

Integrated Postharvest Management of Anthracnose in Dragon Fruits Using Plant Extracts



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for the degree of
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DEDICATION

I dedicate this work to my late parents, Mr. Nanabenyin Bordoh and Mrs. Monica Sampson-Eduam Bordoh. I also want to dedicate this work to Mr. Reginald Dzipkor and Mrs. Anna Dzipkor for their prayers, support and believing in me.

ABSTRACT

Integrated Postharvest Management of Anthracnose in Dragon Fruits Using Plant Extracts

The study was conducted to develop an environmentally, biodegradable, non-toxic and sustainable bio-fungicide using the formulation where each plant crude extract (ginger, turmeric and “dukung anak”) at different concentrations were incorporated separately with 10% gum Arabic (GA). The formulation was assessed against a wide range of parameters such as disease incidence (DI) and severity (DS) as well as postharvest quality (physicochemical and nutritional) on dragon fruits during storage at $11\pm 2^{\circ}\text{C}$, 80% RH for up to 28 days. Additionally, the study also investigated a diagnostic tool, thus Loop-mediated isothermal amplification (LAMP), for early detection of anthracnose in dragon fruits caused by *Colletotrichum gloeosporioides* Penz during postharvest storage.

Prior to the formulation, the antifungal effect of each plant extract alone was evaluated *in vitro* (against *C. gloeosporioides*) at concentrations (2.5 mg/ml, 5 mg/ml, 7.5 mg/ml and 10 mg/ml) and *in vivo* (on the fruits to control anthracnose) at concentration (5 mg/ml, 10 mg/ml and 15 mg/ml). *In vitro* antifungal activity of plants extracts alone showed that all plant extracts showed significant antifungal activity against *C. gloeosporioides* that causes anthracnose in dragon fruit. This was evident in inhibition of mycelial growth and conidial germination, which translated to morphological damage such as distortion and shrivelling in the fungal hyphae during scanning electron microscopy (SEM) studies result. Ginger crude extracts at 10 mg/ml showed a promising result in terms of suppressing mycelial growth (88.48%) and conidial germination (87.5%) which was comparable to commercial fungicide (Mancozeb) at 2 mg/ml (80.45%).

In the *in vivo* study, where dragon fruits were coated (treated) with only plant extracts by dipping, fruits treated at 15 mg/ml irrespective of the crude extracts experienced severe disease incidence due to phytotoxicity. However, DI and DS was significantly low in dukung anak-treated fruits irrespective of the concentration used. Postharvest anthracnose caused by *C. gloeosporioides* and its severity was significantly low in dukung anak-treated fruits at 10 mg/ml which was not different in fruits treated with 10 mg/ml of turmeric crude extracts after 28 days of cold storage. The antifungal activity was due to the presence of bioactive compounds gingerol, curcumin and some alkaloids in ginger, turmeric and dukung anak which resulted in electrolyte leakage from fungal cell wall contributing to direct control of anthracnose. While dukung anak extract controlled anthracnose and its severity at 10 mg/ml, DI was more pronounced in ginger-treated fruits even at 5 mg/ml due to phytotoxicity. Overall, disease incidence was highest especially at 15 mg/ml irrespective of plant extract used. Additionally, the external appearance of treated fruits at 10 mg/ml and 15 mg/ml of extracts alone (irrespective of the type of plant used) negatively affected customers appeal and preference due to compromised original smell and colour of the fruit.

To overcome this problem, a formulation comprised of each plant crude extracts especially at higher concentrations (10 mg/ml and 15 mg/ml) and at a lower concentration (5 mg/ml) were incorporated with 10%GA to develop an alternative non-chemical approach that can enhance the efficacy of each plant extract against anthracnose whilst improving other postharvest quality of fruits. Fruits treated with a composite coating of 10%GA+10 mg/ml turmeric crude extracts significantly reduced anthracnose and its severity by about 94% compared to controls. This was not different in fruits treated with 10%GA+15 mg/ml ginger extract after 28 days of storage.

For a rapid detection of postharvest anthracnose caused by *C. gloeosporioides*, LAMP, a diagnostic tool showed that composite coating of 10%GA+5 mg/ml of turmeric markedly reduced the amount of fungal DNA isolated from infested fruits compared to controls. A clear indication that Lamp can be used for detection of anthracnose during postharvest storage. Generally, respiration rate and ethylene production were significantly lower in fruits coated with 10%GA plus either 10mg/ml or 5 mg/ml of ginger crude extracts even though it was markedly low in fruits coated with 10%GA+10 mg/ml of the either turmeric or dukung anak crude extracts. In this study, fruit treated coated with 10%GA+10 mg/ml of any crude extracts especially turmeric crude extract showed the greatest firmness, reduced water loss, delayed colour development, lowered the levels of TSS and maintained a high TA after 21 to 28 days of storage. Furthermore, an composite coating of 10%GA+10 mg/ml of turmeric crude extract helped to maintain higher total antioxidant and nutritional activity in dragon fruits due to the presence of high ascorbic acid content and total phenolic compounds. Similarly, sensory evaluation results proved the effectiveness of 10%GA+10 mg/ml of turmeric crude extract composite coating by maintaining the overall quality of dragon fruits. These findings suggest that 10%GA+10 mg/ml of turmeric crude extracts can be used for extending the storage life of dragon fruits for up to 28 days. Additionally, this study proposes LAMP as a promising diagnostic tool for early detection of postharvest anthracnose in dragon fruits and other tropical fruits during regular checks in transits (sea transports) or at packing houses. By doing so, the onset of postharvest anthracnose could be detected and immediate steps taken to control the disease.

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DECLARATION

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.

Paa Kwesi Bordoh

21st November, 2017

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LIST OF ABBREVIATION

AA- Ascorbic acid

DPPH- 2, 2-Diphenyl-1-Picrylhydrazyl

GA- Gum Arabic

LAMP- Loop-Mediated Isothermal Amplification

NCBI- National Centre for Biotechnology Information

TA- Titratable Acidity

TPC- Total Phenolic Content

TSS- Total Soluble Solids

HPLC- High Performance Liquid Chromatography

10%GA+5 mg/ml- 5 mg/ml of any crude extract incorporated with 10% gum arabic

10%GA+10 mg/ml- 10 mg/ml of any crude extract incorporated with 10% gum arabic

10%GA+ 15 mg/ml- 15 mg/ml of any crude extract incorporated with 10% gum arabic

5 dun- 5 mg/ml of *Phyllanthus niruri* (dukung anak) extract

10 dun- 10 mg/ml of *Phyllanthus niruri* (dukung anak) extract

15 dun- 15 mg/ml of *Phyllanthus niruri* (dukung anak) extract

5 gin- 5 mg/ml of *Zingiber officinale* (ginger) extract

10 gin- 10 mg/ml of *Zingiber officinale* (ginger) extract

15 gin- 15 mg/ml of *Zingiber officinale* (ginger) extract

5 tur- 5 mg/ml of *Curcuma longa* L. (Turmeric rhizome) extract

10 tur- 10 mg/ml of *Curcuma longa* L. (Turmeric rhizome) extract

15 tur- 15 mg/ml of *Curcuma longa* L. (Turmeric rhizome) extract

10%GA+5 dun - 5 mg/ml of dukung anak crude extract incorporated with 10% gum arabic

10%GA+10 dun - 10 mg/ml of dukung anak crude extract incorporated with 10% gum arabic

10%GA+ 15 dun-- 15 mg/ml of dukung anak crude extract incorporated with 10% gum arabic

10%GA+5 gin - 5 mg/ml of ginger crude extract incorporated with 10% gum arabic

10%GA+10 gin - 10 mg/ml of ginger crude extract incorporated with 10% gum arabic

10%GA+ 15 gin-- 15 mg/ml of ginger crude extract incorporated with 10% gum arabic

10%GA+5 tur - 5 mg/ml of turmeric crude extract incorporated with 10% gum arabic

10%GA+10 tur- 10 mg/ml of turmeric crude extract incorporated with 10% gum arabic

10%GA+ 15 tur- 15 mg/ml of turmeric crude extract incorporated with 10% gum arabic

CHAPTER 1

1 INTRODUCTION

Red-fleshed dragon fruits (*Hylocereus costaricensis* (F.A.C. Weber) Britton & Rose) or red-fleshed pitaya is a perennial, epiphytic, climbing plant belonging to the family Cactaceae (Mizrahi et al., 1997). In Vietnam, where it is largely cultivated, the fruit is known and sold as Thanh Long, or 'Green dragon', a description associated with the green colour of the immature fruit, and the dragon-like appearance of the 'scales' or brackets on the fruit surface (Hoa et al., 2006). In Malaysia it is called "Buah naga". It is native to Mexico and widely cultivated worldwide, including Taiwan, Southern China, Israel and more recently in Thailand, Australia, United States of America and Malaysia (Mizrahi and Nerd, 1999). The fruit is a medium to large berry with red peel and green or red fleshy scales. The flesh is sweet and reddish in colour and interspersed with numerous tiny black seeds. The fruits can be consumed fresh or used to make jellies, jams, wine, beverages and marmalades (Wybraniec and Mizrahi, 2002; Chuah et al., 2008). Red-fleshed dragon fruit has is rich source of dietary fibre for diabetic patients (Mahattanatawee et al., 2006). It is also rich in beta-carotene, lycopene and vitamin E, with average concentrations of 1.4, 3.4 and 0.26 µg/100g edible portion, respectively (Charoensiri et al., 2009). These nutritional benefits have significant roles for reducing cholesterol, activating the digestive function and preventing colon cancer and diabetes (Ariffin et al., 2009). Fruits consumption is dependent on two aspects of fruit quality: taste and nutritional benefits. These two factors affect the decision process of consumers, producers and exporters. With the rapid expansion and economic growth in the agroindustry, tropical fruits are becoming increasingly important due to their desirability by consumers (Silva et al., 2014). Due to its rich nutritional and economic value, it has attracted global attention and interest in developing it for fresh fruit

export (Mizrahi and Nerd, 1999; Bellec et al., 2006). Economically, it serves as a source of income to farmers/producers and improves their livelihood. Additionally, it provides foreign exchange to producing countries through exports thereby creating employment for the youth.

It is also reported that European consumers' general attitude towards tropical fruit consumption is positive. They believe that tropical fruits are nutritious, healthy, good in taste, attractive and special (Sabbe et al., 2008; Jani and Tih, 2010). Reports shows that the world import of tropical fruits would reach 4.3 million metric tons by 2010 of which 87% of this demand would come from developed country markets (Jani and Tih, 2010). This gives tropical countries like Malaysia the competitive urge and advantage in the European market when it comes to the production of tropical fruits, especially dragon fruits, even though countries like China and Vietnam have an unprecedented lead over the years as major suppliers to European countries. In a survey conducted in Belgium, 77.5% of 290 respondents did not know about dragon fruit, 13.5% knew it but had not bought it, and only about 10% knew dragon fruit and had bought it (Sabbe et al., 2008). The authors concluded that dragon fruit can be considered as exotic tropical fruit that is unknown to the majority (>60%) of the respondents, and it is unlikely for its inclusion in the European consumers' daily diet without a properly planned successful market introduction campaign (Sabbe et al., 2008).

Some studies also show that because of increasing foreign competition and declining returns to traditional agricultural commodities, many growers in South Florida have embarked on an aggressive search for viable alternative agricultural commodities, and one commodity that has gained attention is dragon fruits (Evans et al., 2010). Dragon fruit have steadily become popular with growers and consumers alike (Lobo and Bender, 2008). Lobo (2007), reported that in California, there is a demand for a new and exotic crop, and dragon fruit have a great potential

as an exotic crop for growers. Easter (2015), reported that dragon fruit leads Vietnamese exports and Vietnam has topped US\$ 600 million fruit and vegetable exports in the first five months of 2015. Dragon fruit was the leading exported fruit with 900,000 metric tons shipped during the five months, followed by watermelon, longan, and bananas. Major export markets include the US, the Netherlands, China and Japan, as well as South Korea, Russia, and Singapore.

There have been limited studies focused on extending the postharvest quality of dragon fruit (Nerd et al., 1999; To et al., 2002; Chien et al., 2007a; Wall and Khan, 2008; Chutichudet and Chutichudet, 2011). Due to the nutritional value to human health and the potential economic benefits to growers and exporters, it has attracted international attention especially researchers to develop non-chemical alternative postharvest managements as opposed to synthetic fungicides to maintain the fruit quality and increase fruit availability.

As a non-climacteric fruit, it is best eaten when harvested ripe, however, its quality deteriorates during storage (Nerd and Mizrahi, 1999; Nerd et al., 1999; Chien et al., 2007a). Like any tropical fruit, dragon fruit experiences transient changes in the phytochemical and physicochemical composition. In recent years, its growth and yield have been affected by some biotic and abiotic factors; paramount among the biotic factors is anthracnose caused by the *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc, the most devastating disease in dragon fruit plants (Masyahit et al., 2009). The disease can affect fruit during cold storage and transportation. Some studies have attributed the decline of these phytochemical composition to pathogen attacks due to poor postharvest management (Hien and Tung, 2003; Nguyen et al., 2013). However, conclusive reports suggest that proper postharvest treatment can significantly improve the phytochemical and physicochemical composition of dragon fruits and reduce postharvest anthracnose whilst extending the shelf life (Ali et al., 2013; Zahid et al., 2013).

Modified atmosphere, cold storage and pre-cooling are common technologies 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; Hien and Tung, 2003; Phong et al., 2010). 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, these technologies are becoming of increasing importance (FAO, 2010). As a result, there are increasing concerns on the importance and impact of using state of the art postharvest tools to control this tropical disease whilst maintaining the nutritional content of fresh produce and their shelf life.

The ever-increasing rate of population growth and a global improvement of living standards has resulted in a steady increase in the world fruit market shares. Studies have shown significant positive correlation between the consumption of tropical fruits containing various phytonutrients with enhanced protection from chronic diseases such as cardiovascular diseases, cancers and dyslipidemia (Mohd Adzim Khalil et al., 2009). Despite this positive correlation between fruits consumption and their health benefits to humans, their consumption is generally below the recommended levels in numerous nations especially in developing and under-developed countries due to food losses along the supply chain (Mohd. Adzahan & Benchamaporn, 2007). Like many Southeast Asian countries, Malaysia boasts of rich tropical fruits and many Malaysian producers and exporters are targeting the European market and, in terms of market demand, the United Kingdom is the largest followed by France and Germany (Medium-term Prospect, 2003; Tih, 2010). The relative decline of Malaysian presence in the European market is due to shortcomings in food quality which have been on the increase (Mohd. Adzahan and Benchamaporn, 2007). Fruits grown in the tropics have high bioactive

compounds such as polyphenols, flavonoids, ascorbic acid, anthocyanins, minerals resulting in their significant nutritional properties (Clerici and Carvalho-ilva, 2011; Dembitsky et al., 2011). However, these perishable commodities are often sporadically cultivated in small farms, impeding their potential for commercialization (Mohd. Adzahan and Benchamaporn, 2007). Conclusive reports show that pre- and postharvest losses in Southeast Asia are estimated at around 20 – 50% and this has a negative impact on food security as well as economic development (Senik, 1995; ASEAN, 2006). Ability to reduce the hindrances within the food supply chain can help to enhance food quality and ensure global food security. This can be achieved by embarking on cost-effective, environmentally friendly and cheap postharvest technology to reduce food losses with the aim of combating the imbalances between consumption increases and huge demands of increased food production (Wills et al., 2007; FAO, 2010). Thus, postharvest technologies play a pivotal role in reducing food losses and waste. By so doing, we can increase in amount of food availability to farmers for their own consumption or for sale, reduce the likelihood that small-holder farmers become net food buyers. Additionally, this lowers expenditure of processors and retailers per unit food grown or harvested and finally lowers household expenditure per unit food consumed and finally maintains nutritional value of food (Lipinski et al., 2013).

It is estimated that roughly one-third of the food produced for human consumption is lost or wasted globally, which amounts to 1.3 billion tons per year (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 medium and high-income countries, food is to a significant extent wasted at the consumption stage, whilst in low-income countries like Malaysia, food is lost mostly during the early and middle stages of the food supply chain; much less food is

wasted at the consumer level (Gustavsson et al., 2011). It is estimated that on an overall per-capita basis, much more food is wasted in the industrialized world than in developing countries. The per capita food waste by consumers in Europe and North America is 95-115 kg/year, while in Sub-Saharan Africa and South/Southeast Asia it is only 6-11 kg/year (Gustavsson et al., 2011). Some notable causes of food losses in low-income countries are connected to financial, managerial and technical limitations in harvesting techniques, and postharvest techniques (storage facilities, infrastructure, and packaging) and marketing systems (Gustavsson et al., 2011; FAO, 2010). For instance, due to the humid and hot weather conditions in Malaysia, tropical fruits like dragon fruits encounter major losses during transportation and storage stemming from fungal decay and rapid maturation. According to Food and Agriculture Organization, it is estimated that 32% (weight basis) of all food produced in the world was lost or wasted in 2009 (Lipinski et al., 2013). When converted into calories, global losses represent approximately 24% of all food produced. Gustavsson et al. (2011), reported that 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. This can be attributed to challenges in the food supply chain due to improper postharvest storage and handling of fruits (inadequate infrastructures such as cold storage and packaging facilities) (FAO, 2010). The consequence of this problem causes food losses which significantly affects farmers and consumers alike since it generates tremendous and unstable hikes in pricing which can lead to rural impoverishment through its direct effects on farmers' livelihoods.

Generally, fruits like dragon fruit are perishable and require coordinated activity by growers, storage operators, processors and retailers to maintain quality and reduce food loss and waste. The extent of coordination can vary greatly from loose in the case of local food supplies to

complex for global supply chains (Mahajan, et al., 2014). Fresh fruits and vegetable attributes such as appearance, texture, flavour and nutritional value have been the traditional quality criteria, but increasingly safety characteristics (chemical, toxicological and microbial), and traceability are important for all the role players along the supply chain, from the farm to consumers. After harvest and during the distribution chain, fresh produce passes through many hands and at relatively large quantities and if proper postharvest treatment is not applied accordingly, the end results will be severe losses due to diseases during the supply chain (Paul, 1999). Owing to multiple uncertainties along the supply chain such as microbial contamination leading to spoilage as well as postharvest losses at any of the stages in the continuum from farm to consumer, growers and exporters should take pragmatic steps by ensuring proper postharvest management practices are employed to minimize microbial spoilage and contamination of fresh fruits and vegetables (Olaimat and Holley, 2012).

Traditionally growers use synthetic fungicides such as benomyl, carbendazim, propineb and difenoconazole to control anthracnose (Gopinath et al., 2006; Boonyapipat, 2013). A chemical known as “Umikai” is also used for postharvest treatment in Vietnam (Nyugen et al., 2013) before export. However, consumer concerns of using synthetic fungicides due to their toxicological effects to human health have resulted in a rise in public awareness to abolish their use. Additionally, negative environmental effects of these synthetic fungicides such as pollution of underground water as well as the build of fungal resistance to these fungicides (Ali et al., 2010a; Tietel et al., 2012), has necessitated the development of non-toxic bio-fungicide alternatives to synthetic fungicides in controlling postharvest anthracnose in dragon fruit. Recently, a non-toxic alternative approach using an ethanolic extract of propolis and submicron chitosan dispersion have been reported to control postharvest anthracnose in dragon fruit (Ali et

al., 2013; Zahid et al., 2013). A detailed literature review on alternative approaches to synthetic fungicides on the postharvest management of dragon fruit anthracnose is found in chapter 2 of this report.

In this research, crude extracts from *Zingiber officinale* Roscoe (ginger), *Curcuma longa* Linn. (turmeric) and *Phyllanthus niruri* Linn. (locally called in Malay as “dukung anak”) incorporated with gum arabic (GA) as a non-toxic alternative approach to synthetic fungicides in the postharvest management of dragon fruit anthracnose, since information of using these plant crude extracts is lacking. A diagnostic tool for early detection of the pathogen during postharvest storage of fruits was investigated. This diagnostic tool involves using a Loop-mediated isothermal amplification (LAMP) for early detection of *C. gloeosporioides* (due to their latency at postharvest). By doing so, we can quickly detect and apply the necessary postharvest treatment, at the recommend dose and time during storage to reduce the infection process to barest minimum. LAMP (Nagamine et al., 2001; Notomi et al., 2000) is a method for specific amplification of target nucleic acid sequences without thermal cycling, the use of which has been demonstrated and reported for detection of various plant pathogens (Tomlinson and Boonham, 2008; Tomlinson et al., 2010a, 2010b; Hodgetts et al., 2011; Tomlinson et al., 2013). Despite several studies on the efficiency of the LAMP technique, no study has been reported on its diagnostic ability for early detection of *C. gloeosporioides* that causes anthracnose in dragon fruits post harvest.

As a medicinal plant, *P. niruri* is used intensively in pharmacological and many clinical practices to prevent various form of human diseases caused by wide spectrum of human pathogens (Bagalkotkar et al., 2006; Paithankar et al., 2011; Mathur et al., 2012). Very little or no studies on the antifungal activity of *P. niruri* have been conducted on phytopathogen such as

C. gloeosporioides. Essential oils and curcuminoids of *C. longa* is used as an insect repellent to control some field crops postharvest (Saju et al., 1998; Tripathi et al., 2002) despite its broad spectral effect on clinical pathogens to control diseases in humans, (Sindhu et al., 2011). Additionally, antifungal activity of turmeric has been reported in phytopathogens such as *Colletotrichum acutatum* J.H Simmonds, *Colletotrichum fragariae* A.N Brooks, *Alternaria alternata* (Fr.) Keissl., *Phytophthora infestans* and *C. gloeosporioides* (Mont.) de Bary (Radwan et al., 2014; Wongkaew and Sinsiri, 2014). Like *C. longa* and *P. niruri*, ginger extracts are reported to possess significant antifungal effect against phytopathogens such as *Sclerotinia sclerotiorum* (Lib.) de Bary, *Rhizoctonia solani*, *Colletotrichum musae* (Berk. & M.A Curtis) Arx., *Fusarium oxysporum* (Schlecht) emend. Snyder & Hansen, *Alternaria radicina* Meier, Dreschsler & Eddy, *Fusarium solani* (Mart.) App. and Wr. Emend. Snyder & Hans., *Macrophomina phaseolina* (Tassi) Goid., *Phoma destructiva* Plowr., *Sclerotium rolsii* Sacc. and *C. gloeosporioides* (Dissanayake and Jayasinghe, 2013; Ademe et al., 2014; Al-Askar et al., 2014; Ojaghian et al., 2014). All these plants have high medicinal and antimicrobial properties, Generally Regarded as Safe (GRAS), non-toxic to humans and cheap. They are also biodegradable and have no direct environmental effect or causes pollution.

Gum arabic (GA) is a common polysaccharide frequently used in industry as a food additive (Motlagh et al., 2006). The joint FAO/WHO Expert Committee on Food Additives has approved GA as a safe compound (Anderson and Eastwood, 1989). GA is the most extensively used hydrocolloid in industry because it has emulsifying, film-forming, and encapsulating properties (Nisperos-Carriedo, 1994; Motlagh et al., 2006). Even though it has no antifungal activity, it helps to protect fruit against anthracnose by providing a film on the fruit surface, thereby creating a modified atmosphere of CO₂ and O₂, that delays the ripening process (Dang

et al., 2008). Incorporation of GA with the extracts may provide multiple effects in the control of anthracnose on dragon fruits and further extend the shelf life.

Major data gaps in understanding food losses, which is an albatross in the supply chain still exist. Attempting to arrest and solve the problem will pave the way for better postharvest technologies, which will ensure high-quality food products to reach the market. Understanding the different processes that make up the supply chain and the value that they add will inform us about their significance in the overall operation (Whicker et al., 2009). A similar concept applies to the use of postharvest strategies, through the understanding of their applications as well as the efficiency of these tools and their value in the supply chain will be grasped. In most cases, postharvest losses due to diseases occur during cold storage at the packing house or transportation. As tropical fruits are increasingly transported over long distances, it is essential to understand the responses of these fruits to postharvest treatments. Therefore, understanding the roles of postharvest tools requires an initial understanding of the physiology of fruits and their responses to postharvest treatment encountered throughout the supply chain. Using crude extracts from ginger, turmeric and “dukung anak” incorporated with gum arabic provides an alternative novel approach to synthetic fungicides in controlling postharvest anthracnose in dragon fruits. This will then inform us to consider these plant extracts as a potential postharvest tool that can be developed and promoted as a natural antimicrobial agent to control postharvest anthracnose on major tropical fruits since these materials are cheap, biodegradable, non-toxic, environmentally friendly and edible.

The Objectives of this research therefore:

- To investigate the antifungal effect of plant extracts; ginger, “dukung anak” and turmeric rhizome crude extracts to control postharvest anthracnose caused by *C. gloeosporioides*.
- To investigate the various bioactive compounds responsible for the antifungal activity against *C. gloeosporioides* that causes postharvest anthracnose.
- To investigate the mechanism of plant crude extracts against *C. gloeosporioides* to control postharvest anthracnose
- Formulation to enhance the efficacy of each plant crude extracts (ginger, turmeric and dukung anak) by incorporation with gum arabic on disease incidence and severity.
- Develop a diagnostic tool using Loop-mediated isothermal amplification (LAMP) technique for rapid detection of *C. gloeosporioides* on fruits during postharvest storage
- Investigate the efficacy of the formulation, thus each plant crude extract plus gum arabic on postharvest quality of red-fleshed dragon fruits.

CHAPTER 2

2 LITERATURE REVIEW

2.1 General overview of anthracnose

Anthracnose caused by *Colletotrichum* spp. is a cosmopolitan disease for many tropical and subtropical fruits (Cai et al., 2009; Cannon et al., 2012). It is recorded widely as both a pre-harvest and postharvest cause of crop loss (Hadden and Black, 1989; Bosland and Votava, 2003). *Colletotrichum* spp. is among the most devastating plant pathogens worldwide causing severe losses to a wide range of hosts including cereals, legumes, vegetables, perennial crops and tree fruits (Bailey and Jeger, 1992) reducing marketable yield from 10% to 80% of the crop production in some developing countries. As a postharvest disease, anthracnose caused by *C. gloeosporioides* can affect developing immature fruit in the field and damage mature fruits during storage or transportation. Anthracnose exhibit the phenomenon of quiescence in which the symptoms only appear after ripening because of the latency of the pathogen in the early ontogeny of the fruits (Bailey and Jeger, 1992). *Colletotrichum* species can infect many hosts and may adapt to new environments (Sanders and Korsten, 2003, Photita et al., 2004), leading to serious cross infection problems in plant production. The cosmopolitan nature of the *Colletotrichum* spp. is attributed to the fact that, in the *Colletotrichum* pathosystem, several strains of *Colletotrichum* spp. can be associated with anthracnose of the same (single) host (Simmonds, 1965; Freeman et al., 1998; Cannon et al., 2000) or a single species occurring on multiple hosts (Sander and Korsten, 2003).

It is in this regard that understanding the etiology of the disease and the pathosystem of the fungus is important so that relevant strategies to control postharvest anthracnose can be developed. Naturally, *Collectotrichum* spp. produce micro-sclerotia to allow dormancy in the

soil during the winter or when subjected to stressful conditions. These micro-sclerotia can survive for many years (Pring et al., 1995). They can also survive in and on seeds as acervuli and micro-sclerotia (Manandhar et al., 1995; Pernezny et al., 2003). In most cases, postharvest fungal infection initiation occurs when conidia from acervuli and micro-sclerotia in the field are dislodged during irrigation or heavy rains to healthy fruit and leaves especially in humid, warm and wet periods. Once dislodged, the conidia from the inoculum colonise the seed coat and peripheral layers of the endosperm and embryo, showing the disintegration of parenchymatous layers of the seed coat and depletion of food material in the endosperm and embryo (Chitkara et al., 1990). In fruits, the dislodged conidia attach to the fruit surface and begin to germinate into appressoria. The appressoria develop into pegs, which then penetrate the fruit epidermis. The fungal infection may remain quiescent as a biotroph at this stage. Activation of quiescent infections occurs during ripening and senescence of the fruit after harvest (Prusky and Lichter, 2007), followed by colonization of fruit tissue, production of acervuli and sporulation (Bailey and Jeger, 1992; Prusky et al., 2000). The known developmental stages where the fungus might become inactive are during penetration, initiation of germination, germ tube elongation, formation of appressorium or subsequent establishment. It was widely accepted that failure of spores to germinate or develop beyond any subsequent stage is due to temporary adverse physiological conditions imposed by the host directly on the fungus or indirectly by exhibiting temporary resistance against the fungus (Swinburne and Brown, 1983). These temporary resistances against the fungus could be due to application of postharvest treatment on fruit surface before storage which inhibit some these known developmental stages of the fungus. Temperatures around 27°C and high humidity (a mean of 80%) are optimum for anthracnose disease development. Other reports also state that temperatures of 20 and 25°C favour colony

growth and sporulation (Mello et al., 2004). In dragon fruit, reddish hard lesions with chlorotic haloes, small, depressed soaked lesions that are sub-circular or angular with translucent light brown margins, characterize symptoms of postharvest anthracnose caused by *C. gloeosporioides* (Masyahit et al., 2009).

2.2 General practices on postharvest handling of dragon fruits

Dragon fruits are harvested while ripe and matured. The fruit is usually hard after harvest but soften slowly afterwards and in some cases quicker leading to postharvest losses if proper measures are not taken. For commercial purposes, dragon fruits are harvested by hand and then placed in plastic crates before transportation by truck to pack house. At the pack house, stem trimming and cleaning of blossoms ends of fruit to remove debris is carried out. Fruits are then washed with clean water and dried, but in some cases, they are soaked in a chemical solution called “Umikai” solution (Nguyen et al., 2013) for a while followed by fan drying before being packed in a perforated polyethylene bag (PE). Once kept in PE bags, they are packed in a carton box (1 layer) (9 fruits/box), then temporarily stored (at least 2 days) at 2-10°C before loading into refrigerated containers for export (Nguyen et al., 2013). Pre-cooling is a common postharvest management used by most dragon fruit producers to control fruit rots in dragon fruits before long voyages especially by sea (Nguyen et al., 2013). In a survey conducted in Vietnam, pre-cooled fruits (14°C for approximately 18 hours) before storage at 5°C during a 22 days’ voyage from Vietnam to Holland saw fruit with better quality in terms of fresh green colour and lower incidence of rots compared to non-pre-cooled fruit. Nguyen et al. (2010) reported that pre-cooling under low temperature prior to storage at 3°C can extend the quality of fruits up to 6-8 weeks. However, reports show that without pre-cooling, some cultivar of dragon fruits (white-fleshed dragon fruits) stored at an optimal storage temperature of 5°C maintained

fruit quality up to 4 weeks (Hien and Tung, 2003). On the shelf (at supermarkets or homes) where temperatures are above 14 or 15°C, the fruits shelf life is approximately 3 to 4 weeks depending on postharvest treatment. Rejections of fruit at the market are partly due to improper postharvest management practices coupled with improper storage temperature management results in main fruit rot. Therefore, investigating alternative postharvet treatment practices in combination with storage at the recommended temperatures can further extend the shelf life of fruits.

Proper handling of fruits during packaging and transportation, use of recommended packages and storage temperature as well as proper postharvest treatment are crucial in minimizing postharvest diseases during the food supply chain. Ali et al., (2016) in their review stated that synthetic fungicides such as propiconazole, difenoconazole, carbendazim, benomyl, maneb and captain have been used traditionally in the pre-harvest control of anthracnose. Even though these fungicides are pre-requisitely used pre-harvest in the postharvest control of anthracnose in some tropical vegetables such as chilli, these synthetic fungicides have been reported to cause severe health problems ranging causing eye defects and other birth related problems (APVMA, 2009). Additionally, long term usage of these fungicides negatively impacts the environment causing soil and water pollution (Wilson et al., 1999; Northover and Zhou, 2002). These reasons coupled with concerns raised by stakeholders in the food processing and export business have informed governments to ban their use. Because of this, researchers are working tirelessly to investigate non-toxic alternative approach to control the postharvest disease without health problems to humans and the environment.

2.3 Current control management of postharvest anthracnose

2.3.1 Conventional practices

2.3.1.1 Traditional control

Cultural practices are carried out as pre-requisites to control postharvest diseases in fruits and vegetables due to the etiology of the pathogen or pest. As a precautionary measure, these practices are implemented to minimize the rate of infection thereby reducing the infection pressure even before fruits are mature and harvested. Fruit flies, mealy bugs, stink bugs are major pests that attack dragon fruits and serves as vectors for *C. gloeosporioides* causing anthracnose (Hoa, 2015). Management programs such as destroying or chopping infected plant parts into pieces for faster decomposition; avoidance of unclean water for canopy irrigation and using disease-free planting materials are required to minimise infection (Hoa, 2015). Additionally, cuttings of dragon fruit should be as near as possible to the pole or stake and tight; ensuring open-center pruning to encourage good ventilation; weeding carefully around the succulent stems to avoid open wounds (good medium for pathogen or pest attacks); and finally burning and destroying diseased branches or plant parts that served as inoculum without leaving them on farm to prevent build-up of pathogens are all good cultural practices (Nelson, 2008; Zainudin, 2011).

At the pack houses, proper drainage systems to channel waste water or sewage after fruit washing should be constructed (Than et al., 2008). If any history of anthracnose outbreak or any diseases that could be detrimental to dragon fruit existed on the field due to previous cultivation anthracnose-prone crops, such fields should be rotated with other crops as suggested in chilli, corn and soybean anthracnose management practice (Lipps, 1983; Bergstrom and Nicholson, 1999; Roberts et al., 2001). By doing so, the life cycle of the pathogen is disrupted and the

chances of infection reduced since previous crop which may serve as inoculum is changed. Despite its contribution as a pre-requisite to postharvest anthracnose management, cultural practices like crop rotation require a lot of skill to understand the nutrient regime of the land, since nutrient requirement varies with crop in rotation and this may result in the alteration of nutrient management programs and weed control practices. The alteration of nutrients if not properly monitored may even increase the farmer's cost of production by using more fertilizers to improve the fertility of the soil before the beginning of the cropping season.

2.3.1.2 Chemical control

Fungicides that belong to the benzimidazole (thiabendazole and benomyl), imidazole (prochloraz), bisdithiocarbonate (EBDC) groups, registered fungicides of the group strobilurins (azoxystrobin) and the benzonitrile compounds (chlorothalonil) as well as mancozeb, difenoconazole, propiconazole, maneb, calcium chloride and captan are applied to control anthracnose on the field (Gopinath et al., 2006; Ghani et al., 2011; Bautista-Baños et al., 2013; Boonyapipat, 2013; Hoa et al., 2015). These fungicides are applied as prerequisite to control postharvest anthracnose and other related diseases in dragon fruit.

For postharvest management practices, calcium chloride is reported not only to control anthracnose, but also improve the shelf life and postharvest qualities of fruits (Awang et al., 2011). Reports also show that “Umikai” solution used by some exporters significantly controls dragon fruit diseases before exports to the European countries (Nguyen et al., 2013). Sodium bicarbonate, a common food additive at 2% significantly controlled anthracnose in papaya caused by *C. gloeosporioides* by 60% compared to control (Hasan et al., 2012a). However, it is imperative to note that some of these synthetic chemicals like benomyl (not registered or approved in Australia) and its associated fungicides carbendazim and thiophanate methyl (both

of which are registered) has raised major health concerns such as causing eye defects, and other birth-related effects by disrupting the process of cell division making their use unacceptable and dangerous (APVMA, 2009). Additionally, the emergence of resistant strains of *Colletotrichum* spp. against benomyl, which is cross-resistant to thiophanatemethyl and carbendazim was reported in Malaysia and Trinidad (Sariah, 1989; Ramdial and Rampersad, 2014).

As an alternative to using synthetic fungicides preharvest, the application of submicron chitosan dispersions (SCD), a natural product can effectively control preharvest anthracnose. This can also control the disease during postharvest storage without leaving any residual effect on fruits (Ali et al., 2014; Zahid et al., 2014; Zahid et al., 2015). Curative applications of submicron chitosan dispersions (SCD) of 600 nm droplet size of 1.0% chitosan to dragon fruit resulted in 15.65% disease reduction with a disease severity score of 1.60 whilst enhancing the growth of the plants (Zahid et al., 2014). The tremendous reduction in disease incidence, disease severity as well as enhancement of plant growth was due to the possible mechanisms of enhanced inducible compounds, production of pathogenesis-related proteins and plant-defence related enzymes such as PPO, PO, PAL, β -1-3 glucanase, chitinase activity and lignin when fruit were treated with SCD of 600 nm droplet size at 1.0% (Ali et al., 2014; Zahid et al., 2015). However, resistance may decrease with the passage of time which could be due to the highly stressed environment of the plants (Zahid et al., 2015).

The current trend by traditional farmers to control pre- and postharvest anthracnose in the dragon fruits relies heavily on the use of synthetic fungicides and there have been very little studies on using natural products. The long-term usage of synthetic chemicals is known to have a negative impact on the environment especially soil and water resources (Wilson et al., 1999; Northover and Zhou, 2002). The increasing health concerns expressed by consumers and the

intention of governments to regulate pesticide use and their residues in fresh produce have necessitated the development of non-toxic alternative management techniques for anthracnose control.

2.3.2 Alternative management practices

2.3.2.1 Physical control

In this section physical control methods such as hot water treatment, vapour heat, forced hot air and irradiation will be discussed. Hot water treatment of fruit following harvest has been demonstrated to protect horticulture produce against postharvest decay (Wills et al., 1989; Lurie, 1998; Ferguson et al., 2000; Fallik et al., 2001; Vicente et al., 2002; Lana et al., 2005). Heat treatment and irradiation technologies are relatively simple and non-chemical alternative approach to synthetic fungicides. They are used as quarantine technique against insect pests by killing eggs and some pathogens on fruit surfaces of perishable commodity thereby controlling postharvest diseases (Lum et al., 2011; Bautista-Baños et al., 2013). Some varieties of dragon fruit (Binh Thuan' dragon fruits) proved to be tolerant to hot air treatment and at the lower heat treatment at 46.5°C for 20 min and fruit quality was not significantly different in non-heated fruits after storage for up to 4 weeks (Hoa et al., 2006). Hoa et al. (2006) provided evidence that some varieties of dragon fruits are a suitable candidate and tolerant against fruit fly disinfection by hot air heat treatments, and that fruit quality during or after storage was not affected. It is also known that different temperatures during hot water treatment, submergence time and storage duration can affect the postharvest quality and shelf life of dragon fruit (Lum et al., 2011). Hot water treatment (60°C) with a submergence time of 60 min for dragon fruits significantly caused severe weight loss with a concomitant decline in other postharvest quality and shelf life after 15 days of storage at 25°C. However, fruits treated with hot water at 35°C for

60 min recorded the highest titratable acidity (TA), were much firmer and extended the shelf life of dragon fruits after 15 days of storage at room temperature (Lum et al., 2011). It is reported that the mode of action of the hot water treatments may be through the impact of the heat to kill the pathogens directly or indirectly on horticulture commodities, thus by acting directly on viable spores on fruit surface or beneath, hence delaying conidia germination, growth and sporulation (Lurie et al., 1996; Fallik, 2004). Another mechanism in controlling these pathogens is because of associated up-regulated proteins such as beta-1,3glucanase, Class III chitinase, 17.7 kDa heat shock protein and low molecular weight heat-shock protein in fruits tissues which are expressed and act as defensive mechanism for host tissue to fight against the pathogen when heat treatment is applied (Yun et al., 2013).

The USDA's Animal Plant Health Inspection Services (APHIS) allows export of dragon fruit from Hawaii after irradiation with a 400-Gray (Gy) dose and 150-Gy dose and inspection for mealy bugs that causes diseases in dragon fruits (APHIS, 2008). As a quarantine treatment, X-ray irradiation on dragon fruit at doses of 800 Gy or less (400 or 600-Gy) recorded minimal softening which was reflective of the absence or minimal fruit decay after 12 days of storage at 10°C (Wall and Khan, 2008). However, such irradiation dose (0, 200, 400, 600, or 800-Gy) didn't affect total soluble contents, titratable acidity or fructose concentrations after 12 days of storage at 10°C (Wall and Khan, 2008). As an environmentally-friendly strategy to extend the postharvest life of fresh vegetables (Civello et al., 2006), UV-C radiation induces beneficial physiological effects in plant tissues, delays ripening and senescence-associated changes (Bu et al., 2013), activates defence-related genes (Pombo et al., 2011) and induces the accumulation of phytoalexins (D'hallewin et al., 1999).

Despite evidence suggests the use of these physical methods to control anthracnose, more physical studies need to be carried out. However, there are several limitations with some physical control methods. For instance, inappropriate exposure (high temperature and longer exposure time) of fruits to hot water or heat treatment can damage fruit tissues, cause abnormal softening or ripening, lack of starch breakdown and severe cases infection, while excessive exposure of fruits to irradiation can cause phytotoxic effect to fruits or could be toxic to humans (Wills et al., 1989; Fallik et al., 2001; Hoa et al., 2006; Wall and Khan, 2008; Kumah et al., 2011).

2.3.2.2 Modified and controlled atmosphere

Modified and controlled atmosphere (MA and CA) technologies can substitute the atmospheric gas inside the package or container for a mixture of gases that help to extend the shelf life of some fruits and vegetables (Bautista-Baños et al., 2013). Chandran (2010) reported that modified atmosphere (MA) packages using polymeric film with different permeability for O₂, CO₂, other gases and H₂O maintained RH, reduction of water loss and reduction of contamination in dragon fruits. Contrary to the above assertion of controlled atmosphere (MA) extending the shelf life of fruits, some studies report that higher temperatures and the use of a perforated plastic bag increased decay incidence in dragon fruits, as well as severity after cold storage and shelf life conditions at room temperature (Freitas and Mitcham, 2013). Dragon fruits at 5°C without a perforated plastic bag showed no decay incidence after storage and shelf life conditions while there was 10% decay in fruit stored at 5°C in a bag after 20 days of storage (Freitas and Mitcham, 2013). At a relatively high temperature compared to 5°C, the use of perforated bags also decreased the percentage of fruit showing decay incidence during storage at 7°C and 10°C, and this reduction was pronounced at 10°C (Freitas and Mitcham, 2013). Very

few studies on MA and no studies on CA have been conducted on postharvest control of dragon fruits, therefore more studies need to be conducted since this technology leaves no harmful residual effect on the fruits for human consumption.

2.3.2.3 Natural compounds

Crude extracts, essential oils and other natural bioactive compounds produced by plants have antimicrobial activity that is present in different organs such as the seeds, roots, leaves and flowers (Bautista-Baños et al., 2013). These antimicrobial compounds synthesized by plants might be a very good alternative for controlling diseases of tropical fruits and vegetables since there have been several conclusive studies of their significant antimicrobial activity against several *Colletotrichum* spp. *in vitro*. As an alternative approach to controlling postharvest anthracnose in dragon fruits, natural compounds such as chitosan and propolis are the major reported products successfully used to control postharvest anthracnose (Ali et al., 2013b; Zahid et al., 2013).

In vitro studies have shown the efficacy of certain medicinal herbs or plant extracts against *Colletotrichum* spp. (Charigkapakorn, 2000; Begum et al., 2007; Nduagu et al., 2008; Johnny et al., 2011; Saravanakumar et al., 2011; Ajith et al., 2012). Aqueous leaf extract of *Plumbago indica* L. had a significant suppressive effect on *C. gloeosporioides* in terms of conidial germination (98.9%) and radial mycelial growth (98.8%). Botanicals or plant extracts from *Catharanthus roseus* L. G. Don., *Coleus aromaticus* Benth., *Manilkara zapota* L. P. Royen., and *Azadirachta indica* A. Juss significantly inhibited the radial mycelial growth of *Colletotrichum* spp. responsible for anthracnose disease in bell peppers (Ajith et al., 2012). Singh and Khirbat (2014), reported on the efficacy of aqueous extract of three wild plants viz., siras (*Albizia lebbek* L. Benth.), babul (*Acacia Arabica* Lam.) and Clerodendron

(*Clerodendron infortunatum* L.) against *Colletotrichum* spp. that causes anthracnose in chilli peppers. The leaf extracts of ‘siras’, ‘babul’ and ‘clerodendron’ caused significant inhibition of spore germination in *Colletotrichum capsici* with percentages of inhibition of 100%, 86.6%, and 73.2% respectively. Additionally, crude extracts of turmeric, ginger and “dukung anak” has reportedly shown significant antifungal activity against mycelia growth and conidia germination of *C. gloeosporioides* (Singh et al., 2007:2008; Ademe et al., 2014; Radwan et al., 2014).

Based on the significant effect of these plant extracts against *Colletotrichum* spp. *in vitro*, some reports confirm their efficacy to control postharvest anthracnose in some vegetables like chilli (Alves et al., 2015). In postharvest management of dragon fruit, fruits treated with only ethanolic extracts of propolis (0.50%), resulted in reduced weight loss, firmer fruits, maintained soluble solid concentrations and shown high titratable acidity after 20 days of storage at $20\pm 2^{\circ}\text{C}$ (Zahid et al., 2013). Although disease incidence was not investigated, Chutichudet and Chutichudet (2011) reported that dragon fruits treated with 3% chitosan had least attributes of stomatal conductance, stomata size; and the fruits showed lowest wilting percentage as well as a maximal postharvest life of 8.17 days at 27°C , 88% Relative Humidity (RH). As a postharvest control of anthracnose in dragon fruit plant, submicron chitosan dispersions at 1.0% with 600 nm droplet size, delayed the onset of postharvest anthracnose and maintained the quality of dragon fruits for up to 28 days of storage at $10\pm 2^{\circ}\text{C}$, $80\pm 5\%\text{RH}$ (Ali et al., 2013b). Plant products or natural products should be used in moderation during postharvest treatment of fruits, as high doses or concentrations can cause phytotoxicity to fruits thereby reducing their shelf life, though such high concentration may not be harmful to humans (Zahid et al., 2013). It is imperative to say that; more studies need to be done with natural products either alone or in combination with other natural products such as gum arabic. Several reports suggest a

significant antimicrobial effect of plant extracts such as turmeric, ginger and “dukung anak” against *C. gloeosporioides*. These plant extracts are environmentally friendly, biodegradable, water soluble and non-toxic to humans and no study of each extract (ginger, turmeric and “dukung anak”) incorporated with gum arabic has been investigated in the postharvest control of anthracnose in dragon fruit.

2.4 Postharvest importance of gum arabic on tropical fruit quality

Gum arabic (GA) (also known as Acacia gum, chaar gund, char good, or meska) is a natural gum, edible, dried exudate from stems and branches of *Acacia senegal* (L.) Wildenow and *Acacia seyal* Delile that is rich in non-viscous soluble fiber (Williams and Phillips, 2000). It is a branched-chain, complex polysaccharide, either neutral or slightly acidic and found as a mixed calcium, magnesium and potassium salt of a polysaccharidic acid (Ali et al., 2008). GA is defined by the FAO/WHO Joint Expert Committee on Food Additives (FECFA) as a dried exudate from the stem of *A. senegal* or closely related species of Acacia (family Leguminosae) (FAO, 1999). It is water soluble and consists of a complex mixture of glycoproteins and polysaccharides from which the original source of the sugars arabinose and ribose were obtained (Renard et al., 2006; Ali et al., 2008). The mixture of polysaccharides and glycoproteins gives it the glue-like binding property that is edible to humans. Pharmaceutical drugs and cosmetics use the gum as a binder, emulsifying agent, and a suspending or viscosity agent (Smolinske, 1992). In the wine industry, it is used as wine fining agent (Vivas et al., 2001). In the food industry is used as an emulsifier and a thickening agent in icing, fillings, chewing gum and other confectionery treats (Rinsky and Rinsky, 2009).

In the field of postharvest, GA has been reported to have a synergistic effect when incorporated with other natural or synthetic (fungicides) products where it contributed to

controlling diseases by maintaining the physiochemical properties of fruits or combination with other natural products. Maqbool et al. (2010a; 2010b), reported 70%-80% reduction of anthracnose in banana fruits after treatment with 10%GA+0.75% or 1% Chitosan after 28 days of storage at 13⁰C. The combination of GA incorporated with cinnamon essential oil at 0.4% significantly reduced anthracnose by 80% in banana (at 13±1⁰C) and 71% in papaya (at 13±1⁰C) after 28 days followed by 5 days of shelf life at room temperature (Maqbool et al., 2011). Recently when 10%GA was incorporated with a synthetic fungicide (3% Calcium chloride) and applied on mango fruits; it significantly alleviated decay incidence (Khaliq et al., 2016). Furthermore, 10%GA alone or combined with 3% Calcium chloride effectively reduced weight loss, soluble solids concentrations and colour changes but maintained a high fruit firmness, titratable acidity and ascorbic acid. Additionally, 10%GA+3% Calcium chloride reduced respiration rate and ethylene production after 28 days of storage at 6±1⁰C and after 5 days of shelf life at 25±2⁰C. As an edible coating, 10%GA delayed the ripening process of tomatoes and significantly preserved the total antioxidant activity, lycopene content, total phenolic and total carotenoids of tomatoes up to 10 days of storage at 20⁰C (Ali et al., 2013a) and reduced browning of sliced tomatoes (Eltoum and Babiker, 2014). Similarly, Al-Juaimi also reported significant high firmness, high total soluble solids and titratable acidity and overall acceptance of tomato fruits treated with 5-20% GA after 16 days of storage at 10±1⁰C. When 10%GA was applied as an edible coating on papaya fruits, the fruits showed a significantly higher antioxidant activity at day 7 during a 15 day of storage (Addai et al., 2013). Addai et al. (2013), also confirmed that 10%GA significantly maintained the physicochemical properties (total soluble solids, pH, titratable acidity, colour, and moisture) of papaya fruits after 15 days of storage at 13±1⁰C. It is evident that GA plays a significant role in enhancing the postharvest

and nutritional quality of fruits while extending their shelf life. Its emulsifying, film-forming, and encapsulating properties (Nisperos-Carriedo, 1994; Motlagh et al., 2006) synergistically helps to protect fruit against diseases by providing a film on the fruit surface, thereby creating a modified atmosphere of CO₂ and O₂, thus delaying fruit ripening (Dang et al., 2008).

2.5 Bioactive compounds and antimicrobial properties of plant extracts

2.5.1 *Curcuma longa* Linn

Turmeric use dates back nearly 4000 years to the vedic culture in India, where it was used as a culinary spice and had some religious significance (Prasad and Aggarwal, 2011). It is reported to have reached China probably by 700 AD, East Africa by 800 AD, West Africa by 1200 AD, and Jamaica in the eighteenth century. Turmeric has a long history of medicinal use in South Asia according to Sanskrit medical treatises, Ayurvedic and unani systems (Kapoor, 1990; Prasad and Aggarwal, 2011). Cultivated widely in the tropics, it has different names in different cultures and countries. “Haldi” in North India and “manjal” in South India. In Malaysia, it is called Kunyit. Turmeric rhizome (*Curcuma longa* Linn. family; Zingiberaceae) produces turmeric. It is an herbaceous perennial plant with tough brown skin and a deep orange colour. It is native to tropical South Asia where they are widely cultivated and its centre of domestication is certainly the Indian subcontinent (Dahal and Idris, 1990; Narrrayana, et al., 2000). It is also produced in the Caribbean and Latin America (Dahal and Idris, 1990; Weiss, 2002). More than 100 components have been isolated from the turmeric plant. The main components of the rhizome are volatile oils, containing turmerone, and other colouring agents called curcuminoids (Mohammadi et al., 2005; Menon and Sudheer, 2007; Hatcher et al., 2008; Singh and Jain, 2012; Rohman, 2012). These curcuminoids (curcumin, desmethoxycurcumin, and

bisdemethoxycurcumin) are compounds responsible for the yellow colour and are rich in antioxidants where curcumin forms the major bioactive component of turmeric (Ruby et al., 1995; Selvam et al., 1995; Bunyaprapatr and Chokchaichareonporn, 1996; Akram et al., 2010; Singh and Jain, 2012). In a conventional or normal form, turmeric contains moisture (>9%), curcumin (5–6.6%), extraneous matter (<0.5% by weight), mould (<3%), and volatile oils (<3.5%) (Prasad and Aggarwal, 2011). Volatile oils include d- α -phellandrene, d-sabinene, cinol, borneol, zingiberene, and sesquiterpenes (Ohshiro et al., 1990). The rhizomes are also reported to contain four new polysaccharides-ukonans along with stigmasterole, β -sitosterole, cholesterol, and 2-hydroxymethyl anthraquinone (Kapoor, 1990; Kirtikar et al., 1993).

Turmeric is generally regarded as safe (GRAS). It has been used as a spice from the early time of human civilization and is correlated with several biological activities (Nagarajan et al., 2010). Because of its specific flavour and yellow colour, the introduction of turmeric keeps the nutritional value and freshness of food items. As a food additive, turmeric can improve the deliciousness, aesthetic appeal, and shelf life of delicate food products (Joe et al., 2005).

Essential oils and curcuminoids of *C. longa* (turmeric) have been reported for their importance in clinical trials on chemoprevention as well their wide spectrum of biological activities such as anti-inflammatory, immune-stimulatory, anti-diabetic, anti-psoriatic, anti-atherogenic, antioxidant, anti-carcinogenic, anti-mutagenic, anti-coagulant, anti-fertility, anti-bacterial, anti-protozoal, anti-viral, anti-fibrotic, anti-venom and anti-ulcer (Huang et al., 1992; Piper et al., 1998; Antony et al., 1999; Heng et al., 2000; Ramí'ez-Bosca' et al., 2000; Arun and Nalini, 2002; Singh et al., 2002; Martin-Cordero et al., 2003; Chattopadhyay et al., 2004; Kohli et al., 2005; LoTempio et al., 2005; Aggarwal et al., 2007; Menon et al., 2007; Kunnumakkra et al., 2008; Aggarwal and Harikumar, 2009; Kalpravidh et al., 2010; Sindhu et

al., 2011; Singh and Jain, 2012; Ferreira et al., 2013). Some studies also suggest that it contributes to the incorporation of the healing process of dermal wound (Gopinath et al., 2004), helps in the prevention of Alzheimer's disease (Lim et al., 2001; Park, 2002) and treatment of cardiovascular diseases (Tilak et al., 2004; Aggarwal and Harikumar, 2009).

Despite its vast use in pharmacopeia, *in vitro* studies have also shown their antifungal effect against several phytopathogens. Turmeric methanolic crude extracts is reported as a bio-fungicide against several plant diseases such as barley powdery mildew (*Erysiphe graminis*), tomato late blight (*Phytophthora infestans* (Mont.) de Bary, rice sheath blight (*Rhizoctonia solani* Kühn), cucumber gray mold (*Botrytis cineria* Pers), wheat leaf rust (*Puccinia triticales* Die. & Holw), rice blast (*Magnaporthe grisea* (Herb.) Barr. at 1000 mg/L (1 mg/ml) or 3 mg/ml (Kim et al., 2003; Cho et al., 2006). Murthy et al. (2013) also reported antibacterial activity of aqueous turmeric extracts (100 mg/ml) against bacterial wilt of tomato caused by *Ralstonia solanacearum* (Smith) Yabuuchi. Similarly, essential oil of turmeric was insecticidal as it significantly controlled rice weevil, lesser grain borer, and red flour beetle by contact or fumigation due to the presence of phenolic compounds (turmerones and ar-turmerones) (Tripathi et al., 2002). *In vitro*, dried turmeric (*C. longa*) methanolic extracts was antifungal against *C. acutatum*, *C. fragariae* and *C. gloeosporioides* and the efficacy was dose dependent (Radwan et al., 2014). Recently, Wongkaew and Sinsiri (2014) reported 65.19 -73.33% inhibitions in daily radial mycelial growth of *C. gloeosporioides* at a concentration range of 10 mg/ml-50 mg/ml using ethanolic rhizome extract of turmeric. Aqueous turmeric extracts at 7.5% resulted in 40.73% inhibition in mycelial growth of *C. gloeosporioides* (Tasiwal et al., 2009). The antifungal activity of this extracts is due to the biological activity of curcuminoids present in the extracts (Radwan et al., 2014).

Curcuminoids are polyphenols. They have poor solubility in water at acidic and physiological pH, and hydrolyze rapidly in alkaline solutions. Curcuminoids are soluble in dimethyl sulphoxide (DMSO), acetone, methanol and ethanol and readily decompose when exposed to bright light, high temperature or oxidative conditions (Schieffer, 2002). As a liposoluble compound, curcumin (the major bioactive) can be extracted with alcohol (methanol or ethanol) and acetone since these organic solvents are considered safe for extraction of food materials (Revathy et al., 2011; Kulkarni et al., 2012; Popuri and Pagala, 2013). Because of their poor solubility in water, it is recommended to re-dissolve crude extracts with some organic solvents such as acetone, DMSO or alcohol before its use. This way, curcumin's bioavailability is maximal for any antifungal assay. Despite success in its use in pharmacopeia, there remains a gap of using crude extracts of turmeric as a postharvest agent in the control of anthracnose in red-fleshed dragon fruits caused by *C. gloeosporioides* and for that matter any tropical fruit.

2.5.2 *Phyllanthus niruri* L.

Phyllanthus niruri is described as a magic herb and it belongs to the family Euphorbiaceae (Paithankar et al., 2011). The flower and the fruit, seems to become one with the leaf, hence the name *Phyllanthus* (meaning “leaf and flower”) (Paithankar et al., 2011). This herb grows up until 40 cm in height and is indigenous to the Amazon rainforest and other tropical areas including Southeast Asia, Southern India and China (Girach et al., 1994). In India and Malaysia it is called “Dukong anak” or “Dukung anak” (Shanmugam et al., 2014; <http://agromedia.mardi.gov.my/MEPIS/index.php/galeri-herba-2/55-dukung-anak>), whilst in Spain it is called Chanca Piedra, meaning stone breaker, because it has been used as an effective remedy to eliminate gallstones, kidney stones and other kidney disorders (Bagalkothar et al.,

2006). Known as a common kharif (rainy season) weed, it is found in both cultivated and wastelands.

In recent times *P. niruri* has been used extensively in ethnomedicine and pharmacological practices all over the world, mainly in Malaysia, the USA, Cuba, Peru, Nigeria, China, the Caribbean, India and Brazil (Paithankar et al., 2011). It has a long history in herbal medicine systems such as Indian Ayurveda, traditional Chinese medicine, and Indonesian jamu. As a medicinal plant, *P. niruri* has a wide variety of phytochemicals with intensive pharmacological and intriguing therapeutic properties in many clinical trials which include anti-hepatotoxic, anti-lithic, anti-hypertensive, anti-HIV, anti-fungal, antibacterial, anti-spasmodic, anti-hypoglycemic, anti-malaria and anti-hepatitis B properties (Tona et al., 1999; Raphael et al., 2002; Cimanga et al., 2004; Ali et al., 2006; Bagalkotkar et al., 2006; Iizuka et al., 2006; Mustofa et al., 2007; Paithankar et al., 2011; Mathur et al., 2012;). Additionally, the whole plant is used as remedies for dysentery, influenza, vaginitis, tumours, diabetes, diuretics, jaundice, stimulating liver, dyspepsia, bronchitis, leprosy, anaemia, urinary discharge, asthma treatment, treatment of hepatotoxicity, hepatitis B, hyperglycaemia, skin diseases and lowering Cholesterol levels (Chopra et al., 1986; Adeneye et al., 2006; Paithankar et al., 2011). It also offers protection against chemical toxins (Wang, 2000; Lee et al., 2006; Chatterjee et al., 2006), liver cancer (Rajeshkumar and Kuttan, 2000). In Malaysia, *P. niruri* is used internally for diarrhea, kidney disorders, gonorrhea and coughs (Burkill, 1996). This herb is generally regarded as safe and is non-toxic even at the highest dose of > 5000 mg/kg. body weight (Asare et al., 2011). In numerous *in vitro* studies, the significant antimicrobial effect of *P. niruri* against vast clinical pathogens has been reported. *In vitro*, aqueous or ethanolic or methanolic “dukung anak” extracts were antibacterial against *E. coli*, *Salmonella typhi*, *P. aeruginosa*, *Lactobacillus* spp,

L. monocytogenes, *Bacillus cereus* Frank. & Frank., *Bacillus anthracis* Cohn., *Methylobacterium hispanicum* Gall., *Streptococcus viridians*, *S. aureus*, *Enterobacter cloacae* (Jord.) Horm. & Ed., *Trichoderma viridae* Pers., *Aspergillus niger* Van Tieg. *Epidermophyton. floccosum* (Harz) Lang. & Milo. (Venugopalan et al., 2010; Kanthimathi and Soranam, 2013; Poh-Hwa et al., 2011; Mathur et al., 2012; Gawai et al., 2013; Shanmugam et al., 2014). However, conclusive reports state that methanolic extract of “dukung anak” gives the best and highest anti-bacterial activity (Shanmugam et al., 2014). Rare reports can be found on antifungal activity of *P. niruri* on phytopathogens such as *C. gloeosporioides*. However, Singh et al. (2007: 2008a) reported the antifungal effect two alkaloids (allosecurinine and securinine) isolated from *Phyllanthus amarus* L. against selected phytopathogens including *Colletotrichum* spp. In their study, allosecurinine and securinine at 600 ppm, 800 ppm and 1000 ppm significantly resulted in reduction of *Colletotrichum* spp as this was evident in reduction in spore (200 to 300 spores) germination after 24 hr of incubation at $25\pm 2^{\circ}\text{C}$ on cavity slides. It is imperative to state that at 1000 ppm of securinine, there was no germination for all *Colletotrichum* spp tested (*C. Musae* and *C. gloeosporioides*). *P. amarus* has been considered as a type of *P. niruri* in the 1990s, this assertion was made during a major reorganization of the *Phyllanthus* genus (Taylor, 2003), and therefore the presence of these alkaloids could be responsible for the antifungal activity on the fungus.

2.5.3 *Zingiber officinale* Roscoe

Ginger (*Z. officinale*, family; Zingiberaceae), is a well-known spice used in the daily diet in many Asian countries and, is generally considered a safe herbal medicine (Surh et al., 1998; Weidner and Sigwart, 2000; Hasan et al., 2012a). Known as Halia in Malaysia, it is a rhizomatous plant grown throughout South-eastern Asia, China and in parts of Japan, Austria, Latin America, Jamaica and Africa. It was known in Germany and France in the ninth century and in England in the 10th century for its medicinal properties (Denim, 2010; Sasidharan and Menon, 2010). Ginger has been widely used in Chinese, Ayurvedic and TibbUnani herbal medicines all over the world, since antiquity, for a wide array of unrelated ailments that include arthritis, rheumatism, sprains, muscular aches, pains, sore throats, cramps, constipation, indigestion, vomiting, hypertension, dementia, fever, infectious diseases and helminthiasis (Ali et al., 2008). Ali et al. (2008), in a review, summarize its pharmacological actions due to the presence of bioactive compounds as antioxidant, anti-cancer, immuno-modulatory, anti-tumorigenic, anti-inflammatory, anti-apoptotic, anti-hyperglycaemic, anti-lipidemic and anti-emetic. It is considered a safe herbal medicine and commonly used as a spice (Afzal et al., 2001). A patented ginger extract EV.EXT 33, when administered by oral gavage groups of 22 pregnant female rats, showed no signs of teratogenic and toxic effects (Weidner and Sigwart, 2000; 2001). Worldwide it also used as a flavouring agent in food, beverage, and confectionery industries who use it for making marmalade, pickles, chutney, ginger beer, ginger wine, liquors, and other bakery products (Wang et al., 2011). In South India, ginger is used in the production of a candy called Injimurappa meaning ginger candy in Tamil (Sebiomo et al., 2011).

Like turmeric and dukung anak, clinical trials have shown antibacterial and antifungal effect of ginger on human pathogens such as *Bacillus subtilis* (Ehrenberg) Cohn), *Proteus vulgaris*

Hauser, *Escherichia coli* (Migula) Castellani and Chalmers, *Pseudomonas aeruginosa* (Schröter) Migula, *Staphylococcus aureus* Rosenbach and *Listeria monocytogenes* (E. Murray) Pirie (Abd-Alrahman et al., 2013), while on phytopathogens, its efficacy against some economic plant pathogens such as *S. sclerotiorum*, *R. solani*, *C. musae*, *F. oxysporum*, *A. radicina*, *F. solani*, *M. phaseolina*, *P. destructiva*, *S. rolsii* and *C. gloeosporioides* has been reported (Dissanayake and Jayasinghe, 2013; Ademe et al., 2014; Al-Askar et al., 2014; Dissanayake, 2014; Ojaghian et al., 2014). At 25%, the methanolic aromatic ginger extract showed a fungistatic inhibition of 91% for *F. oxysporum* while at minimal inhibitory dilution (MID) of 3.13% (v/v), it resulted in 69.00% inhibition of the same fungus (Dissanayake, 2014) after. Other studies showed that, at volatile phase, ethyl acetate and ethanol extracts of ginger meaningfully reduced myceliogenic germination of *Sclerotinia sclerotiorum* sclerotia at contact phase at 10 g/L after incubation at 22°C for a period of 6 days (Ojaghian et al., 2014). Aqueous ginger extracts at 70% resulted in about 40.87% inhibition in mycelial growth of *C. gloeosporioides* after 5 days of incubation at room temperature (Mukherjee et al., 2011). Other studies suggest that at high concentrations of 25% (250 mg/ml), methanolic ginger extracts significantly controlled *F. oxysporum* with 75% inhibition in mycelial growth (Dissanayake et al., 2014). Additionally, 5% (5 mg/ml) to 20% (20 mg/ml) methanolic extracts of ginger resulted in 42% to 75% inhibition in mycelia growth against *F. oxysporum*, 10%-41% against *R. solani*, 34%-61% against *C. musae* (Dissanayake and Jayasinghe, 2013).

Sesquiterpene hydrocarbons, diarylheptanoids and gingerol-related compounds (methyl gingerols, 1-Dehydro-gingerdiones) are major constituents of ginger (Charles et al., 2000; Afzal et al., 2001; Jolad et al., 2005; Joal et al., 2004; Jiang et al., 2005; Jiang et al., 2007; Tao et al., 2009). The antifungal activity of ginger extract may be attributed to the presence of gingerol

and shogaol as active ingredients within methanolic extracts of ginger (Singh et al., 2008b; Hasan et al., 2012b). Gingerol-related compounds can be further classified into five major groups as follows: (1) gingerols, which are homologues of 1-(3-methoxy-4-hydroxyphenyl)-3-keto-5-hydroxyhexane and include the sub-group methyl gingerols (Jiang et al., 2005); (2) shogaols, which are dehydration products of the gingerols; (3) paradols, β -ketone hydroxyl deoxygenation products of gingerols; (4) gingerdiones, which are β -ketone hydroxyl dehydration products of gingerols and include the sub-group 1-dehydro gingerdiones; and (5) gingerdiols, which are ketone-reduction products of gingerols (Charles et al., 2000; Afzal et al., 2001; Jolad et al., 2005; Joal et al., 2004; Jiang et al., 2005). Each ginger group homologues contains an unbranched alkyl chain of varying length and range in mass between 300 and 500 Da. For example, gingerols homologues include 4-, 6-, 8-, 10- and 12- gingerol, and shogaol homologues include 4-, 6-, 8-, 10-, 12- shogaol. Ali et al. (2008), reported that the major components that have been widely implicated in pharmacological and biological activities of ginger extracts are volatile oils, gingerols, and shogaols. These bioactive compounds vary depending on the place of origin and whether the rhizomes are fresh or dry. Over 50 components of volatile oil which form 1% to 3% of ginger extracts have been characterised and these are mainly monoterpenoids [*b*-phellandrene, (+)-camphene, cineole, geraniol, curcumene, citral, terpineol, borneol] and sesquiterpenoids [*a*-zingiberene (30–70%), *b*-sesquiphellandrene (15–20%), *b*-bisabolene (10–15%), (E-E)-*a*-farnesene, arcurcumene, zingiberol] (Ali et al., 2008). The Pungency of fresh ginger is due primarily to gingerols, which are a homologous series of phenols as mentioned earlier (Ali et al., 2008) and the most abundant are 6-gingerols (Afzal et al., 2001). When dried, the pungency of ginger mainly results from shogaols (6-shogaols) (Aktan et al., 2006).

For complete solubility of gingerols in water, research has shown that a co-solvent also known as entrainer is required, and the main co-solvent may be methanol or ethanol. The solubility and bio-availability of these bioactive compounds are achieved at the maximum when ginger extracts are dissolved in hot water between 50⁰C to 100⁰C followed by addition of co-solvent in a recommended and proportional mole fraction of the co-solvent (Jaapar et al., 2014). The authors reported that when the concentration (mole fraction of ethanol) of co-solvent increases, the solubility of ginger bioactive compounds increase, and whilst the temperature increases, the concentration (mole fraction of ethanol) of co-solvent decreases. By adding a small amount of co-solvent, we can enhance their solubility (Abaroudi et al., 2002) by increasing and expanding the range of solvent polarity and solvent power (Peña et al., 2006). Ethanol or methanol is the most efficient solvent for extraction of ginger bioactive compounds since it extracts both the polar and non-polar compounds. Apart from that, the use of these alcohols is also economical feasible and relatively safe in food processing (Hu et al., 2011). No study on the postharvest control of anthracnose in dragon fruits caused by *C. gloeosporioides* has been investigated using ginger extracts.

2.6 Mechanism and mode of natural products against pathogens

Plants produce a diverse array of metabolites, many of which have antimicrobial activities against some pathogenic micro-organisms. Several antimicrobial modes of actions of phytochemicals have been suggested by different researchers. This includes inhibition of microbial growth, inducing cellular membrane perturbations, interference with certain microbial processes, modulation of signal transduction or gene expression pathways (Omojate et al., 2014). Antimicrobial activity of ethanolic extracts of medicinal plant in Kenya against 1EC-6 cells and probable mode of actions (to evaluate interleukin 7 cytokines) of these plant crude

extracts against cells at 0.78-100 mg/ml was investigated. The report shows that these plants had significant bacterial and fungicidal activity against the cells and the mode of actions was due to the cytotoxicity, gene silencing of IL 7 mRNA and immunopotentiality (2 times IL mRNA expression) (Mwitari et al., 2013). Khan and Nasreen (2010), also reported that methanolic plant crude extracts of some selected plants in India exhibited significant antifungal activity against some phytopathogens and the mode of actions was due to degradation of the fungal cell wall and lack of catalase production. Catalase is an essential enzyme in fungi for conversion of hydrogen peroxide (H_2O_2) to oxygen and water. The lack of catalase production by the fungus results in the accumulation of H_2O_2 leading to toxicity to the fungal cell. Gupta and Bilgrami (1970) reported that decoction of bark from *Acacia arabica* and *Casuarina equisetid folia* inhibits the production and activity of cellulolytic enzymes in *Pythium aphanidermatum*. Another mode of action of these natural products or plant extracts on phytopathogenic fungi using scanning and transmission electron microscopy (SEM/TEM) shows swelling (rough and fluffy surface), depression and distortion of spore development (by SEM). Similarly, TEM depicts the mechanisms of action of these natural products as the disintegration of cytoplasm, breaking down fungal cell walls, cellular distortion and intense vacuolization (Bautista-Baños et al., 2012; Aala et al., 2013). Antifungal effect of Dioscin (steroidal saponin) isolated from the dark of wild yam resulted in a significant effect against *Candida albican* cells. The mode of action of dioscin disrupted the plasma membrane of the fungal cells after invasion into the fungal membrane and this was evident by the morphological changes and leakage of ions (Rhodamine leakage of the giant unilamellar vesicles) from the fungal cell wall (Cho et al., 2012). As a mechanism to control anthracnose in dragon fruit plants which may also serve as an inoculum for the *C. gloeosporioides*, application of submicron

chitosan dispersions enhanced the production of plant-defence related enzymes such as polyphenol oxidase (PPO), peroxidase (PO), phenylalanine ammonia-lyase (PAL) which delayed the symptoms of the disease (Zahid et al., 2015).

2.7 Loop-mediated isothermal amplification

Plant pathogens need to be quickly and accurately detected in the field or pack houses to ensure that unsafe and infected food is not shipped to consumers (Niessen, 2015). Loop-mediated isothermal amplification (LAMP) is an easy and reliable method that can be used on-site to detect these pathogens (Tomlinson et al., 2012). In recent times, nucleic acid amplification, an important tool has paved the way for many molecular biologists in application-oriented fields such as clinical medicine for diagnosis and monitoring of infectious diseases, genetic diagnosis, genetic traits and in plant pathology to identify infectious plant diseases (Nagamine et al., 2002; Njiru et al., 2008; Bekele et al., 2011; Tomlinson et al., 2012). Among the established nucleic acid amplification tools used are polymerase chain reaction (PCR), nucleic acid sequence-based amplification, self-sustained sequence replication, strand displacement amplification and Loop-mediated isothermal amplification (LAMP) (Saiki et al., 1985; Saiki et al., 1988; Guatelli et al., 1990; Compton, 1991; Walker et al., 1992a; Walker et al., 1992b; Notomi et al., 2000; Nagamine et al., 2001). Whereas PCR is the most extensively used technique, promotes the next round of DNA synthesis by heat denaturation of double-stranded DNA products, other signal amplification methods such as branched DNA (bDNA) amplification, Invader, and rolling circle amplification (RCA) are known target amplification methods (Erice et al., 2000; Lizardi et al., 1998; Lyamichev et al., 1999). However, LAMP provides a pronounced advantage over all these amplification methods due to its ability to directly amplify specific sequences of DNA under isothermal conditions (Notomi et al., 2000)

and most importantly does not require denaturation of DNA template (Nagamine et al., 2001). The LAMP reaction requires a DNA polymerase with strand displacement activity and a set of four specifically designed primers, termed inner and outer primers to improve specificity.

2.7.1 Steps of LAMP technique

Nagamine et al. (2002) describe the steps of the LAMP technique. As the first step, a stem-loop DNA structure in which the sequences of both DNA ends derived from the inner primer is constructed as starting material. Subsequently, one inner primer hybridises to the loop on the product in the LAMP cycle and initiates strand displacement DNA synthesis, yielding the original loop and a new stem-loop DNA with a stem twice as long. Stem-loop DNAs with several inverted repeats of the target DNA and cauliflower-like structures with multiple loops, amplifying the amount to 10^9 copies of the target are the final products. This brings the whole reaction of the original LAMP to less than an hour. To make the time shorter for rapid detection and genomic diagnosis, the LAMP reaction needs to be accelerated by the addition of two loop primers (Loop F and Loop B), bringing the whole primer set for LAMP to six (Nagamine et al., 2002). These loop primers hybridize to the stem-loops except for the loops that had been hybridised by the inner primers. The mechanism and expected reaction steps are illustrated (Nagamine et al., 2002). The authors concluded that the addition of additional loop primers makes the LAMP technique very efficient for rapid detection and diagnosis of genetic point-of-care testing.

2.7.2 Importance of LAMP technique

Despite the polymerase chain reaction (PCR) being the main method for DNA amplification *in vitro*, this method has been shown to have features that make it not ideal for use in the field (Niessen et al., 2015). LAMP is a simple, cost-effective and rapid method for specific DNA-based detection (Tao and Cai, 2013). It is cheaper to run than PCR and requires a smaller machine than PCR; hence the equipment would be more efficient for field use (Parida et al., 2008; Zhang et al., 2013). It also has an advantage over other amplification methods due to its ability to directly amplify specific sequences of DNA under isothermal conditions and optimized for rapidness and sensitivity (Notomi et al., 2000). Most importantly it does not require denaturation of DNA template (Nagamine et al., 2001). Additionally, reverse transcription LAMP (RT-LAMP) allows rapid detection of amplification products using lateral flow devices containing antibodies specific to the incorporated labels, avoiding the need for fluorescence detection or gel electrophoresis (Tomlinson et al., 2012). The use of loop primers during a LAMP reaction means the whole process of amplification and detection of the products can take roughly 30 minutes whereas PCR takes a long time and this may be the time that is not available on the site as a decision may be needed quickly (Ushikubo, 2004). LAMP has been used by plant pathologist as a potential application tool for diagnosis of pathogenic fungi and surveillance of plant diseases without requiring sophisticated equipment (Nagamine et al., 2002; Njiru et al., 2008; Bekele et al., 2011; Tomlinson et al., 2012). Despite several studies suggest the use of LAMP technique for clinical diagnosis of diseases as well as in plant pathology on field crops, no study of LAMP technique as a diagnostic tool for detection of postharvest anthracnose in dragon fruits has been investigated.

CHAPTER 3

3 GENERAL MATERIALS AND METHODS

In the first part of the experiment, only crude extracts of ginger, turmeric and “dukung anak” were used as treatment *in vitro* and *in vivo*. Whiles in the second part of the experiment a formulation of each plant extract at different concentration incorporated with gum arabic served as treatment.

3.1 Materials

3.1.1 Dragon fruit

Disease free, medium and uniform red-fleshed dragon fruit (*Hylocereus costaricensis*) (

Figure 3-1) at matured stage (purplish pink colour) of weight 420-480g were purchased from a commercial farm called Golden River farm located at Sg. Sompo in Lenggeng, Malaysia. The fruits were washed with clean water to get rid of debris and soil and then disinfected with 1% sodium hypochlorite. Fruits were washed with distilled water after disinfection and air-dried at room temperature.



Figure 3-1 Fresh-cut and whole piece of red-fleshed dragon fruits. Picture dimension is 1.5cm.

3.1.2 Treatment materials

Plant crude extracts turmeric (*Curcuma longa* Linn.), ginger (*Zingiber officinale* Roscoe cv. “Bentong”) and Dukung Anak” powder (*Phyllanthus niruri*) at different concentrations incorporated separately with gum arabic (GA) served as treatments. GA powder (500g) CP grade was purchased from Sigma-Aldrich. Disease-free turmeric rhizomes (*Curcuma longa* Linn.) (Figure 3-2) and ginger (*Zingiber officinale* Roscoe cv. “Bentong”) (Figure 3-3) were purchased from commercial farms. Ginger was purchased from a commercial farm in Bentong along the Titiwangsa mountain. Turmeric was bought from a commercial supplier at Farm Price SDN.BHD in Johor, Malaysia where most turmeric is grown. “Dukung Anak” powder (*Phyllanthus niruri*) (Figure 3-4) was purchased from Ethnoherb resources, Malaysia. All treatment was disinfected with 1% sodium hypochlorite, followed by washing with distilled water before oven-drying or drying at room temperature and crude extraction.



Figure 3-2 Whole piece, cross-sectional view and powder of turmeric rhizome



Figure 3-3 Whole piece, cross-sectional view and powder of ginger



Figure 3-4 Whole plant and powdered form of “dukung anak”

3.1.3 Crude extraction of plants

Turmeric rhizome, ginger and “Dukung anak” were extracted by maceration using 95% methanol according to Ademe et al. (2014) with slight modification. Before extraction, ginger, and turmeric rhizome were prepared into a powdered form according to Kulkarni et al. (2012). Fresh rhizomes of turmeric and ginger were disinfected with 1% sodium hypochlorite followed by washing with distilled water. The washed tissues were sliced into pieces, sun-dried for one week followed by oven-drying at 50°C in a hot air oven for 6 h to achieve a moisture content of 11.54±12%. Dried rhizomes (turmeric and ginger) were then pulverised separately to a coarse powder, approximately 0.5 mm diameter using ultracentrifugal mill ZM 200 prior to extraction (Puenghian and Sirichote, 2007). Powdered samples of 200-250g depending on the plant were soaked in a 1L conical flask containing 800 ml of methanol for 24 h. Extractions (5-6x) were done and the total extract solution was pooled and stored at room temperature in a 1L conical flask. Extract solutions obtained were filtered through a muslin cloth to remove impurities before rotatory evaporation. The collected solution was concentrated and dried under reduced pressure on a rotary evaporator at 40°C water bath to obtain the crude extracts. Each crude extract yield was calculated and the extract was tightly covered and stored at 4°C until further use. Turmeric rhizome crude extracts were wrapped in aluminum foil to avoid exposure to light.

3.2 Preparation of crude extract stock solution for antifungal studies

Preparation of extract solutions was carried out according to Jaapar et al. (2014) with slight modification. To improve the solubility and bioactivity of each plant crude extract, each methanolic crude extract obtained in section 3.1.4 was first re-constituted with 0.03-0.05 mole fraction of methanol as the co-solvent followed by addition of purified distilled hot water (60°C) to make a final stock solution. The final solution was stirred (4 rpm) continuously on a hot plate

magnetic stirrer (model CB162; Stuart, Biocote, UK) at 20°C for 30 min (Yang et al., 2014) and filtered with two layers of cheesecloth to remove any undissolved impurities. The final solution was left at room temperature. At this point, the entire methanol was evaporated leaving a final aqueous crude extract solution.

CHAPTER 4

4 EFFICACY OF PLANT EXTRACTS TO CONTROL DRAGON FRUIT ANTHRACNOSE

4.1 Introduction

C. gloeosporioides contributes significantly to preharvest and postharvest losses in fruits. Crop losses caused by *C. gloeosporioides* generally occur as a direct reduction in quantity or quality of the harvested produce (Lakshmi et al., 2011) and most fruits suffers mostly due to latent infection of the pathogen (Freeman and Shabi, 1996). Ginger, turmeric and “dukung anak” possess strong antimicrobial activity against vast economic phytopathogens amongst them is *C. gloeosporioides*. These plants are generally regarded as safe (GRAS), non-toxic to humans, biodegradable, cheap and leaves no residual effect on fruits making them a good candidate as a postharvest agent to control anthracnose on dragon fruits. Studies have shown that methanolic ginger extracts significantly controlled *F. oxysporum*, *R. solani*, and *C. musae* (Dissanayake and Jayasinghe, 2013; Dissanayake et al., 2014). The efficacy of methanolic “dukung anak” has also been reported to significantly inhibited the growth of several clinical pathogens (Mathur et al., 2012; Gawai et al., 2013), whiles aqueous turmeric extracts inhibited mycelial growth of *C. gloeosporioides* (Tasiwal et al., 2009). Despite studies shows their efficacy *in vitro* on some clinical and phytopathogens, studies on their ability to control postharvest anthracnose in dragon fruit caused by *C. gloeosporioides* is still lacking. The significance of this study was to investigate the *in vitro* and *in vivo* antifungal activity of ginger, turmeric and “dukung anak” crude extracts against *C. gloeosporioides* that causes anthracnose in dragon fruits. Concentrations range between 2.5 mg/ml to 10 mg/ml and 5 mg/ml to 20 mg/ml were selected for *in vitro* and *in vivo* antifungal studies respectively. The lowest bio

extracts concentration with highest inhibition percentage was taken as minimum inhibition concentration (MIC) in both *in vitro* and *in vivo* studies.

The objective of this study is

- To screen for the best concentrations of each extract and investigate the antifungal effect of each extract against *C. gloeosporioides* that causes anthracnose in dragon fruits.
- To evaluate the efficacy of extracts on disease incidence and disease severity on dragon fruit.
- To investigate the limitations associated with the use of plant extract on fruits external qualities (appearance and smell) based on consumer preference.

4.2 Materials and methods

4.2.1 Materials

Aqueous crude extracts of ginger, turmeric and “dukung anak” obtained and prepared (section 3.2 and 3.3) were used as treatment. Red-fleshed dragon fruits purchased and prepared (section 3.1.1) was the specimen sample for this experiment. Concentrations at 2.5, 5, 7.5 and 10 mg/ml were selected for the *in vitro* studies and 5,10 and 15 mg/ml for *in vivo* studies. These concentrations were selected based on preliminary trials which shown that concentrations between 7.5 and 10 mg/ml of some plant extracts significantly controlled the fungus *in vitro* by almost 80%. Even though above 15 mg/ml, the plant extracts was fungistatic against the fungus *in vitro*, it negatively resulted in phytotoxicity during the *in vivo* trials for all the plant crude extracts. The above reason informed the choice of selecting a MIC (2.5 and 5 mg/ml) in

addition to 7.5 and 10 mg/ml for the *in vitro*, while 5 and 10 mg/ml were chosen as MIC for the *in vivo* in addition to high concentration of 15 mg/ml for the *in vivo* studies.

4.2.2 *In vitro* antifungal assay of extracts against *C. gloeosporioides*

4.2.2.1 Isolation of fungus

C. gloeosporioides isolation and culture conditions were performed using an infested anthracnose peel tissue of dragon fruit and surface sterilized according to Maqbool et al. (2010a). Small sections of infected peel tissue were cut and surface sterilized using 1% (w/v) sodium hypochlorite for 5 min. After three washings in sterile purified water, the tissue pieces were placed on sterilized Petri plates (9 cm diameter) containing potato dextrose agar (PDA; Difco, Sparks, MD, USA) and incubated at room temperature (25°C). Once mycelial growths were observed, the colonies were subcultured on fresh PDA plates to obtain pure cultures.

4.2.2.2 Cultural and molecular identification of fungus

Pure-culture of fungus was identified based on cultural or morphological characteristics (Barnett and Hunter, 1972) as well as molecular identification using PCR assay (Cano et al., 2004).

DNA extraction; DNA of the pure fungal culture obtained in section 4.2.2.1 was extracted using Qiagen DNeasy Plant Mini Kit. Fungal identification was carried out by PCR followed by gel electrophoresis (Cano et al., 2004) before purified samples were sent for sequencing.

Primer design; Cgbt F3 (Forward; 5' AATATCATACTTGCGAGGAC-3') and Cgbt B3 (Reverse; 5'CTCCAAATCGACGAGGAC-3') with specificity to *C. gloeosporioides* beta-tubulin allele from genomic DNA were designed after amplification by PCR and comparing

with numerous *Colletotrichum* spp in the GenBank database according to Buhr and Dickman, (1994).

PCR protocol; PCR was performed with DNA extracted from the fungal culture using the two ribosomal primers above. In total 8 reaction mixes was made for fungal DNA samples. One reaction of the PCR comprised of 10 µl of mango mix (*Taq* polymerase, dNTPs, MgCl₂), 1 µl of each primer (cgbt-F3 and cgbt-B3) and 12 µl of water. A 29 µl aliquot of the reaction mix was then pipetted into each of the 8 tubes of a Gene[®] strip and 1 µl of fungal DNA template added to each tube. In total, 7 samples for fungal DNA were used for the PCR assay and the control tube consisted of only the reaction mix without fungal DNA. The PCR was performed in the S 1000 thermal cycler. The conditions were denaturation for 2 min at 95°C followed by a touchdown procedure consisting of 30 s at 94°C and then annealing for 1 min at a decreasing temperature of 53°C during the first seven cycles. The final step was an extension (elongation) at 72°C for 1 min followed by holding for up to 18 h at 40 C. A total of 36 cycles were performed. Next 5µl of the PCR product was electrophoresed on a 1% agarose gel in the presence of ethidium bromide and visualised under UV light. A 100-bp DNA ladder marker was used as the size standard. The products were purified using Qiagen QIA quick PCR purification Kit and then sent to Eurofilms for sequencing for the final results. Sequenced results were blasted using online standard Nucleotide Blast, and the obtained results compared to GenBank database.

4.2.2.3 Pathogenicity test

This was carried out by a wound/drop technique according to Stanghellini and Aragaki (1966) and Kanchana-udomkan et al. (2004). A circle of 3-4 cm diameter was drawn with a marker pen on the fruit surface previously surface sterilized by dipping in 1% sodium hypochlorite for 4 min and rinsing in sterile water. The circled area was wounded with a sharp sterile scalpel to a depth of 1 mm. One ml of inoculum (10^5 spore/ml) obtained from the 10-day old pure in section 5.2.1.3. was dropped onto the wounded tissues of each marked circle. Fruit (3) were then incubated at room temperature 25°C for 8 days in a plastic bag and examined daily for lesion development.

4.2.2.4 Preparation of crude extracts solution

A working concentration of each extracts at 2.5, 5, 7.5 and 10 mg/ml was prepared from stock solution of concentration (15 mg/ml) (w/v) (

Figure 4-1). The stock solution per plant crude extract was prepared according to section 3.2. The working concentration per plant crude extract was prepared based on the poison food technique by amending a freshly prepared potato dextrose agar (PDA) with stock solution from each plant crude extract in a ratio of (PDA: extract solution) as 3:7 (v/v) (Dhingra and Sinclair (1985). The amended mixture was vortexed for 30 s at minimal speed (4 rpm) using vortex mixer (model: SA8; Stuart, Biocote, UK) and then dispensed into a sterile 9 cm Petri dish and allowed to solidify.

2.5 mg/ml	• Ginger • Turmeric • Dukung anak
5 mg/ml	• Ginger • Turmeric • Dukung anak
7.5 mg/ml	• Ginger • Turmeric • Dukung anak
10 mg/ml	• Ginger • Turmeric • Dukung anak
Negative control	• Only PDA
Postive control	• Commercial fungicide (Mancozeb- 2 mg/ml)

Figure 4-1 Concentrations of plants extracts used *in vitro* study.

4.2.2.5 Inhibition of radial mycelial growth of *C. gloeosporioides*

Inhibition of fungal growth was performed based on percentage inhibition in radial mycelial growth of *C. gloeosporioides* on amended PDA with each extract at different concentrations using the poison food technique (as described in section 4.2.2.3) (Dhingra and Sinclair 1985). An agar disc (5 mm diameter) obtained from a 10-day old pure culture in section 4.2.2.1. was placed in the centre of a Petri plate containing amended PDA with extracts at different concentrations. Petri plate containing freshly prepared PDA alone and a commercial fungicide (Mancozeb- 2mg/ml) were used as negative and positive control respectively (

Figure 4-1). To check if methanol used for extract had any effect 0.05 mole fraction of methanol amended with PDA was tested. Preliminary results showed that the 0.05 mole fraction of methanol did not have any significant antifungal effect, all the methanol vaporised at room

temperature during the solidification of the mixture in a Petri plate. All Petri plates containing agar disc of fungus were incubated at 25°C and experiment ended when mycelial growth in the control reached the edge of the plate. The percentage inhibition in radial mycelial (MI) growth was calculated using the formula described by Al-Hetar et al. (2010). Ten replicates of each treatment and controls were arranged according to a completely randomized design (CRD).

$$MI (\%) = \frac{C_1 - C_2}{C_1}$$

Where C_1 = radial mycelial growth in control Petri dish, C_2 = radial mycelial growth in treated Petri plate.

4.2.2.6 Preparation of fungal spore suspension

Fungal spore suspension was carried out according to Zhang et al. (2013) with slight modification. Ten ml purified water was poured into a 10-day old culture of *C. gloeosporioides* obtained from section 4.2.2.1 and then surface scraped lightly with a bent glass rod. Fungal spore suspension was then filtered through a double layer of sterilized muslin cloth. Concentration of spore suspension was adjusted to (10^5 spores/ml) by the addition of distilled water using improved Neubauer haemocytometer (model: Neubauer, Germany).

4.2.2.7 Inhibition of conidial germination of *C. gloeosporioides*

The conidial germination inhibition test was carried out according to Al-Hetar et al. (2010) with slight modification using the cavity slide technique. An aliquot of 50 µl each extracts at concentrations (2.5 mg/ml, 5 mg/ml, 7.5 mg/ml and 10 mg/ml) was pipetted onto cavity slides. Twenty microliters (20 µl) of a freshly prepared conidial suspension of *C. gloeosporioides* (10^5 spores/ml) was pipetted and dispensed into each cavity slide containing extracts at different concentrations, covered with a glass slide and kept in a dark for 48 h in the dark at room

temperature ($25 \pm 2^{\circ}\text{C}$). Cavity slides containing only purified water served as control. Sodium azide (2.0%) was added at the end of the 48-h incubation to kill conidia from further germination and samples were observed under a light microscope (Leica DM1000, Leica microsystems) at 40x magnification. The number of germinated conidia was counted in 10 microscopic fields in 10 replicated dishes and presented as percent inhibition. A conidium was considered germinated if its germ tube was longer than the conidium itself. The percentage inhibition of germination was calculated by the method of Cronin et al. (1996). Images of inhibition in conidia germination in both control (only PDA) and treated (PDA amended with crude extract) Petri plates were taken after using the protocol according to Al-Hetar et al. (2010). To do this, a thin layer of the amended PDA with extracts and only PDA with each containing evenly spread 20 μl of conidia suspension (10^5 spores/ml) was placed on a glass slide. Images of conidia germination were viewed under a light microscope (Leica DM1000, Leica microsystem) fitted with a camera at 40x magnification.

4.2.2.8 Scanning Electron microscopy

Scanning electron microscope (SEM) was used to observe the changes in hyphal morphology of test pathogens according to Al-Hetar et al. (2010) and Maqbool et al., (2011) with slight modification. Samples of 1.5 mm^3 from control and treated Petri plates with each evenly distributed with 20 μl of *C. gloeosporioides* (10^5 spore/ml) after 48 h incubation in the dark were taken. The samples were then mounted onto aluminum stubs and viewed using a scanning electron microscope (FEI Quanta 400 FE-SEM) fitted with camera

4.2.3 *In vivo* antifungal assay of extracts to control anthracnose

In vivo antifungal effect of each crude extracts (obtained from section 3.1.3 and 3.1.4) on disease incidence was investigated in dragon fruits at 5,10 and 15 mg/ml and their ability to reduce the disease severity was performed at a weekly time point.

4.2.3.1 Preparation of plant extracts and fruit

A stock solution of 30 mg/ml for each plant crude extract obtained in section 3.2 was prepared. To improve the solubility and bioactivity of all crude extracts, a slight modification according to Jaapar et al., (2014) was used. The working concentrations used for DI and DS analysis were 5 mg/ml, 10 mg/ml and 15 mg/ml (

Figure 4-2). Tween 80 (0.1%) was added as an emulsifier to each working concentration per plant crude extract. The pH of each working solution was adjusted to 5.6 by adding 1 M NaOH using a digital pH meter (Model CyberScan pH 510; Eutech Instruments Pte.Ltd. Singapore). The new aqueous solution obtained was stirred continuously on a hot plate magnetic stirrer (model CB162; Stuart, Biocote, UK) at 30⁰C for 30 min, a slight modification from Maqbool et al. (2010a). The stirred aqueous solution was filtered through two layers of cheesecloth to remove any undissolved impurities.

Dragon fruits of uniform size, colour (purplish pink colour) and with no deformity were washed with sodium hypochlorite (1%), rinsed with purified water and air-dried at room temperature (25±2⁰C). Firstly, all fruits were inoculated by dipping for 2 min in a spore suspension of *C. gloeosporioides* (10⁵ spore/ml) with 0.1% (v / v) Tween 80 and air-dried completely at ambient (25±2⁰C). After inoculated fruits were air-dried, they were then dipped for 2 min in 5 mg/ml, 10 mg/ml and 15 mg/ml for each crude extract and left to dry again

completely at room temperature. Fruits dipped in spore suspension (10^5 spore/ml) with 0.1% (v / v) Tween 80 for 2 min served as control. All inoculated treated and untreated fruits (control) were allowed to dry completely followed by packaging in commercial packaging cartons and stored at $11\pm 2^\circ\text{C}$, 80% RH for 28 days (Ali et al. (2010). For disease incidence and severity, a total of 300 fruits were used. For each plant extract, a total of 90 fruits were used (30 fruits per treatment). Thus 3 replicates and each replicate contain 10 fruits (Figure 4-3).

5 mg/ml	• Ginger • Turmeric • Dukung anak
10 mg/ml	• Ginger • Turmeric • Dukung anak
15 mg/ml	• Ginger • Turmeric • Dukung anak
Control	• Inoculated fruits (10^5 spore/ml+0.1% Tween 80)

Figure 4-2 Treatments used to test efficacy of extracts to control anthracnose

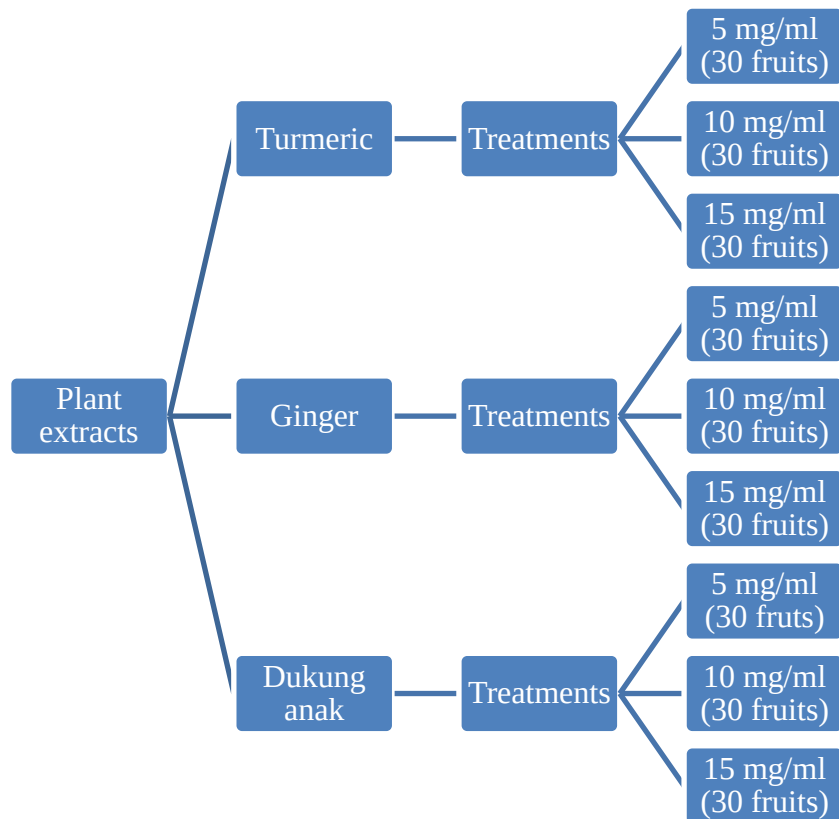


Figure 4-3 Total number of fruits used per plant crude extracts for disease incidence and severity analysis

4.2.3.2 Disease incidence and disease severity

The effect of crude extracts on disease incidence and disease severity was evaluated weekly for 28 days during cold storage. Disease incidence data are presented as the percentage of fruit showing anthracnose out of the total number of fruit in each treatment, while disease severity was scored following the scale (1 = 0% of fruit surface rotten; 2 = 1–25%; 3 = 26–50%; 4 = 51–75% and 5 = 76–100% rotten) (Sivakumar et al., 2002).

4.2.3.3 Limitations of plant extracts on external fruit appearance

To further understand consumer preference at the marketing level on fruit external quality (appearance and smell), an observational study was conducted to ascertain which fruit had the best consumer appeal after fruits were treated with each plant extract. This was conducted through observation by 20 participants. The parameters observers/participants were asked to look for are smell and colour changes (appearance) that affected or compromised the original colour of dragon fruit.

4.3 Statistical analysis

Efficacy of extracts against fungus (inhibition of mycelial growth and conidia germination) and their (extracts) ability to control anthracnose (disease incidence and disease severity) on dragon fruits was conducted using completely randomised design (CRD) in triplicates. Analysis of data was performed by two-way analysis of variance (ANOVA) using computer software GenStat version 18th Edition (VSNI product). Means were separated using Fisher unprotected test at ($P < 0.05$). Disease incidence data were transformed (arcsine of the square root of the proportion of affected fruit) before analysis. Experiments were repeated twice and data was pooled.

4.4 Results

4.4.1 Effect of extracts against *C. gloeosporioides*

4.4.1.1 Isolation and identification of *C. gloeosporioides*

The fungi isolated from infected dragon fruits, cultured on PDA medium at room temperature for 12 days were identified firstly based on their cultural and morphological characteristics from previous studies (Masyahit et al., 2009; Gautam, 2014). The presence of whitish-orange, woolly or cottony colonies coupled with acervuli having spore cells bearing a capsule shape clearly suggest that the fungus as *Colletotrichum* spp. (Figure 4-4 a and b). Its morphology was the same as that described by Masyahit et al. (2009) and Gautam (2014).

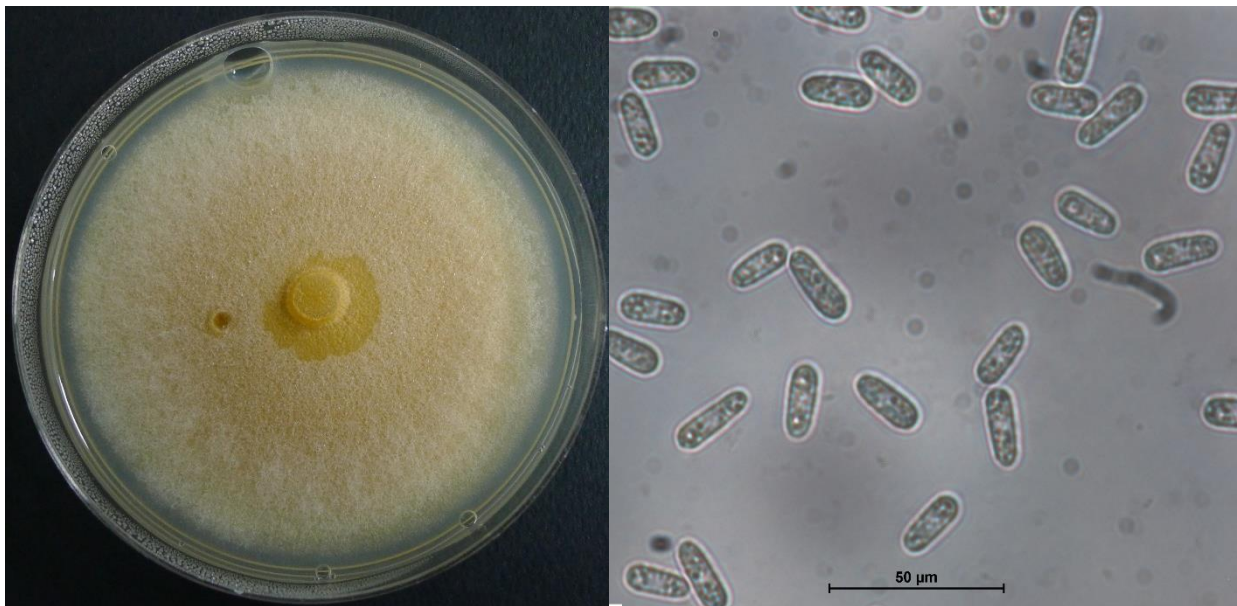


Figure 4-4. a) Pure culture of *C. gloeosporioides* and (b) conidia isolated from dragon fruit

To further clear the doubt regarding the authenticity of the fungus, PCR was performed using genomic DNA of the pure culture. The amplification using primers Cgbt F3 and B3 was positive confirming the presence of *C. gloeosporioides* and species-specificity of primers. The

DNA fragments generated approximately 280 bp during gel electrophoresis (Figure 4-5) with accession number KU743283.1 from the GenBank database after purified samples were sent for sequencing. Primer sets were specific and targeted the Beta-encoding gene [beta-tubulin (*TUB2*)] which is highly conserved in *Colletotrichum* spp in GenBank (Buhr and Dickman, 1994; Akgul and Awan, 2016). Beta-tubulin gene is a housekeeping gene that is expressed differentially in conidia, conidiating mycelia, and vegetative mycelia such as hyphae, especially hyphal tips (Buhr and Dickman, 1994). Thus, this gene is required for or immediately after spore germination (Buhr and Dickman, 1994). Both the cultural, morphological characteristics as well as molecular identification, confirmed the fungus as *C. gloeosporioides* as the cause anthracnose in dragon fruits and the most economically important fungus causing latent infections in dragon fruits during postharvest storage.

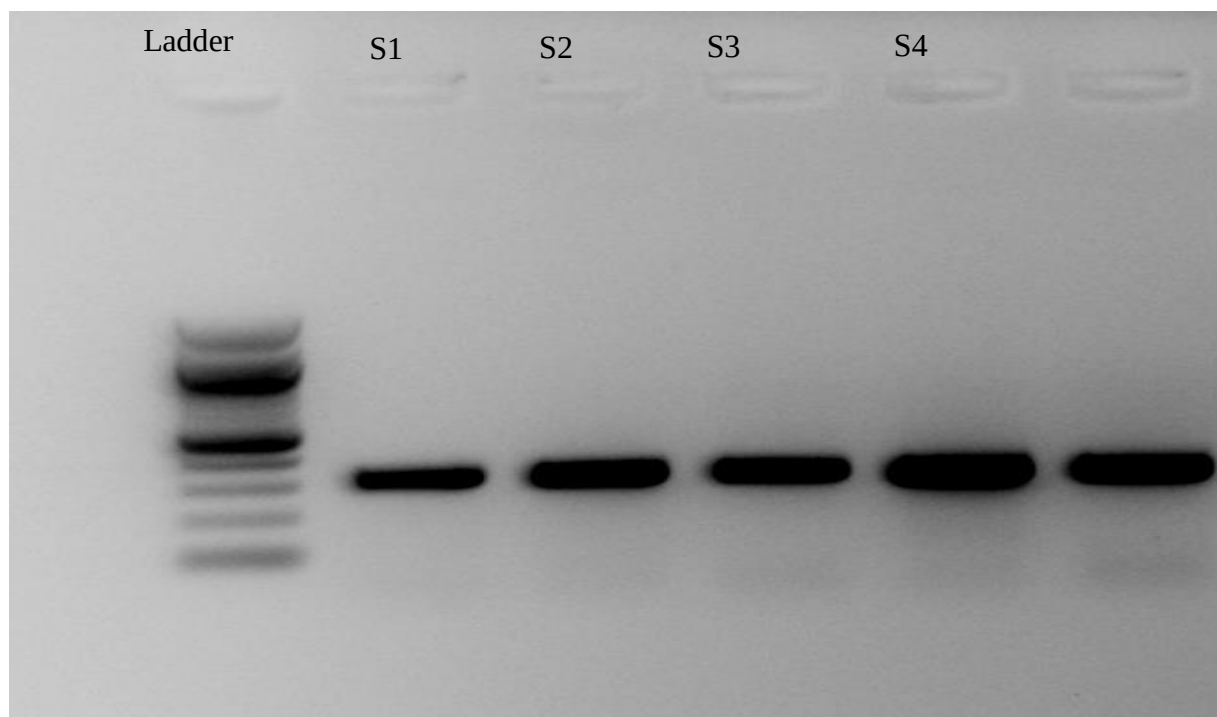


Figure 4-5. The amplification products from fungal DNA of pure *Colletotrichum gloeosporioides*. Gel electrophoresis on 1% agarose gel was performed on the PCR products. S1, S2, S3, S4, and S5 are products amplified with primer Cglt F3 and B3.

4.4.1.2 Pathogenicity test

Dragon fruits developed sunken, water-soaked lesions which were round to oval with brownish red to black spots as described by Guatam, (2014). These lesions, had coalesced to rot after 3 days of inoculations and increased in concentric circular formation with whitish mycelial growth by day 7 (Figure 4-6a, b).

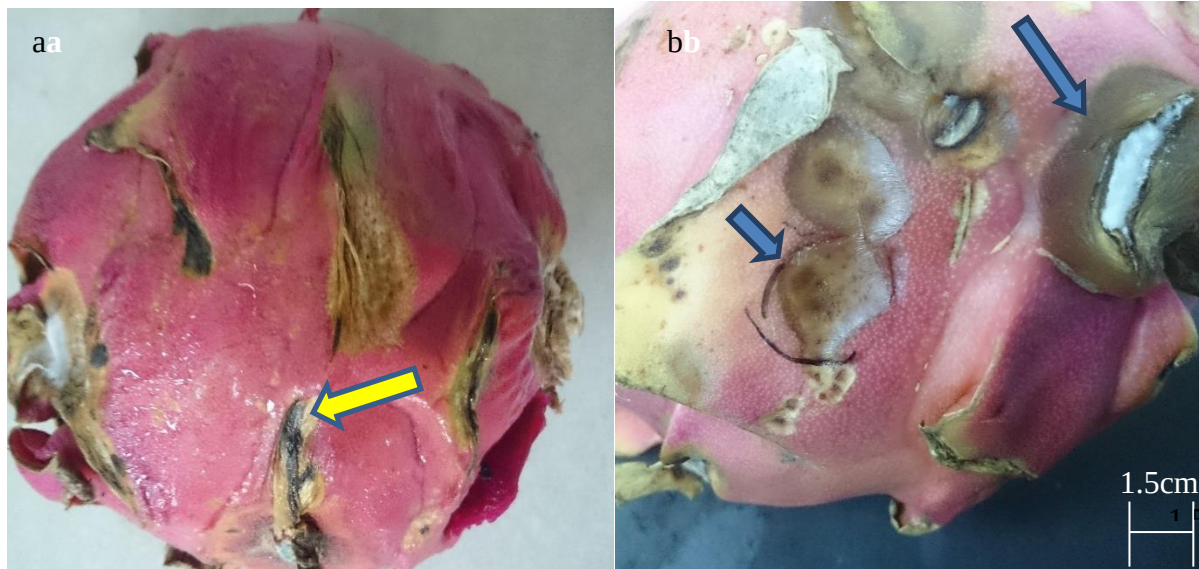


Figure 4-6. Pathogenicity test showing lesions of fruits with anthracnose a) 3 days (yellow arrow) and b) 7 days (blue arrow) after inoculation and storage at room temperature. The scale for each picture is 1.5 cm

Anthrachnose symptoms occurred as brown to black sunken spots with white masses of mycelial growth (Masyahit et al., 2009). Our test confirms that *C. gloeosporioides* is the causal organism of anthracnose in dragon fruit based on previous reports (Awang et al., 2010; Ghani et al., 2011; Zahid et al., 2014). Reports show that latent infections occur in the field on immature fruit but that the pathogen remains quiescent until the postharvest stage (Zahid et al., 2014).

4.4.1.3 Efficacy of plant extracts on mycelial growth and conidia germination of *C. gloeosporioides*

There was a significant difference ($P < 0.05$) between plant extracts at different concentrations (treatment) on percentage inhibition of fungal mycelial growth (Appendix 0-1). The highest inhibition ($P < 0.05$) in radial mycelial growth was observed in Petri plates amended with 10 mg/ml of ginger extracts (88.46%) after 12 days of incubation at room temperature (Figure 4-7; Table 4-1). Inhibition of radial mycelial growth (%) was dose-dependent and was significantly highest ($P < 0.05$) at 10 mg/ml irrespective of the plant extracts. Ginger extracts at 7.5 mg/ml (78.59%) was somewhat comparable to commercial fungicide Mancozeb at 2 mg/ml (80.45%) Figure 4-8. Additionally, the results also showed that methanol used in the extraction had evaporated during treatment preparation as our data shows no percentage inhibition (Figure 4-7). This suggest that its use for the extraction was not responsible for inhibiting mycelial growth. overall, ginger extracts significantly ($P < 0.05$) recorded the highest inhibition in mycelial inhibition followed closely by turmeric after 12 days of incubation (Table 4-2). Despite, all plant extracts showed a similar dose-dependent effect on mycelial growth, Pictorial image of the efficacy of ginger crude extract was shown because it gave the highest inhibition (Figure 4-9).

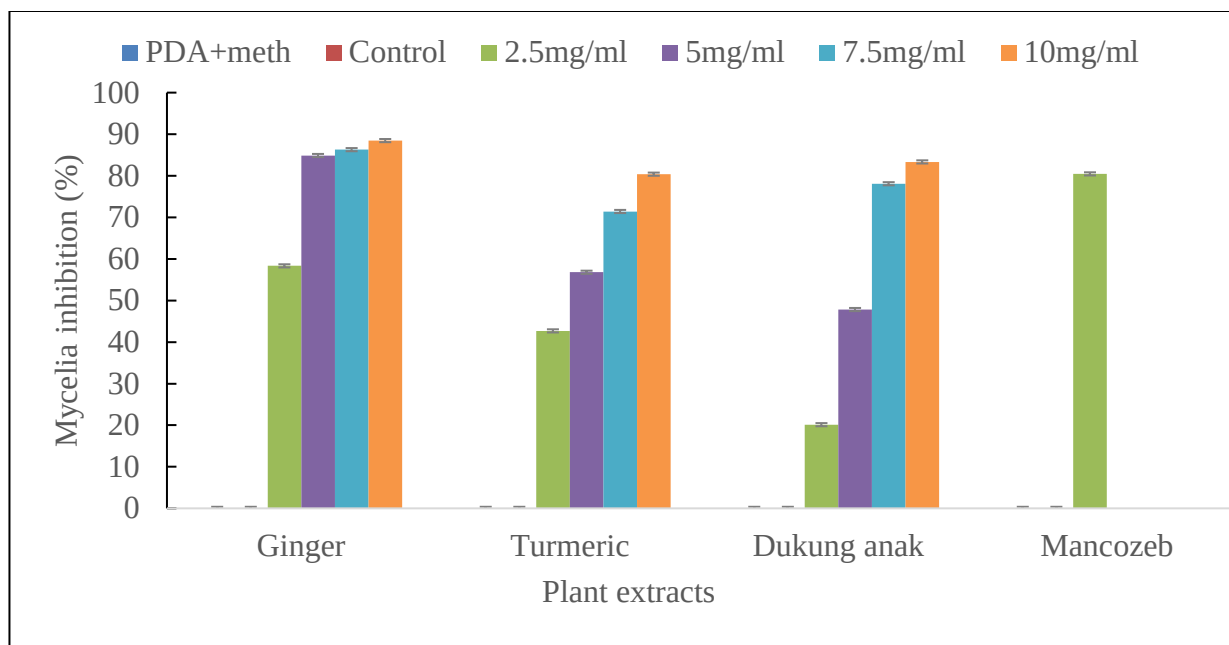


Figure 4-7 Effect of plant extracts at different concentration on radial mycelial growth of *C. gloeosporioides* after 12 days of incubation at room temperature. Control (only PDA), Mancozeb = 2 mg/ml.

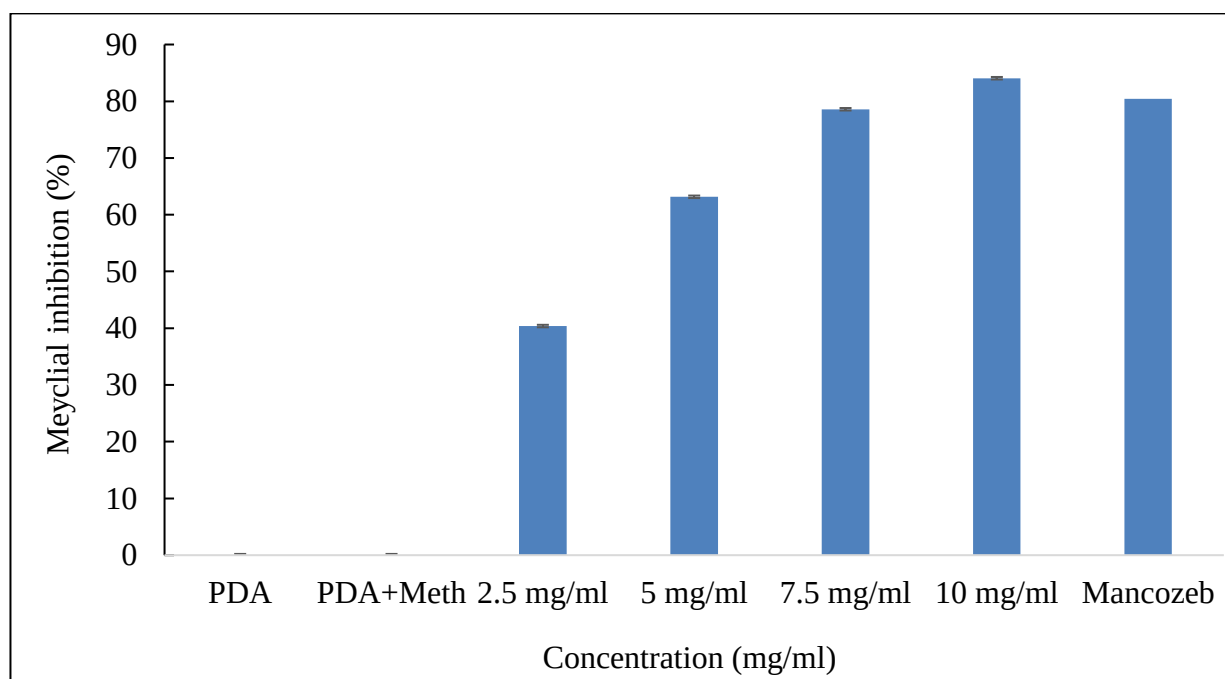


Figure 4-8. Effect of concentration of all extracts on inhibition of mycelial growth of *C. gloeosporioides* after 12 days of incubation at room temperature. Meth = Methanol, PDA = Potato dextrose agar (PDA).

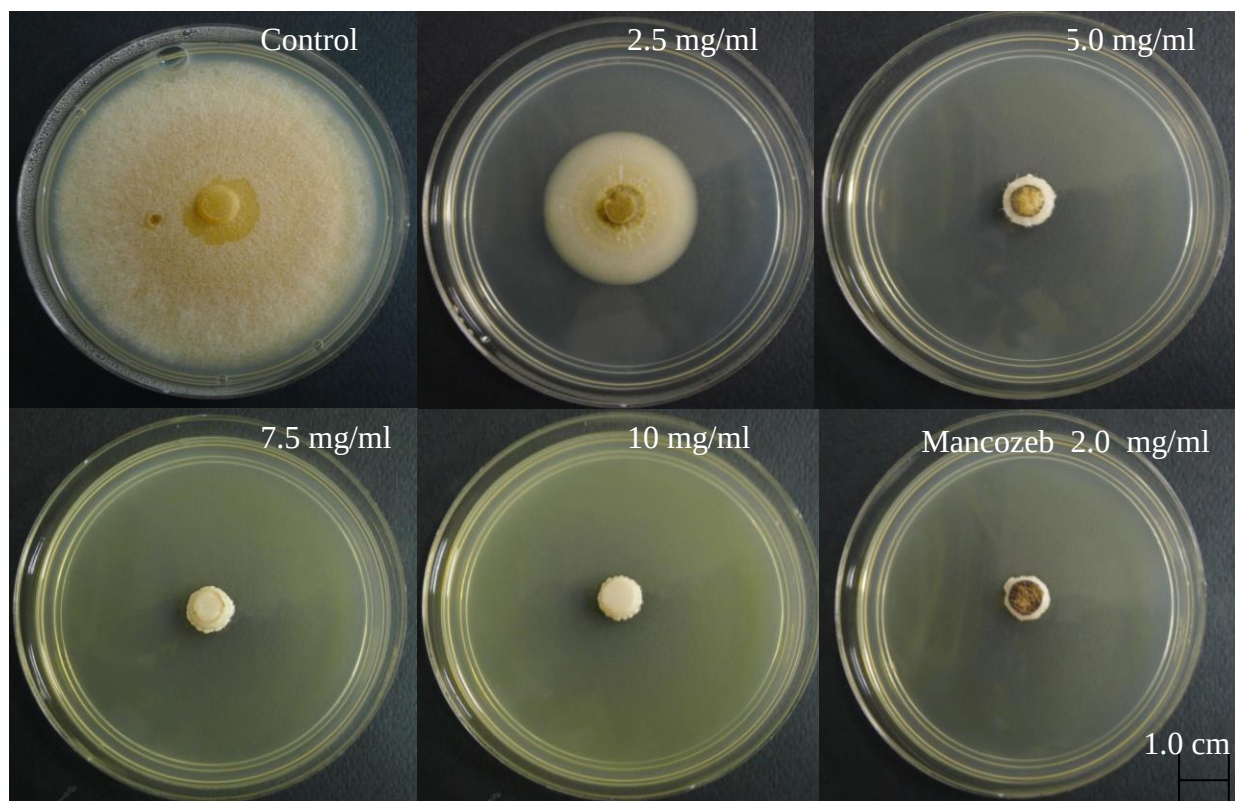


Figure 4-9 Effect of ginger extract at different concentration on inhibition of radial mycelial growth after 12 days of incubation at room temperature. Numbers on each Petri plate are the concentrations (mg/ml). The scale for each picture per Petri plate is 1.0 cm.

A significant effect ($P < 0.05$) of treatments on inhibition of conidial germination (%) was observed after 48 h incubation at room temperature (Appendix 0-2). The highest inhibition of conidial germination was observed in Petri plates amended with 10 mg/ml of ginger extracts (87.5 %) (Figure 4-10; Table 4-1). Like inhibition of mycelial growth, conidial inhibition (%) was dose-dependent and this was significantly highest ($P < 0.05$) at 10 mg/ml irrespective of the plant extracts, and lowest at 2.5 mg/ml (Figure 4-11). Light microscope images showed germination of conidia into germ tube in control, whilst germination was significantly inhibited or reduced at 7.5 mg/ml or 10 mg/ml irrespective of plant extracts used (Figure 4-12). Overall, ginger extracts significantly ($P < 0.05$) recorded the highest inhibition in conidial germination followed closely by turmeric after 48 h incubation at room temperature (Table 4-2). Despite all

plant extracts showed a similar effect on conidial germination, Pictorial image of efficacy of ginger crude extract was shown because it gave the highest inhibition (Figure 4-12).

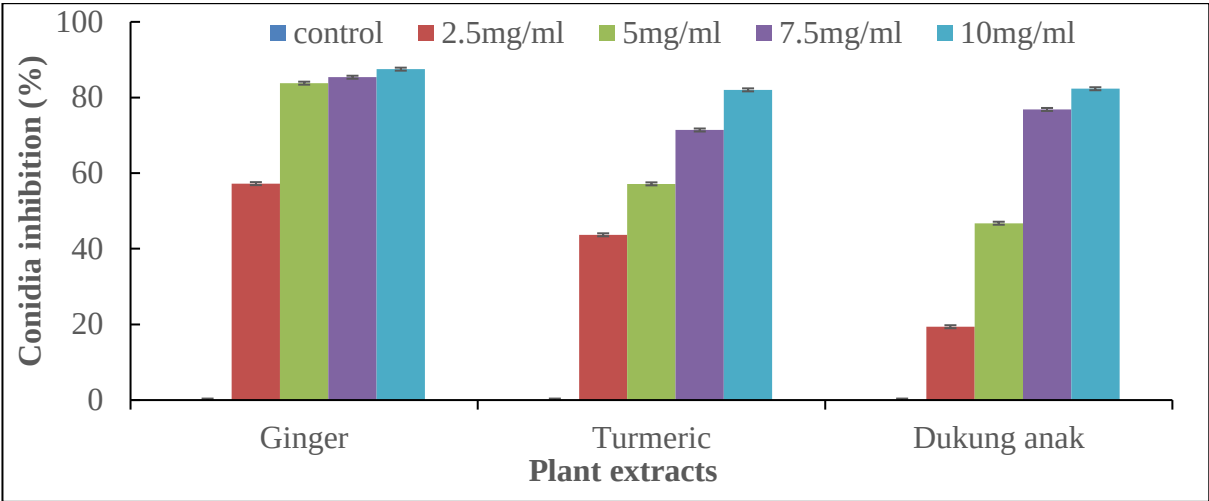


Figure 4-10 Effect of plant extracts at different concentration on inhibition of conidial germination of *C. gloeosporioides* after 48 h of incubation in the dark at room temperature. Control = only PDA

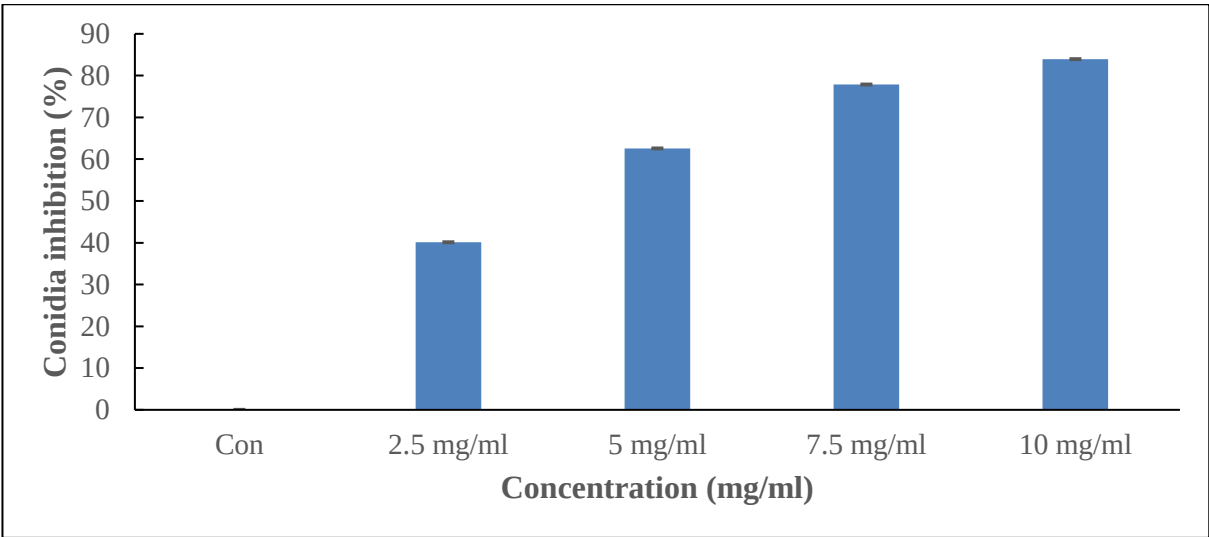


Figure 4-11 Effects of concentration on inhibition of conidial germination after 48 h incubation in the dark at room temperature. Con = Control (only PDA)

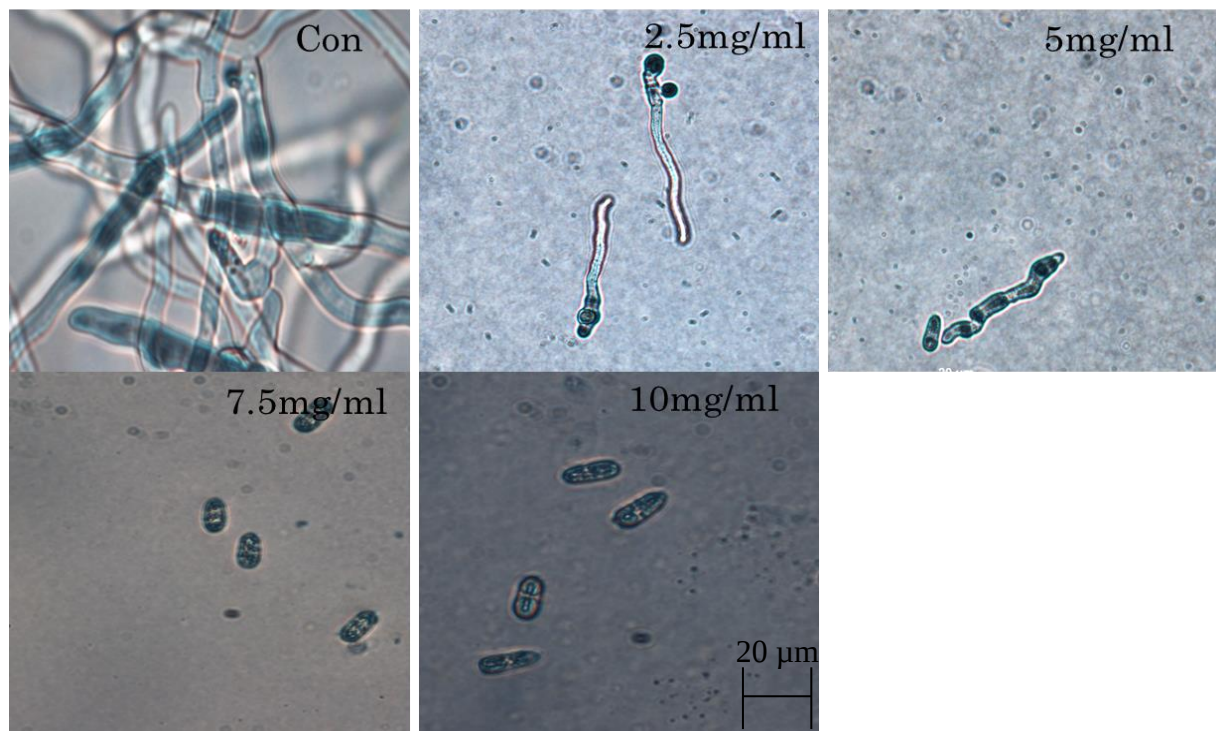


Figure 4-12 Effect of ginger extract on conidial germination after 48 h incubation in the dark at room temperature. The scale for each picture is 20µm

Table 4-1 Two-way Anova for effect of plant extracts at different concentrations on inhibition of mycelial growth and conidial germination.

	Mycelia inhibition (%)			Conidial Inhibition test (%)		
	Extracts (E)			Extracts (E)		
Treatment (T)	Turmeric	Dukung anak	Ginger	Turmeric	Dukung anak	Ginger
Only PDA	0n	0n	0n	0k	0k	0k
PDA+Methanol	4.23m	4.23m	4.23m	N/A	N/A	N/A
2.5mg/ml	20.13l	58.33h	42.69k	43.7i	19.4j	57.23g
5mg/ml	56.79i	47.82j	84.87c	57.17g	46.76h	83.79c
7.5mg/ml	71.41g	78.08f	86.28b	71.41f	76.84e	85.36b
10mg/ml	80.38e	83.33d	88.46a	82.02d	82.32d	87.5a
Sem (T x E)	0.3806			0.395		
P-value (T x E)	0.001			0.001		

Levels connected vertically by different letters are significantly different. N/A= not applicable.

Table 4-2. One-way Anova table for the overall effect of plant extracts on inhibition of mycelial growth and conidial germination after 12 days of incubation at room temperature.

Extracts (E)	Mycelia inhibition (%)	Conidial inhibition test (%)
Turmeric	42.59b	50.86b
Dukung anak	38.93c	45.05b
Ginger	53.7a	62.77a
Sem (E)	0.1554	0.77
P-value (E)	0.001	0.001

Levels connected vertically by different letters are significantly different.

4.4.1.4 Morphological changes of fungal hyphae

It was evident that all the plant extracts at the different concentrations caused some level of morphological damage to fungal hyphae as this evident in the inhibition of mycelial growth and conidial germination. The effect of this was more pronounced in ginger extracts. Even though at 2.5 mg/ml, all treatment did not significantly inhibit conidial and mycelial germination using visual and light microscope respectively, SEM studies revealed morphological hyphal damage especially in ginger crude extracts. The SEM studies revealed that at 2.5 mg/ml, hyphal growth in ginger-amended PDA was distorted and swollen compared to the control (Figure 4-13b). Hyphae in the control looked healthy, elongated and not distorted (Figure 4-13a). A similar trend of distortion and swelling of fungal hyphae was observed at 5 mg/ml in “Dukung anak” and turmeric rhizome (Figure 4-133b). There was slight inhibition in conidial germination even though signs of germ tube formation in ginger-treated Petri plates at 5 mg/ml was observed (Figure 4-14a). At 7.5 mg/ml or 10 mg/ml, there was little (Figure 4-14b) or no germination (Figure 4-14c) of conidia, however the conidia were distorted and swollen (Figure 4-14b, c) irrespective of the crude extracts used. This clearly shows that the highest concentrations significantly inhibited the fungal growth.

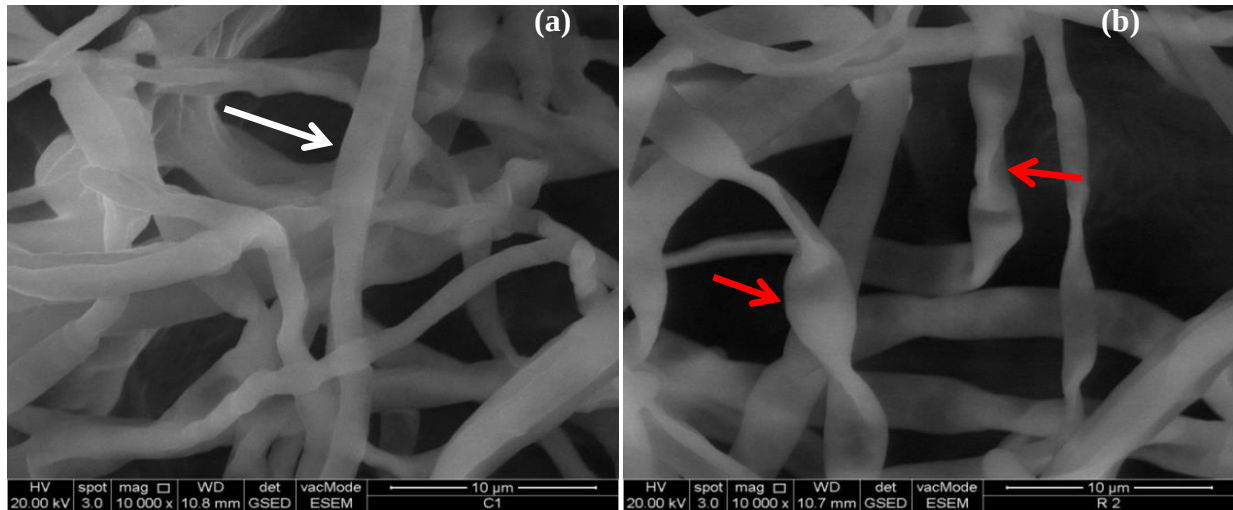


Figure 4-13 Scanning electron micrographs of (a) normal hyphae in control (white arrow) and (b) unhealthy hyphae (distorted, shrunk and swollen) of *C. gloeosporioides* as shown in red arrow.

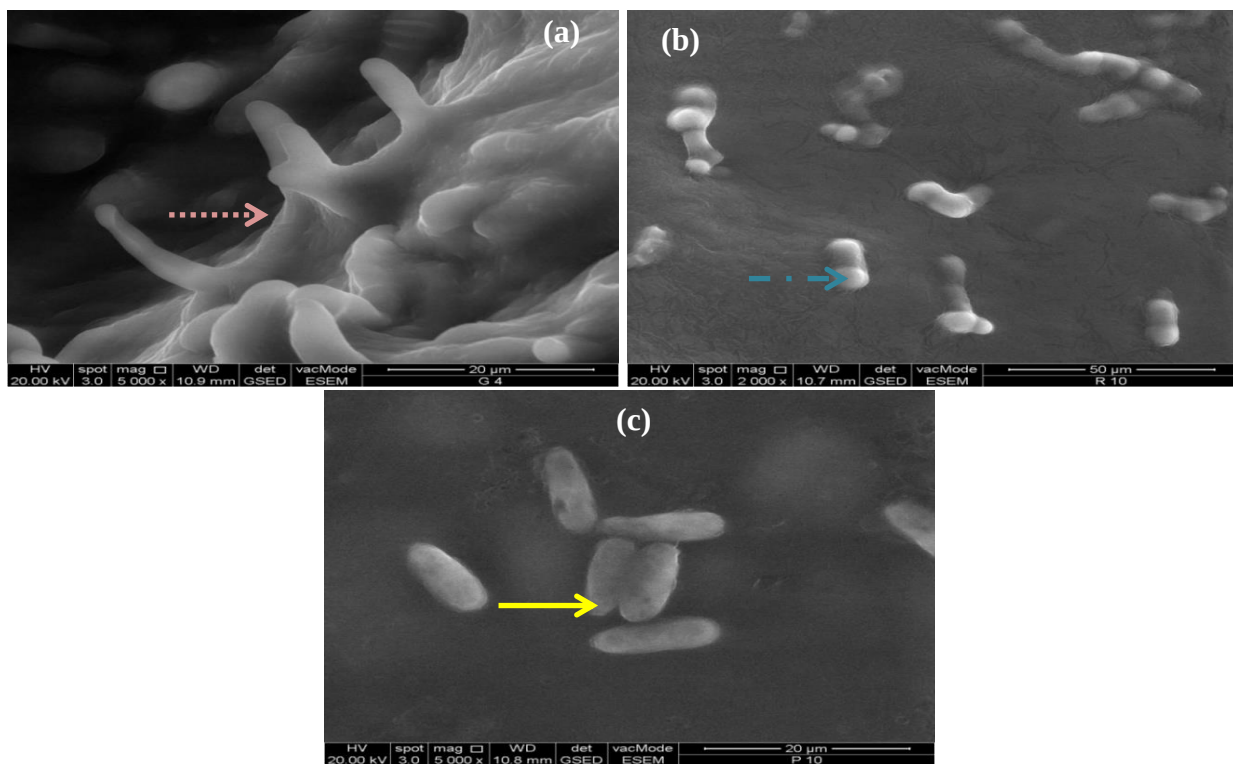


Figure 4-14. Scanning electron micrographs of conidia spores (a) inhibition of conidial germination at 5 mg/ml of ginger extracts (b and c) inhibition of conidial germination at 7.5mg/ml or 10 mg/ml. Brown broken arrows = beginning of germ tube formation. Blue broken arrow = distorted conidia at 7.5 mg/ml. Continuous yellow arrow = complete inhibition of conidia at 10 mg/ml.

4.4.2 Effect of extracts on anthracnose disease *in vivo*

By day 21, DI was between 6-80% and DS score (1.2-5) depending on the treatment used. For further observation, fruits were kept for another week (day 28) and it was evident most of the treated fruits including the control recorded between 90-100% DI with high DS (almost 4.0) depending on the type of treatment used (Figure 4-15). Images for DI and DS was taken for fruits treated at day 21 since most of fruits by the end of the 28 days storage were severely infested with anthracnose with signs of secondary infection (

Figure 4-18).

4.4.2.1 Disease incidence

. There was a significant effect ($P < 0.05$) between plant extracts at different concentrations (treatment) and storage time on disease incidence (DI) after 28 days of cold storage (

Appendix **0-3**). DI increased with storage time irrespective of the plant extracts (Figure 4-15). On the contrary ginger-treated fruits recorded the highest DI (100%) especially at 10 mg/ml or 15 mg/ml compared to control (93.33%) (Figure 4-16;

Figure **4-18**), whilst dukung anak- fruits treated at 5 mg/ml or 10 mg/ml recorded the lowest DI (46.67% or 40%) followed by turmeric treated fruits at 5 mg/ml (80.00%) or 10 mg/ml (73.33%) compared to control (93.33%) at day 28 of cold storage (Figure 4-16; Figure 4-17).

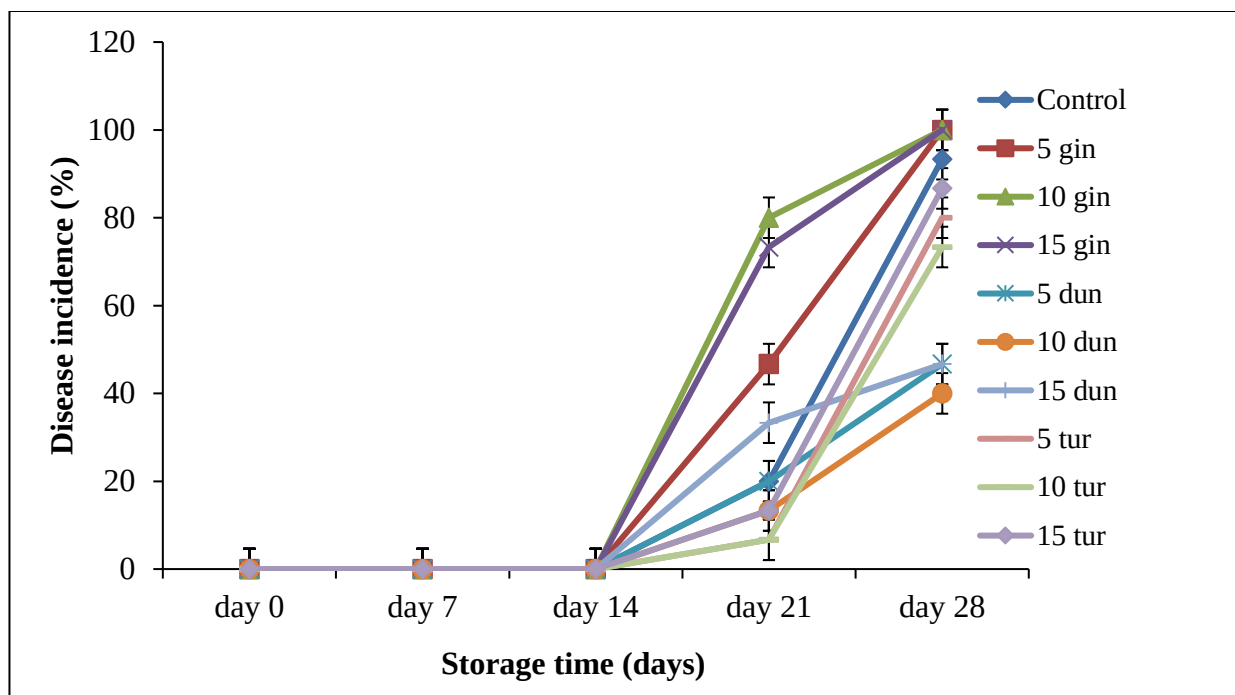


Figure 4-15 Effect of plant extracts at different concentrations on disease incidence during cold storage for 28 days.

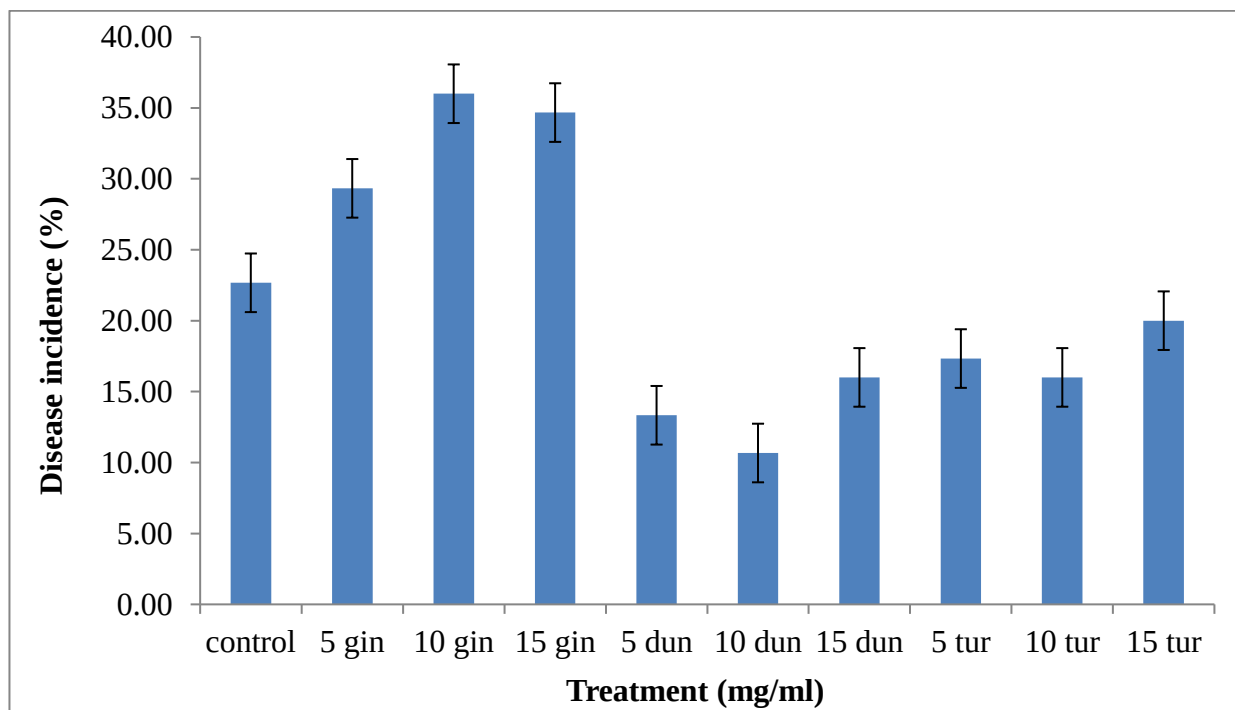


Figure 4-16 Effect of treatment on disease incidence after 28 days of cold storage.

At the end of the 28 days of cold storage, dukung anak-treated fruits at 10 mg/ml showed a significant ($P<0.05$) effect in controlling DI and this is was not different in turmeric- treated fruit at 10 mg/ml, however ginger-treated fruits at 15 mg/ml recorded the highest DI (Figure 4-16;Table 4-3).

Table 4-3 Anova table for the overall effect of plant extracts on disease incidence and disease severity after 28 days of cold storage based on three-way Anova analysis.

Extracts (E)	Disease incidence (%)	Disease severity score
Turmeric	19.00b	1.22b
Dukung anak	15.67c	1.14c
Ginger	30.67a	1.54a
Sem (E)	0.981	0.01678
P-value (E)	0.001	0.001

Levels connected vertically by different letters are significantly different.

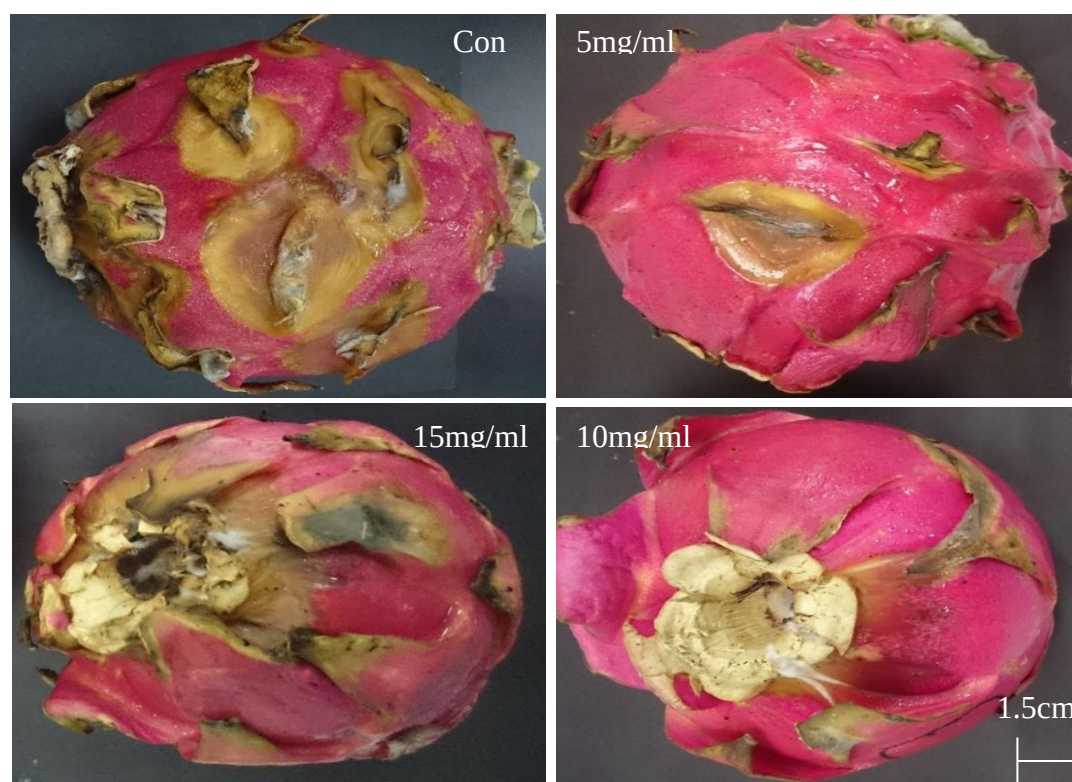


Figure 4-17 Effect of dukung anak extracts at different concentrations on disease incidence and severity of dragon fruits after 21 days of cold storage. Fruits showing symptoms of anthracnose. The scale for each picture is 1.5 cm.

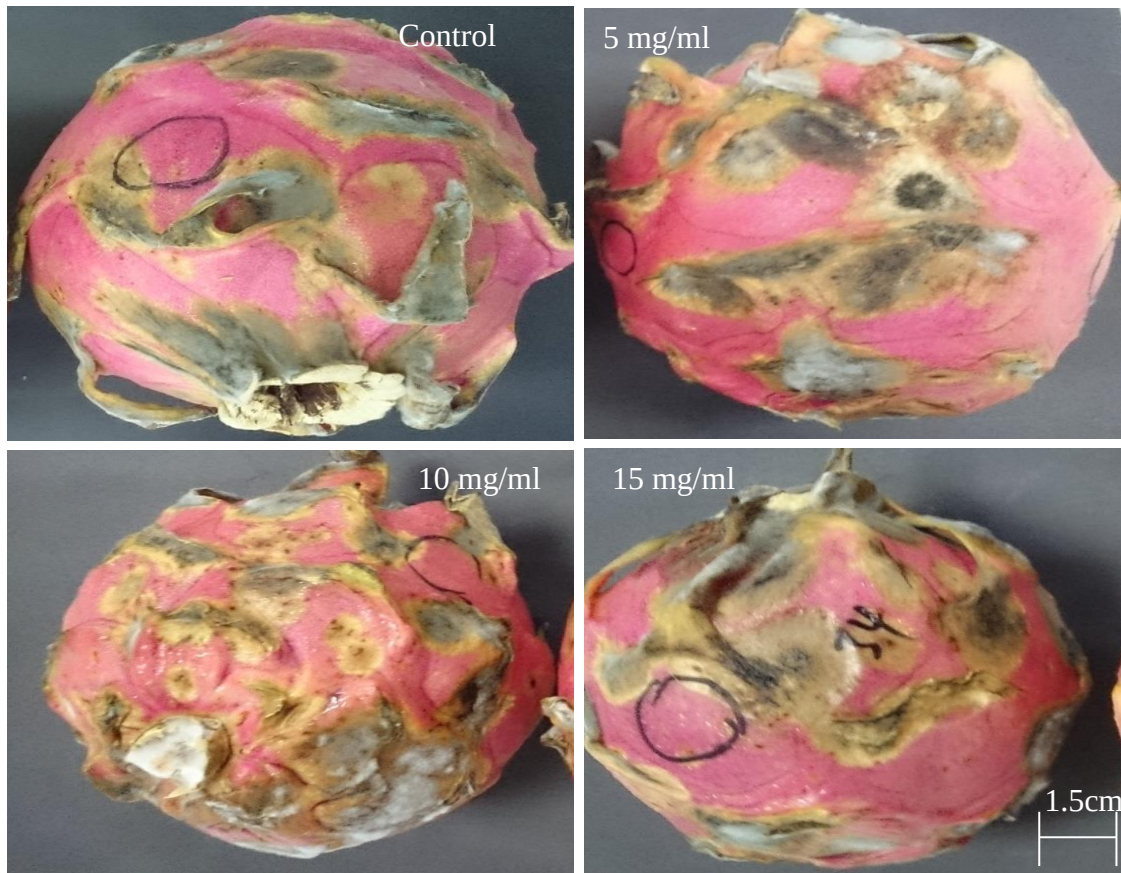


Figure 4-18 Effect of ginger extracts at different concentrations on disease incidence and severity of dragon fruits after 21 days of cold storage. Fruits showing symptoms of anthracnose with secondary infections especially in control fruits. The scale for each picture is 1.5 cm.

4.4.2.2 Disease severity

There was a significant effect ($P < 0.05$) between treatment and storage time on DS after 28 days of cold storage (

Appendix 0-4). DS increased with time irrespective of the plant extract (Figure 4-19). It was evident that high DI resulted in a concomitant increase in DS. Like DI, ginger-treated

fruits recorded a significantly ($P<0.05$) high disease severity (DS) especially at 10 mg/ml or 15 mg/ml (DS score of 3.26 or 3.13) compared to control (2.66) at day 28 of storage (

Figure **4-18**; Figure 4-20), whilst dukung anak-fruits treated at 5 mg/ml or 10 mg/ml recorded the lowest (DS score of 1.46 or 1.40 respectively) (Figure 4-17; Figure 4-20) which was not different in turmeric-treated fruits at 10 mg/ml (DS score of 1.93).

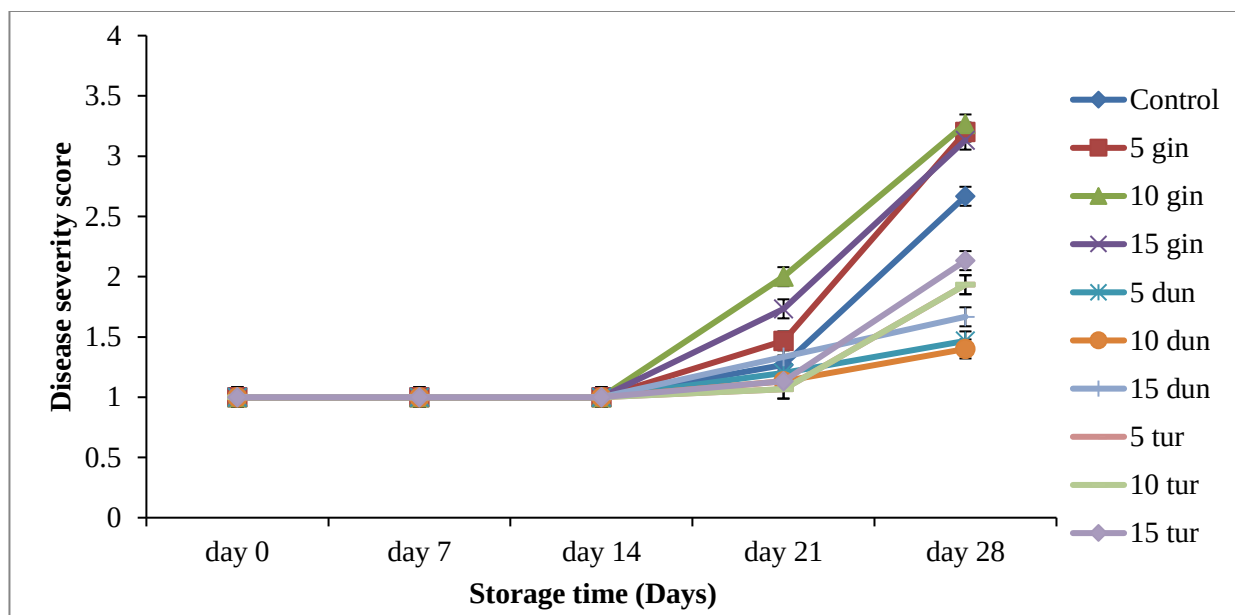


Figure 4-19 Effect of different plant extracts at different concentrations on disease severity caused by anthracnose on dragon fruits during cold storage for 28 days.

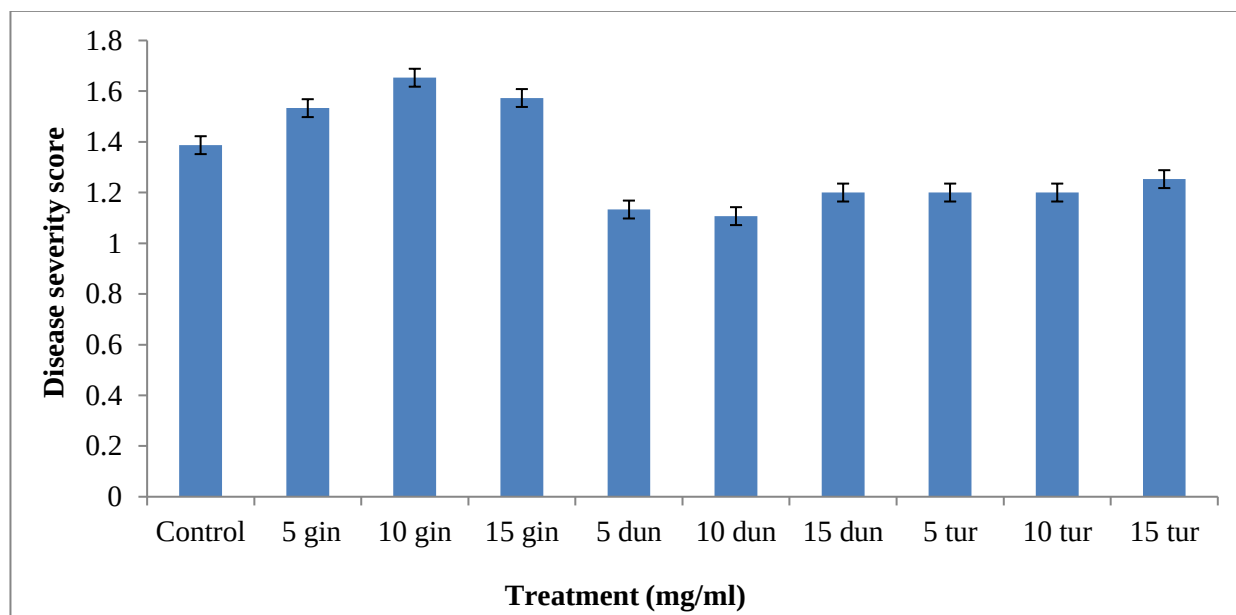


Figure 4-20 Effect of different plant extracts at different concentrations on disease severity caused by anthracnose on dragon fruits after 28 days of cold storage.

In general, dukung anak extracts significantly lowered DS especially at 10 mg/ml as was evident in lower DI which was not different in turmeric-treated fruits at 10 mg/ml. The highest

DS was observed in ginger-treated fruits especially at 15 mg/ml due to higher DI after 28 days of cold storage (Figure 4-20; Table 4 3).

4.4.2.3 Limitations of plant extracts on external appearance of fruits

The observational study revealed that, 18 out of the 20 observants preferred fruits treated at 5 mg/ml irrespective of extract used or control fruits as opposed to the ginger or turmeric-treated fruits at 10 mg/ml or 15 mg/ml. The main reasons that informed their preference was that, the original pinkish peel colour of dragon fruits was compromised by an intense yellow and brownish colour due to the turmeric extract and ginger extract respectively that was used to coat (treat) the fruits at 10 mg/ml or 15 (Figure 4-21). Additionally, the strong smell of each plant extracts (ginger, turmeric and dukung anak) at 10 mg/ml or 15 mg/ml also compromised the original smell of dragon fruit. All these affected consumer's appeal of the fruits for consumption.

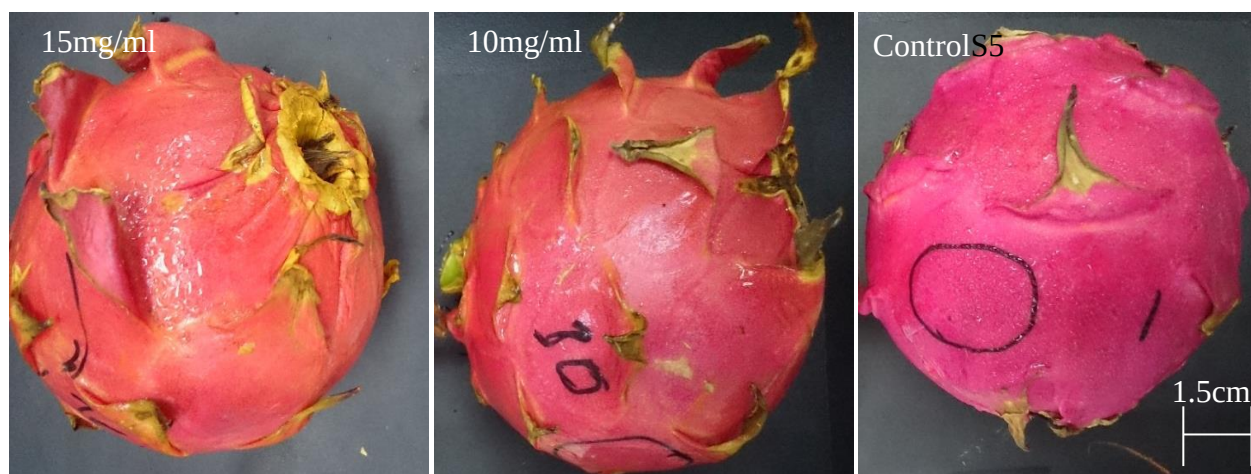


Figure 4-21 External appearance of turmeric-treated fruits showing intense yellow colour at high concentrations compared to control. The scale for each picture is 1.5 cm.

4.5 Discussion

4.5.1 Efficacy of extracts against mycelial and conidial germination

In this present study, crude extracts of ginger, turmeric, and dukung anak crude were significantly effective against the control of *C. gloeosporioides* as was evident in inhibition of mycelial growth and conidial germination. The efficacy of extracts to control *C. gloeosporioides* was dose dependent irrespective of the plant extracts used but it was more pronounced in ginger at 10 mg/ml (88.46%) which was not very different to the commercial fungicide (Mancozeb at 2 mg/ml) (80.45%). Our study confirms Ojaghian et al. (2014) where ethanolic ginger extracts (contact phase) at 10 mg/ml resulted in 84.5% reduction in myceliogenic and carpogenic germination of sclerotia in isolate 2 of *Sclerotinia sclerotiorum* that causes carrot rot. Dissanayake (2014) also confirmed the fungistatic effect of methanolic crude ginger extracts resulting in 91% inhibition of mycelia growth of *F. oxysporum* at 25 mg/ml. Radwan et al. (2014) reported a dose-dependent effect of methanolic turmeric extracts on *C. gloeosporioides* which resulted in 5.5 mm and 10.5 mm in mycelia inhibitory zone at a minimum inhibitory concentration (MIC) at 80 µg/spot and 160 µg/spot respectively. In other studies, 5 mg/ml and 7.5 mg/ml aqueous turmeric rhizome resulted in 37.72% and 39.64% inhibition of mycelial growth of *C. gloeosporioides* confirming a dose dependent effect (Tasiwal et al., 2009). MIC at 0.002- 0.020 g/L using dimethyl sulphoxide (DMSO) turmeric rhizome extracts also showed an antibacterial activity against *R. solanacearum* resulting in 20-26 mm zone of mycelial inhibition (Murthy et al., 2013). Previous antifungal studies have been performed using pure bioactive compounds isolated from dukung anak on the tested fungus. Singh et al. (2007; 2008) reported *in vitro* antifungal effect of securinine and allosecurinine isolated from *Phyllanthus* spp. (dukung anak) on *Colletotrichum* spp. which resulted in 0%

spore germination at 1 mg/ml. Similarly, 10.16% spore germination was observed when ent-norsecurinine was applied on *Colletotrichum* species at 1mg/ml (Goel et al., 2002).

In all cases, the efficacy of extracts to control the fungus was dose dependent and this is due to the presence of bioactive compounds in the extracts. The antifungal activity of extracts against *C. gloeosporioides* could be due to the presence of bioactive compounds such as gingerols (in ginger), curcumin (turmeric) and any of these alkaloids (securinine, allosecurinine or ent-norsecurinine in dukung anak (Goel et al., 2002; Ficker et al., 2003; Kim et al., 2003; Singh et al., 2007; 2008; Martins et al., 2008; Michielin et al., 2009; Hasan et al., 2012b; Ferreira et al., 2013; Radwan et al., 2014). However further studies to ascertain this by HPLC is required. It is fair to say that, as a polar solvent, methanol may have extracted more of the polar bioactive compounds in ginger as opposed to polar compounds in dukung anak or turmeric, hence making ginger treatment possesses a strong antifungal activity against the *C. gloeosporioides* even at the lowest concentration used in this study.

4.5.2 Morphological changes of hyphal growth due extracts

In our study, treatments at 2.5 mg/ml or 5 mg/ml caused shrivelling, distortion and swelling of the fungal hyphae as was observed in the SEM, whiles at 7.5 mg/ml and 10 mg/ml, it caused severe damage to conidia resulting in little and no conidial germination respectively. This study confirms similar effect when chitosan and essentials oils obtained from different plants resulted in morphological damage of hyphae of several phytopathogens such as *F. oxysporum* f. sp. *gladioli*, *R. stolonifer*, *C. gloeosporioides*, *Alternaria alternata*, *C. musa* (Kendra and Hadwiger, 1987; Muñoz et al., 2009; Rozwalka et al., 2010; Sánchez-Domínguez et al., 2011; Baños-Bautista et al., 2012). The mechanism these plant extracts employed to cause morphological damage to fungal hyphae could be due to breaking of cytoplasm, intense vacuolization and the

breakdown of fungal cell wall (Bautista-Baños et al., 2012; Aala et al., 2013). Another mode of action that resulted in the hyphae morphological changes could be due to disruption of the plasma membrane and leakage of ions from the fungal cell wall (Cho et al., 2012), the latter (ion leakage) confirms our study where we observed electrolyte leakage from the fungal cell wall. Details of ion leakage study from fungal cell wall can be found in the next chapter.

4.5.3 Efficacy of extracts on disease incidence and disease severity

In this study, fruits treated with dukung anak at 5 mg/ml or 10 mg/ml recorded very low DI which accounted for less DS after 28 days of cold storage. However, anthracnose control was more pronounced at 10 mg/ml even though it was not significantly different in turmeric-treated fruits at 10 mg/ml compared to control. On the contrary, DI and DS were compounded in ginger treated-fruits irrespective of the concentration used. The efficacy of dukung anak and turmeric extracts at 5 mg/ml or 10 mg/ml demonstrated that these concentrations are optimum to control anthracnose on fruits without any phytotoxic effect. Several studies suggest the presence of curcuminoids (particularly curcumin) in turmeric and securinine, ent-norsecurinine and allosecurinine in dukung anak as the main compounds that may have accounted for inhibiting *C. gloeosporioides* (Bunyaprapatr and Chokchaichareonporn, 1996; Goel et al., 2002; Singh et al., 2007; 2008; Akram et al., 2010; Selvam et al., 1995; Singh and Jain, 2012; Radwan et al., 2014) and it could be that these concentrations were not phytotoxic to the dragon fruits.

The pronounced DI and DS in ginger-treated fruits at 5 mg/ml and above was due to phytotoxic effect which the fruit peel could not comprehend. This was evident in the peel tissue damage culminating in DI after 28 days of cold storage. Reports show that aqueous crude ginger extract was phytotoxic at 8 mg/ml due to the presence of some water-soluble allelochemicals that inhibited seed germination, seedling growth, water uptake and lipase activity of soyabean

and chives (Han et al., 2008). Allelochemicals such as phenolic acids (vanilic acid, syringic acid, p-hydrobenzoic acid, p-coumaric acid and ferulic acid) and coumarins were found in stems, leaves and rhizomes of ginger (Han et al., 2008) and studies suggest that ginger has an auto toxic potential especially at 8 mg/ml when applied on ginger seedlings and could cause phytotoxicity when used as a bio-fungicide on the next cropping of ginger seedling on the same farm (Han et al., 2012). It is imperative to state that reducing ginger concentrations could reduce DI and DS as was reported in a study where 2 mg/ml significantly reduced carrot rot in carrots caused by *S. sclerotiorum* (Ojaghian et al., 2014). Our study confirms Han et al. (2012) study that, at 5 mg/ml and above, ginger extracts could cause phytotoxicity. The concentration of 15 mg/ml irrespective of the crude extracts resulted in severe disease incidence due to phytotoxicity. This could be due to the presence of some bioactive compounds which interacted with the host cells and tissues resulted in tissue damage in the host allowing the fungus to attack fruit and infest them.

4.6 Concluding remarks

Molecular identification by PCR using specific primers Cglt F3 and Cglt B3 as well as pathogenicity test confirmed that the fungus as *C. gloeosporioides* that caused postharvest anthracnose in dragon fruit. *In vitro* studies showed that all plant extracts significantly controlled *C. gloeosporioides* but this was highest in ginger crude extracts at 10 mg/ml as this evident in inhibition of mycelial and conidial growth. During the *in vivo* assay, 15 mg/ml (irrespective of the crude extracts) resulted in severe DI due to phytotoxicity. However, at 5 mg/ml or 10 mg/ml of fruits treated with either dukung anak or turmeric, anthracnose and its severity was significantly low and this was more pronounced in dukung anak-treated fruits at 10 mg/ml. However, the external quality appearance (smell and colour) of dragon fruits was

compromised especially when fruits were treated with 10 mg/ml and above mostly in turmeric and ginger crude extract during the observation despite phytotoxicity at 15 mg/ml. Therefore, further study to correct this anomaly and enhance the efficacy of extracts by combining GA should be investigated as captured in chapter 6. Despite some studies suggest low concentrations of ginger extracts (2 mg/ml) for instance in the case of carrot, such concentrations may not have significant effect to control disease in dragon fruits due to differences in skin (peel) thickness between of these two produces. We conclude that crude extracts of turmeric and dukung anak alone can be used as bio-fungicide to control anthracnose in dragon fruits and other tropical fruits at optimal concentration of 10 mg/ml, whiles below 5 mg/ml, fruits treated with ginger extracts can also help to alleviate anthracnose and its severity. However, further studies also need to be conducted to ascertain the bioactive compounds responsible for the antifungal activity and the mechanism these plant crude extracts exhibit against the *C. gloeosporioides* that causes anthracnose (see chapter 5).

CHAPTER 5

5 DETECTION AND QUANTIFICATION OF BIOACTIVE COMPOUND IN PLANT EXTRACTS AND MECHANISM OF PLANT EXTRACTS AGAINST FUNGUS

5.1 Introduction

Natural products from medicinal plants, either as pure compounds or as standardized crude extracts alone or in combination, provide opportunities for controlling disease during postharvest storage because of the availability of chemical diversity as well the health and safety concerns to humans. Plants like ginger, turmeric, and dukung anak crude extracts contain various types of bioactive compounds which account for their antifungal activity and identification of such bioactive compounds has been determined by using High Performance Liquid Chromatography (HPLC) (He et al., 1998; Natarajan et al., 2012; Sintov, 2015). In some identification processes using HPLC analysis, the retention time is used as a key indicator to identify the bioactive compounds in a crude extract after the retention time of the pure compound is known. Comparing the retention time between a pure compound and retention time from different peak resolution in crude extracts can help to propose the presence of the bioactive compound in the extracts. Ginger extracts were found to inhibit the growth of several phytopathogens including *C. gloeosporioides* and this was due to the presence of gingerols (Dissanayake and Jayasinghe, 2013; Ademe et al., 2014; Al-Askar et al., 2014; Ojaghian et al., 2014). Studies indicate that the antifungal activity of turmeric due to the biological activity of curcuminoids present in them (Radwan et al., 2014) and curcumin forms the major bioactive curcuminoid (Ruby et al., 1995; Singh and Jain, 2012). Singh et al. (2007:2008) reported the antifungal effect of *Phyllanthus* spp against selected phytopathogens including *Colletotrichum* spp., and this was due to the presence of two alkaloids (allosecurinine and securinine). To

identify and confirm the presence of these bioactive compounds present in the extracts (ginger, turmeric and dukung anak), the retention time of the peak resolution between an authentic standards and crude extracts are compared using HPLC. Based on this, the bioactive compound is confirmed or proposed to be present in the extracts (He et al., 1998; Ficker et al. 2003, Hasan et al., 2012b; Mishra et al., 2013)

Disruption of plasma membranes of fungal cells resulting in morphological damage, leakage of ions from fungal cell wall i.e., affecting their permeability and release of intracellular constituents (e.g. ribose, Na glutamate) and interfere with membrane functions (electron transport, nutrient uptake, protein, nucleic acid synthesis and enzyme activity) (Rozwalka et al., 2010; Sánchez-Domínguez et al., 2011; Baños-Bautista et al., 2012; Cho et al., 2012) are some of the prominent mechanism of natural products against fungi. Additionally, the lack of catalase enzyme production by the fungus resulting in the accumulation of hydrogen peroxide (H_2O_2) leading to toxicity to the fungal cell as well as inhibition of production and activity of cellulolytic enzyme in the fungus are some mechanism these extracts exhibit against the fungus (Gupta and Bilgrami, 1970; Khan and Nasreen, 2010).

The objectives of this study under this chapter are:

- To identify and quantify the main bioactive compound in ginger, turmeric and dukung anak crude extract responsible for the antifungal activity against *C. gloeosporioides* by HPLC.
- Investigate the mechanism of the extracts against *C. gloeosporioides* through leakage of electrolytes from the fungal cell wall.

5.2 Materials and methods

Crude extracts obtained in section 3.3 were used for the HPLC analysis.

5.2.1 HPLC analysis for detection and quantification of bioactive compounds

5.2.1.1 HPLC instrumentation for detection of 6-gingerol

HPLC analytical run was performed according to He et al. (1998) with slight modification in column temperature. An HPLC 1260 infinity (Agilent technologies) with a photodiode array detector set at 280 nm was used. Chromatographic conditions were: column, Zorbax 300SB-C18, 5 μ m, 4.6 x 250 mm (Agilent technologies); eluent (A) water and (B) acetonitrile. The gradient elution had the following profile: 0–8 min, 45–50% B; 8–17 min, 50–65% B; 17–32 min, 65–100% B; 32–38 min, 100% B; 38–40 min, 100–45%B. Flow rate, 1ml/min, the temperature at 25⁰C.

5.2.1.2 HPLC instrumentation for detection of securinine

HPLC analytical run was performed according to Natarajan et al. (2012) with slight modification. An HPLC 1260 infinity (Agilent technologies) with a photodiode array detector set at 254 nm was used. Chromatographic conditions were: column, Zorbax 300SB-C18, 5 μ m, 4.6 x 250 mm (Agilent technologies); eluent (A) water and (B) methanol. The column was used for isocratic resolution using methanol: water (50:50 v/v) as the mobile phase at a flow rate of 1.0 ml/min and the column temperature was set at 25⁰C.

5.2.1.3 HPLC instrumentation for detection of curcumin

This was carried out according to Sintov, (2015) with slight modification. Aliquots of 20 µl of each sample were injected into an HPLC system, equipped with a prepacked column, Zorbax 300SB-C18, 5 µm, 4.6 x 250 mm (Agilent technologies), which was constantly maintained at 30°C. An HPLC 1260 infinity (Agilent technologies) with a photodiode array detector at 424 nm was for the quantification curcumin. The samples were chromatographed using an isocratic mobile phase consisting of acetonitrile 0.2% acetic acid solution (50:50) at a flow rate of 1 ml/min.

5.2.1.4 Sample preparation and HPLC analysis

5.2.1.4.1 Standard curve

A standard curve for 6-gingerol and securinine was carried out according to Zick et al. (2010) with slight modification whilst for curcumin, it was carried according to Fonseca-Santos et al. (2016). A standard stock for 6-gingerol and securinine (Sigma Aldrich, HPLC grade) was prepared separately by dissolving 6-gingerol and securinine in methanol (HPLC grade) until a final stock concentration of 0.62 mg/ml, whilst for curcumin (Sigma-Aldrich, HPLC grade) until a final stock concentration of 2.5 mg/ml. Calibration curve was prepared using working concentrations of 0.02, 0.03, 0.06, 0.18, 0.30, 0.43 and 0.55 mg/ml for securinine and 6-gingerol, whilst for standard curcumin a working concentration between 0.0005 to 0.0075 mg/ml (0.5 to 7.5 µg/ml) was used. A standard curve was determined for each pure compound based on their respective instrumentation protocol.

5.2.1.4.2 Sample preparation and quantification by HPLC

For quantification of the bioactive compounds (6-gingerol, securinine, and curcumin) present in each extract at different treatment concentrations, a stock concentration of 17 mg/ml was prepared for each crude extract. This was done by dissolving weighed crude extract in HPLC grade methanol from which a working concentration of 5 mg/ml, 10 mg/ml and 15 mg/ml of each extract was prepared. Each working concentration (samples) per plant crude extract were filtered with nylon mesh (0.45µm) to remove any debris prior to HPLC analysis. This was done for both standard and sample solutions. All solutions prepared were stored at -20°C until use. Before the HPLC run, an aliquot of 10µl of each working concentration (samples) per extract were analyzed using the respective HPLC specification above and quantified using standard curve obtained from the HPLC analysis from each respective pure compound. Quantified samples were expressed as mg/100 ml.

5.2.2 Effect of extracts on electrolyte leakage of *C. gloeosporioides*

To investigate the mechanism the plant crude extract employed to control the fungus, electrolyte leakage from fungal cell wall at concentrations 0.2 mg/ml, 2.5 mg/ml and 5 mg/ml (from *in vitro* studies in section 4.2.2.4) were used. A lowest concentration of 0.2 mg/ml was included to see the effect of extracts at extremely low concentration on electrolyte leakage on fungal cell wall.

5.2.2.1 Sample preparation

The tested crude extracts were added to a conical flask containing sterile liquid potato dextrose broth medium to give concentrations of 0.2, 2 and 5 mg/ml of the medium until a final volume of 30 ml. The flasks were then inoculated with mycelial discs (6 mm diameter) of *C.*

gloeosporioides. Five flasks were used for each treatment and other five flasks without crude extracts were used as control. All flasks were then incubated at 20°C for 10 days after which the mycelium was filtered off and washed thoroughly with deionized water (Ouf et al., 2008). The experiment was done in triplicate.

5.2.2.2 Measurement of leakage of electrolytes

Measurement of electrolyte leakage was carried out according to Manoj-Kumar et al. (2015) with slight modification. Harvested fresh mycelia from the broth were then filtered, washed continuously with purified distilled water and dried at room temperature. One gram of thoroughly washed and dried mycelium was transferred to a test tube containing 30 ml bidistilled water and incubated in a shaking water bath at 20°C for 10 h period (Ouf et al., 2008). Initial conductivity (C_0) was measured with a conductivity meter after subjecting the samples to incubation at 20°C in 20 ml bidistilled water for 2 h with continuous shaking at 100 rpm. Conductivity (C_f) was measured for each sample every 2 h for a period of 10 h. The conductivity of bidistilled water was also measured and referred to as C_w . The percentage of electrolyte leakage (EL %) was calculated as follows;

$$EL\% = [(C_0 - C_w) / (C_f - C_w)] \times 100$$

5.3 Statistical analysis

Quantification of bioactive compounds by HPLC and electrolyte leakage (%) was done in triplicate. Two-way Anova was performed in both cases using computer software GenStat version 18th Edition (VSNI product). Means were separated using Fisher unprotected test at ($P < 0.05$).

5.4 Results

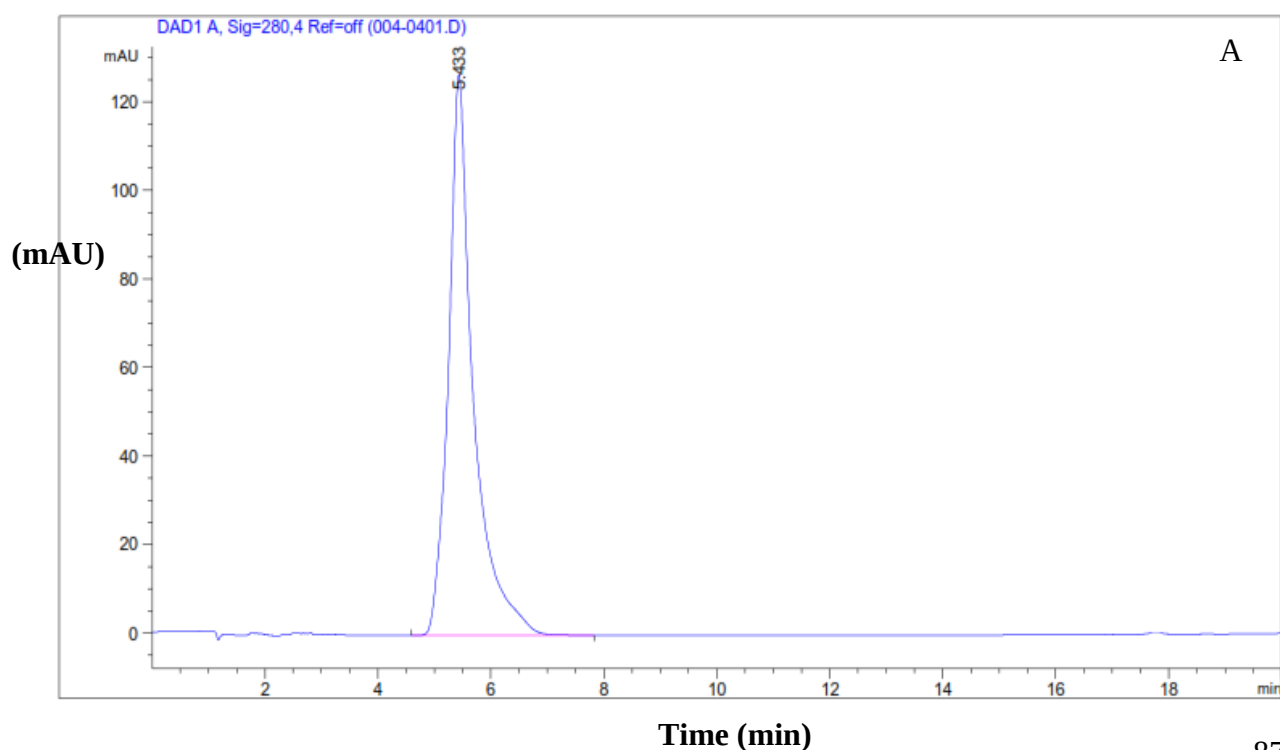
5.4.1 Detection and quantification of bioactive compounds by HPLC

Figure 5-1A, Figure 5-3A and Figure 5-5A shows chromatographs of all standards, thus 6-gingerol, curcumin, and dukung anak respectively, while Figure 5-1B, Figure 5-3B and Figure 5-5B shows chromatograms of typical methanolic crude extracts of ginger, turmeric, and dukung anak respectively. The gradient runs were optimised using aqueous acetonitrile for ginger extract and 6-gingerol extracts; aqueous methanol for securinine and dukung anak extract and isocratic run using aqueous acetonitrile with a typical modifier (0.2% acetic acid) for curcumin and turmeric extract, and comparable retention times were obtained from both the standard compounds and each extract. The composition of the solvent system was initially varied through optimisation (results not shown) until the resolution of all standards were satisfactory which was further used for HPLC analysis in the crude extracts. Calibration curves based on linear regression analysis were determined by plotting the peak areas of all standards, thus 6-gingerol, securinine, and curcumin against the corresponding concentrations 0.02- 0.55 mg/ml, 0.02-0.55 mg/ml and 0.5-7.5 µg/ml respectively. All three bioactive compound standards showed a good linearity ($r^2 > 0.99$) (Appendix 0-5;

Appendix 0-6; Appendix 0-7) in the range of the concentrations tested for the optimised gradient. This shows that the analytical method established for HPLC allows for the quantification of the specific bioactive compounds if present in the crude extract of each plant. In this analysis, we identified the major bioactive compounds that are known to be abundant in these extracts to possess strong antimicrobial activity and this includes 6-gingerol, curcumin, and securinine in ginger, turmeric, and dukung anak respectively.

5.4.1.1 6-gingerol

Figure 5-1A and Figure 5-1B shows chromatographs of 6-gingerol and crude extracts of ginger respectively. There were several peaks observed for ginger extracts that were unidentified (Figure 5-1B). The characterised bioactive compound found in ginger crude extracts consisted mainly of 6-gingerol (with the highest peak) (Figure 5-1B) as this was evident and similar in the chromatograph from the pure compound (Figure 5-1A) with both having the similar retention time of approximately 5.40 min.



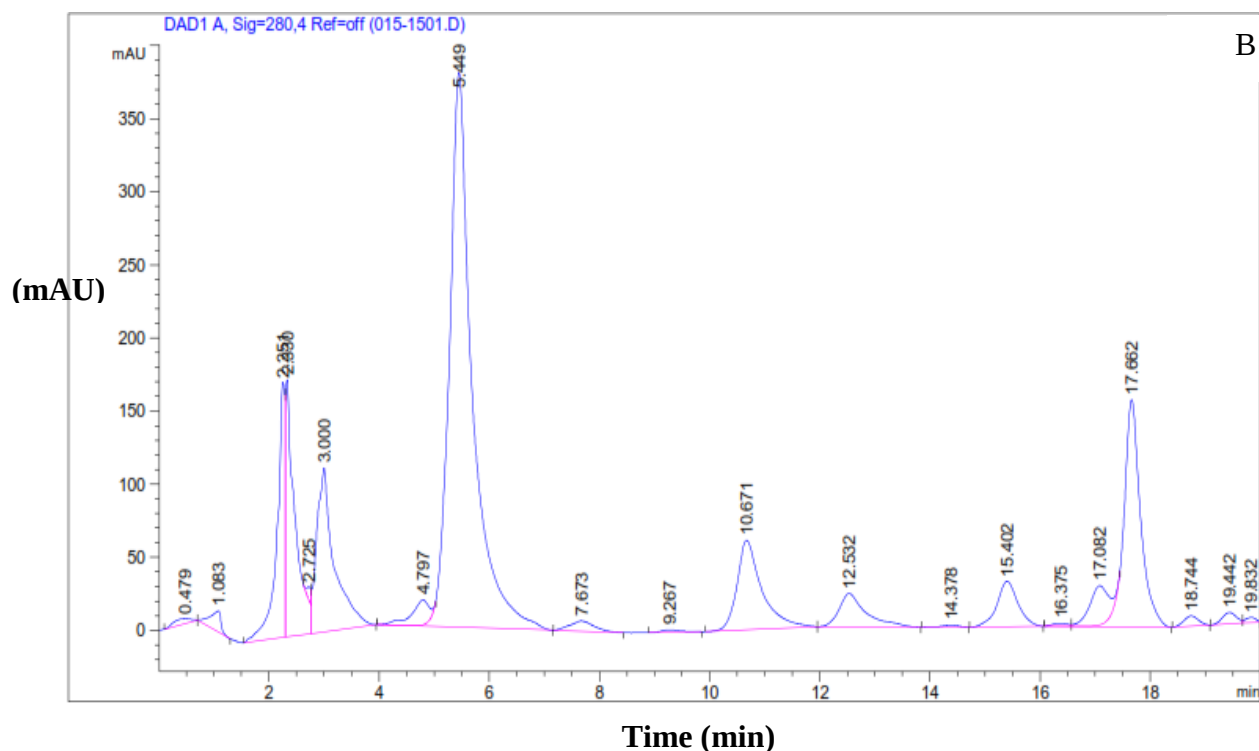


Figure 5-1 Simultaneous HPLC–UV chromatograms of (A) standard 6-gingerol and (B) ginger methanol extract identification based on retention time.

In terms of quantification, the amount of 6-gingerol in crude extract increased with the concentration of the extract. The highest amount of 6-gingerol ($P < 0.05$) was recorded at 15 mg/ml (119.65 mg/100 ml) of ginger crude extract whiles the lowest was at 5 mg/ml (44.35 mg/100ml) (Figure 5-2).

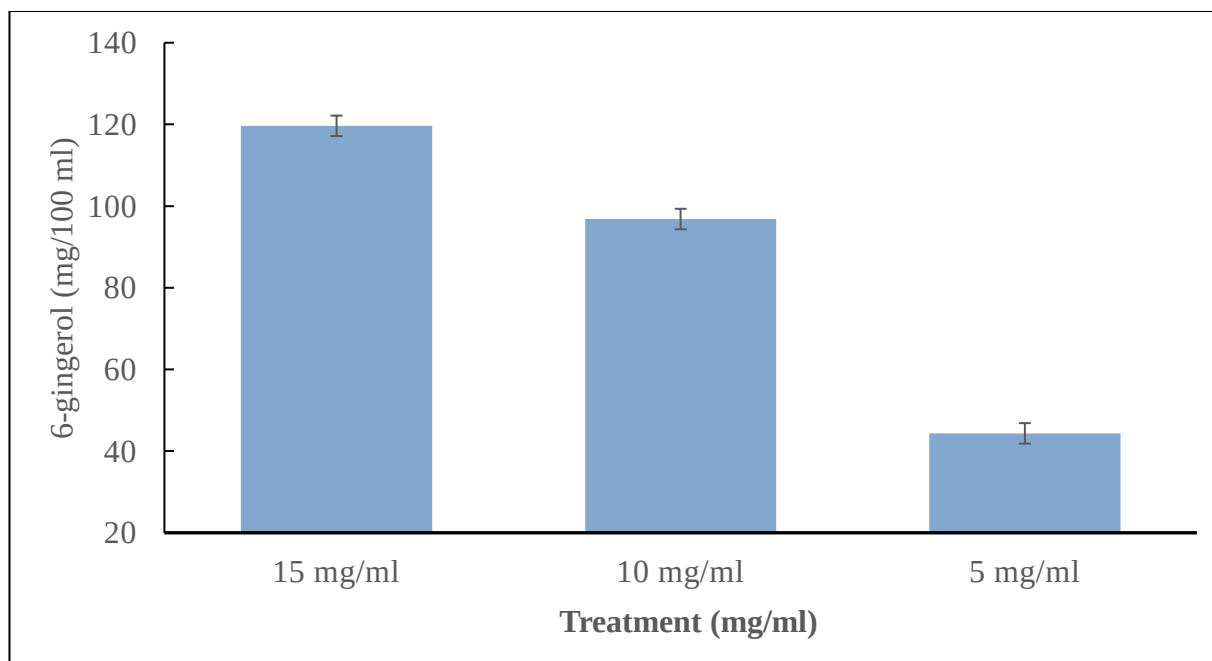


Figure 5-2 Amount of 6-gingerol analyte present in ginger crude extract based on the treatment

5.4.1.2 Curcumin

The presence of curcumin was observed as the active compound present in methanolic turmeric crude extract as shown in the respective chromatographs and this was based on the retention time in both the pure compound and the turmeric crude extracts (Figure 5-3A and B). Several peaks were observed for turmeric crude extract that were unidentified (Figure 5-3B). The characterised bioactive compound found in turmeric crude extract consisted mainly of curcumin (with the highest peak) (Figure 5-3B) as this was evident and similar in the chromatograph from the pure compound (Figure 5-3A) with both having the similar retention time of approximately 5.92 min.

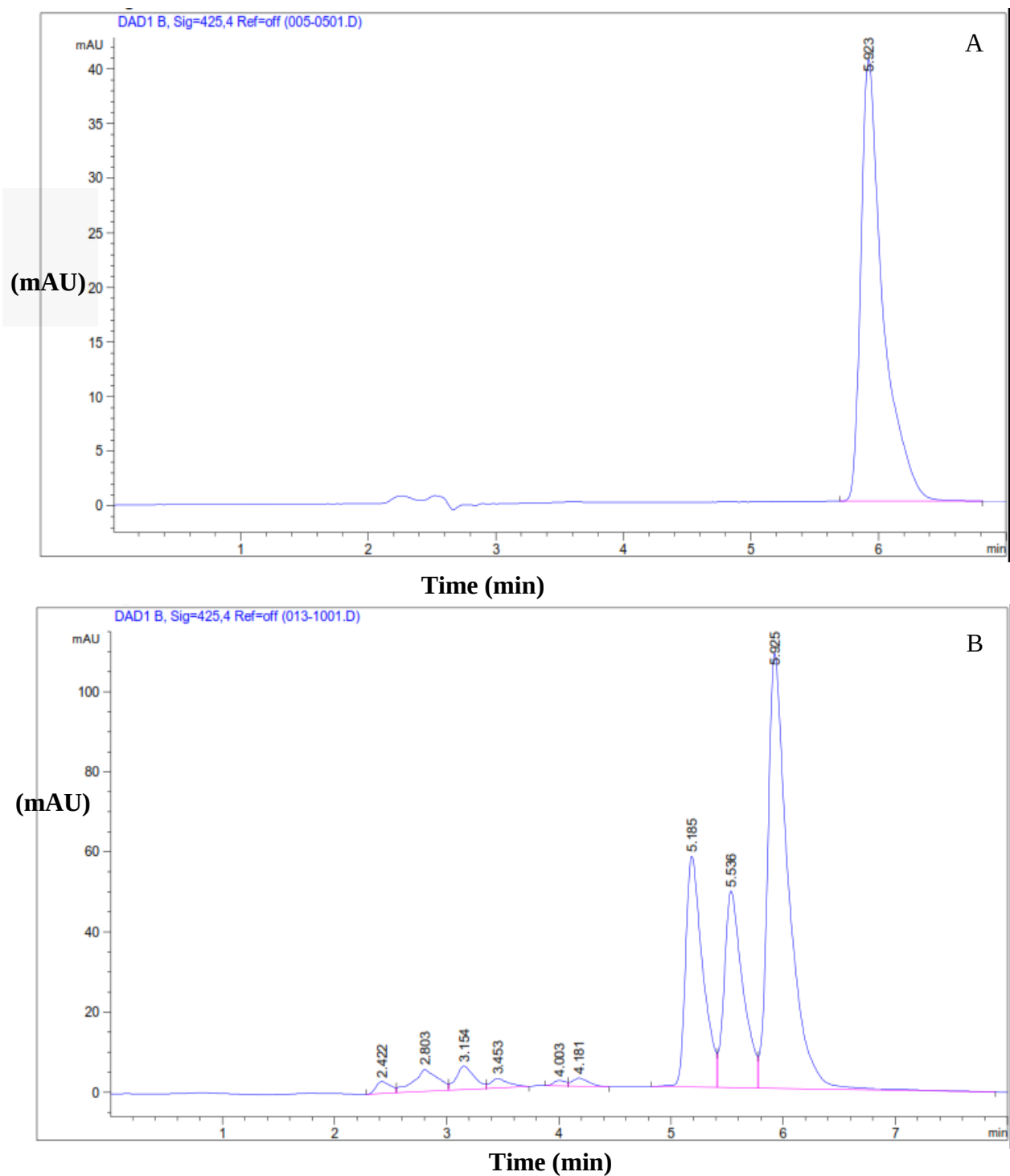


Figure 5-3 Simultaneous HPLC–UV chromatograms of (A) standard curcumin and (B) turmeric methanol extract identification based on retention time.

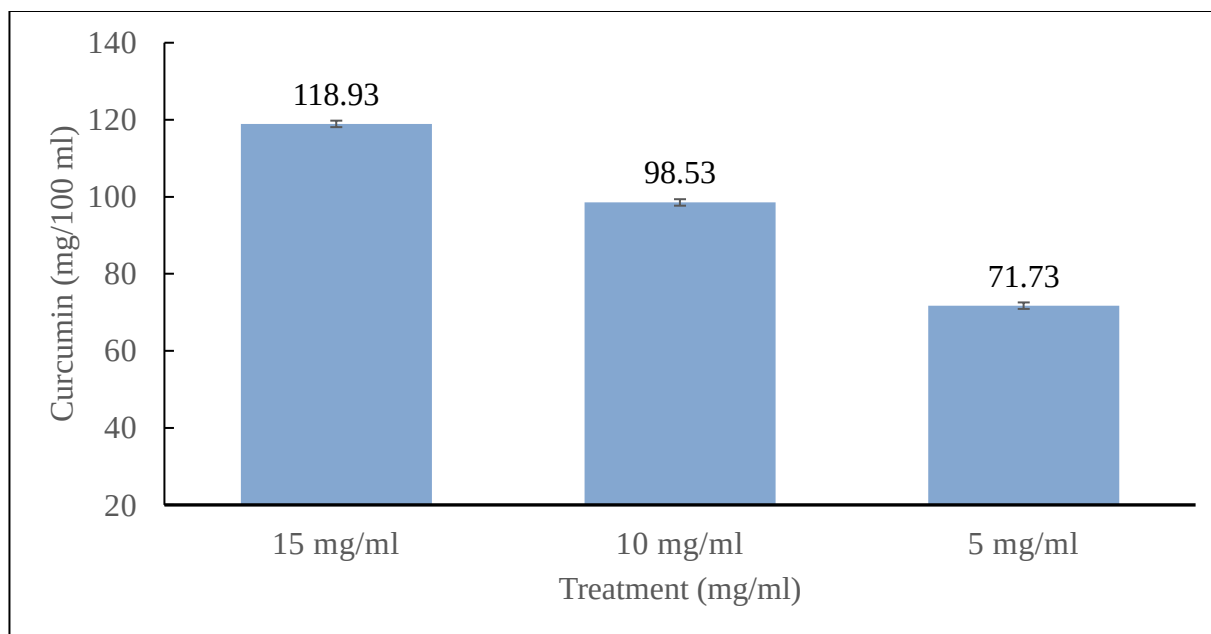


Figure 5-4 Amount of curcumin analyte present in turmeric rhizome crude extract based on the treatment

The amount of curcumin present in turmeric crude extracts increased with the concentration of the extracts used for the experiment. The highest amount of curcumin ($P < 0.05$) was observed at 15 mg/ml (118.93 mg/100 ml) in turmeric crude extracts while the lowest was recorded at 5 mg/ml (71.73 mg/100 ml) (Figure 5-4).

5.4.1.3 Securinine

Several unidentified peaks were observed in dukung anak crude extracts, and none of these peaks could be identified as securinine. It was evident that securinine was not identified in dukung anak extracts and this was due to the difference in retention time in the chromatograph of pure securinine and dukung anak extract (Figure 5-5A and B). Therefore, the present study cannot confirm that securinine in dukung anak crude extracts was responsible for the antifungal activity that occurred *in vitro* and *in vivo*. Further study to ascertain which bioactive compound responsible for this antifungal activity need to be investigated.

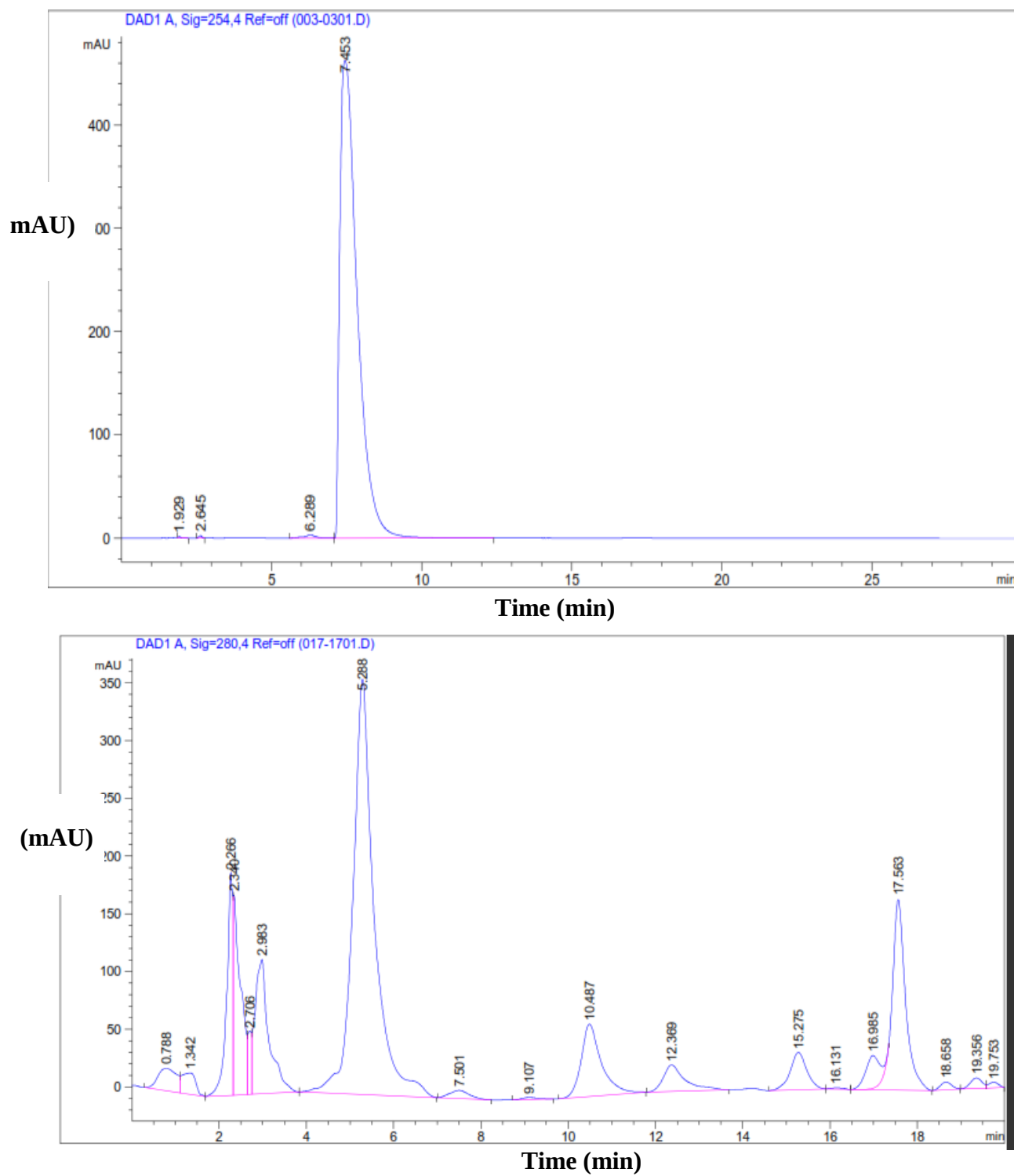


Figure 5-5 Simultaneous HPLC–UV chromatograms of (A) standard securinine and (B) dukung anak methanol extract identification based on retention time.

5.4.2 Effect of extracts on electrolyte leakage

Total electrolyte leakage was investigated in only ginger crude extract using the above concentrations since ginger crude extracts showed a significant effect in controlling *C. gloeosporioides in vitro*. There was a significant effect ($P < 0.05$) of extracts on electrolyte leakage after 8 h incubation in the water bath at 20°C (

Appendix 0-8). The leaked electrolyte varied in amount according to the concentration used. Total electrolyte leakage increased after 4 h and reached a plateau at the end of the 8 h incubation ((Figure 5-6).

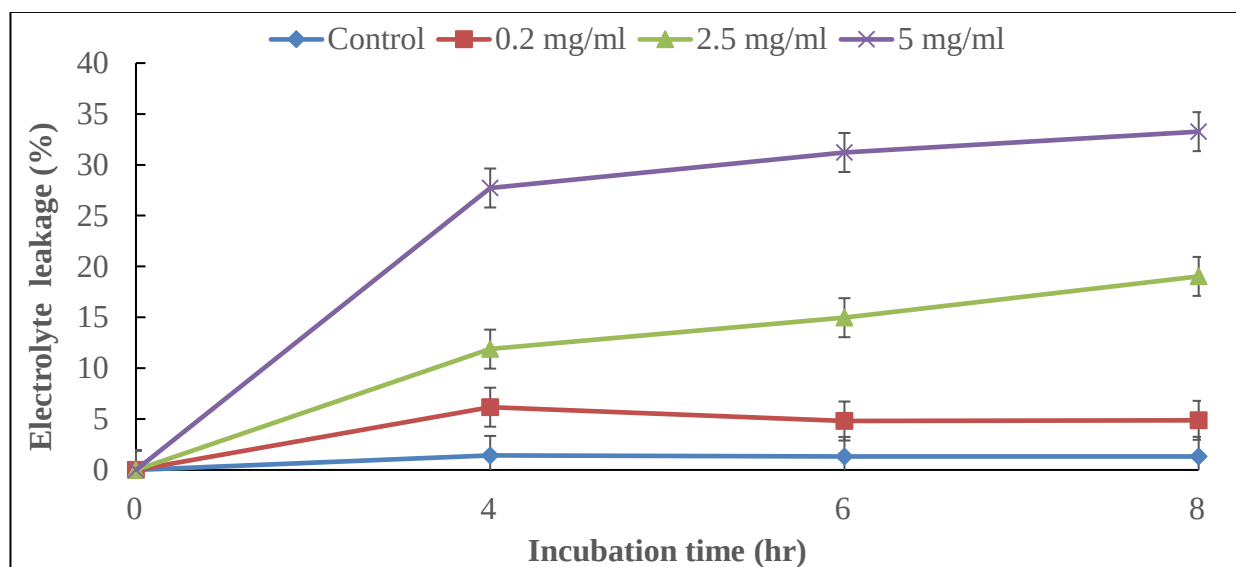


Figure 5-6 Total electrolyte leakage from fungal mycelia treated with ginger extracts at different concentrations during an 8 h incubation in a water bath at room temperature.

The leakage of electrolytes from the fungal mycelia reached the highest (30.73%) at 5 mg/ml after 8 h compared to control (1.37%) (Figure 5-7). The leakage of electrolyte leakage was

dose-dependent and at the end of 8 h incubation, total electrolyte leakage was highest especially at 5 mg/ml.

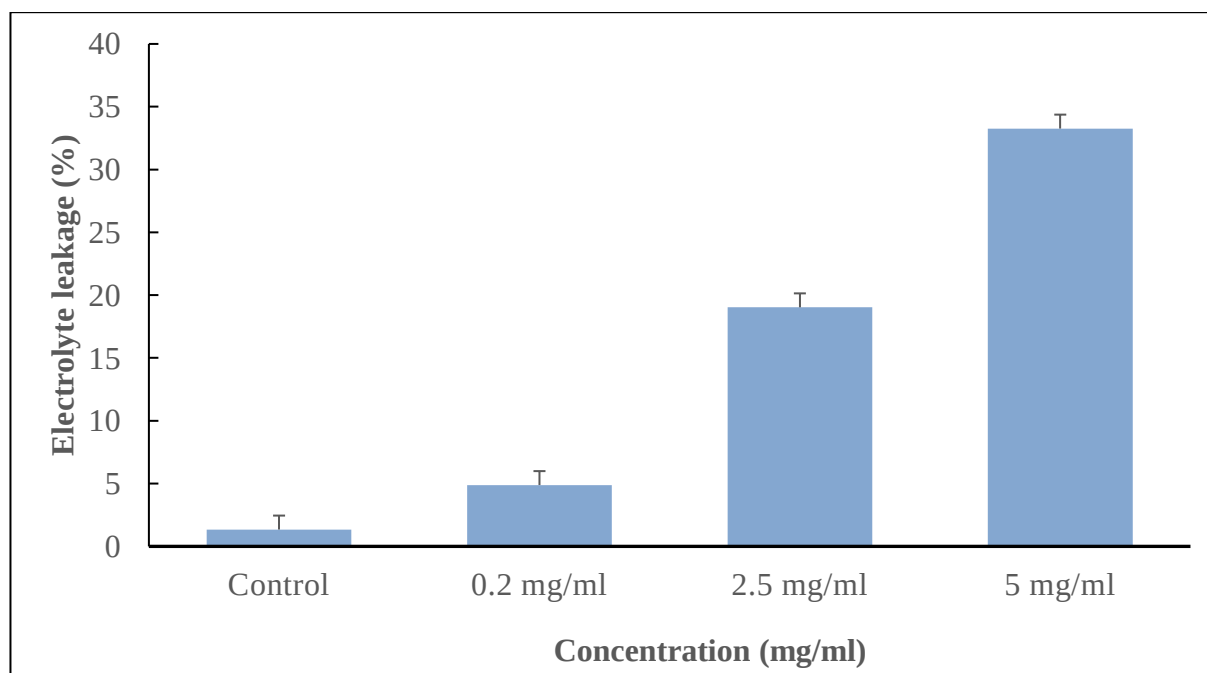


Figure 5-7 Effect of ginger concentration on total electrolyte leakage from fungal mycelia at the end of 8 h incubation in a water bath at room temperature.

5.5 Discussion

5.5.1 Bioactive compounds responsible for antifungal activity

Identification of the main bioactive compound in extracts was carried out by HPLC. Presence of bioactive compound in extract was confirmed based on retention time between standard compounds and compounds present in extracts. This study did not confirm the presence of securinine as the main antifungal bioactive compound in dukung anak. *P. amarus* has been considered as a type of *P. niruri* in the 1990's, an assertion made during a major reorganisation of the *Phyllanthus* genus (Taylor, 2003). The presumption that securinine found in *P. amarus* that resulted in antifungal effect against *Colletotrichum* spp (Singh et al., 2007; 2008) could be

the same bioactive compound responsible for antifungal effect by *P. niruri* against *C. gloeosporioides* was not confirmed in this present study. We propose other alkaloids such as ent-norsecurnine a derivative of securinine in dukung anak could be responsible for this antifungal activity, as *in vitro* studies reported the efficacy of this compound against some phytopathogens including *Colletotrichum* spp. (Goel et al., 2002).

However, this study confirmed the presence of 6-gingerol (ginger) and curcumin (turmeric) as the main antifungal compounds against *C. gloeosporioides* that caused dragon fruit anthracnose, based on retention time between standard and crude extract. This confirms other studies where gingerol and curcumin were reported as the major bioactive compounds in ginger and turmeric respectively (Chattopadhyay et al., 2004; Singh et al., 2008; Michielin et al., 2009; Elaissi et al., 2011; Hossain et al., 2011; Singh and Jain, 2012; Ferreira et al., 2013; Radwan et al., 2014). Hasan et al. (2012b) confirmed the presence of gingerols in methanolic ginger extracts which was antifungal against several clinical pathogens. Ficker et al. (2003) further confirmed the presence of 6-gingerol among other n-gingerol (6-, 8- and 10-gingerol) as one of the effective bioactive compounds against several filamentous fungi. Additionally, our study confirms Al-rahmah et al. (2013) reports, where *Z. officinale* extracts were strongly active against several phytopathogens with Minimal Inhibitory Concentration (MIC) of 4 mg/ml and Minimum Fungicidal Concentrations (MFC) of either 8 mg/ml or 16 mg/ml (depending on the fungus) and they further confirmed that this was due to the presence of gingerol (46.85%), the main bioactive compound present in ginger.

Conclusive reports further confirmed that curcuminoids showed significant antibacterial as well as antifungal activity compared to volatile oil in turmeric (Singh and Jain, 2011; Radwan et al., 2014). Clinical trials have shown a significant effect of curcumin against many clinical

pathogens (Martins et al., 2008; Neelofar et al., 2011; Khalil et al., 2012; Ferreira et al., 2013). Additionally, Kim et al. (2003) reported a fungicidal effect of n-hexane turmeric rhizome crude extracts against several phytopathogens at 1000 mg/L and this was due to the presence of curcumin. This present study confirms the report by Kim et al. (2003) where turmeric extracts significantly controlled *C. gloeosporioides* due to the presence of curcumin. However, other studies also report the presence of bisdemthoxycurcumin in turmeric extracts as an effective bioactive compound in controlling *Colletotrichum* spp. (Cho et al., 2006; Radwan et al., 2014).

5.5.2 Effect of extracts on total electrolyte leakage of fungus

In this study, the mechanism that these plant extracts especially either ginger or turmeric extracts employed in controlling the fungus was due to electrolyte leakage from the fungal cell wall since both extracts showed a significant antifungal effect against the fungus *in vitro*. We confirm that the presence of 6-gingerol and curcumin in ginger and turmeric may have accounted for this effect by interacting with the fungal cell wall leading to the leakage. Thus, these compounds might have several invasive targets which could lead to the inhibition of pathogens (Bajpai et al., 2009). Poeloengan (2011), reported that the antibacterial effect of ginger is due to the presence of gingerol. As a major phenolic compound in ginger with certain surface tension reducer activity, gingerol can create a detergent-like effect (MDIdea, 2009). This allows gingerol to rupture bacterial outer cell membranes leading to loss of cell permeability properties, which in turn cause leakage, including nucleic acid, metabolites, and ions (Poeloengen, 2011). Oonmetta-aree et al. (2006), also confirmed with our report that higher ginger extract concentrations resulted in higher cell materials, including nucleic acid, critical molecules, and ion loss.

Similarly, the antifungal mechanism of curcumin against several clinical pathogens showed that curcumin induced leakage of intracellular components, particularly ions leading to fungal cell death and growth (Lee and Lee, 2014). In untreated *C. albicans* cells, 17.50% of the potassium ions were released from cells, whereas curcumin-treated cells showed a 50.40% release of potassium ions (Lee and Lee, 2014). Cellular homeostasis is central to life, and in microbial pathogens. It is attained by controlling the permeability of the cytoplasmic membrane to ions and solutes. The potassium ion gradient is an important determinant of growth and survival through its role in regulating cytoplasmic pH and cell structure (Roosild et al., 2010). Loss of cytoplasmic potassium would, therefore, lead to cell death in fungi (Pena et al., 2013). Leakage of potassium ions can be used in the evaluation of membrane-lytic events in fungi and liposomes as the internal ionic environment in cells is usually potassium rich (Orlve et al., 2002). The loss of the potassium gradient across the cell membrane because of membrane damage can lead to compromise of membrane functions and eventually cell death.

Our study confirms other studies where bioactive compounds in some natural products (chitosan) or extracts attacked the fungal cell walls and membranes i.e., affecting their permeability and release of intracellular constituents (e.g. ribose, Na glutamate) and interfere with membrane functions (electron transport, nutrient uptake, protein, nucleic acid synthesis and enzyme activity) (Rozwalka et al., 2010; Sánchez-Domínguez et al., 2011; Baños-Bautista et al., 2012). Ouf et al. (2008) also confirm our report, where leakage of electrolyte from several phytopathogenic hyphae was directly proportional to the concentration of biocides

5.6 Concluding remarks

All the tested plant extracts significantly inhibited the growth criteria of *C. gloeosporioides* that causes anthracnose in dragon fruit. This was evident in inhibition of mycelial growth,

conidial germination which translated to morphological damage in the fungal hyphae during SEM studies. The efficacy was highest in ginger crude extracts followed closely by turmeric based on the *in vitro* studies. Antifungal activity observed during the *in vitro* study at 7.5 mg/ml or 10 mg/ml especially in ginger extract was very significant against *C. gloeosporioides* and is comparable to the synthetic fungicide (mancozeb) at 2 mg/ml. We propose that the presence of mainly 6-gingerol in ginger and curcumin in turmeric was responsible for the controlling *C. gloeosporioides in vitro* and its corresponding effect in causing anthracnose *in vivo*. Additionally, the mechanism these plant extracts employed to control the fungus was through the leakage of electrolyte from fungal cell wall which resulted in inhibition of fungal growth to control anthracnose in dragon fruit.

In the earlier study (chapter 4), it was observed that, the original colour and smell of dragon fruit was compromised when fruits were treated with turmeric and ginger at 10mg/ml or 15 mg/ml coupled with phytotoxicity problems especially at 15 mg/ml. Therefore, a recommended suggestion and approach to enhance the efficacy of the crude extracts while reducing the problem of phytotoxicity is to incorporate the extracts with edible coatings such as GA, which studies show serves as a carrier to bioactive compounds, modify the internal atmosphere of fruit and enhance external quality appearance.

CHAPTER 6

6 FORMULATION OF CRUDE EXTRACT INCORPORATED WITH GUM ARABIC ON ANTHRACNOSE DISEASE OF DRAGON FRUIT

6.1 Introduction

Gum Arabic has been reported to have a synergistic effect when incorporated into other natural products/synthetic fungicides or alone in controlling diseases and maintaining the physiochemical properties of fruits. It is generally regarded as safe and leaves no harmful residual effect when applied as a treatment on fruits (Maqbool et al. 2010a; 2010b). The combination of GA incorporated with cinnamon essential oil at 0.4% significantly reduced anthracnose by 80% in banana (at $13\pm 1^{\circ}\text{C}$) and 71% in papaya (at $13\pm 1^{\circ}\text{C}$) after 28 days followed by 5 days of shelf life at room temperature (Maqbool et al., 2011). Other studies also reported 70%-80% reduction of anthracnose in banana fruits after treatment with 10%GA+0.75% or 1% Chitosan after 28 days of storage at 13°C (Maqbool et al. (2010a; 2010b). Additionally, incorporation of 10%GA with 3% calcium chloride significantly alleviated anthracnose in mango fruits (Khaliq et al., 2016). It is confirmed that GA has no antifungal effect but plays a significant physicochemical role in maintaining fruits quality postharvest (Maqbool et al., 2010a). Therefore, incorporation of ginger, turmeric and dukung anak crude extracts with GA at 10% may provide multiple effects in controlling anthracnose on dragon fruits by providing better modified atmosphere around the fruit which is good for fruit maintenance and further extend the shelf life of dragon fruits. As a film-forming material, GA is

reported to help reduce phytotoxicity (Maqbool et al., 2011) and serve as a carrier for bioactive compounds in plant crude extracts. Therefore, incorporation of each plant crude extract with GA will help reduce the phytotoxic problems since our previous study showed that at high concentrations (15 mg/ml), there were toxicity problems on the fruits which resulted in severe anthracnose infestation and other secondary infections (See chapter 4).

The objective of the study is;

- Prepare a formulation by incorporating ginger, turmeric and dukung anak crude extract separately with 10% gum arabic.
- Investigate the efficacy of each formulation on disease incidence (anthracnose) and disease severity of dragon fruit caused by *C. gloeosporioides*.

6.2 Material and methods

6.2.1 Formulation of Gum arabic incorporated with plant extracts

6.2.1.1 Preparation of Gum arabic

GA solution was prepared as per protocol of Maqbool et al. (2010a). About 150 g of gum arabic powder was dissolved in 1500 ml of ULTRA pure water (PURELAB ELBA system Option-R&BP; Veolia Water Systems, High Wycombe, UK) to obtain 10%GA. The GA solution was stirred constantly at low heat (40⁰C) using a hot plate magnetic stirrer (Model LHS-HTS-1003; Bunkyo-Ku, Tokyo, Japan) for 60 min. The solution was then filtered through four layers of cheesecloth to remove any undissolved impurities. Each plant crude extract obtained was then incorporated separately with 10%GA to make the formulation whiles stirring on magnetic stirrer until a uniform solution is obtained.

6.2.1.2 Incorporation of plant crude extract with gum Arabic.

A measured weight of each crude extract (ginger, turmeric, and dukung anak) was incorporated with 10%GA solution obtained in section 6.2.1.1 to prepare a stock concentration of 22.17 mg/ml from which the working concentrations (treatments) for the experiment was prepared (Figure 6-1). To prepare the stock solution, each known weight of crude extract was re-dissolved with 0.05 mole fraction of methanol as the co-solvent (Jaapar et al., 2014) and the final stock solution was made to 22.17 mg/ml by addition of 10%GA solution (v/v). A working concentration (treatments) of 5 mg/ml+10%GA, 10 mg/ml+10%GA and 15 mg/ml+10%GA per plant extract was prepared by diluting the stock with already prepared 10%GA solution (Figure 6-1). The pH of each working solution was adjusted to 5.6 by adding 1 M NAOH using a digital pH meter (Model CyberScan pH 510; Eutech Instruments Pte.Ltd. Singapore). Each working concentration was left a magnetic stirrer for 2-3 hours under low heat of 40°C. The un-dissolved materials (residues) were filtered with three layers of cheesecloth and discarded. The filtrated was used for DS and DI as well postharvest quality parameters (as mentioned in next chapter 7).

6.2.2 Efficacy of formulation of disease incidence and severity

6.2.2.1 Preparation of fungal spore suspension

Fungal spore suspension was carried out according to Zhang et al. (2013) with slight modification. Ten ml purified water was poured into a 10-day old culture of *C. gloeosporioides* obtained from section 4.2.2.1 and then surface scraped lightly with a bent glass rod. Fungal spore suspension was then filtered through a double layer of sterilized muslin cloth. Concentration of spore suspension was adjusted to 10^5 spores/ml using improved Neubauer

haemocytometer (model: Neubauer, Germany) after addition of purified distilled water until a volume of 1500 ml before the *in vivo* test.

6.2.2.2 Plant extract incorporated with gum arabic application on fruits

Dragon fruits of uniform size, colour and with no deformity were washed with sodium hypochlorite (1%), rinsed with purified water and air-dried at room temperature ($25\pm 2^{\circ}\text{C}$). All fruits were then inoculated [(dipped for 2 min in a spore suspension of *C. gloeosporioides* (10^5 spore/ml)) and air-dried completely at ambient ($25\pm 2^{\circ}\text{C}$). Air-dried fruits were dipped for 2 min in each formulated treatment (Figure 6-1) and left to dry completely at room temperature. Inoculated fruits and those treated with fungicide (mancozeb-2 mg/ml) for 1 min served as negative and positive control (Figure 6-1). Previous results from *in vitro* studies had shown that the entire methanol had evaporated at room temperature when 0.05 mol fraction of methanol was used as a co-solvent in preparation of crude extracts and therefore methanol was not added in the controls. All fruits were then allowed to dry completely followed by packaging in commercial packaging cartons and stored at $11\pm 2^{\circ}\text{C}$, 80% RH for 28 days (Ali et al. (2010)). For disease incidence and severity, a total of 330 fruits were used. For each plant extract, a total of 90 fruits were used (30 fruits per treatment). For each treatment, there are 3 replicates and each replicate contain 10 fruits. Positive and negative controls were made of 30 fruits each, bringing the total fruits for DI and DS to 330 fruits.

10%GA+5 mg/ml	• Ginger • Turmeric • Dukung anak
10%GA+10 mg/ml	• Ginger • Turmeric • Dukung anak
10%GA+15 mg/ml	• Ginger • Turmeric • Dukung anak
Negative control	• Inoculated fruits (10^5 spore/ml)
Positive control	• Fungicide (Mancozeb-2 mg/ml)

Figure 6-1 Treatments used to control anthracnose during postharvest storage of dragon fruit.

6.2.2.3 Disease incidence and disease severity

The effect of crude extracts on disease incidence and disease severity was evaluated weekly for 28 days during cold storage. Disease incidence data were presented as the percentage of fruit showing anthracnose out of the total number of fruit in each treatment, while disease severity was scored following the scale (1 = 0% of fruit surface rotten; 2 = 1–25%; 3 = 26–50%; 4 = 51–75% and 5 = 76–100% rotten) (Sivakumar et al., 2002).

6.2.3 Effect of formulation on external appearance

Based on the limitation that was encountered when fruits were treated with only plant crude extracts (chapter 4, section 4.4.2.3), we sort to further investigate and enhance the external quality appearance by incorporation of plant extracts with GA. This was to further understand consumer preference at the marketing level on fruit external quality (appearance and smell). An observational study was conducted using 20 participants to ascertain which fruit had the best consumer appeal after fruits were treated with formulation prepared in section 6.2.1.3. Participants were asked to ascertain whether fruits treated at 10%GA+10 mg/ml and 10%GA+15 mg/ml enhanced fruit external appearance (smell, glossiness and peel colour) irrespective of plant extract (ginger or turmeric) used.

6.3 Statistical analysis

Two-way Anova was performed for the efficacy of extracts on disease incidence and disease severity using computer software GenStat version 18th Edition (VSNI product). Means were separated using Fisher unprotected test at ($P < 0.05$). Disease incidence data were transformed (arcsine of the square root of the proportion of affected fruit) before analysis. Experiments were repeated twice and data pooled.

6.4 Results

6.4.1 Efficacy of formulation on disease incidence

There was a significant effect ($P < 0.05$) of fruits coated with 10%GA plus plant extracts at different concentrations on disease incidence (DI) during the 28 days of cold storage (Appendix 0-9). DI increased progressively with storage time in both control and treated fruit (**Error! Reference source not found.**). The onset of DI began after day 14 and increased until day 28

where all the fruits in both treated and control recorded 100% DI (**Error! Reference source not found.**). It is imperative to state that the efficacy of the formulation on dragon fruit DI and DS was best reduced at day 21 since by day 28, all the fruits have rotten showing severe symptoms of anthracnose and other secondary infections. At day 21, fruits treated with 10%GA+10mg/ml of turmeric crude extracts significantly ($P<0.05$) recorded lowest DI which is somewhat comparable to fungicide mancozeb followed closely by fruits treated with 10%GA+10 mg/ml of ginger crude extract, while the control fruits recorded the highest DI control.

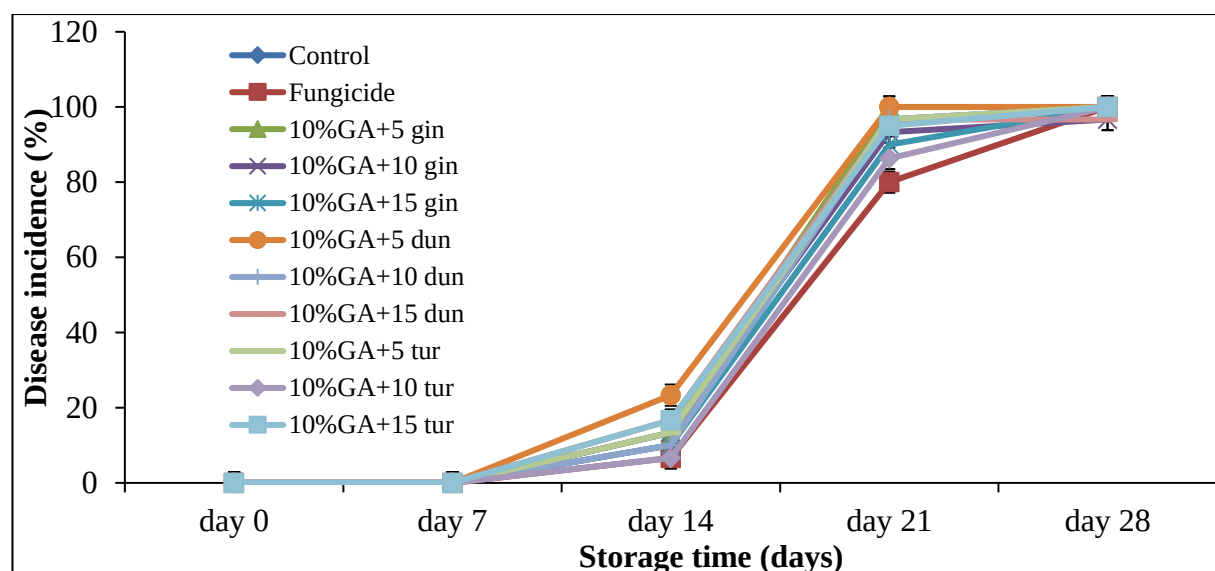


Figure 6-2 Effect plant crude extract at different concentrations incorporated with of 10%GA on disease incidence during cold storage for 28 days.

Generally, high DI was observed in fruits treated with 10%GA plus either 15 mg/ml or 5 mg/ml of crude extracts which was not different in control fruits after 28 days of storage (Figure 6-3).

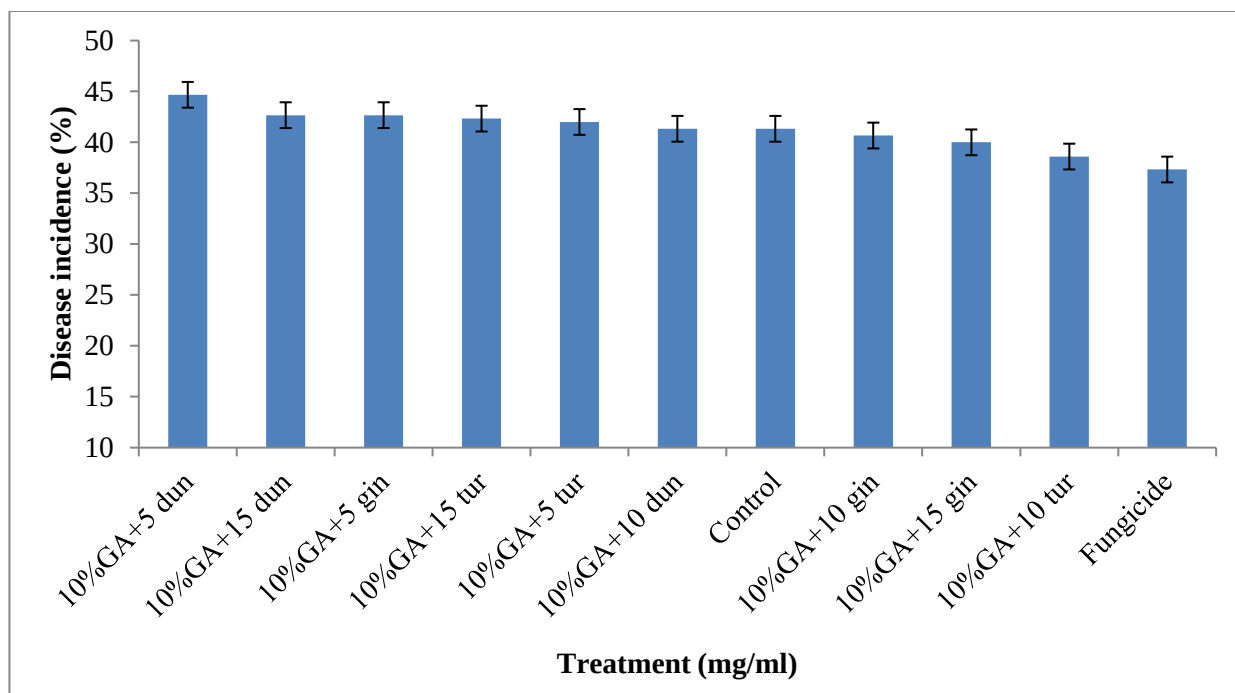


Figure 6-3 Effect of 10%GA incorporated with plant extracts at different concentrations on disease incidence after 28 days of cold storage.

At the end of the 28 days of storage, fruit coated with 10%GA plus 10 mg/ml of turmeric recorded ($P < 0.05$) the lowest DI (Figure 6-3; Figure 6-4) which was not different in fruits coated with 10%GA plus either 10 mg/ml or 15 mg/ml of ginger extracts compared to control (Figure 6-3; Figure 6-5). Fruit coated with 10%GA plus dukung anak extracts didn't show any significance to control DI irrespective of concentration used (Figure 6-3).

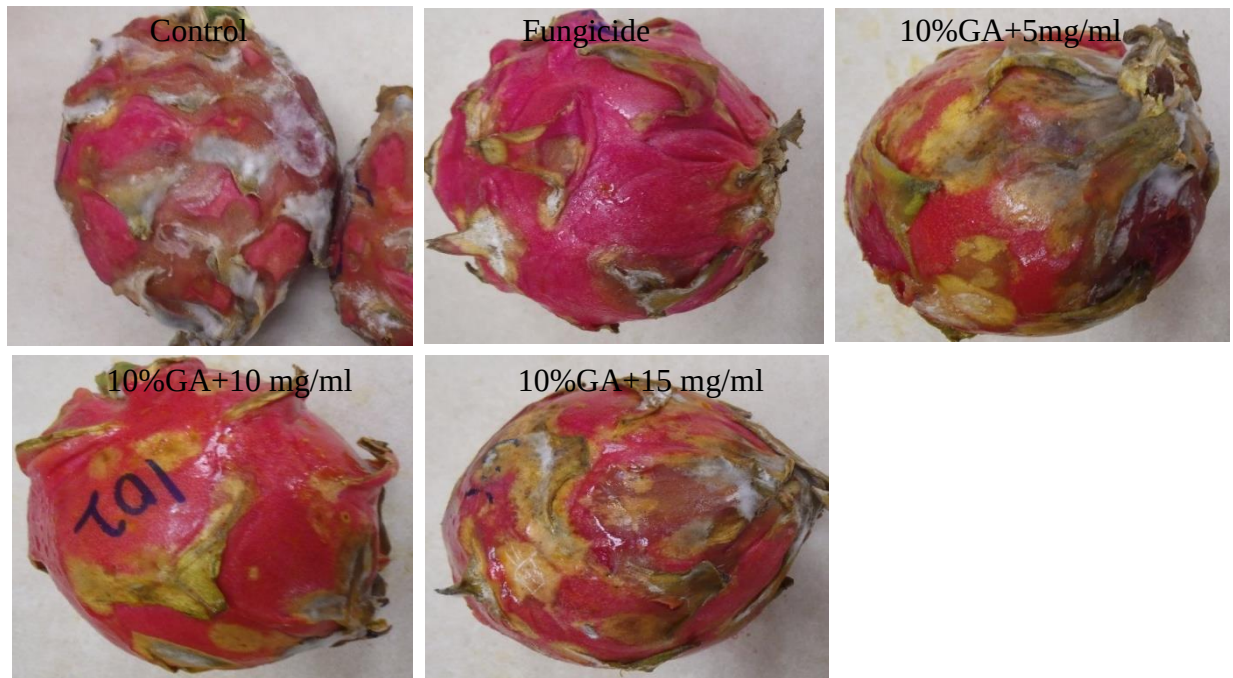


Figure 6-4 Effect of turmeric extract at different concentrations incorporated with 10%GA on disease incidence and severity of dragon fruits after 28 days of storage.

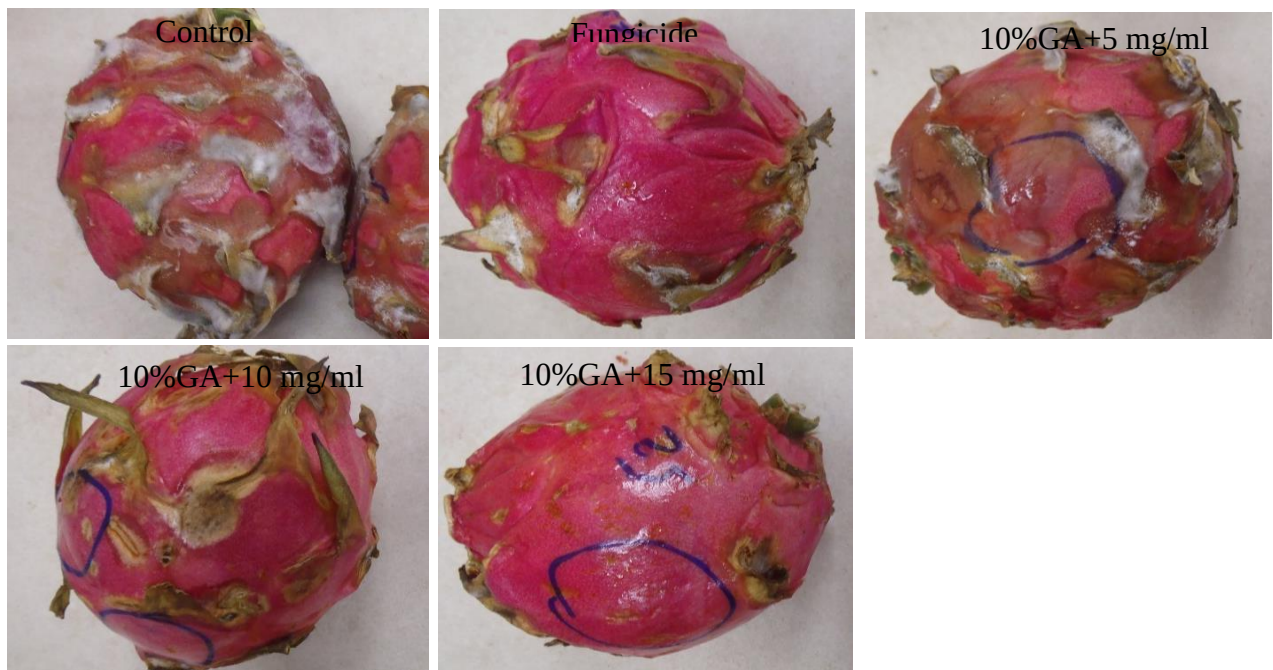


Figure 6-5 Effect of ginger extracts at different concentrations incorporated with 10%GA on disease incidence and severity of dragon fruits after 28 days of cold storage.

6.4.2 Efficacy of formulation on disease severity

A significant effect ($P<0.05$) of fruits coated with 10%GA plus crude extracts at different concentrations on disease severity (DS) was observed after 28 days of cold storage (

Appendix 0-10). Disease severity progressed significantly ($P<0.05$) with storage time and it was low in treated fruits compared to control fruits (Figure 6-6).

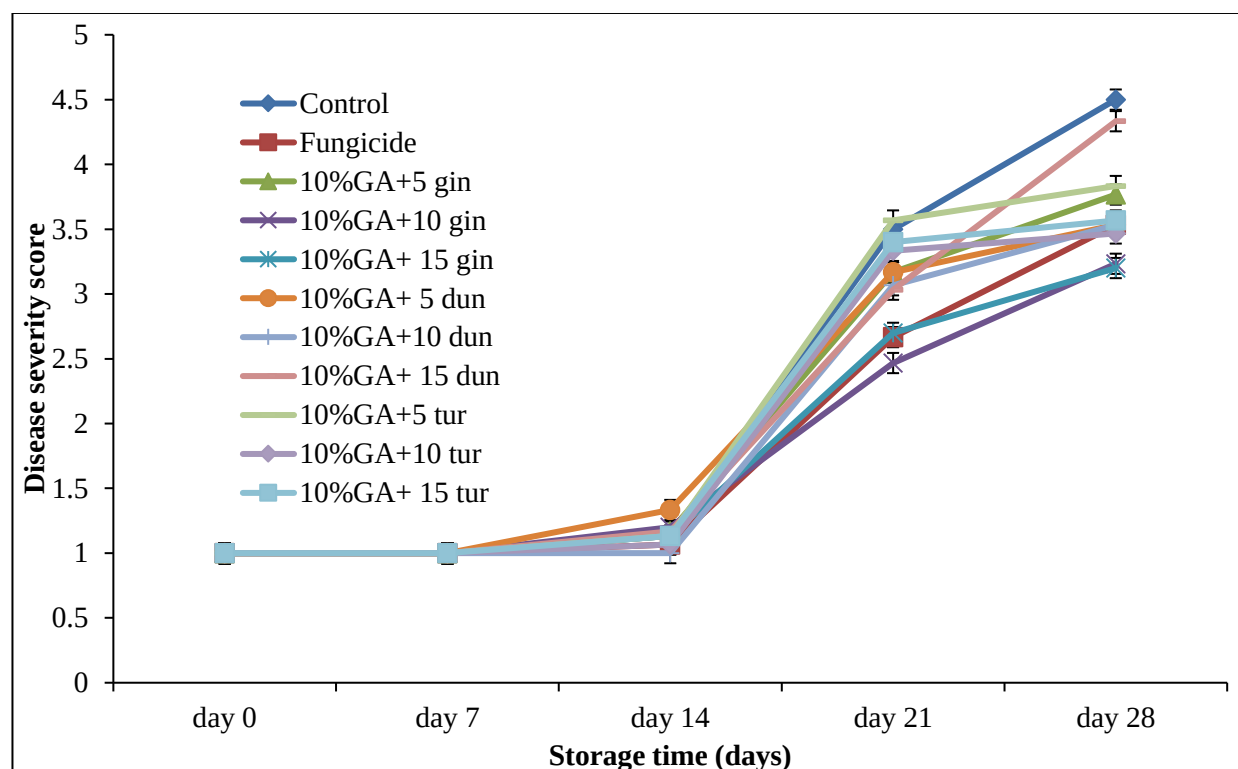


Figure 6-6 Effect of plant extracts at different concentrations incorporated with 10%GA on disease severity during cold storage for 28 days.

A similar trend in DI was observed in DS. A DS score- 1 (an indication of no DS) was observed until after day 14 and remained high throughout the 28 days of storage. At day 21 which marks the best storage time of treatment on DS, 10%GA+10 mg/ml of ginger-treated extracts significantly reduced DS (DS score- 2.45) compared to control (DS score- 3.5). The reduction in DS was due to relatively low DI recorded in fruits treated with 10%GA+10mg/ml

of ginger extract. At the end of the 28 days storage, DS was significantly ($P<0.05$) low in fruits treated with 10%GA+10 mg/ml of ginger extracts (DS score- 3.23), while control fruits had a DS score- 4.5 (Figure 6-6). It was also observed that DS in fruits coated with 10%GA+10 mg/ml of turmeric crude extracts (DS score- 3.46) or 10%GA+10 mg/ml of dukung anak (DS score-3.53) were low and comparable to fungicide (Mancozeb) (DS score-3.53) after 28 days of storage. This was because such treatments recorded relatively low DI.

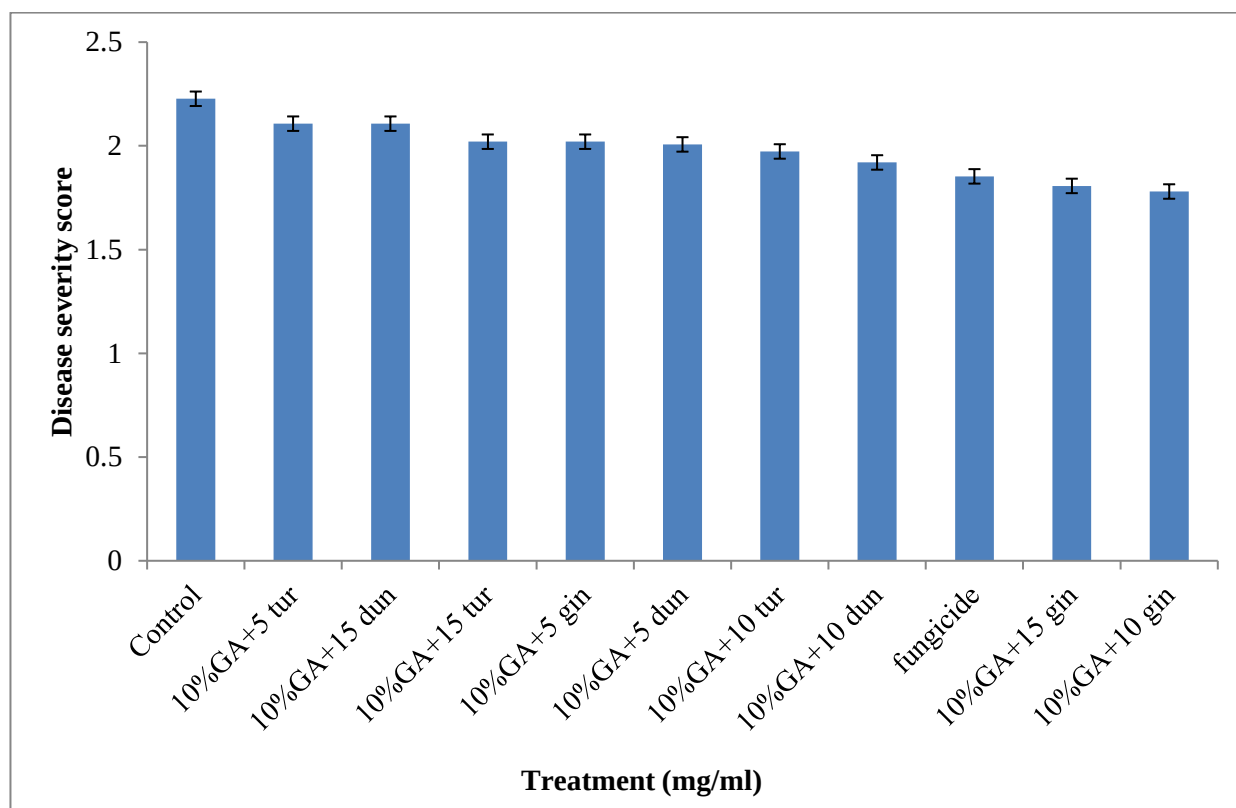


Figure 6-7 Effect of plant extracts at different concentrations incorporated with 10%GA on disease severity after 28 days of cold storage

overall, 10%GA+10 mg/ml of ginger significantly ($P<0.05$) reduced DS which was not different from the fungicide-treated fruits after 28 days of storage (Figure 6-7). Fruits coated with 10%GA+5mg/ml or 10%GA+15 mg/ml of either dukung anak or turmeric (Figure 6-7) didn't significantly reduce DS (Figure 6-7).

6.4.3 External appearance of fruit quality after formulation application

Incorporation of 10%GA with plant extracts markedly enhanced the external appearance of fruits, even at the highest concentrations. All treated fruit irrespective of the plant extracts had an enhanced external glossy appearance after incorporation with 10%GA (Figure 6-8; bottom row 10%GA+15 mg/ml and 10%GA+10 mg/ml). Additionally, the dragon fruits original colour was maintained when 10%GA was incorporated with crude extracts especially in turmeric or ginger-treated fruits (Figure 6-8; Bottom row 10%GA+15 mg/ml and 10%GA+10 mg/ml) compared to when only the extracts were applied in fruits (Figure 6-8; Upper row 15 mg/ml and 10 mg/ml).

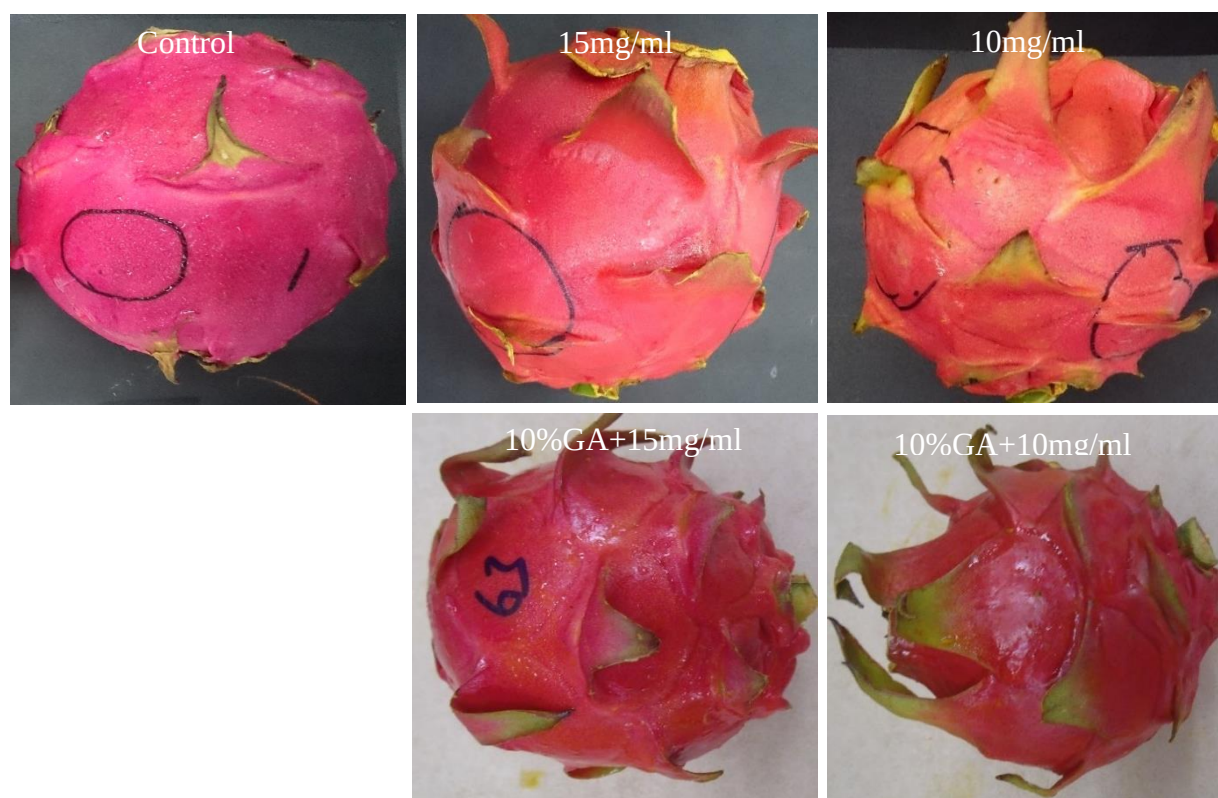


Figure 6-8 External appearance of turmeric-treated fruits alone (upper row) and after incorporation with 10%GA (bottom row) at day 7.

Incorporation of 10%GA with plant extracts to some extent reduced the intensity of crude extracts smell especially in ginger and turmeric-treated fruit at 10 mg/ml or 15 mg/ml allowing dragon fruits to somewhat maintain its original smell and colour. This was based on observation from the 16 panelists out of the 20.

6.5 Discussion

6.5.1 Efficacy of formulation on disease incidence and severity

Composite coating solution of 10%GA plus 10 mg/ml of either ginger or dukung anak or turmeric controlled anthracnose and its severity. However, this was pronounced in 10%GA plus 10 mg/ml of turmeric crude extract which is comparable to the fungicide. Whiles GA is the most extensively used hydrocolloid in the food industry (Vivas et al., 2001; Motlagh et al., 2006; Rinsky and Rinsky, 2009), no study on its antifungal or fungitoxicity has been reported (Maqbool et al., 2010a). Nonetheless, crude extracts of ginger, turmeric and dukung anak are known to possess strong antifungal effects against several phytopathogens including *C. gloeosporioides* (Singh et al., 2007; Singh et al., 2008; Radwan et al., 2014; Dissanayake and Jayasinghe, 2013; Ademe et al., 2014; Al-Askar et al., 2014; Ojaghian et al., 2014; Wongkaew and Sinsiri, 2014).

In this present study, composite coatings of 10%GA plus 10 mg/ml of turmeric or ginger delayed anthracnose and its severity. We proposed a synergistic effect of 10%GA and the crude extracts played a vital role to achieve this result. The 10%GA served as carrier for the crude extracts allowing the slow release of bioactive compounds in crude extracts to control anthracnose on fruits during the postharvest storage. Additionally, 10%GA also provided a good film-forming property around the fruit surface, creating a modified atmosphere of carbon

dioxide and oxygen, which helped to delay the ripening process and senescence (Dang et al., 2008), whilst the 10 mg/ml of especially turmeric crude extracts was the optimum concentration that was antifungal against *C. gloeosporioides*. Pectate lyase is one of the most dominant enzymes and is responsible for the maceration of fruit tissue leading to decay, and consequently produce symptoms of infection and lesion (Prusky et al., 2001). The secretion and activity of this enzyme is influenced by pH of the host. To cope with this problem, Ammonia (NH₃) is secreted by the fungus which would act as a buffer, resulting in host tissue alkalinisation which elevates the pH of the fungal inoculated fruit allowing fungal attacks on fruit tissue (Awang et al., 2011). Therefore, the presence of 10 mg/ml crude extract of turmeric extracts (which contains bioactive compounds) may have synergistically reduced fungal attacks by delaying the secretion of ammonia as a buffer from the fungus, thereby reducing dragon fruit tissue alkalinisation and decreasing the pH of the fruit. It is imperative to say that the presence of bioactive compounds (in the 10 mg/ml of extracts) especially gingerols (ginger) and curcumin (turmeric) and some alkaloids in dukung anak (Singh et al., 2007; Singh et al., 2008; Awang et al., 2011; Radwan et al., 2014; Dissanayake and Jayasinghe, 2013; Ademe et al., 2014; Al-Askar et al., 2014; Ojaghian et al., 2014; Wongkaew and Sinsiri, 2014) resulted in the antifungal effect against *C. gloeosporioides* that causes postharvest anthracnose. Whiles our study confirms 6-gingerol (in ginger) and curcumin (in turmeric) as the main bioactive compounds responsible for the antifungal activity during the HPLC analysis, securinine in dukung anak cannot be confirmed as the bioactive compound responsible for its activity against *C. gloeosporioides* that caused anthracnose in dragon fruit during storage. The presumption that securinine found in *P. amarus* that resulted in antifungal effect against *Colletotrichum* spp (Singh et al., 2007; 2008) could be the same bioactive compound responsible for antifungal

effect by *P. niruri* against *C. gloeosporioides* was not confirmed in this present study. We propose other alkaloids such as ent-norsecurnine a derivative of securinine in dukung anak could be responsible for this antifungal activity, as *in vitro* studies reported the efficacy of this compound against some phytopathogens including *Colletotrichum* spp. (Goel et al., 2002). Our study confirms findings from Maqbool et al. (2010a; 2010b); Maqbool et al., (2011), Khaliq et al., (2016) where natural products such as chitosan, essential oils incorporated with GA synergistically helped to control anthracnose in banana, papaya, and tomatoes while extending their shelf life.

6.6 Concluding remarks

Application of either turmeric or dukung anak extracts alone at 5 mg/ml or 10 mg/ml significantly controlled anthracnose for 21 even though to some extent until 28 days as reported earlier in chapter 4 depending on the plant extract used and the concentration. Nonetheless, there was a drawback due to the external quality appearance caused by the crude extracts which affected consumer preference during the observation analysis in terms of fruits smell and intense colour. Additionally, when ginger extracts alone were applied at 5 mg/ml and above, phytotoxicity was observed. To address the problem of unattractive external quality appearance of fruits that affected consumer selection as well as reduce phytotoxicity, each extract at their respective concentrations were incorporated with 10%GA. The present study showed that fruits treated with a composite coating of 10%GA plus 10 mg/ml (for all extracts) or 10%GA plus 15 mg/ml (ginger extract) significantly reduce anthracnose and its severity. However, the reduction of DI and DS was more pronounced in 10%GA+10mg/ml turmeric or ginger-treated fruits.

This chapter clearly shows that plant extracts at 15 mg/ml incorporated with 10%GA clearly reduced the phytotoxicity on fruits while enhancing their external quality. Since anthracnose is one of the major economic disease postharvest during the supply chain, proper diagnostic tool for early detection of the disease is very important to reduce postharvest losses. Using diagnostic tool such as Loop-mediated isothermal amplification (LAMP) can help overcome this problem.

CHAPTER 7

7 LOOP-MEDIATED ISOTHERMAL AMPLIFICATION: A DIAGNOSTIC TOOL FOR EARLY DETECTION OF *C. gloeosporioides* ON INFESTED DRAGON FRUIT

7.1 Introduction

The detection of phytopathogens needs be quick and accurate in the field to ensure unsafe, infected foods are not shipped to consumers (Niessen, 2015). Diseases like anthracnose result in about 50% postharvest losses in dragon fruits, and it affects the fruits during storage and transportation. Diagnosis of *Colletotrichum* spp. have been performed traditionally over the years using classical mycological methods involving isolation from host materials followed by morphological and cultural identification (Abang et al., 2002; Barnett and Hunter, 1972). With the existence of species-specific primers based on nucleotide sequences of internal transcribed spacer region 1 (ITS 1) of rDNA, PCR among other techniques has become a tool to aid in the identification of *Colletotrichum* species (Brown et al., 1996). Other molecular techniques that have been developed and implemented for more accurate characterization and identification of some pathogens such as *Colletotrichum* spp. include restriction fragment length polymorphism (RFLP) analyses of mitochondria DNA (Sreenivasaprasad et al., 1992), ELISA (Hughes et al, 1997), AT-rich analyses (Freeman et al., 2000), amplified fragment length polymorphism (AFLP) (Rodriquez-Guerra et al., 2003), random amplified polymorphic DNA (RAPD) (Whitelaw-Weckert et al.,2007) and branched DNA (bDNA) amplification, Invader, and rolling circle amplification (RCA) methods (Erice et al., 2000; Lizardi et al., 1998; Lyamichev et al., 1999). Although useful, these methods are time consuming and are unable to yield accurate

results under specific short time. Also, these methods have been shown to have features that make it not practically ideal for the field or on-site (Niessen et al., 2015). Additionally, due to the quiescent nature of the *C. gloeosporioides* in causing the disease to the host (dragon fruit), the importance of speed and efficiency of diagnostic techniques for early detection of *C. gloeosporioides* should be of economic importance in controlling postharvest anthracnose in dragon fruit. Therefore, early detection is essential to control emerging, re-emerging or any infectious diseases since the spread of diseases are easier overcome at early stages (Morse, 2007). As a simple, cost-effective and rapid method for specific DNA-based detection, LAMP is used by plant pathologist as a potential application tool for diagnosis of pathogenic fungi as well as surveillance of plant diseases without requiring sophisticated equipment (Nagamine et al., 2002; Njiru et al., 2008; Bekele et al., 2011; Tomlinson et al., 2012). The sensitivity of LAMP is high, with 8×10^{-2} pg/ μ l genomic DNA as the lowest detectable concentration (Tao and Cai, 2013). In recent times, its application has been used for detection of cassava brown streak virus that causes cassava brown streak disease in cassava (Tomlinson et al., 2012).

Despite several studies suggesting the use of the LAMP technique for clinical diagnosis of diseases as well as in plant pathology on field crops, no study of LAMP technique as a diagnostic tool for detection of *C. gloeosporioides* that causes anthracnose in dragon fruits during postharvest storage has been investigated.

The objectives of this research are:

- To confirm the presence of *C. gloeosporioides* with specific Lamp primers on infested dragon fruits using PCR of treated fruits during storage

- To develop a *C. gloeosporioides* specific LAMP assay, and to use this assay as a quantitative measure for direct and early detection of *C. gloeosporioides* in naturally infested dragon fruits after treatment application during postharvest storage.

7.2 Materials and methods

7.2.1 Spore suspension preparation and treatment application.

In this experiment, separate fruits, thus different from those used for the postharvest quality analysis as well as disease incidence and disease severity was used. Prior to application of treatment, a spore suspension was prepared using the method from section 6.2.1.3. The fruits were inoculated followed by treatment application and stored according to section 6.2.1.4. For this part of the experiment, a treatment concentration that significantly reduced disease incidence and severity as well as enhanced most part of postharvest quality was used, and it was compared to the untreated coated fruits (control). Hence, fruits were treated with 10%GA plus 10 mg/ml of each extract and 10%GA plus 5 mg/ml of each extract before storage (Table 7-1). Control fruits were inoculated with only spore suspension. For each treatment, five fruits were randomly selected. Three sample point thus, middle, stem and blossom end that were either infested with anthracnose were taken as replicates per fruit in each treatment for the Lamp assay and PCR. A total of 35 fruits were used including the control. Samples were then analysed for the presence of *C. gloeosporioides* using PCR, as well as by detection and quantification by LAMP assay every week (day 7, day 14 and day 21) for a period of 21 days.

Table 7-1 Number of fruits used for LAMP assay and PCR during cold storage for 21 days

Treatments (mg/ml)	Number of fruits
10%GA+5 dun	5
10%GA+10 dun	5
10%GA+5 tur	5
10%GA+10 tur	5
10%GA+5 gin	5
10%GA+ 10 gin	5
Control (inoculated fruits)	5

The full meaning of each treatment can be found in the list of abbreviation.

7.2.2 Polymerase Chain Reaction

7.2.2.1 Fungal DNA extraction from dragon fruit skin

Fungal DNA extraction from anthracnose infested dragon fruits was carried out using Qiagen DNeasy 96 Plant Mini Kit. Infested dragon fruits with symptoms of anthracnose were randomly selected from each treatment. A small piece of infested tissue was excised which includes; the peel and small portion of the pulp of the fruit. About 2-3 g of this tissue was homogenised using pestle and mortar in liquid nitrogen. About 100 mg of homogenised tissue was used for extraction of fungal DNA using the mini kit.

7.2.2.2 PCR assay

To confirm that *C. gloeosporioides* were responsible for causing anthracnose, PCR was performed using fungal DNA extracted from infested fruits (see section 7.2.2.1). The protocol used for the PCR assay for molecular identification of the pure culture at Section 4.2.2.2 was the

same protocol used in the PCR assay of extracted fungal DNA from either infested or not infested treated and non-treated dragon fruits at each time point (weekly) of postharvest storage. The sequences were compared with those deposited in NCBI GenBank database. The primers used for the PCR assay was specific to *C. gloeosporioides* (Table 7-2). In all, a total of 27 samples were used for PCR assay based on the time point.

7.2.3 Loop Mediated Isothermal Amplification

7.2.3.1 Lamp primer design

Lamp primers (Table 7-2) were designed with specificity to *C. gloeosporioides* beta-encoding region of the beta-tubulin operon using LAMP designer 1.10 (Premier Biosoft).

Table 7-2 Oligonucleotide primers used for LAMP based on ribosomal DNA gene of *C. gloeosporioides*

Assay	Primer	Type	Length	Sequence (5'-3')
PCR	Cgbt F3	Forward	18mer	AATATCATACTTGCGAGGAC
	Cgbt B3	Backward	20mer	CTCCAAATCGACGAGGAC
LAMP	Cgbt F3	Forward outer	18mer	AATATCATACTTGCGAGGAC
	Cgbt B3	Backward outer	20mer	CTCCAAATCGACGAGGAC
	Cgbt FIP	Forward inner primer	41mer	AGCTGTTCGAGTTTAGCGGTCCAGCA ATGGAGTGTATGTCA
	Cgbt BIP	Backward inner primer	44mer	GCATGAGCGTCTACTTCAACGAGGTC AGTACTCGTCAATATGTT
	Cgbt LoopF	Loop forward primer	22mer	AACCACCAATGTGGACAGATAA
	Cgbt LoopB	Loop backward	20mer	CCTTATAGCCCCAAGAGTGC

		primer		
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7.2.3.1 LAMP assay

LAMP was performed using a Genie® III machine (OptiGene) whiles graphs obtained from the assay were with plotted Genie® Explorer v 2.0.6.3. The concentration of primers used was 100 pmol/μl. A primer mix containing 152 μl of water, 4 μl of F3 primer, 4 μl of B3 primer and 20 μl of each of the other 4 primers was made. From this, a reaction mix for 8 LAMP reactions was made containing 46 μl of water, 23 μl of the primer mix and 115 μl of the isothermal master mix, which contains a fluorescent dye, from Optigene. Twenty (20) μl of this reaction mix was then pipetted into each 8 Genie® Strip tubes. The DNA used in the assays were from extracted inoculated treated fruits with symptoms of anthracnose (1st to 6th of the Genie® Strip tube), whiles the 7th Genie® Strip tube containing only fungal genomic DNA from a pure culture and 8th Genie® Strip containing only the reaction mix served as positive control and negative control respectively (Figure 7-1). Measurement was taken at weekly for a period of 21 days. The Genie® Strip was then placed in the LAMP machine for roughly 30 minutes. The products were amplified at 63°C for 30 minutes and then annealing was measured between 98°C and 70°C. Amplification and annealing times are quantified in real-time LAMP as fluorescence is monitored. Each treatment was done in duplicate per one Genie® Strip.

A1	A2	B1	B2	C1	C2	+	-
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Figure 7-1 Layout of Genie Strip used during the LAMP assay. Where A, B, and C stand for treatments. 1 and 2 represents replicate. + = positive control (genomic DNA of pure culture), - = negative control (only the reaction mix).

7.2.3.2 Detection and quantification of fungal DNA from inoculated fruits by LAMP

To confirm the specificity of the LAMP primers as well as the detection of fungus isolated from extracted treated and control (only inoculated) fruits, DNA of pure *C. gloeosporioides* was used as standard and later compared with fungal DNA extracted from inoculated fruits either treated or untreated every week during cold storage. A stock concentration of 1µg/µl genomic DNA of pure culture was prepared and diluted into a 10-fold series or 3x dilutions (1×10^0 to 1×10^{-3}). A standard curve was constructed according to Zhang et al., (2013) with slight modification by serial dilutions of extracted genomic DNA of the pure culture. Quantification of fungal DNA extracted from inoculated treated and untreated fruits was performed using standard curve from pure culture after data was obtained from LAMP assay. An aliquot of 1µg/µl of each DNA sample extracted from inoculated treated and untreated fruits was prepared and used for the LAMP assay before quantification.

7.3 Statistical analysis

Two-way Anova was performed for the efficacy of extracts on the effect of fungal DNA extracted from inoculated treated and untreated fruits using computer software GenStat version 18th Edition (VSNI product). Means were separated by Fisher unprotected test at ($P < 0.05$). Experiments were repeated twice and data pooled before analysis.

7.4 Results

7.4.1 PCR assay

The PCR assay successfully identified all seven fungal DNA samples extracted from both treated and untreated inoculated infested fruits *C. gloeosporioides* when compared to genomic DNA of pure culture, which served as positive control (Table 7-3). All purified DNA samples after sequencing showed 99% similarity of *C. gloeosporioides* isolate with accession number KU836641 (Table 7-3) from GenBank database. The amplification of all DNA shown that, the primers were specific to the pathogen and these primers (Cgbt F3 and B3) encoded the *TUB2* region of *C. gloeosporioides*. A fragment size band of between 212-250 bp (day 7), 212-252 bp (day 14) and 211-249 bp (day 21) (Table 7-3) of beta-tubulin gene was detected when the PCR products were run on 1.0% agarose gel and stained with ethidium bromide (Figure 7-2). In short, PCR results showed the presence of *C. gloeosporioides* in both inoculated treated and non-treated fruits at day 7 and throughout the 21 days of cold storage (Table 7-3) and confirm the specificity of the primer on DNA template of fungus isolated from infested fruit.

Table 7-3 Table showing fungal DNA from treated and untreated fruits and details after sequencing

Days	Fungal DNA samples from treated and untreated fruits	Fragment size	Remarks	Accession number	Gene encoded
Day 7	Untreated fruits	243	+	KU836641	Beta-tubulin gene
	10%GA+5 dun	250	+	KU836641	Beta-tubulin gene
	10%GA+10 dun	243	+	KU836641	Beta-tubulin gene
	10%GA+5 gin	244	+	KU836641	Beta-tubulin gene
	10%GA+10 gin	212	+	KU836641	Beta-tubulin gene
	10%GA+5 tur	245	+	KU836641	Beta-tubulin gene
	10%GA+10 tur	212	+	KU836641	Beta-tubulin gene
Day 14	Untreated fruits	212	+	KU836641	Beta-tubulin gene
	10%GA+5 dun	252	+	KU836641	Beta-tubulin gene
	10%GA+10 dun	252	+	KU836641	Beta-tubulin gene
	10%GA+5 gin	267	+	KU836641	Beta-tubulin gene
	10%GA+10 gin	231	+	KU836641	Beta-tubulin gene
	10%GA+5 tur	212	+	KU836641	Beta-tubulin gene
	10%GA+10 tur	244	+	KU836641	Beta-tubulin gene
Day 21	Untreated fruits	249	+	KU836641	Beta-tubulin gene
	10%GA+5 dun	212	+	KU836641	Beta-tubulin gene
	10%GA+ 10 dun	212	+	KU836641	Beta-tubulin gene
	10%GA+5 gin	243	+	KU836641	Beta-tubulin gene
	10%GA+10 gin	211	+	KU836641	Beta-tubulin gene
	10%GA+5 tur	245	+	KU836641	Beta-tubulin gene
	10%GA+ 10 tur	211	+	KU836641	Beta-tubulin gene
	Pure culture	249	+	KU836641	Beta-tubulin gene

+ = Presence of *C. gloeosporioides*, - = Absence of *C. gloeosporioides*

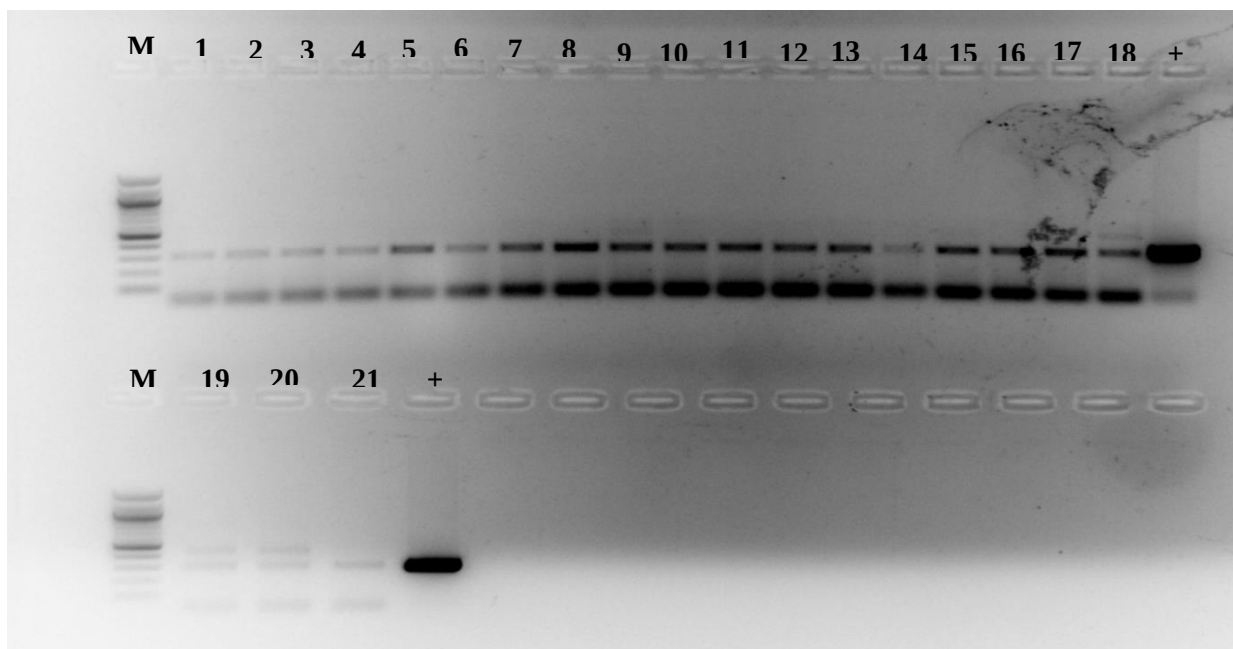


Figure 7-2 Agarose gel (1.0%) analysis of PCR assay amplicon showing specificity of PCR assay using primer Cgbt F3 and B3 for detection of *C. gloeosporioides* from inoculated treated and untreated fruits at each time point for 21 days of cold storage. M = Ladder, Lane 1-7= Amplified fungal DNA extracted from treated and untreated inoculated fruit at day 7, Lane 8-14 = Amplified fungal DNA extracted from treated and untreated inoculated fruit at day 14 and Lane 15-21 = Amplified fungal DNA extracted from treated and untreated inoculated fruit at day 21. + = genomic DNA of pure culture of *C. gloeosporioides* (as control).

7.4.2 Detection and quantification of LAMP assay

7.4.2.1 Standard curve and pathogen detection

LAMP assay from genomic DNA of pure culture of *C. gloeosporioides* was used to derive the standard curve by serial dilutions before the quantification of fungal DNA extracted from inoculated dragon fruits. Serial dilutions were made as low as $1 \times 10^{-3} \mu\text{g}/\mu\text{l}$ which was detectable during the assay (

Figure 7-3).

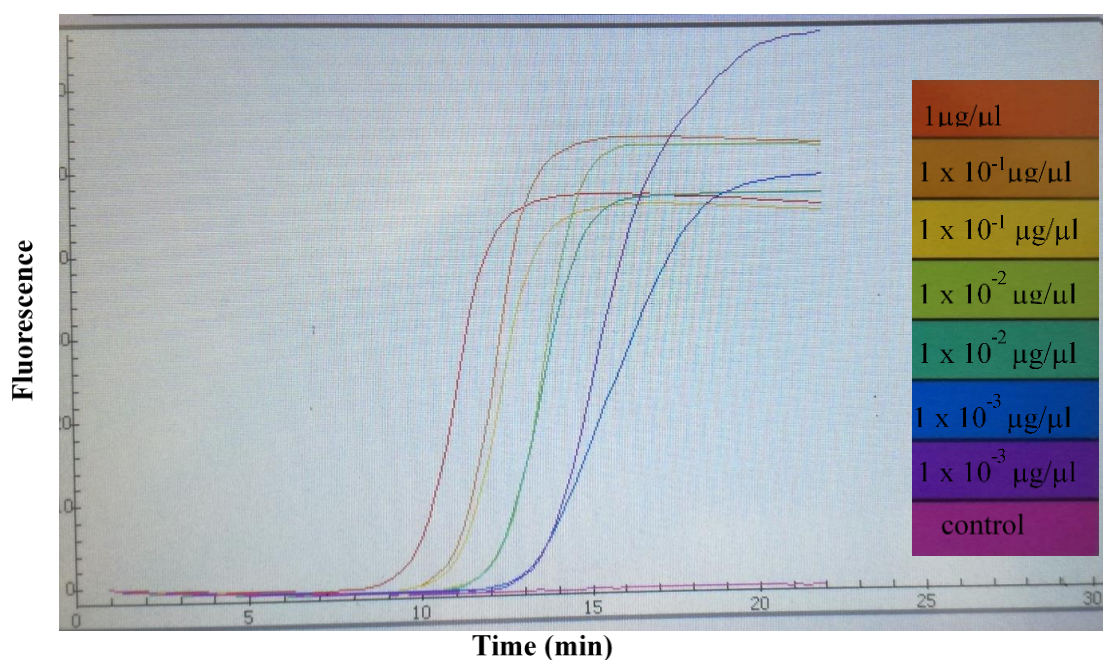


Figure 7-3 Sensitivity test of LAMP assay of serial 10-fold dilutions of *C. gloeosporioides* genomic DNA ranging from 1.0 µg/µl to 1 x 10⁻³ µg/µl. The fluorescence units vs. time amplification curves plotted automatically using Genie® Explorer v 2.0.6.3.

A good linearity between the threshold time of application and the serial dilutions of template genomic DNA of *C. gloeosporioides* was observed from standard curve ($R^2 = 0.99$). This confirms the reliability of the standard curve obtained from the pure genomic DNA of fungus for quantification of fungal DNA extracted from inoculated-treated and untreated dragon fruits. Thus, a standard curve between the amplification time and amount of serial dilutions of template genomic DNA of pure culture was constructed (Figure 7-4). The maximum amplification time of fungal detection in pure culture occurred in 14.38 min at $1 \times 10^{-3} \mu\text{g}/\mu\text{l}$ and the minimum time was 11.00 min at $1 \mu\text{g}/\mu\text{l}$ (Figure 7-4).

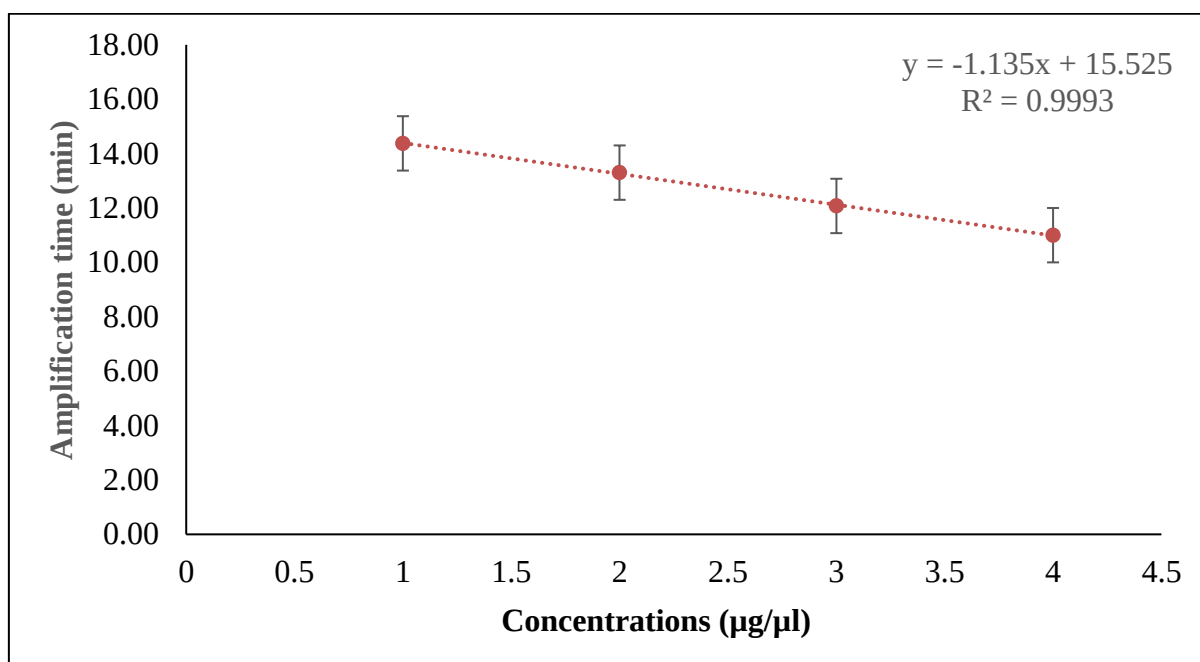
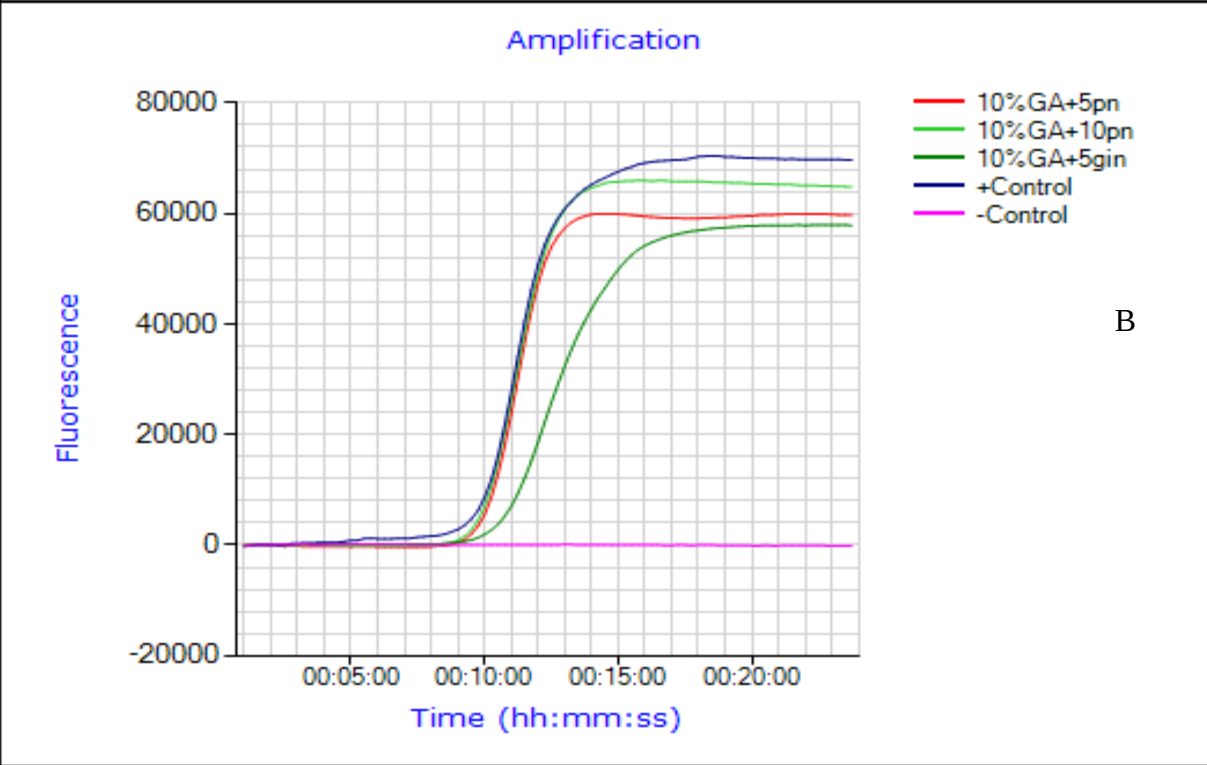
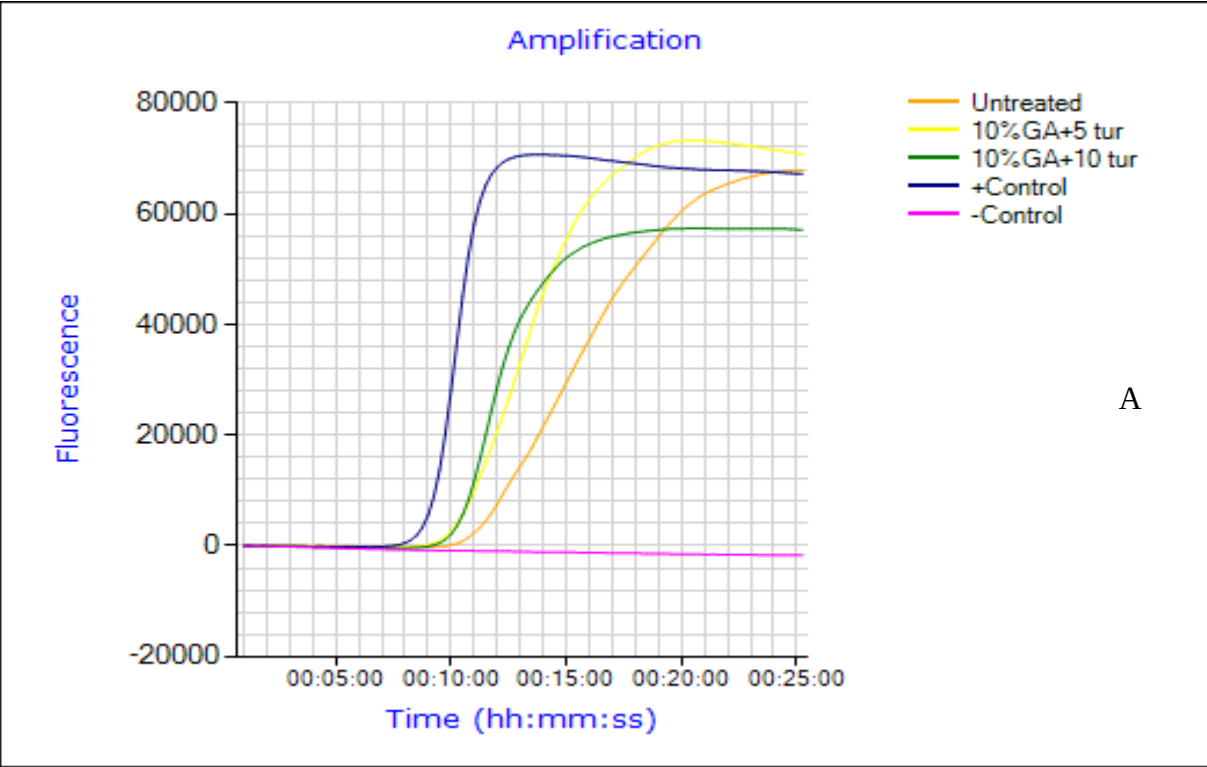


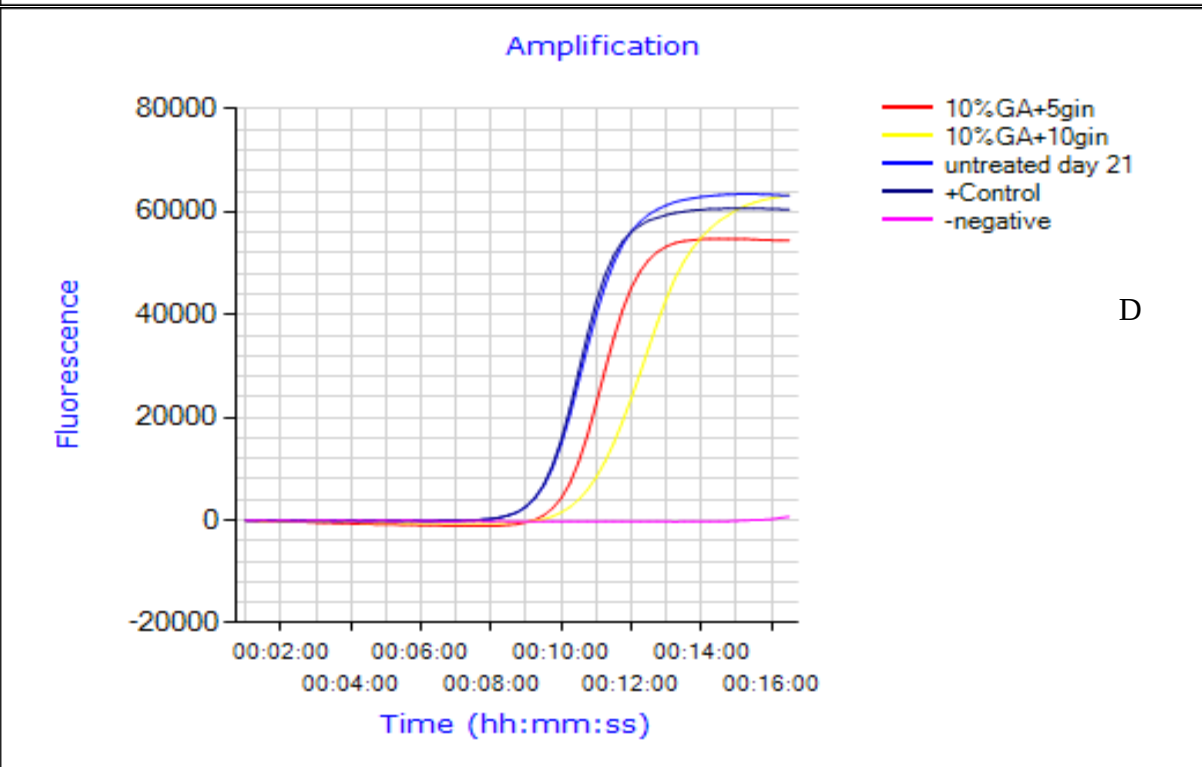
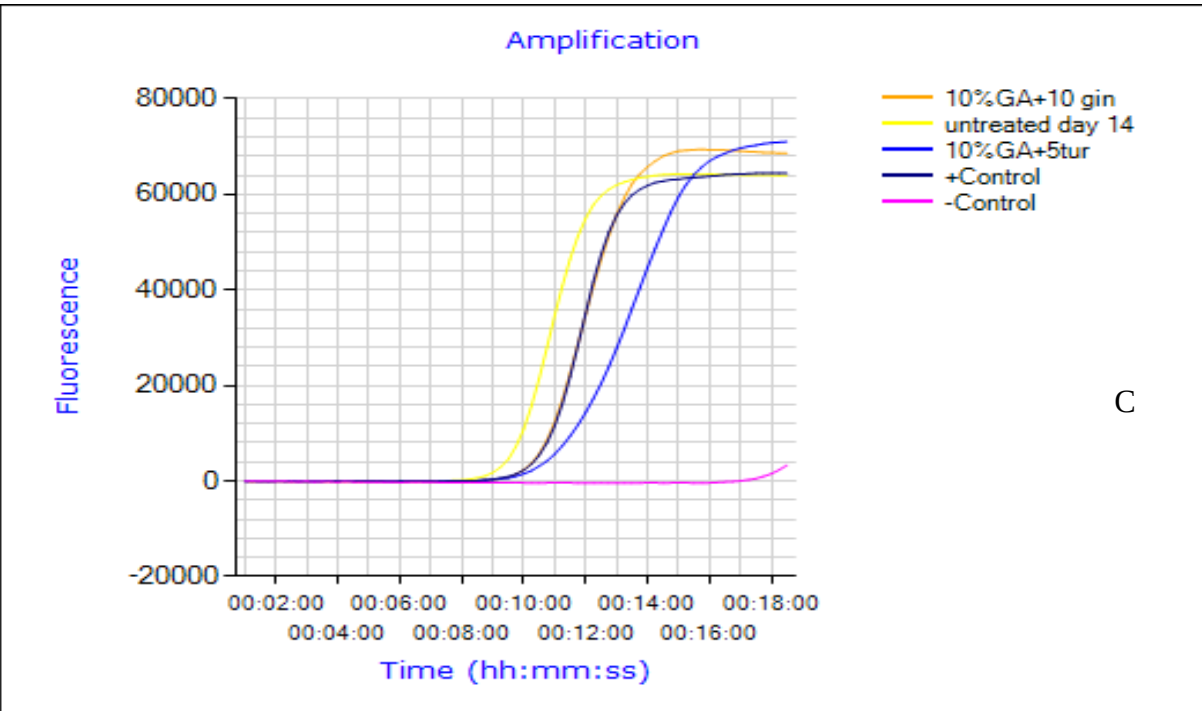
Figure 7-4 The sensitivity of LAMP assay standard curve of 10-fold serial dilutions of *C. gloeosporioides* genomic DNA ranging from $1.0 \mu\text{g}/\mu\text{l}$ to $1 \times 10^{-3} \mu\text{g}/\mu\text{l}$. Error bars represent sem from triplicate reactions.

7.4.2.2 Quantification of fungal DNA extracted from inoculated fruits

During quantification, $1 \mu\text{g}/\mu\text{l}$ of fungal DNA extracted from inoculated fruits (treated and untreated) was used each week during the 21 days of storage. Irrespective of the treatments, an

average detection time of 11.20 min, 11.33 min, and 11.96 min was recorded at day 7, day 14 and day 21 respectively (Figure 7-5A, B, C, D and E).





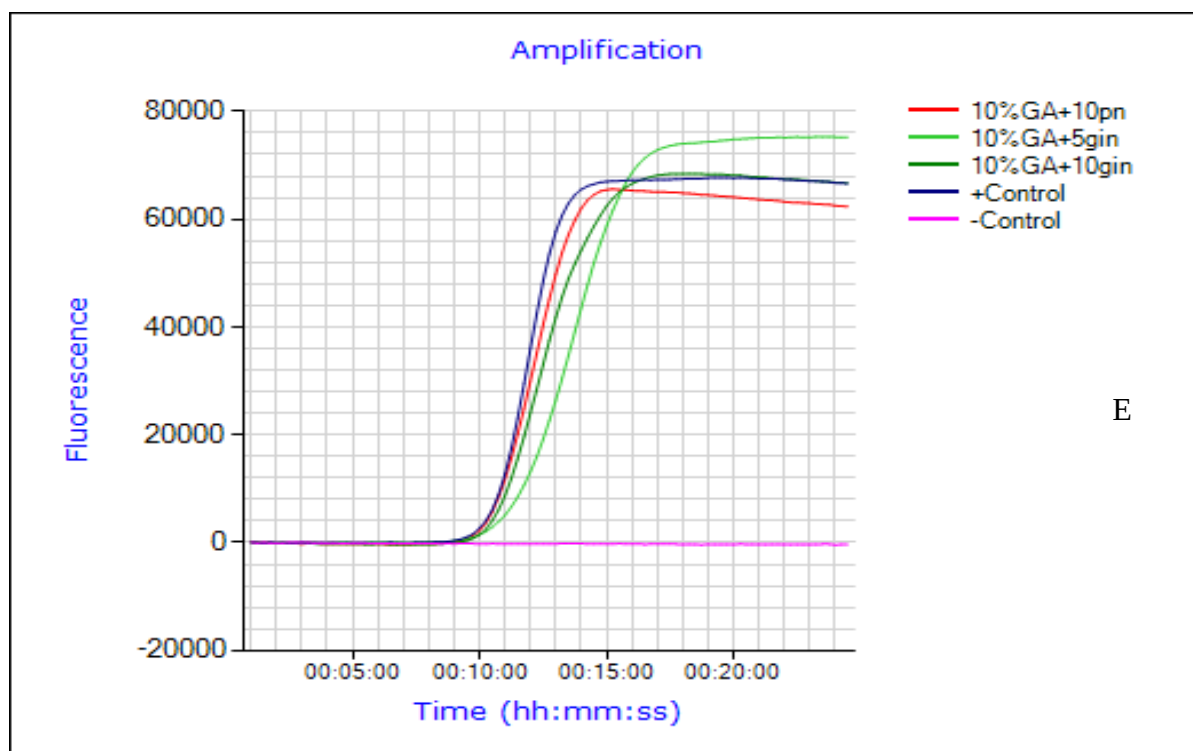


Figure 7-5 Sensitivity test of LAMP assay of *C. gloeosporioides* DNA (1µg/µl) extracted from some selected inoculated treated and untreated fruits at day 7 (A, B, and C), day 14 (C and D) and day 21 (D and E). The fluorescence units vs. time amplification curves plotted automatically using Genie® Explorer v 2.0.6.3. Untreated = Fungal DNA extracted from untreated fruits after storage at day 7. Untreated day 14= Fungal DNA extracted from untreated fruits after storage at day 14 and untreated day 21= Fungal DNA extracted from untreated fruits after storage at day 21.

The amount of fungal DNA extracted from all inoculated treated (coated) fruits was markedly low compare to controls (inoculated untreated fruits) after 21 days (Appendix 0-11). The lowest ($p<0.05$) amount of fungal DNA was recorded in fruits treated with 10%GA plus 5 mg/ml of turmeric crude extracts (3.30 µg/µl) followed closely by fruits treated with 10%GA plus 10 mg/ml of the same extract (3.392 µg/µl) compared to controls (3.95 µg/µl) (Figure 7-6; Table 7-4).

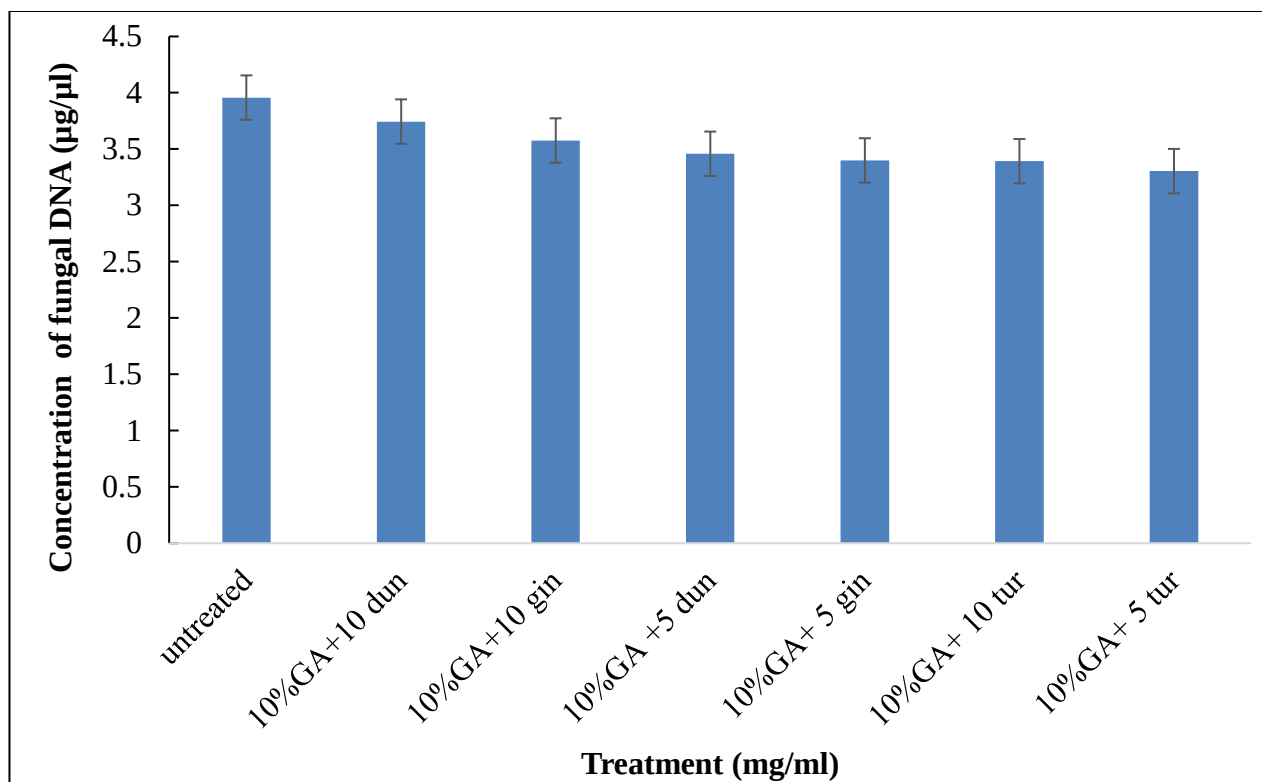


Figure 7-6 Quantified amount of fungal DNA extracted from treated and untreated fruits after 21 days of cold storage based on amplification time.

Table 7-4 Two-way Anova table on the effect of formulation on amount of fungal DNA extracted from treated and untreated fruits after 21 days of storage.

Concentration (C)	Extracts (E)		
	Turmeric	Dukung anak	Ginger
Con		3.95a	
5mg/ml+10%GA	3.304b	3.458ab	3.399ab
10mg/ml+10%GA	3.392a	3.576ab	3.576ab
Sem (T*)		0.1971	
P-value (T*)		0.269	

Levels connected vertically by different letters are significantly different.

C= Different concentrations for each plant extracts incorporated with 10%GA.

T* (Treatment) = Combination of each extracts (E) at different concentrations (C) after two-way Anova.

7.5 Discussion

7.5.1 PCR assay of fungal DNA extracted from infested fruits.

C. gloeosporioides is a ubiquitous tropical fruit pathogen, which causes postharvest anthracnose in dragon fruit. The specificity of Cbgt F3 and B3 were validated by PCR assay, which detected the fungus extracted from inoculated treated and untreated fruits as *C. gloeosporioides*. The amplification of the fungal DNA was specific to Cbgt F3 and B3 and they encoded the *C. gloeosporioides* beta-tubulin region of the TUB2 allele from genomic DNA. Beta-tubulin gene is a housekeeping gene that is expressed differentially in conidia, conidiating mycelia, and vegetative mycelia such as hyphae, especially hyphal tips (Buhr and Dickman, 1994). Thus, this gene is required for or immediately after spore germination (Buhr and Dickman, 1994).

7.5.2 Detection and quantification of fungal DNA on infested fruits by LAMP

Rapid and reliable detection of the pathogen is essential for undertaking appropriate and timely disease management during postharvest storage of fruits. Apart from classic real-time PCR widely used in the quantitative detection of soil-borne pathogens, an alternative technique termed loop-mediated isothermal amplification (LAMP) is also available used for quantitative analysis. The generation of LAMP products can also be monitored in real-time by measuring the increased amplified DNA concentration, allowing quantitative detection of the target (Mori et al., 2001; Mori et al., 2004; Tomlison et al., 2010; Bekele et al., 2011). Accordingly, Genie® Explorer v 2.0.6.3. used to monitor the DNA synthesis reaction using fluorescence showed that the LAMP primers were specific to amplify the target DNA sequence.

The sequence of the smallest fragments of DNA from inoculated treated and untreated fruits after 21 days of storage had 99% sequence identity to the ITS region of *C. gloeosporioides* in GenBank from the PCR assay (accession number KU836641). Anthracnose is a disease caused by *C. gloeosporioides*. In this study, the generation of LAMP products (fungal DNA isolated from infested treated and untreated fruits after inoculation) were monitored in real-time by measuring the increased amplified fungal DNA concentration, allowing quantitative detection of the target (Tomlison et al., 2010; Bekele et al., 2011). The Genie® Explorer v 2.0.6.3. showed the DNA synthesis reaction using fluorescence which clearly depicted that the LAMP primers were specific to amplifying the target DNA sequence. Hence showing the presence of *C. gloeosporioides* that causes anthracnose. The presence of fungal DNA at day 7 indicates the quiescent nature of the pathogen and the slight similarity in the controls and treated fruits could be due to the method used in the extractions of fungal DNA which probably did not allow maximum extraction of fungal DNA from infested fruits.

7.6 Concluding remarks

It was observed that composite coating of 10%GA plus either 5 mg/ml or 10 mg/ml reduced the amount of fungal DNA of *C. gloeosporioides* and this was more pronounced in turmeric crude extracts at 10%GA+5 mg/ml. This confirms a similar trend where a composite coating of 10%GA plus 10 mg/ml of either extract (turmeric or ginger or dukung anak) delayed anthracnose and its severity (section 6.4.1 and 6.4.2). It is evident that the reduction of the amount of fungal DNA extracted from fruits was due to these treatments, which resulted in the control of postharvest anthracnose caused by *C. gloeosporioides* after 28 days of cold storage. In this present study, we conclude that LAMP can be used as a diagnostic tool (qualitative and

quantitative analysis) for direct early detection of *C. gloeosporioides* in naturally infested dragon fruits during postharvest storage *vis- a- vis* long voyage (sea transport).

By doing so, we can detect and take necessary preventive measures by incorporating plant crude extracts especially 10 mg/ml of turmeric and/or ginger with 10%GA to control the anthracnose in dragon fruit or any tropical fruit before it becomes an epidemic during postharvest storage. Apart from early detection of the disease to reduce postharvest losses, further study to understand the efficacy of the formulation on postharvest quality and their ability to maintain if not enhance the nutritional quality of the fruit need to be investigated.

CHAPTER 8

8 EFFICACY OF PLANT EXTRACT INCORPORATED WITH GUM ARABIC ON POSTHARVEST QUALITY OF DRAGON FRUIT

8.1 Introduction

Maturity at harvest is the most important factor that determines storage life and final fruit quality (Kader, 1999). However, matured fresh produce after harvest is still alive and as a result undergoes a certain stage of development where the fruits will continue their ontogeny even if detached (physiological maturity) or a stage of development where the fruits possess prerequisite for utilisation by consumers for a particular purpose (horticultural maturity) (Kader, 1999). The physiological maturity during maturation is as a result of fruit ripening that occurs from the latter stages of growth and development through the early stages of senescence and that results in characteristic aesthetic and/or food quality, as evidenced by changes in fruits firmness (texture and softness of fruits), colour, weight loss, or other sensory and chemical attributes such as total soluble solids (TSS), titratable acidity (TA) and pH of fruit (Kader, 1999; Valero and Serrano, 2010; Deaquiz et al., 2014). Additionally, increased rates of respiration and ethylene production are also associated with physiological or biochemical changes that occur during ripening and can adversely affect the nutritional quality of fresh produce leading to postharvest losses if not controlled (Dhat and Mahajan, 2007). Therefore, to maintain the overall quality of fruits, ripening, which involves a complex physiological process resulting in softening, colouring, sweetening and increase in aroma compounds, should be delayed by restraining respiratory and other metabolic or physiological activities of fruits (Kays, 1991; Dhat and Mahajan, 2007).

Several techniques have been developed to minimise the rates of respiration and transpiration in dragon fruits using low temperature and controlled atmosphere to reduce postharvest losses, but there exist some limitations (Chandran, 2010; Bautista-Baños et al., 2013; Freitas and Mitcham, 2013). As a non-climacteric fruit, dragon fruit has no peak of ethylene or carbon dioxide production (Le Bellec et al., 2006). The fruit stay fresh for a few days at ambient temperature but its physical appearance deteriorates with the onset of disease infestation leading to spoilage within 7 days. Reports show that dragon fruits stored at 10°C with 90% RH remained disease free but deteriorations started after day 15 (Lau et al., 2008). However, at a relatively high temperature of 22°C, inoculated dragon fruits exhibited 100% anthracnose infection after 3 days of incubation (Awang et al., 2001).

GA alone or incorporation of other natural or synthetic chemicals has been reported to improve and enhance the postharvest quality of some tropical fruits and vegetables. For instance, studies report that 10%GA alone or combined with 3% calcium chloride effectively reduced weight loss, soluble solids concentrations, and colour changes; maintained a high firmness, titratable acidity, and ascorbic acid; reduced the rate of respiration and ethylene production after 28 days of storage at 6±1°C and after 5 days of shelf life at 25±2°C. When 10%GA was used as an edible coating, it delayed the ripening process of tomatoes and significantly preserved the total antioxidant activity, lycopene content, total phenolic and total carotenoids of tomatoes up to 10 days of storage at 20°C (Ali et al., 2013a) and reduces browning of sliced tomatoes (Eltoum and Babiker, 2014). Al-Juaimi (2014) also reported significant effect improvement in the physicochemical properties (firmness, high total soluble solids, and titratable acidity) and overall acceptance of tomato fruits treated with 5-20% GA after 16 days of storage at 10±10°C. When 10%GA was applied as an edible coating on papaya

fruits, the fruits showed a significantly higher antioxidant activity at day 7 during a 15-day storage (Addai et al., 2013). Addai et al. (2013) also confirmed that 10%GA significantly maintained the physicochemical properties (total soluble solids, pH, titratable acidity, colour, and moisture) of papaya fruits after 15 days of storage at $13\pm 1^{\circ}\text{C}$. It is evident that GA plays a significant role in enhancing the postharvest and nutritional quality of fruits whilst extending their shelf life.

Therefore, the objectives of the study in this chapter are to:

- To investigate the efficacy of plant extracts at different concentrations incorporated with 10%GA on physicochemical qualities of dragon fruit during storage.
- To investigate the efficacy of plant extracts at different concentrations incorporated with 10%GA on gas exchange of dragon fruit during storage.
- To investigate the efficacy of plant extracts at different concentrations incorporated 10%GA on nutritional and sensory quality of dragon fruit during storage.

8.2 Materials and methods

Preparation of GA, its incorporation with each plant extract at different concentrations and the application treatment on dragon fruits was based on protocols in section 6.2.11, 6.2.1.2 and 6.2.1.3 respectively. However, plant extracts at different concentrations incorporated with 10%GA served as treatment whilst fruits washed in only distilled water served as control.

8.2.1 Effect of treatment on physicochemical quality of dragon fruits

For the physical quality analysis; weight loss, colour, and fruit firmness, a total of 15 fruits were used for each treatment (each plant extract at different concentration). The experiment was done in triplicate and each replicate per treatment contained 5 fruits, whilst for the chemical

quality analysis such as total soluble solids (TSS), titratable acidity (TA) and pH, a total of five fruits randomly sampled per treatment. The physiochemical quality analysis was conducted every week for a period of 21 to 28 days.

8.2.1.1 Weight loss

The weight of samples was measured using a digital balance (Model GF-6100, A&D Co. Ltd., Japan) at day 0 and at the end of the 7-day interval measurement for a period of 28 days. The difference between initial and final fruit weight each week was considered as total weight loss during that storage interval and calculated as a percentage on fresh weight basis. In total, 15 fruits were used for each treatment and each replicate containing 5 fruits.

8.2.1.2 Colour changes

Peel colour was analyzed using the Hunter Lab System, Miniscan XE Plus colorimeter (Model: 45/0-5, Reston Virginia, USA). A total of 15 fruits were used per treatment and this comprise 5 fruits per replicate. Blossom, middle and stem end of each fruit were measured and the average taken to represent the colour variation for each fruit. The colorimeter was equipped with a measuring head that had an 8-mm diameter measuring area and calibrated with standard black and white tiles. The Miniscan XE Plus colorimeter was calibrated using black and white tiles with values of $X=79.0$, $Y=83.9$, and $Z=87.9$. The peel colour determination was expressed in chromaticity values of L^* , C^* , and h° . The L^* coordinate represented the lightness of colour with values ranging from 0 = black to 100 = white. $C^* = (a^{*2} + b^{*2})^{1/2}$, which indicates the hypotenuse of a right triangle with values ranging from 0 = least intense to 60 = most intense. h° (hue angle) = $\arctan(-b^*/a^*)$, where 0° = red purple, 90° = yellow, 180° = blue-green and 270° = blue (Maqbool et al, 2011).

8.2.1.3 Fruit firmness

Instron Universal Testing Machine with a 5.00 mm plunger tip, single-column model (Instron 2519-104, Norwood, MA) interfaced with a computer was used to determine the fruit firmness, by measuring the amount of force (N) to puncture a hole in the fruit on each sampling day. The machine was set for maximum compression with a speed of 20 mm/min. Measurements were taken for each of the 15 fruits at the stem end, mid-region, and blossom end using the probe, and the average of three readings was recorded as the fruit firmness per fruit (Maqbool et al, 2011).

8.2.1.4 Total soluble solids

TSS was determined according to Dávila-Aviña et al. (2011) with slight modification. A total of five fruits which represented 5 replicates per treatment were used. The average was calculated as the overall TA and TSS value. Fruit pulp (10 g) was frozen for 24 hr, homogenised using a kitchen blender with 40 ml of purified distilled water. The solution was centrifuged at 5000 x g for 10 min using Eppendorf centrifuge 5810 R (Eppendorf AG, Hamburg). The obtained filtrate (fruit juice) was used for TSS and TA analysis. TSS (°Brix) was determined using a Palette Digital Refractometer (Model: PR-32 α Atago Co., Ltd. Japan). An aliquot of filtrate was placed on the prism glass of the refractometer which has already been calibrated with distilled water prior to taking readings. For each fruit, three droplet measurements were taken and averaged to obtain the overall TSS per each of the 5 fruits selected.

8.2.1.5 Titratable acidity

The Titratable acidity (TA) of fruits was performed and calculated using the titration method of Ranganna (1986). An aliquot of 5 ml of fruit juice obtained from section 7.2.1.4 was placed in a beaker and two drops of 0.1 % phenolphthalein (R & M Chemicals, UK) was added as an indicator and titrated against 0.1 N NaOH (Merck, Germany) until an endpoint colour of pink (pH 8.1). Three beakers containing the fruit juice and all necessary reagents for titration served as replicate for each of 5 fruits used, and then averaged to obtain the overall TA per fruit. The results were expressed as the percentage of citric acid as stated below:

$$\frac{\text{Titre value} \times \text{NaOH normality} \times \text{milliequivalent factor of citric acid equivalent (0.064g)}}{\text{the volume of sample used for titration} \times \text{wt of the sample (g)}} \times 100$$

8.2.1.6 pH

The acidity level of fruit juice was determined weekly using bench pH meter- (model 3505-Jenway) by inserting a probe into the juice obtained in section 7.2.1.4 to record the pH of the juice. Prior to measurement, the pH meter was calibrated using a buffer of pH=7.

8.2.2 Effect of plant extracts incorporated with GA on gas exchange

The rates of respiration and ethylene evolution were measured according to the method of Maqbool et al. (2011) with slight modification. Three medium-sized dragon fruits of approximate weight of 540 g were placed in a 2 L plastic container representing a replicate. The experiment was done in triplicate per treatment, and time point for measurements was every week until the end of the storage period.

8.2.2.1 Respiration rate

Respiration rate as indicated by CO₂ production was measured by placing three-medium sized dragon fruits in a 2 L plastic container for 2 h, and 1 ml of gas sample was withdrawn from the headspace with a gastight hypodermic syringe and analyzed with a gas chromatograph (GC) (Clarus-500, Perkin-Elmer, USA) equipped with a stainless-steel column (Porapak R 80/100). Helium served as the carrier gas at a flow rate of 20 ml/min. Temperatures were 60, 100, and 200 °C for the oven, injector, and thermal conductivity detector (TCD), respectively. One millilitre of CO₂ gas (1.0%) (Scotty Gases, Bellefonte, PA, UK) was used as the external standard for calibration. The amount of CO₂ production was expressed in millilitres of CO₂ per kilogram per hour and was calculated using the formula below:

$$\text{ml CO}_2 \text{ Kg/hr} = \frac{\text{CO}_2 \text{ produced by sample (\%)} * \text{volume of Void (ml)} * 1}{100 * \text{Weight of sample (Kg)} * \text{incubation time (hr)}}$$

8.2.2.2 Ethylene production

Ethylene evolution was measured by taking a 1 ml gas sample from each jar (as described for respiration rate in section 7.2.2.1) using a hypodermic syringe and injecting it into a GC. The GC was equipped with a stainless-steel column (Porapak T, 100/120) and a flame ionization detector (FID). Nitrogen, hydrogen, and air flow rates were 20ml/min. Nitrogen served as a carrier gas. Temperatures were 150, 200, and 200°C for the oven, injector, and FID, respectively. One millilitre of 10 µl/ml pure ethylene gas (Scotty Gases, Bellefonte, PA, UK) was used as an external gas standard for calibration. The amount of ethylene was expressed in microliters of ethylene per kilogram per hour.

$$\mu\text{l C}_2\text{H}_4 \text{ Kg/hr} = \frac{\text{Ethylene produced by sample}(\mu\text{l/ml}) * \text{Volume of void (ml)} * 1000}{\text{Weight of sample (Kg)} * \text{incubation time (hr).}}$$

8.2.3 Nutritional quality

For nutritional quality, this research looked at the antioxidant levels in fruits based on total phenolic content (TPC), radical scavenging activity using the 1, 1-Diphenyl-2-picryl-hydrazyl (DPPH) assay and the amount of vitamin C which was indicative as ascorbic acid (AA) content using the titration method. All analyses were done in triplicate. A total of five fruits from each treatment were sampled for both TPC and DPPH assay. During the spectrophotometric analysis, three cuvettes were used as replicate per fruits for each parameter (TPC and DPPH assay) analysed.

8.2.3.1.1 Sample preparation and extraction for TPC and DPPH assay

The nutritional quality such as the total phenolic content in fruits (pulp) as well as the total antioxidant activity (DPPH assay) was carried out according to Nurliyana et al. (2010) with slight modification. About 20 g of the pulp which was frozen for 24 hours to reduce the moisture content was crushed and homogenized with 70% ethanol using kitchen blender to a paste-like state for 1 min. The homogenised sample was filtered using a muslin cloth and poured into 250 ml volumetric flasks. About 40 ml of the filtrate was poured into a 50-ml test tube and centrifuged for 5 min at 4000 rpm using a centrifuge (Eppendorf centrifuge 5810 R-Eppendorf AG, Hamburg). Samples were then used for DPPH and TPC assay.

8.2.3.1.1.1 Total Phenolic Content

Antioxidant level for each sample was measured by TPC assay using Folin-Ciocalteu's method based on modified method by Nurliyana et al. (2010). A volume of 0.3 ml of each treatment (extract at different concentration incorporated with 10%GA) was mixed with 1.5 ml of 50% Folin-Ciocalteu's reagent (prepared by with distilled water) and 1.2 ml of sodium

carbonate (7.5% w/v) in a test tube. The tube was then vortexed and covered by parafilm. The tube was incubated in dark for 30 min at room temperature. After that, absorbance readings were taken by measuring the sample using Multiskan Go UV/Vis spectrophotometer (Thermo Fisher Scientific Oy, Ratapiikatie 2, FI-01620 Vantaa, Finland) at an absorbance of 765 nm. A standard calibration curve was prepared using gallic acid with concentrations 0.05 mg/ml, 0.10 mg/ml, 0.15 mg/ml, 0.20 mg/ml and 0.25 mg/ml by dissolving standard gallic acid in 70% ethanol. TPC was expressed in gallic acid equivalents, GAE in mg per 100 g fresh fruit. Both samples (treatments) and gallic acid were measured against 70% ethanol which was used as blank. All samples and readings were measured in triplicate in a 96 well plate reader.

8.2.3.1.1.2 DPPH assay

The radical scavenging activity of each sample was measured using the method of Nurliyana et al. (2010). Each sample was prepared in a series of dilution (0.2 mg/ml, 0.4 mg/ml, 0.6 mg/ml, 0.8 mg/ml and 1.0 mg/ml) with final volume of 10 ml. A volume of 1 ml of each solution was then mixed with 2 ml of 0.1 mM DPPH reagent and mixed thoroughly. After the tubes were incubated at room temperature in the dark for 30 min, the absorbance of the mixture was measured spectrophotometer Multiskan Go UV/Vis spectrophotometer (Thermo Fisher Scientific Oy, Ratapiikatie 2, FI-01620 Vantaa, Finland) at 517 nm using against only 2 ml of 0.1 mM DPPH which was used as blank. Each sample treatment was measured in triplicate and expressed in mean average. The radical scavenging activity was calculated accordingly: -

$$\% \text{ inhibition} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100$$

A_{control} (DPPH), = Absorbance reading of control (only DPPH), A_{sample} = Absorbance reading of the sample.

8.2.3.1.1.3 Ascorbic acid content

This was carried out according to Ranganna, (1986). Reagents made of 3% Metaphosphoric acid (HPO_3), standard L-ascorbic acid (1mg/ml) and a dye solution, made of 50 mg of sodium salt dissolved in 2, 6-dichloropophenol-indophenol in approximately 150 ml of hot glass distilled water containing 42 mg of sodium bicarbonate. Before analysis at weekly time point, dye solution was standardized by titration against a mixture of 5 ml standard ascorbic acid and 5 ml of HPO_3 to a pink colour which persisted for 15 sec. A dye factor, thus mg of ascorbic acid per ml of the dye was established and later for calculation of ascorbic acid content in fruits using the formula:

$$\text{Dye factor} = \frac{0.5}{\text{Titre}}$$

For sample preparation and assay, 10 g of sample was blended with 3% HPO_3 and the final volume made up to 100 ml with 3% HPO_3 . Samples were then centrifuged for 5 min at 4000rpm using Eppendorf centrifuge 5810 R (Eppendorf AG, Hamburg). An aliquot of 2 ml of 3% HPO_3 extract samples was titrated against with standard dye to a pink end-point which persisted for 15 s. Ascorbic acid was estimated as mg of ascorbic acid / ml, and was determined by the following equation:

$$\text{mg of ascorbic acid / ml} = \frac{\text{Titrate vol. (ml of dye used)} * \text{dye factor} * \text{vol. made up} * 100}{\text{Aliquot of sample taken for estimation} * \text{weight of sample}}$$

8.2.4 Sensory evaluation

Sensory evaluation of the fruit for taste, pulp colour, texture, aroma and overall acceptability for all the samples was performed at the end of 21 days of storage using the method of Bai et al. (2003). Fruits used in this evaluation were stored separately from fruits used in the other postharvest quality analysis. For sensory evaluation, the best coating treatments that

significantly controlled disease incidence and maintained nutritional and physicochemical quality were used. Such coating treatments were made of 10%GA plus 10 mg/ml and 10%GA plus 5 mg/ml for each plant crude extracts. For each treatment, six fruits were randomly selected and sliced into pieces before the analysis. Control fruit were fruits washed with distilled water. A total of forty-two fruits was used for sensory evaluation. Panelists were asked to score the difference among the samples by allotting numbers on appearance, pulp colour, aroma, sweetness, texture and overall acceptability. Each panellist was asked to rate each parameter base on the rating as follows; 1-extreme dislike, 2 = dislike, 3 = acceptable, 4 = good and 5 = excellent.

8.3 Statistical analysis

Two-way Anova was performed for the efficacy of extracts on postharvest quality of fruits except for sensory evaluation where one-way Anova was performed using computer software GenStat version 18th Edition (VSNI product). Means were separated by Fisher unprotected test at ($P < 0.05$). Experiments were repeated twice and data pooled before analysis.

8.4 Results

Plant extracts at different concentrations incorporated with 10%GA served as treatment.

8.4.1 Efficacy of formulation on external physical quality of fruit

8.4.1.1 Weight loss

There was a significant effect ($P < 0.05$) between fruits coated with plant extracts at different concentrations incorporated with 10%GA (treatment) on weight loss (%) after 28 days of storage (Appendix 0-12). Weight loss increased progressively ($P < 0.05$) with storage time in

both treated and control fruits, and it was highest in controls after 28 days (Figure 8-1; Table 8-1).

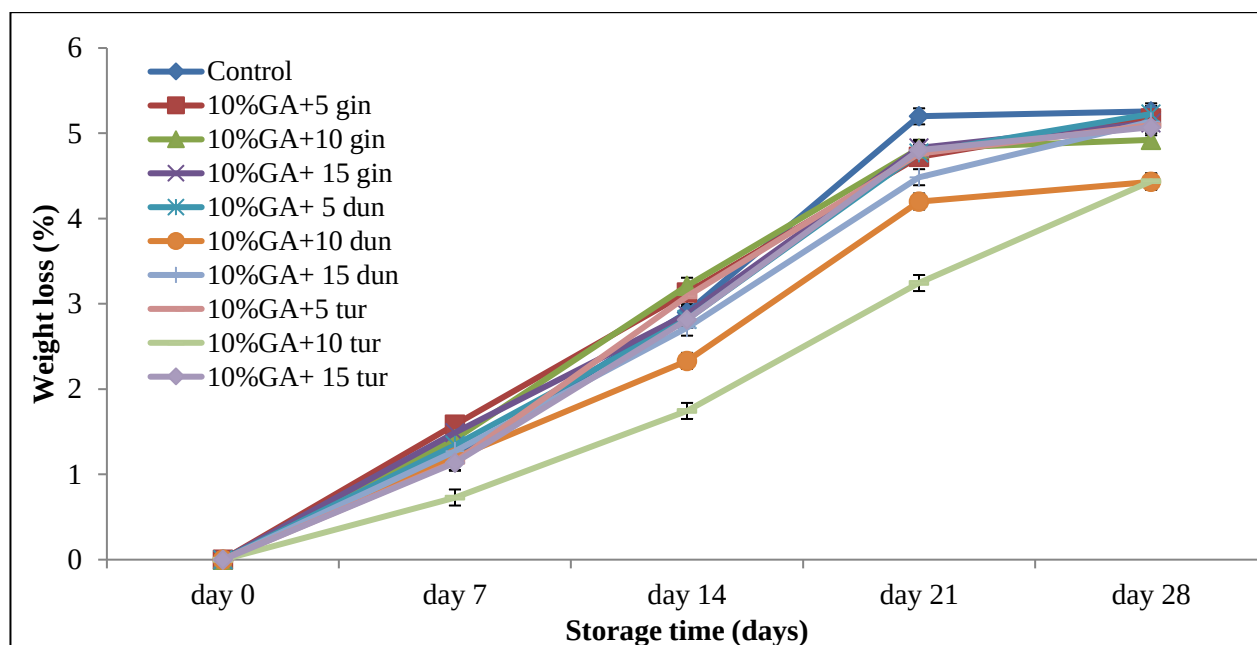


Figure 8-1 Effect of plant extracts at different concentrations incorporated with 10%GA on weight loss (%) for 28 days' cold storage.

At day 21 (recommended edible stage), fruits coated with 10mg/ml+10%GA of turmeric significantly ($P<0.05$) recorded lower weight loss (3.24%) compared to control (5.19%). However, fruits coated with 10%GA plus either 5 mg/ml or 15 mg/ml of crude extracts especially in ginger recorded high weight loss which was not different in control fruits after 28 days of storage.

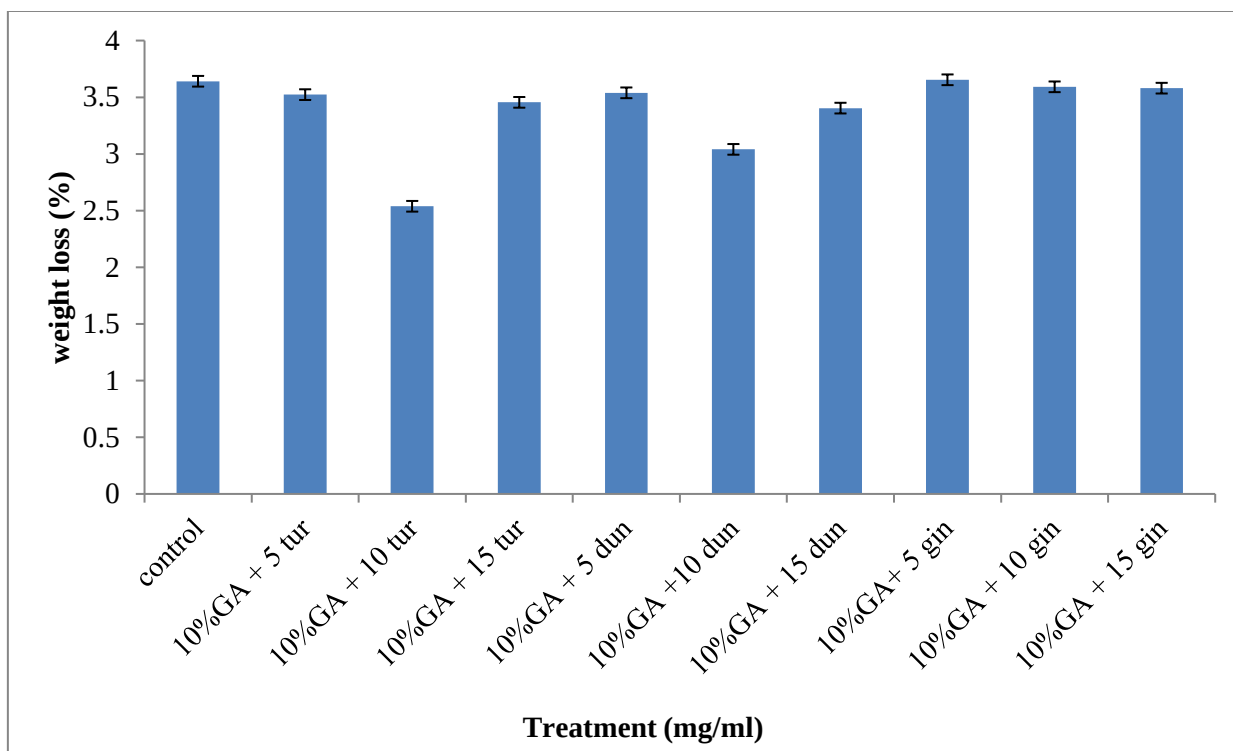


Figure 8-2 Effect of plant extracts at different concentrations incorporated with 10%GA on weight loss after 28 days of cold storage.

At the end of the 28 days of storage, fruits treated with a composite coating of 10%GA+10 mg/ml of turmeric crude extracts recorded the lowest weight loss followed closely by dukung anak coated fruits at 10%GA+10 mg/ml compared to control fruits (Figure 8-2). However, fruits coated with 10%GA plus either 5 mg/ml or 15 mg/ml of crude extracts especially in ginger recorded high weight loss which was not different in control fruits after 28 days of storage (Figure 8-2).

8.4.1.2 Firmness

There was a significant effect ($P<0.05$) between treatment and storage time on firmness (Appendix 0-13). Like weight loss, firmness decreased ($P<0.05$) with storage time in both treated (coated) and control fruits after storage (Figure 8-3).

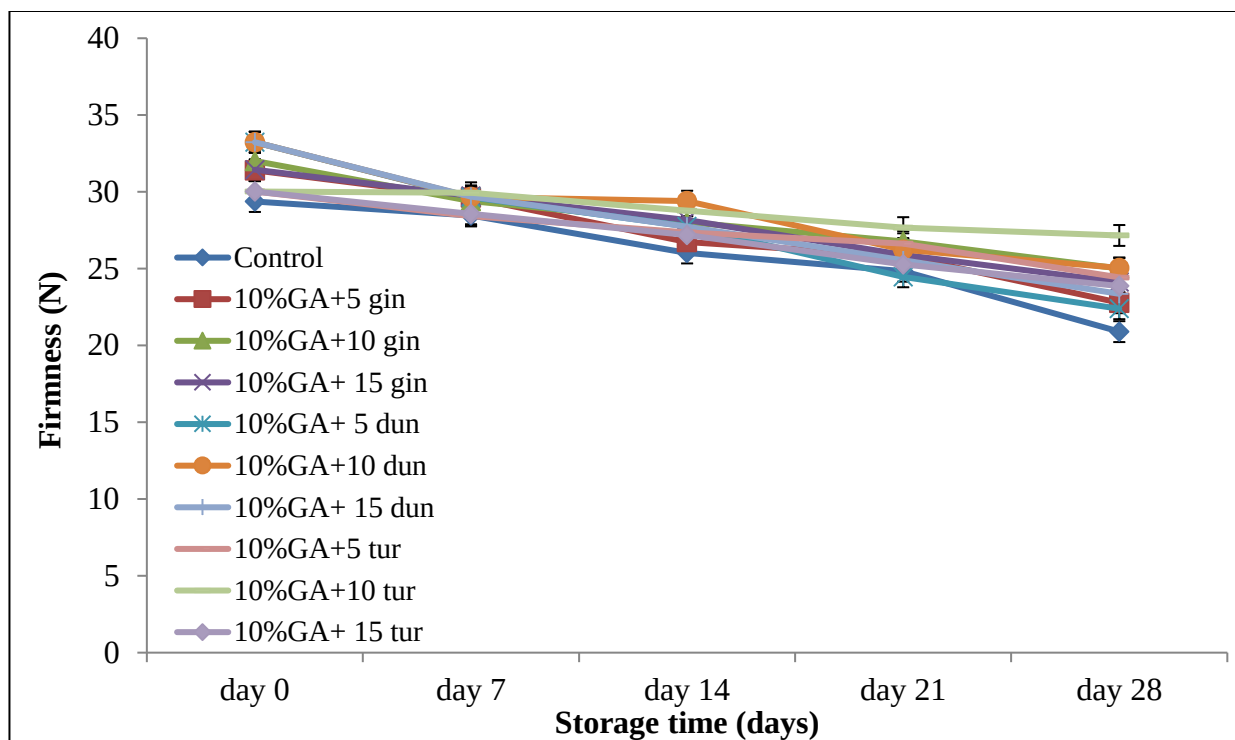


Figure 8-3 Effect of plant extracts at different concentrations incorporated with 10%GA on firmness (N) during 28 days' cold storage.

Despite a decline in fruit firmness, fruits were firmer in treated fruits compared to control fruits (Figure 8-4).

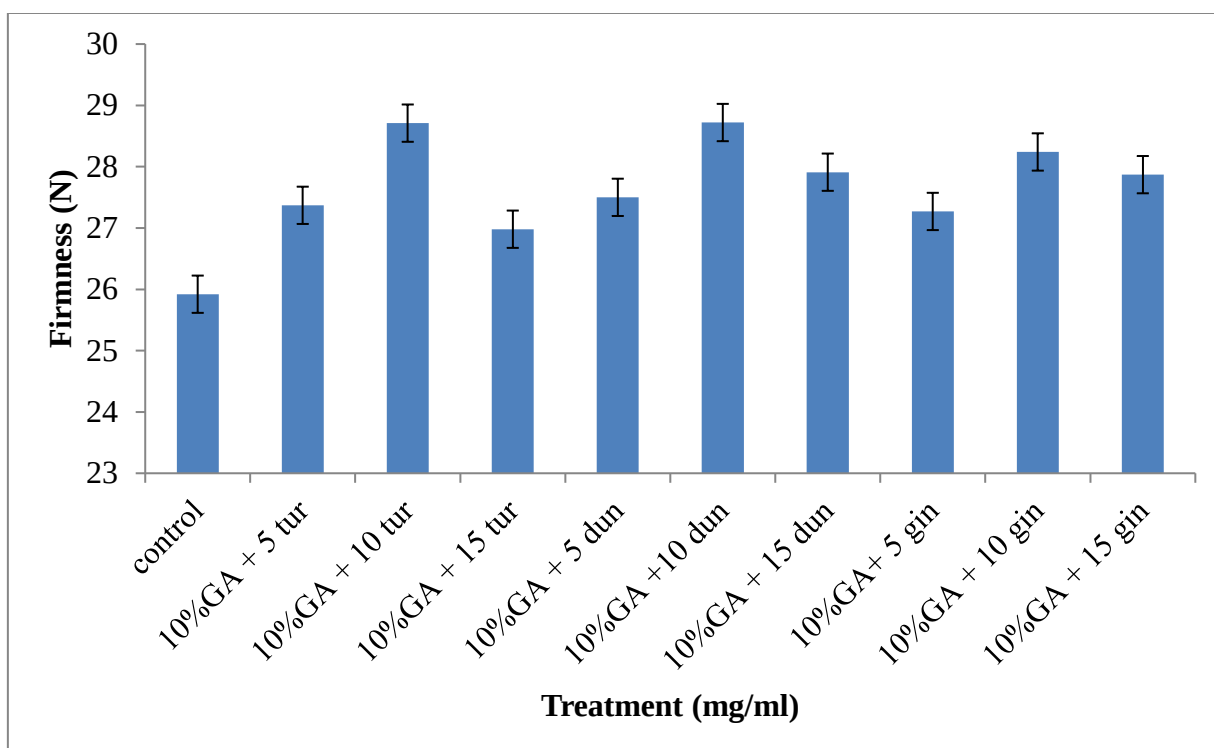


Figure 8-4 Effect of plant extracts at different concentrations incorporated with 10%GA on firmness after 28 days of cold storage

It was evident that composite coating of 10%GA+10 mg/ml of any crude extracts (either turmeric or ginger or dukung anak) significantly ($P<0.05$) helped to maintained fruit firmness. However, Fruits were firmer and maintained especially in dukung anak-coated fruits at 10%GA+10 mg/ml which was not significantly different in turmeric-coated fruits at the same concentrations, whilst the control fruits recorded the lowest weight loss after 28 days of storage (Figure 8-4).

8.4.1.3 Fruit colour development

Firmness analysis, which is a more destructive parameter, and weight loss which didn't require any reflectance measurement on fruit peel, were easy to measure until day 28. Unlike firmness and weight loss, colour changes were observed until day 21 instead of day 28. This is

because most of the peel tissue of fruit were completely damaged as result of DI. At day 28, there was severe DI making it difficult to use the colorimeter due to poor and unstable reflectance which affected the accuracy of hue angle readings.

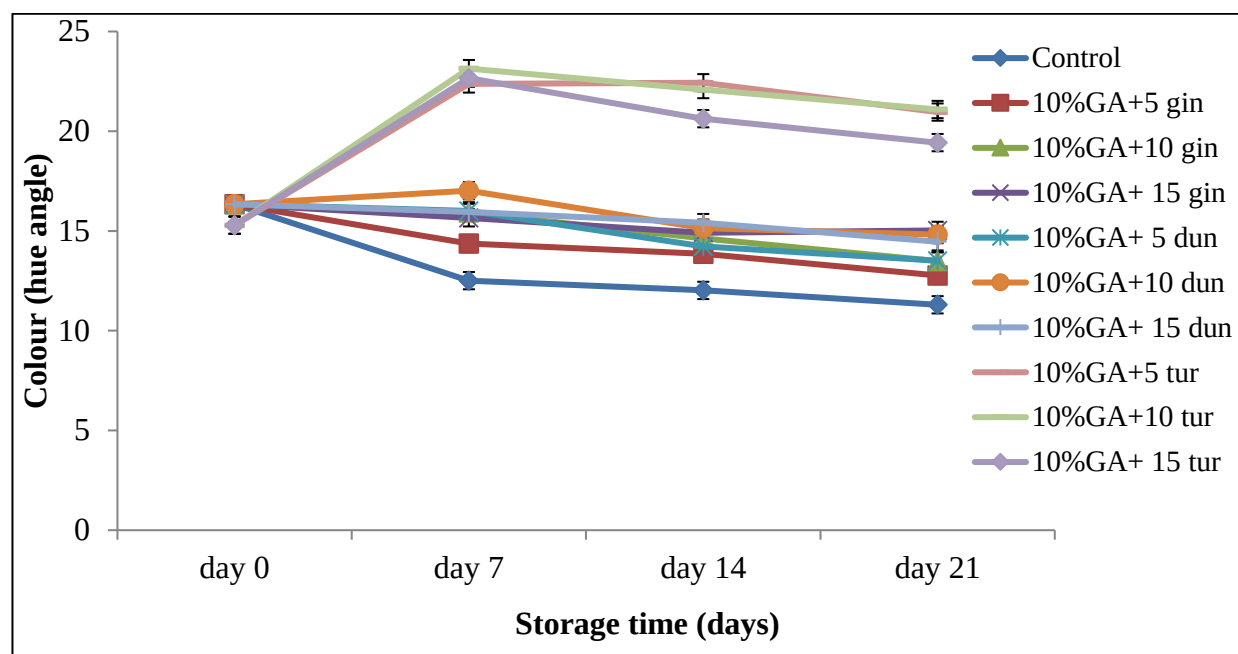


Figure 8-5 Effect of plant extracts at different concentrations incorporated with 10%GA on colour development during 28 days' cold storage.

Hunter lab colorimeter results showed a maintained low hue angle (h^0) in dragon fruit peel throughout the 21 days of storage, however h^0 was lower in control fruits compared to treated fruits (Figure 8-5). Low values of h^0 indicated fruit senescence and ripening. This was evident as dragon fruit peel had a dark colour due to the development of reddish colour in peel as pigmentation process occurred during the postharvest storage (Phe et al., 2009).

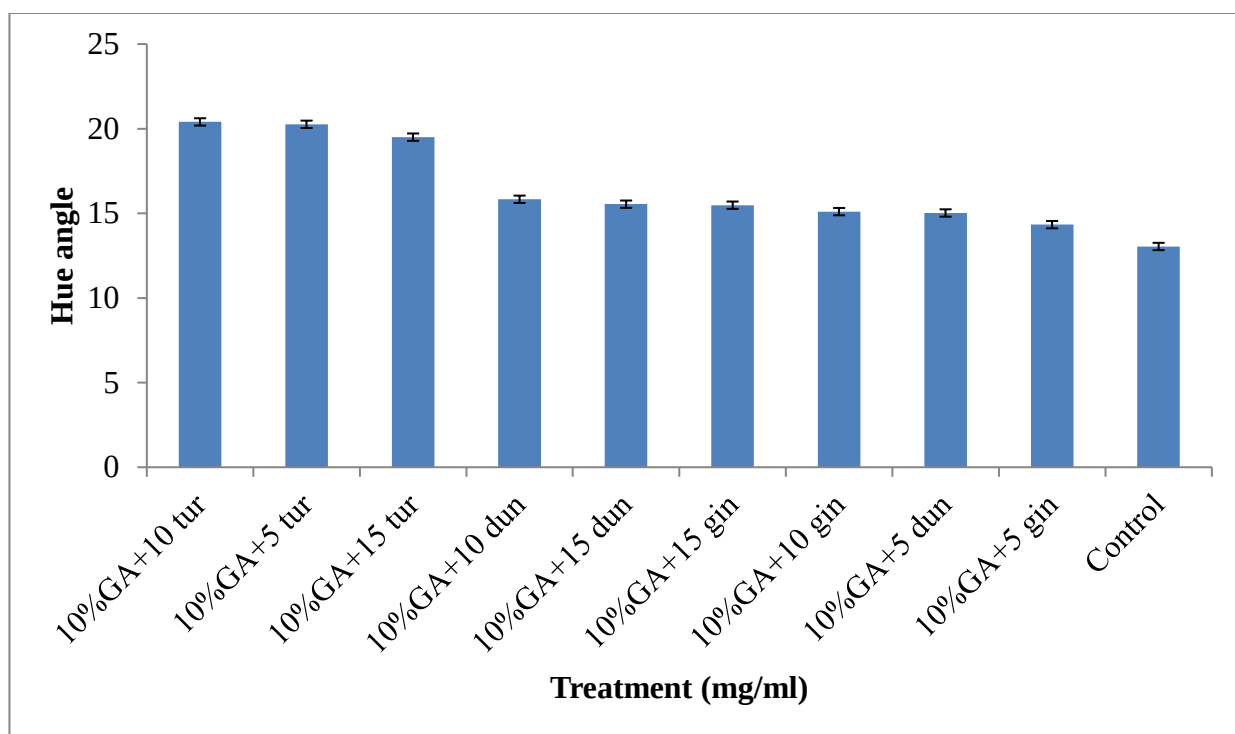


Figure 8-6 Effect of plant extracts at different concentrations incorporated with 10%GA on colour changes (hue angle) after 28 days of cold storage.

A significant effect ($P < 0.05$) between treatment and storage time on colour changes was observed after 21 days of storage (Appendix 0-14). Ripening was delayed ($P < 0.05$) in fruits coated with 10%GA plus turmeric crude extract at any concentration but this was more pronounced at 10%GA+10 mg/ml of turmeric extract compared to control (Figure 8-6). Additionally, a composite coating of 10%GA+10 mg/ml of either Dukung anak or ginger coated crude extracts also markedly delayed colour changes in fruits.

8.4.2 Efficacy of formulation on chemical quality of fruit

TSS, TA, and pH were determined until day 21 since most of the fruits were not edible by the end of the 28 days of cold storage due to severe anthracnose infestation.

8.4.2.1 Total Soluble Solids

There was a significant effect ($P<0.05$) between treatment and storage time on TSS (Appendix 0-15). At day 21, TSS was significantly ($P<0.05$) low at 10%GA+10 mg/ml of ginger coated fruits (2.04^0 brix) which was not different in composite coating of dukung anak coated fruits at 10%GA+10 mg/ml (2.12^0 Brix) or turmeric-coated fruits at 10%GA+10 mg/ml (2.14^0 Brix) compared to control (2.60^0 brix) (Figure 8-7).

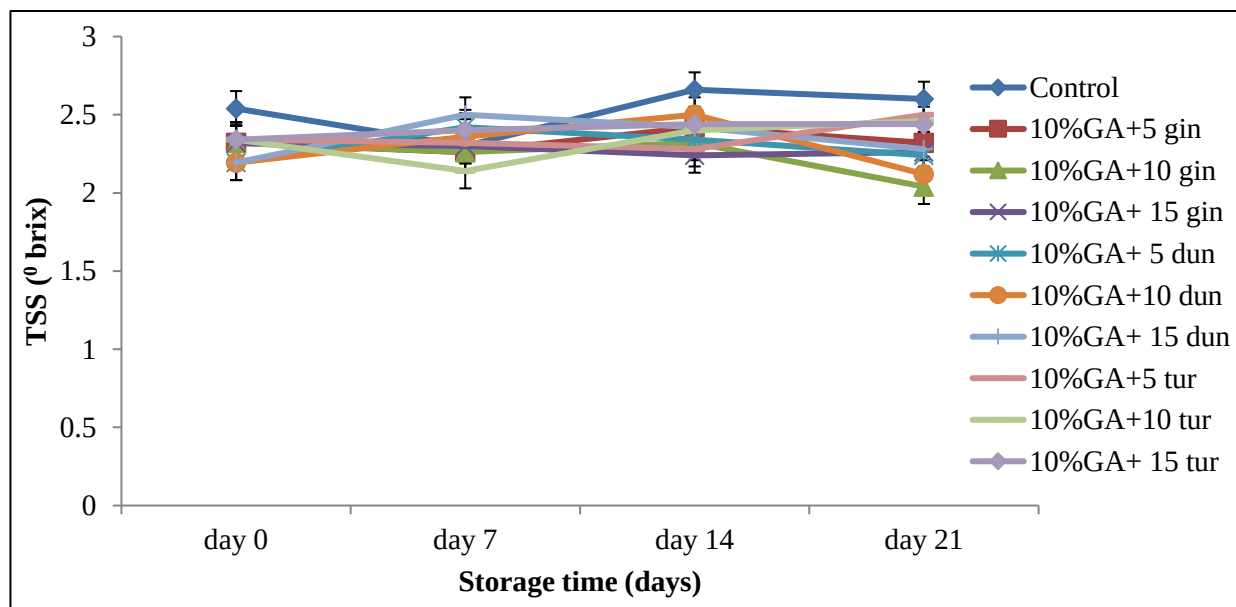


Figure 8-7 Effect of plant extracts at different concentrations incorporated with 10%GA on TSS during 21 days' cold storage.

TSS increased steadily ($P<0.05$) but was maintained in treated fruits, which were lower especially at 10%GA+10 mg/ml in ginger coated fruits compared to controls after 21 days of

storage (Figure 8-8). However, this was not different in turmeric treated fruits 10%GA+10 mg/ml.

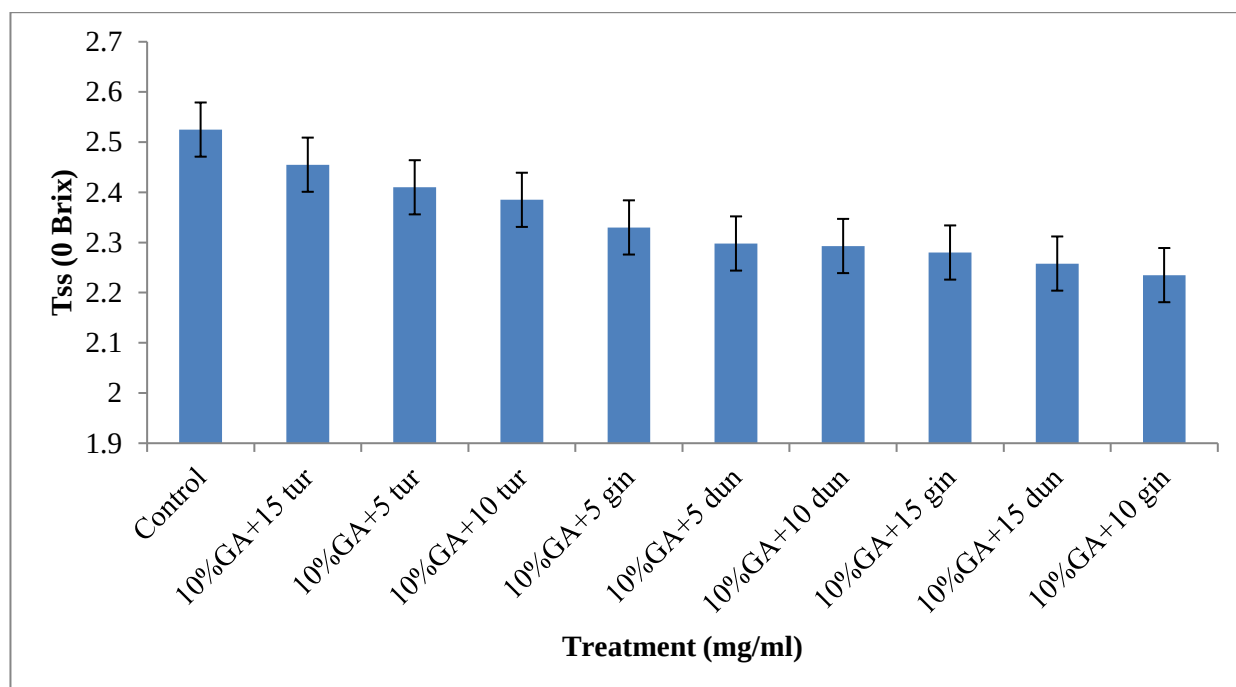


Figure 8-8 Effect of plant extracts at different concentrations incorporated with 10%GA on TSS after 21 days of cold storage.

8.4.2.2 Titratable acidity

A significant effect ($P < 0.05$) between treatment and storage time on TA was observed after 21 days of storage (Appendix 0-16). TA decreased steadily with storage time in both treated and control fruits; however, TA was relatively high in treated fruits compared to controls (Figure 8-10). Unlike TSS, TA was significantly lower ($P < 0.05$) in control fruits (0.064%) at day 21 but was highest in composite coating of 10%GA+10 mg/ml of dukung anak -treated fruit (0.11%), followed closely by composite coated fruits at 10%GA+ 15 mg/ml ginger crude extract (Figure 8-8) which was not different in turmeric treated fruits at 10%GA+10 mg/ml.

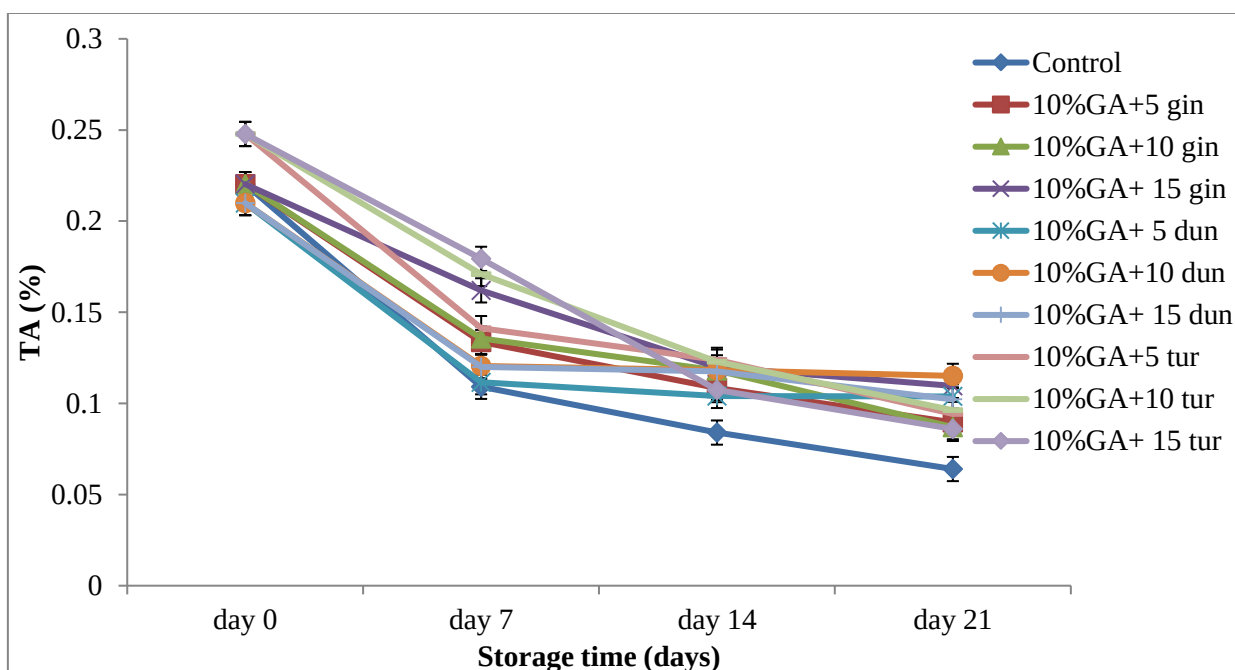


Figure 8-9 Effect of plant extracts at different concentrations incorporated with 10%GA on TA for 21 days' cold storage.

At the end of the 21 days of cold storage, TA was highest in fruits coated with 10%GA+10 mg/ml of turmeric crude extract which was not different in fruits coated with 10%GA+15 mg/ml of ginger or 10%GA+10 mg/ml of dukung anak-coated fruits compared to control (Figure 8-10).

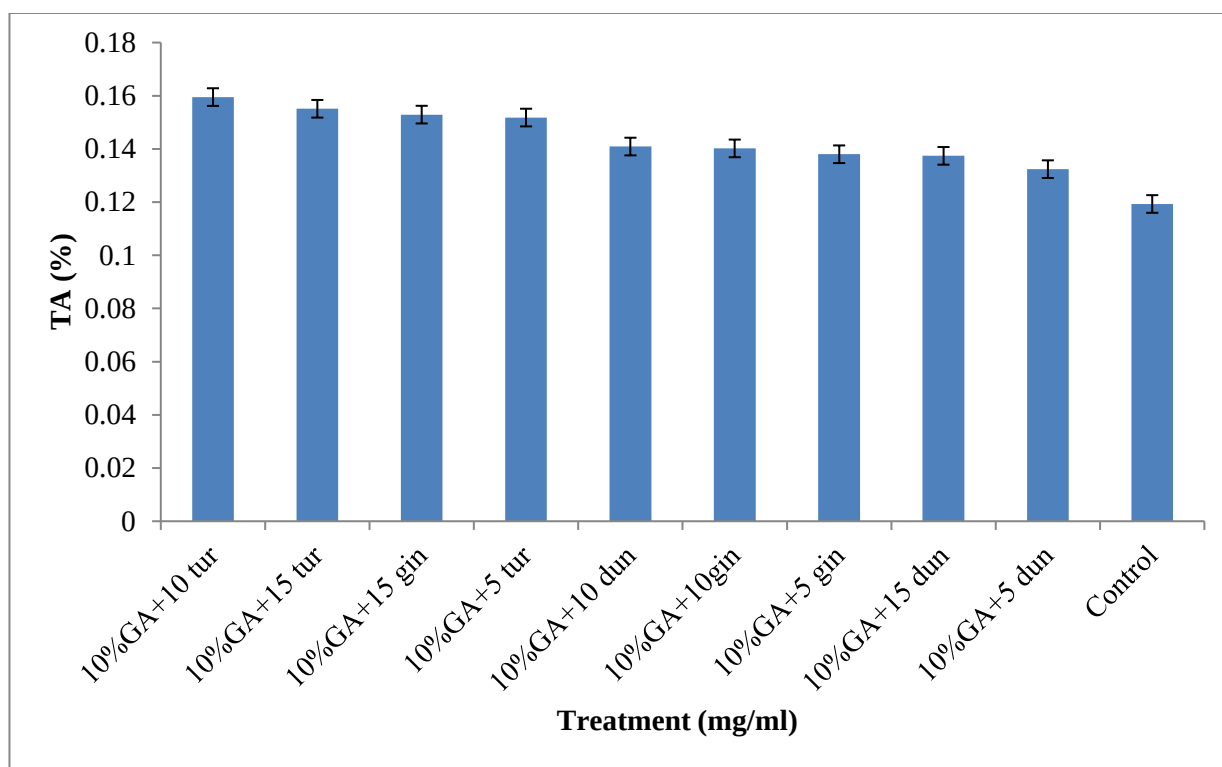


Figure 8-10 Effect of plant extracts at different concentrations incorporated with 10%GA on TA after 21 days of cold storage.

8.4.2.3 pH

pH increased steadily ($P < 0.05$) with storage time in both treated and control fruits; however, it was relatively low in treated fruits compared to controls after 21 days of storage (Figure 8-11). There was a significant interaction ($P < 0.05$) between treatment and storage time after 21 days of cold storage on pH (Appendix 0-17).

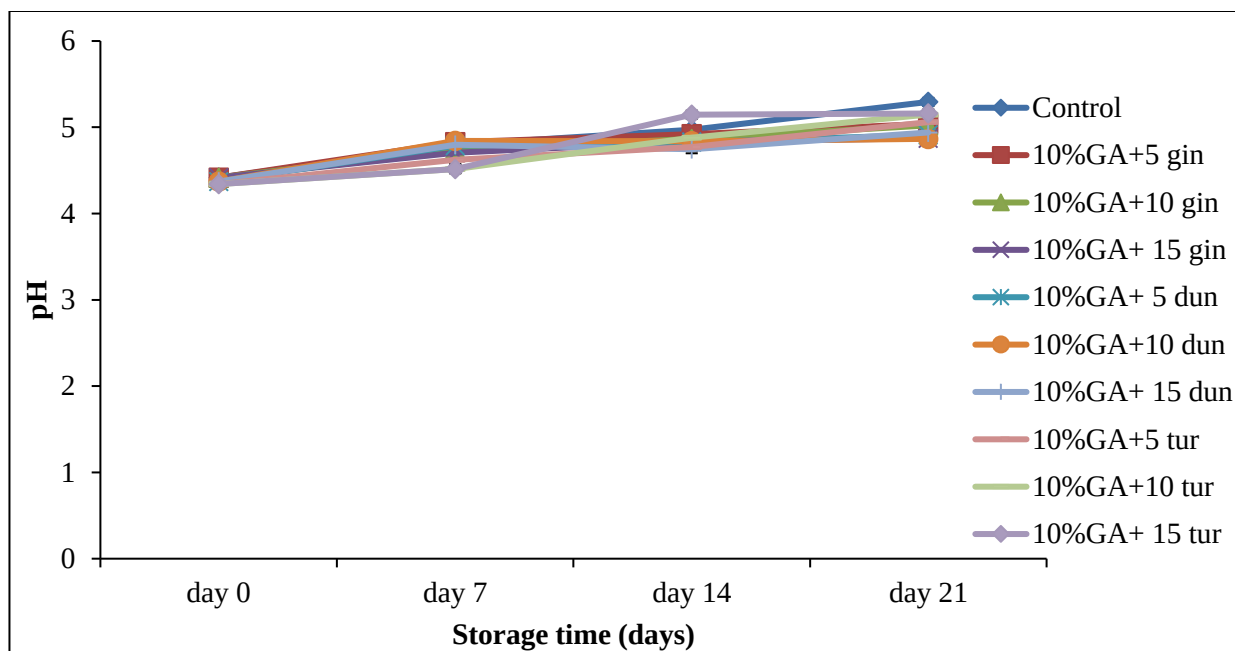


Figure 8-11 Effect of plant extracts at different concentrations incorporated with 10%GA on pH during 21 days' cold storage.

At day 21 fruits coated with 10%GA+15 mg/ml of dukung anak crude extract recorded the lowest pH (4.86) ($P<0.05$) which was not different in fruits coated with 10%GA+15 mg/ml ginger crude extract (4.87) compared to control (5.29) (Figure 8-11). At the end of the 21 days of cold storage, fruits coated with 10%GA+15 mg/ml of ginger crude extract recorded the lowest pH ($P<0.05$) which was not different in fruits coated with either 10%GA+10 mg/ml of turmeric or 10%GA+15 mg/ml of dukung anak compared to control which recorded the highest pH.

8.4.3 Efficacy of formulation on gas exchange

8.4.3.1 Respiration rate

Significant effect ($P < 0.05$) between treatment and storage time was observed in respiration rate (

Appendix 0-18). A continuous decline in respiration rate occurred in all coated treated fruit compared to control. A sudden decrease in respiration rate was observed on the 7th day and was maintained until day 14 but increased afterward until day 21 (Figure 8-12).

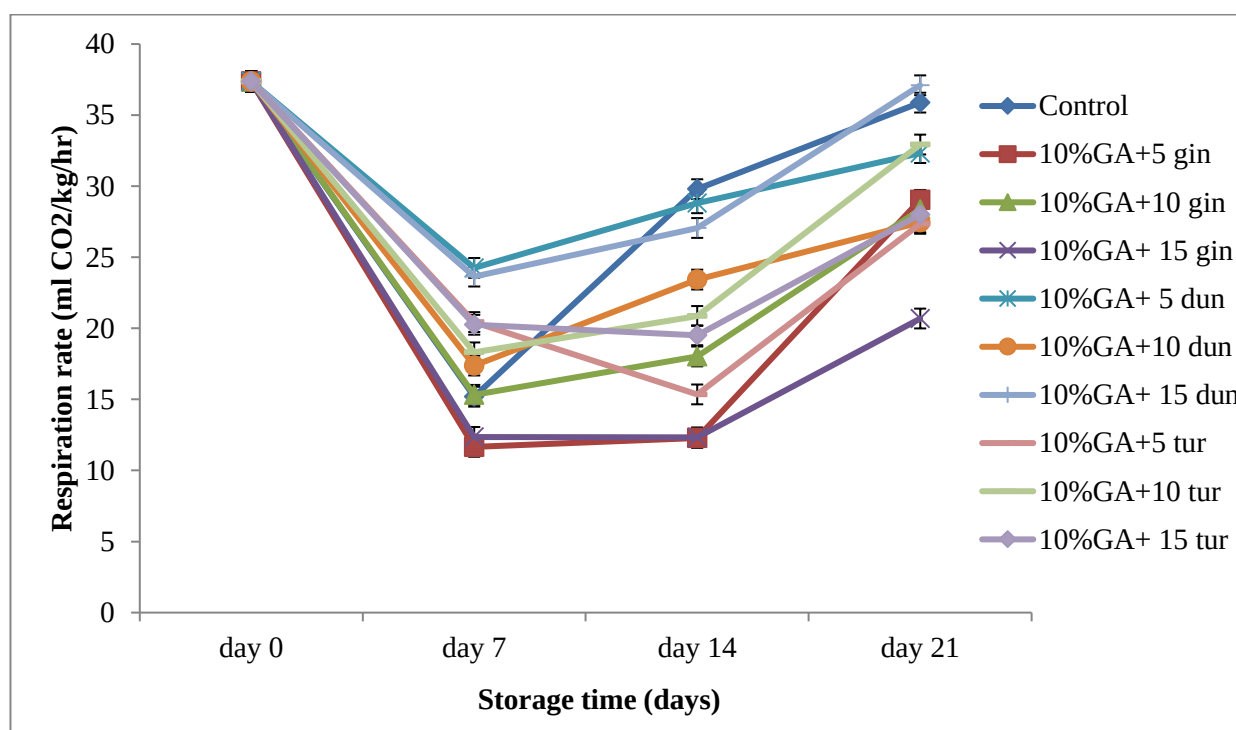


Figure 8-12 Effect of plant extracts at different concentrations incorporated with 10%GA on respiration rate during 21 days' cold storage.

At day 21, the treatment that significantly reduced ($P < 0.05$) respiration rate during storage was composite coating of 10%GA+5 mg/ml of turmeric crude extract (27.35 ml CO₂/kg/hr.),

followed closely by 10%GA+ 10 mg/ml of dukung anak crude extract (27.47 ml CO₂/kg/hr.) compared to control (35.88 ml CO₂/kg/hr.) (Figure 8-12).

At the end of 21 days of storage, respiration rate was highest ($P<0.05$) in control fruits even though not different in dukung anak coated fruits at 10%GA+15 mg/ml while it was lowest ($P<0.05$) ginger coated fruits at 10%GA+5 mg/ml. In the case of turmeric treated fruits, respiration rate was low at 10%GA+5mg/ml of turmeric crude extract.

8.4.3.2 Ethylene production

There was a significant effect ($P<0.05$) between treatment and storage time on ethylene production (

Appendix 0-19). Ethylene production decreased with storage time without any peak or drastic increase and the decline maintained throughout until day 21 (Figure 8-13) contrary to respiration rate where it increased drastically after day 14 until the end of 21- day storage (Figure 8-13). This phenomenon suggests the non-climacteric behaviour of dragon fruit.

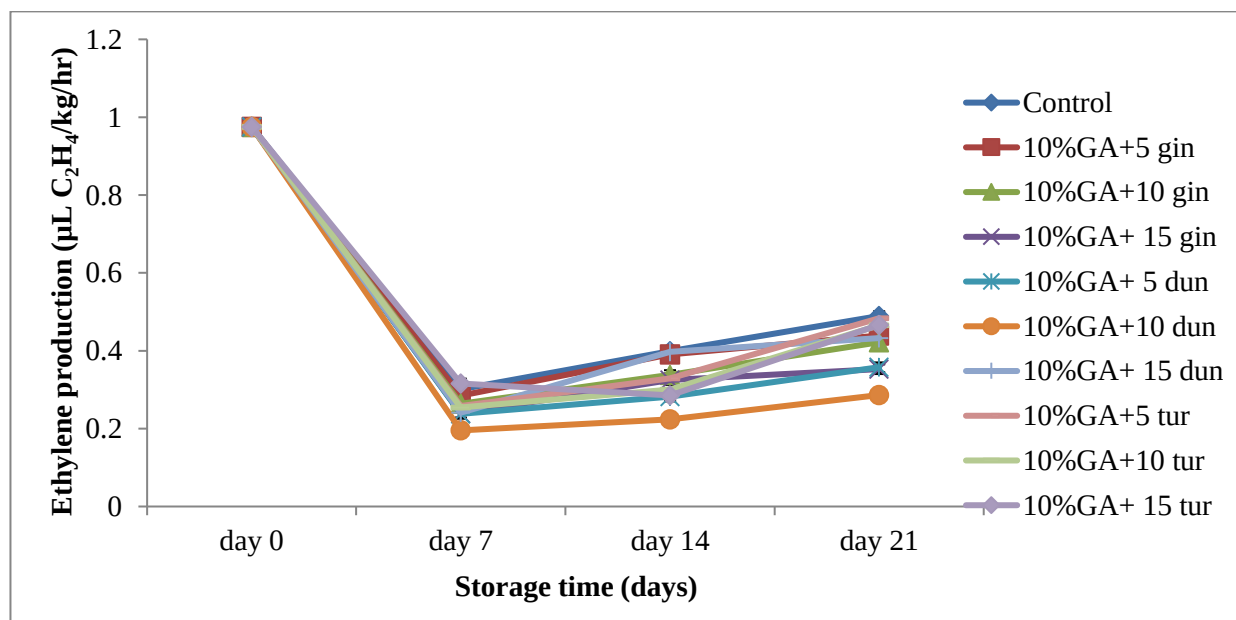


Figure 8-13 Effect of plant extracts at different concentrations incorporated with 10%GA on ethylene production during 21 days' cold storage.

Ethylene production was significantly lower in composite coating of fruits coated with 10%GA+10 mg/ml of dukung anak crude extract ($0.28 \mu\text{l C}_2\text{H}_4/\text{kg/hr.}$), ginger coated fruits at 10%GA+15 mg/ml followed closely with ethylene production of $0.35 \mu\text{l C}_2\text{H}_4/\text{kg/hr.}$ compared to controls ($0.48 \mu\text{l C}_2\text{H}_4/\text{kg/hr.}$) at day 21 cold storage (Figure 8-13). For turmeric composite coating, fruits treated with 10%GA+10 mg/ml recorded slightly lower ethylene production of $0.46 \mu\text{l C}_2\text{H}_4/\text{kg/hr.}$ compared to control at day 21.

After 21 days of cold storage, ethylene production was significantly low ($P<0.05$) in fruits coated with 10%GA+10 mg/ml of dukung anak extract compared to control. In turmeric-coated fruits, ethylene production was markedly low at 10%GA+10 mg/ml compared to control.

8.4.4 Effect of formulation on nutritional and antioxidant quality of fruit

Table 8-1 Two-way Anova table on the effect of formulation on nutritional qualities after 21 days of storage.

	TPC (mg GAE/100g)			DPPH (%)			AA (mg of AA/100g)		
	Extracts (E)								
Concentration (C)	Turmeric	Dukung anak	Ginger	Turmeric	Dukung anak	Ginger	Turmeric	Dukung anak	Ginger
Con	178.0fg			23.05e			11.87e		
5mg/ml+10%GA	256.9a	177.1g	188.5de	34.34bc	29.03d	28.56d	16.62a	14.03c	11.71e
10mg/ml+10%GA	251.4a	201.3c	178.9efg	38.03a	33.98c	32.91c	16.17a	14.83b	12.08de
15mg/ml+10%GA	236.2b	194.4cd	188.8de	37.84ab	27.53d	30.97cd	16.40a	14.09c	12.67d
Sem (T*)	3.86			1.271			0.249		
P-value (T*)	0.001			0.001			0.001		

Levels connected vertically by different letters are significantly different.

C= Different concentrations for each plant extracts incorporated with 10%GA.

T* (Treatment) = Combination of each extracts (E) at different concentrations (C) after two-way Anova.

8.4.4.1 Total phenolic content

TPC assay was expressed as gallic acid equivalent (i.e. x mg of gallic acid per 1 g of the extract). TPC of treatment was calculated from the calibration curve of gallic acid where the calibration equation was determined to be $y = 4.6988x + 0.001$ ($R^2 = 0.9916$), whereby y = absorbance at 765nm and x = concentration of total phenolic compounds in mg per 1 ml of the extract. We observed that reaction mixture of pulp extracts of the fruit was light blue in colour that visually indicated the presence of phenolic content. The light blue colour was due to the use of diluted Folin-Ciocalteu's reagent (50%). Under high concentration of this reagent, the colour of extract after reaction becomes dark blue, signifying the presence of high total phenolic content (Nurliyana et al., 2010). In this study, we used 50% Folin-Ciocalteu's reagent, hence the light blue colour.

A significant effect ($P < 0.05$) between fruits coated with 10%GA plus crude extracts at different concentrations and storage time on TPC (Appendix 0-20). A decline in TPC was observed until day 14 but increased slightly afterward until the end of storage irrespective of plant extract used (Figure 8-14). TPC was high in all treated fruits, and this was more pronounced ($P < 0.05$) in fruits coated with 10%GA+10 mg/ml of turmeric crude extracts (254.8 mg GAE/100g FW) which was not different in the same turmeric coated fruits at 10%GA+5 mg/ml (254.9 mg GAE/100g FW) compared to control (187.3 GAE/100g FW) at day 21 (Figure 8-14).

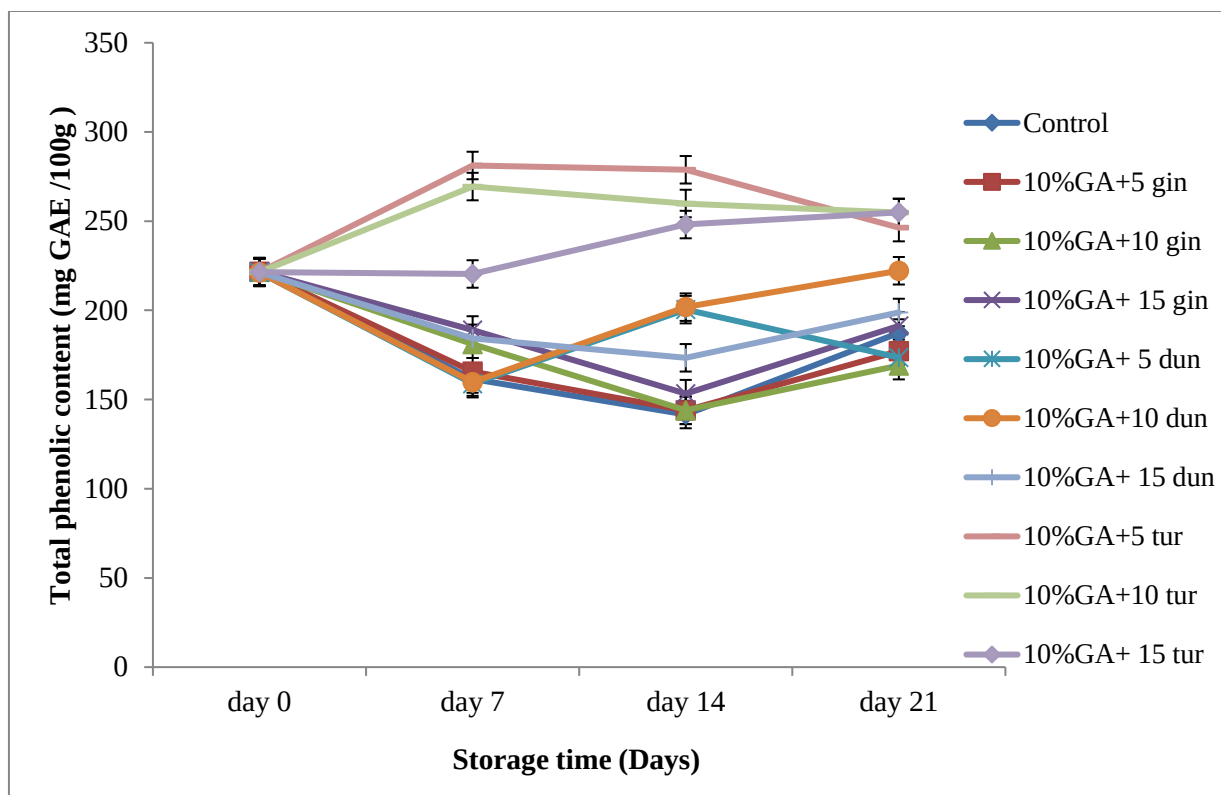


Figure 8-14 Effect of plant extracts at different concentrations incorporated with 10%GA on TPC during 21 days' cold storage

At the end of the 28 days of storage, fruit coated with 10%GA+5 mg/ml turmeric crude extract recorded the highest TPC which was not different at 10%GA+10 mg/ml of the same extract compared to control (178 mg GAE/100g FW) after 21 days of storage (Table 8-1).

8.4.4.2 DPPH Free Radical Scavenging Activity

A significant effect ($P < 0.05$) between treatment and storage time was observed in DPPH free radical scavenging activity (

Appendix 0-21). Free radical scavenging activity declined throughout the storage time (Figure 8-15). However, it was relatively high in treated fruits compared to control fruits.

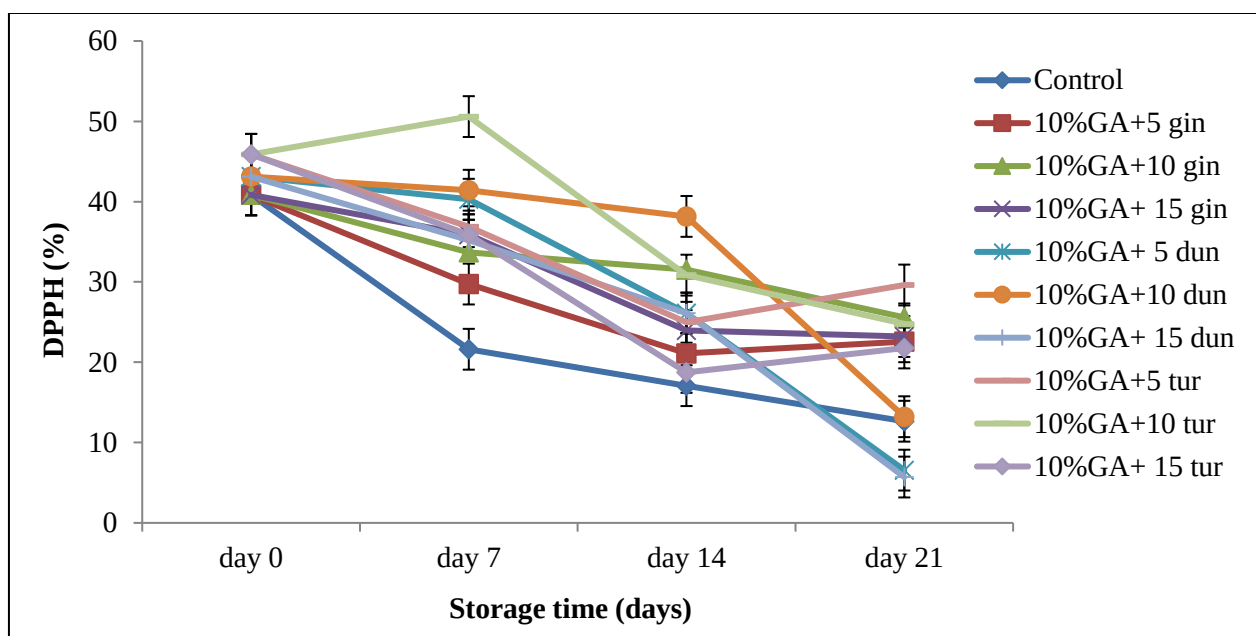


Figure 8-15 Effect of plant extracts at different concentrations incorporated with 10%GA on DPPH activity during 21 days' cold storage.

At the end of the 21 days, all turmeric-coated treated fruits showed high DPPH activity and this was more pronounced ($P<0.05$) at 10%GA+10 mg/ml compared to control (Table 8-1). Similarly, DPPH was high in dukung anak and ginger-coated fruits at 10%GA+10 mg/ml and 10%GA+10 mg/ml respectively compared to controls (Table 8-1).

8.4.4.3 Ascorbic acid content

Ascorbic acid (AA) content in fruits decreased with storage time in both treated and control fruits during the 21 days of cold storage (Figure 8-16). However, AA levels in treated fruits were higher than in control fruits (Table 8-1). There was a significant interaction ($P<0.05$) between fruits coated with 10%GA plus crude extracts at different concentrations and storage time in AA content of fruits after 21 days of storage (Appendix 0-22).

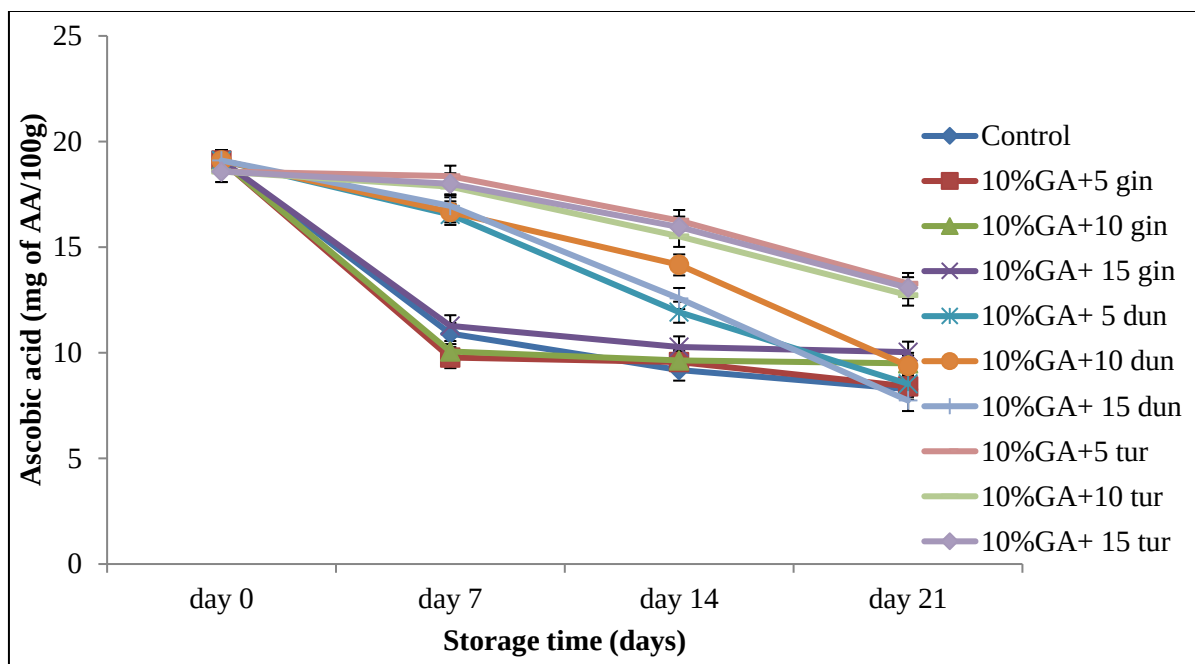


Figure 8-16 Effect of plant extracts at different concentrations incorporated 10%GA on ascorbic acid content during 21 days' cold storage

Like TPC and DPPH activity, AA was high in all the turmeric coated fruits and this was more pronounced ($P < 0.05$) at 10%GA+15 mg/ml (13.08 mg of AA/100g) compared to control (8.28 mg of AA/100g), even though it was not different at 10%GA plus 5 mg/ml (12.73 mg of AA/100g) of the same extract at day 21 day. In Dukung anak and ginger coated fruits, AA was high at 10%GA+10 mg/ml (9.38 mg of AA/100g) and 10%GA+10 mg/ml (12.08 mg of AA/100g) respectively compared to controls (Figure 8-16).

At the end of the 21 days of storage, turmeric-coated fruits at 10%GA+5 mg/ml recorded the highest AA levels which was not different in 10%GA+10 or 10%GA+15 mg/ml of the same extract compared to control (Table 8-1).

8.4.4.4 Sensory evaluation

Sensory evaluation of coated and uncoated (control) dragon fruits at the end of storage period revealed significant ($P < 0.05$) differences in appearance, pulp colour, aroma, sweetness, texture and overall acceptability.

Table 8-2 Sensory evaluation of dragon fruits treated with plant extracts at different concentrations incorporated with 10%GA.

Treatment	Sensory quality parameters					
	Appearance	Pulp colour	Aroma	Sweetness	Texture	Overall Acceptability
Control	2.17a	2.33c	3.67a	1.67d	3.17ab	2.00c
10%GA+ 5 dun	3.33a	3.0b	3.17b	3.16bc	2.67bc	3.17a
10%GA+10 dun	3.33a	3.5ab	3.33b	3.00bc	3.17ab	3.17a
10%GA+5 tur	3.50a	3.67a	3.33a	3.50a	3.33ab	3.50a
10%GA+ 10 tur	3.67a	3.67a	4.17a	4.67a	3.83a	3.83a
10%GA+ 5 gin	3.50a	3.67a	3.33b	3.67bc	2.83bc	3.33a
10%GA+ 10 gin	3.50a	3.50ab	2.83bc	2.50c	3.00abc	3.00a
Sem	0.252	0.2218	0.27	0.287	0.309	0.303
P-value	0.001	0.001	0.001	0.001	0.021	0.007

Means with different letters in a column are significantly different at $P < 0.05$ using fisher unprotected test. Storage conditions; 21 days at $11 \pm 2^\circ\text{C}$, 80% RH (n= 42 fruits).

There was a varied opinion by a panellist on all parameters during sensory evaluation. However, fruits coated with 10%GA plus 10 mg/ml of turmeric attained the highest score (between 3.83 to 4.67) in terms of good external appearance, pulp colour, aroma, sweetness, texture and overall acceptability during the sensory evaluation (Table 8-2). This indicated that fruits treated at 10%GA plus 10 mg/ml of turmeric was widely accepted and good to the panellist compared to control.

8.5 Discussion

8.5.1 Efficacy of formulation on physicochemical quality of dragon fruits

8.5.1.1 Weight loss

In this study, weight loss increased progressively ($P < 0.05$) with storage time irrespective of treatment used. Composite coating of 10%GA+10 mg/ml of turmeric extracts significantly reduced weight loss in dragon fruits, followed closely by fruits coated with 10%GA+10 mg/ml of dukung anak extracts compared to control. Overall, fruits coated with 10%GA+10 mg/ml of any crude extracts reduced weight loss while those coated with 10%GA incorporated with 15 mg/ml or 5 mg/ml of any extracts reportedly recorded higher weight loss which was not different in control after 28 days of storage. Once ripened, matured dragon fruits are harvested and the fruits remain normal for a few days or weeks during which several physiological and metabolic changes occur. During this time, the fruits continue to respire and transpire, losing some of its substrate and water which results in weight loss. It is reported that the weight loss from fresh produce is through the peel by vapour pressure differentials, which can cause flesh softening, fruit ripening and senescence by metabolic reactions (Yaman and Bayoindirli, 2002; Ghasemnezhad et al., 2010). Generally, respiration and moisture evaporation through the skin are correlated with fruit weight loss. The thickness of the fruit skin determines its susceptibility to rapid water loss resulting in shriveling and deterioration (Hernandez-Munoz et al., 2008). Hence, using edible coatings maintain the fruit quality by maintaining the turgidity through water retention in the fruit (Chien et al., 2007b). In the present study, the reduction in weight loss especially in fruits coated with either 10%GA+10 mg/ml of turmeric or dukung anak was probably due to the synergistic effect of 10%GA and 10 mg/ml of extract. The 10%GA served

as a semi-permeable barrier against oxygen, carbon dioxide, and moisture, thus reducing respiration, transpiration and oxidation reactions (Baldwin et al., 1999; Park, 1999) while the crude extract (especially turmeric) at 10 mg/ml provided antifungal effect in reducing disease incidence caused by the fungus. Normally, the advent of DI generates peel tissue damage which increases respiration, resulting in excessive weight loss and decrease in firmness. Our results support findings of Maqbool et al. (2010a) where composite coatings of 10%GA combined with 0.75% chitosan extended the storage life of banana for up to 33 days by reducing weight loss since GA plays a semi-permeable barrier to this effect despite chitosan also exhibiting a semi-permeable apart from its antifungal effect. Composite coatings of GA combined with other natural products (cinnamon oil and propolis) or synthetic chemicals (calcium chloride) or silver nanoparticles delayed fruit ripening in terms of reduction in weight loss in banana, mangoes, green bell pepper and papaya (Maqbool et al. 2010b; Maqbool et al., 2011; Ali et al., 2014; Hedayati and Niakousari, 2015; Khaliq et al., 2016) confirmed our study.

Composite coatings of 10%GA plus either 5 mg/ml or 15 mg/ml of especially ginger crude extract recorded significantly higher weight loss, which could be explained by the thickness of composite coatings. A coating of 10%GA+5 mg/ml of ginger crude extract was not thick enough to provide enough barriers against water loss, whereas a 10%GA+15 mg/ml composite coating was so thick that it completely blocked the lenticles (Maqbool et al., 2011) due to the presence of excess gingerol (oils). Alternatively, the high weight loss, especially in composite coated fruits at 10%GA+15 mg/ml, could be due to excessive heat generation due to 10%GA plus relatively high amount of lipids in ginger at 15 mg/ml that led to an increased anaerobic respiration (Weichmann, 1987; Maqbool et al. 2011). Similarly, Zahid et al. (2013) reported

higher weight loss of fruits, which they concluded, was due to the heat production of fruits treated with higher concentration of ethanolic propolis as an edible coating on dragon fruits. Ghasemnezhad et al. (2010) also reported a similar trend of high weight loss in high chitosan coating in apricot. Maqbool et al. (2011) also reported high weight loss in the composite coating of 20% GA plus 1.0% CH in banana and concluded that composite coating on banana fruits might be due to the generation of heat and production of end products from anaerobic fermentation.

8.5.1.2 Firmness

One of the physical appeals of fruits that affect a customer's decision to buy is firmness. However, during postharvest storage, fruits ripening and other physiological activities affect firmness. In this study, firmness decreased with storage time irrespective of treatment used despite treated fruits were much firmer ($P < 0.05$) compared to control. Overall, fruits were firmer when coated with 10%GA+10 mg/ml of dukung anak extracts which was not significantly different in turmeric and ginger coated fruits at the same concentrations compared to control after 28 days of storage. Fruit softening is commonly due to the damage of the cell structure and the weakening in cell wall conformation during ripening (Seymour et al., 1993; Deytieux-Belleau et al., 2008). As a biochemical process, fruit softening involves the hydrolysis of pectin and starch by hydrolases in fruit cell walls (Seymour et al., 1993). As fruits ripens, there is depolymerisation or decrease of pectin chain length culminating to increased activities of pectin esterase and polygalacturonase leading to reduction of fruit firmness (Yaman and Bayoindirli, 2002; Bai et al., 2003). Maqbool et al. (2011) reported that the reduced respiration in coated bananas could be responsible for delaying ripening, which

could be due to low levels of oxygen and relatively high levels of carbon dioxide restricting the activities of cell wall degrading enzymes thereby retaining fruit firmness during storage.

In this study the maintenance of fruit firmness in dragon fruit coated at 10%GA+10 mg/ml of dukung anak or turmeric composite coating could be due to synergistic effect of extract at 10 mg/ml and 10%GA. The 10 mg/ml resulted in higher antifungal activity to control disease infection, since the advent of DI results in peel tissue damage which facilitates high weight loss and reduction in firmness through respiration, therefore the 10%GA provided an additional film-forming property to reduce transpiration and respiration as well as slow down the ripening, hence reducing firmness during storage. Our study confirms other studies where composite coating of chitosan and gum arabic application on banana, composite coating of ethanolic extract of propolis and gum arabic application on papaya and application of ethanol extract of propolis on dragon fruit resulted in modified atmosphere in fruit surfaces, reduced moisture loss and consequently retained the firmness of fruits during the physiological and horticultural maturity of fruit (Ali et al., 2014; Maqbool et al., 2011; Zahid et 2013). Fruits coated with 10%GA plus 5 mg/ml of any of the three-crude extract could not maintain fruit firmness probably due to the low antifungal activity against the host-pathogen at 5 mg/ml, thereby leading to disease infection, which in turn increased weight loss and decreased firmness. Another possible reason could be that 10%GA+5 mg/ml of any crude extract could not significantly modify gaseous exchange composition thereby not being able to delay ripening resulting in reduction on fruit firmness. Whilst at 10%GA plus 15 mg/ml, we propose the decrease in firmness could be due to anaerobic respiration leading to disease infestation, severe weight loss and to some extent decrease in firmness since this composite coating was too thick thereby completely covering the surface of the fruit and blocking the lenticels. Crude

extracts like ginger and turmeric especially at high concentrations (15 mg/ml) contains high amount of lipids that could have contributed to the above effect. This confirm a study by Maqbool et al. (2011) where reduction in fruit firmness was highest in the composite coating at 20% GA plus 1.0% CH in banana, and the authors concluded that composite coating on banana fruits might be due to the generation of heat and production of end products from anaerobic fermentation.

8.5.1.3 Colour

Dragon fruits develop reddish dark colour as pigmentation process occurs and this is evident in decreasing hue angle values (Phebe et al., 2009). The continuous reddish dark colour developed at horticultural maturation during storage is a sign of fruit senescence and therefore needs to be controlled. Reddish dark pigmentation in fruit peel increased significantly with storage time irrespective of coated treatment used and it was significantly lower in turmeric-coated fruits compared to controls. Overall, fruits coated with 10%GA plus 10 mg/ml of crude extracts significantly delayed peel colour changes though not different in fruits coated at 10%GA plus 15 mg/ml of extracts after 21 days of storage. Overall, colour saturation was significantly lower (delayed) in fruits coated with 10%GA plus 10 mg/ml or 5 mg/ml of turmeric even though the delay was pronounced at 10mg/ml compared to controls.

The possible explanation for the colour change in fruit peel was associated with the enzymatic degradation of chlorophyll as fruit ripeness increased (Ding et al., 2007). This process involved the dissolution of thylakoids and stroma within the chloroplasts which caused the loss of structure of chloroplasts accompanied by degradation of chlorophyll. Thereby greatly reducing absorption of the red and blue region in the electromagnetic spectrum as chlorophyll absorbs light strongly in the red and blue regions (Taiz and Zeiger, 2006), hence,

loss of green colour in fruit peel. According to Nerd and Mizrahi (1997), the changes of peel colour to pale green then to reddish of pitaya fruit was due to the changes in peel chlorophyll content during fruit development. Additionally, as degreening occurs or colour development increases during ripening, there is loss of chlorophyll which leads to decrease in fruit firmness due to activities of cell wall degrading enzymes, leading to excessive weight loss, drastic changes in gaseous exchange around the fruits commencing disease infestation in fruits (Pelayo et al., 2003).

In this study fruits coated with 10%GA plus 10 mg/ml of turmeric had reduced reddish to dark colour saturation. This could be attributed to the slower rate of respiration and reduced ethylene production due to the synergistic effect of 10%GA that modified the atmosphere around the fruit, whilst turmeric extracts concentration at 10 mg/ml helped to reduce fungal infection that could have resulted in excessive weight loss and fruit firmness. This phenomenon helped to retard the ripening process and senescence of the fruits, ultimately resulting in reduced colour change and fruit firmness (Maqbool et al., 2011). Despite colorimeter is used objectively to determine physiological changes such as colour development during fruit ripening, some factors may affect its accuracy. For instance, when fruits are treated (coated) with products that exhibit intense colour (in this case, yellowish colour of turmeric crude extract), they tend to skew the reflectance readings obtained from the colorimeter in one direction. Therefore, in such instances, other physiological parameters such as weight loss, fruit firmness should be considered during the postharvest quality analysis. In this study we showed that, 10%GA+10 mg/ml did not only delayed ripening base on colour changes but it also helped to reduce weight loss and firmness.

The results from this study agree with those of Hedayati and Niakousari (2015) where 10%GA combined with silver nanoparticles as a composite coating significantly delayed colour changes in green bell pepper for 21 days during cold storage. Similarly, Ali et al. (2014) also confirmed our study when the application of an ethanolic extract of propolis (1.5%) combined with 10%GA reduced occurrence of anthracnose and delayed colour changes in papaya for 28 days of cold storage.

8.5.1.4 Total soluble solids

Composite coatings are reported to provide an excellent semi-permeable barrier around the fruit, hence modifying the internal atmosphere by reducing oxygen and elevating carbon dioxide levels and suppressing ethylene evolution (Maqbool et al., 2011). It is reported that as fruits respire quicker, they ripened faster as well which leads to high level of TSS and low level of TA due to the breakdown of carbohydrate into sugar and use of organic acids as an energy source (Wills et al., 1998). Additionally, higher enzyme activity during respiration is also responsible for starch hydrolysis to soluble sugar (Hassan et al., 2014).

In this study, TSS was low in ginger-coated fruits at 10%GA+10 mg/ml which were not significantly different in turmeric-coated fruits at 10%GA+10 mg/ml or dukung anak coated fruits at 10%GA+10 mg/ml compared to control fruits. The increase of TSS in dragon fruits is due to the hydrolysis of various structural polysaccharides, such as starch, pectin's and other oligosaccharides in the cell wall, which when solubilised in the aqueous phase, become part of the cellular juice (Menéndez et al., 2006). Similarly, starch accumulation during maturation gets degraded into sugars through enzymatic action of α -amylase, β -amylase and starch phosphorylase, increasing TSS (Kays, 1997). Some reports also show that the reduction of TSS and TA are associated with the utilization of sugar and acid contents as carbon skeleton

sources for the fungus (Awang et al., 2011). Therefore, a suppressed respiration rate will slow down the synthesis and use of metabolites, resulting in lower soluble solids contents due to the slow hydrolysis of carbohydrates to sugars (Rohani et al., 1997). In this study, the lowest TSS recorded in a composite coating of 10%GA+10 mg/ml of ginger which not different in 10%GA+10 gm/ml of turmeric-coated was due to the synergistic effect of 10%GA that modified the internal atmosphere of the fruits providing an excellent semi-permeable membrane around the fruits to reduce respiration rate and evolution of ethylene production. This resulted in a lower TSS due to slow hydrolysis of carbohydrates to sugars. The 10 mg/ml of ginger extracts gave the optimum antifungal effect to control the fungus that caused anthracnose, thereby reducing fungus ability to secrete cell wall degrading enzymes to break down the fruit cell wall during infection (Fernando et al., 2001). Additionally, the 10 mg/ml ginger or turmeric extract also inhibited *C gloeosporioides* utilisation of sugars in the dragon fruits as their nutrient source for growth (Amarante et al., 2001). Our study confirms Ali et al. (2014), where a delay in ripening in terms of reduction of soluble solids concentrations when papaya fruits were coated with composite coatings of ethanolic extract of propolis (1.5%) and 10%GA during cold storage at 13°C for 28 days. Kamilova et al. (2006) also reported the amount of sugar was reduced approximately by half of concentration of the uninfected tissue of tomato. Therefore, infected fruits would eventually have lower sugar and acid contents. Wang and Galletta (2002) confirms this when they reported that anthracnose infected fruits contained lower soluble solid concentrations and TA than healthy fruits.

8.5.1.5 Titratable acidity

Citric acid is the major organic acid in matured ripe dragon fruit (Esquivel et al., 2007). TA was expressed as citric acid content in dragon fruit and it decreased steadily with storage time in both control and coated fruits. Unlike TSS, TA was significantly lower ($P < 0.05$) in control fruits whilst it was high in coated fruit. TA was highest in turmeric coated fruits at 10%GA+10 mg/ml and this was not different in fruits coated with 10%GA+10 mg/ml of ginger after 21 days of storage. The decline in TA during postharvest storage is because organic acids, which served as respiratory substrates declined rapidly as the fruits transpired and respiratory rate increased (Rodriguez et al., 2005), thereby converting organic acids to sugars (Wills et al., 2007). It is established that edible coatings reduce the rate of respiration and therefore delay the utilization of organic acids (Yaman and Bayoindirli, 2002). The low levels of TA in the control fruits compared to the coated fruits suggest that the 10%GA+10 mg/ml or 15 mg/ml of either turmeric or ginger crude extract, synergistically delayed the ripening process. The 10%GA provided a semipermeable film around the fruits to reduce respiration and ethylene productions which maintained relatively high levels of TA during the break down of organic acids (Wills et al., 1998). Whiles the optimum concentrations (10 mg/ml or 15 mg/ml) were antifungal against *C. gloeosporioides* thereby inhibiting the production of cell wall degrading enzyme by the fungus to break down the fruit cell wall during infection (Fernando et al., 2001). Several reports confirm this study where a composite coating of 10%GA plus 0.75% chitosan; and 1.5% ethanolic extract of propolis plus 10%GA delayed ripening in fruits such as banana and papaya during cold storage for 28 days (Maqbool et al 2010a; Ali et al., 2014).

8.5.1.6 pH

pH increased drastically ($P < 0.05$) especially in control fruits compared to coated fruits. In this study, fruits coated with 10%GA plus 5 mg/ml of turmeric recorded lowest pH levels despite this pH levels were not different in fruits coated with 10%GA plus 10 mg/ml of either extract (turmeric or dukung anak or ginger) compared to controls after storage. The increase in pH is because of a decrease in TA in fruits during postharvest storage. The higher levels of TA, which corresponds to the relatively low pH in coated fruits, may have been due to protective oxygen barrier or reduction of oxygen supply to the internal fruit surface inhibiting respiration rate (Jiang and Li, 2001). In this present study, composite coating especially 10%GA plus 5 mg/ml of turmeric or 10%GA plus 10 mg/ml of any crude extract, helped to reduce the pH levels and this is due to 10%GA's ability to create and modified the internal atmosphere of the fruits to reduce respiration. Additionally, the concentration at 10 mg/ml was optimum to significantly controlling any fungal attacks, which could have breached or increased the respiratory mechanism.

8.5.2 Efficacy of formulation on respiration rate and ethylene production

Fruits and vegetables are living commodities and their rate of respiration and ethylene production is of key importance to the maintenance of quality. It is a common phenomenon that greater respiration rate shortens the postharvest life of fruits (Aked, 2002). Several studies show that fruits with surface covering had a reduction in respiration rate (Hernandez-Munoz et al., 2008; Eum et al., 2009; Ali et al., 2010; Shao et al., 2012). Ginger coated fruits recorded the lowest rate of respiration irrespective of the concentration used, and this was more pronounced at 10%GA+5 mg/ml even though not different at 10%GA +10 mg/ml of the same crude extract compared to control. Thus, coating 10%GA+5 mg/ml or 10 mg/ml of ginger

providing a semi-permeable membrane helped to reduce the breakdown of carbohydrate (starch) which serve as a respiratory substrate during storage. In the case of turmeric coated fruits, respiration rate was low at 10%GA+5 mg/ml while for dukung anak coated fruits it was lowest at 10%GA+10 mg/ml compared to controls after 21 days of storage. Similarly, ethylene production decreased without any peak throughout storage time and it was low in all treated fruits especially in fruits coated with 10%GA+ 10 mg/ml of dukung anak extracts compared to control fruit.

No respiration peak and very little ethylene were detected during the early storage time from day 7 to day 16, a phenomenon that describes the non-climacteric nature of dragon fruits (Jamaluddin et al., 2011). However, the sudden peak in respiration rate at the end of the storage was due to tissue damage (wounding) caused by fungal attack (Pérez-Tello et al., 2001; El Ghaouth et al., 1991; Jiang and Li, 2001). This supports an earlier finding by El Ghaouth et al. (1991); Jiang and Li, (2001) where an increase in respiration rate in strawberries and Longan fruit due to disease infection was observed. In this study, a composite coating of 10%GA plus 15 mg/ml of ginger reduced respiration and ethylene production. The decrease, which was maintained from day 7 until day 14, could be due to the synergistic effect of 10%GA that modified the internal atmosphere of the fruits thereby reducing respiration rate and ethylene whilst crude extract of ginger at 15 mg/ml provided an antifungal barrier against fungal attack.

8.5.3 Efficacy of formulation on nutritional and antioxidant quality of fruits

8.5.3.1 Total phenolic content

Phenolic compounds are common compounds found in the plant kingdom and have been exploited extensively due to their multiple biological activities such as antimutagenicity, anticarcinogenicity, antiaging and antioxidant (Kosem et al., 2007; Nurliyana et al., 2010). One such typical phenolic compound, which is highly correlated with an antioxidant, is phenolic acid (gallic acid) (Bertoncelj et al., 2007). Folin-Ciocalteu assay depends on the basic mechanism of oxidation and reduction reaction (Verzelloni et al., 2007; Ajila et al., 2007) and this mechanism refers to the redox properties of compounds present in the samples, which are called antioxidants. The reaction of compounds with Folin-Ciocalteu allows the measurement of the phenolic concentration (Nurliyana et al., 2010). A study shows that increase in TPC is related to the enhancement of antioxidant capacity (Reye and Cisneros-Zevallos, 2003). Also, higher respiration rate, which results in an increase in water loss, reduced cellular turgidity and eventually possible fungal infection, have been reported to cause a decline in TPC (El Ghaouth et al., 1991; Saladié et al., 2007).

In this study, a decline in TPC was observed until day 14 but increased slightly afterwards until the end of the storage. TPC was high in all coated treated fruits but this was more pronounced ($P < 0.05$) in turmeric coated fruits at 10%GA+5 mg/ml which was not different in fruits coated with 10%GA plus 10 mg/ml of the same crude extract compared to control. The high amount of TPC in fruits coated with 10%GA+10 mg/ml of especially at turmeric crude extract might be due to maintaining higher amounts of antioxidants in fruit due to the presence high ascorbic acid at 10%GA plus 10 mg/ml of turmeric extracts compared to controls. This phenomenon was possible in the composite coating of 10%GA plus 10 mg/ml because 10%GA

synergistically provided a semipermeable membrane to reduce the respiration rate, which caused a reduction in moisture loss thereby maintaining a turgid cellular structure, and maintaining TPC due to less degradation of phenolic content. While the crude extract, especially in turmeric at 10 mg/ml, was the optimum concentrations that provided an antifungal effect against fungal attacks, which also attributed to enhancing the TPC in fruits; by reducing any form of wounding due to disease infestation, which could have increased fruit respiration (Day, 2001; Ali et al., 2013a). This result confirms the earlier study Ali et al. (2013a) where GA coated tomatoes recorded high amount of TPC due to the presence of high antioxidants and reduction in respiration rate and Zahid et al. (2013), where TPC in dragon fruit coated with ethanolic extract of propolis was highest due to reduction of respiration rate by propolis, which provided a semi-permeable to modify the internal atmosphere of fruit. The decrease in TPC, which resulted from the degradation of certain phenolic compounds in the control fruit, could be due to high respiration rate and infection caused by *C. gloeosporioides*. Another possible reason could be due to senescence and breakdown of cell structure due to reduced cellular turgidity (Day, 2001; Ali et al., 2010).

8.5.3.2 DPPH Free Radical scavenging activity

The antioxidant can be categorised into two main types, namely primary and secondary antioxidants and each is responsible for a particular mechanism (Lim et al., 2007). Whereas primary antioxidant acts to scavenge free radicals to inhibit chain initiation and break chain propagation by donating hydrogen atoms or electrons that convert them into a more stable product, secondary antioxidant functions by suppressing formation of radicals and protect oxidative damage (Lim et al., 2007). DPPH assay measures the primary antioxidant activity by removing or scavenging free radicals (Wang et al., 2008) and this is visually noticeable as the

colour of dragon fruit juice changes from purple to yellow due to hydrogen donating ability (Ajila et al., 2007). A rapid decrease in absorbance signifies a more potent primary antioxidant activity (Siddhuraju et al., 2002). In this study, purple bleaching reaction in all samples was observed, indicating the presence of compounds responsible as free scavengers, which reduced the initial DPPH. DPPH assay is used to measure the non-enzymatic antioxidant capacity of fruits.

In this study, free radical scavenging activity which translates as the primary total antioxidant activity decline throughout the storage time which complements earlier findings of Ghasemnezhad et al. (2010) and Nurliyana et al. (2010). DPPH radical scavenging activity was significantly high in treated fruits compared to control after storage. Fruits coated with 10%GA+10 mg/ml of any crude extracts recorded a high DPPH scavenging activity, and it was significantly pronounced in turmeric-coated fruits. The increased antioxidant activity in the composite coating of 10%GA+10 mg/ml of any crude extracts (especially in turmeric) is possibly due to the existence of natural antioxidants such as ascorbic acid, and this was observed when fruit treated with 10%GA'+ 10 mg/ml of crude extracts especially in turmeric recorded significantly high amount of ascorbic acid. Positive correlation between phenolic content and total antioxidant activity has been reported (Artés-hernandez et al., 2007) and among the major antioxidants in fruits includes carotenoids, ascorbic acid and phenolic compounds (Giovanelli et al., 1999). Earlier reports by Addai et al. (2013), Ali et al. (2013a), Khaliq et al. 2016 and Zahid et al. (2013) confirm our study, where a maintained higher DPPH scavenging activity of GA treatment on papaya, tomatoes, mangoes and dragon fruits compared to control. It may, therefore, be considered that 10%GA plus 10 mg/ml of crude

extracts decreased oxidative damage in dragon fruit by stimulation of DPPH radical scavenging activity.

8.5.3.3 Ascorbic acid content

As a water-soluble and a strong antioxidant, ascorbic acid (AA) acts to prevent and reduce the damage caused by reactive oxygen species in fruits. It is the main reactive oxygen species-detoxifying compound scavenging hydroxyl radicals, superoxide anion and reducing hydrogen peroxide to water through ascorbate peroxidase reaction (Blokhina et al., 2003). In the detoxification process of different reactive oxygen species, O_2 , H_2O_2 , and OH , ascorbic acid plays an important role as an antioxidant and hence reduces the oxidative damage (Khaliq et al., 2016). Factors such as high temperatures and wounding accelerate ascorbic acid loss (Lee and Kader, 2000). Also, postharvest technologies such as modified storage or edible coatings that slow down respiration can reduce ascorbic acid loss (Lee and Kader, 2000; Khaliq et al., 2016). In this study, AA decreased gradually with storage time in both control and coated fruit. However, coated fruit inhibited ascorbic acid loss as compared to control fruits and this complements a similar study by Khaliq et al. (2016), where a composite coating of 10%GA plus calcium chloride inhibited ascorbic acid loss in mango. Like TPC analysis and DPPH activity, AA was significantly high in turmeric coated treated fruits at 10%GA+5 mg/ml of extracts though it wasn't different in fruits coated at 10%GA plus 10 mg/ml of the same extract compared to controls (which was lowest).

The composite coating of 10%GA plus either 5 or 10 mg/ml of turmeric extracts that reduced the AA in dragon fruits was due to the synergistic effect where 10%GA served as a semipermeable to reduce respiration rate, hence maintaining high ascorbic acid. Concentrations at 10 mg/ml or 5 mg/ml slowed down fungal attacks on fruits caused by disease infestation or

any form of wounding during packaging which could have increased the respiration and inhibited fruit senescence. In the control fruit, there was not a protective barrier to control the internal gas exchange of fruits resulting in high respiration rate, leading to high loss of ascorbic acid. Our study also confirms reports by Eltoum and Babiker (2014) in which ascorbic acid losses reduced with GA coating in tomato slices and alginate plus antioxidant in fresh cut mango (Robles-Sánchez et al., 2013). A study by Khaliq et al. (2016) confirmed our study where there was a reduction in ascorbic acid in the composite coating of GA plus calcium chloride in coated mango fruits compared to controls.

8.6 Concluding remarks

The effect of treatment on postharvest quality of fruit varied with quality parameters measured. Generally, respiration rate and ethylene production were significantly low in fruit coated with 10%GA plus either 10 mg/ml or 5 mg/ml of ginger crude extracts even though it was markedly low in fruits coated with 10%GA plus 10 mg/ml of the other crude extracts. Despite the significant effect of this composite coating in reducing the internal gas exchange in ginger coated fruits, it did not help to maintain the qualities of dragon fruits during the postharvest storage of fruits. In the present study, fruit treated with 10%GA plus 10 mg/ml of turmeric crude were firmer, reduced water loss, delayed colour development, lowered levels of TSS with and maintained a high TA after 21 to 28 days of storage. Additionally, composite edible coating of 10%GA plus 10 mg/ml of turmeric crude extract helped to maintain a higher total antioxidant due to the presence of high ascorbic acid content and total phenolic compounds. Similarly, sensory evaluation results proved the effectiveness of 10%GA plus 10 mg/ml of turmeric crude extract composite coating by maintaining the overall quality of dragon fruits. Fruits treated with 10%GA+10 mg/ml of turmeric crude extract attained the best

external appearance, pulp colour, aroma, sweetness, texture and overall acceptability during the sensory evaluation.

These findings suggest that 10%GA plus 10 mg/ml of turmeric crude extracts can be used for extending the storage life of dragon fruits for up to 28 days based on the overall effect it exhibited in controlling anthracnose and its severity as well the ability of the treatment to maintain and enhance most of the physicochemical, nutritional and antioxidant qualities of the fruit.

GENERAL DISCUSSION

As a tropical commodity, dragon fruits can be preserved and the shelf life extended for up to 28 days at $11\pm 2^{\circ}\text{C}$, 80% RH depending on treatments applied. However, in this study where plant crude extracts incorporated with 10%GA was used, the recommended storage life for dragon fruit is 21 days at $11\pm 2^{\circ}\text{C}$, 80% RH. High postharvest losses are due to various microorganisms, with anthracnose being one of the major problems associated with the marketing of high-quality fruits in many dragon fruits production areas. Traditionally, application of fungicides is the most common method for controlling anthracnose pre-harvest and postharvest. Commonly used postharvest practices in managing anthracnose in dragon fruits are controlled atmosphere and proper storage temperature or a combination with fungicides especially during shipments or export. Synthetic chemicals used to control anthracnose include broad-spectrum contact fungicides such as benomyl, carbendazim. Some of these fungicides (benomyl) have been discouraged due to fungal resistance and their negative impact on the environment and human health. However, in some countries, its registration has not been abrogated and it is still produced. It is suggested that its use or production in such countries should be discouraged due to health concerns, for instance, the treatment of dragon fruits with benomyl might have a negative effect on a producer's ability to export these fruits due to health concerns.

At the postharvest level, Alternatives such as plant crude extracts of turmeric, ginger and “dukung anak” promise a significant effect to control postharvest anthracnose since there is enough evidence that these plant crude extracts are antifungal against vast pathogens such as *C. gloeosporioides* that cause anthracnose. This research identified that the use of ginger rhizome, dukung anak and turmeric rhizome crude extract alone played a significant role in

controlling anthracnose caused by *C. gloeosporioides* especially at 10 mg/ml in dukung anak crude extract which was not so different in turmeric crude extract at the same concentration. However, at 5 mg/ml or above (15 mg/ml) there was severe phytotoxicity to the fruits especially when ginger crude extract was used. Despite the phenomenal antifungal activity amongst all plant extracts, there were some limitations. Paramount among which was the phytotoxic effect on fruit that occurred at the highest concentration (15 mg/ml) coupled with the poor external appearance (colour and smell) of the dragon fruit that was compromised by the extracts alone at both 10 mg/ml or 15 mg/ml in all extracts affecting customer appeal. The antifungal activity that occurred *in vitro* was due to the presence of 6-gingerol, curcumin and other alkaloids in ginger, turmeric and dukung anak respectively, despite ginger crude extract at 10 mg/ml significantly controlled *C. gloeosporioides*. The best antifungal activity against anthracnose *in vivo* was the presence of other alkaloids in dukung anak and not securinine, since securinine was not detected in dukung anak during the HPLC analysis. Therefore, further studies need to be conducted to ascertain the specific alkaloid responsible for this effect since alkaloids are most abundant compounds in dukung anak. Therefore, the presence of bioactive compounds (gingerol and curcumin) accounted for most antifungal activity against the fungus during HPLC analysis and the mechanism these plant extracts employed to control the fungus was through the leakage of electrolyte from fungal cell wall, which resulted in inhibition of fungal growth to control anthracnose in dragon fruit.

To further enhance the efficacy of only the crude extracts by reducing the phytotoxic effect as well as improving the external appearance of the dragon fruits, a formulation of crude extracts of each plant (ginger, turmeric and dukung anak) were incorporated with gum arabic (10%GA). This formulation showed a bio-fungicide property since it helped to control postharvest anthracnose while maintaining the physicochemical properties of dragon fruits, as well the nutritional and antioxidant activity of the fruits during postharvest storage. Crude extracts of especially turmeric crude extract at 10 mg/ml incorporated with 10%GA helped to maintain the fruit firmness, reduced weight loss and as well slowed down colour development during postharvest storage. Additionally, it also helped to maintain TA and TSS of the fruits and significantly controlled anthracnose and its severity.

For early detection during storage, LAMP technique shown a promising effect for early detection and quantification of *C. gloeosporioides* isolated from treated and untreated fruits during postharvest storage. Composite coating of 10%GA+ either 5 mg/ml or 10 mg/ml of turmeric crude extracts reduced the amount of fungal DNA of *C. gloeosporioides* on fruit tissue confirming the efficacy of this formulation as the best treatment that delayed anthracnose and its severity (section 6.4.1 and 6.4.2). This makes it an important diagnostic tool for detection of fungal pathogens during postharvest storage of fruits either during sea or road transport. It is therefore recommended that for early detection of fruits especially during transport (sea) or storage, regular checks for detection of fruits should be employed. This check can help to reduce or overcome any form of a disease epidemic. Using LAMP machine has shown their specificity and ability to identify pathogens during postharvest storage and will help fruits producers, exported to reduce any losses since detection time of disease using this technique is very short.

As a recommendation, the antifungal activity of any plant extracts requires the recommended organic solvents that best extract the major bioactive compounds for effective result. Additionally, since these plant extracts consist of vast bioactive compounds and some may very destructive to the fruits, it is important to use relatively low concentrations of crude extracts since very high concentrations can be phytotoxic to the fruits, leading to tissue and hence postharvest losses. Therefore, it is recommended that a combination of extracts with film-forming natural products like GA can help overcome this problem. Due to the specificity of LAMP, using generic primers like ITS-primers could be deceptive in pathogen identification, since these generic primers could pick up other pathogens and not the target pathogen under study. This may affect proper postharvest regime or treatment to be carried during postharvest storage in the supply chain as also may result in wrong diagnosis of diseases. The end results could be severe postharvest losses. Therefore, designing a specific primer for detection of target pathogen is recommended and LAMP is the best technique due to its primer specificity.

Finally, our finding suggests that, incorporation of 10%GA plus 10 mg/ml of crude extracts especially turmeric is an inexpensive method with the potential to control postharvest anthracnose of dragon fruit during cold storage and transport (sea or road). Future studies should be conducted to ascertain if by chance, the treatment diffused from the dragon fruits peel into the fruits pulp which could have otherwise compromised the nutritional quality of fruits at the genetic level. Finally, commercial testing of the formulation by dragon fruits growers and exporters on dragon fruits and other range of tropical fruits should also be conducted.

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APPENDICES

Appendix 0-1. Two-way analysis of variance (ANOVA) for effect of plant extracts at different concentrations (treatment) on percentage inhibition in radial mycelia growth after 12 days of incubation at room temperature

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment (T)	5	200768.3	40153.67	27725.9	<.001
Extracts (E)	2	7096.227	3548.113	2449.95	<.001
T x E	10	9197.878	919.788	635.11	<.001
Residual	162	234.614	1.448		
Total	179	217297.1			

Appendix 0-2. Two-way Anova for effect of plant extracts at different concentrations (treatment) on percentage inhibition in conidia germination after 12 days of incubation at room temperature

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment (T)	4	139282.2	34820.54	22279.65	<.001
extracts (E)	2	8153.872	4076.936	2608.6	<.001
T x E	8	7666.828	958.354	613.2	<.001
Residual	135	210.99	1.563		
Total	149	155313.9			

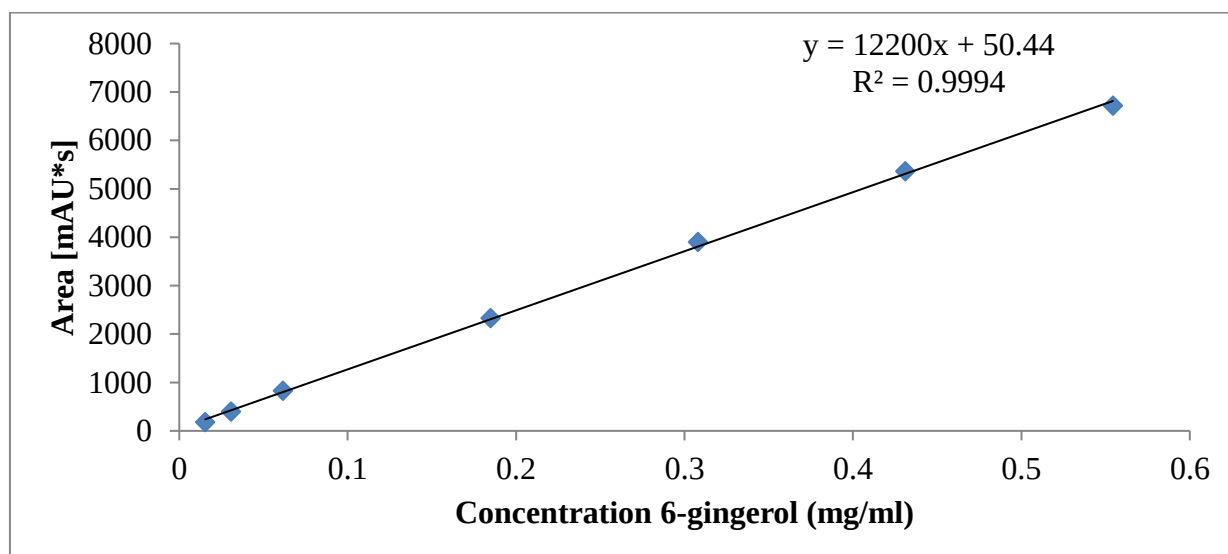
Appendix 0-3. Two-way Anova for effect of treatment and storage time on disease incidence after 28 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
storage time (S)	4	173053.3	43263.33	748.79	<.001
Treatment (T)	3	355.56	118.52	2.05	0.11
Extracts (E)	2	7444.44	3722.22	64.42	<.001
S x T	12	4333.33	361.11	6.25	<.001
S x E	8	15600	1950	33.75	<.001
T x E	6	2884.44	480.74	8.32	<.001
S x T x E	24	6426.67	267.78	4.63	<.001
Residual	120	6933.33	57.78		
Total	179	217031.1			

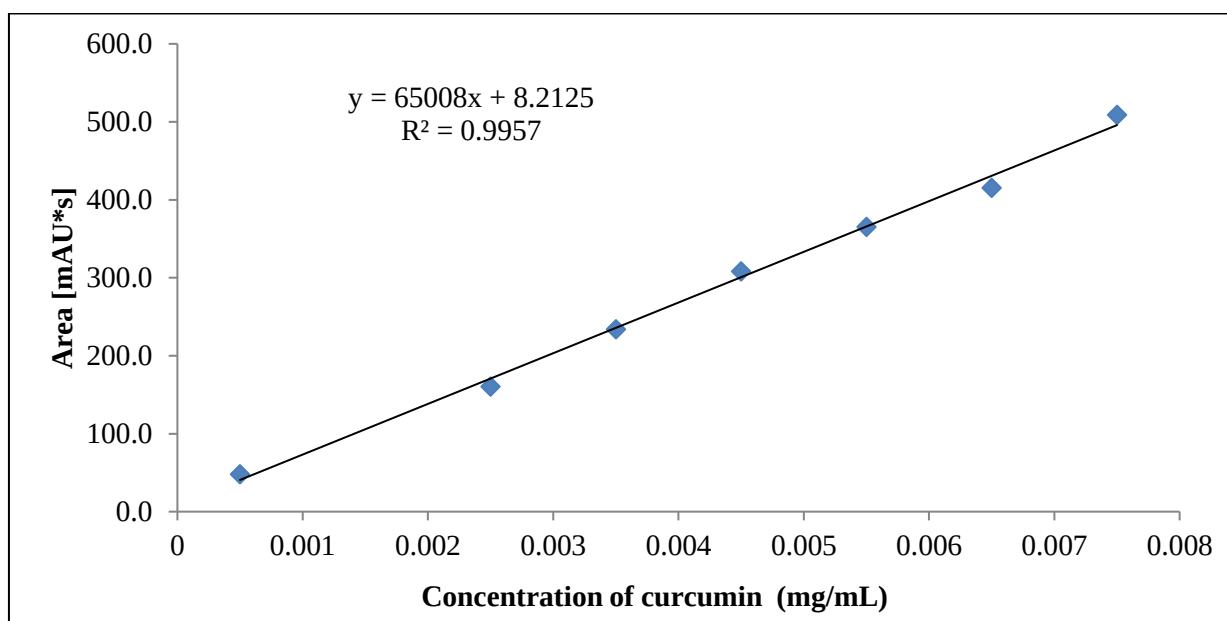
Appendix 0-4. Two-way Anova for effect of only plant crude extracts and storage time on disease severity after for 28 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Storage time (S)	4	48.80533	12.20133	741.97	<.001
Extract (E)	2	3.76578	1.88289	114.5	<.001
Treatment (T)	3	0.22822	0.07607	4.63	0.004
S x E	8	7.992	0.999	60.75	<.001
S x T	12	1.28178	0.10681	6.5	<.001
E x T	6	1.40044	0.23341	14.19	<.001
S xT x E	24	3.01956	0.12581	7.65	<.001
Residual	120	1.97333	0.01644		
Total	179	68.46644			

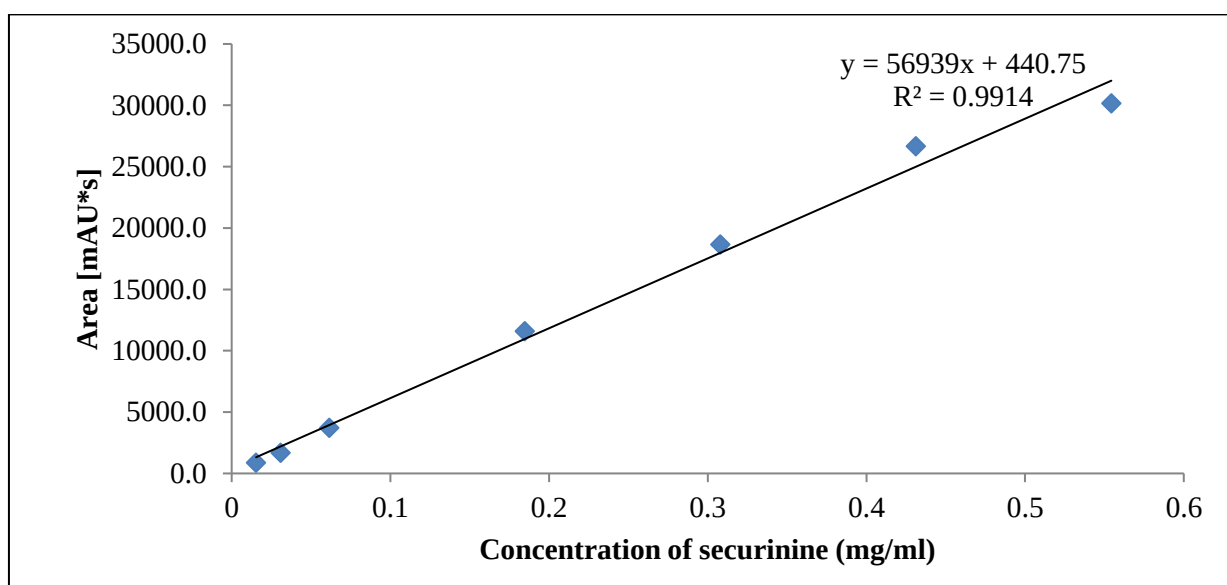
Appendix 0-5. 6-gingerol standard curve for detection of 6-gingerol in ginger crude extracts



Appendix 0-6. Curcumin standard curve for detection of curcumin in turmeric crude extracts



Appendix 0-7. Securinine standard curve for detection of securinine in dukung anak crude extracts



Appendix 0-8. Two-way Anova effect of extract at different concentrations and storage time on total electrolyte leakage of *C. gloeosporioides*

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Storage time (S)	2	39.327	19.664	6.05	0.007
Treatment (T)	3	392.218	130.739	40.21	<.001
S x T	6	4804.355	800.726	246.27	<.001
Residual	24	78.035	3.251		
Total	35	5313.935			

Appendix 0-9. Two-way Anova on the effect plant extract at different concentrations incorporated with 10%GA and storage time on disease incidence after 28 days of storage.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment (T)	10	625.52	62.55	2.6	0.007
Storage time (S)	4	342862.2	85715.55	3566.08	<.001
T x S	40	1242.06	31.05	1.29	0.15
Residual	110	2644	24.04		
Total	164	347373.8			

Appendix 0-10. Two -way Anova on effect plant extract at different concentrations incorporated with 10%GA and storage time on disease severity during cold storage for 28 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment (T)	10	2.79648	0.27965	15.28	<.001
Storage time (S)	4	223.4482	55.86206	3052.07	<.001
T x S	40	6.32776	0.15819	8.64	<.001
Residual	110	2.01333	0.0183		
Total	164	234.5858			

Appendix 0-11. Two -way Anova effect of treatment and storage time on fungal DNA extracted from inoculated fruits after 21 days of cold storage.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment (T)	6	1.9261	0.321	1.38	0.269
Storage time (S)	2	3.5678	1.7839	7.65	0.003
T x S	12	10.304	0.8587	3.68	0.004
Residual	21	4.8953	0.2331		
Total	41	20.6932			

Appendix 0-12. Two-way Anova on effect plant extracts at different concentrations incorporated with gum arabic and storage time on weight loss during cold storage for 28 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment (T)	9	13.21854	1.46873	55.23	<.001
Storage time (S)	3	268.2729	89.42429	3362.76	<.001
T x S	27	3.68759	0.13658	5.14	<.001
Residual	80	2.1274	0.02659		
Total	119	287.3064			

Appendix 0-13. Two -way effect of on effect plant extract at different concentrations incorporated with 10%GA and storage time on firmness during cold storage for 28 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment (T)	9	160.913	17.879	7.73	<.001
Storage time (S)	4	1691.815	422.954	182.95	<.001
T x S	36	171.519	4.764	2.06	<.001
Residual	200	462.366	2.312		
Total	249	2486.614			

Appendix 0-14. Two -way Anova on effect plant extract at different concentrations incorporated with 10%GA and storage time on colour (hue angle) during cold storage for 21 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Trt	9	739.4377	82.1597	145.89	<.001
days	3	60.3455	20.1152	35.72	<.001
Trt.days	27	358.0938	13.2627	23.55	<.001
Residual	80	45.0526	0.5632		
Total	119	1202.93			

Appendix 0-15. Two -way Anova on effect plant extract at different concentrations incorporated with 10%GA and storage time on TSS during cold storage for 21 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
trt	9	1.41047	0.15672	2.54	0.01
days	3	0.2056	0.06853	1.11	0.347
trt.days	27	2.1474	0.07953	1.29	0.171
Residual	160	9.88267	0.06177		
Total	199	13.64613			

Appendix 0-16. Two -way Anova on effect plant extract at different concentrations incorporated with 10%GA and storage time on TA during cold storage for 21 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment (T)	9	0.026705	0.002967	13.47	<.001
Storage time (S)	3	0.503716	0.167905	762.51	<.001
T x S	27	0.02873	0.001064	4.83	<.001
Residual	160	0.035232	0.00022		
Total	199	0.594382			

Appendix 0-17. Two -way Anova on effect plant extract at different concentrations incorporated with 10%GA and storage time on pH during cold storage for 21 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment (T)	9	0.52827	0.0587	5.26	<.001
Storage time (S)	3	11.69043	3.89681	349.35	<.001
T x S	27	1.66788	0.06177	5.54	<.001
Residual	160	1.78471	0.01115		
Total	199	15.67129			

Appendix 0-18. Two -way Anova on effect plant extract at different concentrations incorporated with 10%GA and storage time on respiration rate during cold storage for 21 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment (T)	9	1674.324	186.036	95.67	<.001
Storage time (S)	3	9486.041	3162.014	1626.06	<.001
T x S	27	1326.662	49.136	25.27	<.001
Residual	120	233.35	1.945		
Total	159	12720.38			

Appendix 0-19. Two -way Anova on effect plant extract at different concentrations incorporated with 10%GA and storage time on ethylene production during cold storage for 21 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment (T)	9	0.173005	0.019223	31.39	<.001
Storage time (S)	3	12.80952	4.269841	6971.48	<.001
T x S	27	0.148105	0.005485	8.96	<.001
Residual	120	0.073497	0.000613		
Total	159	13.20413			

Appendix 0-20. Two -way Anova on effect plant extract at different concentrations incorporated with 10%GA and storage time on TPC during cold storage for 21 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment (T)	9	173298.3	19255.4	64.73	<.001
Storage time (S)	3	22509.9	7503.3	25.23	<.001
T x S	27	93465.5	3461.7	11.64	<.001
Residual	160	47592.1	297.5		
Total	199	336865.8			

Appendix 0-21. Two -way Anova on effect plant extract at different concentrations incorporated with 10%GA and storage time on DPPH activity during cold storage for 21 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment (T)	9	4021.28	446.81	13.82	<.001
Storage time (S)	3	20042.32	6680.77	206.71	<.001
T x S	27	5999.83	222.22	6.88	<.001
Residual	160	5171.08	32.32		
Total	199	35234.51			

Appendix 0-22. Two -way Anova on effect plant extract at different concentrations incorporated with 10%GA and storage time on AA content during cold storage for 21 days

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment (T)	9	664.858	73.873	59.55	<.001
Storage time (S)	3	2116.761	705.587	568.78	<.001
T x S	27	490.106	18.152	14.63	<.001
Residual	160	198.483	1.241		
Total	199	3470.207			

Appendix 0-23. Hedonic scale for sensory evaluation

Hedonic scale rating

Product: Red fleshed dragon fruits coated with 10%GA incorporated with different plant extract

Date:

Panellist number:

Signature:

Instruction: (please read the instructions carefully before filling blanks)

1. This sensory analysis form for the evaluation of different treatments
2. Please follow the numerical system for scoring the samples
 - I. extreme dislike
 - II. dislike
 - III. acceptable
 - IV. good
 - V. excellent

Please do not disturb the sequence of the samples provided

Please rinse your tongue before testing next sample, with water provided

Samples	Appearance	Pulp color	Aroma	Sweetness	Texture	Overall acceptability
A						
B=4G						
C=10G						
D=5PN						
E=10PN						
F=5T						
G=10T						

A= non treated fruits, B = Ginger coated treated fruits at 10%GA+4mg/ml, C = Ginger coated treated fruits at 10%GA+10 mg/ml, D = Dukung anak coated treated fruits at 10%GA+5 mg/ml, E = Dukung anak coated treated at 10%GA+10 mg/ml, F = Turmeric coated treated fruit at 10%GA+5 mg/ml, G = Turmeric coated treated fruits at 10%GA+10 mg/ml.