 6 °C. Thus, textural changes were selected as a determining factor for the length of storage period for mangosteen, and the fruits were analysed for a total period of 12 days at two-day intervals.

Meanwhile, rib browning was selected as the determining factor for the onset of chilling injury of carambola and was used for establishing the end of storage period (Perez-Tello et al. 2001). Preliminary results had shown that carambola showed first symptoms of rib browning within 16 days of storage, which was subsequently selected as the storage period for carambola in these experiments. Dragonfruit does not exhibit a distinct symptom to chilling injury, but it has a reasonably short shelf life due to the high respiration of the fruit, resulting in more rapid occurrence of ripening-related changes (Zahid et al. 2013). The 3 % weight loss was used for selecting the end of the storage period which was established at 21 days from preliminary results.

Thus, the fruits were exposed to doses of MeJA and SA for their respective storage period at 6 °C and ripening-related physicochemical changes were assessed at the established regular intervals. Weight loss, colour evolution, textural changes and changes in the soluble solids content and titratable acidity of the fruits were studied to assess the changes in the quality factors and marketability of the fruits that occur in response to the applied stresses.

3.2 Materials and methods

Carambola, dragon fruit and mangosteen were subjected to the treatments described in Sections 2.2.1 and 2.2.2 and used for assessing the physicochemical responses of the fruits to the stress treatment. Carambola was assessed at three-day intervals for a storage period of 16 days, mangosteen was subjected to 12 days of cold storage and assessed at two day intervals. Meanwhile, dragonfruit was stored for 21 days and the quality was assessed at weekly intervals. Four biological replicates were used for each fruit to assess each parameter.

3.2.1 Weight loss

Weight loss was measured using a digital balance (EK-600H, Japan) at day 0 and at each sampling day. Same fruits were used for weight loss until the end of storage period. The difference between final weight and initial weight were considered as weight loss during storage and calculated as percentage weight loss using the following formula (3.1) (Ali et al., 2010):

$$weight\ loss = (initial\ weight - final\ weight)/initial\ weight \text{ x } 100\% \quad (3.1)$$

3.2.2 Colour

Colour was determined using Hunter Lab System, MiniScan XE Plus Colourimeter, (Model: 45/0-5, Reston Virginia, USA). The meter was equipped with a measuring head that had an 8 mm measuring diameter and calibrated with standard black and white tiles with values of X = 79.0, Y = 83.9 and Z = 87.9. Data were recorded as $C^* = (a^{*2} + b^{*2})^{1/2}$,which represented the hypotenuse of a right triangle with the values ranging from 0 = least intense to 60 = most intense, and hue angle ($h°$) was the angle of tangent-

[1] b*/ a* [*h*° represents red-purple at an angle of 0°, yellow at 90°, bluish green at 180°, and blue at 270°]. The mean values of C^* and *h*° were obtained for four fruits in each treatment from two opposing ribs along the equator for carambola, and at three points along the equator for dragonfruit and mangosteen.

3.2.3 Firmness

Fruit firmness was determined before the biochemical analyses were carried out, using an Instron Universal Testing Machine (Instron 2519-104, Norwood, MA) equipped with an 8 mm plunger tip. Firmness was assessed by subjecting fruits to puncture test at a constant speed of 20 mm/min. The maximum amount of force (N) required to penetrate the fruits was recorded. For carambola, the fruit ribs were cut and firmness was determined for two ribs at two points along their surface (Ali et al. 2004). Meanwhile, for dragonfruit and mangosteen the firmness was determined at three points along the equator of the fruit.

3.2.4 Soluble solids content (SSC)

Fruit samples were homogenized in a blender. Soluble solids content (SSC) was determined using a Palettle Digital Refractometer (Model: PR-32α, Atago Co., Ltd. Japan) calibrated against sucrose, with a sensitivity range of 0 – 32^{o}Brix. 100 µl of the fruit juice was placed on the prism glass of refractometer to obtain SSC reading. The refractometer was standardised with distilled water before analysis. The results were then expressed in percentage (Ali et al., 2010).

3.2.5 Titratable acidity

Titratable acidity was determined by titrating 5ml of the fruit homogenate against 0.1N NaOH using 0.1% phenolphthalein as an indicator. Results were expressed as percentage of titratable acidity (% TA) determined by the following formula

(Ranganna, 1977) (3.2). The equivalent weight of citric acid (64) was used to calculate titratable acidity in mangosteen and dragonfruit, while the equivalent weight of oxalic acid (63) was used to calculate titratable acidity for carambola.

$$\%\mathrm{TA} = \frac{\text{titre value} \times \text{volume made up} \times \text{Eq. weight of acid} \times 100}{\text{sample weight} \times \text{aliquot of sample used} \times 1000} \quad (3.2)$$

3. 3 Results

3.3.1 Carambola (*Averrhoa carambola* L.)

3.3.1.1 Weight loss

Weight loss is an indicator of the metabolic rate of fruit, as increased weight loss represents high metabolic activity of the fruit. The effect of the different stresses on the metabolic rate of the fruit was thus determined by measuring the changes in weight loss throughout the storage period. A non-significant interaction ($p > 0.05$) (Appendix B 3.1) was exhibited between time and treatment for weight loss, nonetheless weight loss of carambola was significantly reduced ($p < 0.05$) for the fruits that were exposed to the lowest and highest dose of MeJA, 0.01 and 0.5 mM MeJA respectively (Figure 3.1). Moreover, while the application of 0.2 mM MeJA did not have a significant effect ($p < 0.05$) on weight loss in comparison to the control, the application of 0.1 mM MeJA significantly increased ($p < 0.05$) weight loss.

Similarly, no significant interaction ($p > 0.05$) (Appendix B 3.2) between time and treatment was observed for carambola treated with SA (Figure 3.2). However, the effect of the treatment was significant ($p < 0.05$) and reduction in weight loss was positively correlated to the concentration of SA applied, with the lowest weight loss observed for the highest SA treatments. The percentage weight loss of the control was significantly lower than the weight loss observed for the control in the MeJA experiment (Figure 3.1). Similarly, the weight loss of the MeJA treated fruits was all higher than the weight loss observed for the control in the SA treatment.

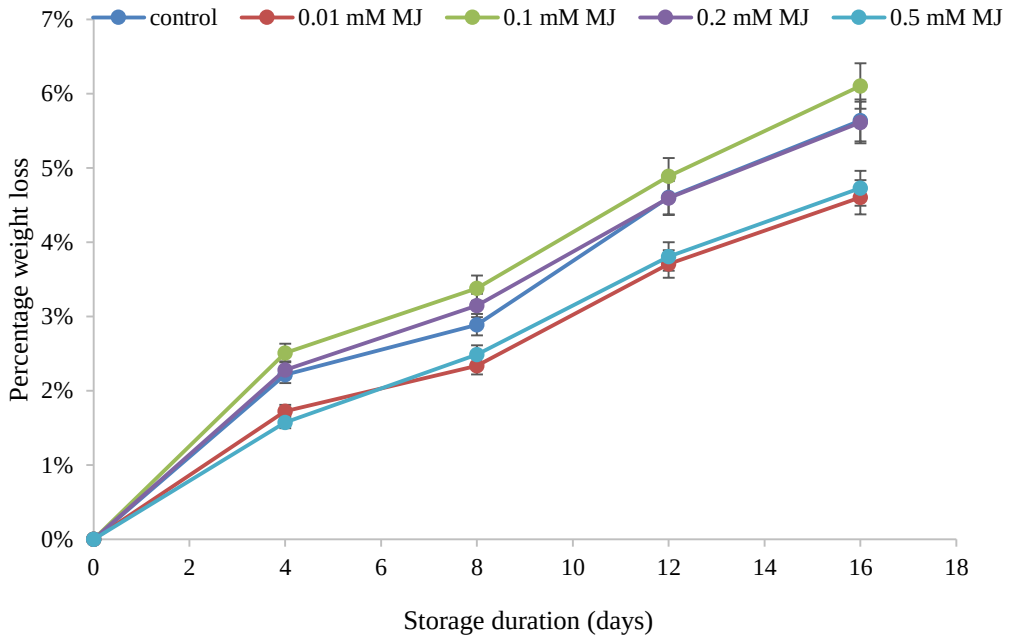


Figure 3.1 Effect of different concentrations of methyl jasmonate on weight loss of carambola stored at 6°C for 16 days. Values are the means ± SE.

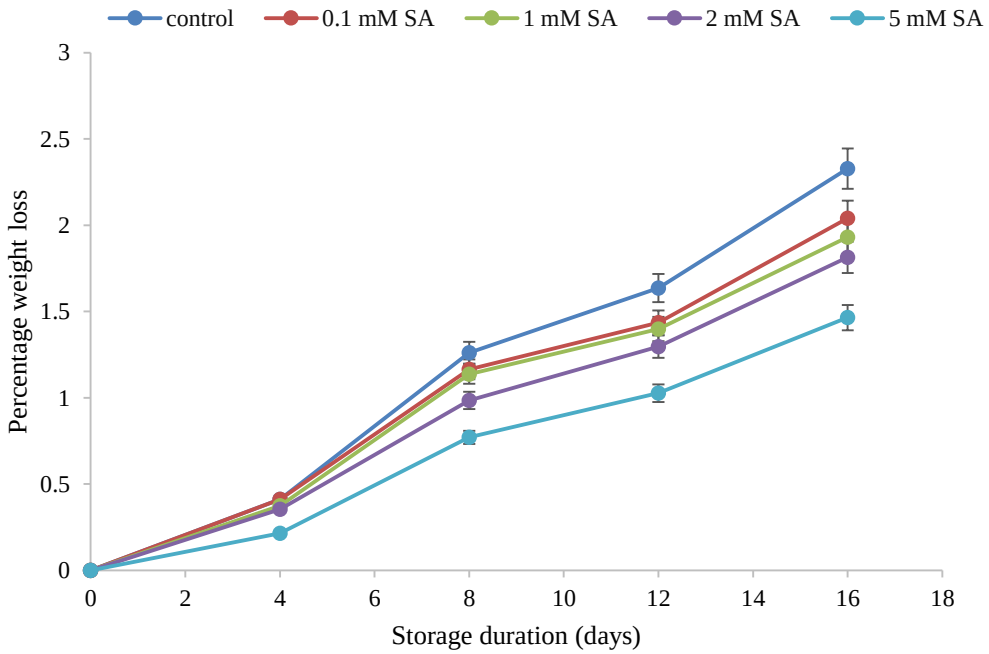


Figure 3.2 Effect of different concentrations of salicylic acid on weight loss of carambola stored at 6°C for 16 days. Values are the means ± SE.

3.2.1.2 Colour

Colour was assessed as L*, C* and *h*. As carambola ripens, it acquires a yellowish-orange hue and the green colour dissipates. There was no significant difference ($p > 0.05$) between the different treatments for both C* and *h*, with the exception of the 0.01 mM MeJA treatment (Table 3.1). Fruits treated with 0.01 mM MeJA exhibited significant colour change during the storage period.

Previous studies demonstrated that MeJA interacts with the ethylene-signalling pathway in the promotion of colour change. This has prompted the use of MeJA for degreening fruits such as apples (Fan et al. 1998). However, the promotion of colour change was only observed for the fruits that were exposed to the lowest dosage of MeJA. Higher doses of MeJA did not elicit changes in the development of colour of carambola.

Fruits that were subjected to the exogenous SA did not exhibit a significant change ($p < 0.05$) in development of L* during the storage period (Table 3.2). Meanwhile, changes in the chromaticity of the fruit were significantly enhanced for the fruits treated with 2 mM SA while the C* value was maintained at the same level the other treatments throughout storage. There was a decline in hue for all the treatments during the storage period, which was accelerated for the 0.1 and 1 mM SA treatments.

Table 3.1 Effect of different concentrations of methyl jasmonate on colour, presented as L*,C and *h*, of carambola stored at 6°C for 16 days. Values are the means ± SE.

Time (days)	Control	0.01 mM MeJA	0.1 mM MeJA	0.2 mM MeJA	0.5 mM MeJA
L* (SE = 2.35)					
0	37.60	37.60	37.60	37.60	37.60
4	27.01	28.15	30.27	30.71	29.37
8	32.80	35.06	30.00	32.40	28.68
12	32.67	34.14	35.73	31.23	34.60
16	34.51	34.83	35.04	27.52	29.93
Chromaticity (SE = 1.30)					
0	10.16	10.16	10.16	10.16	10.16
4	9.30	8.19	9.85	8.78	9.54
8	8.60	8.20	8.99	8.77	9.71
12	9.49	8.02	9.47	8.76	9.78
16	9.76	8.32	9.86	8.66	8.82
Hue (SE = 0.53)					
0	1.89	1.89	1.89	1.89	1.89
4	2.38	1.05	1.38	2.30	2.65
8	1.50	0.70	1.98	1.96	1.94
12	1.46	0.39	1.37	1.63	1.20
16	1.26	0.40	1.17	1.23	1.32

Table 3.2 Effect of different concentrations of salicylic acid on colour, presented as L*,C and *h*, of carambola stored at 6°C for 16 days. Values are the means ± SE.

Time	Control	0.01 mM MeJA	0.1 mM MeJA	0.2 mM MeJA	0.5 mM MeJA
L* (SE = 2.14)					
0	37.23	37.23	37.23	37.23	37.23
4	43.18	40.04	37.50	37.38	36.51
8	38.25	37.52	40.35	41.98	37.54
12	35.61	33.15	37.73	34.71	35.26
16	38.69	37.79	39.08	39.15	36.81
Chromaticity (SE = 0.82)					
0	9.88	9.88	9.88	9.88	9.88
4	7.46	9.59	9.78	10.82	9.73
8	9.18	9.53	9.25	11.27	9.46
12	8.60	8.31	8.92	10.08	8.98
16	8.90	8.23	9.05	10.25	9.22
Hue (SE = 0.60)					
0	2.34	2.34	2.34	2.34	2.34
4	1.62	0.87	0.91	1.24	1.33
8	1.60	1.10	0.93	1.16	1.27
12	0.67	0.87	0.76	1.19	0.87
16	1.92	0.62	0.61	0.96	0.92

3.2.1.3 Firmness

Fruit softening is one of the primary indicators of the onset of ripening. Thus, textural changes of carambola were assessed throughout the storage period. Throughout the storage period the firmness of the fruit fluctuated (Figure 3.3), however the differences observed were not significant for the interaction between treatment and time ($p > 0.05$) (Appendix B 3.3).

Similarly, no significant difference ($p < 0.05$) (Appendix B 3.4) was observed in the SA experiment (Figure 3.4). The exception was the 2 mM SA treatment, which was significantly lower ($p < 0.05$) than the other treatments on the final day of storage. This was the same treatment that exhibited enhanced changes in chromaticity, which could be an indicator of accelerated ripening changes compared to the other treatments upon exposure to the stress hormones.

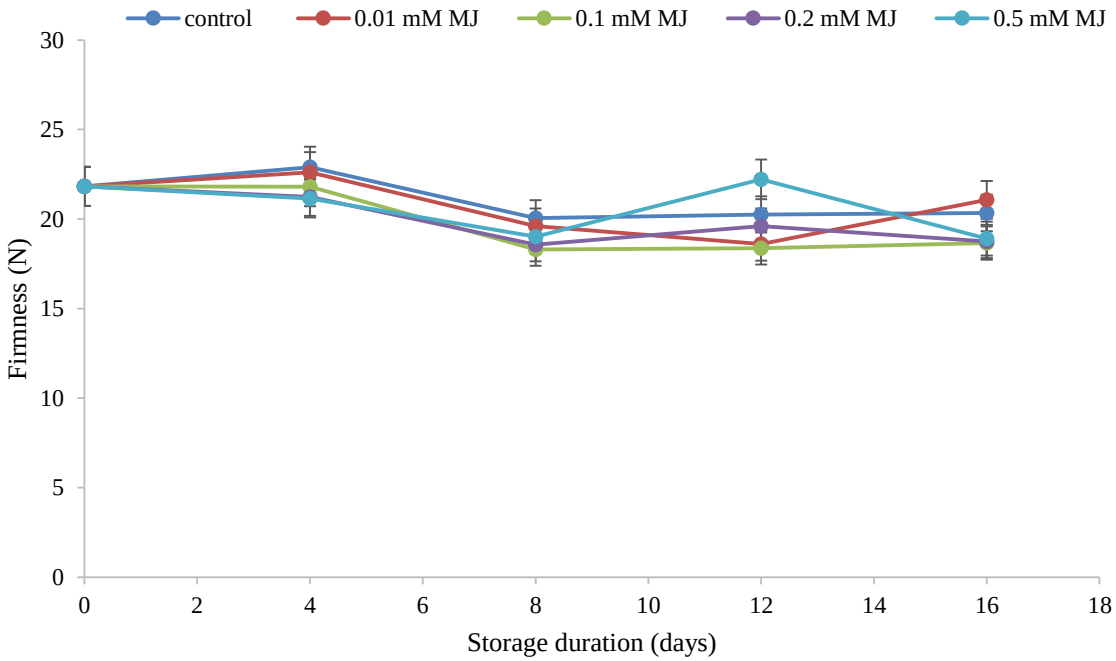


Figure 3.3 Effect of different concentrations of methyl jasmonate on firmness of carambola stored at 6°C for 16 days. Values are the means ± SE

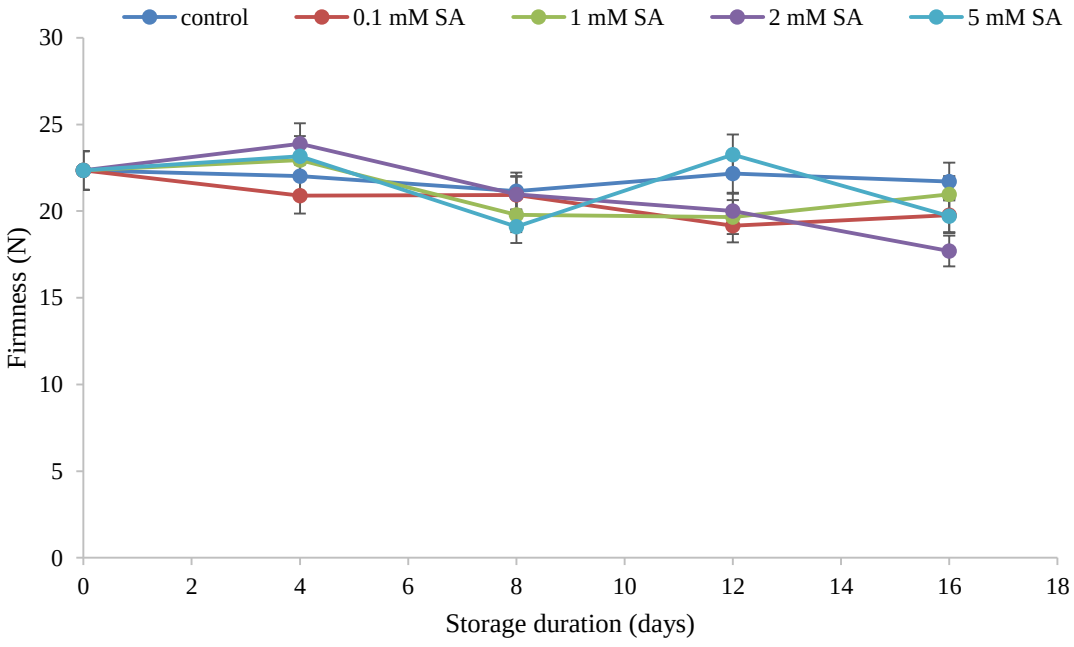


Figure 3.4 Effect of different concentrations of salicylic acid on firmness of carambola stored at 6°C for 16 days. Values are the means ± SE

3.2.1.6 Soluble solids content

Soluble solids content (SSC) were assessed as an indicator of the metabolic status of the fruit. Application of MeJA induced a significant ($p < 0.05$) (Appendix B 3.7) response for SSC. A 2-fold increase in SSC was observed for the control on the 8th day of storage (Figure 3.5). Meanwhile, the fruits treated with MeJA experienced delayed and lowered peaks on the 12th day of storage. The peaks observed for fruits treated with 0.01 and 0.1 mM MeJA were at par ($p > 0.05$) with the control. Moreover, as the concentration of MeJA applied on the fruits increased, the SSC values declined with the lowest values observed for the 0.5 mM MeJA treatment.

Meanwhile in the SA experiment, the same general trend for SSC was observed for all of the treatments (Figure 3.6). Nonetheless, an earlier peak was observed for the control as compared to the other treatments. The fruits treated with 5 mM SA maintained significantly lower ($p < 0.05$) SSC values as the fruit continued to ripen. However, on the final day of storage the fruits treated with 5 mM SA were at par ($p > 0.05$) with the control and exhibited the highest levels of SSC.

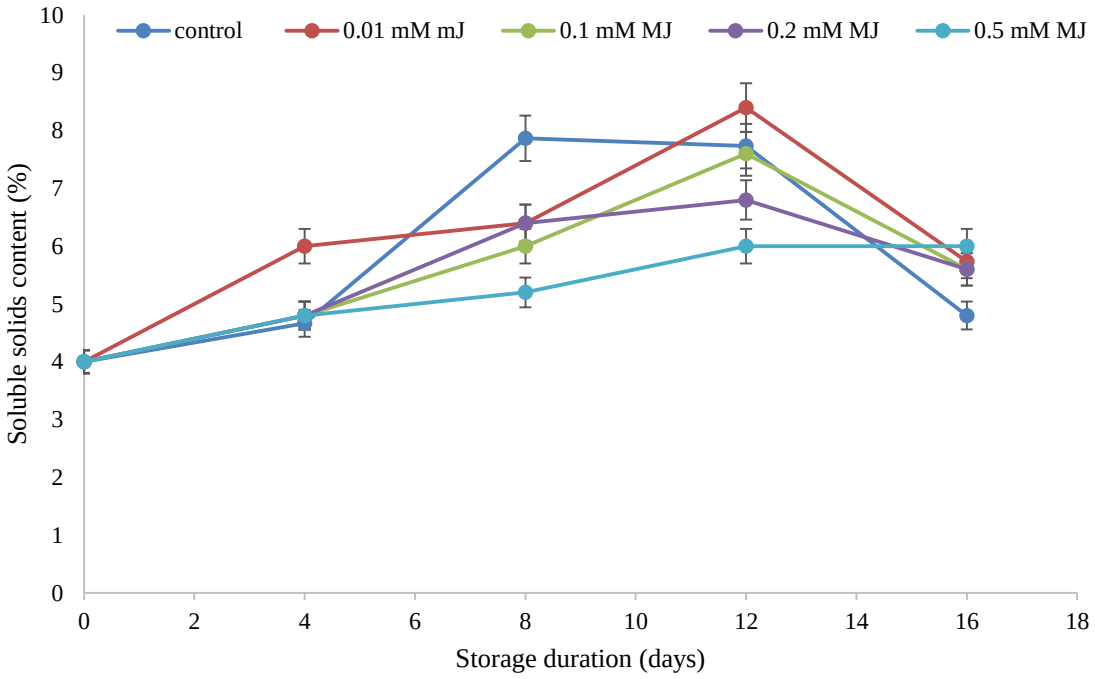


Figure 3.5 Effect of different concentrations of methyl jasmonate on soluble solids content of carambola stored at 6°C for 16 days. Values are the means ± SE.

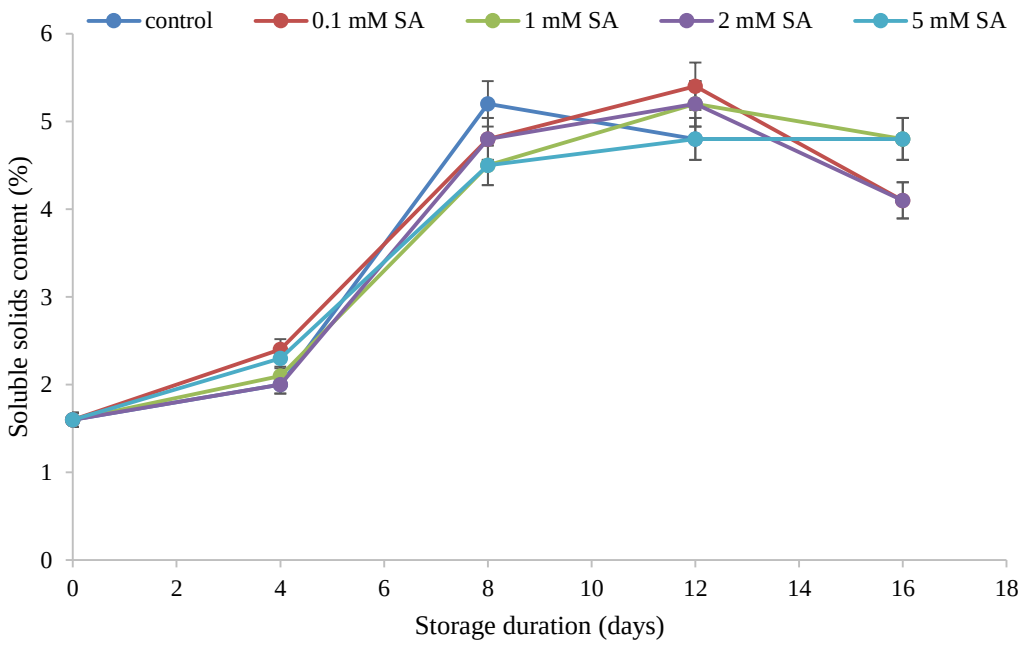


Figure 3.6 Effect of different concentrations of salicylic acid on soluble solids content of carambola stored at 6°C for 16 days. Values are the means ± SE.

3.2.1.5 Titratable acidity

The titratable acidity (TA) reflects the organic acid content of the fruit, which are also used during ripening as energy reserves. Carambola possesses a significant amount of oxalic acid, thus the equivalent mass of oxalic acid was used to determine TA (Dembitsky et al. 2011). A similar trend to SSC was observed for TA where a significant interaction ($p < 0.05$) (Appendix B 3.5) between treatment and time was observed, and an earlier peak was observed for the control (Figure 3.7). The peak for the control was observed on the 8th day of storage, while for the treated fruits it was observed on day 12. The increase in TA was inversely related to concentration of MeJA applied, as the peak observed for the highest MeJA treatment was significantly lower ($p < 0.05$) than the other treatments.

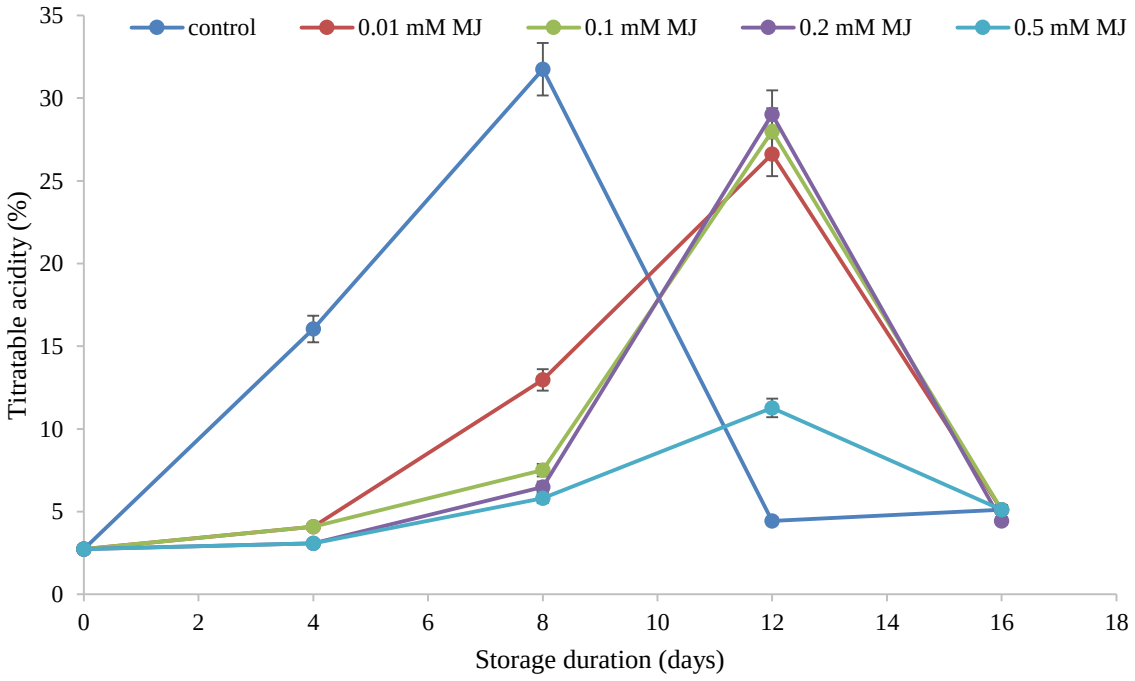


Figure 3.7 Effect of different concentrations of methyl jasmonate on titratable acidity of carambola stored at 6°C for 16 days. Values are the means ± SE.

A significant interaction was also observed for treatment and time for fruits treated with SA ($p < 0.05$) (Appendix B 3.5). The TA values for the SA treated fruits were generally maintained during the storage period (Figure 3.8). Although a peak was observed for the fruits treated with 5 mM SA during the initial days of storage, this treatment maintained the lowest TA value as the fruit continued to ripen. The lowest TA values throughout the entire storage period were observed for the 2 mM SA treatment, which was at par ($p < 0.05$) with the 5 mM SA treatment during the final days of storage.

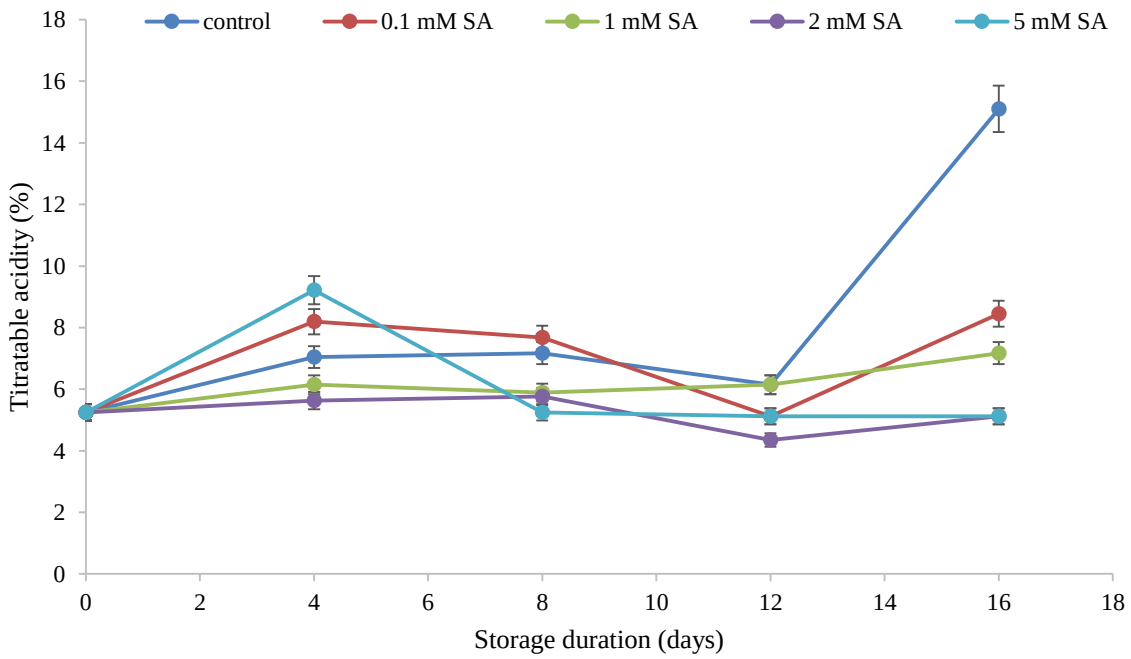


Figure 3.8 Effect of different concentrations of salicylic acid on titratable acidity of carambola stored at 6°C for 16 days. Values are the means ± SE.

3.2.2 Dragon fruit (*Hylocereus polyrhizus* L.)

3.2.2.1 Weight loss

Dragonfruit is characterised with rapid ripening, resulting in significant changes in the physiological and biochemical quality factors of the fruit (Ali et al., 2013). Weight loss of dragon fruit was monitored over three weeks of cold storage (Figure 3.9). The storage period and the levels of the treatment applied had a significant effect ($p < 0.05$) (Appendix B 3.9) on the rate of weight loss. As the fruit continued to ripen, the control maintained the lowest levels of weight loss. This is an indication of higher metabolic activity in the MeJA treated fruits, which accelerated the weight loss of the fruit.

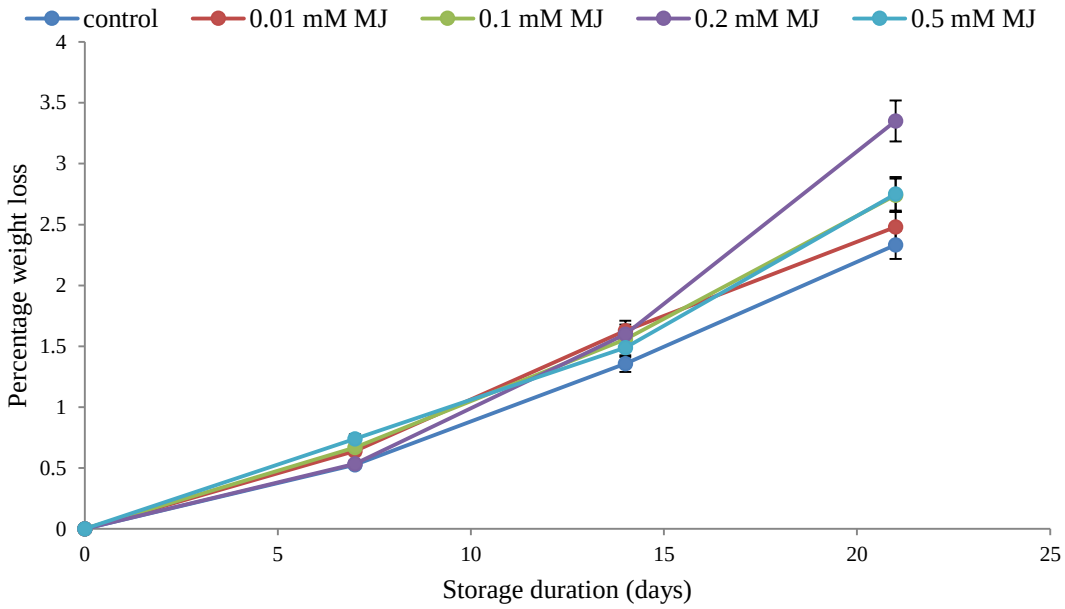


Figure 3.9 Effect of different concentrations of methyl jasmonate on weight loss of dragonfruit stored at 6°C for 21 days. Values are the means ± SE.

Meanwhile in the SA treated fruits, weight loss was initially significantly higher for the control (Figure 3.10). However, by the final day of storage, there was no significant difference ($p > 0.05$) between the control and the other treatments. Throughout the storage period all of the treatments were at par ($p > 0.05$), indicating that exposure to high SA does not exhibit adverse effects on the fruit.

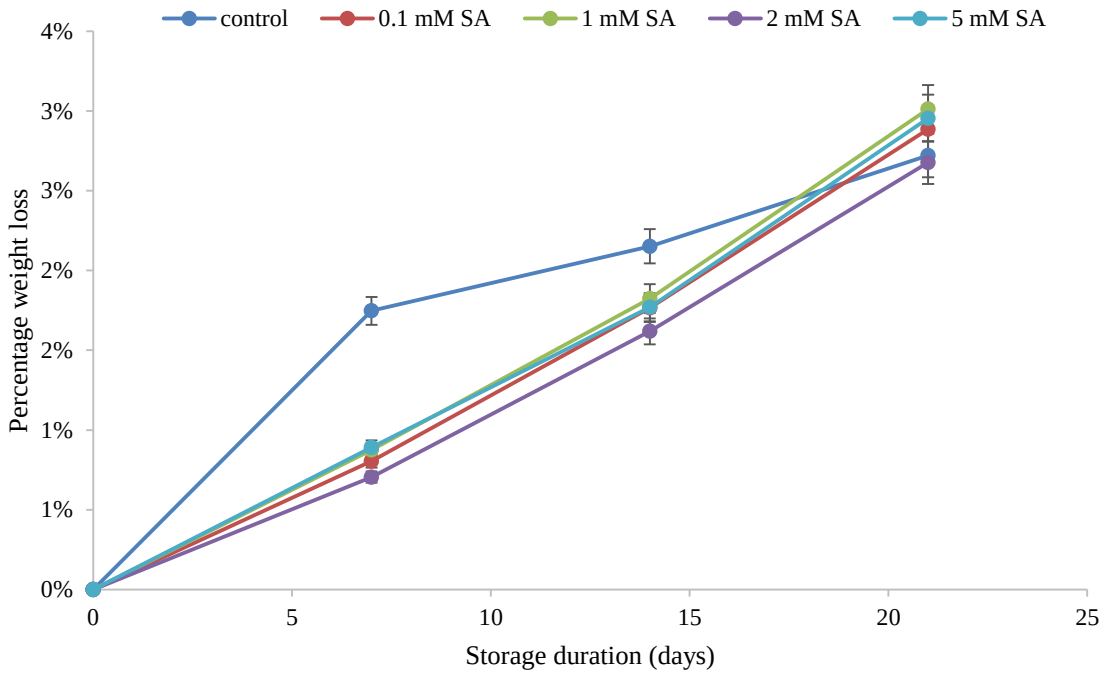


Figure 3.10 Effect of different concentrations of salicylic acid on weight loss of dragonfruit stored at 6°C for 21 days. Values are the means ± SE.

3.2.2.2 Colour

Table 3.3 Effect of different concentrations of methyl jasmonate on colour, presented as L*,C and *h*, of dragonfruit stored at 6°C for 21 days. Values are the means ± SE.

Time	Control	0.01 mM MeJA	0.1 mM MeJA	0.2 mM MeJA	0.5 mM MeJA
L* (SE = 4.02)					
0	54.45	54.45	54.45	54.45	54.45
7	51.07	52.08	47.47	51.80	51.72
14	45.02	48.20	45.97	47.65	46.53
21	57.95	58.82	53.95	55.35	55.33
Chromaticity (SE = 0.04)					
0	0.23	0.23	0.23	0.23	0.23
7	0.26	0.35	0.21	0.23	0.23
14	0.22	0.29	0.18	0.27	0.27
21	0.27	0.34	0.23	0.29	0.26
Hue (SE = 2.29)					
0	43.16	43.16	43.16	43.16	43.16
7	41.79	38.65	44.86	42.89	41.54
14	40.80	39.36	42.23	40.44	39.10
21	43.77	44.59	46.66	46.43	45.46

Changes in the colour of dragonfruit were monitored as the fruit ripened (Table 3.3). No significant difference ($p > 0.05$) for L* was observed throughout the storage period, as the lightness of the fruit was generally maintained. Similarly, no significant difference ($p > 0.05$) was observed for the hue values which were maintained throughout the storage period. Chromaticity slightly increased for the control and the

two highest MeJA treatments (0.2 and 0.5 mM MeJA). Moreover, the highest C* values were observed for 0.01 mM MeJA, which promoted colour change of the fruit. Fruits treated with 0.1 mM MeJA exhibited C* values that were significantly lower ($p < 0.05$) than the control.

Table 3.4 Effect of different concentrations of salicylic acid on colour, presented as L*,C and *h*, of dragonfruit stored at 6°C for 21 days. Values are the means ± SE.

Time	Control	0.01 mM MeJA	0.1 mM MeJA	0.2 mM MeJA	0.5 mM MeJA
L* (SE = 4.10)					
0	54.45	54.45	54.45	54.45	54.45
7	58.35	48.78	52.72	49.41	49.50
14	49.19	49.48	48.49	45.08	45.40
21	57.92	54.77	58.27	53.78	57.25
Chromaticity (SE = 0.04)					
0	0.23	0.23	0.23	0.23	0.23
7	0.31	0.33	0.37	0.34	0.31
14	0.33	0.26	0.31	0.29	0.28
21	0.25	0.25	0.27	0.30	0.32
Hue (SE = 2.85)					
0	43.16	43.16	43.16	43.16	43.16
7	40.22	38.55	40.11	41.53	41.64
14	38.19	38.57	38.51	39.53	40.68
21	47.23	44.49	44.42	44.27	48.16

There was an initial decline in the lightness of all the fruits in the SA experiment (Table 3.4). Chromaticity increased in all of the SA treated fruits as ripening progressed, and a delayed peak was observed for the control. Nonetheless, by the final day of storage the highest C* values were observed for the 2 mM and 5 mM SA treatments. Although a slight increase in hue angle was observed throughout the storage period, no significant difference ($p < 0.05$) was observed between the different treatments.

3.2.2.3 Firmness

As ripening progresses, a general increase in the firmness of the fruit was observed (Figure 3.11). During the initial storage period, no significant difference ($p > 0.05$) was observed between all treatments, including the control. By the final day of storage control and the fruit treated with 0.1 mM and 0.5 mM MeJA maintained the lowest values of firmness, while fruits treated with 0.01 mM MeJA exhibited a significant increase ($p < 0.05$) in firmness.

Firmness also increased in the SA experiment as the fruits ripened (Figure 3.12). The highest values for firmness were observed for the control during the second week of storage. This was followed by a decline in the firmness for the control, while the SA treated fruits maintained the same firmness throughout the storage period. By the final day of storage, all of the treatments were at par ($p < 0.05$).

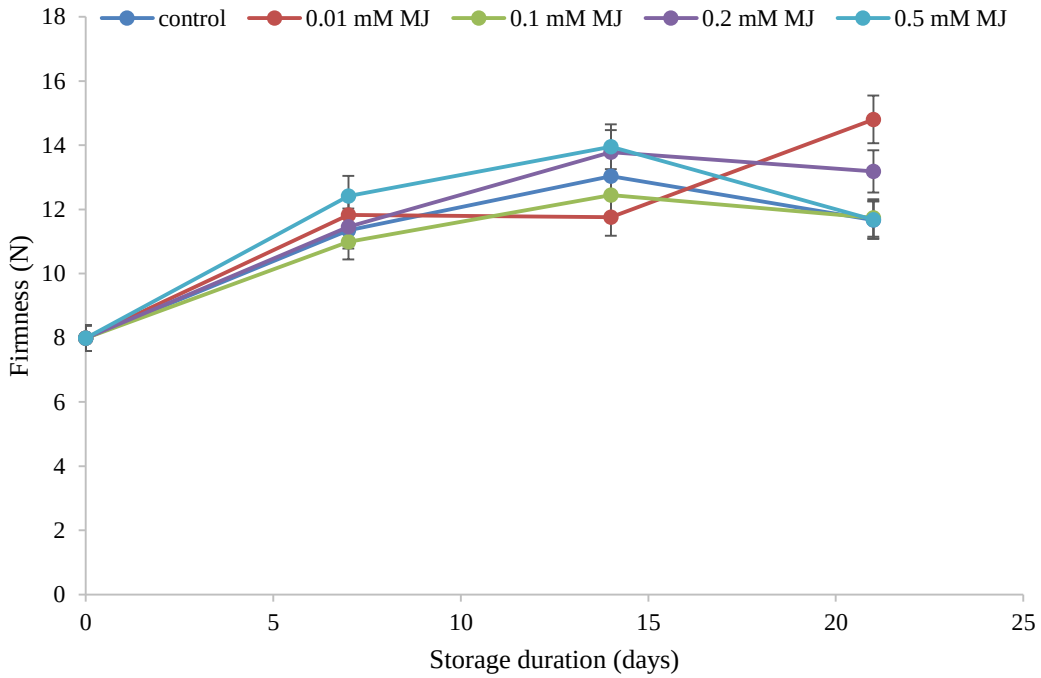


Figure 3.11 Effect of different concentrations of methyl jasmonate on firmness of dragonfruit stored at 6°C for 21 days. Values are the means ± SE.

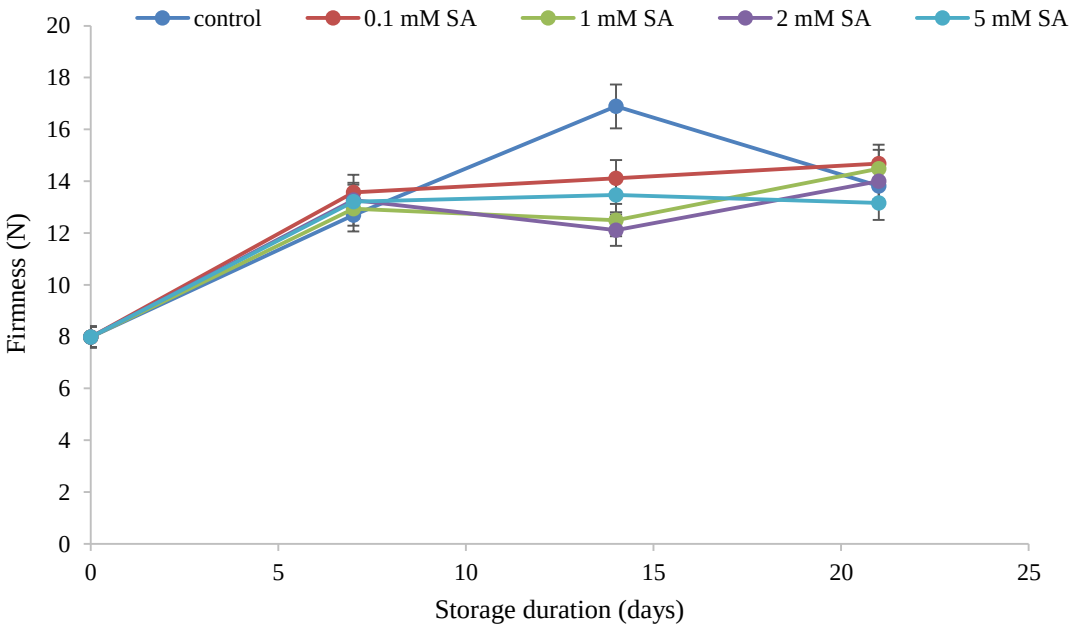


Figure 3.12 Effect of different concentrations of salicylic acid on firmness of dragonfruit stored at 6°C for 21 days. Values are the means ± SE.

3.2.2.4 Soluble Solids Content

The soluble solids content (SSC) of the dragonfruit declined significantly ($p < 0.05$) (Appendix B 3.13) during the storage period (Figure 3.13). Although by day 14 the highest MeJA treatments exhibited the lowest SSC values ($p < 0.05$), on the final day of storage this set of treatment exhibited the highest values. Changes in SSC represent the metabolic status of the fruit, as the energy reserves are mobilised and utilised to fuel metabolic activities of the plant cell. The initial levels of SSC indicate the intrinsic sugar levels of the fruits, and rate of decline in the SSC values of the fruit are an indication of the fruits' metabolic activity. In this context, it can be observed that the higher doses of MeJA were initially promoting the metabolic activity of the fruit.

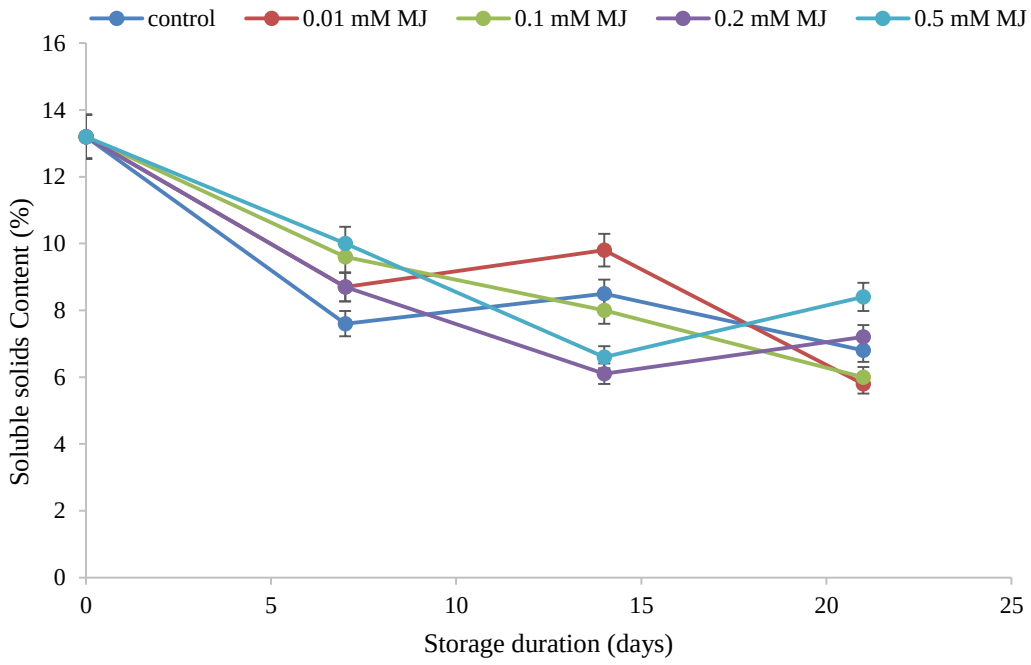


Figure 3.13 Effect of different concentrations of methyl jasmonate on soluble solids content of dragonfruit stored at 6°C for 21 days. Values are the means ± SE.

For the fruits subjected to SA, a general decline in SSC was also observed throughout the storage period (Figure 3.14), however the interaction was not significant ($p > 0.05$) (Appendix B 3.14). The higher hormonal treatments (2 mM and 5 mM SA) exhibited the same trend that was observed in the MeJA experiment. SSC declined at a significantly higher rate ($p < 0.05$) for these two treatment during the initial two weeks of storage. Nonetheless, by the final day of storage the SSC values increased for these two treatments and no significant difference ($p > 0.05$) was observed between them and the control.

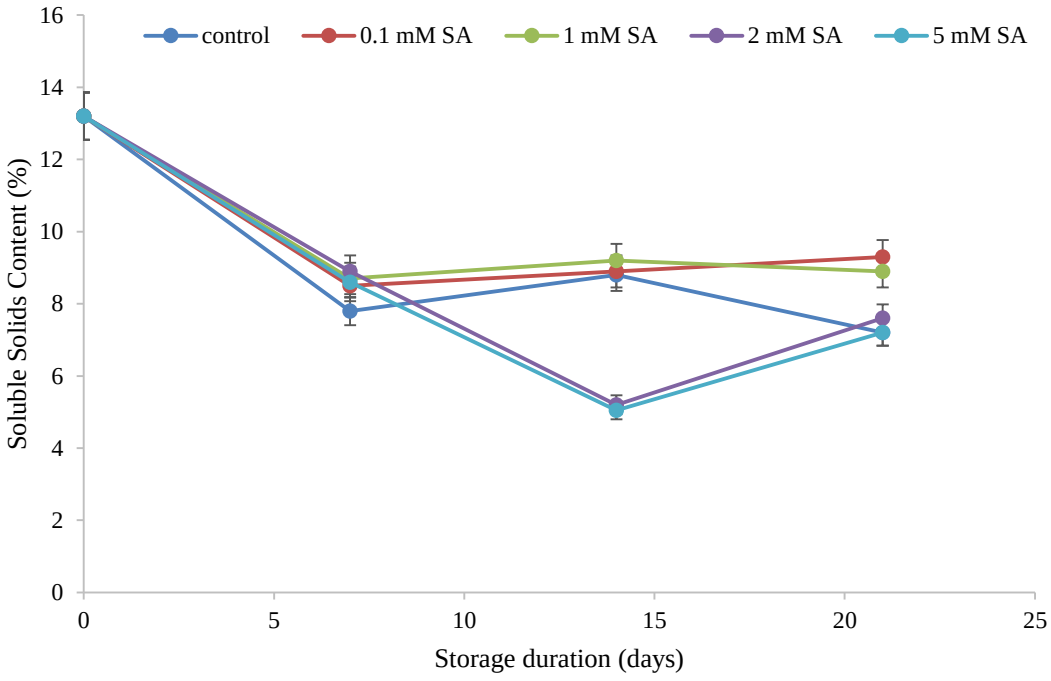


Figure 3.14 Effect of different concentrations of salicylic acid on soluble solids content of dragonfruit stored at 6°C for 21 days. Values are the means ± SE.

3.2.2.5 Titratable acidity

As ripening progressed, a slight reduction in TA was observed for the SA treated fruits (Figure 3.15). The control and 0.1 mM MeJA treatment exhibited a significantly lower ($p < 0.05$) decline in TA during the initial storage period. However, by the end of the storage period, there was no significant difference ($p > 0.05$) between the control and the higher MeJA treatments (0.1 mM, 0.2 mM and 0.5 mM MeJA). The lowest TA values were observed for the lowest MeJA treatment on the final day of storage, which was at par ($p > 0.05$) with the lowest value observed for the 0.5 mM MeJA treatment during the first week of storage.

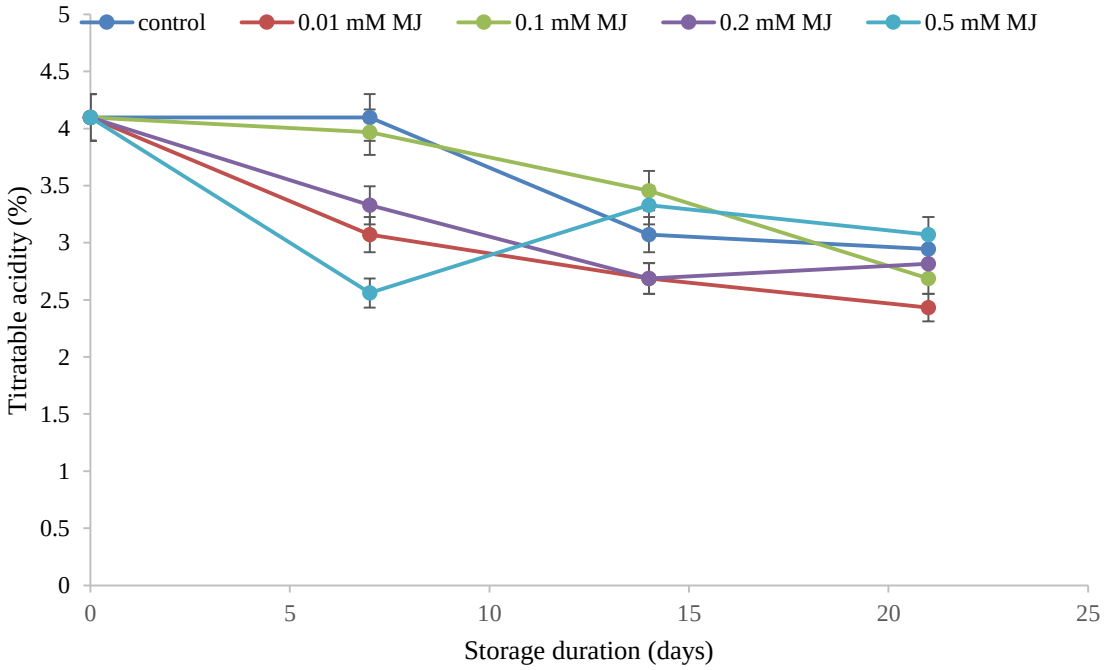


Figure 3.15 Effect of different concentrations of methyl jasmonate on titratable acidity of dragonfruit stored at 6°C for 21 days. Values are the means ± SE.

A slight decline in TA was observed during the storage period for the control and the two highest SA treatments (2 mM and 5 mM SA) (Figure 3.16). TA values for the two lowest SA treatments (0.1 mM and 1 mM SA) dropped at a faster rate, with the lowest values observed for 1 mM SA treatments. However, after the first week of storage an increase in TA values was observed for these two treatments. By the final day of storage, fruits that were treated with 1 mM SA exhibited the highest TA values, while the TA values for the two highest SA treatments were the lowest.

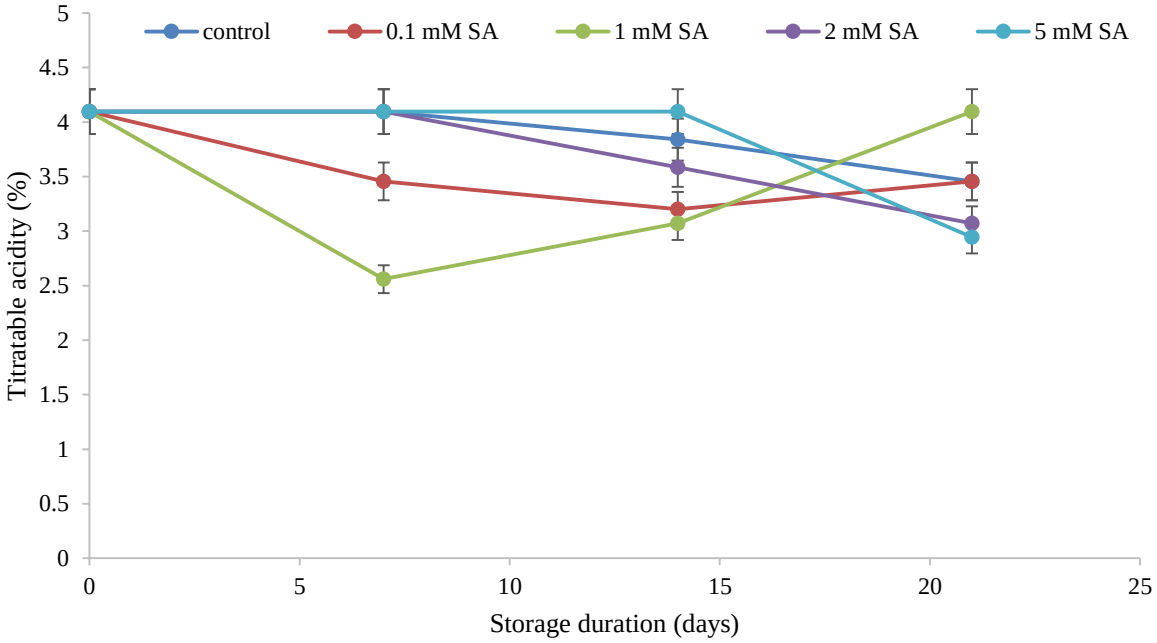


Figure 3.16 Effect of different concentrations of salicylic acid on titratable acidity of dragonfruit stored at 6°C for 21 days. Values are the means ± SE

3.2.3 Mangosteen (*Garcinia mangostana* L.)

3.2.3.1 Weight loss

Treatment level had a significant effect ($p < 0.05$) on percentage weight loss, as fruits treated with MeJA exhibited significantly lower weight loss (Figure 3.17). Nonetheless, fruits treated with 0.01 mM MeJA were at par ($p > 0.05$) with the control during the storage period. Treatment with 0.2 mM MeJA had the most significant effect on reducing the weight loss of mangosteen as the fruit ripened. Mangosteen is characterised by a short shelf-life, which is apparent in the control fruits exceeding the 3% weight loss threshold by the 12th day of storage. The lower rate of weight loss indicates that exposure of the fruit to the MeJA treatment played a role in delaying the onset of fruit senescence.

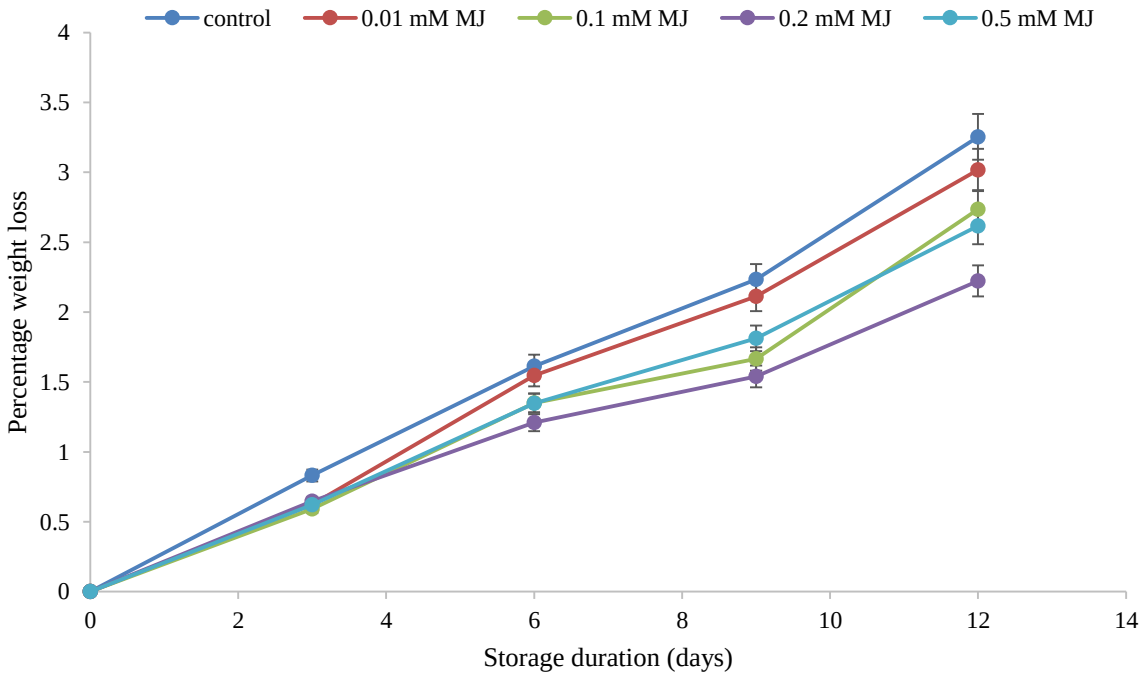
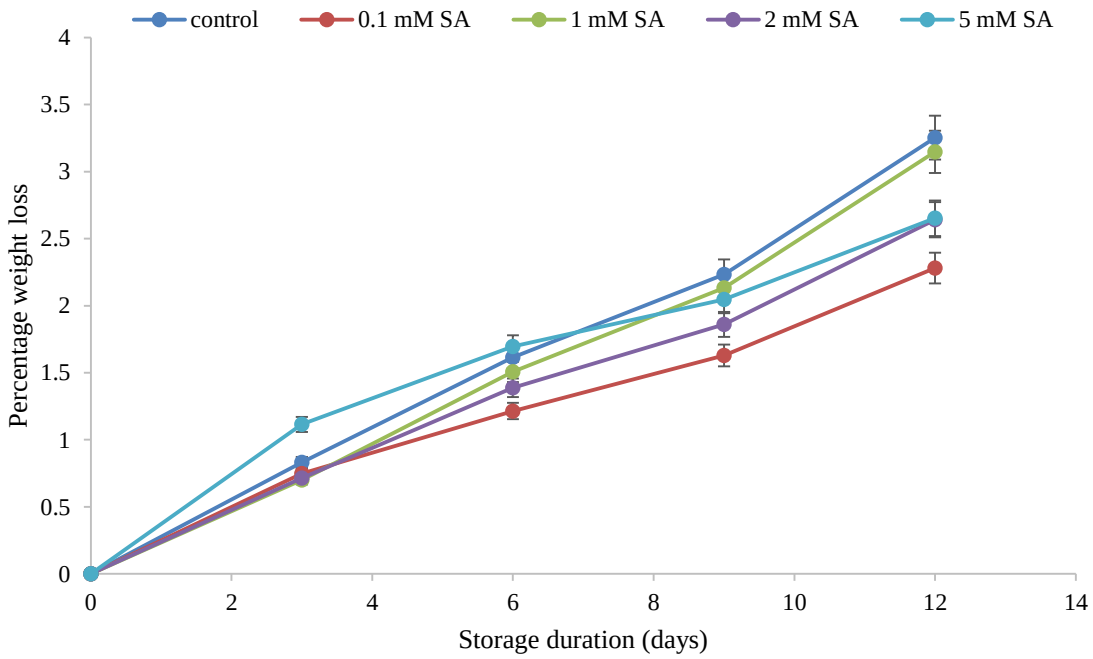


Figure 3.17 Effect of different concentrations of methyl jasmonate on weight loss of mangosteen stored at 6°C for 12 days. Values are the means ± SE

Treatment of mangosteen with SA significantly reduced ($p < 0.05$) (Appendix B 3.16) weight loss of the fruit, however the interaction between treatment and time was not significant ($p > 0.05$). The lowest rate of weight loss was observed for the fruits treated with 0.1 mM SA. This was followed by fruits treated with 2 and 5 mM SA, which were all significantly lower ($p < 0.05$) than the control. Although weight loss was initially accelerated in the fruits exposed to 5 mM SA, the rate of increase in percentage weight loss was slower towards the final days of storage. Fruits treated with 1 mM SA were at par ($p > 0.05$) with the control by the final day of storage. However, an interesting interaction is apparent between the SA concentration applied and the effect it has on metabolic activity of the fruit, as the lowest and highest doses of SA reduced weight loss while 1 mM SA did not have a significant effect on weight loss.

Figure 3.18 Effect of different concentrations of salicylic acid on weight loss of



mangosteen stored at 6°C for 12 days. Values are the means ± SE

3.2.3.2 Colour

As mangosteen ripens it acquires a darker hue, due to the accumulation of anthocyanins in the fruit peel. Assessment of the peel colour is a good indicator of the ripening stage of the fruit. Application of MeJA significantly influenced the colour of the fruit (Table 3.5). The lowest dose of MeJA (0.01 mM MeJA) significantly increased ($p < 0.05$) L*, C and *h* of the fruit, which was closely followed by 0.1 mM MeJA. Meanwhile, the application of 0.2 mM MeJA resulted in the least changes in all aspects of colour of the fruit. MeJA at this level maintained colour changes related to ripening. Changes in the hue angle of the fruit were very pronounced for the control.

Exposure of the fruits to SA also had a significant effect on the colour changes during the fruit ripening (Table 3.6). Although no changes were observed for L* throughout the storage period, there were pronounced changes observed in the chroma and hue angle of the fruit. Application of 2 mM SA significantly enhanced ($p < 0.05$) changes in the chromaticity of the fruit, as the changes were significantly higher ($p < 0.05$) than those observed for the control. The fruits that were exposed to 1 mM SA did not experience significant changes ($p > 0.05$) in chromaticity from day zero to the final day of storage. Meanwhile, most pronounced changes in the hue angle were observed for the fruits that were subjected to 0.1 mM SA. Nonetheless, by the final day of storage the lowest values for hue angle were observed for the two lowest SA treatments (0.1 and 1 mM SA) and the highest SA treatment (5 mM SA).

Table 3.5 Effect of different concentrations of methyl jasmonate on colour, presented as L*,C and *h*, of mangosteen stored at 6°C for 12 days. Values are the means ± SE.

Time	Control	0.01 mM MeJA	0.1 mM MeJA	0.2 mM MeJA	0.5 mM MeJA
L* (SE = 2.73)					
0	25.17	25.17	25.17	25.17	25.17
3	26.86	26.73	26.41	24.32	28.16
6	22.07	22.30	20.18	19.84	21.34
9	27.90	28.99	28.28	25.61	28.93
12	21.93	24.82	23.47	21.04	22.92
Chromaticity (SE = 4.01)					
0	13.10	13.10	13.10	13.10	13.10
3	34.01	38.46	40.27	26.76	45.96
6	28.28	46.99	35.81	22.78	21.86
9	32.98	70.43	47.43	32.13	42.55
12	32.18	67.43	55.82	32.03	45.49
Hue (SE = 0.01)					
0	0.22	0.22	0.22	0.22	0.22
3	0.51	0.36	0.36	0.31	0.44
6	0.55	0.39	0.32	0.41	0.25
9	0.10	0.45	0.39	0.29	0.38
12	0.40	0.51	0.50	0.30	0.44

Table 3.6 Effect of different concentrations of salicylic acid on colour, presented as L*,C and *h*, of mangosteen stored at 6°C for 12 days. Values are the means ± SE.

Time	Control	0.01 mM MeJA	0.1 mM MeJA	0.2 mM MeJA	0.5 mM MeJA
L* (SE = 2.02)					
0	25.17	25.17	25.17	25.17	25.17
3	22.73	22.51	22.66	22.97	22.04
6	21.94	23.05	23.84	24.06	22.48
9	27.18	27.61	27.23	25.90	25.99
12	21.65	20.36	21.73	24.27	21.05
Chromaticity (SE = 4.15)					
0	13.10	13.10	13.10	13.10	13.10
3	48.05	34.86	19.00	57.66	26.54
6	46.82	45.31	25.03	57.39	34.00
9	63.22	42.46	20.97	66.09	46.10
12	47.31	41.89	24.25	55.76	41.49
Hue (SE = 0.01)					
0	0.22	0.22	0.22	0.22	0.22
3	0.34	0.65	0.34	0.28	0.22
6	0.37	0.12	0.45	0.39	0.28
9	0.52	0.37	0.34	0.28	0.25
12	0.43	0.34	0.37	0.54	0.33

3.3.3.3 Firmness

As mangosteen ripens, the firmness of the fruit increases due to increased lignification of the fruit, however the increase observed was not significant ($p > 0.05$) (Appendix B 3.17). Increased lignification of the fruit was witnessed for fruits subjected to the higher doses of MeJA (Figure 3.19). The application of 0.5 mM MeJA resulted in an initial rapid increase in firmness that was significant ($p < 0.05$) on the 6th day of storage. Meanwhile, an increase in the firmness of fruits that were exposed to 0.1 mM MeJA was only observed on the 9th day of storage. The lowest dose of MeJA (0.01 mM) maintained the firmness of the fruits and delayed the increase in firmness throughout the storage period.

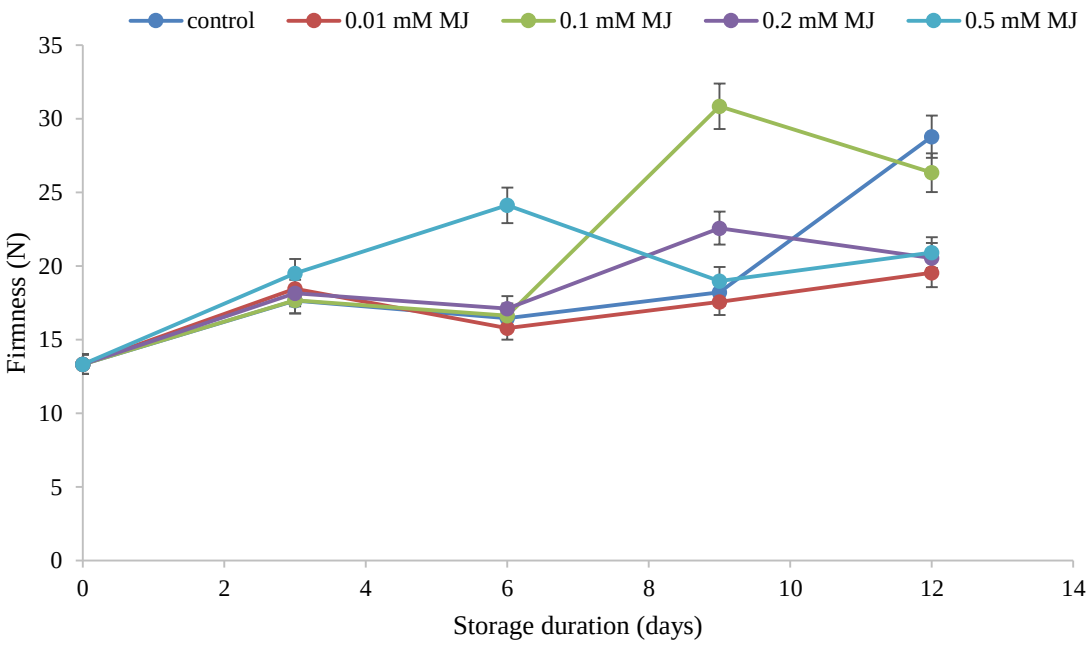


Figure 3.19 Effect of different concentrations of methyl jasmonate on firmness of mangosteen stored at 6°C for 12 days. Values are the means ± SE

Although the higher doses of SA resulted in an initial increase in firmness of the fruit, the highest firmness was observed for the control on the final day of storage (Figure 3.20). A similar trend to the lowest dose of MeJA was observed for the lowest dose of SA, as the firmness of the fruits was maintained throughout most of the storage period. Pericarp hardening is an undesirable change in the fruit that is associated with over-ripened fruits (Dangcham et al. 2008). It indicates the end of the shelf-life of the fruit, thus lower doses of plant stress hormones can extend the shelf life of the fruits by delaying the increase in lignification of the fruit.

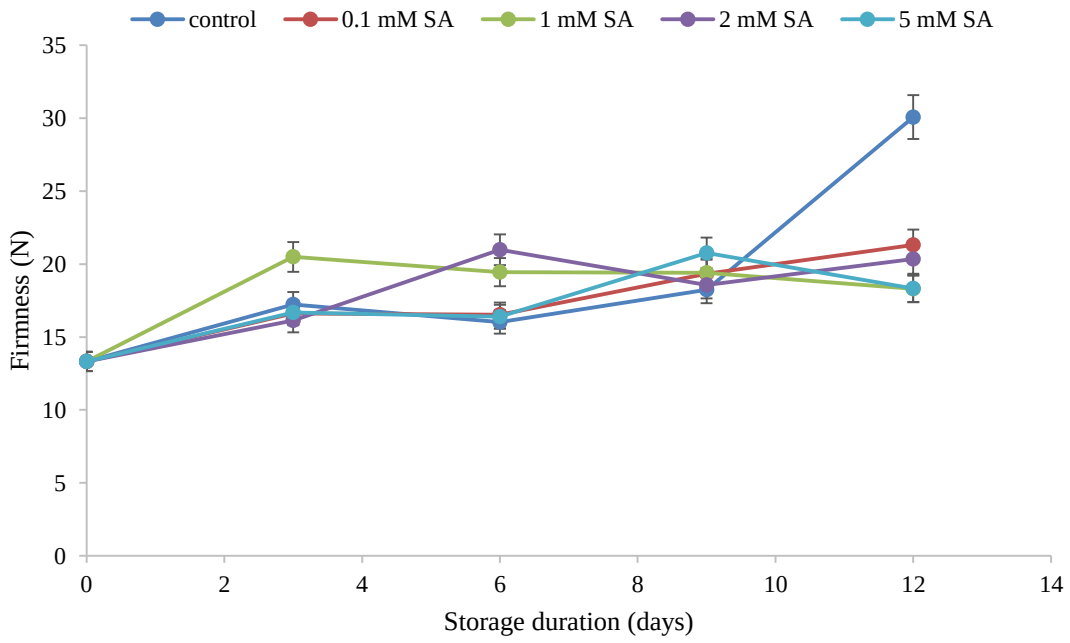


Figure 3.20 Effect of different concentrations of salicylic acid on firmness of mangosteen stored at 6°C for 12 days. Values are the means ± SE

3.3.3.4 Soluble solids content

As mangsoteen ripened, a significant ($p < 0.05$) (Appendix B 3.21) decline in SSC was observed (Figure 3.21). The application of MeJA resulted in a more rapid decline in the SSC of the fruit, which was more prominent in the fruits subjected to 0.01 mM MeJA. Nonetheless, by the final day of storage a sudden increase in SSC was observed for all of the MeJA treated fruits, and the control fruits had the lowest value for SSC, which was significantly lower ($p < 0.05$) than the fruits that were exposed to 0.01 and 0.5 mM MeJA. SSC content is a good indicator of the metabolic activity of the fruit, as it represents the changes in assimilates of the fruit. However, changes in the weight loss of the fruit indicated that metabolic activity of the fruit was reduced by hormonal treatment.

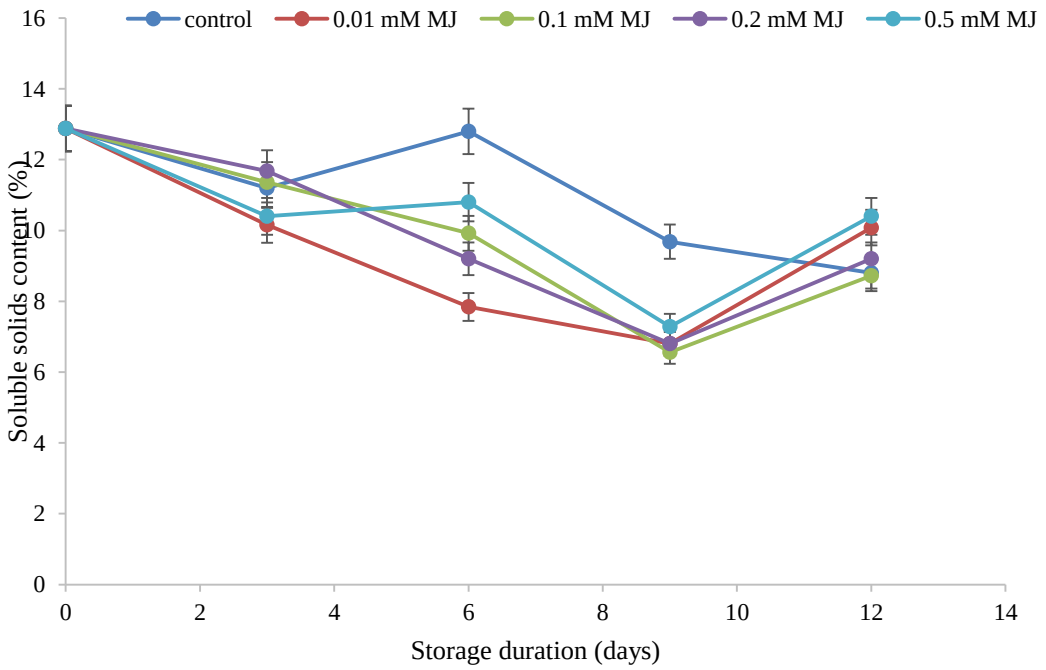


Figure 3.21 Effect of different concentrations of methyl jasmonate on soluble solids content of mangosteen stored at 6°C for 12 days. Values are the means ± SE

A general decline in the SSC of the fruit was witnessed, although the interaction observed was not significant ($p > 0.05$) (Appendix B 3.22). The decline in SSC was gradual for the SA treated fruits, while the control fluctuated with a drastic decline observed on the final day of storage (Figure 3.22). By the final day of storage all of the treatments, including the control, were at par ($p > 0.05$).

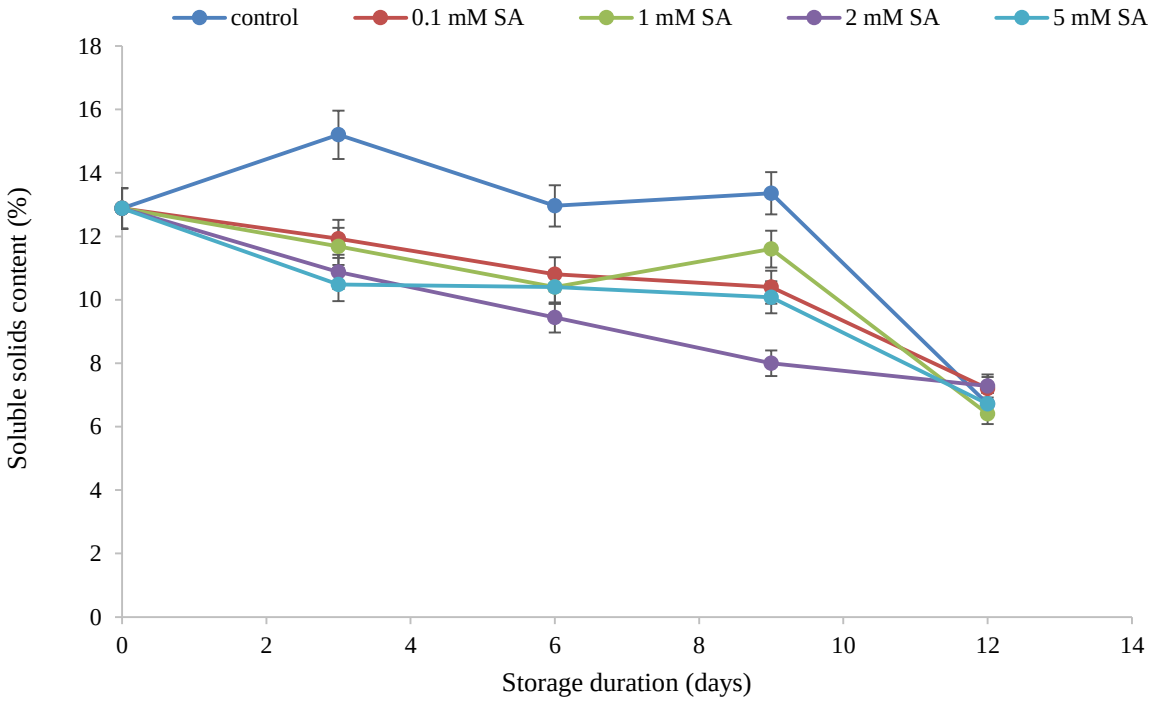


Figure 3.22 Effect of different concentrations of salicylic acid on soluble solids content of mangosteen stored at 6°C for 12 days. Values are the means ± SE

3.3.3.5 Titratable acidity

The TA of mangosteen declined significantly ($p < 0.05$) (Appendix B 3.19) during the storage period (Figure 3.23). Similar to SSC, the MeJA treatment initially enhanced this decline in TA of the fruit, as the control was significantly higher ($p < 0.05$) than all of the MeJA treated fruits until the ninth day of storage. A rapid decline in TA of the control was observed leading to the ninth day of storage, and on the final day of storage the control fruits exhibited the lowest TA values, which was at par with fruits treated with 0.01 and 0.2 mM MeJA ($p > 0.05$).

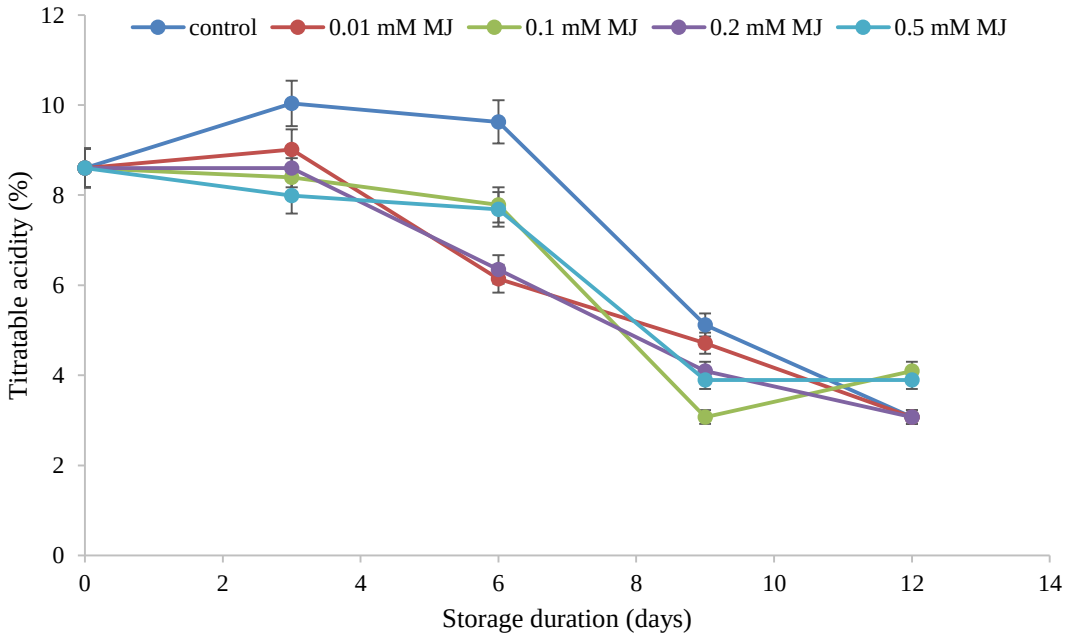


Figure 3.23 Effect of different concentrations of methyl jasmonate (MeJA) on titratable acidity (TA) of mangosteen stored at 6°C for 12 days. Values are the means ± SE

The decline in TA for SA treated fruits was also similar to the trend observed for the MeJA treated fruits (Figure 3.24; Appendix B 3.20). As the TA values for the control fluctuated throughout the storage period, a more linear decline was observed for the SA treated fruits. Moreover, by the final day of storage, all of the treatments with the exception of 2 mM SA were at par ($p > 0.05$).

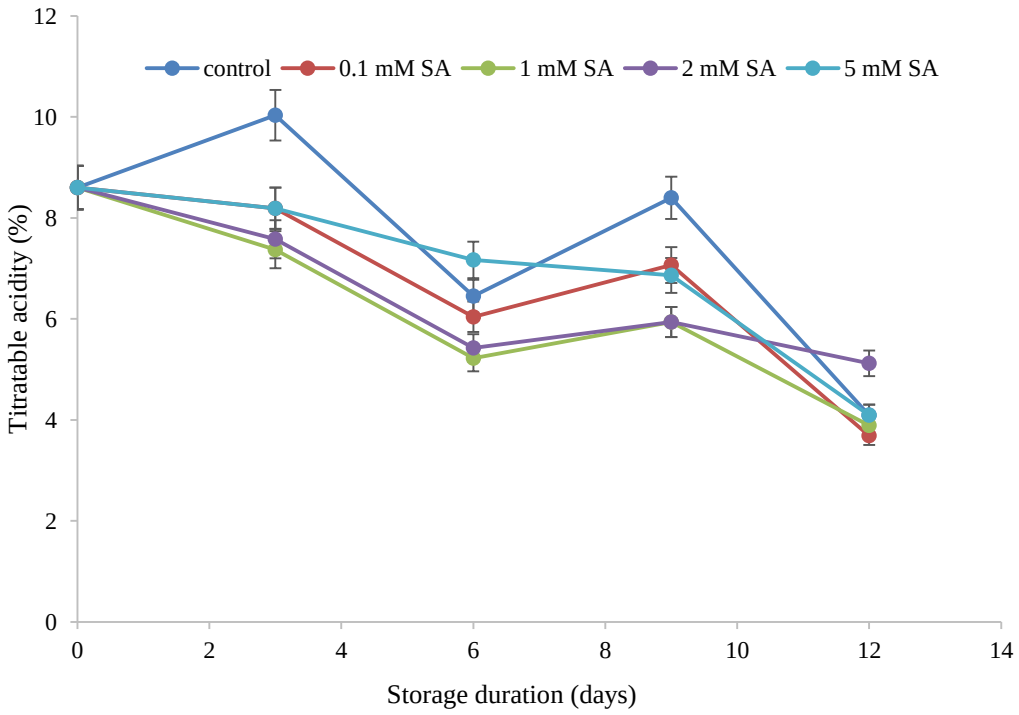


Figure 3.24 Effect of different concentrations of salicylic acid (SA) on titratable acidity (TA) of mangosteen stored at 6°C for 12 days. Values are the means ± SE

3.4 Discussion

3.4.1 Ripening profile of tropical fruits

In spite of the increasing interest in nutritional composition of fruits, consumers readily judge fruits by the appearance, texture and flavour. These parameters experience progressive changes, which are often measurable, during ripening. The quality parameters are controlled by intrinsic and extrinsic factors, such as storage temperature and hormonal levels. An estimation of the shelf life of fruits can be achieved by measuring the on-going changes in the quality of the fruit, and the effect of the environmental and biological factors on ripening.

Ripening of fruits is a complex process that involves several interlinked changes in the quality of the fruits. Breakdown of starch reserves into simple hexoses occurs as ripening progresses, which could increase the soluble solids content of the fruit. On the other hand, the hydrolysis of insoluble polysaccharides into sugar could result in a decline in the SSC of fruits (Vyas et al. 2015). Thus, a rapid decline in SSC could be an indication of higher respiratory activity. Organic acids are also energy reserves that are utilised during respiration and broken down, resulting in lower titratable acidity of the fruit (Valero & Serrano 2010).

The pectin and hemicellulose polymers are also degraded, which weakens the cell wall and loosens the cells' attachments to each other during ripening. Protopectin is an insoluble parent of pectins, which is generally large and crosslinked with other polymers, such as sugars and phosphates (Wills et al 2007). It is also broken down during ripening into molecules of lower molecular weight and weaker cross-linkages. This is a natural process resulting in softer, sweeter and more desirable fruits. However,

the prolonged degradation of these polymers may result in the eventual disintegration of the fruit material (Wills et al. 2007).

Fruits ripen quickly, and although cold storage can delay the onset of ripening it is not always a promising tool to preserve fruit quality (Vyas et al. 2015). Various cellular processes have different sensitivities to extremely low storage temperatures, which might induce chilling injury to occur. This is attributed to loss of cellular compartmentalisation and imbalanced metabolism of the plant cells. Thus, it will impact ripening related changes which will differ based on the specific ripening characteristics of the commodity in question (Wills et al. 2007).

3.4.1.1 Carambola

Carambola is susceptible to chilling injury at temperatures below 10 °C. Chilling injury was found to be inversely related to the maturity of the fruit at the time of harvest with more mature fruits being less susceptible to chilling injury (Ali et al. 2004). Prolonged exposure to cold storage exacerbates the chilling injury symptoms, as Ali et al. (2004) reported pronounced symptoms after transferring carambola from 10 °C to 28 °C after 10 or 14 days. In comparison, fruits kept in cold storage for 7 days were found to ripen normally. Thus storage of the fruits for 16 days was sufficient to exhibit chilling injury symptoms.

Chilling injury in carambola is associated with membrane dysfunction and disrupted commercialisation within the cells, resulting in increased water permeability (Ali et al., 2004). This would increase water loss from the plant tissue. The water content of carambola is 91% (data not shown), so a 3% loss in water content would account for significant reduction in the quality of the produce. An increase in the activity of ß-galactosidase and pectinesterase are also associated with chilling injury

(Ali et al. 2004). Thus, a decline in firmness of the fruit was expected under cold storage. Nonetheless, Perez-Tello et al. (2001) reported negligible changes in the membrane integrity of carambola and determined it not to be a suitable indicator for assessing tissue damage during chilling injury. That is in agreement to the results observed here, as textural changes were not a clear indicator of ripening related changes carambola during cold storage.

Degreening involves the breakdown of chlorophyll, which is attributed to three main factors: pH changes due to leakage of organic acids from the cell vacuoles, the action of chlorophyllases and the oxidative system. Carotenoids exist concomitantly with chlorophylls, but are usually masked in the presence of the chlorophylls (Wills et al. 2007). Upon the degradation of chlorophylls, the colour of the carotenoids are unmasked accounting for the colour change in fruits such as carambola. Carotenoids are generally made up of 40 carbon atoms with one or more oxygen atoms bonded, and are usually stable even during senescence.

The maturity stage at which the fruit was subjected to cold storage was a major factor influencing the effect of the induced postharvest stresses. There were notable differences observed between the two sets of experiment where one set involved storing the fruits on the first day of harvest, while the other set was stored after 20 hours of storage at room temperature.

Previous studies have also demonstrated a similar effect between MeJA and SA on weight loss (Al-Qurashi & Awad 2012). Carambola in the SA experiment were washed and transferred to cold storage immediately after the treatment, while in the MeJA experiment the fruits were kept at room temperature for 20 hours before transferring to cold storage. Thus, the fruits were transferred to cold storage at different

maturity states for the SA and MeJA experiment, which would demonstrate different responses to cold storage.

SSC is a measure of sweetness and an important attribute of fruit flavour. It is largely made up of soluble carbohydrates, of which sugar is the main component. The sugar content of carambola generally increases as the fruit ripens, and starts to drop as senescence commences (Zainudin et al. 2014). Although fructose is reportedly the principal sugar in carambola, the most significant changes in sugar content were observed for the sucrose content of carambola. Invertase activity is high in immature fruits, hydrolysing sucrose to glucose and fructose. This reduces the sucrose levels in the fruits while balancing the osmotic pressure within the plant cells. However, as the fruit matures the invertase activity declines and the sucrose levels increase once more (Villanueva 2004). Coupled with the conversion of starch into simple sugars during ripening, a massive increase in sucrose levels is observed as the fruit ripens. Thus, since the fruits used in the MeJA experiment were at an advanced maturity stage compared to the SA experiment, it was expected that the SSC values would be initially higher for the MeJA experiment.

As fruits ripen, organic acids are used as energy reserves, resulting in a decline in acidity which is related to enhanced sweetness making the fruit more desirable. Thus it can be observed that the fruits in MeJA experiment had reached this stage before the fruits in the SA experiment. Moreover, both hormonal treatments played a role in reducing the metabolic activity of the fruit, which was more pronounced at higher doses of the hormone.

3.4.1.2 Dragonfruit

Essential physiological properties of the fruit that are used to judge the quality and marketability of the fruit include weight, SSC and TA. Although dragonfruit is characterised with a short shelf life due to the high respiration activity of the fruit (Ali et al. 2013) its shelf life was the longest compared to mangosteen and carambola. The chemical composition of the fruit changes rapidly, in terms of weight loss and decline in SSC and TA. Exposure of the fruit to the hormonal treatments did not inhibit or delay the ripening-related changes.

Dragonfruit is characterised with a thick peel that could have inhibited the absorption of the exogenous hormonal application into the fruit system. Moreover, fruits such as dragon fruit are commercially harvested at higher ripening stages to ensure better quality for consumers (Wang et al. 2010). This is in contrast to most researches which have been focused on immature fruit that are characterised with low ethylene production and incomplete quality attributes. Thus, the dragonfruit in this experiment exhibited decline in SSC as the storage period advanced, indicating progression of ripening in a mature fruit of desirable quality attributes.

An odd observation in the experiment was the slight increase in firmness observed for all of the treatments during the storage period. It is reported that the fruit softens along with increases in the levels of the water soluble pectin, which would increase the SSC values of the fruit (Wanitchang et al. 2010). However, as ripening progressed, SSC of the fruit steadily declined while firmness increased. This could indicate a response of the fruit to the cold storage involving increased accumulation of lignin as a defence strategy. The following chapter (Chapter 4) will explore changes in the total phenolic content of the fruit, as well as the characteristic betacyanin content, of dragonfruit.

3.4.1.3 Mangosteen

Mangosteen is usually marketed at late mature stage, when it attains a dark purple hue (Piriyavinit et al. 2011). When the fruit reaches the end of storage period, it is characterised by pericarp hardening and calyx wilting. Pericarp hardening is experienced by the fruit upon exposure to stresses in addition to prolonged storage (Kamdee et al. 2014). This is a unique stress response that adversely affects the marketability of the produce and severely shortens the shelf life of the produce. Pericarp hardening occurs due to the increased production of lignin, which is also related to depletion of the phenolic content of the fruit (Kamdee et al. 2014). Chilling injury is manifested in mangosteen through pericarp hardening, which becomes more apparent upon transfer to ambient storage.

Moreover, maturity is another factor that affects the responses of the fruit to stresses induced by cold storage. More mature fruits are reportedly more resistant to chilling injury than immature fruits (Dangcham et al. 2008). Reduced activity of enzymes that are involved in the synthesis of lignin, such as peroxidase and laccase, can lower the rate of pericarp hardening. Moreover, synthesis of lignin depends on the presence of oxygen and hydrogen peroxide (Kamdee et al. 2014). Thereby, conditions that reduce the presence of oxygen or hydrogen peroxide, such as SA regulation of hydrogen peroxide metabolism, can reduce the rate of pericarp hardening.

3.4.2 Influence of methyl jasmonate on fruit ripening

Methyl jasmonate has been demonstrated to reduce the incidence of chilling injury on various commodities including mango (González-Aguilar et al. 2000), tomato (Ding et al. 2001), guava (González-Aguilar et al. 2004) and loquat (Sayyari et al. 2011; Cao et al. 2010). It also promotes ethylene activity in various commodities, such as strawberry

(Mukkun & Singh 2009), pear (Kondo et al. 2007) and tomato (Yu et al. 2011). The exposure of cold stored carambola to MeJA exhibited various effects on the fruit ripening; including delayed and lower ripening peaks which was dependent on the concentration of MeJA applied. The lowest percentage weight loss was observed for fruits that were exposed to the lowest and highest MeJA dose, 0.01 and 0.5 mM respectively.

The mode of action of MeJA involves modification of the redox status of the fruit. It induces the accumulation of hydrogen peroxide and ascorbic acid in the plant tissue (Cao et al. 2010). Increase in hydrogen peroxide levels within a tissue are a stress indicator that stimulates stomatal closure through the stimulation of increase Ca^{+} content of the guard cells (Zhang et al. 2001). Thus, water loss from the fruit will be reduced, which would further minimise weight loss (Jubany-Marí et al. 2010). Nonetheless, cells have a rapid response to changes in the intracellular redox state through the accumulation of ascorbic acid (Zhang et al., 2001). Ascorbic acid is involved in regulating stomatal opening through hydrogen peroxide scavenging. This stress induced stomatal closure is reversed by the presence of ascorbic acid at concentrations as low as 0.1 mM, inducing slight stomatal opening (Zhang et al. 2001). Meanwhile, the results observed for the highest MeJA dose show that ripening was generally delayed, as observed in other quality parameters. Thus, MeJA at a concentration of 0.01 mM could be sufficient to produce this effect and reduce weight loss.

MeJA is an important regulator of cell-based processes such as senescence and stress alleviation. Application of MeJA on climacteric fruit such as apple enhances ethylene biosynthesis and ripening-associated changes such as colour change and anthocyanin accumulation. Degreening along with transient enhanced anthocyanin

accumulation is also observed in non-climacteric fruits such as strawberry. Moreover in other non-climacteric fruits such as raspberry soluble solid content and anthocyanin content increased in response to MeJA (Concha et al. 2013). MeJA has been well-demonstrated to induce ripening. It is a stimulant of ethylene biosynthesis in climacteric fruits such as tomato and apple (Concha et al. 2013). It induces the expression of ethylene biosynthesis genes, 1-aminocyclopropane-1- carboxylate synthase (ACS) and 1-aminocyclopropane-1-carboxylate oxidase (ACO). In non-climacteric fruits MeJA enhances depolymerisation of hemicelluloses, resulting in loss of firmness.

Organic acid content, specifically malic, oxalic and tartaric acid, decline during the ripening of carambola (O'Hare 1993). Changes in TA and SSC represent changes in the metabolism rate of the fruit. As the concentration of MeJA was increased, both TA and SSC declined significantly for carambola, which demonstrates that at the higher levels of induced stress the metabolism rate was suppressed. The highest dosage of MeJA (0.5 mM) showed lowest levels of TA and SSC. This attests to an inhibitory effect of exogenous MeJA on fruit ripening, when applied at high levels.

Supplementation of MeJA with sucrose was demonstrated to enhance the expression of certain genes associated with the phenylpropanoid pathway such as CHS and UFGT (Belhadj et al., 2008). The promotion of ripening and defence mechanisms upon exposure to MeJA involves the up-regulation of ethylene and MeJA related genes. MeJA treatment was found to increase the expression of these three genes and, consequently, the concentration of JA, indicating positive feedback of JAs on their own biosynthesis pathway during fruit ripening (Ziosi et al. 2008).

3.4.3 Influence of salicylic acid on fruit ripening

Salicylic acid and its derivatives are bioactive compounds with nutraceutical properties and have received the generally recognised as safe (GRAS) status by FDA (Sayyari et al. 2011; Sayyari et al. 2009). It reduces the incidence of chilling injury and general quality deterioration, as has been demonstrated on tomato (Ding et al. 2001), loquat (Cai et al. 2006), peach (Cao, Hu, et al. 2010), plum (Luo et al. 2011) and pineapple (Lu et al. 2011) pomegranate (Sayyari et al. 2009). This study assessed the effect of SA application on the quality parameters of cold stored tropical fruits.

Exposure of fruits to non-toxic concentration, and even at high concentrations, has been reported to lower the metabolism rate of fruit material (Luo et al. 2011; Zhang et al. 2003). Delayed ripening was associated with maintained texture of the fruit under study. Zhang et al. (2003) reported a decline in endogenous SA levels during kiwifruit ripening, which were accompanied with rapid softening. However, exogenous application of a derivative of salicylic acid increased the intrinsic levels of ethylene, and delayed ripening and softening of the fruit. This was also in agreement with Srivastava and Dwivedi (2000) who reported a delay in softening of banana upon exposure to salicylic acid.

The effect of SA on reducing metabolism rate has also been associated with a postponed respiration climacteric (Luo et al. 2011). SA treatment reduced ethylene production in fruits in a dose-dependent manner in banana (Srivastava & Dwivedi 2000) and strawberry (Babalar et al. 2007) and kiwi (Zhang et al. 2003). Application of SA decreases the activity of the ethylene synthesis enzymes 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase and ACC synthase (Vyas et al. 2015; Zhang et al., 2003). Zhang et al. (2003) established a link between the endogenous SA levels and softening, reporting increased softening rates are related to lower SA levels. Tareen et

al (2012) also reported a decrease in ethylene levels associated with general maintenance of fruit quality during storage.

Respiration rate and ethylene production are closely related, as an increase in one factor is accompanied by an increase in the other (Babalar et al., 2007). SA was reported to lower respiration rate through the inhibition of ethylene production, resulting in firmness retention (Tareen et al. 2012). Moreover, lowered metabolic rates are also associated with decreased weight loss and sugar depletion, ultimately delaying fruit senescence (Babalar et al., 2007). Through the regulation of ethylene production and respiration of the fruit, SA has the capacity to regulate the ripening-related changes of the fruit, which influence the quality and shelf life of the fruit. Nonetheless, Lu et al (2011) and Sayyari et al (2009) reported that SA did not influence the SSC and TA of the fruits used in the experiment. This indicates variation in the responses of fruits to SA treatment, depending on the biology of the fruit under study.

Although a dose-dependent inhibition of metabolism rate has been reported in response to SA treatment, different concentration dependent responses have been reported as well. Babalar et al. (2007) reported that SA activity was enhanced by increasing the concentration from 1 to 2 mM, however adverse effects were observed on the fruit at 4 mM SA.

Moreover, another mode of action of SA involves the induction of the intrinsic defence mechanisms of the plant. SA enhances the defence mechanisms of plants and stimulates antioxidant activity, reducing the incidence of postharvest fungal diseases (Sayyari et al. 2009; Xu & Tian, 2008; Chan & Tian, 2006). This defence mechanism can also play a big role in maintaining the quality parameters of the tropical fruits.

During cold storage, SA application offers chilling tolerance through the reduced accumulation of hydrogen peroxide (Tareen et al. 2012). The regulation of

hydrogen peroxide metabolism contributes to the closure of stomata, as was reported in mandarin, reducing weight loss (Tareen et al. 2012; Zheng & Zhang, 2004). Moreover, the reduction in weight loss also contributes to texture retention of the fruit and maintenance of overall quality, as higher values of SSC and organic acids were observed.

3.5 Concluding remarks

There is a clear intrerplay between the stresses that the fruits experienced from the cold storage and the stress hormones. Physicochemical changes are genetically programmed and render the fruit more palatable. These are the changes that are most noticeable to the consumers and can be qualitatively assessed by them. Metabolic activity of the fruit was repressed by the application of the SA hormone. Meanwhile, MeJA stimulates the production of ethylene resulting in the induction of respiration and ripening related responses. However, at higher doses of MeJA an inhibitory effect on ripening was observed. More elaborate understanding of the changes that fruits experienced can be observed by analysis of the antioxidant changes in the fruits.

An understanding of the impact that postharvest stresses that fruits experience exert on their antioxidant capacity will give a deeper understanding of the forces that are undergoing within the fruits. Complex pathways exist which interlink changes in the physicochemical properties of the fruit with the bioactive content. A key example is the interplay between ascorbic acid and weight loss that was discussed earlier. While a clear reduction in metabolic activity was observed for carambola, this response was not observed for dragonfruit and mangosteen. The hormonal treatments did however delay the incidence of pericarp hardening in mangosteen. Thus, a further study into key bioactive content of carambola, dragonfruit and mangosteen was carried out.

CHAPTER 4: ANTIOXIDANT ACTIVITY OF FRUITS UNDER POSTHARVEST INDUCED STRESS

4.1 Introduction

Autoxidation mechanisms and chain breaking antioxidant activities have been an ongoing topic of research in biological sciences since the early 1940s. Oxidative stress is a state of long-term redox reactions where oxidation is more prevalent than reduction, ultimately causing oxidative damage to the cellular components (Demidchik 2014). Alternatively, it can be defined as a stress factor that is damaging to cells and triggers signalling responses (Demidchik 2014). Oxidative stress is associated with the accumulation of reactive oxygen species (ROS), a class of highly active pro-oxidants found in biological systems. This class includes radical oxygen such as superoxide anion and hydroxyl radical as well as non-radical forms such as hydrogen peroxide and other hydroperoxides (Brigelius-Flohé 2009). The enhanced accumulation of ROS is a typical condition resulting from environmental challenges (Noctor et al. 2014).

ROS are essential in plant systems as they are involved in signalling of various developmental processes, although at relatively lower levels than those associated with stress (Noctor et al. 2014). A multitude of factors can result in excessive ROS levels, including microbial or fungal infection, severe cold or heat and wounding or infection (Noctor et al. 2014). Antioxidants offer cells and organisms protection against oxidative damage by repressing or inhibiting oxidation through the direct or indirect termination of oxidation chain reactions (Zengin et al. 2010). However, only compounds that can be oxidised to a stable product can be considered as effective antioxidants. Several of these antioxidants are oxidised to an irreversible form, thus require continuous re-synthesis to allow effective control of the oxidative stress (Noctor et al. 2014).

Antioxidative systems for the control of oxidative stress operate through two main pathways (Noctor et al. 2014). The first pathway involves enzymes catalysing reactions between primary ROS that ultimately convert ROS into stable species. Meanwhile, in the second pathway ROS are reduced at the expense of metabolites, which are sacrificially oxidised into stable compounds. An example of the second pathway is the ascorbate-glutathione system. This is a reversible reaction as ascorbic acids are regenerated through an enzyme-dependent manner. Enzymes such as ascorbate peroxidase are powerful enzymes and are directly involved in hydrogen peroxide metabolism (Vanderauwera et al. 2011; Rizhsky et al. 2002).

Various metabolites found in plant cells can react with ROS and decrease their levels. They play a key role in delivering nutritional properties to fruits and vegetables. Fruit nutraceutical properties are a strong factor for attracting consumers and increasing the marketability of fruits. The increasing popularity of fruits is attributed to their nutraceutical properties and ability to prevent numerous degenerative diseases (Rautiainen et al., 2012; Marnewick et al., 2011; Park et al., 2003). Consequently, the antioxidant properties of fruits have received significant interest and a multitude of methods have been developed for quantification of antioxidant activity.

Antioxidants function through direct chain breaking activities or secondary activities involving reduction of chain initiation reactions. Their activity is attributed to three main activities: radical scavenging, metal ion binding and peroxide decomposition (Moure et al. 2001). Quantification of antioxidant activity in *in vitro* assays involves measurement of the antioxidant capacity to inhibit activities of non-biological radicals generated within the system itself (Gutteridge & Halliwell 2010). *In vivo* assays of antioxidant capacity are frequently under question with regards to the reliability of the system to predict accurately the antioxidant capacity within the system without prior

understanding of the bioavailability, absorption, metabolism and pharmacokinetics of the antioxidant.

Assays for estimation of antioxidant activity have been developed, that follow different mechanisms. The Folin-Ciocalteu assay is based on the mechanism of sequential proton loss / electron transfer (SPLET) which provides an estimation of the antioxidant activity of compounds that follow this mechanism, ideally phenolic compounds (Klein & Lukes 2006). The second mechanism assesses the hydrogen donating capacity of the antioxidant, such as DPPH assay. In the DPPH assay, the purple DPPH* compound is reduced to the yellow diphenyl pricryhydrazine by the donation of hydrogen from the antioxidant (Molyneux 2004). Meanwhile, the third mechanism assesses the electron donation property of the antioxidant compound (Rojano et al. 2008). An example for this mechanism is the FRAP assay which involves assessing the capacity of antioxidants to donate electrons for the reduction of the Fe^{3+} TPTZ complex into the blue Fe^{2+} TPTZ complex (Moon & Shibamoto 2009).

Phenolic compounds are responsible for a large share of the antioxidant activity of fruits and vegetables. They are characterised with several hydroxyl rings which allow it to donate hydrogens to ROS for radical quenching (Rice-Evans et al. 1997). Thus, the availability of an electron-donating structure within the phenolic component is accountable for their antioxidant activity. Estimation of the total phenolic content can be carried out through the application of Folin-Ciocalteu assay. Although this assay has been reported to overestimate the phenolic content, due to interferences from sugar content, it is still generally accepted as a repeatable and rapid assay for estimating phenolic content of fruits and vegetables (Ikram et al. 2009).

Chilling injury in plants is associated with oxidation of phenolic compounds, resulting in degradation of membrane integrity. Chilling tolerance in various plants has

been attributed to the regulation of oxidative stress within the biological system (Gonzalez-Aguilar et al., 2004). The activity of phenolic compounds and other antioxidants play a key role in the acclimation to cold stress. Increased accumulation of these compounds has been reported during chilling tolerance, thus they are considered an integral part of the plant defence systems (Chen et al. 2006).

Tropical fruits are characterised by distinct nutraceutical properties that are attributed to the antioxidant activity of the fruits (Dembitsky et al. 2011). Carambola contains an abundance of phenolic compounds that include hydroxycinnamic acids and proanthocyanidins (Leong & Shui 2002). Meanwhile, dragonfruit owes its distinct purple colour to the presence of betacyanins, although it is also rich in phenolic compounds (Wu et al. 2006). Although mangosteen is characterised by potent antioxidant activity as well, most of the activity is attributed to the presence of anthocyanins in the thick purple pericarp (Naczk et al. 2011). It is hypothesized that the stresses induced by cold storage and the stress hormones SA and MeJA would elicit changes in the bioactive content of these fruits.

This experiment aims to assess changes in the antioxidant activity and total phenolic content of carambola, dragonfruit and mangosteen under the postharvest stresses of SA and MeJA. This will explore the role of stress hormones in activating the defence mechanism to elucidate any interaction between ripening and the bioactive content of the fruits used in this study.

4.2 Materials and methods

Carambola, dragon fruit and mangosteen were subjected to the treatments described in Sections 2.2.1 and 2.2.2 and used for assessment of the antioxidant activity of the fruits. Four biological replicates were used for the preparation of the extracts used in this study.

4.2.1 Extraction of fruit bioactive material

Fruit pulp was extracted from carambola, dragon fruit and mangosteen. The fruit materials were pooled together from four biological replicates, and homogenised using a blender and subjected to the extraction method. Extractions were carried out using 60 % methanol in water v/v at 37 ^{o}C for one hour. The ratio of solute to solvent was optimised at 1:10 (w/v). Centrifugation at 10,621 xg and 4 ^{o}C for 15 minutes using Eppendorf 5810R (Hamburg, Germany) was then carried out and the supernatant was filtered using Whatman No 1 filter paper and used for antioxidant analysis.

4.2.2 Ascorbic acid content

Ascorbic acid content was determined using the DCPIP (2,6-dichlorophenol-indophenol dye) titration method (Ranganna, 1977). DCPIP dye (Fisher Chemicals, USA) was prepared by dissolving 42 mg ± 0.1 of sodium carbonate in 150 ml ± 0.1 of hot distilled water followed by adding 50 mg ± 0.1 of DCPIP sodium salt. The solution was then cooled and the volume made up to 200 ml by adding distilled water and refrigerated. 10 g of carambola fruit pulp were extracted using 100 ml of 3 % metaphosphoric acid as the extracting medium (George et al., 2004), and titrated against the DCPIP dye until a faint pink colour that persisted for 15 seconds was observed. Ascorbic acid content was determined using the following equation (4.1):

$$AA\ (\mu g\ per\ mg) = \frac{dye\ factor \times titre\ value \times volume\ made\ up}{sample\ weight \times aliquot\ of\ sample\ used} \quad (4.1)$$

The dye factor was determined daily by standardizing the DCPIP dye. A 5 ml aliquot of standard L-ascorbic acid solution (0.1 mg/ml in metaphosphoric acid) was mixed with 5 ml metaphosphoric acid and titrated against DCPIP dye. The dye factor was then calculated using the following equation (4.2):

$$Dye\ factor = conc.\ ascorbic\ acid\ (mg)/titre\ value\ (ml) \quad (4.2)$$

4.2.3Total phenolic content (TPC)

Total phenolic content was determined using Folin Ciocalteu spectrophotometric method (Singleton & Rossi 1965). The reaction mixture contained 6 ml ± 0.01 of distilled water, 0.1 ml ± 0.01 of sample (extraction in section 2.3) and 0.5 ml ± 0.01 of undiluted Folin Ciocalteu reagent (Sigma-Aldrich, USA). After 5 minutes the reaction mixture was neutralized by adding 1.5 ml ± 0.01 of 7 % sodium carbonate solution. Distilled water was added to the solution to make the volume 10 ml and the mixture was incubated at 37 °C for two hours. The absorbance readings were recorded at 765 nm using Varioskan Flash Multimode Reader (Thermo Scientific, USA) and the blank that was prepared alongside the samples. A standard curve was constructed by using freshly prepared gallic acid solution (0 - 1000 µM) (Appendix A 4.1) and results were expressed as µg gallic acid equivalent (GAE)/g FW.

4.2.4 Betacyanin content

While betacyanins are most soluble in water, it is advisable to extract them in aqueous methanol at 60 % or 80 % (v/v) to denature the protein structures of the endogenous enzymes, thus terminating the activities of enzymes such as polyphenoloxidases,

peroxidases and ß-glucosidases (Stintzing & Carle 2007). Betacyanins were extracted from the pulp in 80 % methanol, and filtered with Whatman no. 1 paper. The filtrate was then mixed with 0.1 M acetate buffer and absorbance was detected at 538 nm using Varioskan Flash Multimode Reader (Thermo Scientific, USA) (Nerd et al. 1999). The betacyanin content was then expressed as betacyanin equivalent [mg/L] (Equation 4.3):

$$Betacyanin\ equivalent\ (mg\ per\ L) = (A * F * MW * 1000)/(\varepsilon * l) \quad (4.3)$$

Where A is the absorbance at 538, F the dilution factor, MW the molecular weight of betacyanin (MW = 550 g/mol), ε the molar extinction coefficient of betacyanin (ε = 60,000 L/ mol*cm), and l the path-length of the cuvette (l = 0.8 for Orange Scientific 96-well microplate with a volume of 300µl) (Herbach et al. 2006).

4.2.5 Ferric reducing antioxidant power (FRAP)

Total antioxidant activity was measured using ferric reducing antioxidant power (FRAP) assay adapted from Benzie and Strain (1996). FRAP reagent was prepared by mixing 2 ml ± 0.1 of acetate buffer (pH 3.6) with 2.5 ml ± 0.01 of 0.01M TPTZ (2,4,6-Tripyridyl-s-triazine) (Sigma-Aldrich, USA) solution and 2.5 ml ± 0.01 of 0.02M iron (III) chloride solution to give a straw coloured solution (Benzie & Strain 1996). The reaction mixture consisted of 20 µl ± 0.01 of the sample mixed with 200 µl ± 0.01 of FRAP reagent. The absorbance was then recorded in triplicate at 593 nm after 4 minutes of incubation at 37 °C using Varioskan Flash Multimode Reader (Thermo Scientific, USA), with the FRAP reagent as a blank. A standard curve was prepared beforehand using Trolox (0 - 1000µM) (Appendix A 4.2) and results were expressed as Trolox equivalent (TE) µM/g.

4.2.6 DPPH radical scavenging capacity

The radical scavenging capacity was determined for the crude methanolic extract using DDPH assay, according to the method of Brand-Williams et al. (Molyneux 2004). An aliquot of sample or blank (30 µl) was mixed with 120 µl of 0.1 M Tris-HCl and 150 µl of 0.3 mM DPPH (Sigma-Aldrich, USA) dissolved in methanol. The mixture was shaken in the dark at room temperature for 20 mins and absorbance readings were taken in triplicate at 517 nm (ODS) using Varioskan Flash Multimode Reader (Thermo Scientific, USA), with a methanol only blank for baseline correction and methanol in DPPH as a control (ODC). The radical scavenging activity was determined based on the change in absorbance (ΔDPPH) as shown in (Equation 4.4). A standard curve was prepared beforehand using Trolox (0 - 1000µM) (Appendix A 4.3) and results were expressed as Trolox equivalent (TE) µM/g.

$$\Delta DPPH = ODC - ODS \tag{4.4}$$

4.3 Results

4.3.1 Carambola (*Averrhoa carambola* L.)

4.3.1.1 Ascorbic acid content

A transient dose response was observed for ascorbic acid in response to MeJA treatment (Figure 4.1). The two highest MeJA treatments (0.2 mM and 0.5 mM MeJA) peaked on the 8th day of storage, with a significantly higher peak ($p < 0.05$) observed for the fruits treated with 0.5 mM MeJA. A peak in ascorbic acid content for the fruits treated with 0.01 and 0.1 mM MeJa was observed on the 12th day of storage, which was at the same level ($p > 0.05$) for both treatments. Although the ascorbic acid content was significantly higher ($p < 0.05$) for the treated fruits throughout the storage period, on the final day of storage the highest levels were observed for the control.

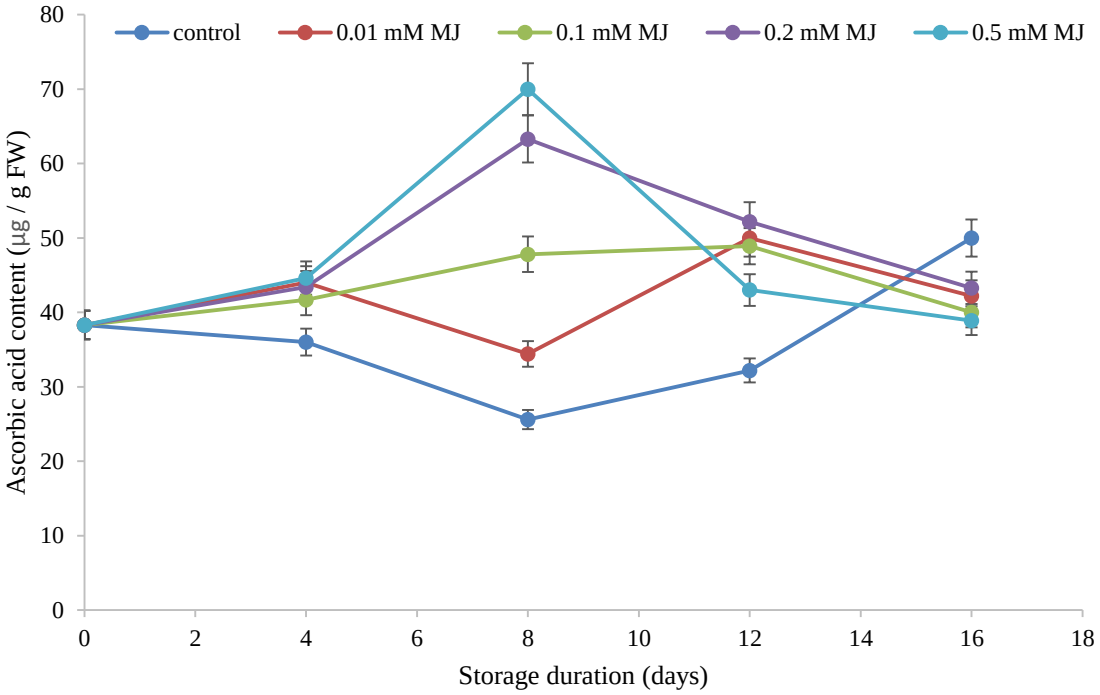


Figure 4.1 Effect of different concentrations of methyl jasmonate on ascorbic acid content of carambola stored at 6°C for 16 days. Values are the means ± SE.

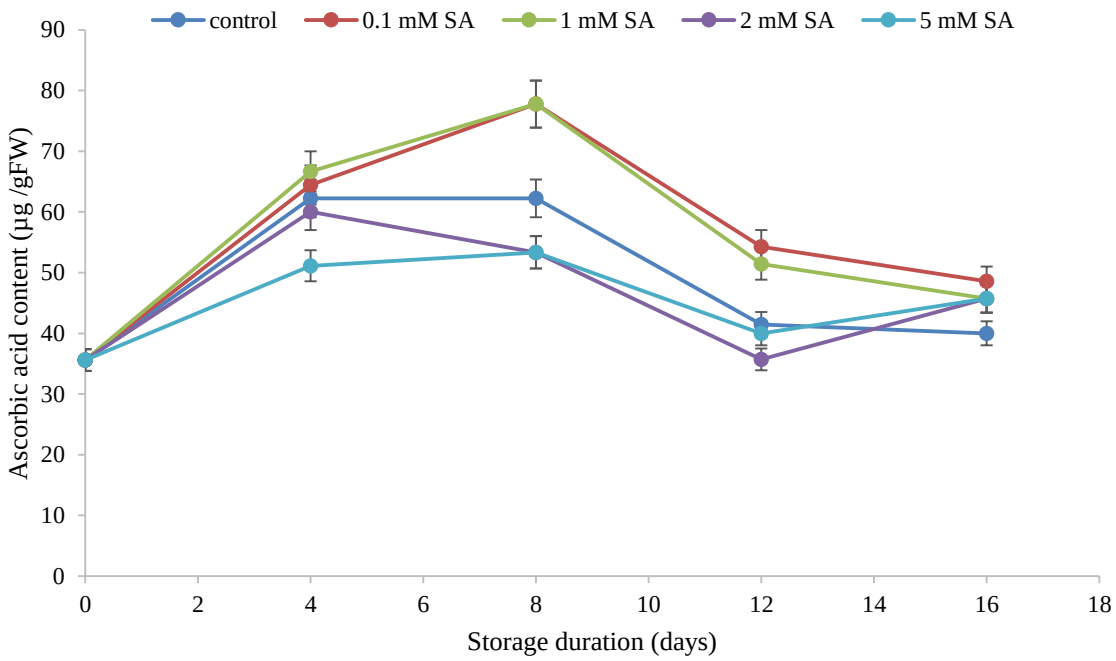


Figure 4.2 Effect of different concentrations of salicylic acid on ascorbic acid content of carambola stored at 6°C for 16 days. Values are the means ± SE.

Ascorbic acid content (Figure 4.2) a significant interaction was observed ($p < 0.05$) (Appendix B 4.2), and the highest content was reported for the lowest doses of SA (0.1 and 1 mM SA). Similar to the effect of treatment with MeJA, a transient peak in ascorbic acid content was observed on the 8th day of storage. However, with the SA treatment the highest SA treatments (2 and 5 mM SA) resulted in reduced ascorbic acid content, at significantly lower levels ($p < 0.05$) than the control. These results were in agreement with (Ali & Jaafar 1992) results on carambola cv. 17 who reported a gradual increase in AA content during ripening.

4.3.1.2 Total phenolic content

A significant interaction ($p < 0.05$) (Appendix B 4.3) was observed between time and treatments for the total phenolic content of the MeJA treated fruits (Figure 4.3). As the storage period increased, the total phenolic content of the control was significantly ($p < 0.05$) higher than the treated fruits. The MeJA treatments resulted in a decline of total phenolic content throughout the storage period. This was most prominent in the highest dose of MeJA (0.5 mM) where a 2.8 fold decline in total phenolic content was reported over the 16 days of storage. Meanwhile, at the final day of storage, the total phenolic content of the lower concentrations of MeJA (0.01, 0.1 and 0.2 mM) were at par ($p > 0.05$) with a 2.2 fold decline from the initial levels recorded at day 0.

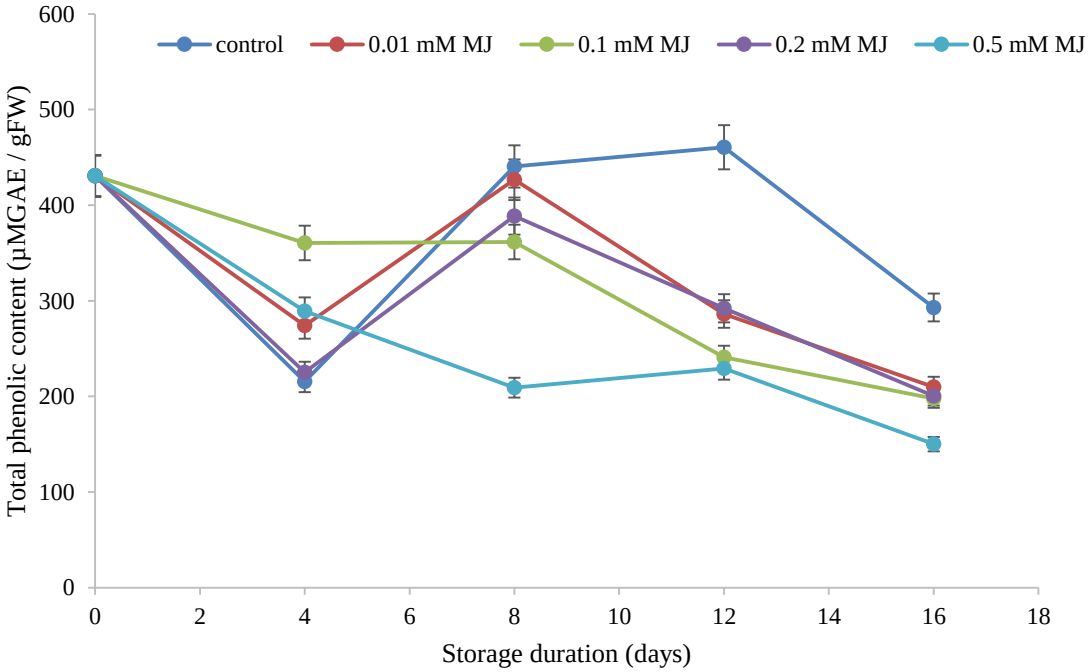


Figure 4.3 Effect of different concentrations of methyl jasmonate on total phenolic content of carambola stored at 6°C for 16 days. Values are the means ± SE.

The total phenolic content was initially highest ($p < 0.05$) for fruits treated with the lowest dose of SA (0.1 mM) at 409 µg GAE/gFW on the 4th day of storage (Figure 4.4). However, during the last day of storage the fruits treated with 2 mM SA maintained the highest phenolic content ($p < 0.05$) at 492 µg GAE/gFW. Throughout the entire storage period, the lowest levels were reported for the 1 mM treatment.

In the SA experiment, the highest reading for total phenolic content was observed at day 8 for the control (Figure 4.4). This level was 1.7 fold higher than the highest level reported for the control in the MeJA experiment. However, the untreated fruits in the MeJA experiment were stored for 16 hours at room temperature, along with the other MeJA treatments, before transferring to cold storage. Thus, the two sets of controls were exposed to cold storage at different maturity stages, which could have influenced the metabolic responses of the fruits to the cold treatment.

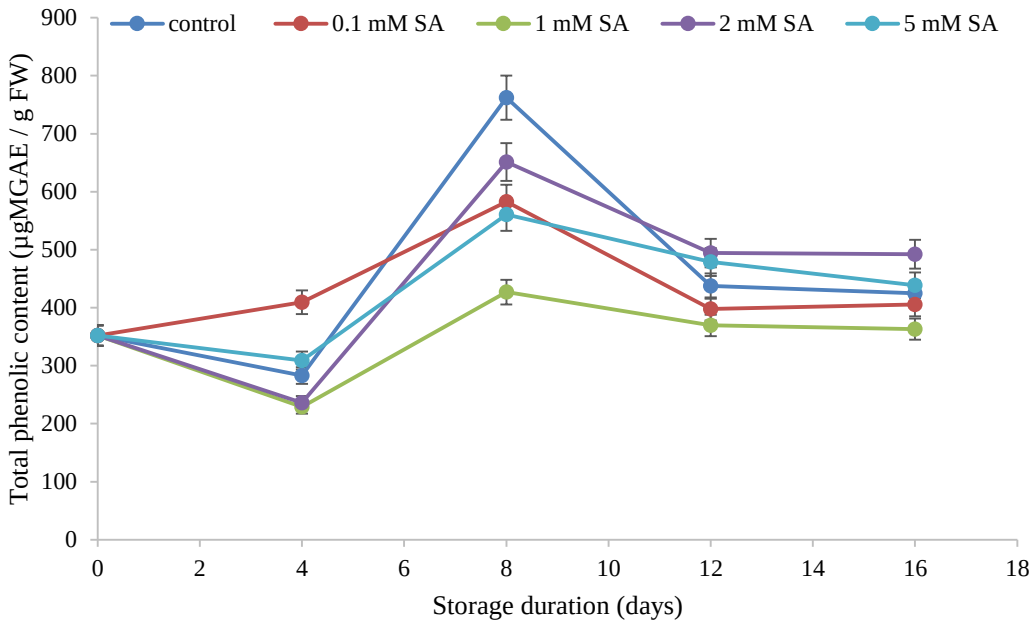


Figure 4.4 Effect of different concentrations of salicylic acid on total phenolic content of carambola stored at 6°C for 16 days. Values are the means ± SE.

4.3.1.3 FRAP Reducing Activity

A significant interaction ($p < 0.05$) (Appendix B 4.5) was observed for total antioxidant activity. Total antioxidant activity was initially highest for fruits treated with 0.1 and 0.5 mM MeJA and lowest for the control (Figure 4.5). However, as the storage period increased, the situation was reversed with the lowest antioxidant activity values observed for the highest MeJA treatment while the highest values were observed for the control. By the end of the storage period, the highest values were reported for control and 0.01 mM MeJA with no significant difference between these two treatments ($p > 0.05$).

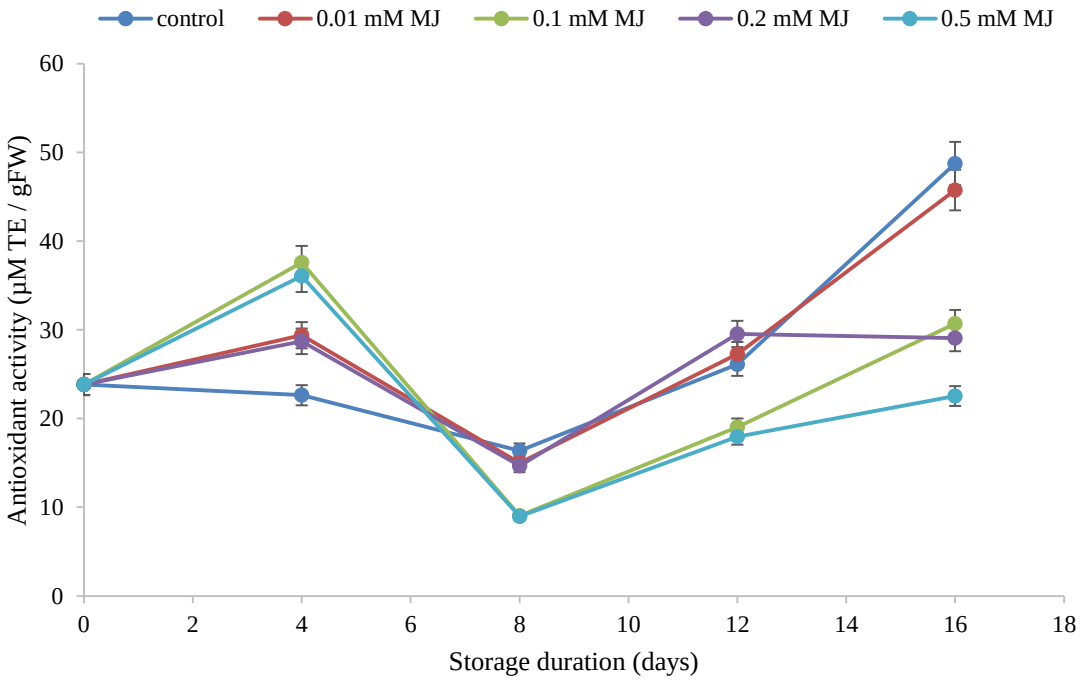


Figure 4.5 Effect of different concentrations of methyl jasmonate on antioxidant activity, determined by FRAP, of carambola stored at 6°C for 16 days. Values are the means ± SE.

The SA treatments induced a significant increase ($p < 0.05$) (Appendix B 4.6) in the antioxidant activity of the fruits, which lasted throughout the storage period (Figure 4.6). Moreover, the antioxidant activity values were positively correlated to the concentration of SA in the treatments. As the concentration of SA increased, the antioxidant activity of the fruits also increased. The highest increase was observed for the highest SA treatment at a 6.7 fold increase, followed by the 2 mM SA treatment at 5.8 fold increase.

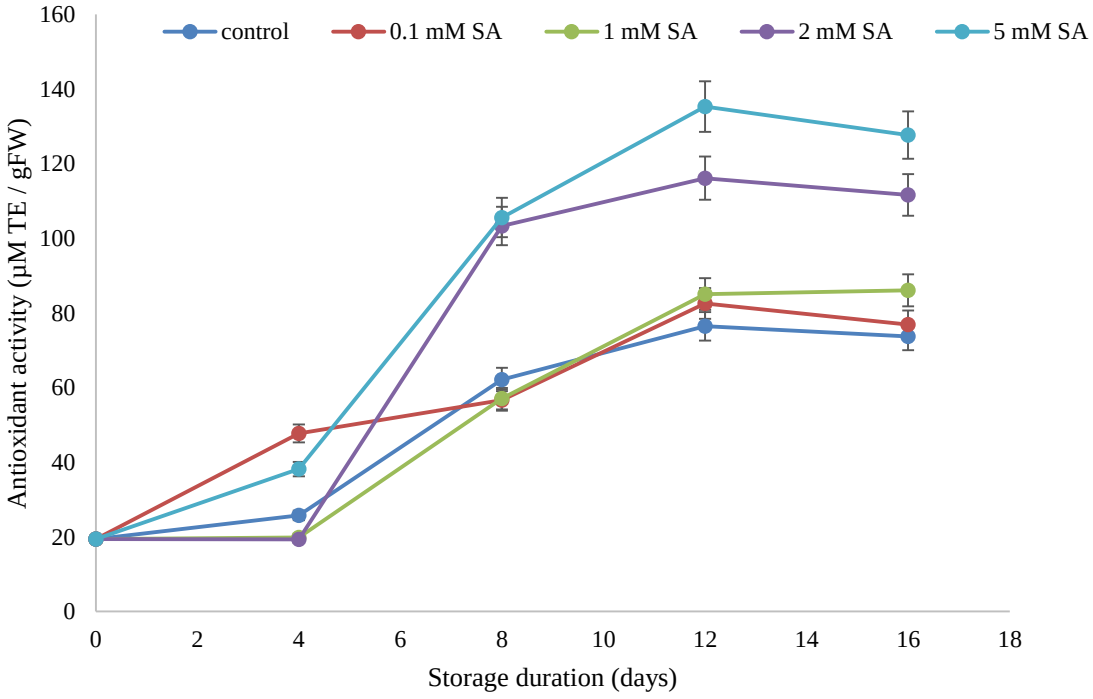


Figure 4.6 Effect of different concentrations of salicylic acid on antioxidant activity, determined by FRAP, of carambola stored at 6°C for 16 days. Values are the means ± SE.

4.3.1.4 DPPH Free Radical Scavenging Activity

The trends in total antioxidant activity observed for DPPH varied greatly from that observed for FRAP. With regards to the control, antioxidant activity was maintained throughout the storage period, with no significant difference ($p < 0.05$) between the levels reported at the initial sampling point and on the final sampling point. Moreover, in response to the MeJA treatments a decline in the antioxidant activity was observed. Antioxidant activity was inversely correlated to the MeJA concentration, with the highest decline (1.8 fold) reported for the 0.5 mM MeJA treatment.

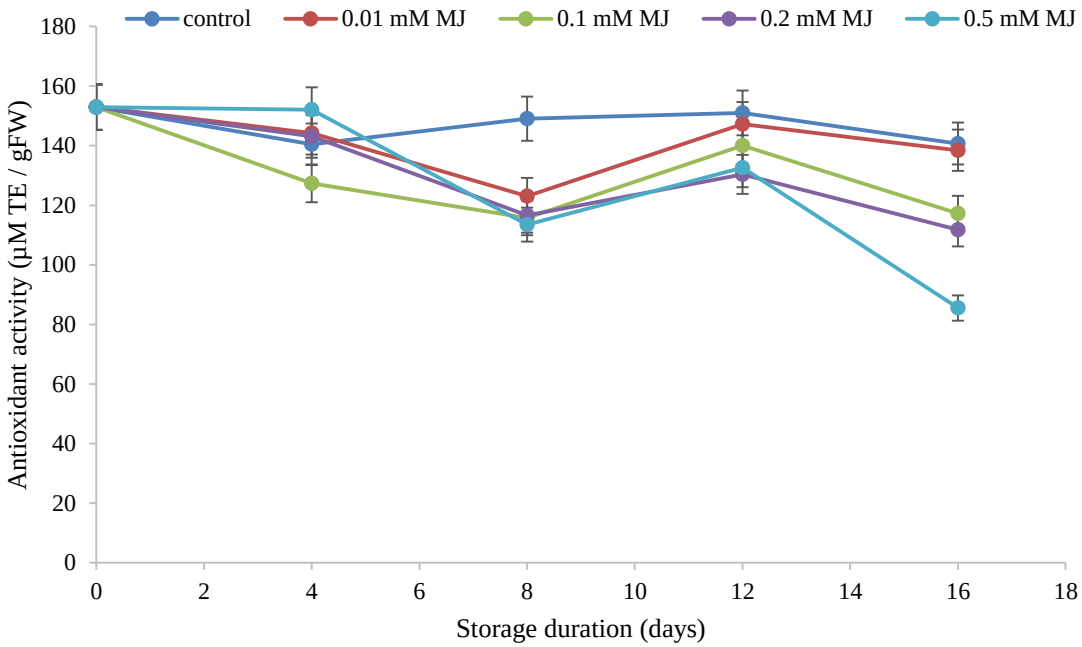


Figure 4.7 Effect of different concentrations of methyl jasmonate on antioxidant activity, determined by DPPH, of carambola stored at 6°C for 16 days. Values are the means ± SE.

Similar to the MeJA treatments, the trend observed in the SA treatments for DPPH was very different from FRAP (Figure 4.8). Antioxidant activity was generally maintained throughout the storage period and no significant interaction was observed ($p > 0.05$) (Appendix B 4.8). Nonetheless, significant difference ($p < 0.05$) was observed between the initial and final values reported in the antioxidant activity for the control and 0.1 mM SA.

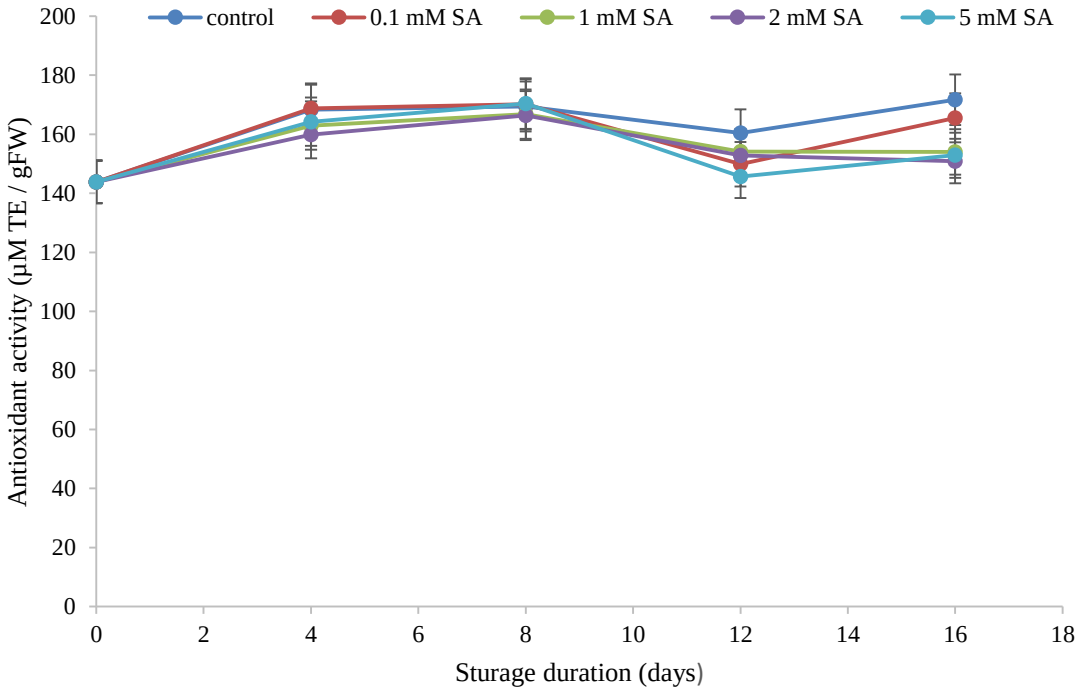


Figure 4.8 Effect of different concentrations of salicylic acid on antioxidant activity, determined by DPPH, of carambola stored at 6°C for 16 days. Values are the means ± SE.

4.3.2 Dragon fruit (Hylocereus spp.)

4.3.2.1 Betacyanin content

Betacyanin content is a characteristic bioactive component of dragonfruit and was measured as an indicator of the changes in the bioactivity (Figure 4.9). Throughout the storage period, the betacyanin content was generally maintained with a significant interaction ($p < 0.05$) (Appendix B 4.9) observed. A peak was observed for the highest MeJA treatment (0.5 mM) on day 14. The two lowest MeJA treatments (0.01 and 0.1 mM) resulted in a gradual increase in betacyanin levels throughout the storage period with the highest values for these two treatments reported on day 21.

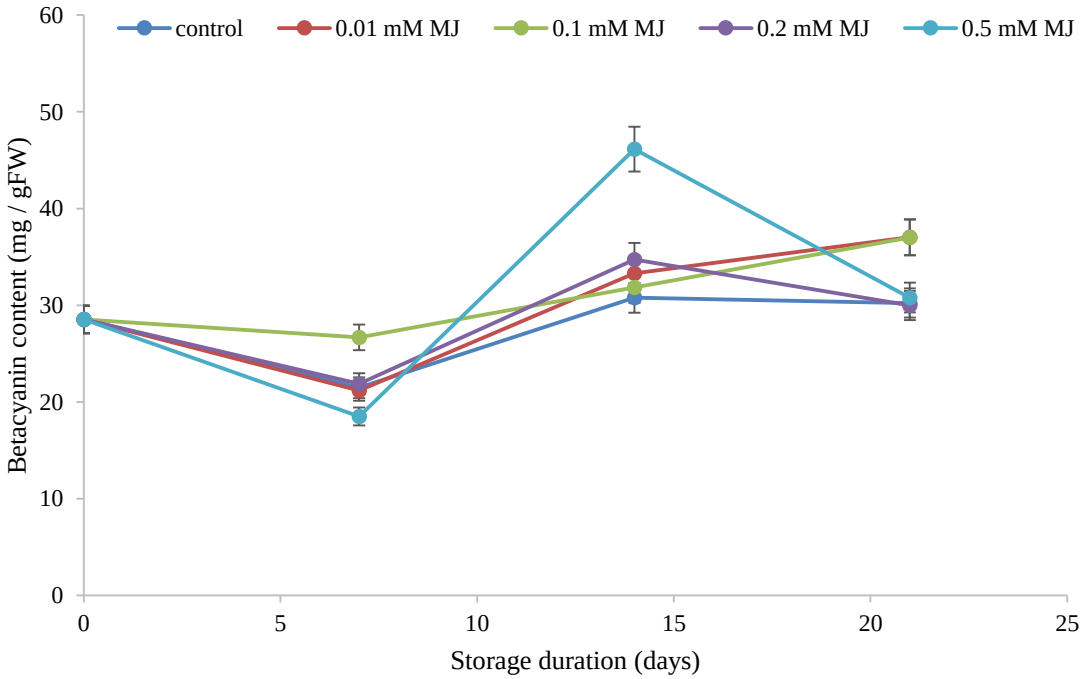


Figure 4.9 Effect of different concentrations of methyl jasmonate on betacyanin content of dragonfruit stored at 6°C for 21 days. Values are the means ± SE.

Meanwhile, treatment with SA resulted in a significant ($p < 0.05$) (Appendix B 4.10) decline in betacyanin content of the fruit during the initial storage period (Figure 4.10). The control in the SA experiment followed a similar trend to that observed for the control in the MeJA experiment, thus maintained a significantly higher ($p < 0.05$) level throughout the first two weeks of storage. On the final day of storage, a slight increase was observed for all of the fruits, and the 1 mM and 5 mM SA treatments were at par ($p > 0.05$) with the control. The effect of SA and MeJA on betacyanin content have not been explored previously, thus these results provide an insight on the interplay between innate betacyanin content and mechanisms for regulation of redox changes within the fruit.

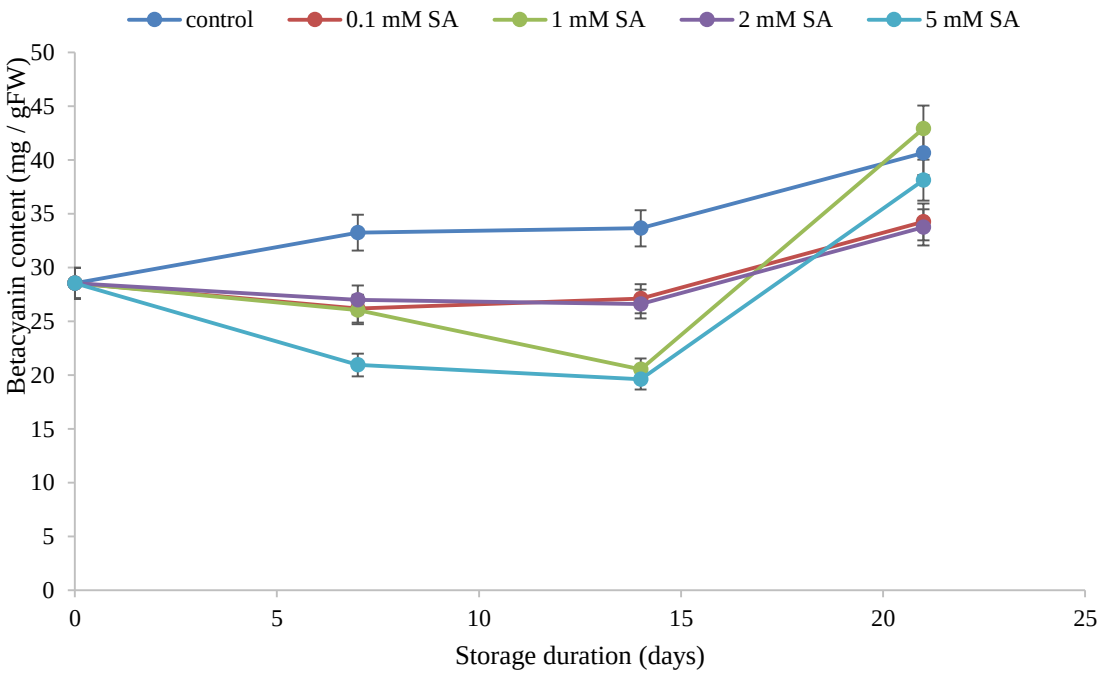


Figure 4.10 Effect of different concentrations of salicylic acid on betacyanin content of dragonfruit stored at 6°C for 21 days. Values are the means ± SE.

4.3.2.2 Total phenolic content

The total phenolic content of dragonfruit declined during the storage period (Figure 4.11). A sharp decline was observed for all treatments during the first week of storage, which was followed by a slight increase over the next two weeks. However no significant interaction was observed between treatment and time ($p > 0.05$) (Appendix B 4.11), as most of the treatments were at par ($p > 0.05$).

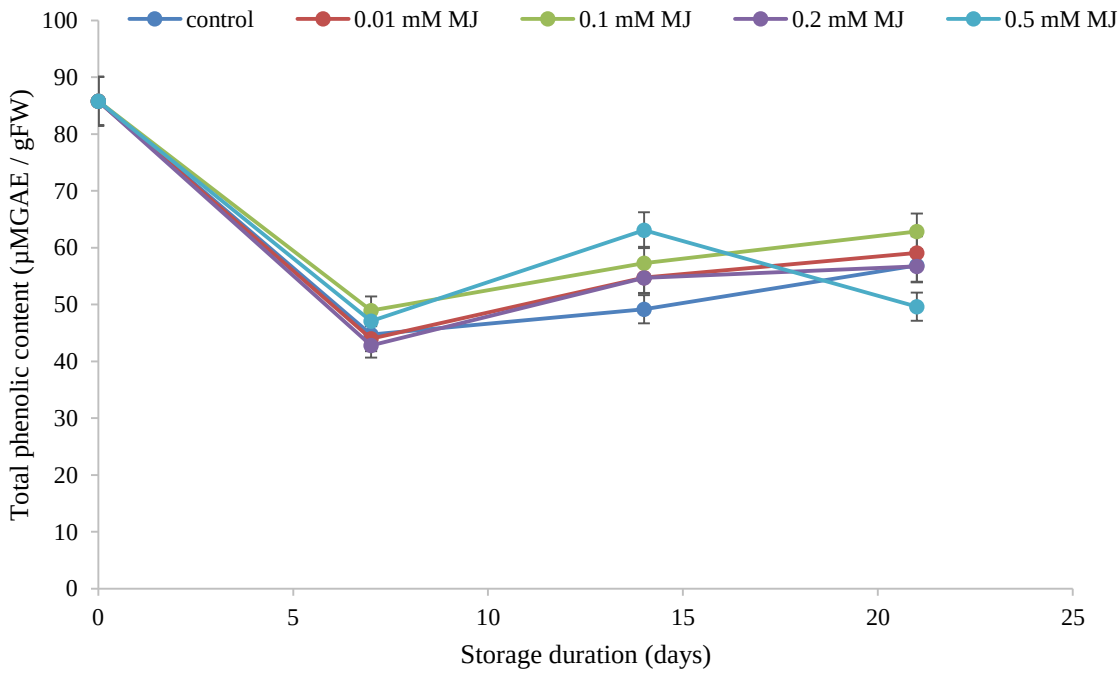


Figure 4.11 Effect of different concentrations of methyl jasmonate on total phenolic content of dragonfruit stored at 6°C for 21 days. Values are the means ± SE.

The changes in the total phenolic content for the SA experiment were similar to the changes observed in the MeJA treatment (Figure 4.12; Appendix B 4.12). However, the decline in total phenolic content was more gradual in the control and the highest treatment of SA (5 mM SA). Nonetheless, hormonal treatment with SA and MeJA did not induce a significant effect ($p > 0.05$) on the total phenolic content of the fruit.

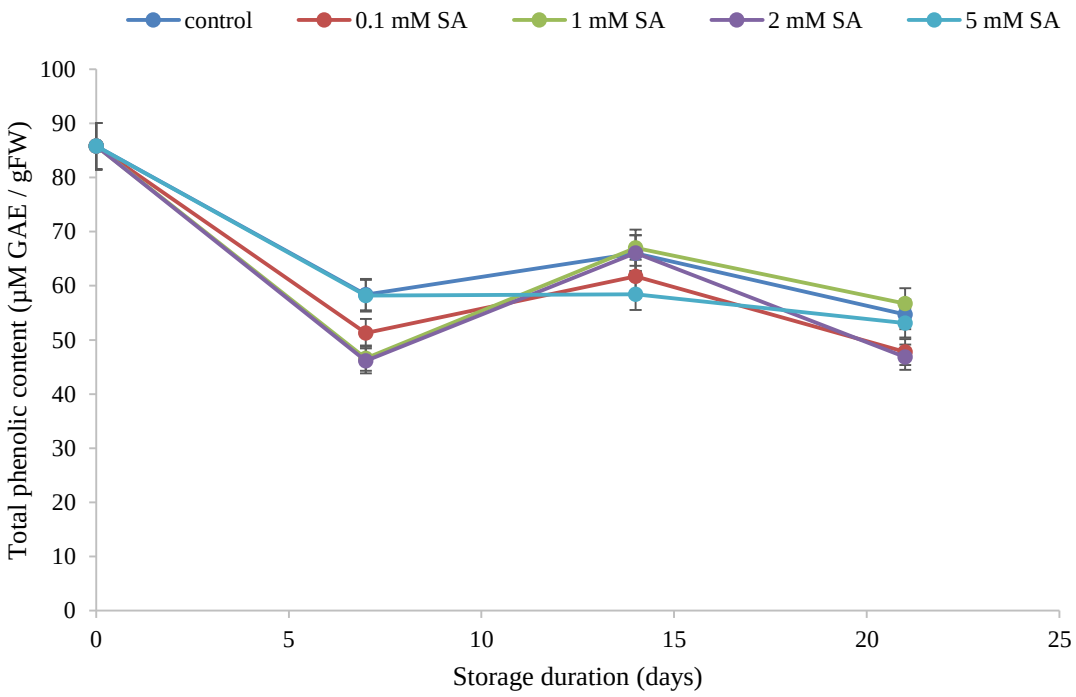


Figure 4.12 Effect of different concentrations of salicylic acid on total phenolic content of dragonfruit stored at 6°C for 21 days. Values are the means ± SE.

4.3.2.3 FRAP reducing activity

Total antioxidant activity of dragonfruit gradually increased during the storage period (Figure 4.13). Significant interaction was observed ($p < 0.05$) (Appendix B 4.13), and a peak was observed for the MeJA treatments on day 14, which was positively correlated to the concentration of MeJA applied. The highest value was reported for 0.5 mM ($p < 0.05$) at a 4.2 fold increase compared to the initial antioxidant values reported. However, by the final day of storage the lowest activity was reported for this treatment. Moreover, no significant difference ($p > 0.05$) was observed between the other treatments and the control on the final day of storage.

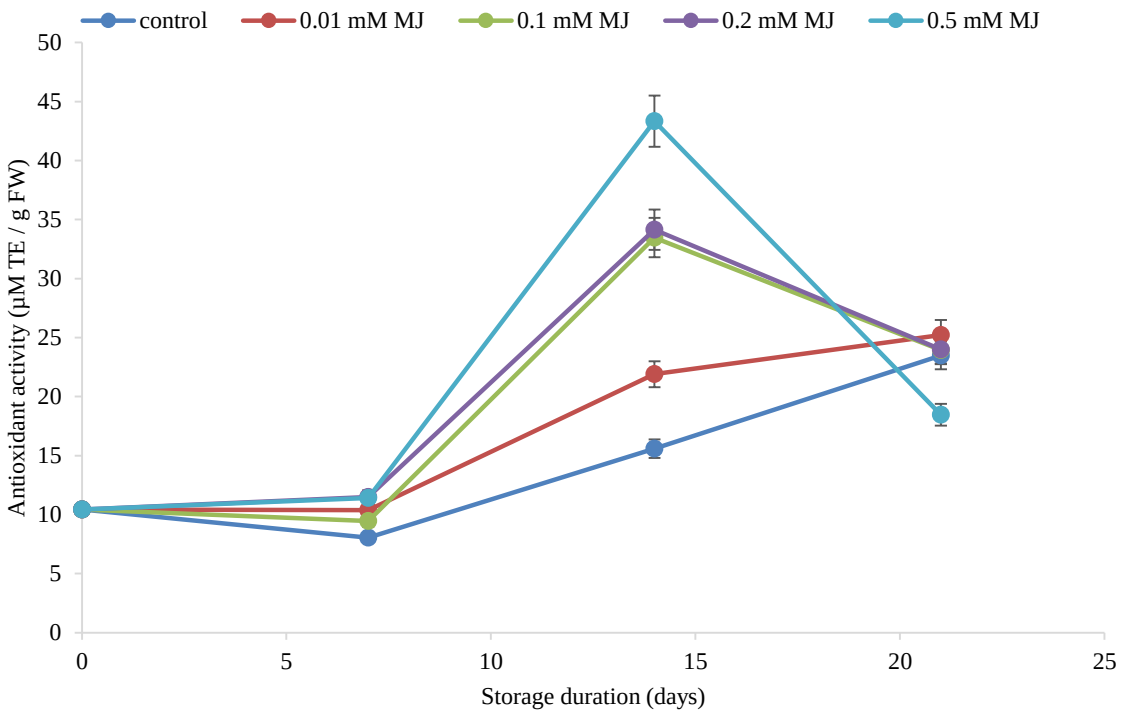


Figure 4.13 Effect of different concentrations of methyl jasmonate on antioxidant activity, determined by FRAP, of dragonfruit stored at 6°C for 21 days. Values are the means ± SE

Antioxidant activity for the SA treatments also increased throughout the storage period for all of the treatments (Figure 4.14). During the first week of storage, antioxidant activity of the control was significantly higher ($p < 0.05$) than the other treatments. However, on the final day of storage the antioxidant activity was positively correlated to the SA treatment with the highest activity observed for the highest concentration; a 2.5 fold, 2 fold, 1.9 fold and 1.6 fold increases from the initial levels were observed for the 5 mM, 2 mM, 1 mM and 0.1 mM SA, respectively.

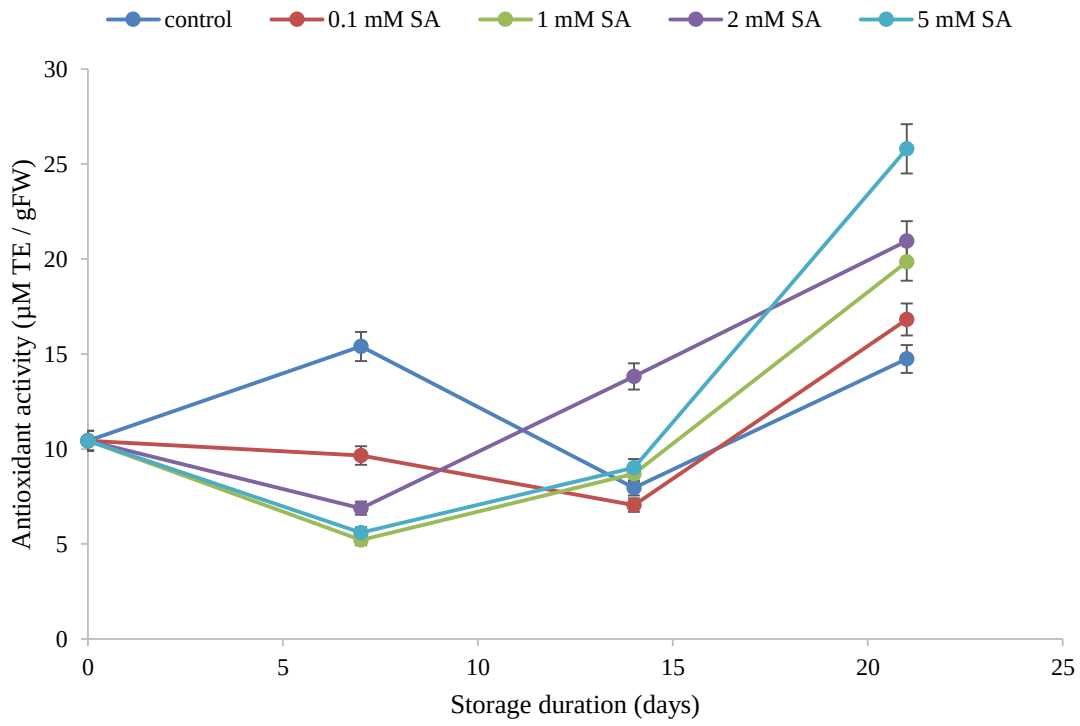


Figure 4.14 Effect of different concentrations of salicylic acid on antioxidant activity, determined by FRAP, of dragonfruit stored at 6°C for 21 days. Values are the means ± SE.

4.3.3.4 DPPH Radical Scavenging Activity

Radical scavenging activity as determined by DPPH increased linearly for dragonfruit throughout the storage period (Figure 4.15). After the first week of storage, the highest level of activity was reported for the control, with the MeJA treated fruits displaying significantly lower ($p < 0.05$) activity. However, no significant interaction ($p > 0.05$) (Appendix B 4.15) between treatment and time was reported for DPPH radicals scavenging activity.

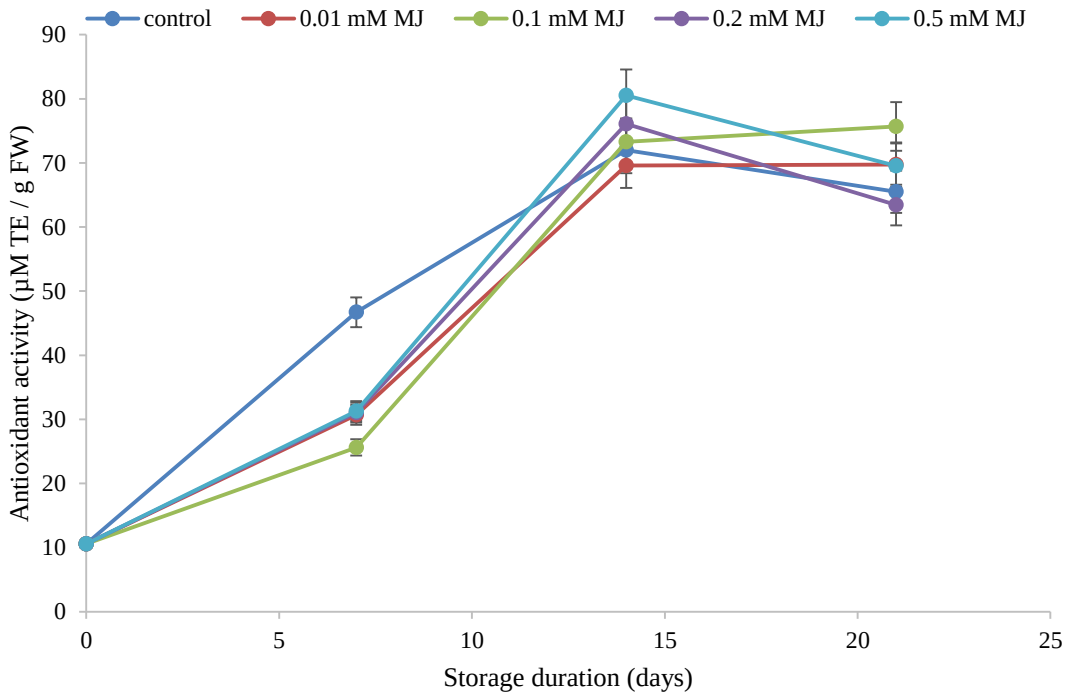


Figure 4.15 Effect of different concentrations of methyl jasmonate on antioxidant activity, determined by DPPH, of dragonfruit stored at 6°C for 21 days. Values are the means ± SE.

In the SA experiment, a significant interaction ($p < 0.05$) (Appendix B 4.16) was observed for the radical scavenging activity, with the control peaking after one week of cold storage (Figure 4.16). Antioxidant activity of treated fruits was significantly lower ($p < 0.05$) than the control. The radical scavenging activity of the fruits after one week of cold storage was inversely related to the SA dosage, as the lowest activity was reported for the highest SA treatment (5 mM SA), which was 3.7 fold lower than the activity of the control on that sampling point. The control maintained highest activity throughout most of the storage period, however by the final sampling point all treatments were at par ($p > 0.05$) with the exception of 1 mM SA, which exhibited the highest activity.

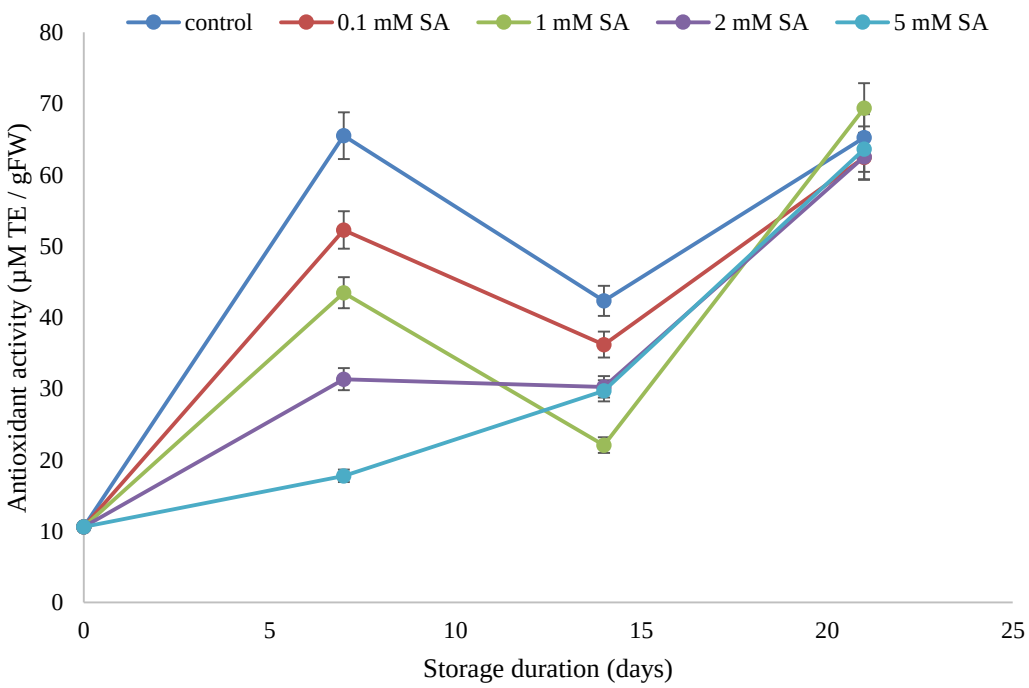


Figure 4.16 Effect of different concentrations of salicylic acid on antioxidant activity, determined by DPPH, of dragonfruit stored at 6°C for 21 days. Values are the means ± SE.

4.3.3 Mangosteen (*Garcinia mangostana* L.)

4.3.3.1 Total phenolic content

Significant interaction was observed for total phenolic content of mangosteen ($p < 0.05$) (Appendix B 4.17). Total phenolic content of fruits treated with 0.2 mM MeJA gradually increased during the storage period, attaining the highest activity by the final day of storage (Figure 4.17). On the final day of storage the lowest activity was reported for the 0.5 mM MeJA treatment.

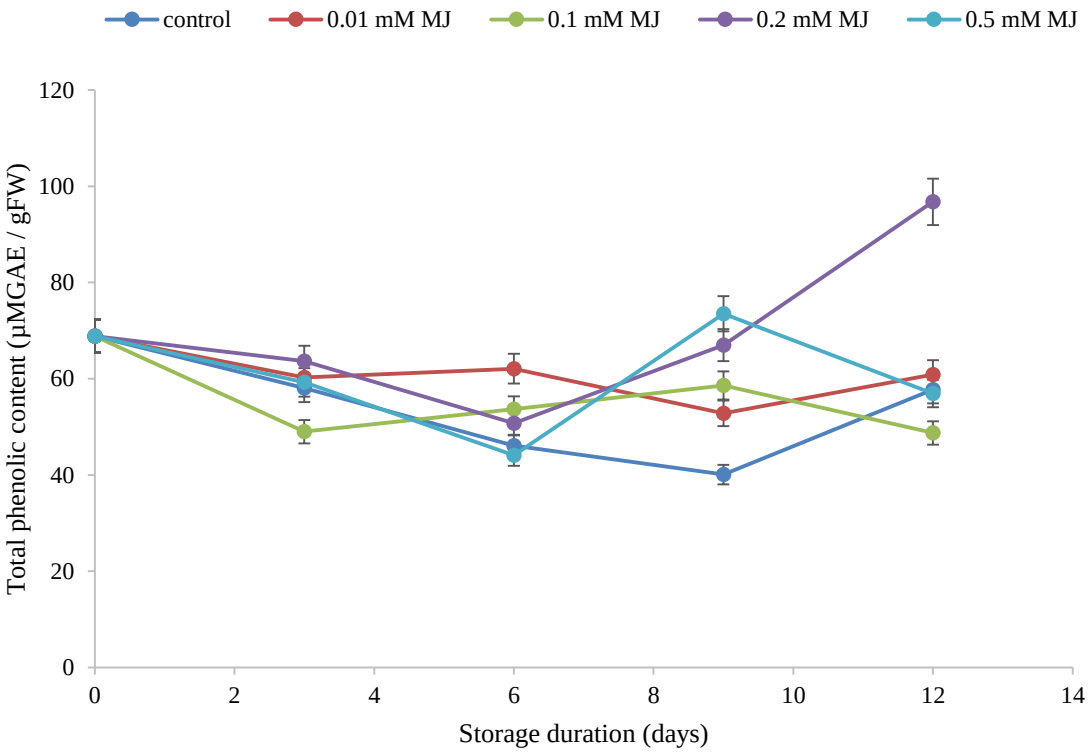


Figure 4.17 Effect of different concentrations of methyl jasmonate on total phenolic content of mangosteen stored at 6°C for 12 days. Values are the means ± SE.

A significant interaction ($p < 0.05$) (Appendix B 4.18) was also reported for the SA experiment. Total phenolic content of the control was maintained throughout the storage period, with slight fluctuations (Figure 4.18). A transient dose response was

observed for the two lowest treatments of SA (0.1 and 1 mM) on the third day of storage. The total phenolic content of these two treatments peaked, with the highest activity was observed for 1 mM SA treatment, which was 2.1 fold higher than the initial levels of phenolic content. Meanwhile, total phenolic content for the highest SA treatment (5 mM SA) increased by the sixth day of storage and was maintained before dropping on the final day of storage.

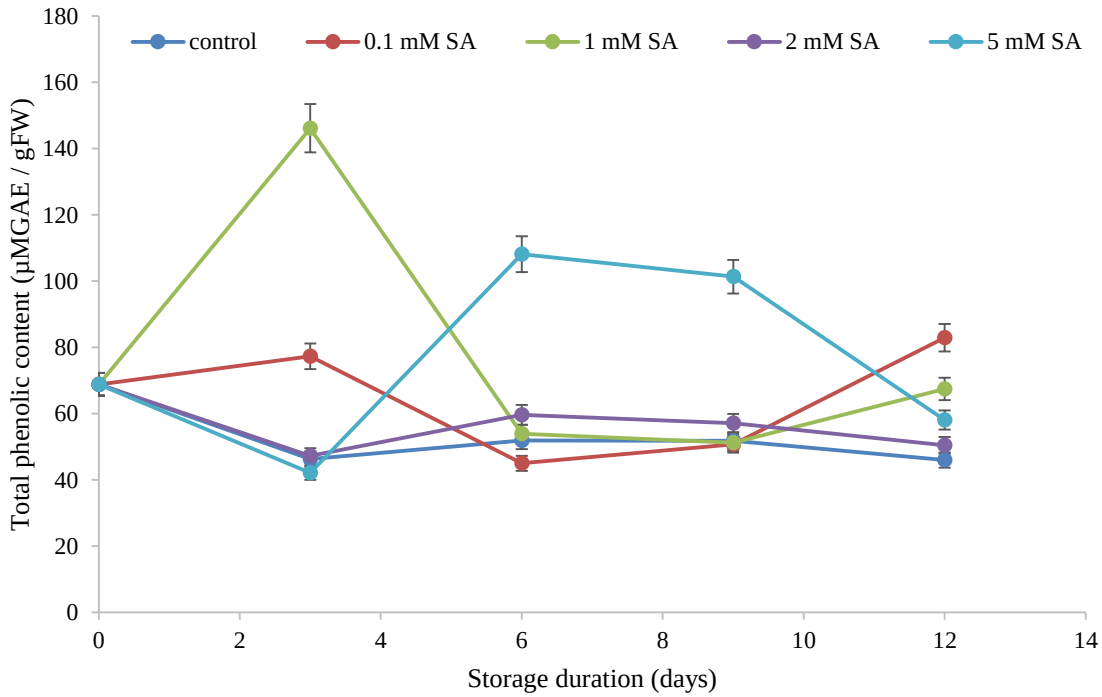


Figure 4.18 Effect of different concentrations of salicylic acid on total phenolic content of mangosteen stored at 6°C for 12 days. Values are the means ± SE.

4.3.3.2 FRAP reducing activity

During the initial days of storage antioxidant activity of the two highest MeJA treatments (0.2 and 0.5 mM MeJA) was significantly higher ($p < 0.05$) than the other treatments (Figure 4.19). By the sixth day of storage, the 0.5 mM MeJA treatment demonstrated significantly higher ($p < 0.05$) activity in comparison to all of the other treatments. This level was maintained by the highest MeJA treatment, which still possessed the highest antioxidant activity ($p < 0.05$) by the final day of storage. The lower doses of MeJA (0.01 and 0.1 mM MeJA) did not have a significant effect ($p > 0.05$) on the antioxidant activity of the fruit during the initial storage period. On the final day of storage, there was no significant difference between the control and the 0.01 and 0.2 mM MeJA treatments.

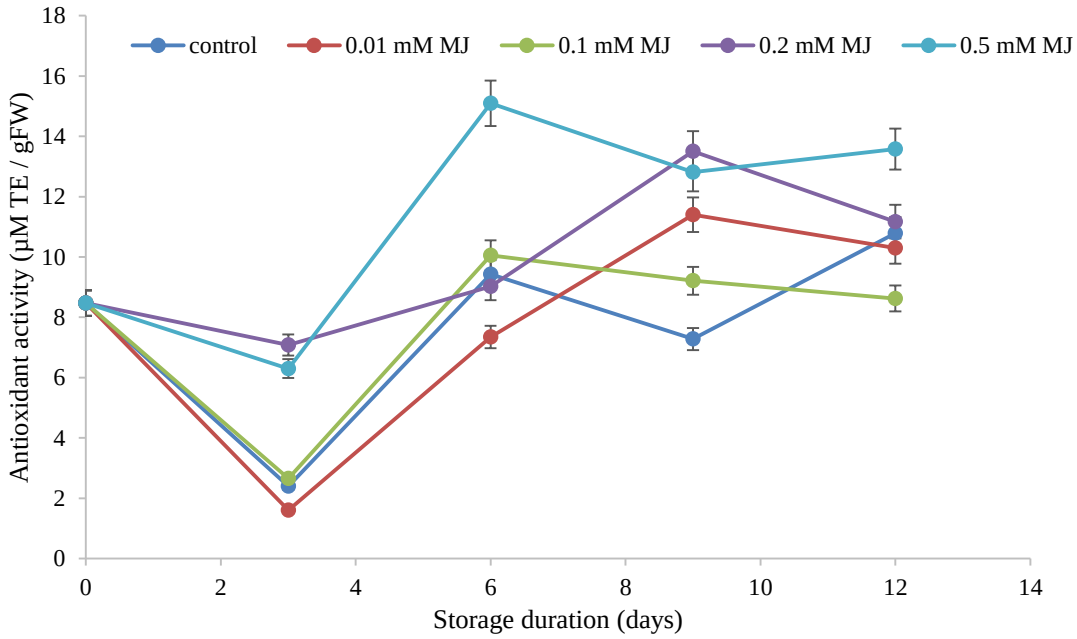


Figure 4.19 Effect of different concentrations of methyl jasmonate on antioxidant activity, determined by FRAP, of mangosteen stored at 6 °C for 12 days. Values are the means ± SE.

A significant ($p < 0.05$) (Appendix B 4.20) decline in the antioxidant activity of the fruits was observed throughout the experiment (Figure 4.20). A peak in the activities of the 1, 2 and 5 mM SA treated fruits was observed on the sixth day of storage, with the highest SA treatment (5 mM SA) inducing the highest antioxidant activity ($p < 0.05$). On the final day of storage, the two highest SA treatments (2 and 5 mM SA) maintained the highest levels, which were still significantly lower ($p < 0.05$) than the initial antioxidant activity reported for the fruits.

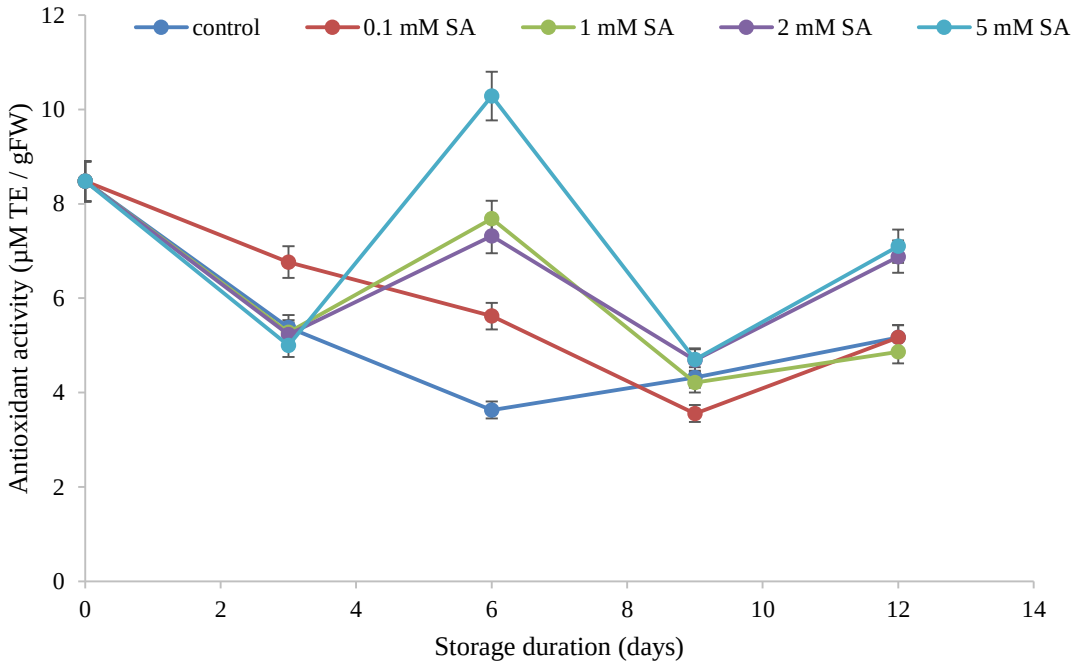


Figure 4.20 Effect of different concentrations of salicylic acid on antioxidant activity, determined by FRAP, of mangosteen stored at 6°C for 12 days. Values are the means ± SE.

4.3.3.3 DPPH radical scavenging activity

The radical scavenging activity of mangosteen significantly ($p < 0.05$) (Appendix 4.21) declined throughout the storage period (Figure 4.21). Nonetheless, the activity was slightly enhanced by the 0.2 and 0.5 mM MeJA treatments on the third day of storage. This was then followed by a rapid decline in the antioxidant activity of these treatments, with the 0.2 mM MeJA treated fruits dropping to levels significantly lower ($p < 0.05$) than the control on the sixth day of storage.

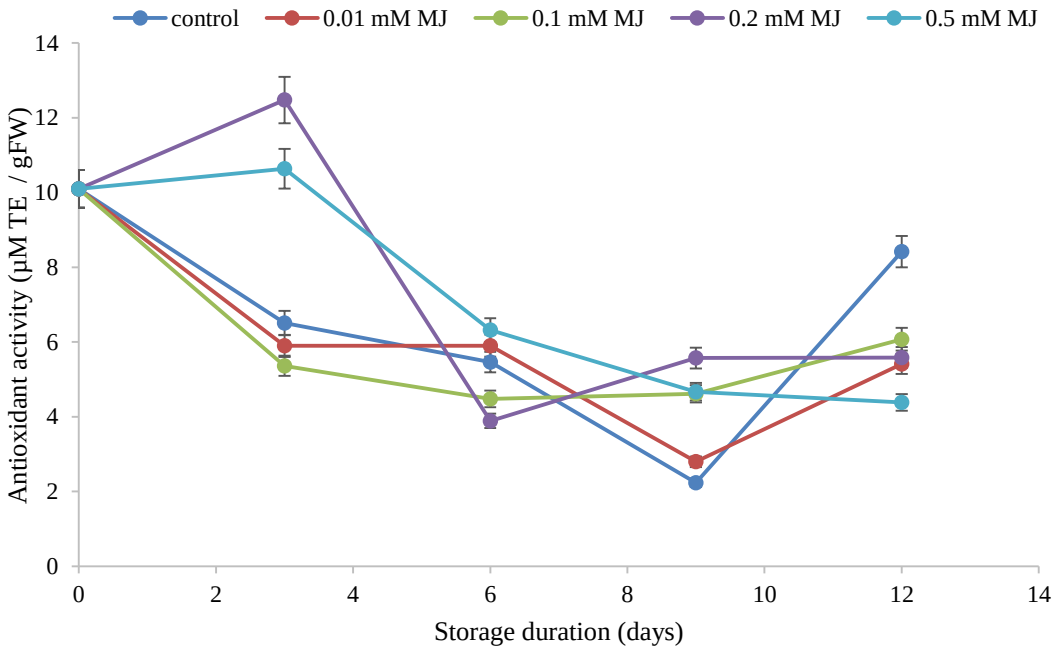


Figure 4.21 Effect of different concentrations of methyl jasmonate on antioxidant activity, determined by DPPH, of mangosteen stored at 6°C for 12 days. Values are the means ± SE.

A significant ($p < 0.05$) (Appendix 4.22) decline in the radical scavenging activity was also observed in the SA experiment as well (Figure 4.22). However, enhanced activity was observed on the third day of storage for the two lowest SA treatments (0.1 and 1 mM SA) in this experiment, which was then followed by a rapid decline in the radical scavenging activity for the rest of the storage period. The lowest SA treatment (0.1 mM SA) induced the highest activity ($p < 0.05$) at 12.04 uMTE/gFW on the third day of storage. Although exposure of mangosteen to SA treatments did not elevate the radical scavenging activity of the fruit, the hormonal treatment prevented a rapid decline as the radical scavenging activity of the treatments was maintained above the control.

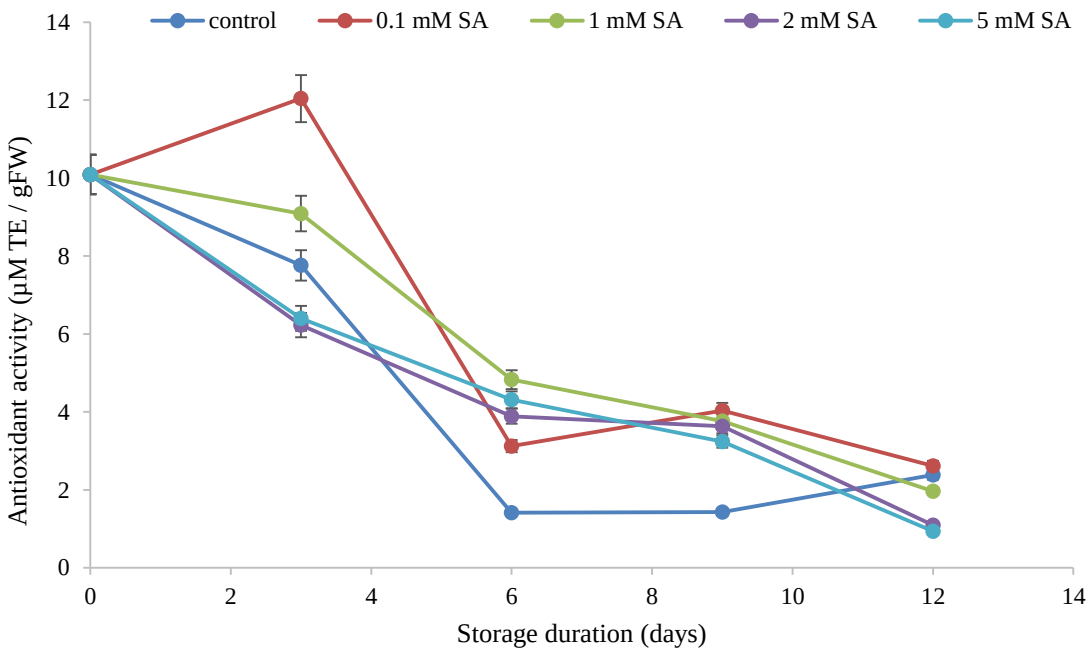


Figure 4.22 Effect of different concentrations of salicylic acid on antioxidant activity, determined by DPPH, of mangosteen stored at 6°C for 12 days. Values are the means ± SE.

4.4 Discussion

4.4.1 Ripening related changes in the antioxidant activity of fruits

Numerous elicitors have been the subject of previous studies, among which MeJA and SA are illustrated as effective enhancers of the antioxidant activity of plants (Flores & Ruiz del Castillo 2014). Nonetheless, there is limited evidence on the role of these stress hormones as postharvest abiotic stresses, but there is literature that indicates that increased phenolic content is a response to these phytohormones. In this chapter, the role of SA and MeJA in enhancing the quality of carambola, dragonfruit and mangosteen under chilling stress was explored by examining their effect on the antioxidant activity.

Low temperature storage induces an oxidative burst, which is perceived by the fruits and activates the phenylpropanoid pathway leading to an accumulation of phenolic acids (Meng et al. 2009). Activation of the phenylpropanoid pathway under cold storage occurs even in chilling tolerant commodities such as grapes resulting in increased anthocyanin content (Sanchez-Ballesta et al. 2007). Phenolic-associated stress responses are very diverse ranging from increased lignification in grape-vine under water stress to accumulation of water-soluble phenolics in frosted rhodendron leaves (Solecka et al. 1999).

The phenolic content and antioxidant activity can be influenced by various factors, including the weight, pH and titratable acidity of the fruit (Flores & Ruiz del Castillo 2014). Abiotic stresses can induce antioxidant activity. Increase in the antioxidant pool has been documented in various commodities such as tomatoes, where a 1.77 fold increase was reported for fruits stored at 5 °C compared to 12 °C storage (Javanmardi & Kubota 2006). However, there were examples, such as apples where no

change in antioxidant activity was observed at low temperature storage (Sanchez-Ballesta et al. 2007).

4.4.1.1 Carambola

Carambola is characterised with high content of ascorbic acid, epicatechin and gallic acid (Shui & Leong 2004). High ascorbic acid content was reported in this study, but it was also coupled with rapid changes. Treatment of carambola with MeJA induced significantly higher ($p < 0.05$) ascorbic acid content and antioxidant activity of the fruit. The highest concentration of ascorbic acid was exhibited under the highest dose of MeJA on the 8^{th} day of storage. Ascorbic acid occupies a key role in the maintenance of the cellular redox state, and previous studies have reported an increase in ascorbic acid content in response to MeJA (Zhang et al. 2001). Enhanced ascorbate-glutathione reducing status was also found to be involved in SA mediated alleviation of chilling injury in mango (Ding et al. 2007).

The increase in AA content of carambola was associated with repressed ascorbate oxidase activity in mature fruits (Zainudin et al. 2014). Although AA may typically function as antioxidants, it can occasionally carry out pro-oxidant activities so a balance is required to avoid oxidative damage. Enhanced expression of the ascorbic acid biosynthesis genes l-galactose-1-phosphate phosphatase, l-galactono-1,4-lactone dehydrogenase and d-galacturonicreductase was reported during fruit ripening (Cruz-Rus et al. 2010). Nonetheless, the turnover of the ascorbate pool is subject to the presence of enzymes and reductants that ensure the efficient turnover of dehydroascorbic acid to ascorbate (Noctor et al. 2014). Under oxidative stress, the turnover is generally rapid, however it can be compromised under very high ROS levels (Davletova et al. 2005). Such oxidative stress promotes the degradation of

dehydroascorbic acid rather than its rapid turnover to ascorbate, reducing their availability as the storage period increased (Noctor et al. 2014).

Previous results reported a decline in TPC of carambola towards late ripening stages, similarly to other fruits such as ciku and pear (Zainudin et al. 2014; Shui & Leong 2004). This was in agreement with the results observed for the TPC of the carambola under SA and MeJA treatment. Zainudin et al. (2014) reported a decrease in antioxidant activity as carambola ripens, which was attributed to the decline in TPC. Phenolic compounds contribute to the major share of the antioxidant activity in carambola, however, increased reducing activity was observed whilst a decline in TPC was occurring (Dembitsky et al. 2011; Leong & Shui 2002). This was in contrast to Zainudin et al. (2014) who reported a concomitant decline in antioxidant activity and total phenolic content of carambola. Carotenoid content increases as chlorophyll is converted to carotenoid during ripening (O'Hare, 1993), thus increases in carotenoid content might account for the increased antioxidant activity.

The ripening stage of the fruit can strongly influence the nutritional value and antioxidant capacity of the fruit (Ali et al. 2004). Thus, similarly to the physicochemical analyses, differences were observed between the antioxidant potential of the fruits in the SA experiment and those in the MeJA experiment. An interesting interplay was observed in this study; the lowest treatment of phytohormones induced increases in antioxidant activity and total phenolic content by the first sampling point, whilst the second treatment repressed these changes. It will be of interest to explore these effects further.

4.4.1.2 Dragonfruit

Interesting responses in the bioactive content of dragonfruit were observed throughout the storage period. Generally, betacyanin content increased for the dragonfruit during storage, while TPC declined. Moreover, the exposure to the stress hormones exhibited no effect on total phenolic content, while it slightly induced betacyanin content. This reflects an interaction between ripening and betacyanin content of dragonfruit. Moreover, An increase in bioactive contents of fruits during ripening as a protective mechanism against stresses is a generally accepted phenomenon, for example anthocyanin content of tomatoes increases and results in delayed ripening (Zhang et al. 2013). Betacyanin is the principal bioactive ingredient of dragonfruit and is responsible for a large share of the fruit's antioxidant and nutritional benefits (Muhammad et al. 2014).

Dragonfruit exhibited significant responses to MeJA treatment, which were not similarly observed for the SA treatment. During the storage period a significant increase in betacyanin content was observed for fruits treated with 0.5 mM MeJA, while SA treatments repressed the increases in betacyanin content. Reducing activity was also significantly elevated in response to MeJA, although an increase on the final day of storage was also observed for the SA treated fruits. Dragonfruit is characterised by a thick peel that could inhibit penetration of substances through the thick peel. MeJA was administered in vapour phase, while SA was administered in liquid phase. Thus, the different responses observed might be as a result of the different modes of delivery.

4.4.1.3 Mangosteen

It is important to note that the edible portions of the fruits were tested in this study, as mangosteen peel is usually characterized with potent antioxidant activity, whilst the edible pulp is not as rich in bioactive content (Palapol et al., 2009; Dembitsky et al., 2011). Thus, antioxidant activity of the pulp did not exceed 16 µMTE/gFW for all of the treatments throughout the entire storage period. Nonetheless, treatment with both MeJA and SA generated significantly higher reducing activity as well a delay in the reduction of radical scavenging activity as the fruit ripened.

A weak correlation, or lack of one, has been reported between TPC and antioxidant capacity as FRAP measure the total reducing activity while DPPH measures the radical scavenging activity (Ikram et al. 2009). This was also demonstrated for mangosteen, thus compounds besides phenolic compounds can be responsible for the increased antioxidant activity observed during ripening. Cold storage of mangosteen was reported to increase pericarp hardening for the fruit samples during the storage period (Section 3.3.3.3). This is associated with increased lignification, which has been linked to a decline in total free phenolics (Ketsa & Atantee 1998). The free phenolic compounds are increasingly incorporated into lignin, which could occur faster than the rate of synthesis of the phenolic compounds during stress (Bunsiri et al. 2003).

The radical scavenging activity of mangosteen was attributed to the presence of phenolic compounds, benzophene compounds, xanthone and epicatechin (Dembitsky et al. 2011). There was a general decline for radical scavenging activity, as determined by DPPH, observed throughout the storage period. This was in agreement with Pothitrat et al. (2009) who reported higher scavenging activity in young fruits compared to more mature fruits. Again, this could be associated to the increased lignification occurring under the chilling stress, which depletes the phenolic content.

Dangcham et al (2008) reported a more rapid decrease in free phenolics of mature fruits versus less mature fruits as they were exposed to cold storage. Thus, the significantly higher levels of TPC observed for the SA treated fruits compared to the MeJA treated fruits could be attributed to the fact that MeJA treated fruits were subjected to cold storage at a more mature stage due to the nature of the treatment. Moreover, the decline of free phenolics in mature fruits was linked to more pronounced CI symptoms in the mature fruits (Dangcham et al. 2008). The SA treatments were more effective in delaying the increase in firmness, which is a key symptom of CI in mangosteen.

4.4.2 Influence of methyl jasmonate on antioxidant activity

Phenolic acids undergo enzymatic oxidation to quinones, as a defence strategy ultimately increasing the incidence of browning. In this context, the lower abundance of phenols within the fruits could be a contributing factor to the lower incidence of chilling injury symptoms in MeJA treated fruits. The mechanism associated with this phenomenon is worthy of exploring, thus further studies will investigate the oxidation enzymes as well as differences in the classes of phenolic acids that constitute the total phenolic content.

MeJA application can enhance antioxidant activity, and has been practiced on various commodities such as blackberry (Szymanowska et al. 2015; Wang et al. 2007). Similarly, MeJA application was observed to induce betacyanin content and antioxidant activity in dragonfruit. Variation in the dose of MeJA was also reported to significantly impact the effect elicited on the fruit antioxidant activity. Kim et al. (2006) reported that low MeJA doses (0.01 mM) did not have a significant impact while higher does (0.1 and 0.5 mM) significantly increased the antioxidant activity of basil. This was also

in agreement with the trend observed for FRAP antioxidant activity of carambola, dragonfruit and mangosteen where the highest activity was induced by 0.5 mM MeJA.

MeJA decreases membrane lipid peroxidation and regulates the cell wall degradation, as protective responses to cold temperatures (Meng et al. 2009). In this context, MeJA plays more of a protective role and does not contribute significantly to enhancing the antioxidant properties of the fruit. Treatments where no changes were detected could indicate that the mechanisms activated are related to resistance rather than stress alleviation (Sanchez-Ballesta et al. 2007).

Jasmonates have been explored in model plants such as tomato and tobacco where they induce the expression of PR proteins and genes involved in the phenylpropanoid pathway. A multitude of other studies demonstrated that jasmonates enhance the accumulation of secondary metabolites such as anthocyanins in strawberry and stilbenes in grapevine (Belhadj et al. 2008). Moreover, MeJA can induce the production of other defence compounds, including salicylic acid.

4.4.3 Influence of salicylic acid on antioxidant activity

The changes in the ascorbic acid content in response to SA treatment differ depending on the fruit commodity. In pomegranates, ascorbic acid was maintained by exposure to SA (Sayyari et al. 2009), while no significant effect was observed on cactus pear (Al-Qurashi & Awad 2012). SA reportedly elevates the reducing status of ascorbic acid and glutathione while concomitantly increasing the intrinsic hydrogen peroxide levels through inhibition of catalase activity (Ding et al. 2007). The elevated hydrogen peroxide levels reduce the availability of ascorbic acid. Hence, a balance between the accumulated hydrogen peroxide levels and ascorbic acid content is required to maintain the ascorbic acid, which was achieved at lower doses of SA (0.1 and 1 mM).

The increases in phenolic content of carambola in response to the treatments avoided a senescence-associated decline. However, the lowest levels of total phenolic content in carambola was observed for fruits treated with 1 mM SA which also accounted for the highest ascorbic acid content. Moreover FRAP and DPPH assess different type of radicals, thus the combined results for all of the antioxidant tests give a more comprehensive picture of the antioxidant activity of the fruit (Moon & Shibamoto 2009).

Salicylic acid is an elicitor that is involved throughout plant growth, development and defence mechanisms. It activates the systemic acquired resistance (SAR), which is associated with increased accumulation of protective compounds, such as phenolics and PR proteins (Chen et al., 2006). SA is involved in transmitting signals that activate reactions for the biosynthesis of these defence compounds, such as phenylalanine ammonia lyase (PAL). Interestingly, PAL is the rate-limiting enzyme in the biosynthesis of SA as well, which is a hydroxybenzoic acid. Thus, an interesting feedback loop is in operation for the regulated production and transduction of the signal molecule SA.

Antioxidants can exist as simple molecules such as ascorbic acid, or more complex enzymes, but they all involve intricate regulation mechanisms. PAL is involved in the phenylpropanoid pathway, so will be a suitable candidate to explore the changes in the phenolic content. Meanwhile, POD and PPO are also important in reducing the oxidative stress, so these enzymes will be assessed as well.

4.5 Concluding remarks

Elicitation is the induction of intrinsic plant defence systems through the exploitation of biotic and abiotic stresses. This has been successfully demonstrated to enhance

production of diverse secondary metabolites with antioxidant properties. In terms of quality and consumer acceptability, it is of importance to assess the overall activity of secondary metabolites. Although individual responses of fruits to the stress hormones varied between crop type and hormone dosage, MeJA was found to generally elicit antioxidant activity of all fruits. Moreover, the role of SA as an elicitor was also exhibited through the increased antioxidant activity for the three fruits, although it was more prominent in carambola.

Changes in the antioxidant responses of carambola were of particular interest, with an interesting interplay observed at different doses of exposure to the phytohormones. Increase in the antioxidant activity of carambola was not matched by increase in total phenolic content. The action of oxidative enzymes reduces levels of phenolic content, which could also result in the reduced levels that were observed for certain treatments. Thus, it is hypothesized that the changes in the total phenolic content and antioxidant activity of the carambola fruit will be associated with altered activity of the phenylpropanoid pathway. It will be of interest to assess the changes in the activity of phenylalanine ammonia lyase (PAL), the gateway to the phenylpropanoid pathway, as well as the action of oxidative enzymes peroxidase (POD) and polyphenol oxidase (PPO).

CHAPTER 5: EFFECT OF POSTHARVEST INDUCED STRESSES ON PHENOLIC METABOLISM OF *AVERRHOA CARAMBOLA*

5.1 Introduction

As static immobile organisms, plants have adapted to their environment and learnt to exploit their inherent production of secondary metabolites (Montanaro et al. 2007).

Plants have developed fairly complex systems to reduce oxidative stress and offer protection from oxidative damage (Anttonen & Karjalainen 2006). Phenolic compounds are a large class of secondary metabolites which are strongly linked to stress tolerance of carambola, as has been seen in Section 4.4.1.1. Thus, the metabolism of these phenolic compounds is of particular interest.

Oxidation is defined as the addition of oxygen or removal of electrons from a system (Gutteridge & Halliwell 2010). A system under prolonged status of oxidation is considered to be under oxidative stress, and this imbalance leaves the system prone to damage (Brigelius-Flohé 2009). This status of oxidative stress is promoted by the presence of pro-oxidants, whilst the presence of antioxidant can inhibit or delay the oxidative damage within a system (Gutteridge & Halliwell 2010).

Some studies associate the increase in the antioxidant pool of plants during stress to enhanced enzyme activity, however this adaptive strategy to stress is still under debate (Oh et al. 2009). Phenylalanine ammonia-lyase (PAL) is considered the gateway to the biosynthesis of phenolic compounds, and some studies have reported enhanced PAL activity under various biotic and abiotic stresses (Oh et al. 2009). The activity of PAL is of particular importance, it serves as the key point where primary metabolism diverges into secondary metabolism, shikimate pathway to phenylpropanoid pathway (González-Aguilar et al. 2004). PAL has been well-characterised as the gateway to the phenylpropanoid pathway catalysing the formation of cinnamic acid from phenylalanine (Flores & Ruiz del Castillo 2014; Siboza et al. 2014; Cao et al. 2009; Oh et al. 2009; González-Aguilar et al. 2004). The activities of PAL along with the products of the phenylpropanoid pathway are closely linked to plant defence responses (Siboza et al. 2014; Cao et al. 2009).

Biotic and abiotic stresses in plants induce the activity of this enzyme (Siboza et al. 2014; Oh et al. 2009; Sanchez-Ballesta et al., 2000). Although PAL activity is light dependent, low temperatures stress was reported to induce PAL activity in the darkness (Sanchez-Ballesta et al. 2007). In various plant species under chilling stress, PAL activity and phenolic content were found to increase, which has been associated with enhanced chilling tolerance (Lafuente et al. 2003; Lafuente et al. 2001).

Peroxidase (POD) and polyphenol oxidase (PPO) are classes of enzymes universally found in all plants and catalyse the oxidation of phenolic compounds to the unstable quinones, which readily polymerise (Yingsanga et al. 2008). This activity results in tissue browning, leading to general fruit deterioration (Siboza et al. 2014; Shetty et al. 2011). Moreover, tissue browning is one of the prominent symptoms of chilling injury, and severely impacts marketability of the fresh produce resulting in postharvest losses (Shetty et al. 2011). Enhanced enzymatic activity has been widely reported as a response to chilling stresses (Siboza et al. 2014).

Browning, a symptom of chilling injury, is equally dependent on the synthesis of phenolic compounds by PAL as well as on the oxidation of phenolic compounds by the activities of PPO and POD (Yingsanga et al. 2008). Low-temperature stress responses were explored extensively, yet there is still knowledge gap with regards to the influence of these stresses on the phenylpropanoid pathway and metabolism within metabolically active tissues. Although PAL activity is commonly considered a marker of the onset of chilling injury, it is still unclear as to how the induction of PAL activity is directly associated with the mechanism of chilling tolerance.

The stress hormones MeJA and SA are closely linked to the activities of these three enzymes. Flores and Ruiz del Castillo (2014) reported an increase in the content of myricetin and quercetin in raspberries subjected to 0.1 mM MeJA. This increase was

associated with enhanced PAL activity and downstream phenylpropanoid pathway. Although SA induces the activity of PAL, PAL is also the rate-limiting enzyme in SA biosynthesis (Sun et al. 2013). Thus, an intricate regulation mechanism exists in the regulation of the activities of PAL, POD and PPO in response to the stress hormones MeJA and SA.

In the previous chapter, potent antioxidant activity was reported for carambola during cold storage, and the stress hormones resulted in enhanced antioxidant activity and ascorbic acid content. However, total phenolic content was only induced during the initial storage period for the treated fruits. Moreover, a relationship between the enzymes involved in the metabolism of phenolic compounds and the occurrence of CI in carambola has been demonstrated by Perez-Tello et al. (2001). Browning is a key symptom of CI in carambola and a major determining factor for the end of shelf life (Ali et al. 2004; Pérez-Tello et al. 2001). Thus, aesthetic value of fruits and nutritonal quality are both linked to the phenolic content of the fruit.

PAL, PPO and POD are all involved in the metabolism of phenolic compounds in fruits, and this interaction becomes even more complex under stresses induced by cold storage and the exposure to stress hormones MeJA and SA. Therefore, the aim of this work was to demonstrate an interaction between metabolism of phenolic compounds and stress responses in tropical fruits using carambola as a model fruit.

5.2 Materials and methods

Carambola fruit was subjected to the treatments described in Section 2.2.1 and 2.2.2 and used for assessment of the enzymatic antioxidant activity of the fruits. Extraction of PAL enzyme (5.2.1) and extraction of POD and PPO enzyme (5.2.1) was carried out using four biological replicates.

5.2.1 Preparation of enzyme extract

For the analysis of PAL activity, fresh fruit tissues (2 grams) were homogenized with 100 mg PVP and 10 ml sodium borate buffer (0.06 M, pH 8.8) followed by centrifugation at 10,000 rpm and 4°C for 10 minutes. The supernatant was used immediately as enzyme extract. Three replicates were used for each extraction.

Meanwhile, the enzyme extract used for assessment of POD and PPO was extracted differently. The enzyme was extracted from the fruit by homogenizing 1 gram of fruit sample with 5 ml potassium phosphate buffer (0.05 M, pH 6.6) and centrifuged at 10,000 rpm and 4 ^{0}C for 10 minutes. Three replicates were used for each extraction.

5.2.2 Phenylalanine ammonia lyase (PAL) analysis

PAL activity was assessed based on the method adapted from Benoit et al. (2000). The assay mixture contained 2 ml sodium borate buffer (0.06 M) and 1 ml phenylalanine (11 mM). 1 ml of the enzyme sample (Section 5.2.1) was mixed in the assay mixture and incubated at 37 °C for two hours. The reaction was stopped by the addition of 0.5 ml of 35 % Trifluoroacetic acid (TFA). Absorbance at 290 nm was monitored by using Varioskan Flash Multimode Reader (Thermo Scientific, USA) for 3 minutes. An increase in absorbance of 0.01 was considered as one unit (Cao et al. 2010), and the units were expressed as changes in optical density per minute per gram fresh weight.

5.2.3 Peroxidase (POD) analysis

The POD activity was determined by assessing the conversion of guaiacol to tetraguaicol, using the method adopted from Macadam et al. (1992). A 0.1 ml aliquot of the enzyme extract (Section 5.2.1) was mixed with 2 ml guaiacol (8 mM in 100 mM sodium phosphate buffer, pH 6.4) and incubated at 30 ^{0}C for 30 minutes followed by the addition of 1 ml hydrogen peroxide (24 mM). The absorbance readings were taken at 460 nm using Varioskan Flash Multimode Reader for 3 minutes and the units were expressed as changes in optical density per minute per gram fresh weight.

5.2.4 Polyphenol peroxidase (PPO) analysis

Assessment of PPO enzyme activity was determined by measuring conversion of catechol to quinine, adapted from Cao et al (2010). The assay mixture contained 2 ml potassium phosphate buffer (0.05 mM, pH 6.6), 1 ml catechol (0. 1M) and 0.2 ml of enzyme extract (Section 5.2.1). Absorbance of the reaction mixture was immediately measured at 410 nm using Varioskan Flash Multimode Reader. The reaction mixture was allowed to react for 3 minutes and absorbance was measured again at 410 nm. The units were expressed as changes in optical density per minute per gram fresh weight.

5.3 Results

5.3.1 PAL activity

The activity of PAL was assessed throughout the storage period for fruits that were exposed to the stress hormones (Figure 5.1). No significant interaction ($p > 0.05$) (Appendix B 5.12) was reported for treatment and time for PAL activity. PAL activity was inversely related to concentration of MeJA. The control fruits peaked at a significantly higher ($p < 0.05$) value than the MeJA treated fruit. These results follow the same pattern observed for phenolic content, where higher values were also observed for the control in comparison to MeJA treated fruits (Figure 4.3). This demonstrates an overall reduction in phenolic content of carambola in response to MeJA induced stress.

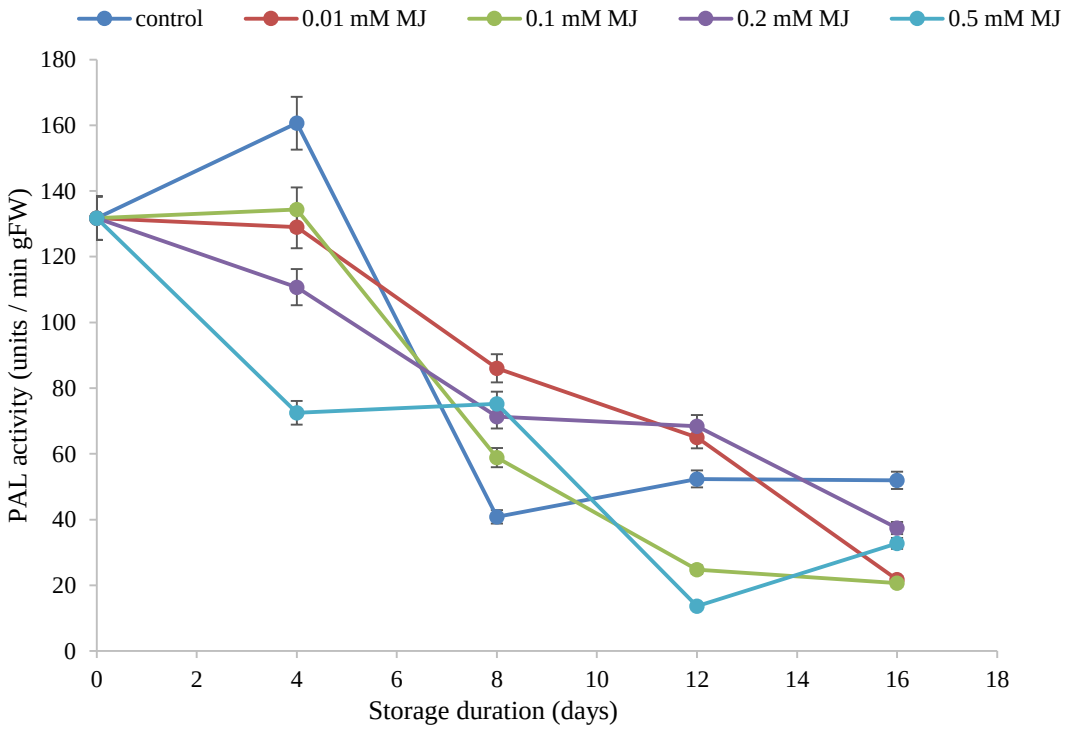


Figure 5.1 Effect of different concentrations of methyl jasmonate on PAL enzyme activity of carambola stored at 6 °C. Values are the means (n=4) ± SE

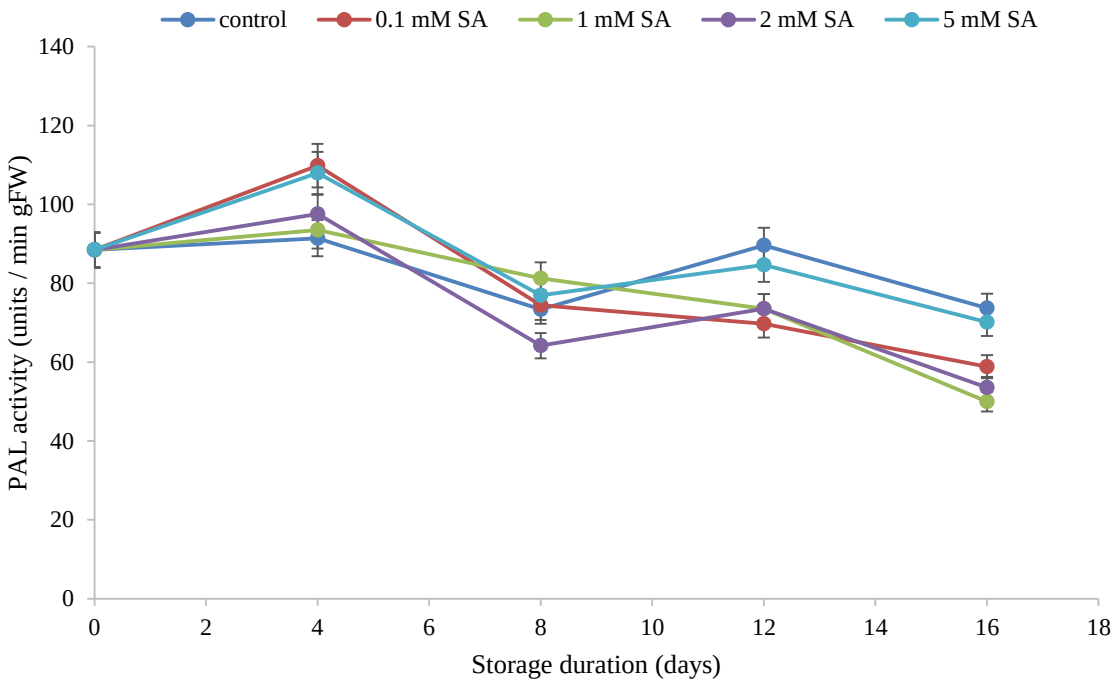


Figure 5.2 Effect of different concentrations of salicylic acid on PAL enzyme activity of carambola stored at 6 °C. Values are the means (n=4) ± SE

No significant interaction ($p > 0.05$) (Appendix B 5.2) was reported for PAL activity in the SA experiment (Figure 5.2). Generally, the control maintained the highest readings, however the standard deviation was large and there was no significant difference ($p > 0.05$) between most treatments. There was a general decline in PAL activity as the storage period increased, with the exception of the control and 5 mM SA treatment. The reported values for total phenolic content (Figure 4.4) followed a similar trend to the PAL activity, and were clustered around the control.

5.3.2 POD activity

A significant interaction ($p < 0.0.5$) (Appendix B 5.3) between time and treatment was reported for POD activity. Initially, the highest activity was observed for fruits treated with 0.5mM and 0.2 mM MeJA, as a transient dose response for these two treatments (Figure 5.3). Towards the end of the storage period, activities of fruits treated with 0.01 mM and 0.1 mM MeJA increased rapidly. No significant difference ($p > 0.05$) was observed between the highest levels of activity for 0.01 mM MeJA and 0.5 mM MeJA. Throughout the storage period, POD activity of the untreated fruits gradually increased to two-fold the initial activity level, but no further change was observed. MeJA might be involved in protective functions that prevent physiological disorders of the membrane systems through the increased accumulation of peroxidases.

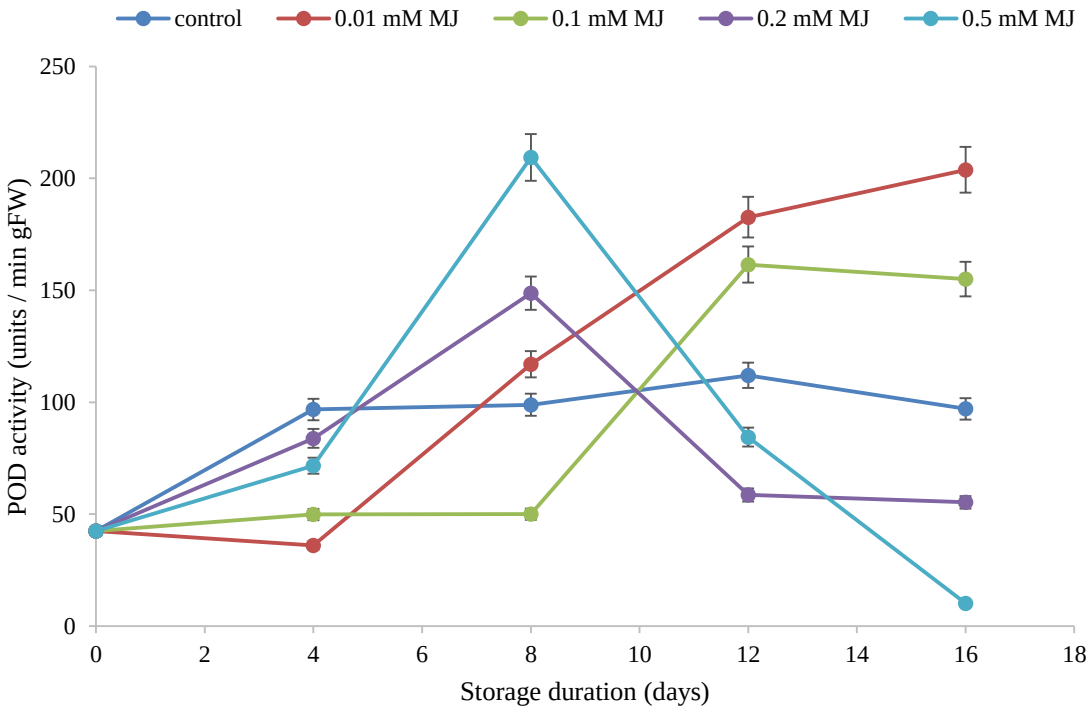


Figure 5.3 Effect of different concentrations of methyl jasmonate on POD enzyme activity of carambola stored at 6°C. Values are the means (n=4) ± SE

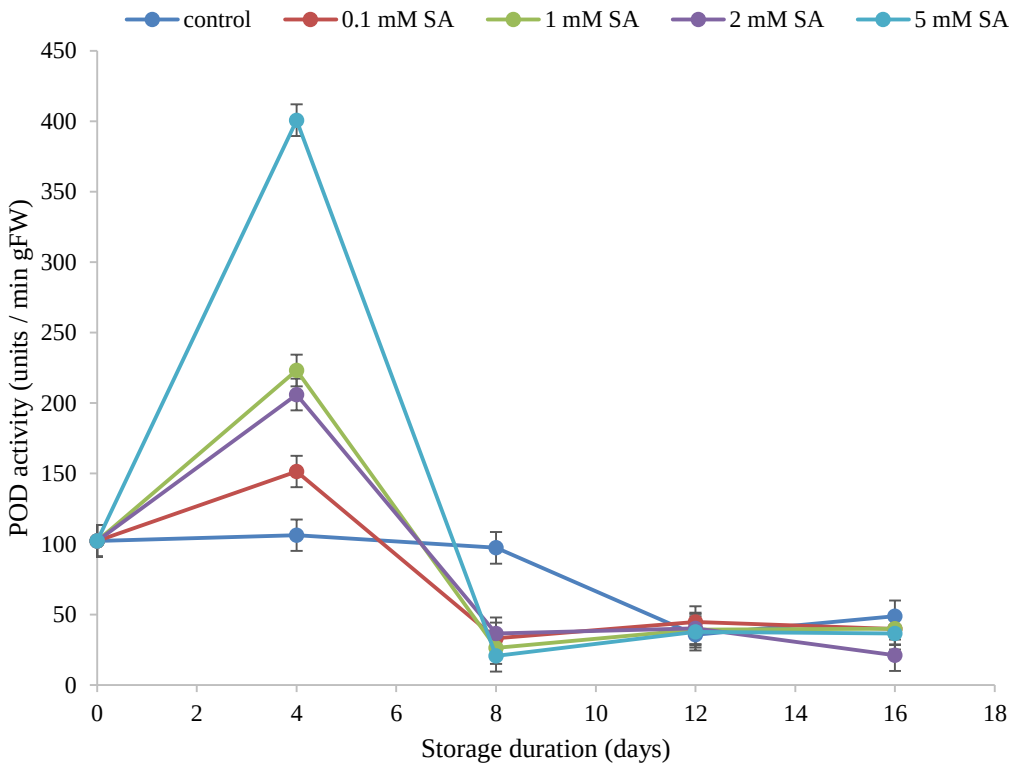


Figure 5.4 Effect of different concentrations of salicylic acid on POD enzyme activity of carambola stored at 6 °C. Values are the means (n=4) ± SE

POD activity was positively correlated to SA concentration (Figure 5.4) with the higher concentrations resulting in significantly higher ($p < 0.05$) (Appendix B 5.4) POD activity. This dose response was exhibited on the 4th day of storage, and appeared to be transient. Studies on tobacco demonstrated that SA induces the expression of peroxidases, which are induced upon infection during development of systemic acquired resistance (Raskin 1992). This is in agreement with the results obtained in this experiment. Thus, the exogenous application of SA induced a stress response similar to infection, increasing the scavenging of ROS.

5.3.3 PPO activity

Meanwhile, PPO activity for the MeJA treated fruits was clustered around the control (Figure 5.5), yet a significant interaction ($p < 0.05$) (Appendix B 5.5) was reported. Activity peaked for all of the treatments on the 8th day of storage, and the highest peak was observed for the two lowest SA treatments (0.01 mM and 0.1 mM MJ). Throughout the experiment, fruits treated with 0.5mM MeJA maintained the lowest activity.

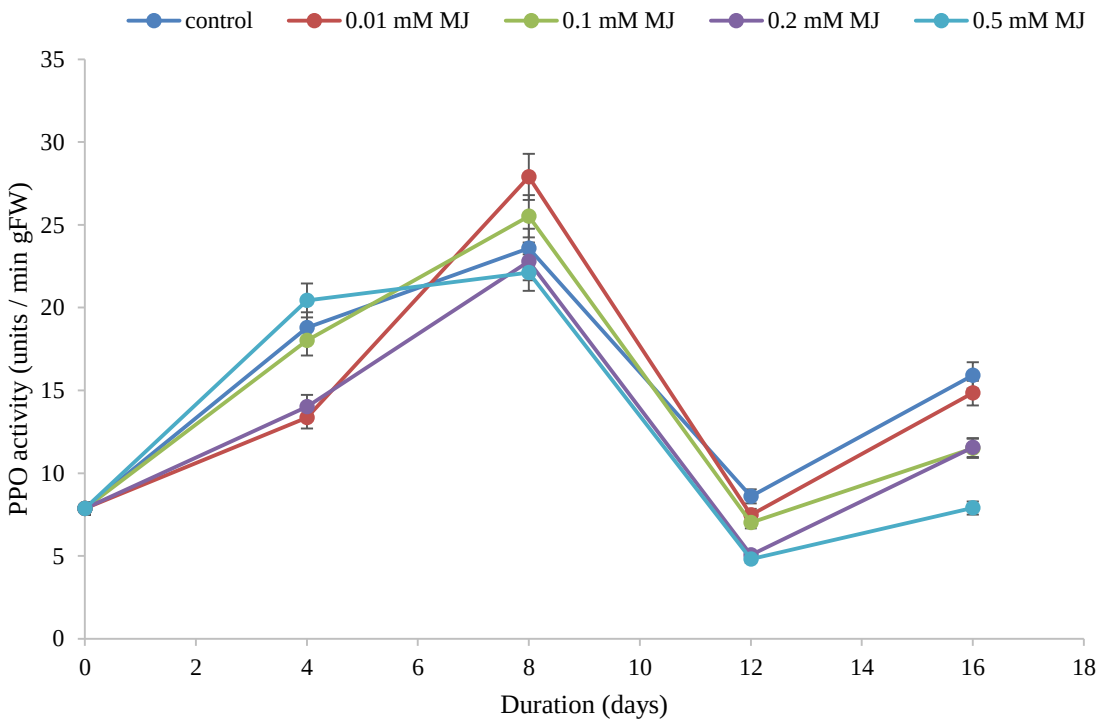


Figure 5.5 Effect of different concentrations of methyl jasmonate on PPO enzyme activity of carambola stored at 6 °C. Values are the means (n=4) ± SE

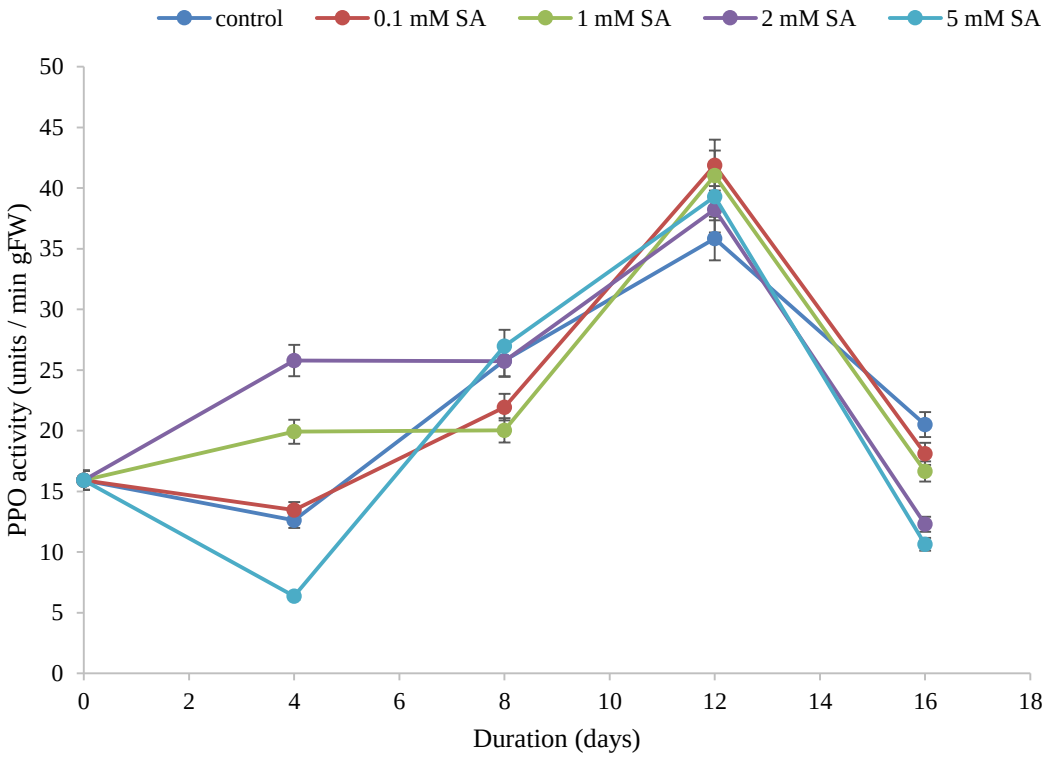


Figure 5.6 Effect of different concentrations of salicylic acid on PPO enzyme activity of carambola stored at 6 °C. Values are the means (n=4) ± SE

With regards to the SA treated fruits, treatment with 1 mM and 2 mM SA resulted in significantly higher ($p < 0.05$) activity (Appendix A 5.6) on the fourth day of storage (Figure 5.6). As the storage period increased, the PPO activity values were clustered around the control, with some treatments having lower activity than the control. However, the lowest activity was observed for 5mM SA during the initial stages of storage as well as at the end of the storage period. This follows the same pattern observed for MeJA whereby the highest dose of phytohormones repressed the enzymatic activity as fruit ripening progressed.

5.4 Discussion

SA and MeJA are universal stress signals, with the capacity to function antagonistically or synergistically in order to overcome biotic and abiotic stresses (Pieterse et al. 2012). These two hormones interact with ethylene, and together are involved in various roles throughout plant growth and development (Shetty et al. 2011). SA and MeJA are generally associated with maintaining fruit quality under chilling stress through the alleviation of chilling injury symptoms and activating intrinsic defence mechanisms. CI is associated with the degradation of membrane integrity allowing the intermixing of enzymes and substrates such as PPO and phenols (Ali et al. 2004). PAL is also prominent in phenolic metabolism, thus is directly involved in acclimation against chilling stress. Thus, the abundance of phenolic compounds and PPO activity, as well as availability of ascorbic acid and POD, are important factors in determining the extent of tissue browning (Meng et al. 2009).

5.4.1 PAL activity and biosynthesis of phenolic compounds

Jasmonates activate the phenylpropanoid pathway in various climacteric and non-climacteric fruits (Concha et al. 2013). Application of MeJA enhances the activity of PAL, as shown in studies conducted on lettuce, potatoes and carrots (Heredia & Cisneros-Zevallos 2009a). In cherries, Yao and Tian (2005) reported enhanced PAL and POD activity, resulting in lignin biosynthesis. SA positively regulates SAR whilst inhibiting octadecanoid mediated defence responses (Shetty et al. 2011). Nonetheless, the defence responses generally associated with SA and MeJA differ slightly between plant species owing to the distinct regulatory elements characterising the specific plant (Shetty et al. 2011). The activities of these enzymes has been assessed in carambola in

response to chilling injury, however, the effect of phytohormones has not been fully explored (Pérez-Tello et al. 2001).

The phenylpropanoid pathway is an integral part of the protection mechanisms of stressed cells. Inhibition of PAL is highly damaging to the plant, as was observed in plants with supressed levels of PAL where spontaneous necrosis was detected (Tamagnone et al. 1998). Reduced PAL activity is linked to reduced accumulation of phenolic compounds, as well as reduced biomass accumulation during growth (Oh et al. 2009). Thus, inhibition of the phenylpropanoid pathway clearly impacts the general well-being of the plant.

The results reported here demonstrate initially high PAL activity, which was followed by a general decline in PAL activity, although a lower decline was observed for the control fruits throughout the storage period. Treatment of carambola with 0.1 mM and 5 mM SA were the only treatments that resulted in a transient increase in PAL activity, while treatment with 0.01 mM MeJA slightly delayed the decline of PAL.

Oh et al (2009) had reported a similar response in lettuce as PAL activity was inherently activated and found to be high in the control plants as well. The elevated PAL activity might be an adaptive response to the stress induced by the cold storage. Temperature is a major influencer of PAL activity, as it is generally elevated under cold stresses allowing acclimation against chilling (Siboza et al. 2014; Tomas-Barberan & Espin 2001).

Antioxidant biosynthesis is enhanced under stress, allowing the accumulation of protective compounds for stress alleviation or prevention (Oh et al. 2009). Hence, deficiency of secondary metabolites or inhibition of PAL activity could produce plants that are increasingly sensitive to environmental stresses. The decrease in the antioxidant pool allows for higher ROS levels which can be observed through pronounced chilling

injury symptoms (Oh et al. 2009). However the physicochemical changes documented for the treated carambola fruits (Section 3.4.1.1) do not reflect the occurrence of chilling injury.

Carambola was characterised with an inherently high abundance of diverse phenolic compounds with varied functions, thus defence strategies under stress might involve a mechanism besides the increased activity of the phenylpropanoid pathway, which could also involve the release of conjugated phenolic compounds. It will be of interest to explore the changes in the specific phenolic content of the fruits that were subjected to the stress hormones to observe any changes in the fruit phenolic profile.

5.4.2 POD and PPO activity and oxidation of phenolic compounds

Generally, stresses such as chilling result in increased PPO and POD activity, which is accompanied with marked symptoms such as pitting and browning (Siboza et al. 2014). As these enzymes catalyse the oxidation of antioxidants, a decrease in the antioxidant pool of the fruit is experienced. Consequently, this could increase the fruit's susceptibility to chilling injury, however since these enzymes increase ROS scavenging in the process, thus the phenomenon of chilling tolerance is much more complex (Siboza et al. 2014; Tomas-Barberan & Espin 2001).

Phenolic compounds are located in vacuoles while PPO is located in plastids, thus PPO mediated browning only occurs upon disruption of intracellular compartmentalisation (Yingsanga et al. 2008). Meanwhile, POD activity is induced by injury and during wound repair and disease resistance. Phenolic compounds are oxidised to the anti-herbivore compound quinone, reducing the nutritional value of plants to herbivores by alkalising essential amino acids found in dietary proteins (Shetty et al. 2011; Thipyapong et al. 2004). Quinones are highly reactive and are directly

involved in the oxidative browning of plant tissue during biotic and abiotic stresses. Moreover, quinones have antimicrobial activity and the hydrogen peroxide signals that induce PPO and POD expression also possess antifungal activity. This indicates a possible cross-talk between biotic and abiotic signalling that involves PPO and POD, with the ultimate aim of ensuring plant survival in unfavourable conditions (Thipyapong et al. 2004).

In carambola fruit, an increase in POD and PPO activity was reported for fruits stored at 2 ^{0}C (Pérez-Tello et al. 2001). In this experiment a significant increase in POD activity was observed for both the SA and MeJA treated fruits. POD activity was inherently high, even for the untreated fruits and this could be attributed to the fruit response to cold storage. Concha et al. (2013) also reported an increase in POD activity upon exposure to MeJA. However, contradictory results were reported in other studies, for example Siboza et al. (2014) reported an inhibition in POD activity, along with PPO and lipid peroxidation in response to 2m M SA and 0.01 mM MeJA.

PPO expression is regulated through the octadecanoid pathway and by the stress signal systemin, and is also associated with the phenylpropanoid pathways (Thipyapong et al. 2004). Early reports demonstrated that MeJA and SA induce the expression of tomato and potato PPO promoter (Thipyapong et al. 1997; Thipyapong et al. 1995). In another study on tomato by Shetty et al. (2011) PPO was induced by the application of MeJA and systemin, where a 70-fold increase in PPO was reported upon exposure to MeJA vapours. The activity of PPO is not limited to stress regulation but also involves defensive responses, as high activity is usually detected in abscission zones along with PR proteins (Thipyapong et al. 2004).

In previous studies on tomato and potato, the overexpression of PPO enhanced resistance against pathogens, while down-regulation of PPO produced highly

susceptible plants (Shetty et al. 2011). Nonetheless, no difference in PPO activity was observed in response to the stress hormones in this study. This was in agreement with Perez-Tello et al. (2001) who reported no significant effect of cold storage on PPO activity of carambola. Thus, PPO activity does not serve as a good indicator of chilling injury for carambola. Moreover, the specific mechanisms that regulate POD and PPO activity are also very different, as can be observed from the differences in the activities of these two enzymes in the same commodity.

5.4.3. Influence of stress hormones on phenolic metabolism

Enzymatic responses to the phytohormones varied depending on the concentration applied. Low concentrations of SA and MeJA applied individually or concomitantly can enhance the expression of both SA and MeJA responsive genes (Shetty et al. 2011). In the MeJA experiment, an increase in PAL activity was observed for the control on the 4^{th} day of storage, and this was followed by an increase in total phenolic content on the 8^{th} day of storage. Meanwhile, the lowest phenolic content was observed for 0.5 mM MeJA, which also exhibited the lowest PAL activity throughout the storage period.

Enhanced PAL activity as a response to exogenous MeJA has been reported on various fruits (Flores & Ruiz del Castillo 2014; González-Aguilar et al. 2004). Siboza et al. (2014) reported that SA at 2 mM and MeJA at 0.01 mM induced cold acclimation, through the induction of PAL and increased accumulation of phenolics. Moreover, Campos-Vargas and Saltveit (2002) also reported increased PAL activity upon exposure to exogenous MeJA. Nonetheless, a general decline in PAL activity during fruit ripening was observed in this experiment. Reduced PAL activity appears to be a mechanism for chilling tolerance, as reduced PAL activity and concomitant decline in

CI was also observed after heat pre-treatment (Chen et al. 2008; Sanchez-Ballesta et al. 2000) and modified atmosphere packaging (Nguyen et al. 2004).

Meanwhile, in the SA experiment, PAL activity was at its highest for the control and 5 mM SA treatment, both of which exhibited high TPC, while the other SA treatments demonstrated lower PAL activity. An inhibition of PAL activity by SA treatment was reported on bamboo (Luo et al. 2012), pomegranates (Sayyari et al. 2009), loquat (Cai et al. 2006), mangosteen (Dangcham et al. 2008), and pineapple (Lu et al. 2010). Thus, the specific involvement of PAL in alleviation of chilling injury is not clearly defined, while various other defence responses might be coordinated to enhance chilling tolerance. Stress hormones might offer chilling tolerance by the inhibition of PAL activity and shifting towards phenolic metabolism rather than biosynthesis, while also enhancing the ascorbate-glutathione reducing status (Section 4.3.1.1).

Moreover, 0.5 mM MeJA exhibited the highest POD and PPO activity during the storage period, which could also account for the low phenolic content. POD and PPO both play antagonistic roles to PAL and phenolic content in the alleviation of chilling injury (Siboza et al. 2014). Thus, different responses were observed for PAL and PPO/POD activity.

On the 4th day of storage the highest TPC was observed for the 0.1 mM SA treatment which also exhibited the lowest POD activity at that time-point. Similarly, fruits treated with 1 mM and 2 mM SA displayed the highest PPO activity on the 4th day of storage as well as the lowest TPC at that time point. Studies by Thaler et al. (2002) reported the antagonistic activity of high doses of SA, while Shetty et al. (2012) reported an inhibition of PPO activity at high SA doses. This is in agreement with the

hypothesis that high doses or prolonged exposure to these elicitors has negative effects on their associated responses.

Cells increasingly turn to reserves of antioxidants such as phenolic compounds or ascorbic acid to maintain internal homeostasis (Zainudin et al. 2014; Sayyari et al. 2009). This creates an interesting interplay of antioxidant reserves as cells synthesise and metabolise the available resources. Thus, dissecting changes in the different compounds that make up the total phenolic compounds might shed some knowledge on the defence mechanisms taking place.

The activities of these three enzymes play a key role in influencing the abundance of phenolic compounds. Changes in the total phenolic content is linked to the expression of the biosynthesis enzymes PAL and the oxidising enzymes PPO and POD. Decreased PAL activity along with the concomitant increase in PPO and POD activity could result in lower abundance of phenolic compounds (Ortega-García & Peragón 2009a; Ortega-García & Peragón 2009b). However, other mechanisms might also be in play for balancing the phenolic content, such as changes in the levels of internal antioxidant enzymes such as SOD or increase turnover of unconjugated phenolic compounds.

5.5 Concluding remarks

Activity of the enzymes PAL, PPO and POD directly influence phenolic metabolism and the onset of chilling injury on the fruits. SA and MeJA are principal signalling molecules that regulate a variety of plant defence responses. The results demonstrated the ability of SA to regulate the activity of PAL, inducing the phenylpropanoid pathway for increased production of protective secondary metabolites under stress. Moreover, through SA induced activity of POD, tolerance to environmental stresses was enhanced

allowing for increased ROS scavenging. Therefore, SA as an elicitor at low levels has the capacity to mimic natural defence responses and promote resistance against stresses.

Thus, it will be interesting to further explore phenolic metabolism by analysing the specific changes that are occurring in the total phenolic content of these fruit samples. To allow a more detailed study, carambola fruits under MeJA and SA stresses will be selected for the further analyses, since the phenolic content is responsible for a significant portion of the fruit's antioxidant activity. The following chapter will only focus on the fruits sampled under immediate stress and fruits that have experienced the induced stresses for 4 days of cold storage, in order to provide a clearer portrayal of the direct response mechanisms that are in play.

CHAPTER 6: CHARACTERISATION OF PHENOLIC CONTENT IN CARAMBOLA FRUIT DURING STORAGE

6.1 Introduction

Products of the phenylpropanoid pathway, of which a number are associated with astringency and unique fruit flavour attributes, are important in fruit development rendering the fruit nutritional and aesthetic value (Fock-Bastide et al. 2014; Tucker 1993). Phenolic compounds have the capacity to function as antioxidants and reduce oxidative stress through the termination of oxidation chain reactions. Thus, the phenylpropanoid pathway is implicated in fruit quality maintenance and is an important pathway in studying the development and ripening of fruits.

The phenylpropanoid pathway plays a key role in stress responses through the biosynthesis of plant secondary metabolites such as lignin (Singh et al. 2010). Lignin biosynthesis is a regulated stress response that offers certain fruits such as mangosteen more protection through enhanced firmness (Seymour et al. 2008; Cai et al. 2006; Ketsa & Atantee 1998). The initial steps of the phenylpropanoid pathway produce hydroxycinnamic acids such as *p*-coumaric acid and ferulic acid, downstream of which lignin biosynthesis and production of other classes of phenolic compounds occurs (Fraser & Chapple 2011; Singh et al. 2010).

Fruits and vegetables are increasingly gaining popularity owing to the numerous health benefits, a few of which are generally attributed to the total phenolic content (Anttonen & Karjalainen 2006; Wen et al, 2005). This has generated vast interest in understanding phenolic compounds, which has driven significant progress in the research and analytical tools for quantification and characterisation of phenolic compounds. These methods involve spectrophotometric methods that are used to

quantify the antioxidant activity of the compounds, as well as reversed-phase high performance liquid chromatography (RP-HPLC) (Wen et al. 2005).

Changes in the metabolite profile of plants can also serve as a good indication of the levels of stress the plant is enduring as well as the plant responses (Noctor et al. 2014). Phenolic acids are one of the main classes of phenols, along with flavonoids, coumarins, tanins and lignin. They are all derived from phenylalanine and tyrosine and can carry out various biological functions such as antioxidants, attractants and antifeedants (Gutteridge & Halliwell 2010). Phenolic compounds commonly function as antioxidant compounds and are involved in necrosis and maintaining cell viability.

The polyphenolic content of tropical fruits is reportedly high, functioning as stress regulators and antioxidants during fruit ripening and senescence. Carambola contains a high level of polyphenolics which correlates with the high antioxidant activity of this fruit (Dembitsky et al. 2011). In Section 4.3.1.2, the polyphenolic content of carambola was reported at above 200 mg GAE/g FW throughout most of the storage period. According to Shui and Leong (2006) the antioxidant activity of carambola residue extract was largely attributed to the proanthocyanidin content of the fruit, although it is also rich in ascorbic acid.

Treatment of the fruit with low doses of phytohormones generated an immediate response on the phenylpropanoid pathway, which was observed on the 4^{th} day of storage. Total phenolic content was elevated for the treated fruits, with the exception of the fruits treated with 1 mM SA where a decline in total phenolic content was observed. Moreover, the results observed for total phenolic content followed the same trend for antioxidant capacity. Thus, fruit samples from the first 4 days of storage were selected for further characterisation of the specific changes in the phenolic content, as an indication of the responses to the stress hormones.

The phenolic content of carambola is largely attributed to the presence of flavonol and hydroxycinnamic acids, which may decline during fruit ripening (Castrejón et al., 2008). Nonetheless, increases in the total phenolic content of fruits and vegetables following stress treatments are largely attributed to changes in the content of specific phenolic compounds. Lettuce leaves subjected to stress treatments for three days were found to accumulate caffeic acids and flavonoids during recovery of the plant material (Oh et al. 2009). Phenolic content of fruits can decline during cold storage which could impair the defence mechanisms of the fruit and enhance the onset of chilling injury (Tomas-Barberan & Espin 2001).

Scavenging of radical oxygen species by phenolic compounds is a key defence strategy that maintains fruit quality (Siboza et al. 2014). Elicitors invoke biotic stresses within the plant and influence physicochemical responses while regulating secondary metabolism (Dong et al. 2010). This ultimately enhances the production of secondary metabolites that serve as antioxidants. Decline in TPC was observed for carambola fruits treated with the stress hormones, which was synchronised with a general decline in PAL activity as well. Nonetheless, the quality of carambola was not compromised during cold storage, thus the defence mechanisms induced by the elicitors might be too intricate to be observed through a general screen of phenolic content. Thus, changes in the specific phenolic content were characterised.

To date, there have been numerous studies on the characterisation of the content and nutraceutical value of fruits, however there is limited information on the specific phenolic compounds and their role during fruit ripening and stress tolerance. In this light, the aim of this study was to establish a better understanding of postharvest stresses, induced by a combination of chilling and plant stress hormones, on the profiles of phenolic compounds in carambola.

6.2 Materials and methods

Carambola fruit was subjected to the treatments described in Section 2.2.1 and 2.2.2 and used for the characterisation of phenolic compounds. Four biological replicates were used for the preparation of the extracts used in this study.

6.2.1 Preparation of extracts

Selected carambola samples (Table 6.1) were selected for this study. The samples were extracted with methanol-water (60:40, v/v), acetone (60:40, v/v), ethyl acetate (60:40, v/v) and hexane (60:40, v/v) at 35°C for one hour (Section 2.3). Extracted fruit material was concentrated using a Buchi Rotavapor R-200 (Buchi, Switzerland) at 50 °C to a final volume of 2ml. Extracts were filtered using 0.45 µM nylon membrane filter (Agilent, Germany) to obtain clear hydrophilic solution, and stored at -20 °C until analysis.

Table 6.1 Carambola fruit samples selected to characterise the phenolic content

Days in storage	**Treatment**	**Assigned name**
0	None	SFD0
4	None	SFD4
4	0.01mM MeJA	SFD4MJ1
4	0.1 mM MeJA	SFD4MJ2
4	0.1mM SA	SFD4SA1
4	1mM SA	SFD4SA2

6.2.2 Antioxidant screening of fractions

Antioxidant activity was determined by total phenolic content (TPC) (Section 4.2.2) and ferric reduction activity potential (FRAP) (Section 4.2.4). TPC was analysed using the Folin-Ciocalteu method and results were expressed as µg gallic acid equivalent

(GAE)/g FW. FRAP antioxidant activity was expressed as Trolox equivalent (TE) µM/g FW.

6.2.3 Solid-phase extraction (SPE)

Solid-phase extraction (SPE) was carried out using Waters Oasis HLB cartridges (Waters, 3 cc/60 mg) according to the method of Savage et al. (2011). The cartridges were conditioned with 4ml of methanol followed by 4ml of deionised water. The sample (S1), at a concentration of 2mg/ml, was loaded at a volume of 4ml and the non-retained compounds were eluted under vacuum to give the first fraction (F1). Deionised water was then used to wash the cartridge (4ml) and collected as the second fraction (F2), before the third and final fraction (F3) was eluted using methanol (4ml). Thus, S1, F1, F2, and F3 were collected and used to compare total phenolic content in the different fractions.

6.2.4 Reversed phase- high performance liquid chromatography (RP-HPLC) analysis

Reverse phased-high performance liquid chromatography (RP- HPLC-UV) analysis was performed using an Agilent HPLC 1260 Infinity (Agilent, Germany) equipped with a quaternary pump, an online vacuum degasser, an autosampler, a thermostated column compartment and a UV/vis detector. A 250 mm x 4.6 mm i.d., 5 µm, Zorbax C18 column (Agilent, Germany) was used at a flow rate of 0.8 mL/min, injection volume of 5 µl and temperature of 25 °C.

HPLC water was prepared from distilled water using a Milli-Q system (Millipore Lab, USA) and HPLC grade methanol (RCI-Labscan, Thailand) was used. The standards tested, which included the hydroxycinnamic acids *p*-coumaric acid,

gallic acid and salicylic acid; as well as flavan-3-ols catechin and epicatechin were purchased from Sigma (Sigma-Aldrich, USA).

Two solvents, namely A (0.1 % phosphoric acid in water) and B (methanol) were used in a gradient run, and detection was at a wavelength of 280 nm. External standards were used for quantification, where calibration equations (Appendix A 6.1 – 6.5) for linear regression were established using the detected peak areas and known concentrations of the standards (50-500 ppm). Spectral data and retention times of the chromatograms from the samples were analysed using Chemstation for LC systems (Agilent, Germany) and compared with the standards to identify the phenolic content. Before starting each run, the HPLC system was warmed up and the baseline was allowed to stabilize. The gradient was varied linearly from 20 % to 22 % B (v/v) in 5 minutes, to 30 % B in 10 minutes, to 35 % B in 8 minutes, to 40 % B in 4 minutes, to 80 % B in 8 minutes and held at 80 % for 5 minutes, before the system was flushed with 85 % methanol for 10 minutes.

6.3 Results

6.3.1 Solvent fractionation

Carambola is rich in polyphenols and vitamin C (Leong & Shui 2002), which is associated with the high activity detected in the hydrophilic extracts of the fruit. Fruit samples were extracted with different solvents and the activity based on FRAP and TPC was assessed (Figure 6.1). The antioxidant activity of carambola methanolic and acetone extracts were significantly higher ($p < 0.05$) in comparison to the other extracts. The recorded antioxidant activity was generally low for the other extracts. A similar trend was also detected for the total phenolic content, where the carambola methanolic and acetone extracts were significantly higher than the other extracts ($p < 0.05$) with the methanolic extracted yielding the highest activity overall. Thus, methanolic extract was used for further analysis of the carambola phenolic content.

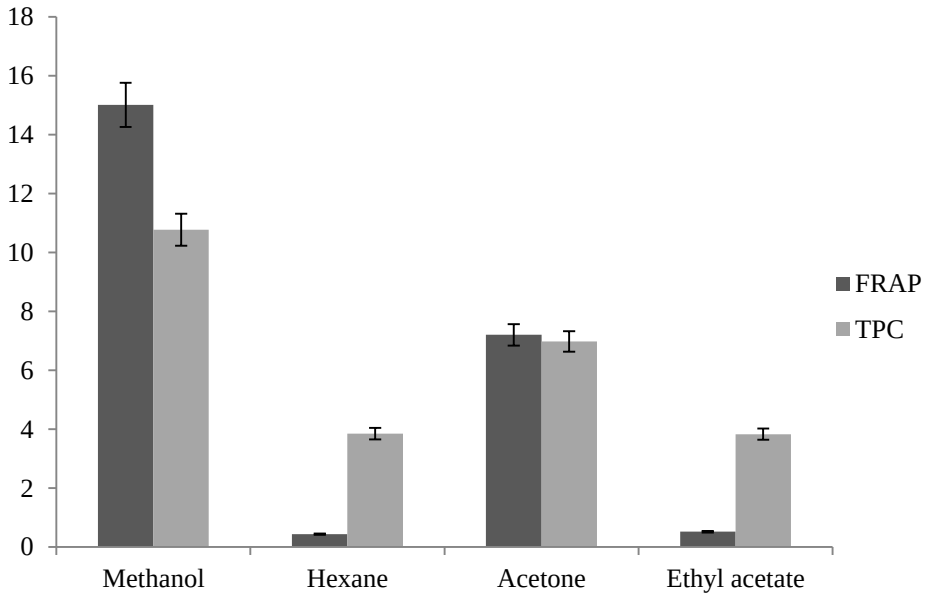


Figure 6.1 Antioxidant activity and total phenolic content of freshly harvested carambola sample extracted with four different solvents; methanol, hexane, acetone and ethyl acetate (n=4)

6.3.2 Solid phase extraction (SPE)

The total phenolic content of carambola was determined after fractionation using solid phase extraction (SPE) columns (Figure 6.2). Carambola samples that were selected from previous results (Table 6.1) were extracted with methanol (S1) and fractionated using Oasis SPE columns to yield three fractions (F1, F2, and F3) and the four fractions were assessed for total phenolic content. The majority of the total phenolic content was eluted in the third fraction (F3) for all of the extracts. The recovery of the total phenolic content was 50 % or less for Day0, Day4 and SAT2 samples. These extracts were used in subsequent analyses comparing the bioactivity of the extracts of the different fruit samples (Chapter 7).

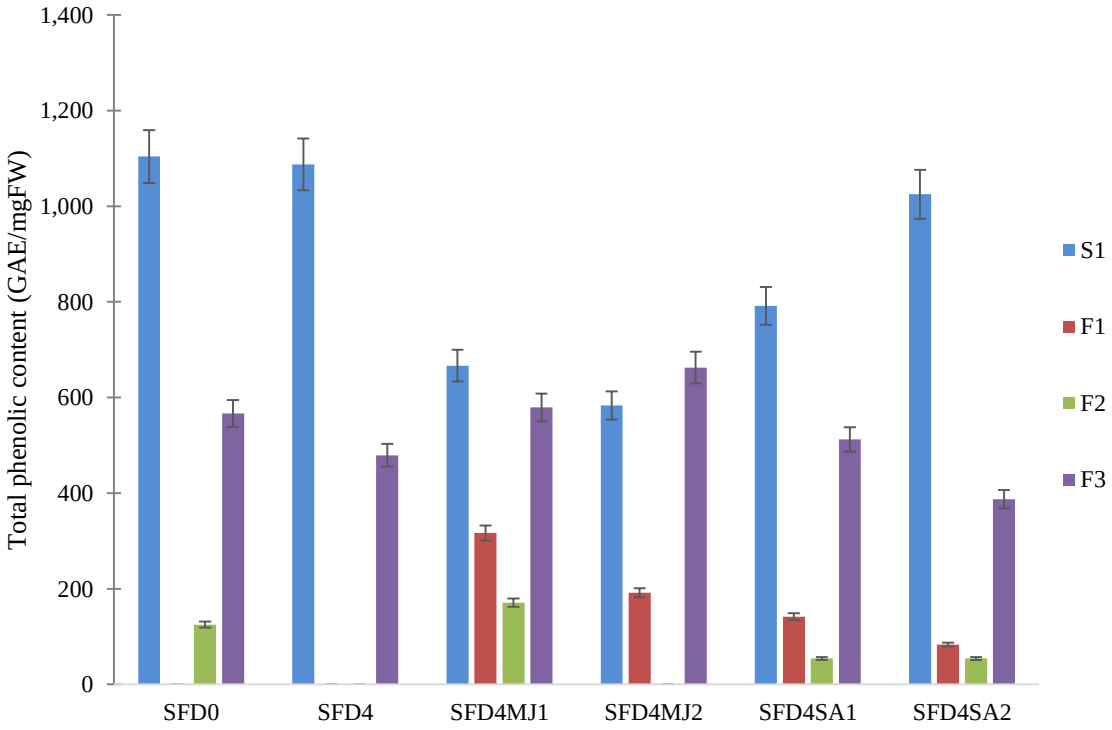


Figure 6.2 Total phenolic content of original extracts at a concentration of 2 mg/ml and resultant SPE fractions (S1, F1, F2 and F3) of carambola samples. Values are means (n=5) ± SE.

6.3.3 Reverse-phased high performance liquid chromatography (RP-HPLC)

Figure 6.3 shows chromatograms of typical samples for carambola extract. The gradient runs were optimized using an aqueous methanol solvent system, and comparable retention times were obtained. The typical modifiers incorporated in the solvent system are sulfuric, formic, acetic or phosphoric acid (Xu et al., 2008; Wen et al., 2005). Phosphoric acid was used in this gradient, with the flow rate adjusted to 0.8 ml/min, for efficient separation of the analytes. The composition of the solvent system was initially varied repeatedly (results not shown) until the resolution of phenolic compounds was satisfactory.

Calibration curves based on linear regression analysis were determined by plotting the peak areas of the phenolic standards (gallic acid, caetchin, epicatechin, *p*-coumaric acid, salicylic acid) against the corresponding concentrations (50 -500 ppm). The five phenolic standards showed good linearity ($r^2 > 0.98$) (A6.1 – A6.5) in the range of concentrations tested for the optimized gradient. This demonstrates that the analytical method established for RP-HPLC allows for the quantification of the specified phenolic compounds, if present in the fruit extract.

There were a number of peaks observed for carambola (Figure 6.3) that were unidentified. The characterised phenolic compounds found in carambola consisted chiefly of the flavan-3-ols catechin and epicatechin, as well as the hydroxycinammic acids gallic acid, *p*-coumaric acid and salicylic acid. Although carambola has been reported to possess cyaniding-3-O-glucoside as well as other anthocyanins such as cyanidin-3,5-O-b-D-diglucoside and apigenin-6-C-b-L-fucopyranoside, cyanidin-3-O-glucoside was not detected in this study (Wei et al., 2014).

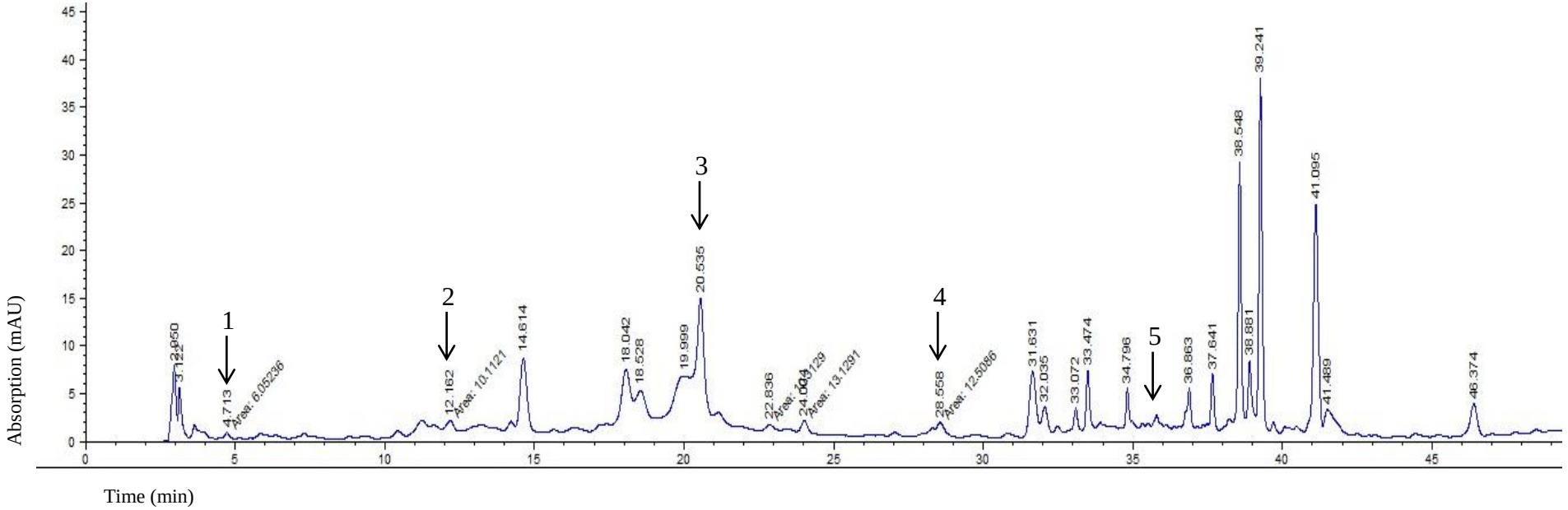


Figure 6.3 HPLC chromatograms of starfruit at UV absorption of 280nm. Peak assignment (1) gallic acid, (2) catechin, (3) epicatechin, (4) *p*-coumaric acid, (5) salicylic acid. HPLC conditions are described in the text.

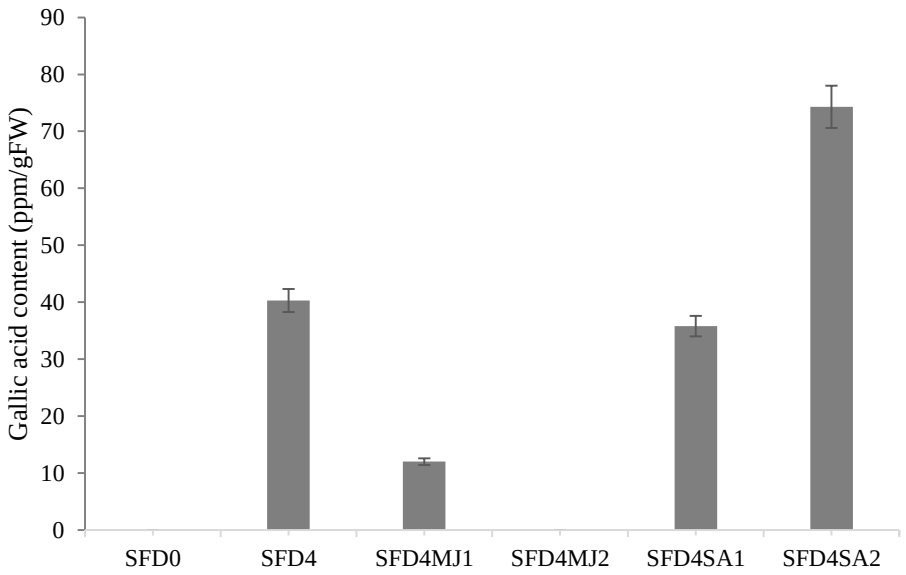


Figure 6.4 Content of gallic acid in carambola in ppm per gram fresh weight, as determined by HPLC UV-VIS analysis.

Gallic acid was not detected for the Day0 carambola samples, but was detected for the untreated Day4 sample (Figure 6.4). Different patterns of changes in gallic acid were observed for the salicylic acid and methyl jasmonate treatment. In the MeJA treated fruits, the gallic acid content dropped for the treated fruits and was undetectable in SFD4MJ2 (0.1 and 0.2 mM MeJA). Meanwhile, treatment with 1 mM SA resulted in a significant increase ($p<0.05$) in the gallic acid content.

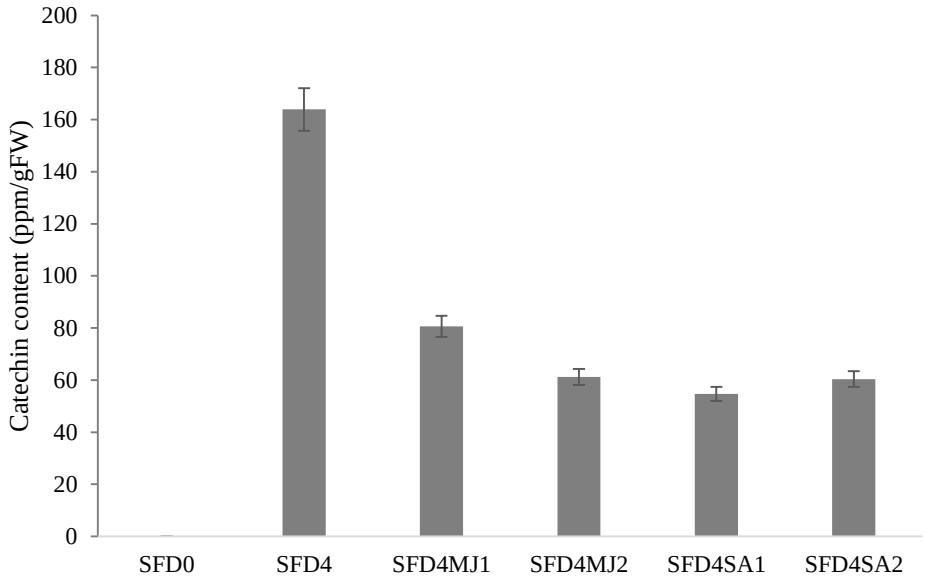


Figure 6.5 Content of catechin in carambola in ppm per gram fresh weight, as determined by HPLC UV-VIS analysis.

Similar to gallic acid, catechin was not detected in the Day0 sample and increased significantly in the untreated Day4 sample (Figure 6.5). Catechin levels dropped significantly ($p<0.05$) for fruits treated with both MeJA and SA. The levels of catechin detected in the MeJA treated fruits were significantly higher ($p<0.05$) than the SA treated fruits. Although higher dose of SA elevated the catechin content of the fruit, the opposite was observed for fruits treated with MeJA as the highest catechin content was observed for fruits treated with 0.01 mM MeJA.

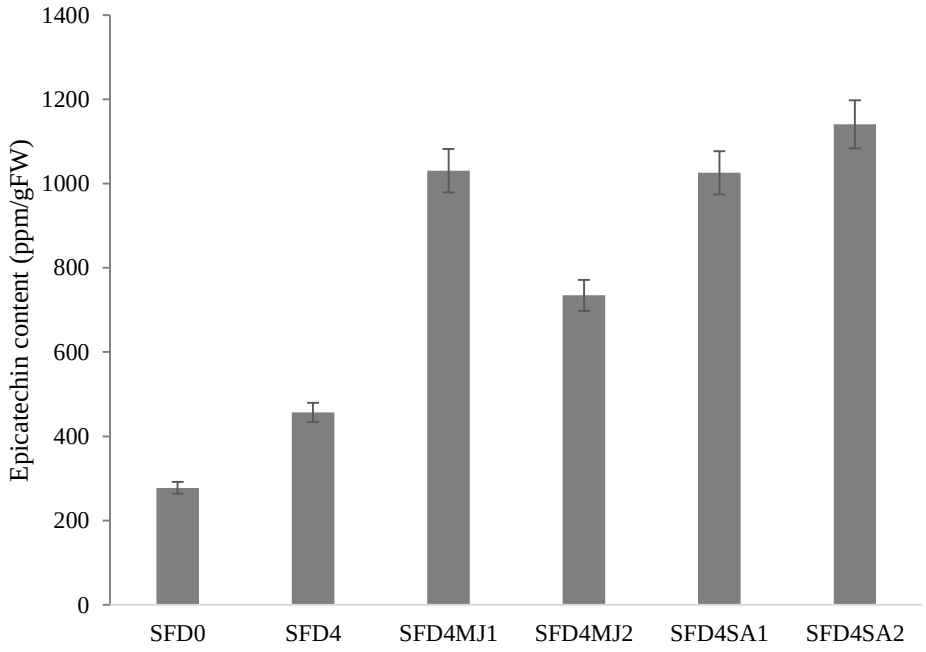


Figure 6.6 Content of epicatechin in carambola in ppm per gram fresh weight, as determined by HPLC UV-VIS analysis.

Epicatechin was detected for all of the samples, at relatively high levels (Figure 6.6). The treated samples contained the highest levels of epicatechin for both the MeJA and SA treatments. Similarly to catechin, MeJA at a dose of 0.01 mM induced the highest levels of epicatechin, four-fold the levels observed for day 0.

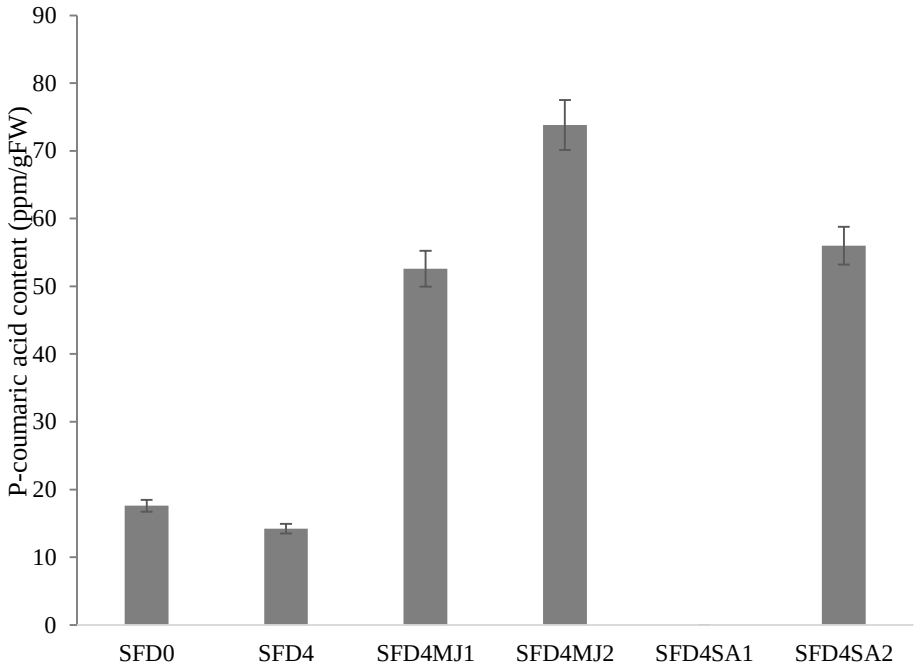


Figure 6.7 Content of *p*-coumaric acid in carambola in ppm per gram fresh weight, as determined by HPLC UV-VIS analysis

The results for *p*-coumaric acid (Figure 6.7) show that it is available at low quantities in the freshly harvested carambola. There was no significant difference ($p<0.05$) betweent the levels observed for Day4 samples and Day0 samples. However, the treatments of SA and MeJA significantly increased the levels of this phenolic acid. The exception to this was 0.1 SA, where *p*-coumaric acid was not detected for this treatment. However, the general trend observed was that higher doses of the phytohormones generally induced higher levels of this hydroxycinammic acid.

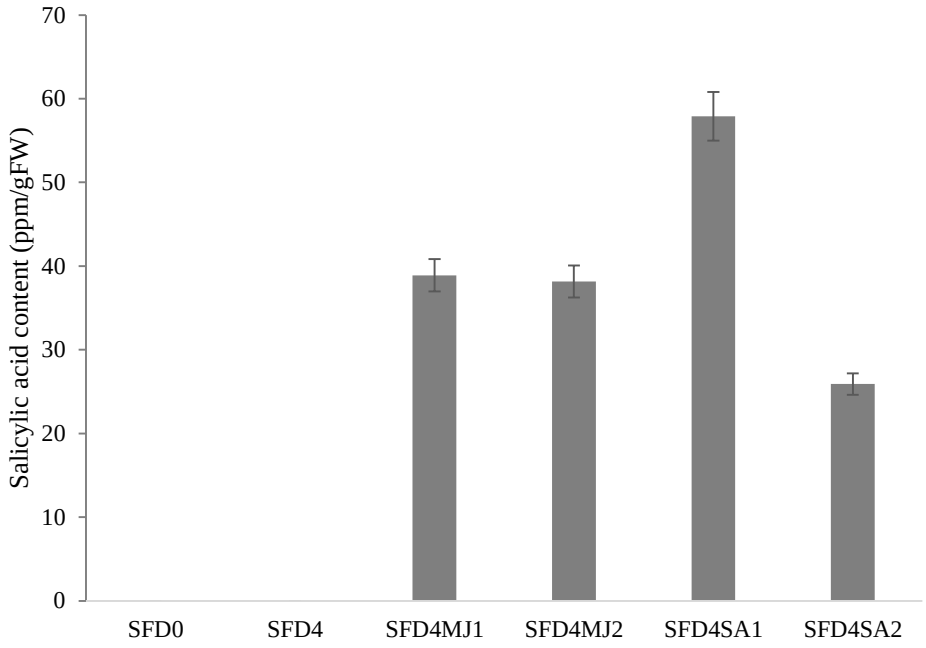


Figure 6.8 Content of salicylic acid in carambola in ppm per gram fresh weight, as determined by HPLC UV-VIS analysis

Salicylic acid (SA) was used in this experiment as a stress inducer, and the levels of this phenolic compound were also analysed using HPLC (Figure 6.8). Fruits exposed to the lowest dose of SA showed the highest levels of salicylic acid in the HPLC analysis, while the highest doses exhibited lowest levels of SA (57.9 and 18.2 ppm / gFW, respectively). Moreover, MeJA treatment also enhanced SA content in the fruit. Although SA is an ubiquitous phenolic compound, it was not detected in the untreated fruit samples.

6.4 Discussion

Although environmental stresses can be used to improve the quality of fresh produce, this has to be carefully deliberated with consideration of the potential unfavourable effects on the quality of the produce. It has been previously demonstrated that plants respond to stress through the increased production of diverse antioxidants, a significant portion of which are secondary metabolites produced through the phenylpropanoid pathway (Oh et al. 2009). These compounds protect cell organelles and membranes against oxidative damage by ROS (Oh et al. 2009; Mittler, 2002). Thus, this chapter was aimed at characterising the changes in the phenolic content of carambola.

6.4.1 Accumulation of the phenolic compounds

The identification of phenolic compounds in plants is a rather complex process due to the presence of thousands of these compounds. Moreover, many phenolic compounds undergo glycosylation with various types of saccharides, which can also occur at multiple sites of the phenolic compound (Lin & Harnly, 2007). This could ultimately generate uncountable variations of the compounds that could be chemically distinguishable. Another challenge for characterization of phenolic compounds is the tissue matrices, which might make it tougher to extract the phenolic compounds from the fruits (Epriliati et al., 2010). A suitable and repeatable extraction method using methanol was established in the preliminary stages of the project and was adopted for subsequent analyses.

Methanol is generally a good choice for the solubilization of phenolic compounds, but it tends to cause precipitation of pectins and does not effectively extract carotenoids (Epriliati et al. 2010). Thus, although carambola contains an abundance of carotenoids (Shui & Leong 2006, 2004) the peaks observed in the chromatogram are

more likely other classes of phenolic compounds rather than carotenoids, and could be further characterized using HPLC coupled with mass spectrometry.

Within phenolic compounds there is a clear distinction between the hydroxybenzoic acids such as vanillic and syringic acids and the hydroxycinnamic acids such as ferulic and coumaric acids. Hydroxycinnamic acids possess more potent antioxidant activity (Veligolu et al. 1998). These natural antioxidants perform various biological functions including antimicrobial, anti-inflammatory and anti-carcinogenic properties (Veligolu et al., 1998). However, there have been several examples of plant materials where factors besides the total phenolic content are responsible for the antioxidant activity and related biological functions of the plant material (Veligolu et al., 1998).

Shui and Leong (2006) previously demonstrated that the residue extract of carambola contained good antioxidant activity and this was attributed to the presence of phenolics and proanthocyanidins. In a study by Wei et al. (2014) on the antioxidant content of carambola, the observed chromatogram peaks comprised chiefly of epicatechin units with a lower abundance of catechin. This is in agreement with the results observed here where the abundance of epicatechin was almost ten folds the amount of catechin detected in carambola.

Elevated levels of *p*-coumaric acid were observed for the MeJA treatments as the concentration increased from 0.01 mM to 0.1 mM MeJA. This hydroxycinnamic acid is an intermediate in the earlier stages of the phenylpropanoid pathway, prior to formation of stress lignin (Fraser & Chapple, 2011). In a study by Solecka et al (1999), *p*-coumaric acid was also found to accumulate under cold stress but two times lower than ferulic acid. Ferulic acid was not detected for the carambola fruit material, while

p-coumaric acid levels were enhanced under the hormonal treatments, with the exception of fruits treated with 0.1 mM SA.

Cao et al (2010) demonstrated that MeJA, is involved in activating the phenylpropanoid pathway, and is capable of inducing the activity of salicylic acid. Salicylic acid was universally induced by all doses of MeJA. However, with regards to gallic acid, only the lowest and highest doses of methyl jasmonate were found to elevate the levels of gallic acid (Figure 6.4a).

6.4.2 Esterification of phenolic compounds

Phenolic compounds can be conjugated to a diverse range of substrates such as choline and malate, subject to their availability (Solecka et al., 1999). Salicylic acid is also stored in the plant in its conjugated form. The conversion is catalysed by the enzyme salicylic acid glucosyl-transferase (SA-Gtase), which increases in activity in the presence of endogenous salicylic acid, following a negative feedback loop (Zhang et al. 2003). It is only after stress reaches a certain threshold that the conjugated form is hydrolysed to release SA, inducing defence responses. Thus, it is possible that in the presence of exogenous salicylic acid at the levels administered, the plant was responding through increased SA-Gtase activity, which would increase the levels of conjugated SA.

Acclimation of plants to cold stresses involves the esterification of phenolic compounds. Ferulic and sinapic acid are both rapidly accumulated under cold, and are usually fully-esterified (Solecka et al., 1999). The availability of phenolic compounds in free bound form is toxic to the plant, thus they are usually esterified and stored in conjugated form to avoid their negative effects when accumulated or during transportation to vacuoles (Solecka et al., 1999). The extraction methods used in these

experiments were effective for extracting free phenolic compounds in free bound form, as conjugated phenolic compounds usually require acidic or alkali treatment for their successful extraction.

Esterified hydroxycinammic acids are usually located in the cell vacuoles where they are substrates of vacuolar peroxidases (Solecka et al., 1999). This peroxidase system serves to protect plants from oxidative damage at the expense of the accumulated phenolic pool. Thus, in this way phenolics protect cells from oxidative damage as substrates of peroxidases, as well as scavenging of ROS or absorption of excess irradiation. However, there is a variation in the rate of oxidation of these phenolic acids, as *p*-coumaric and ferulic acids are more rapidly oxidised by bound peroxidases than other classes such as sinapic acid (Dangcham et al., 2008). Thus, hydroxycinammic acids and their esterified derivatives can serve as screening molecules for the redox status of plants.

Phenolic compounds offer protective benefits for human nutrition as well. Although pure polyphenols have been examined for their anti-proliferative activity on HepG2 a few studies have explored the protective properties of enhanced accumulation of phenolic compounds against HepG2 cancer cells. Ramos et al. (2005) found potent activity from strawberry and plum extract (0.6 and 1.5 mg/ml) and they attributed the highest activity to the presence of quercetin with an IC50 value of 87 µM. Meanwhile, in another study by Yeh et al. (2005) anti-proliferative activity was exhibited with gallic acid and ferulic acid, while no effects were demonstrated for *p*-coumaric acids and hydroxybenzoic acids (Yi et al., 2006). Thus, in the next chapter the antiproliferative activity of the different carambola extracts with different patterns of accumulation for phenolic acids will be assessed.

A key obstacle is the development of a separation method suitable for sufficiently separating the large range of compounds inherently present in the sample (Epriliati et al., 2010; Lin & Harnly, 2007). Using UV detection, the target class of compounds would all be detectable at the same wavelength, thus it is essential to select the appropriate column, solvent and solvent gradient to selectively elute the desired compounds, regardless of abundance levels. This was successfully achieved in this experiment.

UV is most commonly used for detection of chromatographic analytes from HPLC, however when dealing with low concentrations of analytes the sensitivity of this method might not be sufficient. Mass spectrometry (MS) and variations of this tools (such as tandem MS or ion trap MS) equipped with electrospray ionization could be employed for identification and structural characterization of analytes at even low concentrations, as it quantifies based on ion molecule abundance (Epriliati et al., 2010; Lin & Harnly, 2007). MS can then be used to observe dynamic changes in the patterns of metabolite accumulation to elucidate further the changes in the phenylpropanoid pathway. However, since MS sometimes lacks the ability to differentiate between molecules possessing similar molecular masses, it will be advisable to exploit both detector systems (UV and MS) for accurate and reliable identification and quantification of analytes.

7.4 Concluding remarks

In this study, an RP-HPLC method using a UV detector method was adopted for the quantification of phenolic compounds in carambola. Numerous compounds were separated using the established HPLC method, however several compounds were separated which were still unidentified and these ought to be determined which would involve the use of improved analytical techniques. Conclusive identification of the phenolic compounds found in the fruits can be possible through the combination of the UV–visible spectra data (using retention times and authentic standards) along with mass spectra data obtained using an MS detector.

This experiment was designed to elucidate whether the differences in the specific phenolic compounds of fruits under various stresses were significant. Exposure of the fruits to low doses of the salicylic acid was sufficient to trigger responses on the phenylpropanoid pathway. It is hypothesized that a negative feedback loop in response to exogenous salicylic acid is in operation that could minimize the response to higher doses of salicylic acid. MeJA at doses below 0.5 mM was also found to increase levels of intermediates from the phenylpropanoid pathway.

Phenolic compounds are beneficial to human health, which is associated with the antioxidant and anti-proliferative activity of the compounds. Exploring any relations between the changes in the phenolic content of the fruits resulting from the postharvest stresses and the induction of therapeutic properties can offer further insight into the benefits generally associated with the consumption of fruits. Thus, the next stage of experimentation will explore the activity of bioactive fruit material on the inhibition of tumour cell proliferation as well as the antioxidant activity of these active extracts on human cell culture using HepG2 as a model.

CHAPTER 7: ANTI-PROLIFERATIVE AND ANTIOXIDANT PROPERTIES OF CARAMBOLA FRUIT MATERIAL

7.1 Introduction

The phenylpropanoid pathway is responsible for the biogenesis of a range of structurally diverse compounds with important functions (Hemaiswarya & Doble 2013). Some may function as antioxidants by either scavenging free radicals or chelating metal ions (Khonkarn et al. 2010). Some of these compounds have also been demonstrated to inhibit tumour proliferation as well as bacterial and viral replication. They can affect cell signalling and influence the activity of cytochrome P450 and arachidonic acid cascade enzymes (Hemaiswarya & Doble 2013).

Oxidative stress in the human body leads to DNA and protein damage, increasing the risk of chronic degenerative diseases. Epidemiological studies have linked the bioactive contents of fruits and vegetables such as carotenoids, tocopherols and polyphenolics to chemopeventive, anti-inflammatory and cardioprotective benefits (Wu et al. 2006). Consequently, the antioxidant and antiproliferative properties of fruits and vegetables have received significant interest and has been considerably characterised in several fruits and vegetables.

In East Asia, as well as globally, there has been an increasing move towards the development of alternative therapies for cancer based on natural plant products (Park et al. 2012). These can have the advantage of overcoming the side effects of conventional chemotherapies and may also play a key role in the move towards prevention rather than treatment of liver cancer (Newell et al. 2010). Thus, there have been numerous studies on the anti-hepatocellular carcinoma activity of natural products. Natural products induce apoptosis by generating reactive oxygen species

(ROS) and disrupting the redox homeostasis (Lee & Lim 2010). ROS play a lead role in apoptosis signalling, but they can play a dual role in cancer promotion or cancer suppression (Lee & Lim 2010).

Human hepatoma cells (HepG2) have served as a validated model for *in vitro* assays in biochemical and nutritional studies to help elucidate the biological functions of various dietary compounds (Alía et al. 2006; Martin et al. 2008). The liver is an important model for metabolism as it is a direct recipient of bioactive compounds, including antioxidants, from the digestive system (Baeza et al. 2014). Xenobiotics are also absorbed into the liver from the digestive tract, thus the liver is highly susceptible to the activity of xenobiotics (Jaeschke 2002). The detoxification of xenobiotics produces ROS and free radicals, increasing the oxidative stress within this organ (Goya et al. 2009). Thus, dietary antioxidants play an important role in alleviating antioxidant stress within this system.

The HepG2 cell line is representative of xenobiotic metabolism which typically occurs in the liver, although at a lower metabolic capacity than normal hepatocytes, and is capable of activation/detoxicifation of genotoxic pro-carcinogens (Deferme et al. 2013). Moreover, this cell line retains and mimics several liver-specific metabolic responses such as a well-functioning glutathione system (Vidyashankar et al. 2013). HepG2 cells have the added benefit of immortality thus are easier to use than primary cell lines (Deferme et al. 2013).

The advancement of cancer cells is dependent on uncontrolled cell division, which result from abnormalities in the regulation of the stages in cell cycle (Wang et al. 2007). Thus, inhibition of apoptosis and irregular cell proliferation are the key factors leading to development of tumours. Anti-proliferative activity of fruits and

vegetables is linked to the antioxidant capacity of the produce, and is associated with their radical scavenging activity within the cells (Wang et al. 2007).

A lot of emphasis has been placed on the anti-proliferative activity of phenolic compounds, and increasing evidence suggests that polyphenol cocktails rather than individual polyphenols are responsible for the anti-proliferative activity (Yi et al. 2006). Wang et al (2007) reported enhanced anti-proliferative activity of blackberry extract upon hormonal treatment with MeJA, which was associated with the improved radical scavenging capacity of the fruit. It is hypothesized that the diverse combination of polyphenols in carambola can prevent cancer progression, and the postharvest induced stresses could potentially enhance the chemopreventive properties of this fruit.

Results from the previous chapters demonstrated the radical scavenging activity of carambola extract as well as antimicrobial activity of the methanolic extract of the fruit. The key functional ingredients of carambola, such as proanthocyanidins, have been reported to exhibit antiproliferative activity. Nonetheless, the potential induction of bioactive compounds by stress hormones in carambola to enhance its antioxidant and therapeutic properties has not been previously examined. Therefore, the aim of this study was to assess the anti-hepatocellular carcinoma activity of carambola methanolic extract and to determine the potential of enhancing the bioactivity of this fruit by subjection to postharvest stresses.

7.2 Materials and methods

Carambola fruit was subjected to the treatments described in Sections 2.2.1 and 2.2.2 and used for the characterisation of anti-proliferative and antioxidant properties of carambola. Four biological replicates were used for the preparation of the extracts used in this study.

7.2.1 Reagents

Dulbecco's Modified Eagle's Medium (DMEM) high glucose (Sigma D6546, containing 4500 mg glucose/L, 110 mg sodium pyruvate/L, pyridoxine, HCl and $NaHCO_3$) was incorporated with heat inactivated foetal bovine serum (FBS) (10 %) (Gibco), L-glutamine (1 %) (Gibco) and penicillin/streptomycin (1 %) (Sigma). HepG2 cells were obtained from the laboratory stock at the University of Nottingham, which were available from European Collection of Cell Cultures.

7.2.2 Cell and culture media

HepG2 cells were obtained from the stock stored in liquid nitrogen, and 1 ml was thawed and cultured in 13 ml of DMEM media mixed with 10% FBS, 1 % L-Glutamine and 1 % penicillin/streptomycin. The culture was maintained at 37 °C in a humidified atmosphere containing 5 % CO_2 and the medium was changed every 2 days, until cell growth reached 80 % confluence. Upon reaching 80 % confluence, cells were sub-cultured by removing the medium, including floaters, and washing with 10 ml of phosphate buffered saline (PBS), composed of 10 mM PO_4^{3-}, 137 mM NaCl, and 2.7 mM KCl at pH 7.4. The cells were then incubated with 2 ml of 0.25 % trypsin and 1 mM EDTA for 3 minutes, and resuspended in 8 ml of fresh serum-containing DMEM to inactivate the trypsin and centrifuged at 1000 rpm for 5 minutes before transferring

to a new flask at a 1:5 split of the original cell number. Cells were continuously maintained at 37 °C in a humidified atmosphere containing 5 % CO_2 and the medium was changed every 2 days.

7.2.3 Plant material

Lyophilised samples from fruit subjected to two stress hormones (SA and MeJA) along with controls (Table 6.1) were used to prepare plant extracts at a concentration of 50 mg/ml in 60 % methanol. Extractions were carried out at 37 ^{o}C for one hour using a ratio of solute to solvent of 1:10 (w/v). Samples were then centrifuged at 10,621 xg and 4 ^{o}C for 15 minutes and filtered and the methanol extract was dried by rotary evaporation. The resultant dry powder was reconstituted in PBS at a concentration of 50 mg/ml. Final concentrations of 5, 2.5, 1, 0.5, 0.1, 0.05, 0.25 mg/ml of the extract were used for the cytotoxicity assay. These concentrations were selected from prior screening.

7.2.4 Anti-proliferative activity of fruit extract

This assay was performed when cells reached 80 % confluence, which were then trypsinized and suspended in a fresh medium following the method described in Section 7.2.2. Cell numbers were counted using a haemocytometer and was plated at a density of 8 x 10^4 in 100 µl of media into each well of a sterile 96-well microplate and were allowed to attach and grow for 24 hours at 37 °C. The plant extracts, or PBS control, were then added to each well to reach a final concentration of 200 µl. Five replicates were used for each trial sample.

The effect of the extracts on HepG2 cell number was assessed after 1 hour, 24 hours and 48 hours of incubation. At each specified time point, one of the three replicate plates was taken out of the incubator and the media was removed, including floaters,

and each well was washed with 200 µl PBS. Sterile water (100 µl) was added into each well and the plate was incubated at 37 °C for one hour before freezing at -20 °C for one hour. The cells were then subjected to three rounds of thawing and freezing, at room temperature and -20 °C respectively, to rupture the membrane and allow for DNA release. 100 µl of 2 µg/ml Hoechst 33258 dye (Sigma-Aldrich) in 2X TNE buffer (20 mM Tris [pH 7.4], 2 mM EDTA and 0.2 M NaCl) was added into each well and the fluorescence was measured at excitation wavelength of 355 nm and emission of 460 nm (BMG Labtech Fluostar Optima). A standard curve was run using a serial dilution of 0.6 to 40 µg/ml of calf thymus DNA (Sigma).

7.2.5 Production of reactive oxygen species (ROS)

The direct effect of plant extracts on production of ROS by the HepG2 cells was assessed using a method adapted from Zhu, Wang et al (2013) and Baezea et al. (2014). Cells were seeded at a concentration of 8 x 10^4 cells/well in 100 µl of DMEM on a 96 well plate and left to attach overnight. The following day, the media was removed and the cells were washed with PBS. Cells were then treated with 100 ul of 35 µM 2′,7′-dichlorofluorescin diacetate dye (DCFH-DA) (Sigma) in Hanks Balanced Salt Solution (HBSS) (Sigma) with 1 % FBS and 0.25 % Dimethyl sulfoxide (DMSO) for 1 hour at 37 °C, after which the dye was removed and cells were washed with 200 µl PBS. Plant extracts (as in table 7.1) at concentrations of 0.06 - 1 mg/ml were then used to treat the cells, along with an untreated control, and the fluorescence was measured at 485 nm excitation and 530 nm emission, at 0, 30, 60, 90, 120, 150 and 180 min. Nine replicates were used for each trial sample.

The protective effect of the plant extracts on ROS levels in HepG2 cells treated with hydrogen peroxide (H_2O_2) was assessed using an adaptation of the method above.

Seeded cells were pre-treated with the plant extracts at concentrations of 0.06 -1 mg/ml, along with two sets of untreated controls, and left for 20 hours. After removing the media and treating the cells with DCFH-DA for one hour, the cells were then exposed to 6 µM of H_2O_2 in 200 µl HBSS and the fluorescence was measured at 485 nm excitation and 530 nm emission, every 30 min for 3 hours. One set of control was not exposed to H_2O_2 stress and 200 µl untreated HBSS was used instead. Nine replicates were used for each trial sample.

7.3 Results

7.3.1 Optimisation of Hoechst fluorimetric method

Potential interference of the plant extract with the Hoechst dye was determined at various concentrations of the plant extract. Calf thymus DNA was prepared within the desired range for calibration (0.6 - 40 µg/ml) and was combined with 0, 1 and 10 mg/ml of fruit extract in PBS. The fluorimetric readings were then determined and presented in Figure 7.1. Presence of the plant extract had a significant quenching effect on the fluorescence and thus final DNA calculation. Although a linear curve was maintained, the calculated DNA was lowered as the concentration of the plant extract increased. Nonetheless, between the required range of DNA concentrations between 5 to 10 µg/ml, the differences were not significant.

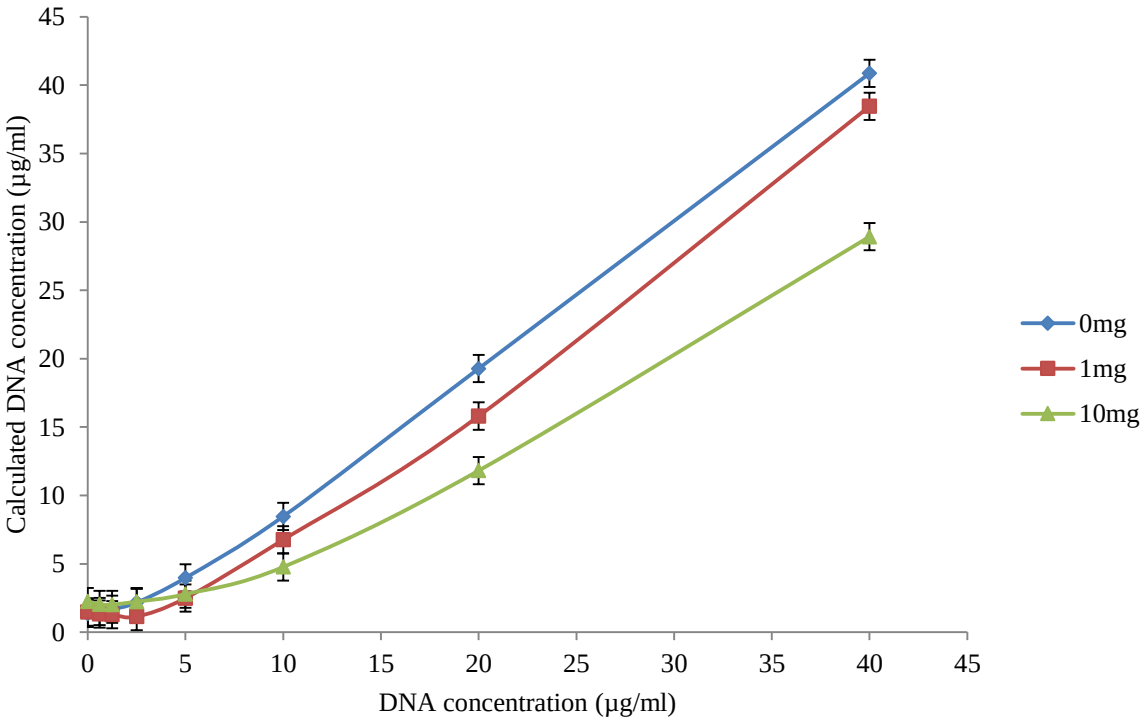
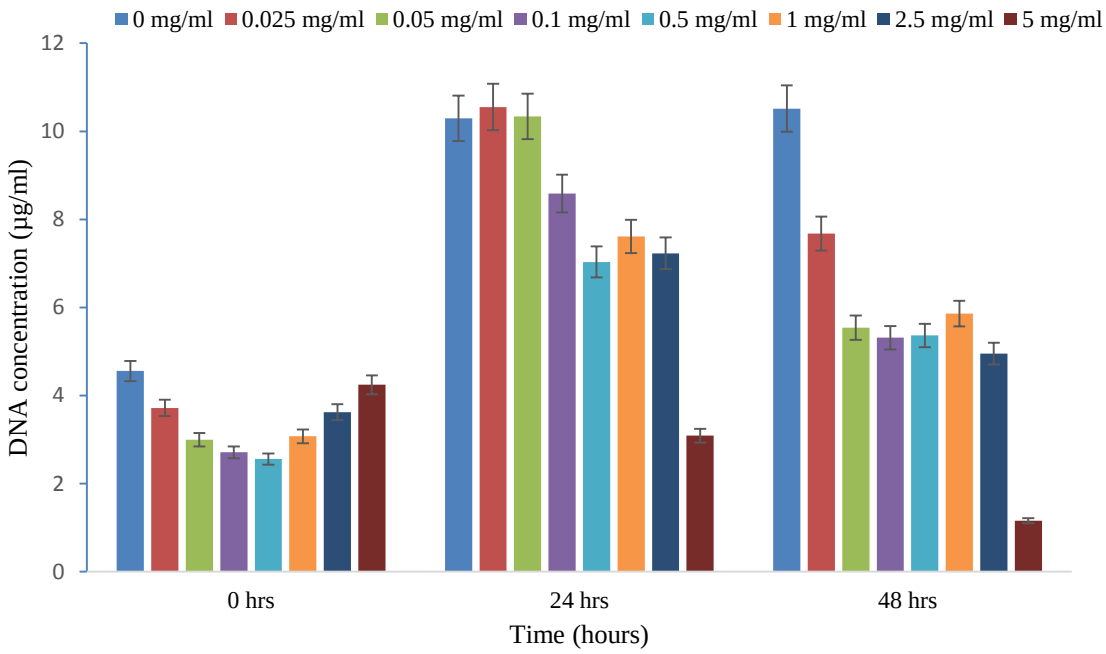


Figure 7.1 Effect of fruit extract (at 0, 1 and 10 mg/ml) on the Hoechst fluorimetric method for determining DNA. Values are means (n=5) ± SE.

7.3.2 Anti-proliferative activity of carambola extracts

To determine the linearity of the Hoechst fluorimetric method, HepG2 cells were seeded at concentrations of 2 x 10^4 to 1 x 10^5 cells per well and the DNA concentration (µg/ml) was determined immediately. A linear increasing relationship was observed for loadings above 5 x 10^4 cells per well (Appendix A7.1). Therefore, 8 x 10^4 cells per well was selected as the optimal initial cell count to be used in the subsequent tests for measuring anti-proliferative activity. Methanolic extracts from carambola fruit at day 0, and after 4 days following treatment with 0.01 mM MeJA, 0.1mM MeJA, 0.1mM SA, 1mM SA or untreated were prepared as described above. These extracts were then tested for anti-proliferative activity at a range of 0.25 to 5 mg/ml. This concentration range was selected following an initial screening using a wider range and determining the range that impacted proliferation. A concentration of 10mg/mL was found to result in cell death (data not shown).

The results are shown in Figures 7.2 to 7.4. In each case the control (no added extract) shows a very clear increase in DNA content over the 48 hours of incubation demonstrating proliferation of the cells. Anti-proliferative activity was detected (Appendix B 7.1 – 7.6) for all of the carambola extracts. A time-dependent dose response was observed for the treatments, whereby after 48 hours of incubation, and in some cases 24 hours, cytotoxic activity was observed. Cytotoxicity was defined as being when the DNA content dropped below the levels measured at time 0.



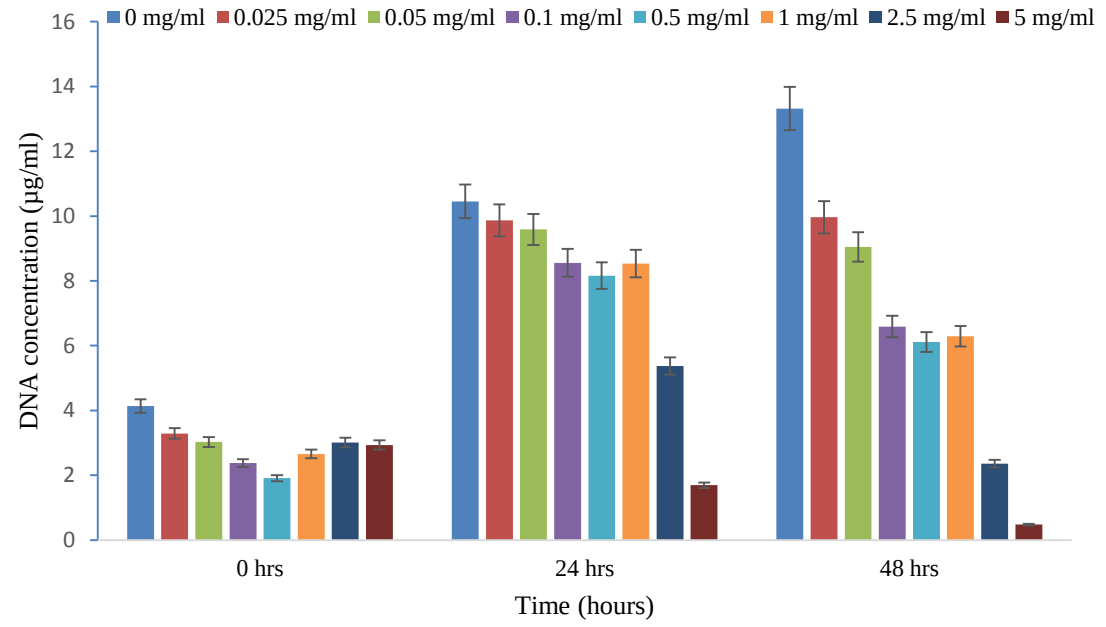
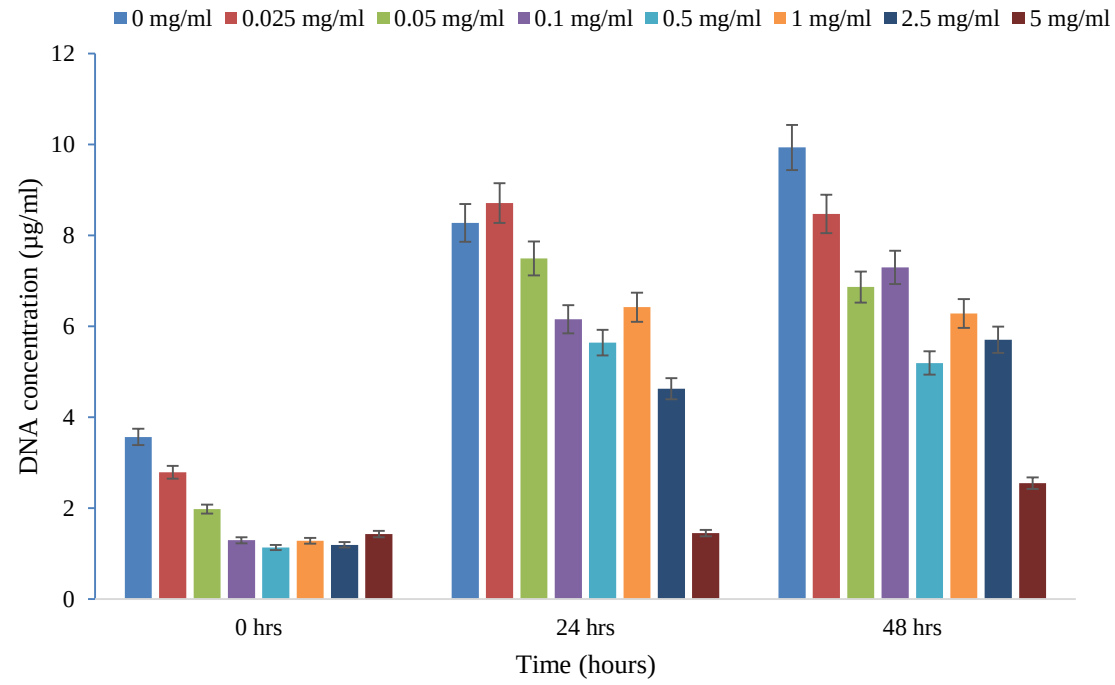


Figure 7.2. Effect of extracts of a) freshly harvested carambola (SFD0) and b) ripening carambola (SFD4) at concentrations of 0.25 to 5 mg/ml on proliferation of HepG2 cells over 48 hours. Values are means (n=5) ± SE.



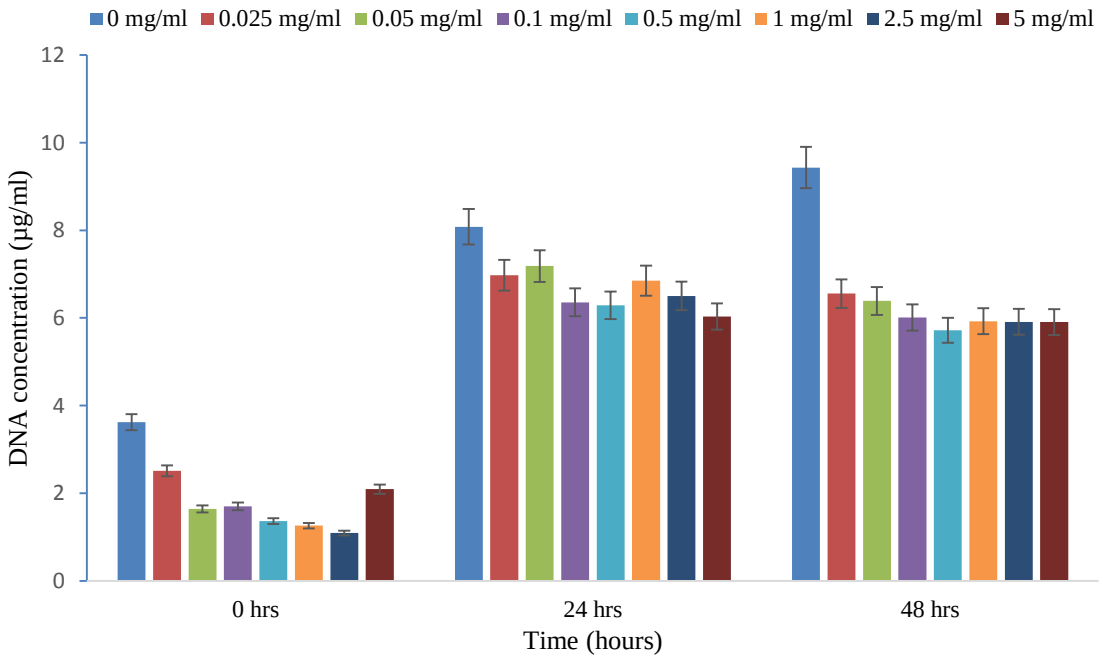
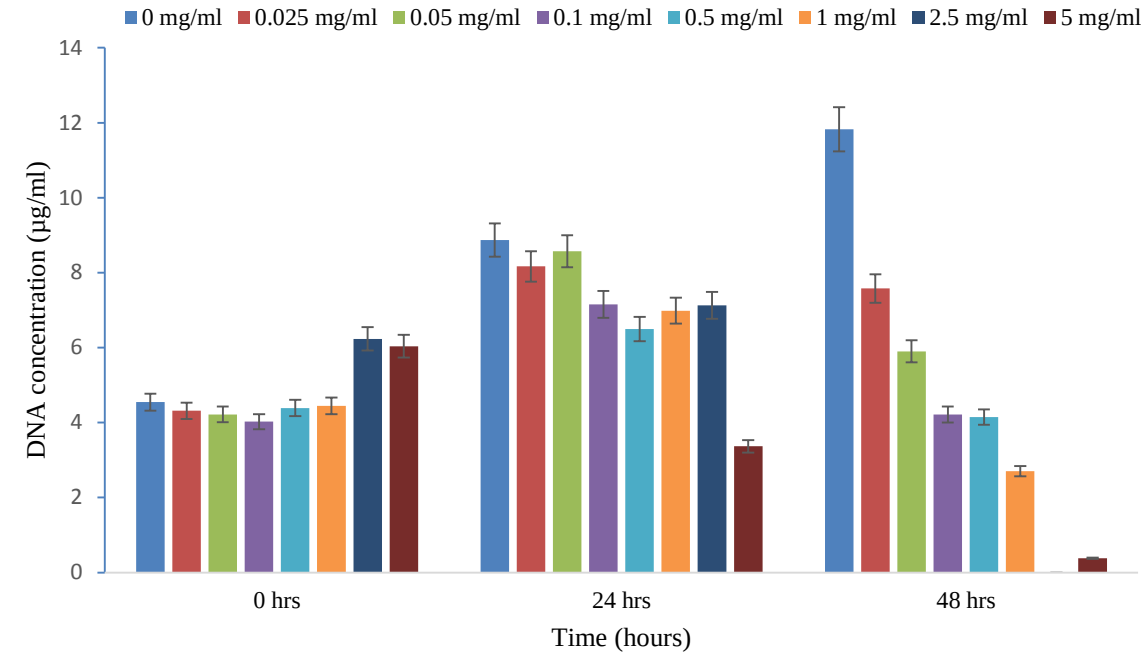


Figure 7.3. Effect of extracts of carambola treated with a) 0.01mM MeJA (SFD4MJ1) and b) 0.1mM MeJA (SFD4MJ2) at concentrations of 0.25 to 5 mg/ml on proliferation of HepG2 cells over 48 hours. Values are means (n=5) ± SE.



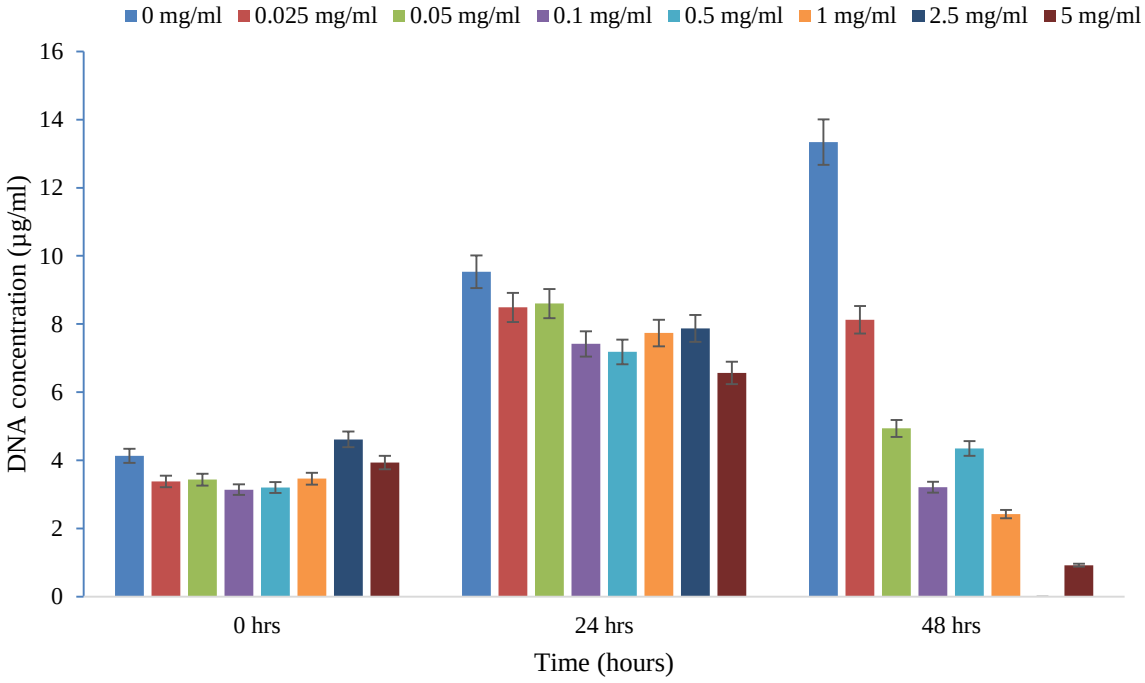


Figure 7.4. Effect of extracts of carambola treated with a) 0.1mM SA (SFD4SA1) and b) 1mM SA (SFD4SA2) at concentrations of 0.25 to 5 mg/ml on proliferation of HepG2 cells over 48 hours. Values are means (n=5) ± SE.

The lowest anti-proliferative activity was observed for the cells treated with SFD4MJ2 where the cell viability was maintained at a high level for all of the concentrations (Figure 7.3b). Nonetheless, the cell viability was significantly lower than the untreated cells after 24 and 48 hours of incubation. Cytotoxic activity was not observed for this treatment, even at higher concentrations.

The fruit anti-proliferative activity increased between day 0 and day 4 (Figure 7.2). Anti-proliferative activity was observed for the two extracts at concentrations of more than 1 mg/ml, however the cytotoxic level for SFD0 was at 3.4 mg/ml while for SFD4 it was at 2.2 mg/ml. This could be an indication of the production of active metabolites within the fruit as it ripens.

Meanwhile, exposure of the fruit to SA also induced higher anti-proliferative activity (Figure 7.4) and was the most active fruit extract. The extract of the fruit subjected to the lower concentration of SA was more potent than the higher concentration. This was similarly observed with the MeJA treated fruits as the lower concentration yielded a more active extract. However, the activity of this fruit extract was lower than the untreated fruit extracts. The observed anti-proliferative levels were more comparable to SFD0 fruit extract. As it is hypothesized that the active metabolites are elevated in the ripening fruit, this could indicate the role of MeJA in delaying the production of active metabolites through mechanisms involving delayed ripening.

7.3.3 Anti-proliferative activity of fruit fractions

A concentration of 2 mg/ml was selected from the previous assay (7.3.2) as a level where the cytotoxic activity of carambola was observed for most of the extracts. Thus the fruit extract was fractionated at this concentration and the different fractions were assessed for their anti-proliferative activity. Solid-phase extraction (SPE) was used to fractionate the carambola extract (section 6.2.3) and the three derived fractions, along with the original extract, were assessed for their total phenolic content (TPC) and anti-proliferative activity on HepG2 cells (Figures 7.5 – 7.7).

Highest TPC were observed in the original extracts, followed by fraction 3 from the SPE as expected. However, for some of the samples some phenolic content was also observed in fractions 1 and 2. Overloading of the column could have accounted for this carry-over into fractions 1 and 2 for some samples, as it was not expected to detect phenolics in these fractions. However, in each case the SPE column has successfully fractionated the phenolics into fraction 3 as expected.

The untreated control exhibited normal cell proliferation while the cells treated with fruit extract at 2 mg/ml (S1) exhibited cytotoxicity as previously observed (Figure 7.5 – 7.7).

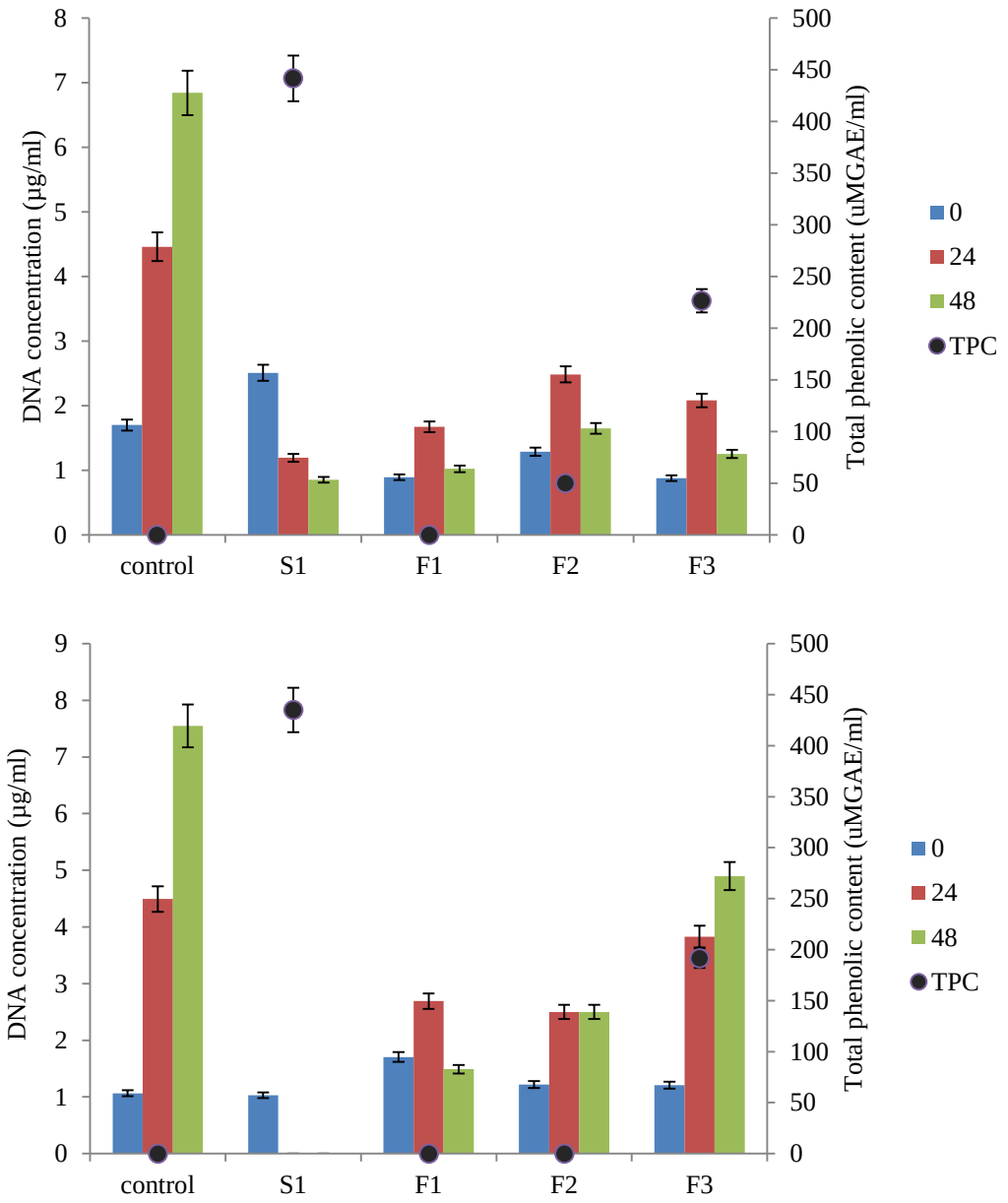


Figure 7.5. Anti-proliferative activity and total phenolic content of original extracts at a concentration of 2 mg/ml and resultant SPE fractions of a) freshly harvested carambola (SFD0) and b) ripening carambola (SFD4) Values are means (n=5) ± SE.

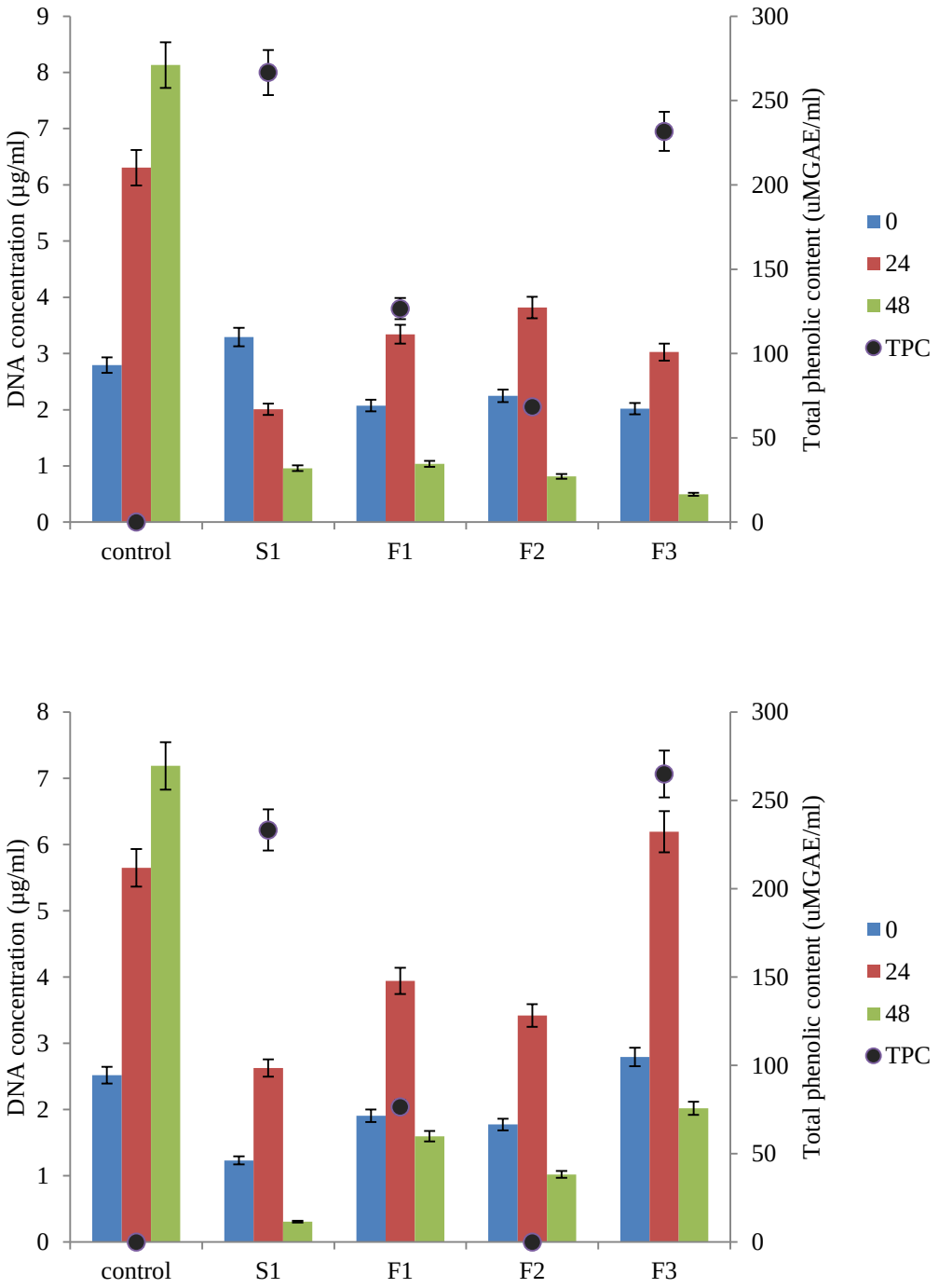


Figure 7.6. Anti-proliferative activity and total phenolic content of original extracts at a concentration of 2 mg/ml and resultant SPE fractions of carambola treated with a) 0.01mM MeJA (SFD4MJ1) and b) 0.1mM MeJA (SFD4MJ2). Values are means (n=5) ± SE.

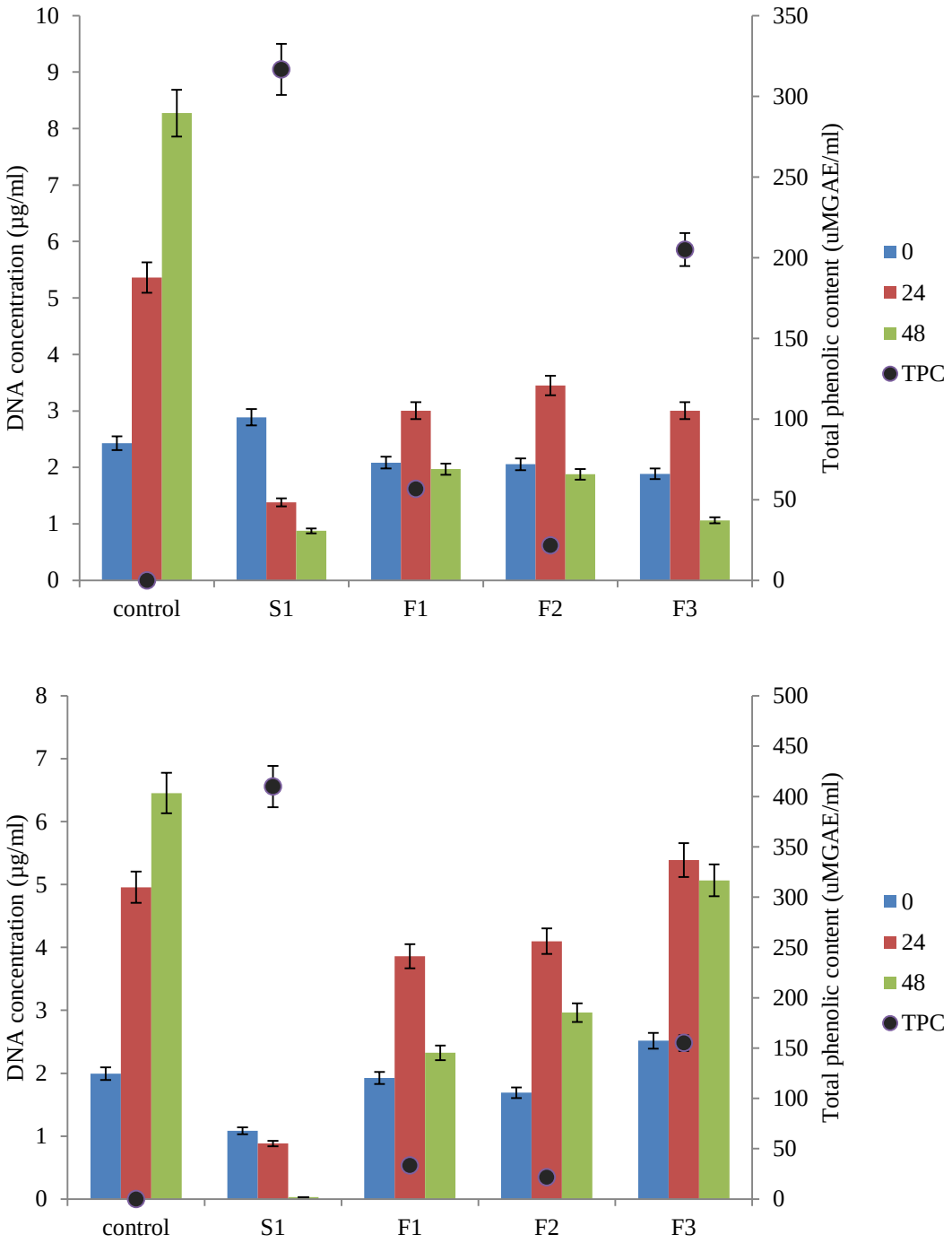


Figure 7.7. Anti-proliferative activity and total phenolic content of original extracts at a concentration of 2 mg/ml and resultant SPE fractions of carambola treated with a) 0.1mM SA (SFD4SA1) and b) 1mM SA (SFD4SA2). Values are means (n=5) ± SE.

Anti-proliferative activity was highest in the original fruit extract, and in general the fractions showed a lower level of anti-proliferative activity compared to the original extract. There was no obvious correlation between phenolic concentration in the fractions and the anti-proliferative activity levels measured. The anti-proliferative activity of fraction 3 was found to be either similar to or in some cases lower than the other fractions.

7.3.4 Cell response to hydrogen peroxide induced stress

Intracellular ROS production was assessed to determine the level of oxidative stress upon exposure to carambola extracts at levels that were not cytotoxic. H_2O_2 is a natural stress inducer, and can be used experimentally to manipulate the cell medium conditions. The susceptibility of HepG2 cells to H_2O_2 within the range of 0 to 24 µM was assessed (Figures 7.8). Intracellular oxidative stress was measured using a DCFH-DA fluorescent probe, which is oxidised to fluorescent DCF by intracellular ROS (Zhu et al., 2013). The inherent ROS levels in HepG2 naturally increased over the time course of 180 minutes, and exposing the cells to H_2O_2 at all concentrations tested elevated the intracellular ROS levels. From this assay, 6 µM was found to induce maximal ROS production, this concentration was thus used for further experiments.

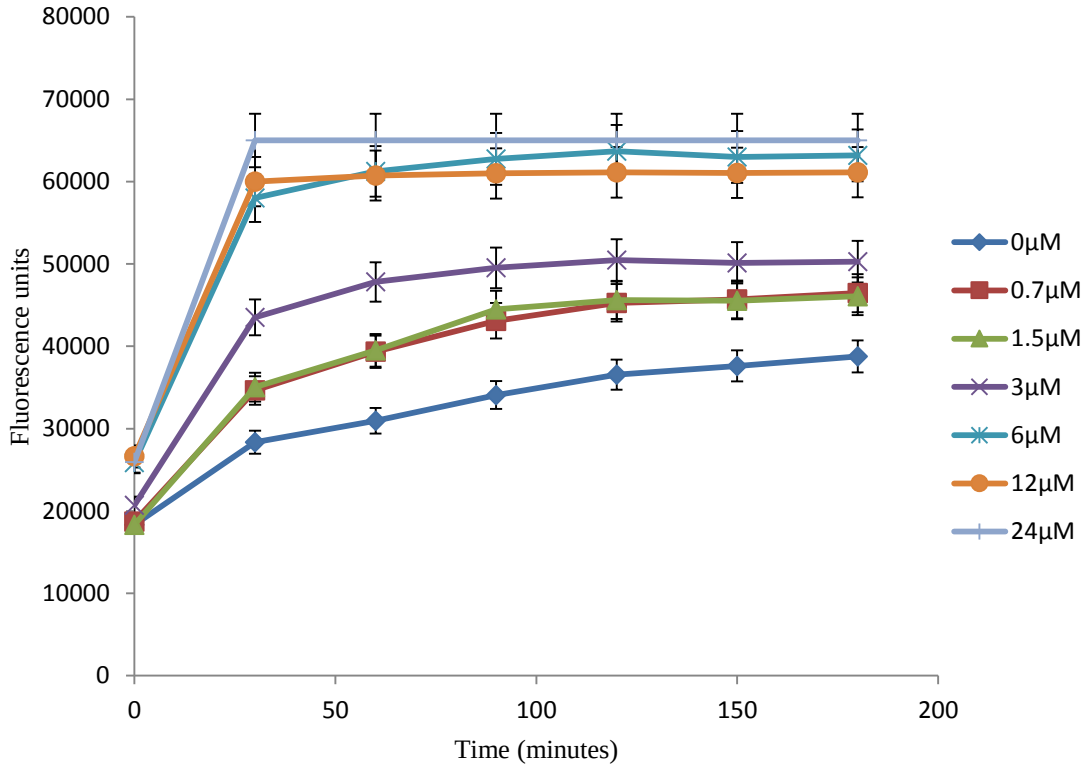


Figure 7.8. Intracellular ROS production of HepG2 cells under H_2O_2 stress (0- 24µM) measured over 180 minutes. Values are means (n=5) ± SE.

7.3.5 Direct effect of extracts on production of ROS in HepG2 cells

The direct effect of the extracts on the production of ROS was assessed over 180 minutes. HepG2 cells were exposed to carambola extract at concentrations of 0.06 - 1mg/ml and the intracellular levels of ROS measured (Figure 7.9 – 7.11). These were compared to the inherent intracellular ROS levels of untreated HepG2 cells.

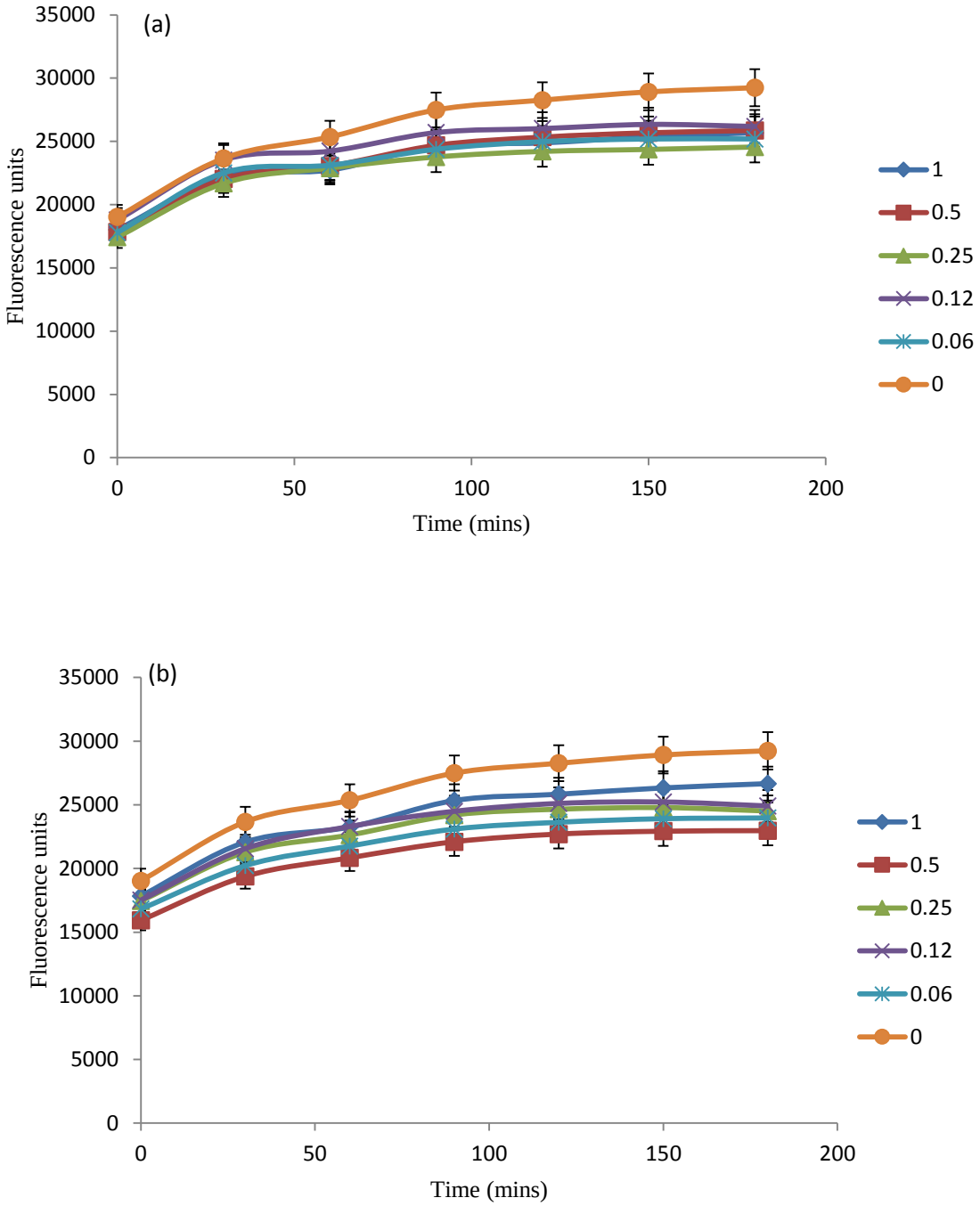


Figure 7.9. Direct effect of a) freshly harvested carambola (SFD0) and b) ripening carambola (SFD4) extracts at 0.06 – 1 mg/ml on the intracellular production of ROS measured over 180 minutes. Values are means (n=5) ± SE.

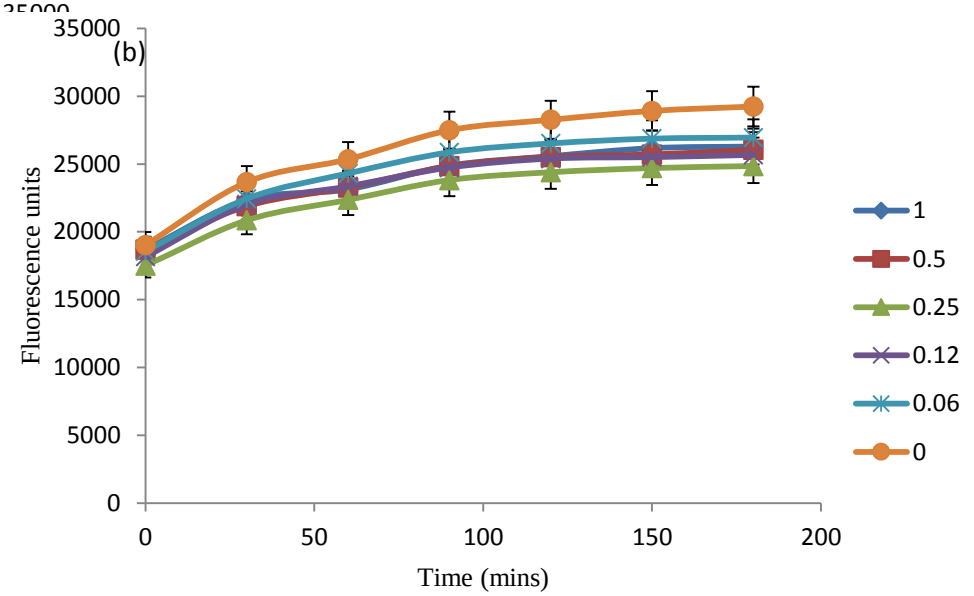
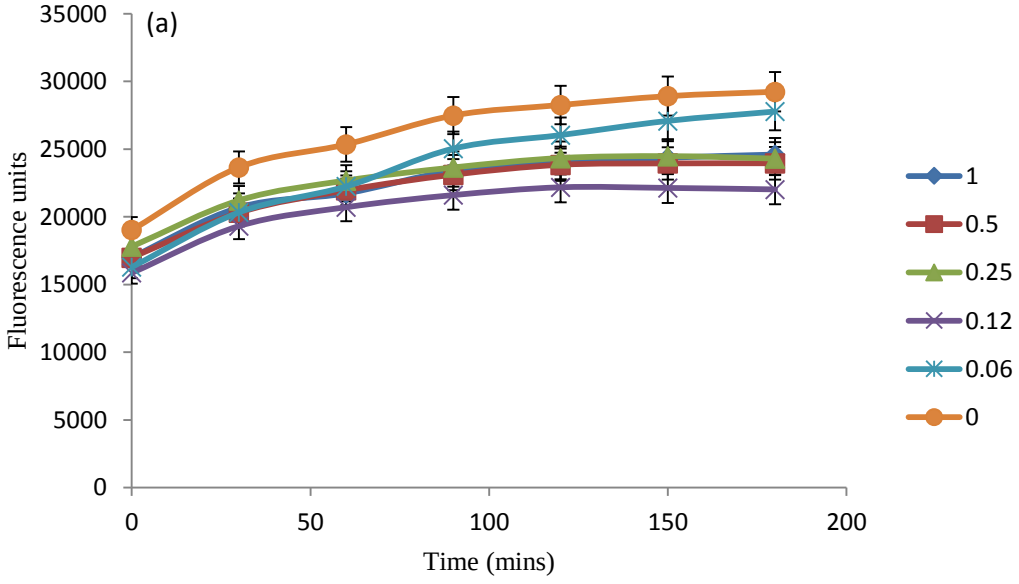


Figure 7.10. Direct effect of extracts of carambola treated with a) 0.01 mM MeJA (SFD4MJ1) and b) 0.1 mM MeJA (SFD4MJ2) extracts at 0.06 – 1 mg/ml on the intracellular production of ROS measured over 180 minutes. Values are means (n=5) ± SE.



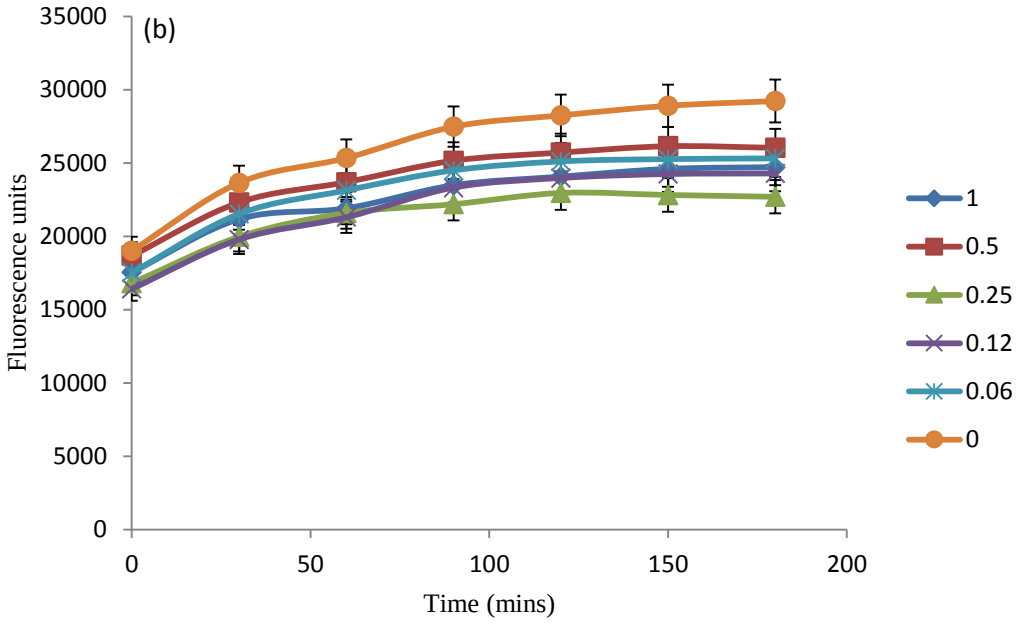


Figure 7.11. Direct effect of the extracts of carambola treated with a) 0.1 mM SA (SFD4SA1) and b) 1 mM SA (SFD4SA2) extracts at 0.06 – 1 mg/ml on the intracellular production of ROS measured over 180 minutes. Values are means (n=5) ± SE.

In all cases carambola extract significantly reduced ($p < 0.05$) (Appendix B 7.13 – 7.18) the intracellular production of ROS (Figures 7.9 – 7.11). ROS levels gradually increased in the untreated HepG2 cells, reaching a level of 29330 fluorescence units by the end of the incubation period. All of the tested carambola extracts were capable of reducing these levels at all the concentrations applied.

There was no significant difference observed between the different concentrations assessed for SFDO (Figure 7.9a). Meanwhile for SFD4, the most active concentration for ROS quenching was 0.5 mg/ml whilst the least active was 1 mg/ml (Figure 7.9b). No significant difference was observed between the lower concentrations that were tested. The observed results for both of the MeJA treated carambola extracts show 0.25 mg/ml with the best ROS quenching activity (Figure 7.10). However, lower

levels of ROS production were observed for SFD4MJ1 in comparison to SFD4MJ2, demonstrating that lower levels of MeJA were inducing more active extracts.

The SA treated carambola extracts also highlight an effect of the hormone treatment on the activity of the fruit extract (Figure 7.11). Although the lowest levels of ROS production detected for SFD4SA1 and SFD4SA2 were not significantly different, the concentrations that yielded these levels were different for the two extracts. The highest ROS quenching activity for SFD4SA1 was 0.12 mg/ml, while for SFD4SA2 it was 0.25 mg/ml.

7.3.6 Protective effect of extracts against H_2O_2 induced ROS in HepG2 cells

The optimal dosage of H_2O_2 for inducing ROS stress was initially determined by exposing the cells to H_2O_2 in HBSS at a concentration of 0 – 24 µM and measuring the production of ROS over 3 hours. HepG2 cells were exposed to carambola extract at concentrations of 0.06 - 1 mg/ml for 24 hours and then cultured on media supplemented with 6 µM H_2O_2 and ROS production was assessed over 180 minutes. These were compared to ROS levels of untreated HepG2 cells cultured on normal media (ct) as well as untreated cells cultured on 6 µM H_2O_2 supplemented media (0 mg/ml).

H_2O_2 at a concentration of 6 µM was demonstrated in section 7.3.4 to induce oxidative stress on cultured HepG2 cells, therefore this concentration was used to assess the potential protective effect of the carambola extracts. There were two groups of controls in this experiment, an untreated control that was not exposed to the H_2O_2 and an untreated control that was cultured under the oxidative stress of H_2O_2. These two groups consistently produced the lowest and the highest levels of ROS, respectively (Figures 7.12 – 7.14). The pro-oxidant H_2O_2 significantly increased the intracellular ROS production in comparison to the non-stressed cells.

Fresh fruit extract displayed minimal protective activity against ROS production (Figure 7.12a), while the ripening fruit extract significantly reduced ($p < 0.05$) the production of ROS (Figure 7.12b). High concentrations (0.25 to 1mg/ml) of the ripening fruit extract were required to have a significant reduction on the ROS levels.

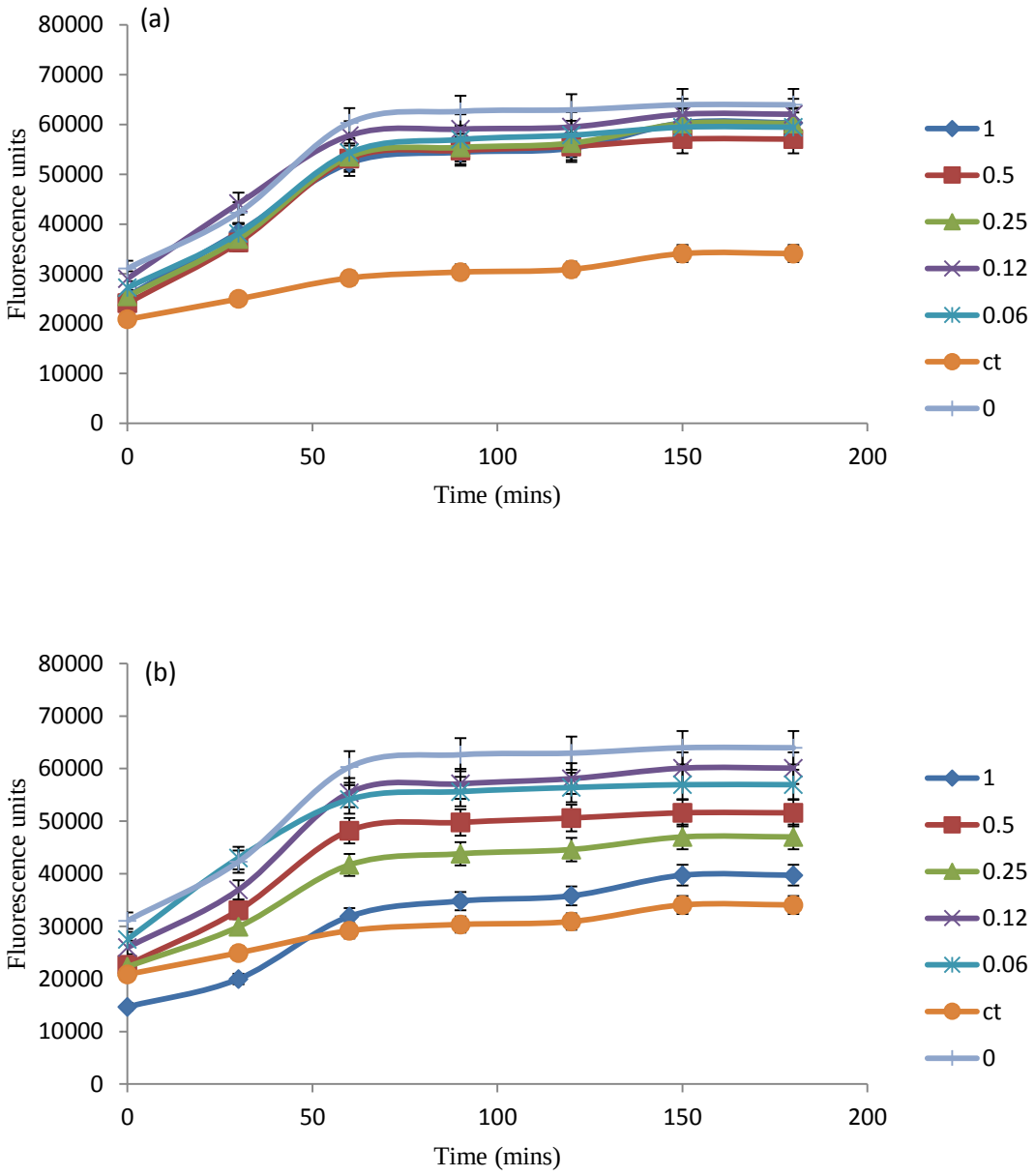


Figure 7.12. Protective effect of a) freshly harvested carambola (SFD0) and b) ripening carambola (SFD4) extracts at 0.06 – 1 mg/ml along with an untreated control (ct) on the intracellular production of ROS measured over 180 minutes. Values are means (n=5) ± SE.

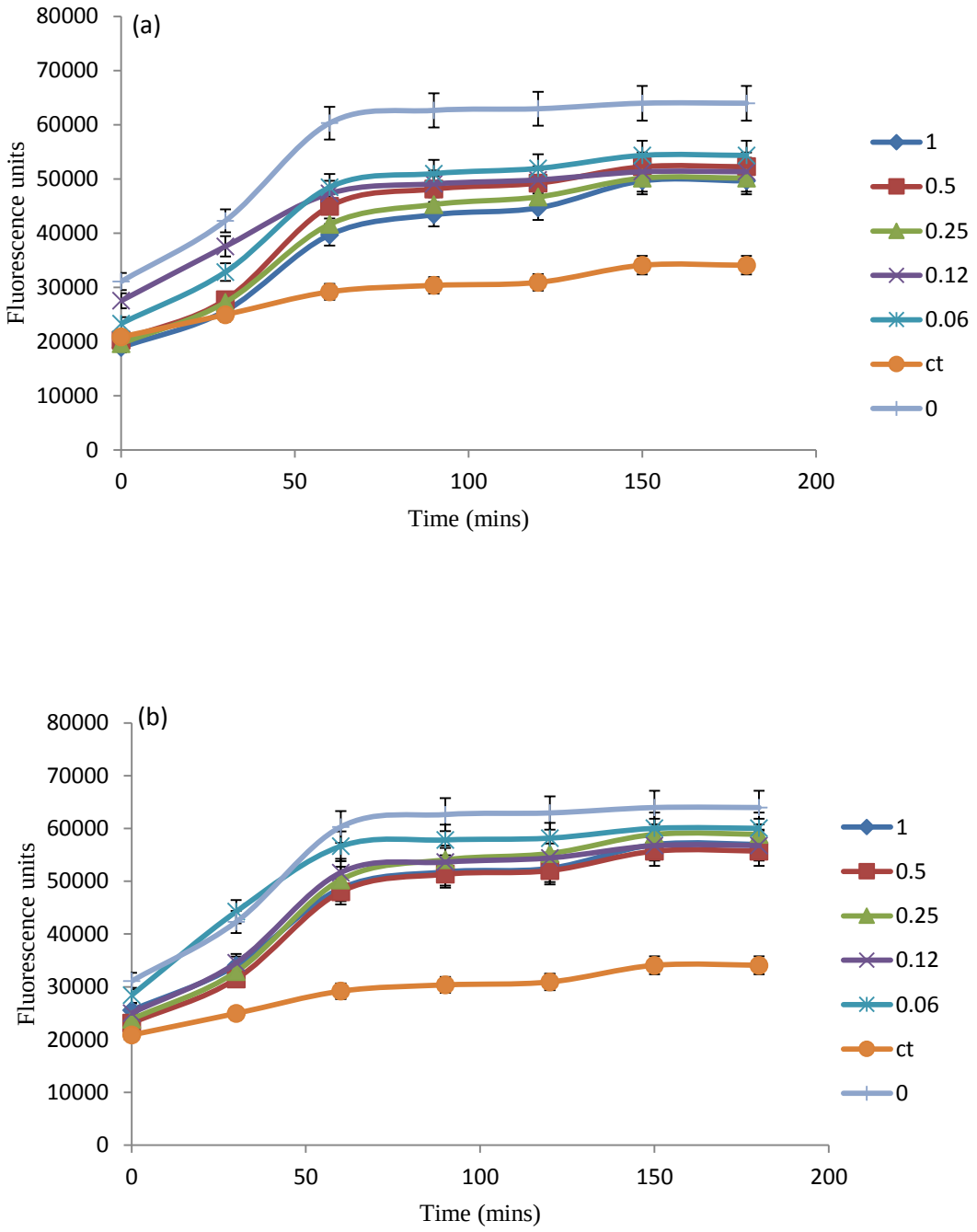
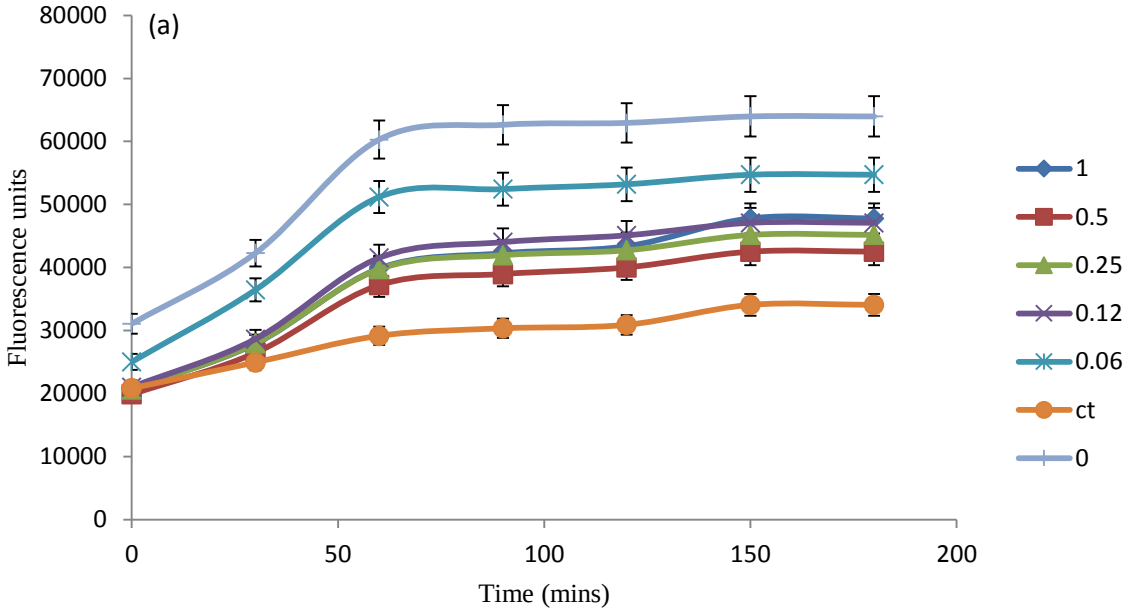


Figure 7.13. Protective effect of the extracts of carambola treated with a) 0.01 mM MeJA (SFD4MJ1) and b) 0.1 mM MJ (SFD4MJ2) extracts at 0.06 – 1 mg/ml along with an untreated control (ct) on the intracellular production of ROS measured over 180 minutes. Values are means (n=5) ± SE.



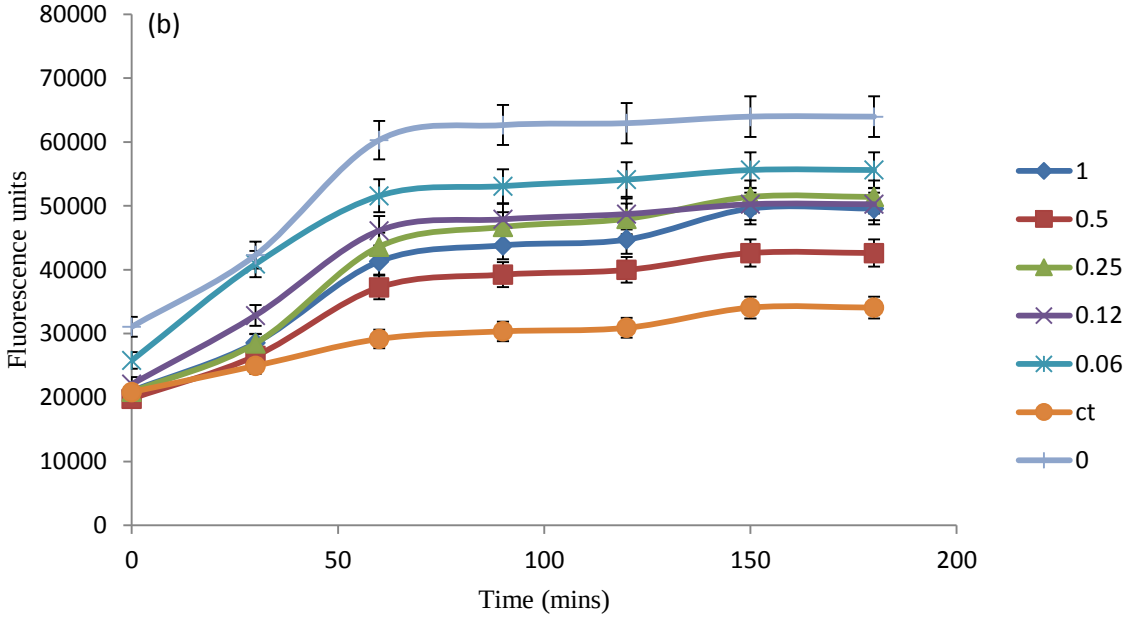


Figure 7.14. Protective effect of the extracts of carambola treated with a) 0.1 mM SA (SFD4SA1) and b) 1m M SA (SFD4SA2) extracts at 0.06 – 1 mg/ml along with an untreated control (ct) on the intracellular production of ROS measured over 180 minutes. Values are means (n=5) ± SE.

Although protective activity against ROS production was observed for the MeJA treated extracts, they displayed lower activity compared to the ripening fruit extract (Figure 7.13). The SFD4MJ1 extract was more active than the SFD4MJ2 extract, which is similar to the results observed for the direct effect of the extracts (section 7.3.5). This again demonstrates the role of the MeJA treatment in delaying ripening of the fruit, and the production of the fruit's active metabolites.

The SA treated fruits displayed a similar effect to the ripening fruit extract (Figure 7.14). Although these extracts demonstrated strong radical quenching activity the activity of the SA treated fruit was slightly lower than SFD4. Unlike the results for cytotoxicity, there was no significant difference observed between the two SA treated fruits in ROS scavenging activity.

7.4 Discussion

In relation to the activity of carambola extract on HepG2 cells, three main findings were demonstrated in this study; the cytotoxic activity of the extract at high doses, the protective antioxidant properties of the extract, and attribution of the anti-hepatoma activity to compounds other than phenolics. These findings will be discussed in lieu of other studies below.

7.4.1 Cytotoxic activity of carambola extract

A clear distinction between the hepatoprotective and hepatotoxic activities of the carambola extract possibly lies at the dose of 1 mg/ml. At levels higher than 1 mg/ml, and depending on the extract used, the cytotoxic activities of the extract were manifested. Cytotoxicity is a key indicator of antitumor activity, and the extracts exhibited cytotoxic activity at high concentrations (Newell et al. 2010). Upon the induction of apoptosis, there is a reduction in the DNA content of the cells as an increasing number of cells are arrested at the G1 phase along with increased diffusion of degraded DNA to the media (Newell et al. 2010). Thus, the cells will not be able to take up the Hoechst stain, used for quantifying cell viability. DNA and protein synthesis is proportional to cell growth rate, while RNA synthesis is proportional to square of cell growth rate (Kjeldgaard & Kurland 1963).

ROS are produced within cells as natural by-products of oxygen metabolism due to the incomplete reduction of oxygen along the electron transport chain (Zhu et al. 2013; Kielland et al. 2009). External environmental factors exacerbate the generation of ROS within the biological system. However, the role of ROS within biological systems is rather complex as it is involved in physiological processes and transcriptional regulation as well as intracellular and extracellular signalling (Kielland

et al. 2009). On the other hand, excessive ROS can disrupt the homeostasis of the redox system leading to oxidative stress (Zhu et al. 2013). This can be damaging to protein and DNA, ultimately causing DNA mutation and disrupting cellular signalling (Kielland et al. 2009). Impaired cellular functionality and damaged DNA is often detected in the cells and repair systems are set in place, or apoptosis may then be induced (Deferme et al. 2013).

Apoptosis is a protective mechanism against cellular damage and tumour development (Newell et al. 2010). Deregulation of apoptosis is a hallmark of cancer, which is characterised by uncontrolled cell division (Lee & Lim 2010). Emerging cancer therapies are increasingly relying on the induction of apoptosis for elimination of damaged cells. ROS is of paramount importance in apoptosis signalling, and apoptosis can be induced by disturbing the inherent redox homeostasis through up-regulation or down-regulation of intracellular ROS (Lee & Lim 2010).

Cancer cells possess an inherently large amount of ROS which function as signalling molecules in the mitogen activated protein kinase (MAPK) pathway (Wang & Yi 2008). The high levels of ROS activate sensitive transcription factors and genes which are crucial for the survival of the cancerous cells. Hence, oxidative stress is a necessity for the cell survival, and disturbances in these favourable redox conditions are a trigger for apoptosis. This was the basis for the establishment of the theory of persistent oxidative stress within tumour cells and the notion that reduced oxidative stress may enhance apoptosis (Wang & Yi 2008). However, it has also been proposed that cancer cell apoptosis might occur at excessively high levels of ROS production due to damage of cellular machinery (Moongkarndi et al. 2004).

Wang and Yi (2008) attributed the chemopreventive activity of the blackberries on human leukemia HL-60 cells to reduced ROS levels, which was in agreement with

several other studies on fruit extracts tested on HepG2 (Malta et al. 2013; Meyers et al. 2003; Sun et al. 2002). This might also be the case in this study, where carambola extract at doses between 1 – 5 mg/ml possessed strong ROS scavenging activity sufficient to induce these effects, whilst below 1 mg/ml the effects were not cytotoxic.

7.4.2 Antioxidant properties of extract

Newell et al. (2010) reported that non-toxic concentrations of free radicals might actually result in cell survival and proliferation. This phenomenon of increased cell survival was apparent at the lower doses, thus it was of particular interest to understand the effect of these doses on the intracellular ROS levels. Nonetheless, the results demonstrated strong radical scavenging activity of the extracts, and this was specifically higher in the extracts that showed more potent cytotoxic activity.

HepG2 cells maintain the specialised functions of hepatocytes, including xenobiotic metabolism, thus are suitable models for studying cytotoxicity as well as hepatoprotective activity of plant extracts (Pareek et al. 2013). Moreover, several aspects of the behaviour of HepG2 are conserved to neoplastic cell behaviour due to presence of the wild-type p53 gene in this cell line (Newell et al. 2010). Nonetheless, effective antioxidant systems of a normal liver are remarkably reduced in diseased or damaged hepatic systems, such as hepatocarcinoma (Vidyashankar et al. 2013).

Oxidative stress is mainly the results of an imbalance between the inherent ROS production and inherent antioxidant defence systems (Zhu et al. 2013). H_2O_2 can cause such an imbalance, causing apoptosis of HepG2 cells (Zhu et al. 2013). The damaging activity of H_2O_2 is associated with the induction of hydroxyl radicals, thereby disrupting the redox homeostasis (Pareek et al. 2013). H_2O_2 is usually produced within biological systems by the activity of SOD converting superoxide radicals. It is then stabilised by

CAT and GSH and reduced to water, however when the ROS generation occurs at a faster rate than the inherent antioxidant systems, oxidative damage and cellular injury occur (Pareek et al. 2013).

Baeza et al. (2014) reported the protective activity of the tested green coffee extracts against t-BOOH induced oxidative stress and attributed it to the prevention of increases in ROS levels, prevention of GSH depletion and restoration of antioxidant enzymes. The antioxidant activity of extracts also involves modulation of antioxidant enzymes and enzymes that activate xenobiotics (Žegura et al. 2011).

7.4.3 Characterisation of bioactive component in extract

Another main finding of this study suggested that phenolic compounds were not responsible for the antihepatoma activity. Although the reported bioactivity of the extracts could be lower than anticipated due to the low purity of the polyphenolic fraction, it is equally possible that the activity is attributed to compounds besides polyphenols (Yi et al. 2006). Similarly, Khonkarn et al., (2010) concluded that the compounds responsible for the antiproliferative activity coconut peel extract were not polyphenols. They found no relation between the antioxidant activity and the antiproliferative activity of the extract, as the yield of the more polar solvents did not demonstrate antiproliferative activity.

Carambola is rich in flavonoids, which are phenolic compounds with antioxidant activity and offer protection against free radicals. Polyphenol activity against cancer has been demonstrated against numerous cancer cell lines when tested in pure form or amongst multiple constituents such as in tea (Newell et al. 2010; Actis-Goretta et al. 2008). Hemaiswarya and Doble (2013) studied the cytotoxic activity of phenolic compounds against the cervical cancer cell line (HeLa) and found ferulic acid

to show the second highest activity after eugenol. Meanwhile p-coumaric acid ranked the sixth amongst the various phenylpropanoids they had tested. While, ferulic acid was not detected in carambola, *p*-coumaric acid was detected at various levels in the different carambola samples assessed (Section 6.3). Moreover, hydroxycinnamic acids from green coffee were found to protect HepG2 cells from oxidative stress by modulating ROS generation, antioxidant defences and cell signalling (Baeza et al. 2014).

Phenolic compounds function directly through the prevention of radical formation and metal ion chelation, while their indirect antioxidant activity is achieved by modulating the activity and expression of antioxidant enzymes (Žegura et al. 2011). The activity of phenolic compounds is strongly dependent on the polarity, bioavailability and hydrophobicity of the compounds, in addition to their antiradical activity (Lima et al. 2006). Thus, it is likely that the phenolic compounds available in the carambola extract are synergistic in nature and require the presence of other compounds available in the first fraction. However, it is possible that the anti-proliferative activity of carambola could also be attributed to other classes of bioactive molecules, such as the lipophilic carotenoids.

Since the anti-proliferative activity of the carambola extract was not wholly attributed to the phenolic content, carotenoids could be responsible for some of this activity. Carotenoids were reported to exert pro-oxidant activity at high oxygen tension or under high concentrations of carotenoids (Jaswir et al. 2012). This was attributed to their depletion by autoxidation and consequent loss of antioxidant activity, thus carotenoids can protect cells from oxidative stress when administered at lower concentrations whilst at higher concentrations the damage to DNA and lipids will be exacerbated, hence inducing cytotoxicity (Jaswir et al. 2012).

Moreover, the cytotoxic activity observed could be attributed to the presence of polysaccharides. Polysaccharides are a structurally diverse class that play numerous roles in living organisms (Ding et al. 2012). Polysaccharides can function as energy reserves, signalling molecules, structural components and defence mechanisms. Anti-tumour and anti-inflammatory activity was exhibited by polysaccharide fractions comprised of galactose and arabinose (Ding et al. 2012). In vitro studies have successfully demonstrated the cytotoxic activity of polysaccharides, although there is still a need for confirmation of these results through in vivo studies (Chien et al. 2015; Zong et al. 2012). Nonetheless, there is still a need for more studies exploring the isolation and characterisation of different polysaccharides found in fruits and dissecting their activity individually or in combination with other bioactive molecules.

7.5 Concluding Remarks

HepG2 as a model illustrated that the carambola extract is a good antioxidant in biological systems. Exposure to concentrations below 1 mg/ml is beneficial as an antioxidant, offering the cells protection against ROS. This study demonstrated the chemopreventive activity of carambola extract, however it also strongly suggests that total phenolic content of the fruit is not the mechanism behind this activity. Natural antioxidants counteract oxidative stress, such as those induced by hepatotoxins, thus there is a constant search for natural antioxidants possessing therapeutic benefits.

From all of the previous results it is apparent that the activity of the fruit extract is changing in response to the different stresses the fruit has faced. Dissecting and understanding the changes occurring within the fruit that are leading to these changes is crucial at this stage. Gene expression studies could help explore the variations in the signalling and metabolic pathways of the phenylpropanoid pathway in response to the stresses that the fruits have been exposed to during the earlier experiments. The key genetic messages of the stressed fruits will be explored in the following chapter to allow subsequent studies elucidating the cellular and developmental processes that have been activated.

CHAPTER 8: EXPLORING STRESS GENETICS OF *AVERRHOA CARAMBOLA*

8.1 Introduction

Various molecules have the capacity to function as elicitors, such as polysaccharides, proteins and lipids, by triggering signalling pathways. The perception of elicitors triggers an influx of calcium that is followed by an oxidative burst, leading to the biosynthesis of stress signals jasmonic acid and salicylic acid (Gonzalez-Aguilar et al., 2010). This triggers the expression of defence related genes regulating the production of compounds with antioxidant and antimicrobial properties, such as phenolic compounds (Belhadj et al. 2008).

Stress is an elicitor resulting in a downstream signalling process. It initially triggers an influx of calcium ions, which is followed by an oxidative burst of reactive oxygen species (ROS) and nitrogen oxide (NO) (Belhadj et al. 2008). The increase in ROS and NO levels triggers signalling pathways involving the signalling molecules salicylic acid and jasmonic acid. This pathway can be simplified as follows:

Stress → Ca^{2+} influx → oxidative burst → signal molecule (JA, SA)→ signal pathway

Numerous enzymes are involved in the regulation of ROS, which involves the timely production and scavenging of the various ROS. These enzymatic activities in *Arabidopsis* are regulated by a total of 152 genes (Mittler et al. 2004). Reduction of oxygen to singlet oxygen is catalysed by various enzymes which include NADPH-dependent oxidases, pH-dependent cell wall peroxidases, germin-like oxalate oxidases

and amine oxidases (Mittler et al. 2004). Genetic studies have assisted immensely in the characterization of the enzymes involved in stress responses.

The downstream response of these various stress-signalling pathways is mitigation of cellular damage. This study has shown that the various stresses investigated influence the quantity and quality of the phenolic content of carambola. Phenolic compounds are involved in the amelioration of cellular damage under biotic and abiotic stresses (Siboza et al. 2014). Phenolic compounds are the product of the phenylpropanoid pathway (Figure 8.1), which produces hydroxycinnamic acids, monolignols, coumarins, benzoic acids, stilbenes, anthocyanins, flavonoids (Fock-Bastide et al. 2014; Ferri et al. 2011).

The core of this pathway is made up of three genes; phenylalalanine ammonia lyase (PAL), cinnamate 4 hydroxylase (C4H) and 4-coumarate coenzyme A ligase (4CL). Moreover, the phenylpropanoid pathway is constantly subject to stress-induced changes. There have been a number of studies on the expression profile of these core genes in relation to the level of phenolic compounds. The complex pathway has been examined at biochemical and molecular level in the model plant Arabidopsis, and less so in crops of agronomic importance (Jadhav et al. 2013). Nonetheless, a large number of these genes can be found in the NCBI database for various crops.

PAL is a cold regulated gene and is induced upon cold storage. It has been studied extensively, especially in relation to low temperature stresses and their incurred responses (Sanchez-Ballesta et al. 2007). Carambola is rich in proanthocyanidins, which are produced downstream of the phenylpropanoid pathway. The core enzyme involved in its biosynthesis is anthocyanidin synthase (ANS)/leucoanthcyano deoxygenase (LDOX), which has been found to be a single copy gene in grapes (Tavares et al. 2013; Ferri et al. 2011). Thus, PAL, C4H and ANS would be important candidates for exploring the stress-related changes the carambola fruit has experienced.

Meanwhile, the anti-proliferative activity observed for carambola extract (section 7.3.3) suggests a probable synergistic effect of the sugars and phenolic content of carambola. Sugars do not only serve as energy sources within plants, but they also modulate gene expression within primary and secondary metabolism such as photosynthesis, respiration and plant defence (Ferri et al. 2011). Sugar levels are detected by biosynthesis and degradation such that high sugar levels direct growth versus photosynthesis and energy mobilisation (Van den Ende & El-Esawe 2013). Moreover, the type of sugars also plays a role in signalling, for example low water-soluble carbohydrates, such as sucrose, glucose and fructose, triggers signal transduction and gene expression (Van den Ende & El-Esawe 2013).

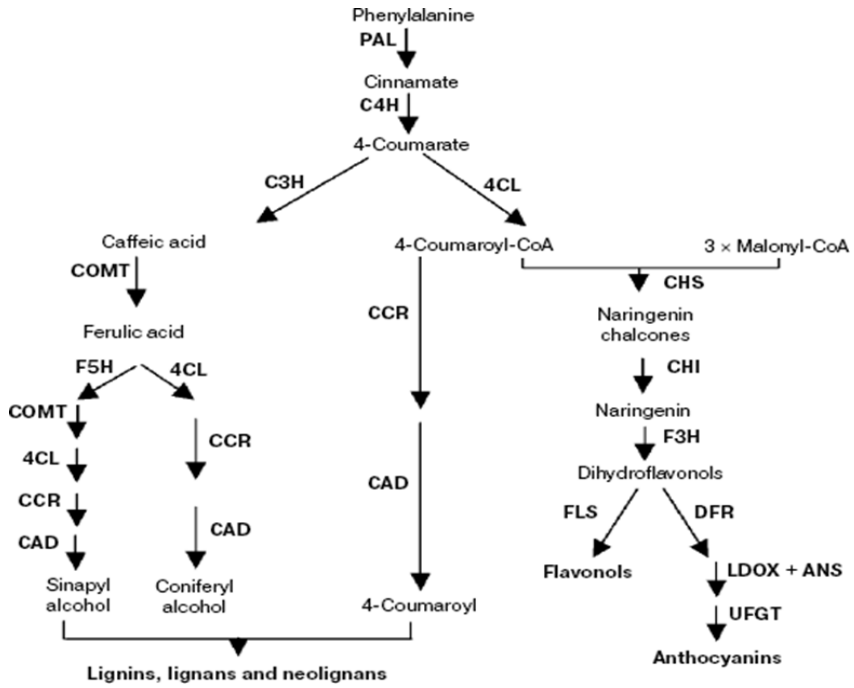


Figure 8.1 Established phenylpropanoid pathway and the downstream products (Gutierrez-Gonzalez et al. 2010).

Fructan is a sugar of particular interest as it has been shown to counteract oxidative stress (van Arkel et al. 2013). Fructan is synthesized through an SA-dependent pathway and it counteracts stress through direct and indirect approaches. The

direct approach involves ROS scavenging, phytoprotection and membrane stabilisation; whilst the indirect approach involves signalling pathways that employ the stress inducible kinase SnrK1 (Sucrose Nonfermenting 1 Protein Related Kinase).

Transcription factors regulate gene expression and this is done by binding to Cis-acting elements in the gene (Harman et al., 2004). There are various differences in the DNA-binding domains and the 3D structure of TFs, which is the key element in their classification into distinct families. SnrK1concomitantly induces the biosynthesis of fructan, whilst inhibiting the activity of transcription factors involved in the biosynthesis of anthocyanin and the core genes of the phenylpropanoid pathway. The stress inducible kinase SnrK1 plays a dual regulation role, so would also be of interest in studying stress regulation in carambola.

Throughout the previous chapters, the developmentally regulated physiological and biochemical changes in carambola, have been explored. These various physiological and biochemical changes control numerous desirable and undesirable characteristics of fruit quality, and are irreversible and often unstoppable. Understanding the pathways involved in ripening and the signalling cascade associated with changes in fruit quality are worth investigating. Thus, there is a need to understand the expression of these genes (ANS, C4H, PAL and SnrK1), which is paralleled with a need to identify the members of these gene families found in carambola. Therefore, in this chapter, the isolation of RNA and identification of these genes in carambola was be carried out.

8.2 Materials and methods

8.2.1 RNA extraction

Two different extraction methods were used for isolating RNA from carambola. Tomato fruit samples were used as a control for comparing the different methods used on carambola. RNA from dragonfruit was extracted using Qiagen RNeasy Plant Mini kit (Qiagen, USA), according to the manufacturer's instructions.

8.2.1.1 Method 1 (LiCl clean up):

The following reagents were prepared for the extraction protocol, 3M sodium acetate, 8M lithium chloride and 80% methanol. Phenol mix was prepared that contained phenol, hydroxyquinoline and m-cresol. The extraction buffer was made up of naphtalenedisulfonic acid disodium salt, 4-aminosalicylic acid and phenol mix, and a phenol-chloroform mixture was also prepared.

The extraction process then took place as follows; 10g of homogenised fruit sample was mixed with 10ml of extraction buffer and 10ml of phenol-chloroform mixture. The mixture was then centrifuged at 10,000 xg for 15 minutes, and the aqueous phase was precipitated overnight at -20 ºC with 2 x volume of 80 % ethanol and 0.1 x volume of 3 M sodium acetate.

On the next day, centrifugation was carried out at 10,000 xg for 10 minutes and the supernatant was removed and pellet re-suspended in 500 µl water. The undissolved debris was removed by centrifugation for 2 minutes. An equal volume of LiCl was added and it was stored overnight at -20 ºC.

The following day the samples were centrifuged at 10,000 xg for 15 minutes and the pellet was redissolved in 450 µl water and undissolved debris was removed.

The sample was then stored overnight at -20 ºC with 3 volumes of ethanol and 0.1 volume of sodium acetate.

On the fourth day, the sample was centrifuged at maximum speed for 10 minutes and the pellet was washed with 80 % ethanol. Complete evaporation of the ethanol was ensured before the pellet was re-suspended in 60 µl water.

8.2.1.2 Method 2 (CTAB clean up):

The following reagents were used for the protocol; phenol mixture made up of phenol, m-cresol, hydroxyquinoline mixed with naphtalenedisulfonic acid disodium salt and 4-aminosalicylic acid. Phenol chloroform was made up off phenol, Tris-HCl, hydroxyquinoline, chloroform and isoamyl alcohol.

The extraction process then took place as follows, 10 g sample was mixed with 10 ml extraction buffer and 10 ml phenol-chloroform and centrifuged at 10,000 xg for 15 minutes. The aqueous phase was transferred to a new tube and mixed with 2.5 x volume 70 % ice cold ethanol and 0.1 x volume sodium acetate. The sample was allowed to precipitate overnight at -20 ºC.

On the next day, the sample was centrifuged at 10,000 xg for 10 minutes and washed with 2ml ethanol. After the ethanol was removed and the pellet was dried, the pellet was re-suspended in 2ml water and mixed with 2ml cetyl-trimethyl ammonium bromide (CTAB) extraction buffer and 2ml CTAB precipitation buffer and centrifuged for 1 hour at 10,000 xg. The supernatant was removed and the previous step was repeated again using a 10 minute spin instead of 1 hour. After removing the supernatant, the pellet was then dissolved in 2 ml of 1.4 M NaCl and allowed to precipitate overnight with 2.5 x volume 70 % ice cold ethanol and 0.1 x volume sodium acetate at -20 ºC.

The following day, the sample was centrifuged at 10,000 xg for 30 minutes and after discarding the supernatant the pellet was washed with 70% ice cold ethanol for 10 minutes at 10,000 xg. The supernatant was then discarded and the pellet dried under vacuum before it was dissolved in 100 µl water and left on ice for 30 minutes.

8.2.2 Assessment of RNA quality

Agilent chip Bioanalyzer (Agilent Technologies, CA, USA) was used to quantitatively and qualitatively analyse the yield of the samples. Promega GoScript reverse transcriptase kit (Promega, MA, USA) was then used to synthesize cDNA from 500 ng of the isolated RNA. The RNA was mixed with 1 µl of oligoprimers and 10mM dNTP and heated at 70 °C for 5 minutes. The reaction mixture was then cooled for 5 minutes and mixed with 4 µl of Buffer, 1 µl of Reverse Transcriptase and 1 µl of RNase inhibitor (Promega, Madison, WI, USA). The reaction was then carried out 42 °C for 60 minutes and stopped by incubation at 72 °C for 15 minutes.

8.2.3 Primer design

Degenerate primers were designed for candidate genes that did not have a known sequence. Aminoacid sequences were sourced from the NCBI protein database (Table 8.1). The conserved regions of target proteins were initially determined by aligning the aminoacid sequences of at least five species using MUSCLE (MUltiple Sequence Comparison by Log- Expectation) (EMBL-EBI). Block Maker on CODEHOP (COnsensus-DEgenerate Hybrid Oligonucleotide Primers) was used to create local multiple alignments (blocks) of the target proteins from the conserved regions. Primers with random nucleotide composition were then designed from the protein blocks using CODEHOP. Primers were selected by confirming 80% identity to the nucleotide sequences of other plants using BLAST (Basic Local Alignment Search Tool).

8.2.4 PCR settings

PCR amplification was set up as follows: 1µl of cDNA template at concentration of 50-100 ng, 50 µM of each primer, 5mM dNTP (Finnzymes, Thermo Scientific, USA), 2.5 µl of Fermentas 10z Taq buffer (Fermentas, Thermo Scientific, USA) and 0.25 µl of Taq 0814 polymerase (Genomic Services, University of Nottingham, UK). $MgCl_2$ was added at concentrations of 0.25, 1.0 and 2.5 mM and DMSO was also tested at three different concentrations of 0, 2 and 5 % (Table 8.2). The final volume was made to 25 µl with distilled water.

The PCR reaction was set at 94 °C for 3 minutes followed by 35 cycles of 94 °C for 30 seconds, 65 °C for 45 seconds and 72 °C for 60 seconds. A final extension at 72 °C for 10 minutes was carried out and samples were then held at 10 °C. PCR amplicons were loaded on a 2 % agarose gel stained with ethidium bromide and the gel was examined and photographed with a UV imager (InGenius 3 GeneSys, Syngene, UK). A blank, reaction mix without genomic template, was included in all tests.

Table 8.1 Protein sequences used for creating protein blocks sourced from the Protein Database on NCBI

Gene description	**Accession number (NCBI)**	**Species**	**Protein length (aminoacids)**	**Assigned name**
ANS	AEI99590.1	*Arabidopsis thaliana*	356	ANS 1
ANS	ACH58397.1	*Brassica juncea*	358	ANS 2
ANS	AAD26205.1	*Malus domestica*	357	ANS 3
ANS	ADK37750.1	*Brassica oleraceae*	358	ANS 3
ANS	ABV82967.1	*Vitis Vinifera*	355	ANS 5
C4H	CAP08845.1	*Arabidopsis thaliana*	505	C4H1
C4H	P37114.1	*Medicago sativa*	506	C4H2
C4H	ABF17874.1	*Brasssica napus*	505	C4H3
C4H	AFG30055.1	*Malus hybrid*	504	C4H4
C4H	AAK54447.1	*Sorghum biocolor*	501	C4H5
PAL	XP_008369679.1	*Malus domestica*	720	PAL1
PAL	AAC18870.1	*Arabidopsis thaliana*	725	PAL2
PAL	ADL09136.1	*Brassica oleraceae*	723	PAL3
PAL	ACM62741.1	*Garcinia mangostana*	718	PAL4
PAL	AFP86474.1	*Brassica rapa*	746	PAL5
SnrK1	Q38997.2	*Arabidopsis thaliana*	535	SnrK11
SnrK1	CDX74460.1	*Brassica napus*	494	SnrK12
SnrK1	XP_009151804.1	*Brassica rapa*	512	SnrK13
SnrK1	ABV49061.1	*Malus hupehensis*	515	SnrK14
SnrK1	ADN44282.1	*Capsicum annuum*	512	SnrK15
SnrK1	ABQ18267.1	*Sorghum bicolor*	509	SnrK16

Table 8.2 DMSO and $MgCl_2$ concentrations used to optimise the PCR reaction conditions

PCR reaction	**DMSO content (%)**	**$MgCl_2$ content (mM)**
A	0	0.25
B	2	0.25
C	5	0.25
D	0	1.0
E	2	1.0
F	5	1.0
G	0	2.5
H	2	2.5
I	5	2.5

8.3 Results

8.3.1 Isolation of RNA from fruit samples

The yield from the LiCl clean-up and the CTAB clean-up method were assessed using Agilent Bioanalyzer, and compared to RNA isolated from tomato samples employing the same methods (Figure 8.2 and 8.3). RNA from tomato was isolated using the LiCl clean-up and CTAB clean-up method with varying degrees of purity (Table 8.3). In addition to the yield from CTAB clean-up method being lower than the LiCl clean-up yield, the 28s RNA was not intact. However, there were no intact RNA bands detected for carambola sample using the LiCl clean-up method, whereas the CTAB method was successful on the carambola fruit sample. Clear bands for 28S and 18S RNA were observed for the carambola sample using the CTAB method.

Table 8.3 Assessment of RNA integrity by Agilent Bioanalyzer

Sample	**RNA area**	**RNA concentration (ng/µl)**	**rRNA ratio (28s/18s)**
Carambola LiCl	9.6	16	0.0
Tomato LiCl	215.7	370	2.0
Carambola CTAB	28.7	64	1.0
Tomato CTAB	69.8	155	0.5

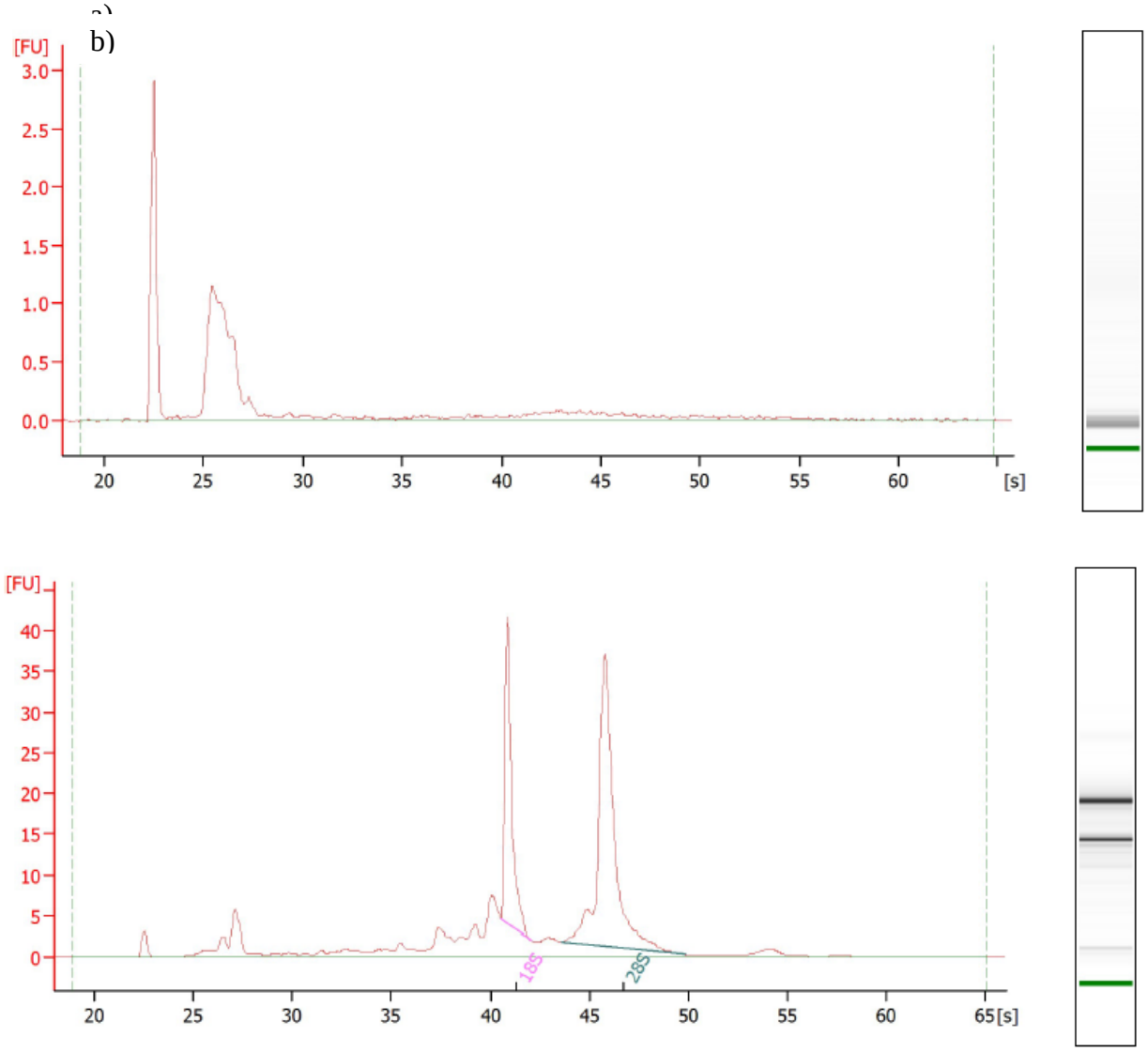


Figure 8.2 Agilent Bioanalyzer electropherograms of RNA isolated from a) carambola and b) tomato using the LiCl clean-up method

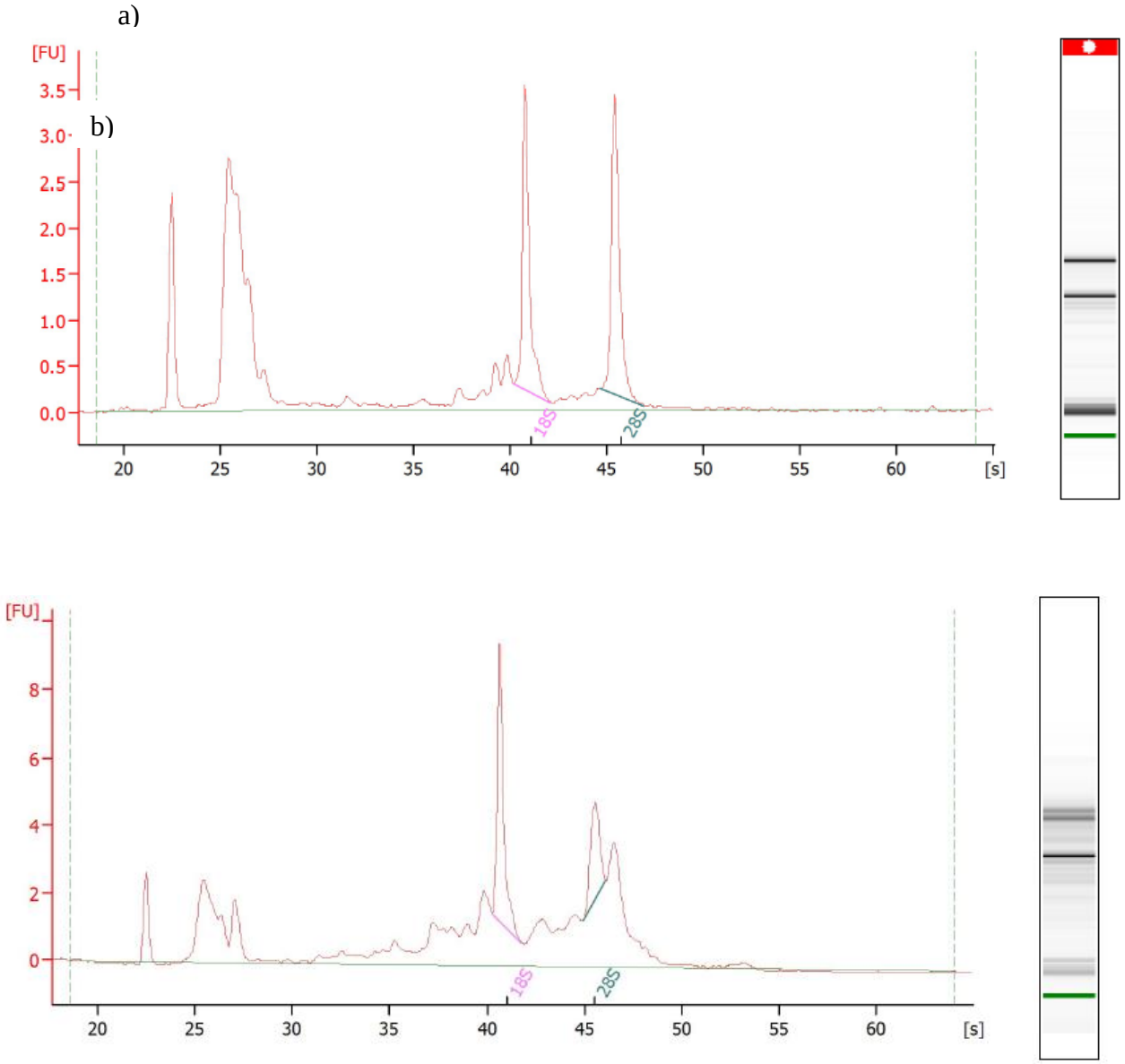


Figure 8.3 Agilent Bioanalyzer electropherograms of RNA isolated from a) carambola and b) tomato using the CTAB clean-up method

The RNA integrity of the isolates from the CTAB method was re-confirmed by running a PCR reaction with ATP synthase primers (Figure 8.4). Although the intensity of the band observed for the carambola PCR amplicon was fainter than the other fruits, bands were detected for all of the fruit samples, confirming the presence of intact mRNA in the samples ideal for further study.

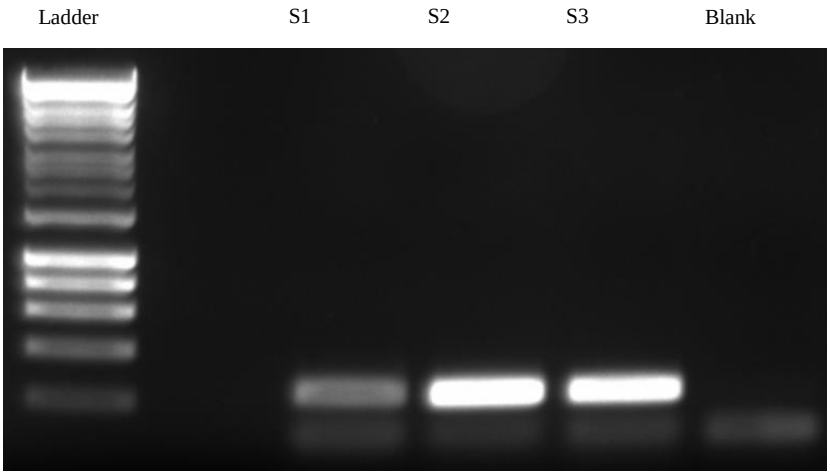


Figure 8.4 PCR amplicons from S1: carambola, S2: dragonfruit, S3: tomato RNA and Blank (no genomic template) using ATP synthase primers.

8.3.2 Designing degenerate primers

Degenerate primers were designed to allow the isolation of distantly related sequences that encode a conserved protein sequence for ANS, C4H, PAL and Snrk1 genes (Rose et al. 2003). The CODEHOP approach was used, whereby protein sequences from at least five species were aligned to determine relation (Figure 8.5), and the conserved regions were then determined using MUSCLE. Blocks of the target protein were created from the conserved regions, which were then used to design primers with random nucleotide composition (Table 8.4)

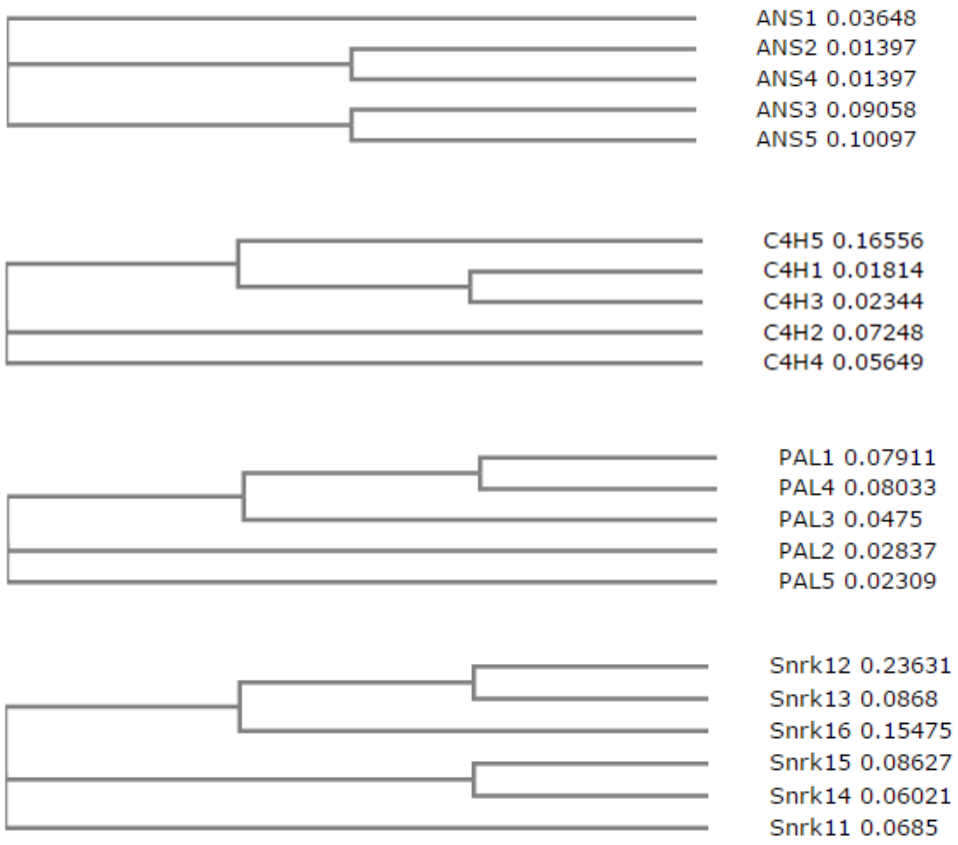


Figure 8.5 Phylogram of protein sequences of a) ANS, b) C4H, c) PAL and d) SnrK

Table 8.4 Degenerate primers designed using CODEHOP software

	Forward	**Reverse**
ANS	AATAATGCTTCTGGACAACTGGAR TGGGARGAYT	AGCCCAAGAAATTCTAACCTTTTC YTTRTTNAC
C4H	TGGTCCATCGAGTGGGGNATHGC	GAGTGGTTCAGGATGTGGATGSW RAAYTGNCC
PAL	GATTATGGATTTAAGGGAGCTGAA ATHGCNATGGC	ACAAATAGGAATAGGAGCTCCRT YCCANYC
SnrK 1	TGGAACTCTGCCTTTTGATGAYGA RAAYAT	TGTGTCAGAAAAGCAGCACAAAR RTCNARRAA

The sequences for each protein (Table 8.1) were selected with a minimum level of 80% similarity, however there were varying levels of relationship as established in the phylogeny tree (Figure 8.5). With regards to the ANS protein, there were two groups of closely related clusters; the first being the two Brassica species and the second was *Malus domestica* and *Vitis vinifera*. The Arabidopsis sequence was of similar level of relation to both clusters. Meanwhile, for C4H the protein sequences were highly conserved between Arabidopsis and *Brassica napus*. A high level of conservation was also observed amongst the PAL protein sequences for apple and mangosteen, with Arabidopsis and the two Brassica species showing a lesser degree of similarity. Moreover, the SnrK1 protein was found to be highly conserved amongst the two Brassica species with Arabidopsis showing lesser similarity as well. Thus, there was a need to include more than five species after the initial analysis of SnrK1 protein, to create the protein blocks for designing the degenerate primers.

8.3.3 Optimising PCR settings

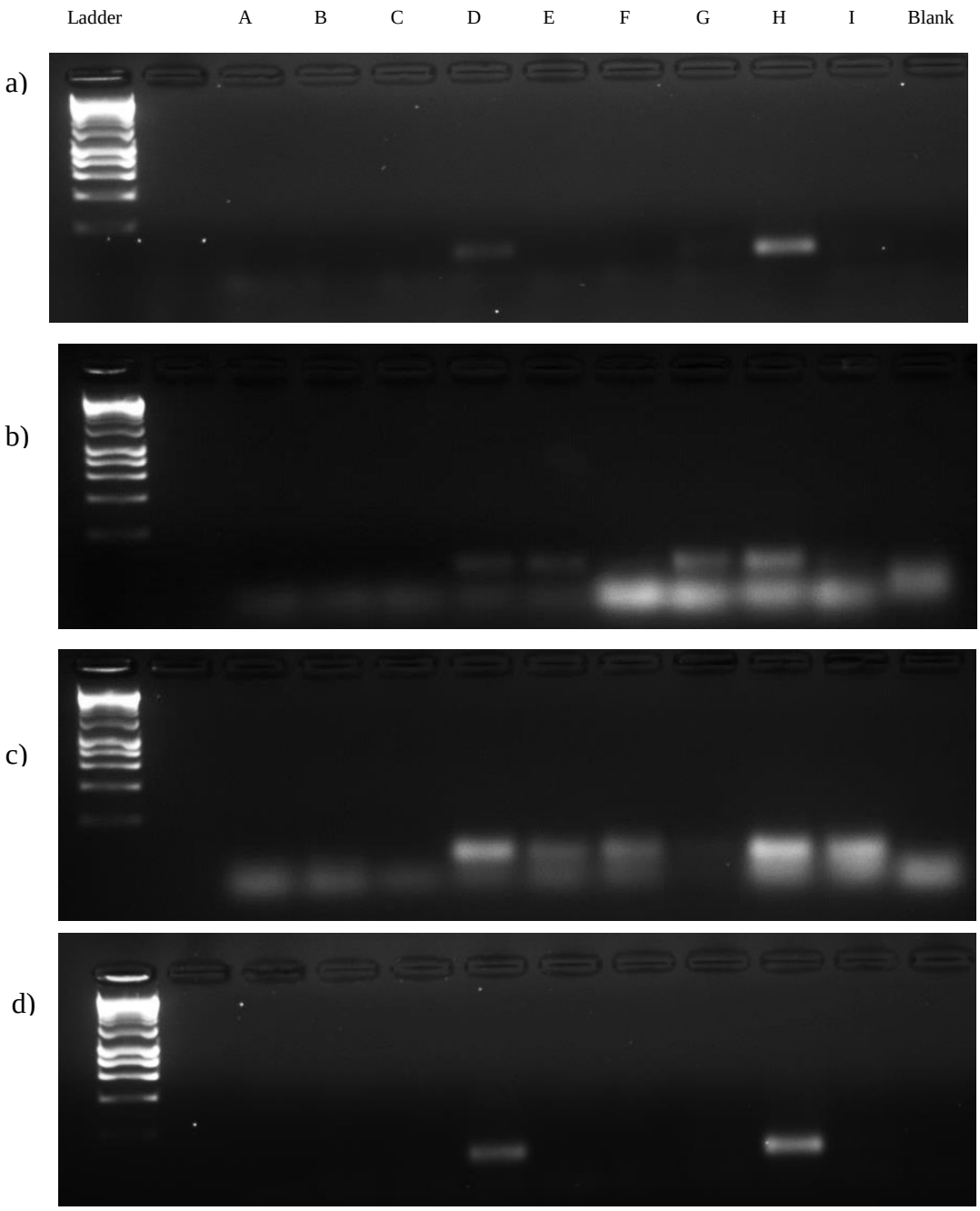


Figure 8.6 PCR experiments illustrating the optimisation of PCR reactions for a) ANS, b) C4H, c) PAL and d) SnrK1 degenerate primers. PCR conditions for DMSO (%) and $MgCl_2$ (mM) are: A 0 % and 0.25 mM; B 2 % and 0.25 mM; C 5 % and 0.25 mM; D 0 % and 1.0 mM; E 2 % and 1.0 mM; F 5 % and 1.0 mM; G 0 % and 2.5 mM; H 2 % and 2.5 mM; and I 5 % and 2.5 mM.

Three different levels of $MgCl_2$ and DMSO (Table 8.2) were tested for the PCR reaction using the degenerate primers on carambola RNA (Figure 8.6). Temperatures were initially optimised for the PCR reactions and 65 °C was selected for the PCR settings. Increasing the cycle number to 70 cycles did not exhibit an effect on the intensity of the bands, thus the cycle numbers was maintained at 35 cycles. The different $MgCl_2$ and DMSO however did play a role in the optimisation of the PCR reactions. PCR reaction H contained 2% DMSO and 2.5mM $MgCl_2$ and clear bands were observed for this reaction condition for all of the tested primers. Clear bands of lesser intensity were also observed for reaction D which contained 0% DMSO and 1.0mM $MgCl_2$.

Whilst, none of the other PCR reactions produced clear bands for ANS and SnrK1 primers, variable results were observed for C4H and PAL. Bands of varying intensities were observed for reactions E,F,G and I, all of which contained variations of DMSO at 0, 2 and 5%. Nonetheless, all of these reactions had higher $MgCl_2$ levels of 1 mM and 2.5 mM. Similarly, reactions A, B and C which all shared the lowest levels of $Mgcl_2$ at 0.25 mM, were not successful PCR reactions. Higher levels of $MgCl_2$ played a main role for these PCR reactions.

8.4 Discussion

8.4.1 RNA isolation from Carambola

Established RNA isolation procedures such as organic extraction protocols as well as lysis extraction methods using TRIzol (Life Technologies, USA) were unsuccessful on carambola. Moreover, even commercial kits such as Qiagen RNeasy Plant Mini Kit (Qiagen, Germany) could not efficiently extract RNA. Phenol-chloroform method with a CTAB clean-up was the only method that successfully yielded intact RNA from carambola samples. Furthermore, variations in the CTAB method, such as addition of an extra CTAB clean-up or exclusion of Tri-isopropyl naphthalene sulphonic acid from the protocol were also found to decrease the RNA yield substantially (results not shown). The specificity of this method for successfully isolating carambola RNA sequences is discussed in context of the carambola fruit material.

There are a number of RNA isolation protocols for fruits involving the use of phenol-chloroform method (Vareli and Frangou-Lazaridis, 1996), hot borate method (Wan and Wilkins, 1994) or polyvinylpyrrolidone (PVP) and ethanol precipitation (Salzman et al. 1999). Application of these methods for fleshy fruits with high levels of polyphenols and polysaccharides such as kiwi, apple and peach were unsuccessful (Hu et al. 2002). Isolation of RNA from fruits is often difficult, and this has often been attributed to the high phenolic and polysaccharide content as well as other secondary metabolites (Asif et al. 2006). These compounds generally bind to the nucleic acids hindering their separation from the nucleic acids during the last steps of resuspension after the genomic material is precipitated with alcohol and salt (Dal Cin et al., 2005).

Carambola is rich in both phenolic compounds and pectins. Polyphenols covalently bind to the genomic material and are almost impossible to break the bonding.

Moreover, as polyphenols are oxidised to quinones when the plant cellular material is broken down, they can bind more readily to the genomic material (Salzman et al. 1999). Thus, it is advisable to prevent the binding during the initial cell lysis. Hence, a number of protocols have resorted to adding detergents such as CTAB, SDS or antioxidants such as b-mercaptoethanol or vinyl-pyrrolidone polymers such as PVP (Salzman et al. 1999).

The numerous additives operate through various mechanisms. Antioxidants decrease the oxidation of polyphenols while detergents solubilise lipids and enzymes, preventing them from forming complexes with the DNA. PVP are the smaller more soluble class of vinyl-pyrrolidone polymers, thus offer more efficient contact with polyphenols. While PVP binds up polyphenols and dissociates the cell membranes, it also disrupts the formation of nucleic acid complexes with insoluble cell debris that would normally be precipitated during the initial stages (Pateraki & Kanellis 2004). Nonetheless, the use of PVP is not compatible with phenol extraction protocols which are preferred for removing proteins (Salzman et al. 1999).

Polysaccharides also bind to genomic material forming a gelatinous residue that obstructs the re-suspension of precipitated genomic material and impede absorbance-based quantification (Azevedo et al., 2003). Acidic polysaccharides such as pectin inhibit enzymes used in downstream processes such as PCR. Pectins encapsulate larger molecules such as nucleic acids, hindering their isolation from the plant material (Asif et al. 2006). These polysaccharides can be precipitated with lithium chloride, salt-alcohol or by outcompeting with high cationinc detergents such as CTAB under high salt conditions (Dal Cin et al. 2005). Thus, the precipitation of the RNA in 1.4M NaCl following a CTAB clean-up was a crucial step for yielding high quality RNA.

There were other parameters to consider for RNA isolation such as avoiding time-consuming steps as it may increase RNA degradation, ultimately decreasing the yield and quality. Attempts to incorporate pectinases to decrease the reaction time, which is generally effective for DNA purification, proved to be unsuccessful in RNA purification as it poses a risk of RNA degradation. Additionally, endogenous ribonucleases increase during ripening and stresses, and also impact the RNA integrity (Azevedo et al., 2003). Moreover, as the fruit ripens, pectin polymers become smaller and tougher to precipitate (Dal Cin et al., 2005). Carambola also contains high water content (91% w/w) thus there is an inherently low concentration of RNA available per mass of sample.

The results demonstrated the presence of intact ribosomal RNA bands for the tomato sample using both methods tested; attesting to the general success attributed to these RNA extraction methods. However, the intact ribosomal RNA were only observed for the carambola sample when the CTAB method was used, as this method could successfully deal with the fruit's high polyphenol, pectin and endonuclease content as well as inherently low RNA levels. The total RNA isolated was of high quality and could be effectively used for cDNA synthesis and subsequent amplification by PCR.

8.4.2 Primer design and PCR reactions

The genes that were the subject of this study are both directly and indirectly related to stress amelioration within plants. These genes were studied in various plant species, thus degenerate primers were designed to assist in the identification of the new gene (Wintermantel & Hladky 2010). The successful use of the degenerate primers suggests

that the sequences of the putative genes share high similarity to the respective gene family.

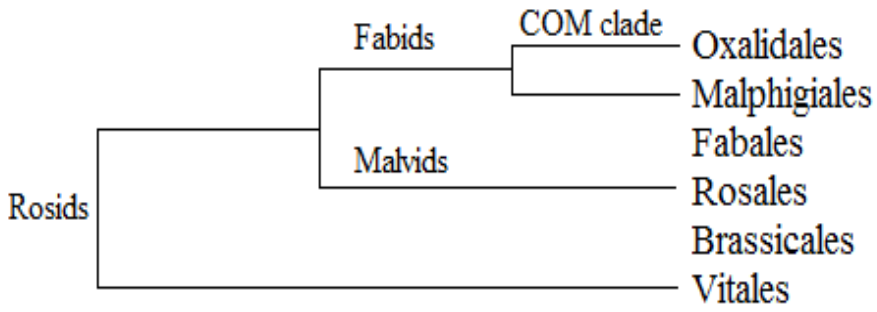
CODEHOP primers have been widely used for the identification of unknown members of a gene family through the alignment of highly conserved short protein sequences (Staheli et al. 2009). They are made up of a short degenerate 3' region along with a longer consensus clamp adjoining the target motif, which consists of the most probable nucleotide sequence. All of primers had a consensus clamp region of a minimum length of 20 bases, with the exception of the C4H forward primer with a clamp region made up of 17 bases.

The 3' region was kept short to limit the degeneracy, minimising the number of individual primers without compromising the broad specificity at initial PCR steps. Meanwhile, the 5' consensus clamp offers stability allowing for higher annealing temperatures with limited degeneracy. Later PCR steps rely on the sequence similarity of the primers and the primed PCR products, thus all primers are continuously used without a need for excessive primers in the reaction (Staheli et al. 2009).

Design of CODEHOP primers is dependent on identifying highly conserved protein blocks within related plant species (Staheli et al. 2009). Carambola is a member of the family Oxalidaceae, part of the order Oxalidales, and it is the most prominent member in its order. There is no available data on carambola's sequence, thus there was the need to explore other plant species with close affinity to carambola. Oxalidales and Malphigiales are members of the same supraordinal group "COM clade" (Figure 8.7). Malphigiales is a large order however the phylogeny has not been explored neither has the sequences of this order. The COM clade is part of the unranked group "Fabids" which is a part of the larger unranked group "Rosids". Rosids are diverse eudicots that

include many flowering plants, therefore it was necessary to use a wider varied selection of members from this group for analysing relationship of the desired protein sequences.

Figure 8.7 Phylogenetic tree illustrating the interrelationship of some order of the unranked order "Rosids", according to the Angiosperm Phylogeny Group (APG III, 2009).



Previous studies have shown that magnesium concentrations can be varied to optimise the PCR conditions (Wintermantel & Hladky 2010). Higher levels of magnesium will be more readily utilised by the Taq polymerase, thus increasing its activity. However this comes at a cost of reduced specificity of the PCR reaction (Wintermantel & Hladky 2010). Therefore, it is highly desirable to maintain the levels of $MgCl_2$ neither excessively high nor low, as well as maintaining standard levels of dNTP and buffer.

The role of various additives such as DMSO in improving yield and specificity has been explored in a number of studies (Frackman et al. 1998). DMSO is an organosulfur compound with the ability to disrupt DNA secondary structure. This is attributed to the high polarity of DMSO allowing it to hydrogen bond with the DNA template and destabilise the helix structure, which is of particular importance in GC-

rich templates (Hardjasa et al. 2010). High GC content in a genomic template increases the hydrogen bond strength favouring the formation of intermolecular secondary structures over primer annealing. However, high DMSO content can interfere with the base-pairing of Taq as well as lead to mismatched base-pairing during primer annealing.

From the results it could be observed that 1mM Mg with 0% DMSO and 2.5 mM Mg with 2% DMSO were optimal for the reaction, with bands of higher intensity observed for the later reaction condition. It is apparent that Taq needed the cofactor Mg, which enhanced the activity of the polymerase. Moreover, at higher levels of activity further enhancement was achieved by unwinding the DNA helix, thus DMSO improved the yield of the PCR reaction which could indicate that the genomic template has a high GC content.

8.5 Concluding remarks

The bulk of the experiments were focused on optimising experimental procedures for RNA isolation from carambola and for identifying unknown members of gene families related to the production of phenolics. The isolated RNA using the CTAB protocol was suitable for synthesizing cDNA and further analyses such as PCR using actin and the degenerate primers. Moreover, the CODEHOP PCR approach provided appropriate specificity for the amplification of the target genes. However, the main question still remains, how does the expression pattern differ as the fruit ripens and encounters elevated level of stresses? As the platform is set for further investigations into this matter, it will also be of interest to explore the relation between phenolic compounds and sugars within the fruit.

CONCLUSION

The main aim of this study was to analyse the effect of postharvest stresses in the form of cold storage in combination with the stress hormones methyl jasmonate (MeJA) and salicylic acid (SA) on the physicochemical responses and changes in the bioactive content of three tropical fruits, carambola, dragonfruit and mangosteen. After an initial investigation of the physicochemical properties and antioxidant activity of the fruits under the postharvest stresses, carambola was selected for further study into the changes in the bioactive content owing to the notable enhanced bioactivity of the samples.

The application of MeJA and SA played an important role in maintaining the quality of the fruit as determined by delayed weight loss and maintaining sugar and organic acid content. Moreover, treatment with phytohormones was also effective in maintaining the antioxidant activity of the tropical fruits, with enhanced antioxidant activity and ascorbic acid content observed for carambola. Moreover assessment of the enzymatic responses to the stress treatments showed enhanced POD activity with inhibited PPO activity. Increased PAL activity was concomitant with phenolic compound accumulation, thus it was hypothesized that the suppressed PAL activity was an adaptive strategy for reducing chilling injury symptoms.

A general decline in total phenolic content of carambola as storage period increased was observed, thus changes in the phenolic acid profiles of carambola fruits after 4 days of exposure to the stress were assessed to provide an understanding of the stress recovery systems in place. This involved the use of HPLC-UV/Vis to characterise the hydroxycinnamic and hydroxybenzoic acids content. Epicatechin is a principal phenolic acid in carambola, and the stresses enhanced the accumulation of this

compound. A similar trend was also noted for the presence of the ubiquitous phenolic compound salicylic acid. Furthermore, essential genes in the phenylpropanoid pathway (PAL, C4H and ANS) that are involved in the synthesis of anthocyanidins and hydroxycinammic acids were identified in carambola. Although the changes in the expression patterns of these genes was not undertaken in this study due to time limitations, these genes were identified for the first time in carambola and can be used in future studies.

This study also assessed the effect of stress related bioactive compounds on human health. Bioactive compounds were identified that offered protection from oxidative stress within the carambola fruit tissue, through the alleviation of chilling injury. These compounds were also found to be beneficial for human use, as they did not only display anti-proliferative activity but also offered protection from oxidative stress induced by hydrogen peroxide on HepG2 cells that were used as an *in vitro* model. Generally, activity of the SA treated fruits was more potent, as was also seen in the anti-proliferative assay. Within the SA treatments, the lowest SA doses (0.1 and 1 mM) were more effective than the higher doses in maintaining fruit quality.

Although the application of stress hormones did elicit the enhanced production of specific phenolic compounds, the activity of the phenol-rich fractions of carambola fruit did not exhibit the highest anti-proliferation activity. This indicates that compounds besides phenolic acids are also involved in the regulation of stress responses in carambola. These compounds can be carotenoids, which increase during the ripening of carambola. Carotenoids are lipophilic, and could possibly be the active ingredients in the plant extract since they would not be eluted out during the fractionation of the extracts.

The sugar content of the fruits was also enhanced in response to the stress responses. Polysaccharides are a class of macromolecules that do not only offer protection during chilling injury, but are also involved in cell signalling and development. Polysaccharides have been gaining recognition for their associated bioactivity that includes antioxidant and anti-proliferative properties. It is highly possible that these compounds work synergistically with other bioactive compounds found in carambola, including the phenolic compounds.

Carambola possesses potent antioxidant activity, and an equally large share of phenolic compounds that were not all identified in this study. A deeper investigation into the phenolic content of carambola can be conducted to further elucidate the on-going changes in the bioactive content of the fruit. Moreover, since it was hypothesized that carotenoids and polysaccharides could be involved in the defence mechanisms of the fruit, it will be of interest to investigate the activity of these compounds individually and in combination with the phenolic compounds. Finally, the platform for effective RNA isolation and gene identification has been established during this study. Further studies into gene expression can assist in providing a better understanding of the variations in the signalling and metabolic pathways of the phenylpropanoid pathway in response to stress.

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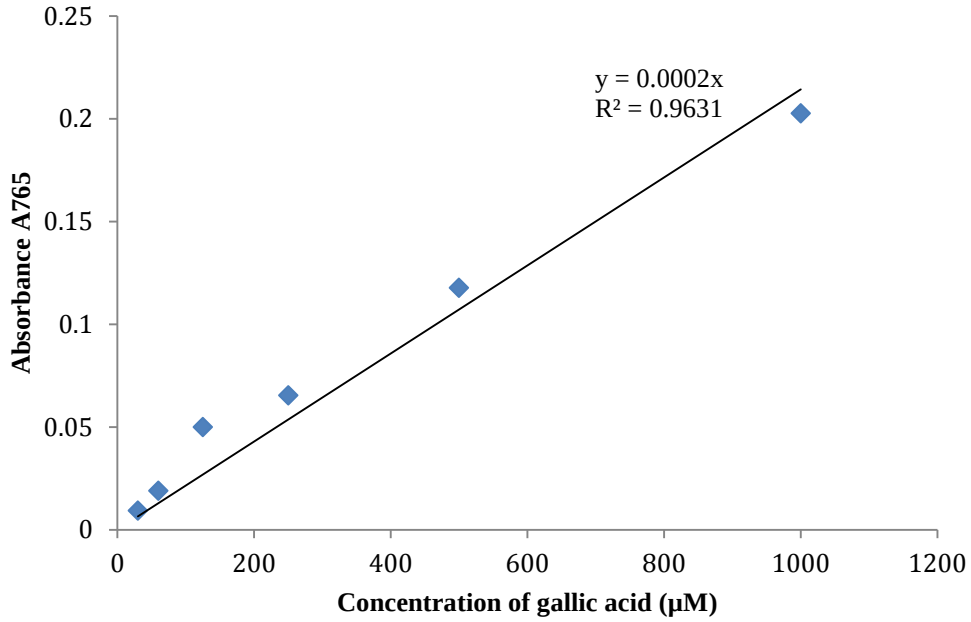
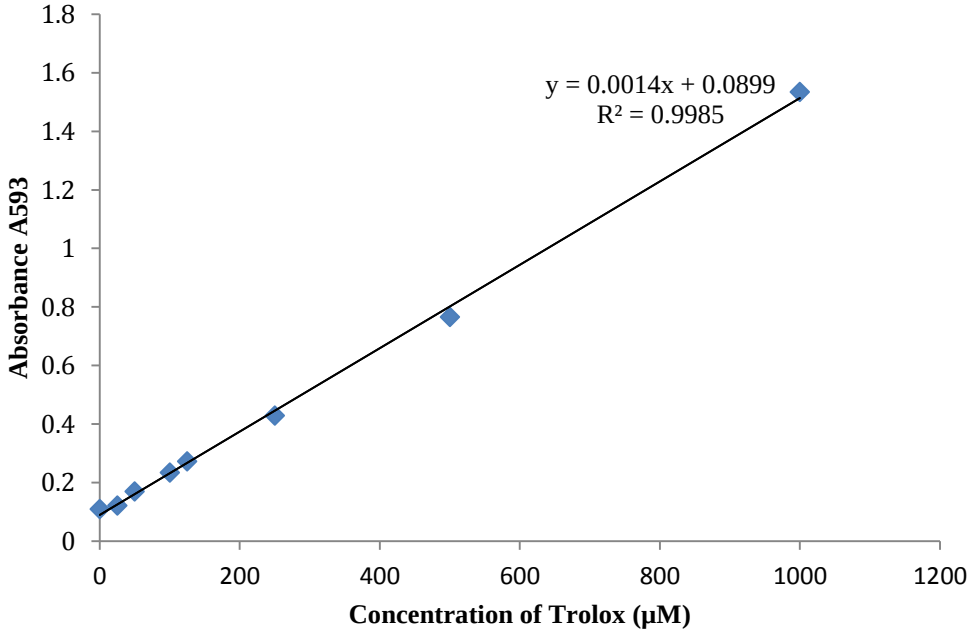
APPENDICES

Appendix A

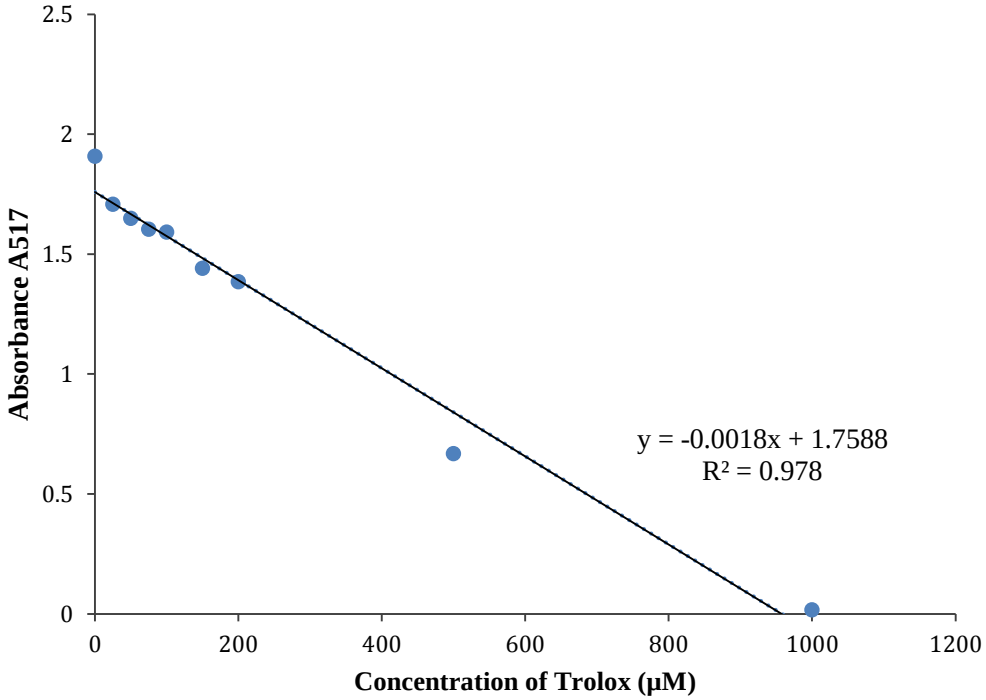
A 4.1 Gallic acid standard curve for total phenolic content assay



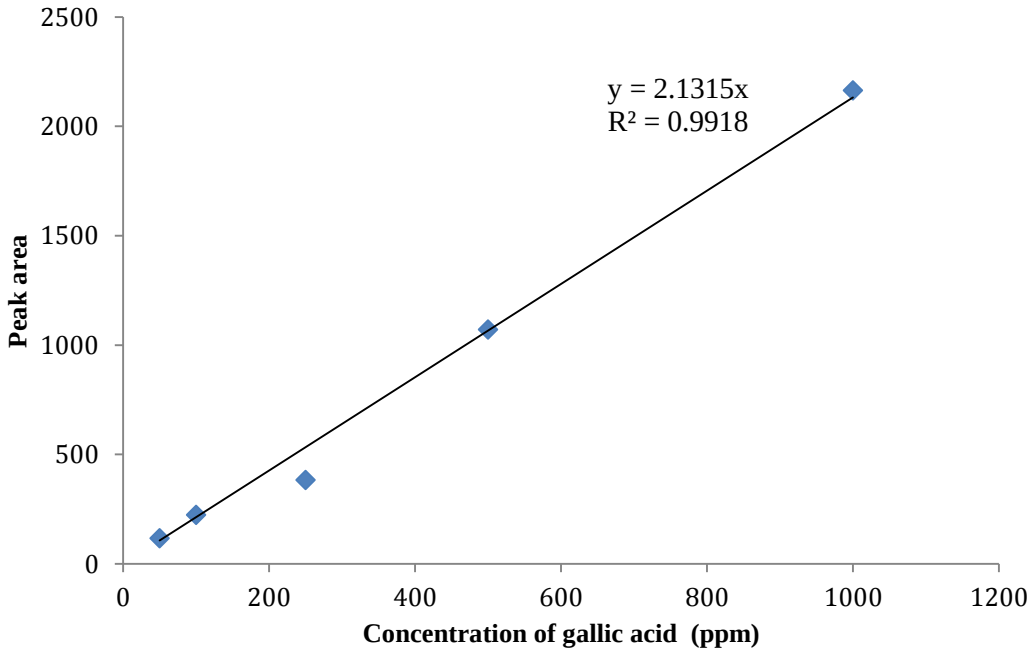
A 4.2 Trolox standard curve for FRAP assay



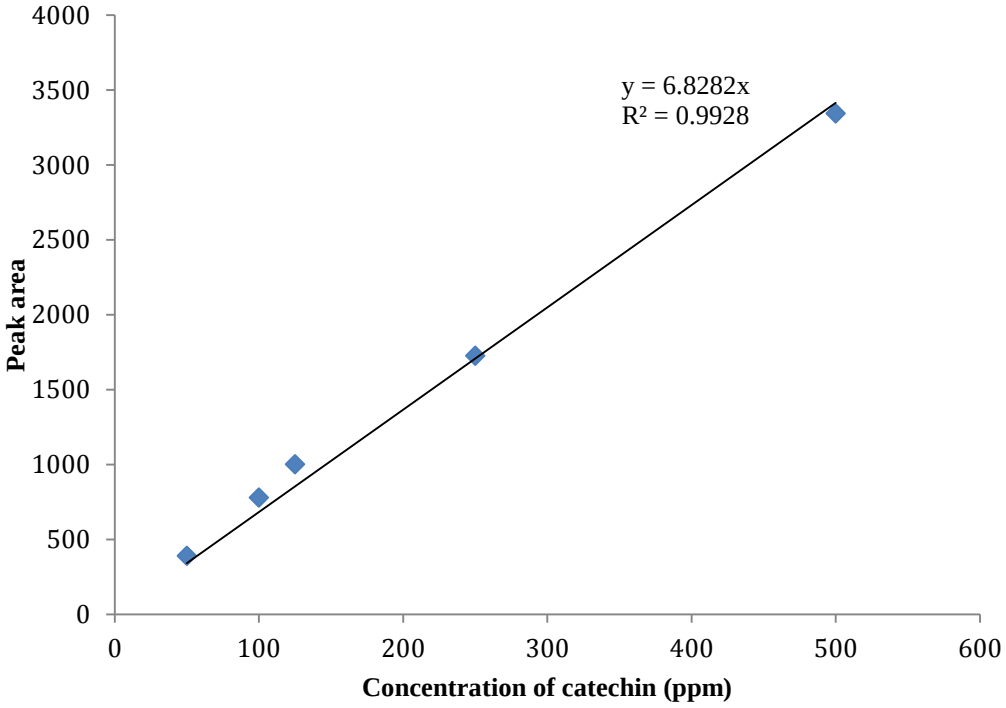
A 4.3 Trolox standard curve for DPPH assay



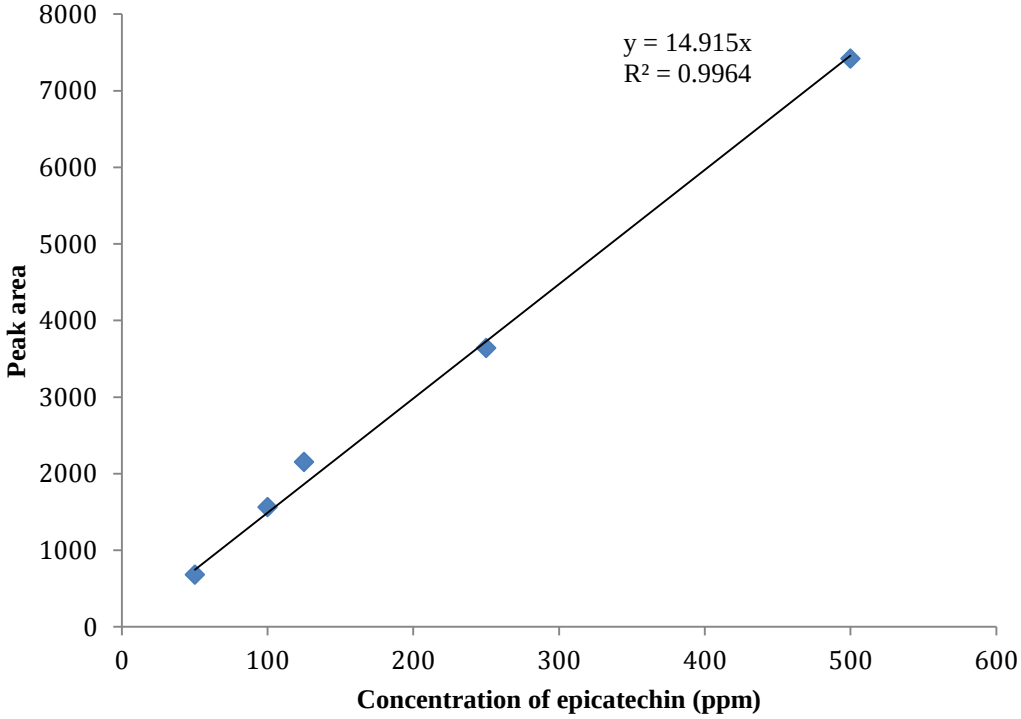
A 6.1 Gallic acid standard curve for estimation by HPLC



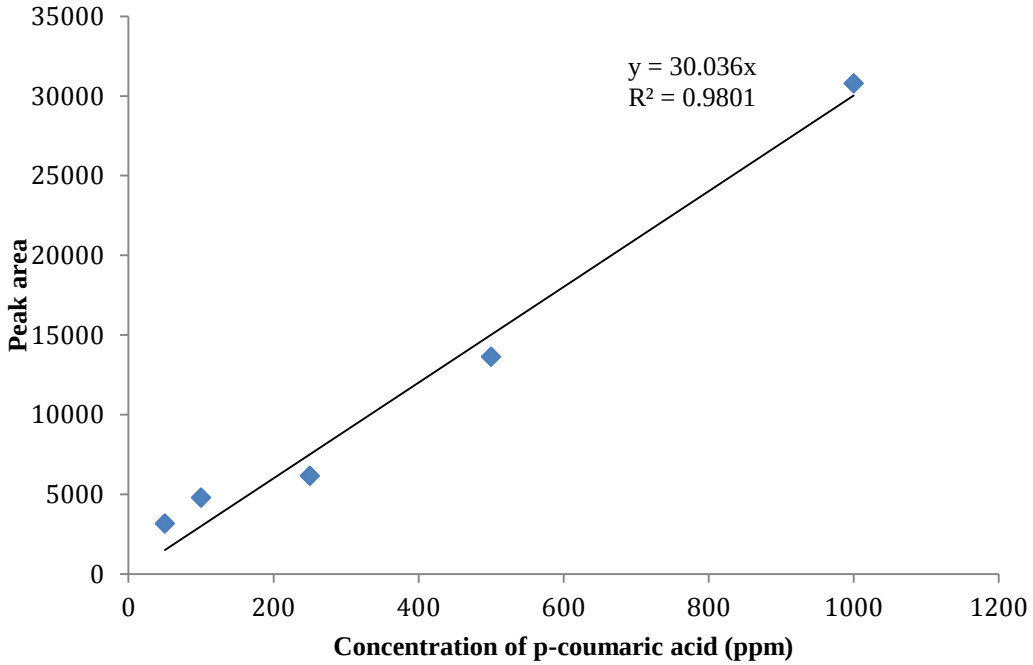
A 6.2 Catechin standard curve for estimation by HPLC



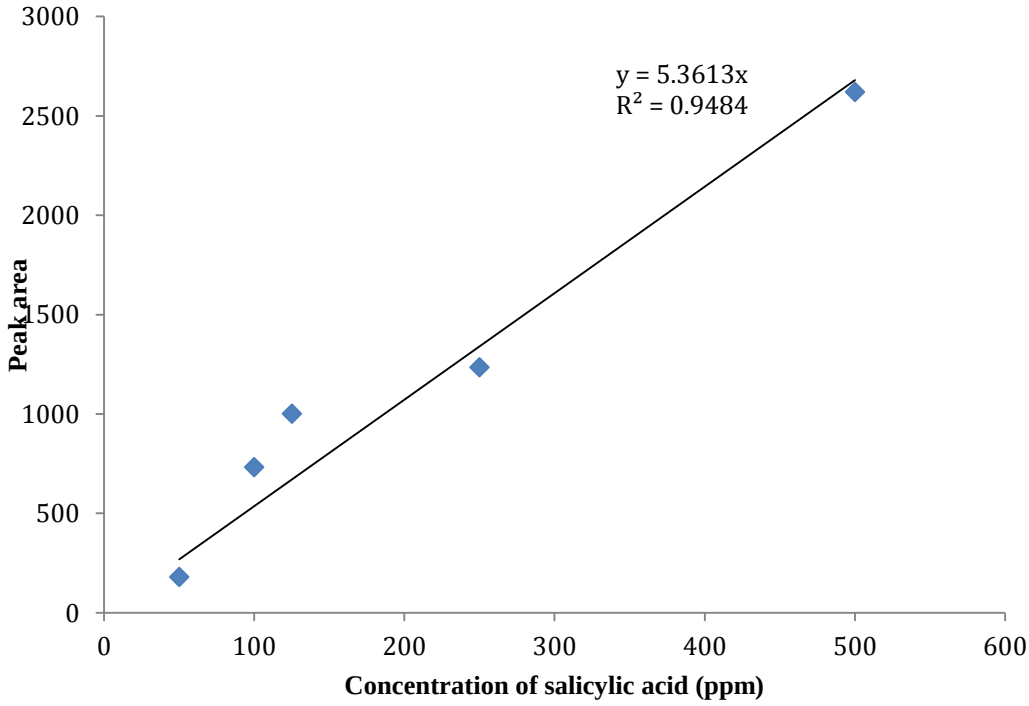
A 6.3 Epicatechin standard curve for estimation by HPLC



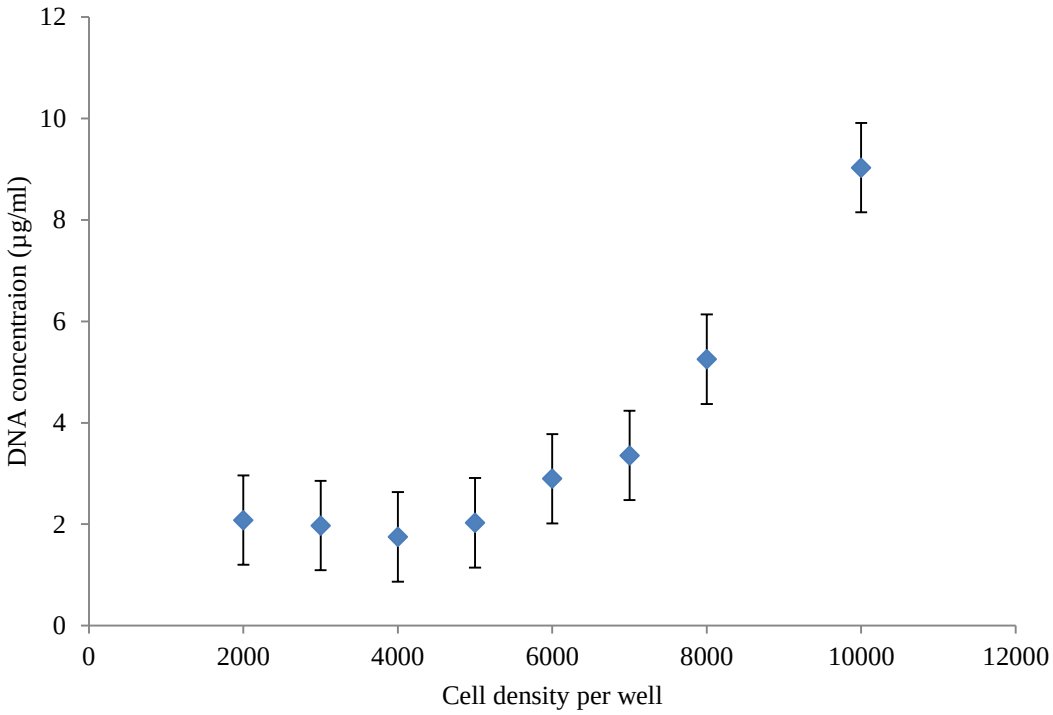
A 6.4 *P*-coumaric acid standard curve for estimation by HPLC



A 6.5 Salicylic acid standard curve for estimation by HPLC



A7.1 DNA concentration (µg/ml) of HepG2 cell densities per well tested (2 x 10^4 to 10 x 10^4 cells per well) determined using Hoechst fluorimetric method, values are means (n = 5) ± SE



Appendix B

B3.1 Analysis of variance for effect of methyl jasmonate on weight loss of carambola stored at 6°C for 16 days

S.O.V	d.f.	S.S.	M.S.	F. Value	Pr>F
trt	4	0.00607888	0.00151972	10.24	<.001
day	5	0.11219154	0.02243831	151.19	<.001
trt.day	20	0.00229792	0.00011490	0.77	0.7369
Residual	90	0.01335725	0.00014841		
Corrected total	119	0.13392559			

B3.2 Analysis of variance for effect of salicylic acid on weight loss of carambola stored at 6°C for 16 days

S.O.V	d.f.	S.S.	M.S.	F. Value	Pr>F
trt	4	0.00038675	0.00009669	7.41	0.006
day	5	0.01009809	0.00201962	154.81	<.001
trt.day	20	0.00019923	0.00000996	0.76	0.7482
Residual	90	0.00110892	0.00001305		
Corrected total	119	0.01183577			

B3.3 Analysis of variance for effect of methyl jasmonate on firmness of carambola stored at 6°C for 16 days

S.O.V	d.f.	S.S.	M.S.	F. Value	Pr>F
trt	4	98.1865267	24.5466317	4.65	0.0025
day	5	207.1943389	41.4388678	7.85	<.001
trt.day	20	152.6367000	7.6318350	1.45	0.1371
Residual	90	2.02790325	0.02253226		
Corrected total	119	43.58751459			

B3.4 Analysis of variance for effect of salicylic acid on firmness of carambola stored at 6°C for 16 days

S.O.V	d.f.	S.S.	M.S.	F. Value	Pr>F
trt	4	18.3107667	4.5776917	0.60	0.6605
day	5	270.2912175	54.0582435	7.14	<.001
trt.day	20	153.0365533	7.6518277	1.01	0.4588
Residual	90	681.651275	7.573903		
Correcred total	119	1123.289812			

B3.5 Analysis of variance for different concentrations of methyl jasmonate on titratable acidity of carambola stored at 6°C for 16 days

S.O.V	d.f.	S.S.	M.S.	F. Value	Pr>F
trt	4	263.758171	65.939543	77.71	<.001
day	5	3300.914766	660.182953	778.03	<.001
trt.day	20	3065.829429	153.291471	180.65	<.001
Residual	90	50.912200	0.848537		
Corrected total	119	6681.414566			

B3.6 Analysis of variance for different concentrations of salicylic acid on titratable acidity of carambola stored at 6°C for 16 days

S.O.V	d.f.	S.S.	M.S.	F. Value	Pr>F
trt	4	1.16533333	0.29133333	12.61	<.001
day	5	25.24266667	5.04853333	218.45	<.001
trt.day	20	8.05066667	0.40253333	17.42	<.001
Residual	90	2.08000000	0.02311111		
Corrected total	119	36.53866667			

B3.7 Analysis of variance for different concentrations of methyl jasmonate on soluble solids content of carambola stored at 6°C for 16 days

S.O.V	d.f.	S.S.	M.S.	F. Value	Pr>F
trt	4	4.5404444	1.1351111	159.63	<.001
day	5	106.8586667	21.3717333	3005.40	<.001
trt.day	20	30.1902222	1.5095111	212.28	<.001
Residual	90	0.4266667	0.0071111		
Corrected total	119	142.0160000			

B3.8 Analysis of variance for different concentrations of salicylic acid on soluble solids content of carambola stored at 6°C for 16 days

S.O.V	d.f.	S.S.	M.S.	F. Value	Pr>F
trt	4	483.712478	120.928119	351.84	<.001
day	5	557.073476	111.414695	324.16	<.001
trt.day	20	1042.725820	52.136291	151.69	<.001
Residual	90	30.932992	0.343700		
Corrected total	119	2114.444766			

B3.9 Analysis of variance for different concentrations of methyl jasmonate on weight loss of dragonfruit stored at 6°C for 21 days

S.O.V	d.f.	S.S.	M.S.	F. Value	Pr>F
trt	4	0.00006276	0.00001569	4.18	0.006
day	3	0.00633752	0.00211251	562.58	<.001
trt.day	12	0.00014275	0.00001190	3.17	0.003
Residual	60	0.00015020	0.00000376		
Corrected total	79	0.00669323			

B3.10 Analysis of variance for different concentrations of salicylic acid on weight loss of dragonfruit stored at 6°C for 21 days

S.O.V	d.f.	S.S.	M.S.	F. Value	Pr>F
trt	4	0.00010504	0.00002626	7.02	<.001
day	3	0.00659666	0.00219889	587.90	<.001
trt.day	12	0.00018080	50.00001507	4.03	<.001
Residual	60	0.00014961	0.00000374		
Corrected total	79	0.00703212			

B3.11 Analysis of variance for different concentrations of methyl jasmonate on titratable acidity of dragonfruit stored at 6°C for 21 days

S.O.V	d.f.	S.S.	M.S.	F. Value	Pr>F
trt	4	2.85896250	0.71474063	0.98	0.4237
day	3	19.27030500	6.42343500	8.83	<.001
trt.day	12	6.61285750	0.55107146	0.76	0.6899
Residual	60	43.62775000	0.72712917		
Corrected total	79	72.36987500			

B3.12 Analysis of variance for different concentrations of salicylic acid on titratable acidity of dragonfruit stored at 6°C for 21 days

S.O.V	d.f.	S.S.	M.S.	F. Value	Pr>F
trt	4	1.93331200	0.48332800	0.73	0.5725
day	3	5.27892480	1.75964160	2.67	0.0554
trt.day	12	11.51467520	0.95955627	1.46	0.1665
Residual	60	39.5182080	0.65863680		
Corrected total	79	58.24512000			

B3.13 Analysis of variance for different concentrations of methyl jasmonate on soluble solids content of dragonfruit stored at 6°C for 21 days

S.O.V	d.f.	S.S.	M.S.	F. Value	Pr>F
trt	4	5.4920000	1.373000	5.99	<.001
day	3	472.15200	157.3840	686.27	<.001
trt.day	12	61.228000	5.102333	22.25	<.001
Residual	60	0.00015020	0.00000376		
Corrected total	79	0.00669323			

B3.14 Analysis of variance for different concentrations of salicylic acid on soluble solids content of dragonfruit stored at 6°C for 21 days

S.O.V	d.f.	S.S.	M.S.	F. Value	Pr>F
trt	4	30.378000	7.5945000	65.94	<.001
day	3	418.68550	139.5618333	1211.82	<.001
trt.day	12	59.382000	4.9485000	42.97	0.144
Residual	60	6.910000	0.1151667		
Corrected total	79	515.3555			

B3.15 Analysis of variance for different concentrations of methyl jasmonate on weight loss of mangosteen stored at 6°C for 12 days

S.O.V	d.f.	S.S.	M.S.	F. Value	Pr>F
trt	4	1.503E-04	3.758E-05	9.47	<.001
day	4	3.439E-03	8.598E-04	216.76	<.001
trt.day	16	9.066E-05	5.666E-06	1.43	0.144
Residual	100	3.967E-04	3.967E-06		
Corrected total	124	4.077E-03			

B3.16 Analysis of variance for different concentrations of salicylic acid on weight loss of mangosteen stored at 6°C for 12 days

S.O.V	d.f.	S.S.	M.S.	F. Value	Pr>F
day	4	9.170 E-04	2.2925 E-04	352.00	<.001
trt	4	2.610 E-03	6.5266E-05	10.02	<.001
day.trt	16	1.826 E-04	1.1415E-05	1.75	0.055
Residual	100	4.884 E-04	6.513E-06		
Corrected total	124	1.0102E-03			

B3.17 Analysis of variance for different concentrations of methyl jasmonate on firmness of mangosteen stored at 6°C for 12 days

S.O.V	d.f.	S.S.	M.S.	F. Value	Pr>F
trt	4	24.07	6.02	0.26	0.901
day	4	709.63	177.41	7.73	<.001
trt.day	16	558.48	34.91	1.52	0.126
Residual	100	1262.53	22.96		
Corrected total	124	2554.71			

B3.18 Analysis of variance for different concentrations of salicylic acid on firmness of mangosteen stored at 6°C for 12 days

S.O.V	d.f.	S.S.	M.S.	F. Value	Pr>F
day	4	4927.2	1231.8	5.21	<.001
trt	4	1173.2	293.3	1.24	0.305
day.trt	16	6101.7	381.4	1.61	0.097
Residual	100	12772.9	236.5		
Corrected total	124	24900.5	319.2		

B3.19 Analysis of variance for different concentrations of methyl jasmonate on titratable acidity of mangosteen stored at 6°C for 12 days

S.O.V	d.f.	S.S.	M.S.	F. Value	Pr>F
day	4	638.0040	159.5010	396.12	<.001
trt	4	20.2249	5.0562	12.56	<.001
day.trt	16	48.5197	3.0325	7.53	<.001
Residual	100	40.2653	0.4027		
Corrected total	124	747.0139			

B3.20 Analysis of variance for different concentrations of salicylic acid on titratable acidity of mangosteen stored at 6°C for 12 days

S.O.V	d.f.	S.S.	M.S.	F. Value	Pr>F
trt	4	24.4444	6.1111	16.80	<.001
day	4	320.9817	80.2454	220.54	<.001
trt.day	16	36.6456	2.2904	6.29	<.001
Residual	100	36.3856	0.3639		
Corrected total	124	418.4573			

B3.21 Analysis of variance for different concentrations of methyl jasmonate on soluble solids content of mangosteen stored at 6°C for 12 days

S.O.V	d.f.	S.S.	M.S.	F. Value	Pr>F
day	4	398.02368	99.50592	2320.57	<.001
trt	4	27.14368	6.78592	158.25	<.001
day.trt	16	98.79552	6.17472	144.00	<.001
Residual	100	4.28800	0.04288		
Corrected total	124	528.25088			

B3.22 Analysis of variance for different concentrations of salicylic acid on soluble solids content of mangosteen stored at 6°C for 12 days

S.O.V	d.f.	S.S.	M.S.	F. Value	Pr>F
trt	4	253.79	63.45	0.90	0.464
day	4	752.91	188.23	2.68	0.036
trt.day	16	1339.69	83.73	1.19	0.286
Residual	100	7012.10	70.12		
Corrected total	124	9358.49			

B4.1 Analysis of variance for different concentrations of methyl jasmonate on ascorbic acid content of carambola stored at 6°C for 16 days

S.O.V	d.f.	S.S.	M.S.	F. Value	Pr>F
day	4	20.4259783	5.1064946	52.22	< .001
trt	5	327.4791967	65.4958393	669.73	< .001
day.trt	20	60.0630117	3.0031506	30.71	< .001
Residual	90	8.8014500	0.0977939		
Corrected total	119	416.7696367			

B4.2 Analysis of variance for different concentrations of salicylic acid on ascorbic acid content of carambola stored at 6°C for 16 days

S.O.V	d.f.	S.S.	M.S.	F. Value	Pr>F
trt	4	4.03125122	1.00781280	44.73	<.001
day	3	30.40952284	6.08190457	269.92	<.001
trt.day	12	7.11883728	0.35594186	15.80	<.001
Residual	90	2.02790325	0.02253226		
Corrected total	119	43.58751459			

B4.3 Analysis of variance for different concentrations of methyl jasmonate on total phenolic content of carambola stored at 6°C for 16 days

S.O.V	d.f.	S.S.	M.S.	F. Value	Pr>F
day	4	24.7742392	6.1935598	9.05	< .001
trt	5	160.5415500	32.1083100	46.92	< .001
day.trt	20	55.8284958	2.7914248	4.08	< .001
Residual	210	143.7005000	0.6842881		
Corrected total	239	384.8447850			

B4.4 Analysis of variance for different concentrations of salicylic acid on total phenolic content of carambola stored at 6°C for 16 days

S.O.V	d.f.	S.S.	M.S.	F. Value	Pr>F
day	4	16.2637051	4.0659263	6.04	< .001
trt	5	385.5121385	77.1024277	114.52	< .001
day.trt	20	96.0280294	4.8014015	7.13	< .001
Residual	210	141.3910596	0.6732908		
Corrected total	239	639.1949327			

B4.5 Analysis of variance for different concentrations of methyl jasmonate on FRAP antioxidant activity of carambola stored at 6°C for 16 days

S.O.V	d.f.	S.S.	M.S.	F. Value	Pr>F
day	4	9.65517750	2.41379438	15.67	< .001
trt	5	80.69157833	16.13831567	104.79	< .001
day.trt	20	25.63349250	1.28167463	8.32	< .001
Residual	210	32.3399500	0.1539998		
Corrected total	239	148.3201983			

B4.6 Analysis of variance for different concentrations of salicylic acid on FRAP antioxidant activity of carambola stored at 6°C for 16 days

S.O.V	d.f.	S.S.	M.S.	F. Value	Pr>F
day	4	2.38891931	0.59722983	21.22	< .001
trt	5	19.26315377	3.85263075	136.90	< .001
day.trt	20	3.63266454	0.18163323	6.45	< .001
Residual	210	4824.06872	34.45763		
Corrected total	239	10951.72304			

B4.7 Analysis of variance for different concentrations of methyl jasmonate on DPPH antioxidant activity of carambola stored at 6°C for 16 days

S.O.V	d.f.	S.S.	M.S.	F. Value	Pr>F
day	4	1.36520583	0.34130146	14.25	< .001
trt	5	6.68495375	1.33699075	55.83	< .001
day.trt	20	2.26779417	0.11338971	4.73	< .001
Residual	210	5.02901250	0.02394768		
Corrected total	239	15.34696625			

B4.8 Analysis of variance for different concentrations of salicylic acid on DPPH antioxidant activity of carambola stored at 6°C for 16 days

S.O.V	d.f.	S.S.	M.S.	F. Value	Pr>F
day	4	0.21156164	0.05289041	1.59	0.1784
trt	5	2.56618005	0.51323601	15.42	< .001
day.trt	20	0.38436816	0.01921841	0.58	0.9254
Residual	210	6.98932800	0.03328251		
Corrected total	239	10.15143785			

B4.9 Analysis of variance for different concentrations of methyl jasmonate on betacyanin content of dragonfruit stored at 6°C for 21 days

S.O.V	d.f.	S.S.	M.S.	F. Value	Pr>F
day	4	259.732158	64.933039	1.88	0.1165
trt	3	4181.239053	1393.746351	40.45	< .001
day.trt	12	1686.683105	140.556925	4.08	< .001
Residual	140	4824.06872	34.45763		
Corrected total	159	10951.72304			

B4.10 Analysis of variance for different concentrations of salicylic acid on betacyanin content of dragonfruit stored at 6°C for 21 days

S.O.V	d.f.	S.S.	M.S.	F. Value	Pr>F
day	4	897.108023	224.277006	7.13	< .001
trt	3	3833.068723	1277.689574	40.60	< .001
day.trt	12	1259.171110	104.930926	3.33	< .001
Residual	140	4406.17904	31.47271		
Corrected total	159	10395.52690			

B4.11 Analysis of variance for different concentrations of methyl jasmonate on total phenolic content of dragonfruit stored at 6°C for 21 days

S.O.V	d.f.	S.S.	M.S.	F. Value	Pr>F
day	4	0.03867317	0.00966829	0.15	0.9612
trt	3	3.56060907	1.18686969	18.82	< .001
day.trt	12	0.13650934	0.01137578	0.18	0.9990
Residual	140	8.76486045	0.06305655		
Corrected total	159	12.50826731			

B4.12 Analysis of variance for different concentrations of salicylic acid on total phenolic content of dragonfruit stored at 6°C for 21 days

S.O.V	d.f.	S.S.	M.S.	F. Value	Pr>F
day	4	0.01242292	0.00621146	0.10	0.9064
trt	3	1.28968429	0.42989476	6.81	< .001
day.trt	12	0.08570806	0.02856935	0.45	0.7164
Residual	140	3.72408952	0.06312016		
Corrected total	159	5.15475841			

B4.13 Analysis of variance for different concentrations of methyl jasmonate on FRAP antioxidant activity of dragonfruit stored at 6°C for 21 days

S.O.V	d.f.	S.S.	M.S.	F. Value	Pr>F
day	4	1004.27118	251.06780	7.22	< .001
trt	3	11009.83520	3669.94507	105.55	< .001
day.trt	12	3084.11037	257.00920	7.39	< .001
Residual	140	4798.13379	34.76909		
Corrected total	159	19933.79581			

B4.14 Analysis of variance for different concentrations of salicylic acid on FRAP antioxidant activity of dragonfruit stored at 6°C for 21 days

S.O.V	d.f.	S.S.	M.S.	F. Value	Pr>F
day	4	108.194257	27.048564	1.72	0.1495
trt	3	2892.897995	964.299332	61.26	< .001
day.trt	12	1188.261197	99.021766	6.29	< .001
Residual	140	2125.045014	15.741074		
Corrected total	159	6308.224815			

B4.15 Analysis of variance for different concentrations of methyl jasmonate on DPPH antioxidant activity of dragonfruit stored at 6°C for 21 days

S.O.V	d.f.	S.S.	M.S.	F. Value	Pr>F
day	4	315.3176	78.8294	0.47	0.7571
trt	3	102012.3053	34004.1018	203.09	< .001
day.trt	12	2974.7743	247.8979	1.48	0.1389
Residual	150	22436.5096	167.4366		
Corrected total	159	127893.3752			

B4.16 Analysis of variance for different concentrations of salicylic acid on DPPH antioxidant activity of dragonfruit stored at 6°C for 21 days

S.O.V	d.f.	S.S.	M.S.	F. Value	Pr>F
day	4	4175.90388	1043.97597	6.79	<.001
trt	3	60221.10293	20073.70098	130.50	< .001
day.trt	12	8104.26816	675.35568	4.39	< .001
Residual	140	20611.70163	153.81867		
Corrected total	159	93160.80913			

B4.17 Analysis of variance for different concentrations of methyl jasmonate on total phenolic content of mangosteen stored at 6°C for 12 days

S.O.V	d.f.	S.S.	M.S.	F. Value	Pr>F
day	4	0.71140	0.17785	8.80	<.001
trt	4	0.49887	0.12472	6.17	<.001
day.trt	16	0.97177	0.06074	3.01	<.001
Residual	174	3.51628	0.02021		
Corrected total	198	5.70067	0.02879		

B4.18 Analysis of variance for different concentrations of salicylic acid on total phenolic content of mangosteen stored at 6°C for 12 days

S.O.V	d.f.	S.S.	M.S.	F. Value	Pr>F
day	4	1.8064	0.4516	1.12	0.347
trt	4	6.9634	1.7408	4.33	0.002
day.trt	16	21.5723	1.3483	3.35	<.001
Residual	174	69.9653	0.4021		
Corrected total	198	100.3200	0.5067		

B4.19 Analysis of variance for different concentrations of methyl jasmonate on FRAP antioxidant activity of mangosteen stored at 6°C for 12 days

S.O.V	d.f.	S.S.	M.S.	F. Value	Pr>F
day	4	1257.33	314.33	19.71	<.001
trt	4	430.14	107.53	6.74	<.001
day.trt	16	452.16	28.26	1.77	0.039
Residual	174	2711.79	15.95		
Corrected total	198	4847.70	24.99		

B4.20 Analysis of variance for different concentrations of salicylic acid on FRAP antioxidant activity of mangosteen stored at 6°C for 12 days

S.O.V	d.f.	S.S.	M.S.	F. Value	Pr>F
day	4	400.416	100.104	16.56	<.001
trt	4	73.233	18.308	3.03	0.019
day.trt	16	185.318	11.582	1.92	0.022
Residual	174	1045.839	6.045		
Corrected total	198	1705.701	8.658		

B4.21 Analysis of variance for different concentrations of methyl jasmonate on DPPH antioxidant activity of mangosteen stored at 6°C for 12 days

S.O.V	d.f.	S.S.	M.S.	F. Value	Pr>F
day	4	9.07125	2.26781	71.09	<.001
trt	4	0.71458	0.17864	5.60	<.001
day.trt	16	3.97574	0.24848	7.79	<.001
Residual	174	5.29572	0.03190		
Corrected total	198	19.13007	0.10068		

B4.22 Analysis of variance for different concentrations of salicylic acid on DPPH antioxidant activity of mangosteen stored at 6°C for 12 days

S.O.V	d.f.	S.S.	M.S.	F. Value	Pr>F
day	4	14.72915	3.68229	91.40	<.001
day	4	0.43098	0.10774	2.67	0.034
day.trt	16	2.87230	0.17952	4.46	<.001
Residual	174	5.84141	0.04029		
Corrected total	198	23.82917	0.14100		

B5.1 Analysis of variance for different concentrations of methyl jasmonate on PAL enzyme activity of carambola stored at 6°C for 16 days

S.O.V	d.f.	S.S.	M.S.	F. Value	Pr>F
day	4	5.3214836	1.3303709	1.21	0.3098
trt	5	124.1763404	31.0440851	28.16	<.001
day.trt	20	20.2549597	1.2659350	1.15	0.3149
Residual	164	189.6217487	1.1024520		
Corrected total	193	336.1487546			

B5.2 Analysis of variance for different concentrations of salicylic acid on PAL enzyme activity of carambola stored at 6°C for 16 days

S.O.V	d.f.	S.S.	M.S.	F. Value	Pr>F
day	4	2282.41872	570.60468	0.68	0.6073
trt	5	29305.54712	5861.10942	6.98	<.001
day.trt	20	10409.15995	520.45800	0.62	0.8943
Residual	164	189.6217487	1.1024520		
Corrected total	193	336.1487546			

B5.3 Analysis of variance for different concentrations of methyl jasmonate on POD enzyme activity of carambola stored at 6°C for 16 days

S.O.V	d.f.	S.S.	M.S.	F. Value	Pr>F
day	4	95651.4191	23912.8548	27.54	<.001
trt	5	536199.0596	134049.7649	154.38	<.001
day.trt	20	622160.5167	38885.0323	44.78	<.001
Residual	164	171921.685	868.291		
Corrected total	193	1422426.624			

B5.4 Analysis of variance for different concentrations of salicylic acid on POD enzyme activity of carambola stored at 6°C for 16 days

S.O.V	d.f.	S.S.	M.S.	F. Value	Pr>F
day	4	42541.225	10635.306	16.37	<.001
trt	5	1017920.135	203584.027	313.43	<.001
day.trt	20	397197.858	19859.893	30.58	<.001
Residual	164	133802.918	649.529		
Corrected total	193	1595540.911			

B5.5 Analysis of variance for different concentrations of methyl jasmonate on PPO enzyme activity of carambola stored at 6°C for 16 days

S.O.V	d.f.	S.S.	M.S.	F. Value	Pr>F
day	4	85.666305	21.416576	1.18	0.3201
trt	5	8979.267533	2244.816883	123.85	<.001
day.trt	20	1097.504693	68.594043	3.78	<.001
Residual	164	3588.82452	18.12538		
Corrected total	193	13762.12316			

B5.6 Analysis of variance for different concentrations of salicylic acid on PPO enzyme activity of carambola stored at 6°C for 16 days

S.O.V	d.f.	S.S.	M.S.	F. Value	Pr>F
day	4	459.99589	114.99897	2.71	0.0317
trt	5	15049.95616	3009.99123	70.95	<.001
day.trt	20	2497.77247	124.88862	2.94	<.001
Residual	164	7552.00759	42.42701		
Corrected total	193	25311.66827			

B7.1 Analysis of variance for freshly harvested carambola (SFD0) extract at concentrations of 0.25 to 5 mg/ml on proliferation of HepG2 cells over 48 hours

S.O.V	d.f.	S.S.	M.S.	F. Value	Pr>F
time	2	426.5156	213.2578	249.50	<.001
trt	7	272.3985	38.9141	45.53	<.001
time.trt	14	142.0816	10.1487	11.87	<.001
Residual	92	74.3628	0.8547		
Corrected total	115	855.8320	7.7803		

B7.2 Analysis of variance for ripening carambola (SFD4) extract at concentrations of 0.25 to 5 mg/ml on proliferation of HepG2 cells over 48 hours

S.O.V	d.f.	S.S.	M.S.	F. Value	Pr>F
time	2	519.593	259.796	154.40	<.001
trt	7	511.423	73.060	43.42	<.001
time.trt	14	284.530	20.324	12.08	<.001
Residual	92	154.801	1.683		
Corrected total	115	1446.864	12.581		

B7.3 Analysis of variance for extracts of carambola treated with 0.01mM MeJA (SFD4MJ1) at concentrations of 0.25 to 5 mg/ml on proliferation of HepG2 cells over 48 hours

S.O.V	d.f.	S.S.	M.S.	F. Value	Pr>F
time	2	490.175	245.087	217.41	<.001
trt	7	281.380	40.197	35.66	<.001
time.trt	14	76.956	5.497	4.88	<.001
Residual	92	91.311	1.127		
Corrected total	115	929.369	8.936		

B7.4 Analysis of variance for extracts of carambola treated with 0.1mM MeJA (SFD4MJ2) at concentrations of 0.25 to 5 mg/ml on proliferation of HepG2 cells over 48 hours

S.O.V	d.f.	S.S.	M.S.	F. Value	Pr>F
time	2	565.613	282.806	199.05	<.001
trt	7	56.304	8.043	5.66	<.001
time.trt	14	16.008	1.143	0.80	0.661
Residual	92	110.819	1.421		
Corrected total	115	753.410	7.460		

B7.5 Analysis of variance for extracts of carambola treated with 0.1mM SA (SFD4SA1) at concentrations of 0.25 to 5 mg/ml on proliferation of HepG2 cells

S.O.V	d.f.	S.S.	M.S.	F. Value	Pr>F
time	2	140.736	70.368	13.21	<.001
trt	7	282.850	40.407	7.59	<.001
time.trt	14	351.732	25.124	4.72	<.001
Residual	92	468.679	5.326		
Corrected total	115	1243.998			

B7.6 Analysis of variance for extracts of carambola treated with 1mM SA (SFD4SA2) at concentrations of 0.25 to 5 mg/ml on proliferation of HepG2 cells

S.O.V	d.f.	S.S.	M.S.	F. Value	Pr>F
time	2	361.002	180.501	69.98	<.001
trt	7	305.048	43.578	16.90	<.001
time.trt	14	351.385	25.099	9.73	<.001
Residual	92	221.818	2.579		
Corrected total	115	1234.907	11.329		

B7.7 Analysis of variance for anti-proliferative activity of original extracts and resultant SPE fractions of freshly harvested carambola (SFD0)

S.O.V	d.f.	S.S.	M.S.	F. Value	Pr>F
time	2	95.4259	47.7130	109.20	<.001
trt	4	63.3979	15.8495	36.28	<.001
time.trt	8	18.3892	2.2987	5.26	<.001
Residual	117	51.1200	0.4369		
Corrected total	131	228.3330			

B7.8 Analysis of variance for anti-proliferative activity of original extracts and resultant SPE fractions of ripening carambola (SFD4)

S.O.V	d.f.	S.S.	M.S.	F. Value	Pr>F
time	2	75.7180	37.8590	69.94	<.001
trt	4	122.7690	30.6922	56.70	<.001
time.trt	8	33.1119	4.1390	7.65	<.001
Residual	117	62.7882	0.5413		
Corrected total	131	294.8944	2.2684		

B7.9 Analysis of variance for anti-proliferative activity of original extracts and resultant SPE fractions of carambola treated with 0.01mM MeJA (SFD4MJ1)

S.O.V	d.f.	S.S.	M.S.	F. Value	Pr>F
time	2	74.2829	37.1415	43.34	< 0.001
trt	4	57.8476	14.4619	16.88	< 0.001
time.trt	8	18.8649	2.3581	2.75	0.008
Residual	117	96.8319	0.8569		
Corrected total	131	250.5305	1.9727		

B7.10 Analysis of variance for anti-proliferative activity of original extracts and resultant SPE fractions of carambola treated with 0.1mM MeJA (SFD4MJ2)

S.O.V	d.f.	S.S.	M.S.	F. Value	Pr>F
time	2	139.183	69.591	68.83	< 0.001
trt	4	100.791	25.198	24.92	< 0.001
time.trt	8	12.855	1.607	1.59	0.136
Residual	117	114.252	1.011		
Corrected total	131	370.525	2.918		

B7.11 Analysis of variance for anti-proliferative activity of original extracts and resultant SPE fractions of carambola treated with 0.1mM SA (SFD4SA1)

S.O.V	d.f.	S.S.	M.S.	F. Value	Pr>F
time	2	76.4490	38.2245	81.78	<.001
trt	4	56.5033	14.1258	30.22	<.001
time.trt	8	93.9120	11.7390	25.11	<.001
Residual	117	54.6892	0.4674		
Corrected total	131	281.5536			

B7.12 Analysis of variance for anti-proliferative activity of original extracts and resultant SPE fractions of carambola treated with 1mM SA (SFD4SA2)

S.O.V	d.f.	S.S.	M.S.	F. Value	Pr>F
time	2	53.9484	26.9742	41.63	<.001
trt	4	97.4800	24.3700	37.61	<.001
time.trt	8	145.1738	18.1467	28.01	<.001
Residual	117	75.8093	0.6479		
Corrected total	131	372.4115			

B7.13 Analysis of variance for effect of freshly harvested carambola (SFD0) extracts at 0.06 – 1 mg/ml on the intracellular production of ROS

S.O.V	d.f.	S.S.	M.S.	F. Value	Pr>F
time	6	1.197E+09	1.995E+08	26.31	<.001
trt	5	2.145E+08	4.290E+07	5.66	<.001
time.trt	30	3.565E+07	1.188E+06	0.16	1.000
Residual	133	9.557E+08	7.585E+06		
Corrected total	174	2.403E+09			

B7.14 Analysis of variance for effect of ripening carambola (SFD4) extracts at 0.06 – 1 mg/ml on the intracellular production of ROS

S.O.V	d.f.	S.S.	M.S.	F. Value	Pr>F
time	6	1.305E+09	2.175E+08	32.55	<.001
trt	5	4.672E+08	9.345E+07	13.98	<.001
time.trt	30	3.103E+07	1.034E+06	0.15	1.000
Residual	133	8.888E+08	6.683E+06		
Corrected total	174	2.692E+09			

B7.15 Analysis of variance for effect of extracts of carambola treated with 0.01 mM MeJA (SFD4MJ1) at 0.06 – 1 mg/ml on the intracellular production of ROS

S.O.V	d.f.	S.S.	M.S.	F. Value	Pr>F
time	6	1.183E+09	1.971E+08	15.76	<.001
trt	5	5.257E+08	1.051E+08	8.40	<.001
time.trt	30	3.779E+07	1.260E+06	0.10	1.000
Residual	133	1.664E+09	1.251E+07		
Corrected total	174	3.410E+09			

B7.16 Analysis of variance for effect of extracts of carambola treated with 0.1 mM MeJA (SFD4MJ2) at 0.06 – 1 mg/ml on the intracellular production of ROS

S.O.V	d.f.	S.S.	M.S.	F. Value	Pr>F
time	6	1.312E+09	2.187E+08	32.07	<.001
trt	5	1.980E+08	3.959E+07	5.81	<.001
time.trt	30	2.606E+07	8.688E+05	0.13	1.000
Residual	133	9.068E+08	6.818E+06		
Corrected total	174	2.443E+09			

B7.17 Analysis of variance for effect of extracts of carambola treated with 0.1 mM SA (SFD4SA1) at 0.06 – 1 mg/ml on the intracellular production of ROS

S.O.V	d.f.	S.S.	M.S.	F. Value	Pr>F
time	6	1.348E+09	2.247E+08	25.97	<.001
trt	5	5.265E+08	1.053E+08	12.17	<.001
time.trt	30	8.418E+07	2.806E+06	0.32	1.000
Residual	133	1.151E+09	8.651E+06		
Corrected total	174	3.109E+09			

B7.18 Analysis of variance for effect of extracts of carambola treated with 1 mM SA (SFD4SA2) at 0.06 – 1 mg/ml on the intracellular production of ROS

S.O.V	d.f.	S.S.	M.S.	F. Value	Pr>F
time	6	1.230E+09	2.051E+08	20.87	<.001
trt	5	4.409E+08	8.818E+07	8.97	<.001
time.trt	30	3.979E+07	1.326E+06	0.13	1.000
Residual	133	1.307E+09	9.827E+06		
Corrected total	174	3.018E+09			

B7.19 Analysis of variance for protective effect of freshly harvested carambola (SFD0) extracts at 0.06 – 1 mg/ml on the intracellular production of ROS

S.O.V	d.f.	S.S.	M.S.	F. Value	Pr>F
time	6	1.850E+10	3.083E+09	79.73	<.001
trt	5	1.342E+10	2.684E+09	69.40	<.001
time.trt	30	1.778E+09	5.926E+07	1.53	0.053
Residual	133	5.143E+09	3.867E+07		
Corrected total	174	3.884E+10			

B7.20 Analysis of variance for protective effect of ripening carambola (SFD4) extracts at 0.06 – 1 mg/ml on the intracellular production of ROS

S.O.V	d.f.	S.S.	M.S.	F. Value	Pr>F
time	6	1.414E+10	2.357E+09	61.10	<.001
trt	5	1.332E+10	2.664E+09	69.05	<.001
time.trt	30	1.479E+09	4.929E+07	1.28	0.175
Residual	133	5.132E+09	3.858E+07		
Corrected total	174	3.408E+10			

B7.21 Analysis of variance for protective effect of extracts of carambola treated with 0.01 mM MeJA (SFD4MJ1) at 0.06 – 1 mg/ml on the intracellular production of ROS

S.O.V	d.f.	S.S.	M.S.	F. Value	Pr>F
time	6	1.522E+10	2.537E+09	34.83	<.001
trt	5	5.984E+09	1.197E+09	16.43	<.001
time.trt	30	1.506E+09	5.021E+07	0.69	0.882
Residual	133	9.688E+09	7.284E+07		
Corrected total	174	3.240E+10			

B7.22 Analysis of variance for protective effect of extracts of carambola treated with 0.1 mM MeJA (SFD4MJ2) at 0.06 – 1 mg/ml on the intracellular production of ROS

S.O.V	d.f.	S.S.	M.S.	F. Value	Pr>F
time	6	1.780E+10	2.966E+09	68.13	<.001
trt	5	1.100E+10	2.201E+09	50.55	<.001
time.trt	30	1.805E+09	6.018E+07	1.38	0.110
Residual	133	5.791E+09	4.354E+07		
Corrected total	174	3.640E+10			

B7.23 Analysis of variance for protective effect of extracts of carambola treated with 0.1 mM SA (SFD4SA1) at 0.06 – 1 mg/ml on the intracellular production of ROS

S.O.V	d.f.	S.S.	M.S.	F. Value	Pr>F
time	6	1.188E+10	1.980E+09	94.47	<.001
trt	5	5.414E+09	1.083E+09	51.65	<.001
time.trt	30	9.212E+08	3.071E+07	1.46	0.074
Residual	133	2.788E+09	2.096E+07		
Corrected total	174	2.101E+10			

B7.24 Analysis of variance for protective effect of of extracts of carambola treated with 1 mM SA (SFD4SA2) at 0.06 – 1 mg/ml on the intracellular production of ROS

S.O.V	d.f.	S.S.	M.S.	F. Value	Pr>F
time	6	1.320E+10	2.200E+09	47.31	<.001
trt	5	6.965E+09	1.393E+09	29.96	<.001
time.trt	30	1.197E+09	3.991E+07	0.86	0.679
Residual	133	6.185E+09	4.650E+07		
Corrected total	174	2.755E+10			

LIST OF PUBLICATIONS

Submissions to Refereed Journals

M.A. Mustafa, G. Seymour, G. Tucker and A. Ali (2015). Revisiting stress responses of tropical fruits under cold storage. *(in progress)*

M.A. Mustafa, G. Seymour, G. Tucker and A. Ali (2015). Exploring the Differential Responses of Mangosteen to Methyl Jasmonate and Salicylic Acid Treatment under Chilling Conditions. *(in progress)*

M.A. Mustafa, G. Seymour, G. Tucker and A. Ali (2015). Treatment of Dragonfruit (Hylocereus polyrhizus) with Salicylic Acid and Methyl Jasmonate improves Postharvest Physico-chemical Properties and Antioxidant Activity during Cold Storage. *(in progress)*

M.A. Mustafa, G. Seymour, G. Tucker and A. Ali (2015). Enhancing the antioxidant content of carambola (*Averrhoa carambola*) during cold storage using salicylic acid and methyl jasmonate treatments. *(in progress)*

Conference Presentations

M.A. Mustafa, G. Seymour, G. Tucker and A. Ali (2014). Inducing Antioxidant Activity Using Stress Hormones in Cold Stored Mangosteen. XXIX International Horticultural Congress, Brisbane, Australia.

M.A. Mustafa, G. Seymour, G. Tucker and A. Ali (2014). The Role of the Ubiquitous Phenolic Compound 'Salicylic Acid' in Chilling Tolerance of Carambola. V International Postharvest Unlimited Conference, Cyprus.

M.A. Mustafa, G. Seymour, G. Tucker and A. Ali (2014). Bioactive Content of Selected Tropical Fruits as Antimicrobial Agents. V International Postharvest Unlimited Conference, Cyprus.

M.A Mustafa, N. Mohd Noh and A. Ali (2013). Incorporation of chitosan with essential oil for controlling anthracnose of bell pepper. Postgraduate Symposium on Plant Protection, organized by Malaysian Plant Protection Society, UNITEN, Malaysia.

M.A. Mustafa, G. Seymour, G. Tucker and A. Ali (2013). Exploiting chilling and methyl jasmonic stresses for enhancing quality of starfruit. International Functional Food Conference, Cyberjaya, Malaysia

M.A. Mustafa, Y.Z. Wei and A. Ali (2012). Application of propolis as a green technology for maintaining the quality of bell pepper. 23rd Malaysian Society of Plant Physiology Conference: Plant Physiology in Addressing Green Economy, Langkawi, Malaysia.

M.A. Mustafa, G. Seymour, G. Tucker and A. Ali (2012). Enhancing quality of export grade carambola by methyl jasmonate induced stress. 23rd Malaysian Society of Plant Physiology Conference: Plant Physiology in Addressing Green Economy, Langkawi, Malaysia.

M.A. Mustafa, A.Ali and S. Manickam (2012). Enhancing the efficacy of chitosan coating on tomato quality by a novel nanoemulsion formulation. 7th International Postharvest Symposium, Kuala Lumpur, Malaysia.