IN VITRO ANTIOXIDANT AND ALPHA-GLUCOSIDASE INHIBITORY ACTIVITIES OF FRUIT EXTRACTS FROM SEVEN SPECIES OF *Citrus*

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by

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LIST OF ABBREVIATIONS

AAE	ascorbic acid equivalent
ABTS	2,2-azino-bis-3-ethylbenzthiazoline-6-sulhonic acid
ANOVA	analysis of variance
cm	Centimetre
CUPRAC	cupric ion reducing antioxidant capacity
DCPIP	2,6-dichlorophenolindophenol
DGPP	glucopyranosylphloretin
DHA	dehydroascorbic acid
DMSO	dimethyl sulfoxide
DPPH	2,2-diphenyl picryl-hydrazyl
EC_{50}	effective concentration at 50% activity
EDTA	ethylene diamine tetracetic acid
FAO	Food and Agriculture Organization
FC	Folin-Ciocalteu
FRAP	ferric reducing antioxidant power
G	Gram
GAE	gallic acid equivalents
He	Herbarium
HORAC	hydroxyl radical antioxidant capacity
HPLC	high performance liquid chromatography
MPA	metaphosphoric acid
ORAC	oxygen radical absorbance capacity
PFRAP	potassium ferricyanide reducing antioxidant power

RNS	reactive nitrogen species
ROS	reactive oxygen species
SD	standard deviation
TPC	total phenolic content
TPTZ	2,4,6-tripyridyltriazine
TRAP	telomeric repeat amplification protocol
USDA	United States Department of Agriculture
UV-VIS	ultraviolet-visible
WHO	World Health Organization

AKTIVITI ANTIOKSIDAN DAN PERENCATAN ALFA-GLUCOSIDASE *IN VITRO* BAGI EKSTRAK BUAH DARIPADA TUJUH SPESIES *Citrus*

ABSTRAK

Aktiviti *in vitro* α-glucosidase dan antioksidan, kandungan fenolik dan vitamin C bagi jus tulen (diperah daripada pulpa), ekstrak kulit dan pulpa daripada enam buah limau yang ditanam di Malaysia, iaitu *Citrus aurantifolia*, *Citrus grandis* var . Tambun (PO52) dan var. Penang Merah (PO5), *Citrus hystrix*, *Citrus latifolia*, dan *Citrus madurensis* bersama *Citrus limon* dari Sepanyol ditentukan dalam kajian perbandingan ini. Ini kerana kajian perbandingan spesies *Citrus* yang didapati di Malaysia tidak pernah dijalankan. Kajian terhadap kulit sering dijalankan tetapi pulpa dan jus kurang mendapat perhatian. Sampel pulpa segar dan kering diekstrak menggunakan metanol, etanol dan air suling manakala 80% metanol digunakan untuk pengekstrakan ekstrak krud daripada kulit dan pulpa kering. Keputusan yang diperolehi daripada ujian 2,2-difenil-1-pikrilhidrazil (DPPH) menunjukkan aktiviti penyingkiran radikal bebas tertinggi (96.14 ± 0.30%) daripada ekstrak metanol kulit kering *C. limon* (pada 100 mg/mL). Ekstrak metanol kulit kering (pada 100 mg/mL) dan jus *C. grandis* var. Tambun (PO52) berkongsi kuasa antioksidan penurunan ferik tertinggi (FRAP) pada 85.03 ± 6.32% dan 84.29 ± 0.40%. Ekstrak etanol kulit segar *C. grandis* var. Penang Merah (PO5) mempamerkan aktiviti pengkelatan logam tertinggi pada 98.85 ± 0.82%. Antara jus, jus *C. grandis* var. Tambun (PO52) memberi jumlah tertinggi kandungan fenolik (TPC) iaitu sebanyak 14956.34 ± 152.65 µg setara asid galik (GAE)/g jus. Ekstrak air suling kulit kering *C. limon* memberikan TPC tertinggi iaitu 33148.87 ± 124.46 µg GAE/g berat segar sampel. Kandungan vitamin C tertinggi dikuantifikasikan daripada jus limon (731.88 ± 0.49 µg setara asid askorbat

(AAE)/g jus). Keputusan juga menunjukkan penurunan dalam aktiviti antioksidan, TPC dan kandungan vitamin C jus semasa penyimpanan. Semua jus tulen tidak termasuk jus *C. grandis*, memberikan aktiviti perencatan α-glucosidase yang tinggi dan aktiviti tertinggi diukur daripada jus limon (96.51 ± 0.26%). Hubungan korelasi sederhana antara aktiviti FRAP dan DPPH semua ekstrak diperhatikan dengan nilai r^2 0.5943 yang signifikan pada $p < 0.001$. Hubungan antara TPC dan FRAP adalah yang tertinggi berbanding dengan aktiviti lain yang diuji pada 0.5649 ($p < 0.001$). Kandungan vitamin C didapati sangat berkorelasi dengan aktiviti perencatan α-glucosidase dengan r^2 0.5164 ($p <0.001$). Apabila ekstrak daripada matriks sampel yang berbeza telah dianalisis secara berasingan, aktiviti DPPH dan TPC bagi ekstrak kulit segar didapati mempunyai korelasi tertinggi pada 0.7947 ($p <0.001$). Kandungan Vitamin C dalam ekstrak segar pulpa dan aktiviti DPPHnya juga sangat berkorelasi pada 0.6810 ($p <0.001$).

IN VITRO* ANTIOXIDANT AND ALPHA-GLUCOSIDASE INHIBITORY ACTIVITIES OF FRUIT EXTRACTS FROM SEVEN SPECIES OF *Citrus

ABSTRACT

The *in vitro* α-glucosidase inhibitory and antioxidant activities, total phenolic and vitamin C contents of the pure juices (squeezed from the pulps), peel and pulp extracts from siix *Citrus* fruits that are grown in Malaysia, which are *Citrus aurantifolia*, *Citrus grandis* var. Tambun (PO52) and var. Penang Merah (PO5), *Citrus hystrix*, *Citrus latifolia*, and *Citrus madurensis* together with *Citrus limon* from Spain were determined in this comparative study. This is owing to the fact that these *Citrus* species that can be found in Malaysia has never been comparatively studied. The peels are mostly studied while the pulps and the juices are given less attention. The fresh and dried peel and pulp samples were extracted using methanol, ethanol and distilled water while 80% methanol was used for the extraction of crude extracts from dried peels and pulps. The result obtained from 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay revealed the highest free radical scavenging activity (96.14 ± 0.30%) of the methanol extract of dried peel of *C. limon* (at 100 mg/mL). The methanol extract of dried peel (at 100 mg/mL) and the juice of *C. grandis* var. Tambun (PO52) shared the highest ferric-reducing antioxidant power (FRAP) at 85.03 ± 6.32% and 84.29 ± 0.40%, respectively. The ethanol extract of fresh peel of *C. grandis* var. Penang Merah (PO5) exhibited the highest metal chelating activity at 98.85 ± 0.82%. Among the juices, the juice of *C. grandis* var. Tambun (PO52) gave the highest total phenolic content (TPC) of 14956.34± 152.65 µg gallic acid equivalent (GAE)/g juice. The distilled water extract of dried peel of *C. limon* gave the highest TPC at 33148.87 ± 124.46 µg GAE/g fresh weight of sample. The highest vitamin C content was quantified from the juice

of *C. limon* (731.88 ± 0.49 μg ascorbic acid equivalent (AAE)/g juice). The results also showed the decrease in the antioxidant activities, TPC and vitamin C contents of the juices during storage. All pure juices excluding the juices of *C. grandis* gave high α-glucosidase inhibitory activities and the highest activity was measured from the juice of *C. limon* (96.51 ± 0.26%). A moderate correlation between FRAP and DPPH activities of all extracts was observed with r^2 of 0.5943 that is significant at $p < 0.001$. The correlation between TPC and FRAP was the highest in comparison with the other tested activities at 0.5649 ($p < 0.001$). Vitamin C content was found to be highly correlated with α-glucosidase inhibitory activity with r^2 of 0.5164 ($p < 0.001$). When extracts from different sample matrices were separately analysed, the DPPH activities and TPC of fresh peel extracts were found to have the highest correlation at 0.7947 ($p < 0.001$). Vitamin C contents of the fresh pulp of extracts and their DPPH activities was also highly correlated at 0.6810 ($p <0.001$).

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Genus *Citrus*, which belongs to Rutaceae family is well-known for having fruits with nourishment and health-promotion potentials. Numerous researches on the biological importance of chemical substances found in *Citrus* fruits and their extracts were carried out in previous years. *Citrus* fruits are commonly accepted by consumers because of their attractive colours, unique flavours and nice aroma. This could no doubt be as a result of increase in production, advancement in storage and processing techniques, and their availability all year-round (Kader and Barrett, 2005; Chalermchai and Noichinda, 2014).

Citrus is one of the world's important fruit crops which is widely grown in most areas with suitable climates (Liu and Deng, 2007) with an annual production of approximately 130 million tons per year (FAO, 2016). Among the *Citrus* fruits, orange is on the top rank with an annual production of 49.6 million metric tons having Brazil as the largest producing country. This is followed by mandarin/tangerine, lemon/lime and grapefruit (USDA, 2017). This represents a significant contribution of *Citrus* commodities to human diet. *Citru*s fruits are mostly consumed as *Citrus*-based beverages, fresh juices and also as deserts (*Citrus* page, 2009). Each *Citrus* species consists of varieties and hybrids resulting from natural selection and artificial cross breeding (Barkley *et al*., 2006).

In Malaysia, different species of *Citrus* are grown in commercial orchards, backyard gardens and by the small holders in various parts of the country. Due to notable genetic diversity, particularly of the pomelo (*Citrus grandis*), *Citrus* collections in Malaysia were established for conservation purposes (Hajivand *et al*., 2009). Among the *Citrus* fruits of Malaysia, *Citrus grandis* is grown commercially in the states of Kedah, Johor, Perak, Kelantan and Melaka with an estimated annual production of 8830 metric tons (Zaini *et al*., 2014; Nur *et al*., 2015).

The antioxidant activities of *Citrus* fruits had been evaluated by many researchers (Proteggente *et al*., 2003; Gorinstein *et al*., 2004; Anagnostopoulou *et al*., 2006; Guimarães *et al*., 2010). The extracts of *Citrus* fruits that exhibited broad biological activities were associated with their phenolic compounds especially their flavonoids (Middleton and Kandaswami, 1994; Samman *et al*., 1996; Montanari *et al*., 1998). Notably, the use of *Citrus* species in the treatment of many life threatening diseases, such as cardiovascular disease, cancer and diabetes has been reported to be linked with the antioxidant activities (Yu *et al*., 2005; Rajendran *et al*., 2014).

Extensive literature survey had revealed a wide array of antioxidants and phenolic constituents in *Citrus* fruits (Guimarães *et al*., 2010; Fattahi *et al*., 2011). *Citrus* fruits are also shown to be rich sources of essential phytochemicals such as coumarins, limonoids, carotenoids, pectins, vitamins and minerals (Zhou, 2012). *Citrus* fruits or its derived products were also reported to have many other biological activities such as anti-inflammatory, anti-carcinogenicity and anti-mutagenicity (Rajendran *et al*., 2014; Ke *et al*., 2015; Zhang *et al*., 2015).

According to the studies of Manthey and Grohmann (2001) and Chau and Huang (2003), *Citrus* by-products particularly the peels contain higher phenolic compounds compared to the edible portions. *Citrus* peels have countless glands that consist of essential oils that might contribute to flavouring and fragrance industries (Goodrich and Braddock, 2006). The peel fibre obtained from orange fruit (*Citrus sinensis*) has been shown to enhance intestinal function and health (Chau *et al*., 2005). Dietary fibres are not only necessary for evading hydrolysis and absorption in the human small intestine and digestion but also functions in increasing faecal bulking efficiency, maintenance of insulin level, intensify colonic fermentation and inhibits pre-prandial cholesterol levels (Champ *et al*., 2003; Fuentes-Zaragoza *et al*., 2010). By-products obtained from fruits, vegetables and whole grains are guaranteed sources of dietary fibres (Larrauri, 1999). According to Drzikova *et al*. (2005), the recommended dietary fibre intake, which can help to overcome the fibre deficit diet is 25–30 g daily. Moreover, dietary fibre has been related to many physiological and metabolic effects. Inclusion of *Citrus* pulp, which is another by-product of *Citrus* fruits in the diet of goats was also found to provide high amount of energy without any harmful effect on milk yield (Salvador *et al*., 2014).

Citrus juices are taken mainly because of their rich nutritional value and unique flavour. The health benefit of the juices is attributed to their vitamin C content; a major vitamin found in fruits (Boudries *et al*., 2012; Rekha *et al*., 2012). According to Zvaigzne *et al*. (2009), ascorbic acid (vitamin C) is the most crucial antioxidant in *Citrus* fruit juices, which has the capability to protect the organism from oxidative stress. The new Institute of Medicine recommends, that an adult men and women need 90 mg and 75 mg of daily vitamin C, respectively. A pregnant woman needs 80 to 85 mg, nursing mothers 115 to 120 mg, while smokers will be needing an extra 35 mg because they deplete their stores of vitamin C faster. It is estimated that half cup of orange contains 50 mg of vitamin C while a medium orange contains 70 mg of vitamin C (webMed, 2000). Orange juice was found to prevent the formation of kidney stone by endowing citrate, which can decrease the acidity of the most prominent compound (uric acid and calcium oxalate) found in kidney stones (Odvina, 2006).

The antioxidant features of phenolics and vitamin C basically lie in their redox potentials, which act as reducing agents, hydrogen donors, singlet oxygen quenchers and metal chelators (Yen *et al*., 2005; Kaviarasan *et al*., 2007). Poor antioxidant defence mechanism results to oxidative stress; that is over production or incomplete removal of highly reactive molecules such as Reactive Oxygen Species (ROS). However, a well maintained balance should exist between production of free radicals and antioxidant defence mechanisms in a healthy individual (Rekha *et al*., 2012).

1.2 Problem statement

Many studies on antioxidant activities of *Citrus* fruits had been carried out. Previous studies put more attention on *Citrus* peels and have shown that the percentage of phenolic compounds in *Citrus* peels is higher than that of their other parts (Gorinstein *et al*., 2001; Guimaraes *et al*., 2010; Lagha-Benamrouche and Madani, 2013). In addition, some other researchers were mainly focused on the antioxidant properties of essential oils from *Citrus* fruit peels (Singh *et al*., 2010). Moreover, several studies of antioxidant activity of *Citrus* juices only have included *Citrus* species, varieties, cultivars and hybrids that can be found easily in their areas such as in China and Thailand (Xu *et al*., 2008; Makynen *et al*., 2013). Furthermore, antioxidants in fruits are considered safer to health, may assist in preventing disease developments, be used in treating some degenerative diseases (for example, diabetes), help to extend the shelf lives of certain products and could delay aging processes (Zhang *et al*., 2015). To date, there is no comparative study on antioxidant activities, α-glucosidase activities and vitamin C content of the common *Citrus* species that can be found in Malaysia especially *Citrus latifolia* that can only be found in Balik Pulau, Penang.

1.3 Objectives of the study

The main purpose of this research is to comparatively evaluate the antioxidant properties of the peels, pulps and juices of seven species of *Citrus* found in Malaysia. The specific objectives of this research include:

1. To determine the best solvent that can be used to extract antioxidants from the fresh and dried peels and pulps of seven *Citrus* fruits.
2. To determine and compare the primary and secondary antioxidant properties (using three different colorimetric assays), total phenolic and vitamin C contents, and alpha glucosidase inhibitory activity of *Citrus* fruit peel, pulp and juice extracts.
3. To monitor and determine the effect of storage on the *Citrus* juices.
4. To analyse the correlation between all studied parameters.

CHAPTER 2

LITERATURE REVIEW

2.1 Genus *Citrus*

The genus *Citrus* belongs to the family Rutaceae, subfamily Aurantioideae, tribe Citreae and subtribe Citrineae (Swingle and Reece, 1967). The subtribe Citrineae contains three groups; primitive *Citrus* fruit, near *Citrus* fruit, and true *Citrus* fruit trees. The third group is formed by the *Citrus* genus and its closely related genera. Within the edible *Citrus*, molecular studies identified four important ancestral taxa which are: *C. medica* (citron), *C. reticulata* (mandarin), C. *grandis* (pomelo), and *C. micrantha* (papeda). All other species including *C. sinensis* (sweet orange), *C. aurantium* (sour orange), *C. paradisi* (grapefruit), *Citrus limon* (lemon) and *C. aurantifolia* (lime) arose from hybridizations between the four basic taxa by reticulate evolution (Nicolosi *et al*., 2000; Garcia-Lor *et al*., 2012; Ollitrault *et al*., 2012).

Genus *Citrus* comprises of mainly trees or sometimes small large shrubs, rarely herbs reaching 5 to 15 meters tall. The stem is erect with thorny branches, woody growth habitat or sometimes climbing. When young the stem is green but grey when old. Leaves are evergreen and are alternately arranged with transparent glands that contain volatile oil. This volatile oil gives the leaves their typical scented smell. Flowers are solitary or in a cluster and measures two to four centimetres (cm) in diameter having five (rarely four) white, yellow or red petals and four to five sepals in green colour. Androecium (male reproductive whorl of the flower) contains eight to ten stamens. Gynoecium (female reproductive whorl) contains three to five carpels.

Filaments are free with two lobed anthers. Fruit is distinctive with hesperidium berry (filled with pulp vesicles), surrounded by a separable leathery rind with the internal fleshy parts divided into 10 to 16 segments (Spiegel-Roy and Goldschmidt, 1996; De Araújo *et al*., 2003; Krueger, 2003; Manner *et al*., 2006; Inyama *et al*., 2015).

The centre of origin and diversity of *Citrus* is believed to be Southeast Asia. The plants are also distributed to northeastern India, north into China and Japan, and south to Australia (Webber 1967; Scora, 1975; Gmitter and Hu, 1990; Anwar and Mirza, 1992; Soost and Roose, 1996; Moore, 2001). According to Cooper and Chapot (1977), almost all *Citrus* cultivars are native to China with the exception of grape fruit and lemon. *C. aurantifolia* (lime) is native to the Indo-Malayan region. *Citrus grandis* (Pomelo) is native to Southeastern Asia and across Malaysia. It was introduced to China around 100 Before Century and mostly cultivated in southern China at Kwang-tung, Kwangsi and Fukien Provinces (Morton 1987; Duarte *et al*., 2016). *Citrus hystrix* (Kaffir lime) is suggested to be native to Southeast Asia including India (Chueahongthong *et al*., 2011). According to Crane (2016), the origin of *Citrus latifolia* is uncertain, although recent reports on genetic analysis of *Citrus* suggests that *C. latifolia* is native to Southeast Asia, specifically North Burma (Myanmar), east and northeastern India, southwest China and eastward through the Malay Archipelago. Furthermore, *Citrus limon* (lemon) is probably native to India and has been cultivated in Asia since ancient times. It was the first fruit to be cultivated into the Mediterranean basin (Pagnoux *et al.*, 2013). Moreover, *Citrus madurensis* (Calamansi) is believed to have originated from China (Cheng *et al*., 2005; Manner *et al*., 2006). According to Yo and Lin (2004), this species is widely distributed in Southeast Asia and are known with many common names including calamansi for the Philippines and calamondin in Taiwan.

According to Rieger (2006), the best climate for *Citrus* cultivation is at the subtropical regions. Some fruits are said to attain their best flesh quality in the subtropical humid climates or Mediterranean climates (drier region) with irrigation. Pomelos can thrive well in tropical and subtropical climates while in the Mediterranean regions, mandarin-like *Citrus* and oranges and its cultivars are grown. The growth elevation of *Citrus* in subtropics is 750 m (2450 ft) above the sea level while in the tropics they grow better at elevation below 1600 m (5250 ft). *Citrus* species grow in climates with winter, summer or bimodal with uniform rainfall (Manner *et al*., 2006). Genus *Citrus* has wide range of soil tolerance from pure sands to richly organic to heavy clay soil (Rieger, 2006). According to Manner *et al*. (2006), *Citrus* grows better in well-drained soils than poorly drained soils and has zero tolerance to waterlogging. Based on soil acidity, it grows in acid to neutral soils but achieve the greatest growth at pH 6-7. It has poor tolerance to drought that can lead to the decrease in productivity. According to Jifon and Syvertsen (2001), light is necessary for an optimal growth of *Citrus* trees as low light level has a strong effect on their production.

Citrus is regarded as one of the most common tree fruit crops in the world. It has great economic and health value (Talon and Gmitter, 2008). From an economic point of view, *Citrus* fruit production rank first and it is estimated above 130 million tons per year (FAO, 2016). Other economic products derived from *Citrus* are as follows:

1. Animal fodder; the pulp, which is a by-product from juice production, is used for feeding cattle. In the United States, the peels and the seeds are dried and processed into pellet forms which serves as cattle feed (Manner *et al*., 2006).
2. Honey; in many parts of the world, *Citrus* is one of the most important honey plants. *Citrus* may not directly benefit from the presence of bees because most *Citrus* varieties are self-pollinating but they are endowed with groves that serve as an important wintering location to rejuvenate and produce new bee colonies. In California, *Citrus* constitute 25% of honey production. Also in Spain honey from *Citrus* plant is an essential ingredient of true Spanish nougat (Morton, 1987; Mabberley, 2004).
3. Essential oil; *Citrus* oil is an ingredient in cleaning products. It has strong bleaching effect and is widely used as an additive in soap and detergent production to remove tough stains and oil (Chueahongthong *et al*., 2011). Essential oil from *Citrus bergamia* (bergamot orange) has been extensively used as a photoprotective agent for protection of human skin against solar radiation (Forlot and Pevet, 2012). According to Manner *et al*. (2006), *Citrus sinensis* oil is used in air freshener, candles and aromatherapy for fragrance.
4. Timber and Craft wood; wood from *Citrus grandis* and *Citrus sinensis* are used in light construction (Clarke and Thaman, 1993). Wood from *Citrus aurantium* is hard with fine grain and valued for cabinetry and turnery design works (Morton, 1987).
5. Flavouring/spice; juices of *Citrus aurantifolia* and *Citrus limon* are usually used to flavour food and to marinate. Leaves of *Citrus hystrx*

(kaffir lime) are used for flavouring in cooked sauces (Manner *et al*., 2006).

All *Citrus* are diploid in nature (2n = 18). In the genus *Citrus*, hybrids are easily raised because seeds can be set without fertilization and through this, desirable forms can easily be propagated (Mabberley, 2004). Somatic and intergeneric hybridization are common among *Citrus* species. Somatic hybridization occurs between/among plants in the same genus while intergeneric is a cross between plants in two different genera from the same family, which are closely related enough that pollination will produce a hybrid, though the seeds of this hybrid are usually sterile. Somatic hybrids usually produce *Citrus* trees smaller than their diploid parents, this is an improvement in size control. It is also, continuously providing access to useful genetic variation that were inaccessible previously (Grosser and Gmitter, 1990; Grosser *et al*., 1995, Grosser and Chandler, 2003; Grosser and Gmitter, 2005). Furthermore, intergeneric hybridization grants an opportunity to combine genomes from distinctly different plants and to introgress traits not found in the main genus of interest (Ladizinsky, 1992). *Citrus madurensis* is an intergeneric hybrid, obtained from a cross between *Citrus japonica* (Kumquat) and *Citrus reticulata* (Mandarin) (Nicolosi *et al*., 2000; Mabberley, 2004; Chen *et al*., 2013). Table 2.1 is a summary of origin and species concept of selected *Citrus* species used in this study.

Table 2.1: Origin and species concept of *Citrus* species used in this study.

Species	Native Origin	Species concept	References
Citrus aurantifolia **(Limau nipis/ Lime)**	Indo-Malayan	Hybrid origin (*Citrus medica* + *Citrus micrantha*)	(Morton, 1987; Nicolosi *et al*., 2000; Curk *et al*., 2014)
Citrus grandis **(Limau bali/ Pomelo)**	Southeast Asia	True species	(Xinwei *et al*., 2013; Duarte *et al*., 2016)
Citrus hystrix **(Limau purut/ Kaffir lime)**	Southeast Asia	Hybrid origin (*Citrus paradisi* + *Citrus aurantifolia*)	(De Araújo *et al*., 2003)
Citrus latifolia **(Limau telur buaya/ Persian lime)**	Southeast Asia	Tri-hybrid origin (*Citrus medica* + *Citrus grandis* + *Citrus micrantha*)	(Crane, 2016; Moore, 2001)
Citrus limon **(Limau susu/ Lemon)**	India	True species	(Garcia-Lor *et al*., 2012; Pagnoux *et al*., 2013)
Citrus madurensis **(Limau kasturi/ Calamansi)**	China	Hybrid origin (*Citrus japonica* + *Citrus reticulata*)	(Mabberley, 2004; Chen *et al*., 2013)

2.1.1 Morphological description of *Citrus* used in this study

Citrus aurantifolia

C. aurantifolia is a perennial evergreen and exceedingly vigorous shrub that can grow to a height of 3–5 m, with many slender spreading branches that usually possesses short and rigid sharp spines or thorns. Its leaves are alternately arranged, elliptic to oval shape measuring 4.5–6.5 cm long and 2.5–4.5 cm wide with minute rounded teeth and narrowly winged petiole 1–2 cm long. It has short and axillary racemes flowers that are white with faintly fragrant. The petals are oblong, 5 in number and is 10–12 mm long. The fruit is round, 4-6 cm in diameter and has green colour but yellow when ripe. The fruit inner layer has white spongy portion called albedo and the number of segments in lime is between 8-11, usually aligned and situated round. It has yellow-green pulp vesicles with seeds (Nithithep and Wannee, 2016).

Citrus grandis

The height of pomelo tree is 5-15 m with somewhat crooked trunk and irregular branches. The young branches are hairly and usually with spines. *C. grandis* has an alternate leaf arrangement, which are ovate, ovate-oblong or elliptic in shape 2-12 cm wide with leathery, dull-green, glossy above and minutely hairy beneath and its petiole is broadly winged (rarely wingless). It has yellow-white fragrant flowers, borne singly or in clusters in the leaf axil or sometimes in terminal racemes with 4 to 5 petals. The fruit is nearly round to oblate or pear-shaped 10-30 cm in diameter. Indeed it is the largest citrus fruit. Peel colour may be greenish-yellow or pale-yellow dotted with tiny green glands. Pulp varies from greenish-yellow or pale-yellow to pink or red divided into 11 to 18 segments (Morton, 1987).

Citrus hystrix

Citrus hystrix is a low tree or shrub whose height measures 2-11 m with many branches and spines, densely crowned and twigy having angular and compressed stem. Leaves are alternately arranged, broadly ovate to ovate-oblong shape 3-11 cm long, and have broad winged petiole. Leaves are leathery, dark green and glossy on the upper side and heavily dotted with glands below and aromatic when bruised. *Citrus hystrix* has densely axillary or terminal yellowish white flowers, shortly stalked with fragrant, petals are usually 4 or 5 in numbers. Fruit is globose, wrinkled and roughen (warty), becomes yellow when ripe. Its peel is yellowish green and thick, while the inner pulp is yellow colour having 10-12 segments with ovoid or oblong seeds (Staples and Kristiansen, 1999; Manner *et al*., 2006).

Citrus latifolia

This is a small tree reaching a height of about 5 m. It has rounded and dense canopy hanging to the ground. Leaves are dark green measuring 9-13 cm long, oval to broadly lanceolate-shaped with narrow petiole. Its white flowers are borne in clusters of 5 to 10 on new shoots, petals are five in numbers. *Citrus latifolia* fruit has an oval shape and short elliptical, dark green at maturity and pale yellow before dropping from the tree, it is 4.7-6.3 cm in diameter having 10-12 segments with no seeds (Crane, 2016).

Citrus limon

Its small trees can reach a height of 3-6 m and often with sharp thorns on the twig. Leaves are dark green (above), light-green (below), oblong, elliptic or long-ovate in shape and are arranged alternately on the stem, jointed to narrowly winged petiole and is about 6-10 cm long. Flowers are white with mild scent and may be solitary or there may be two or more clusters attached to the leaf axil having 4 or 5 petals. Fruits are globose, ovoid to oblong with a nipple-like protuberance at the apex and fruit colour ranges from greenish yellow to bright yellow and measures about 5-8 cm in diameter. Fruit's peel is light yellow with aromatic dotted of oil glands while pulp contains 8-10 segments (Morton, 1987; Chaturvedi and Shrilastava, 2016).

Citrus madurensis

Calamansi is a tree which is erect, slender, usually cylindrical, slightly thorny, densely branched with an extraordinarily deep taproot. Its height ranges from 2-7.5 m high. The evergreen leaves are alternately arranged, dark green, glossy on the surface, yellowish green beneath, aromatic and broad oval in shape 4-7.5 cm long, slightly teethed at the apex with short narrow petioles. Its white flowers are richly fragrant having 5 elliptic-oblong petals borne singly or in 2's or 3's terminally. Fruits are round or oblate with very aromatic, glossy and dotted small oil glands 4.5 cm in size. Fruit peel is orange-red while pulp colour is orange having 6-10 segments (Morton, 1987). Table 2.2 shows the morphological characteristics of the fruits.

2.1.2 Medicinal values of *Citrus* fruits

Malaysians use juice extract from lime in the treatment of scalp dandruff. Its juice was mixed with turmeric, salt and brown sugar to treat stomachache. Concoction of fruit peel from lime, sugar and warm water is drunk to treat vaginal discharges (Ong and Nordiana, 1999). Lime juice and its oil were consumed orally or applied on the skin to rejuvenate the skin, reduce body odour and give the skin protection against infections. Daily intake of lime juice protects the eyes from aging and macular degeneration. The citric acid present in lime is an excellent fat burner; a glass of warm water mixed with lime juice is believed to facilitate in weight loss (Mohanapriya *et al*., 2013).

Orange peel has been traditionally used to relieve stomach upset, skin inflammation, and enhancing the immune system (Shetty *et al*., 2016). According to Parle and Chaturvedi (2012), orange juice assists in the elimination of toxins from the body, maintains hydration, improves appetite and prevents constipation. The pulp of pomelo has been used to improve appetite, neutralizes the action of a poison, improving the action of the heart and to relief for bloating, gas cramps and stomach acidity (Arias and Ramón-Laca, 2005).

Table 2.2: Morphological characteristics of *Citrus* fruits used in this study.

Species	Local name	Size (diameter)	Shape	Peel Colour	Pulp Colour	Number of segments.
Citrus aurantifolia	Limau nipis (Lime)	4–6 cm	Globose to ovoid	Green, greenish yellow	Green	8-11
Citrus grandis	Limau bali (Pomelo)	9–25 cm	Subglobose, globose, oblate to pyriform	Pale green to pale yellow	Pale green to Pinkish	11-18
Citrus hystrix	Limau purut (kaffir lime)	7 cm	Globose, wrinkled and roughen	Green to yellow	Greenish	10-12
Citrus latifolia	Limau telur buaya (Kaffir lime)	5-6.35 cm	Oval or short elliptical	Lightly yellow	Greenish	10-12
Citrus limon	Limau susu (Lemon)	4–7 cm	Globose, ovoid to oblong	Yellow to green	Pale greenish to yellowish	8-10
Citrus madurensis	Limau kasturi (Calamansi)	3-4 cm	Round or oblate	Orange to red	Orange	6-10

References: Morton, 1987; Staples and Kristiansen, 1999; Manner *et al.*, 2006; Etebu and Nwauzoma, 2014; Nithithep and Wannee, 2016; Crane, 2016.

Traditionally, Malaysians use the coarsed fruit skin of kaffir lime for washing their hair and body parts in a washing ceremony called "Mandi berlimau" and in certain occasion for driving out demons (Nor, 1999). Recently, its peel is used for fragrance in shampoos and its leaves for flavouring in "laksa asam" sauce, curries, cakes and tomyam soup, a delicacy for Asean (Wong, 1992). The fruits are used as a digestive stimulant, blood purifier, and for lowering high blood pressure. Furthermore, the fruit juice is used in softening the skin. Also, the mixture of the fruit juice with bath water can be used to eliminate body odour (Stone, 1985). Furthermore, calamansi juice has often been used for seasoning dishes, flavouring and as an additive to improve the absorption of iron. It is an effective medication for coughs and for itches and can be utilized by ladies for lighten spots (Quisumbing, 1978).

Drinking of lemon juice and honey helps in weight loss, lemon juice is recommended for people with urinary tract infection problem because it has the ability to flush high level of uric acid, it helps in skin lightening, lemon juice and olive oil is used to cure gall stones and kidney stones (Chaturvedi and Shrilastava, 2016).

2.1.3 Bioactivity evaluations of *Citrus* fruits

Emam and Ghada (2015), compared the *in vitro* antioxidant activity of different orange peel extracts using different organic solvents (methanol, petroleum ether, ethanol diethylether, hexane and acetone). The result showed that methanolic extract displayed the highest antioxidant activity. Orange peel is therefore considered as a good source of polyphenols and can safely be used for preservation of oils and fats from peroxidation and rancidity instead of synthetic antioxidants.

Kumar *et al*. (2011), reported antibacterial activity of two *Citrus* fruit peels (*Citrus sinensis* and *Citrus limon*) extracted using five different solvents (ethyl acetate, acetone, ethanol, petroleum ether and water) against five pathogenic bacteria, *Staphylococcus aureus, Bacillus subtilis, Escherichia coli*, *Klebsiella pneumonia* and *Salmonella typhi.* The highest antibacterial activity was exhibited by the acetone peel extract of *Citrus sinensis* followed by the ethyl acetate peel extract of *Citrus limon*. The result had suggested that the peel extracts of *Citrus sinensis* and *Citrus limon* can be considered to be as equally potent as the antibiotics, such as metacillin and penicillin.

Mehmet *et al*. (2007), studied the anti-inflammatory activity of *Citrus bergamia* essential oil using carrageenan-induced rat paw oedema model. The result revealed that reduction in the inflammation was 63.39% with 0.100 mL/kg *Citrus bergamia* oil and the median effective dose (ED_{50}) value was found to be 0.079 mL/kg. The results showed that *Citrus bergamia* oil possesses anti-inflammatory potential. Nagaraju *et al*. (2012), found the decrease in ulcer formation with aqueous extract of *Citrus medica* fruit against ethanol-induced ulcer in rats at 250 and 500 mg/kg although reduction was significantly higher at 500 mg/kg.

Chutia *et al*. (2009), investigated antifungal activity of *Citrus reticulata* essential oil against five plant pathogenic fungi namely; *Alternaria alternate Rhizoctonia solani*, *Curvularia lunata*, *Fusarium oxysporum* and *Helminthosporium oryzae*. It was found that 0.2 mL/100 mL was the Minimum Inhibitory Concentration (MIC) for *Alternaria alternata, Rhizoctonia lani, Curvularia lunata* whereas >0.2 mL/100 mL for *Fusarium oxysporum* and *Helminthosporium oryzae*. Fungal sporulation was also completely inhibited at 2 mL/100 mL except for *Curvularia lunata* and *Helminthosporium oryzae*.

Bhavsar *et al*. (2007), studied the protective effect of the ethanol extract of *Citrus limon* fruits on carbon tetrachloride–intoxicated rats. The effect of three doses of the extract (150, 300, and 500 mg/kg) were examined independently. The result showed that 500 mg/kg dose of *Citrus limon* extract has the highest significant decrease in carbon tetrachloride–induced liver damage.

Abirami *et al*. (2014), studied the *in vitro* anti-diabetic activity of *Citrus hystrix*, *Citrus maxima* (Red) and *Citrus maxima* (White) juice extracts. The result revealed that all juices of the studied *Citrus* fruits have high potential α-glucosidase inhibitory activity ranging from 70.68% to 72.83% and α-amylase (75.55% to 79.75%), respectively.

2.2 Antioxidant activities of *Citrus* fruits

Antioxidant is a molecule that is capable of preventing or slowing down the oxidation of other molecules in order to maintain cell structure and activities by effectively clearing free radicals, preventing lipid peroxidation and inhibiting other oxidative damage (Bravo, 1998). Antioxidants have been reported severally to be responsible for the defence mechanisms of the organism against the pathologies associated with the attack of free radicals. According to Lobo *et al*. (2010), a free radical is simply defined as an unstable and highly reactive molecule containing at least one unpaired electron. According to Phaniendra *et al*. (2015), the free radicals, of both the Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS), are generated from both internal sources (such as mitochondria, peroxisomes, endoplasmic reticulum, phagocytic cells) and external sources (such as pollution, alcohol, tobacco smoke, heavy metals, transition metals, industrial solvents, pesticides, certain drugs like halothane, paracetamol, and radiation). These are also generated as a result of normal biochemical reactions, increased rate of exposure to the environment, and higher levels of dietary xenobiotics (Bagchi and Puri, 1998; Satish and Dilipkumar, 2015).

Table 2.3 is the list of some of the ROS. A practical approach to prevent the effect of ROS is by the consumption of antioxidant rich foods (Oboh and Rocha, 2007). At a minimum or moderate concentration, free radicals perform beneficial roles in physiological processes such as energy production, cell growth and differentiation (Poli *et al*., 2004; Lien *et al*., 2008). Free radicals when produced in excess are capable of causing damage to biomolecules such as proteins, lipids DNA/nucleic acid (Valko *et al*., 2006), which may lead to several health disorders such as inflammation, cancer, Alzheimers, arteriosclerosis, diabetes and cardiovascular diseases (Valko *et al*., 2007; Uttara *et al*., 2009). Free radicals have been associated with intracellular killing of bacteria by phagocytes such as macrophages, neutrophils and granulocytes, which are necessary for life. It is also linked with cell signalling processes called redox signalling (Droge, 2002; Pacher *et al*., 2007; Lien *et al*., 2008).

These harmful effects of free radicals can be balanced by the activities of both enzymatic and non-enzymatic antioxidants (Balsano and Alisi, 2009; Anjum *et al*., 2011). Consumption of fresh fruits can act as non-enzymatic antioxidants (Ke *et al*., 2015; Rajendran *et al*., 2014; Zhang *et al*., 2015).

Table 2.3: List of reactive oxygen species by Satish and Dilipkumar (2015)

Symbol	Name
$^1O_2$	Singlet oxygen
$O_2^{\cdot -}$	Superoxide anion radical
$^{\cdot}OH$	Hydroxyl radical
$RO^{\cdot}$	Alkoxyl radical
$ROO^{\cdot}$	Peroxyl radical
H_2O_2	Hydrogen peroxide
LOOH	Lipid hydroperoxide

2.2.1 Methods for determination of antioxidant activity

A number of methods have been developed for the evaluation of both general and specific antioxidant property of various samples (plant extracts, compounds and commercial antioxidants). In practice, various *in vitro* methods have been used (Prior *et al*., 2005). Frankel and Finley (2008), have stated the need for an agreement on standardized test methods which are as follows: (1) to have a guideline for an appropriate application of assays, (2) to allow for relevant comparisons of foods and commercial products, (3) to serve as an avenue for control variation within or between products, (4) to provide quality standards for regulatory issues and health claims. According to Litescu *et al*. (2010), the various analytical methods for evaluation of antioxidant capacity fall into three distinct categories. These categories are described in Table 2.4.

Table 2.4: Categories and methods of antioxidant evaluations by Litescu *et al*., (2010).

Antioxidant capacity assay	**Principle of the method**	**End-product determination**
1. Spectrometry		
DPPH (2,2-diphenyl picryl-hydrazyl)	Antioxidant reaction with an organic radical	Colorimetry
ABTS (2,2-azino-bis-3-ethylbenzthiazoline-6-sulhonic acid)	Antioxidant reaction with an organic cation radical	Colorimetry
FRAP (Ferric reducing antioxidant power)	Antioxidant reaction with a Fe(III) complex	Colorimetry
Metal chelating	Chelation of metal ions by antioxidants in the mechanism of interrupting the formation of ferrous complex through capturing of ferrous ions.	Colorimetry
PFRAP (Potassium ferricyanide reducing antioxidant power)	Potassium ferricyanide reduction by antioxidants and subsequent reaction of potassium ferrocyanide with Fe^{3+}	Colorimetry
CUPRAC (Cupric ion reducing antioxidant capacity)	Cu (II) reduction to Cu (I) by antioxidants	Colorimetry
ORAC (Oxygen radical absorbance capacity)	Antioxidant reaction with peroxyl radicals, induced by AAPH (2,2'-azobis-2-amidino-propane)	Loss of fluorescence of fluorescein.

Table 2.4 continued

Antioxidant capacity assay	**Principle of the method**	**End-product determination**
HORAC (Hydroxyl radical antioxidant capacity)	Antioxidant capacity to quench OH radicals generated by a Co(II) based Fenton-like system	Loss of fluorescence of fluorescein.
TRAP (Telomeric repeat amplification protocol)	Antioxidant capacity to scavenge luminol-derived radicals, generated from AAPH decomposition	Chemiluminescence quenching.
Fluorimetry	Emission of light by a substance that has absorbed light or other electromagnetic radiation of a different wavelength	Recording of fluorescence excitation/emission spectra.
2. Electrochemical techniques		
Cyclic voltammetry	The potential of a working electrode is linearly varied from an initial value to a final value and back, and the respective current intensity is recorded	Measurement of the intensity of the cathodic/ anodic peak
Biamperometry	The reaction of the analyte (antioxidant) with the oxidized form of a reversible indicating redox couple	Measurement of the current flowing between two identical working electrodes, at a small potential difference and immersed in a solution containing the analysed sample and a reversible redox couple
Amperometry	The potential of the working electrode is set at a fixed value with respect to a reference electrode	Measurement of the intensity of the current generated by the oxidation/reduction of an electroactive analyte

Table 2.4 continued

Antioxidant capacity assay	Principle of the method	End-product determination
3. Chromatography		
Gas chromatography	Separation of the compounds in a mixture is based on the repartition between a liquid stationary phase and a gas mobile phase	Flame ionisation or thermal conductivity detection
High performance liquid chromatography	Separation of the compounds in a mixture is based on the repartition between a solid stationary phase and a liquid mobile phase with different polarities, at high flow rate and pressure of the mobile phase.	UltraViolet-Visible (UV-VIS) (e.g. diode array) detection, fluorescence, mass spectrometry or electrochemical detection

2.2.2 Comparative studies previously done on the antioxidant activities of *Citrus* fruits.

Various comparative studies using different methods of antioxidant evaluation have been done on *Citrus* fruits. Some researchers have compared between/among the *Citrus* species juices, peels and pulps. Extraction of bioactive constituent has been shown to be an important step for the determination of antioxidant potential from plants. The extraction solvents such as ethanol, methanol, acetone, water, propanol, ethyl acetate and dimethylformamide in different quantities and ratios have been frequently used for the extraction of antioxidant from fruits (Caetano *et al*., 2011). Nevertheless, the extraction methods chosen for analysis must enable absolute extraction of the compounds of interest and must avoid their chemical modification (Zuo, 2002). Notably, reports have shown the variations of bioactivities of the extracts as well as the extraction yields prepared using different extraction methods (Hayouni *et al*., 2007). Table 2.5 describes some of the *in vitro* comparative studies done on *Citrus* fruits.

Table 2.5: *In vitro* comparative studies on the antioxidant activities of *Citrus* fruits.

***Citrus* fruits**	**Parts used**	**Extraction Solvent**	**Results/References**
Citrus aurantifolia ***Citrus grandis*** ***Citrus macrocarpa***	Juice		In the DPPH assay the juice extract of *Citrus grandis* exhibited the highest free radical scavenging activity while the juice extract of *Citrus aurantifolia* showed the lowest activity. The juice extract of *Citrus grandis* also achieved the best result in the FRAP while *Citrus microcarpa* extract had the lowest activity. For the metal chelating activity, the extract with the best activity in chelating ion was the juice extract of *Citrus microcarpa*. Next to it was the extract of *Citrus aurantifolia* while no activity was observed for *Citrus grandis* (Sulaiman and Ooi, 2014).
Citrus aurantifolia ***Citrus hystrix*** ***Citrus microcarpa*** ***Citrus sinensis***	Juice		*Citrus hystrix* juice extract exhibited the highest antioxidant activity in the DPPH assay at EC_{50} values of 35 mg/100 mL of fresh juice. This was followed by *Citrus aurantifolia* and *Citrus sinensis* while *Citrus macrocarpa* obtained the highest EC_{50} value of fresh juice with the lowest activity. For the FRAP assay, *Citrus hystrix* juice extract also had the highest activity of while *Citrus macrocarpa* juice extract also showed the lowest antioxidant activity in the FRAP assay (Ghafar *et al*., 2010).