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Functional properties of *Phaleria macrocarpa* fruit flesh at different ripeness

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Abstract

Functional property changes in *Phaleria macrocarpa* fruit during ripening on tree were studied. Results showed that juice extracted from fruit flesh had low acidity and soluble solid content. Fruit acidity decreased but soluble solids increased as the fruit ripened. In terms of antioxidant content, ascorbic acid, DPPH free radical scavenging activities and total phenolic content were, however, the lowest in fully ripe fruit flesh while the unripe fruit flesh had the highest. High percentage of these antioxidants was water soluble. This study suggests that the unripe fruits should be harvested for valuable medicinal product development instead of the fully ripe fruits.

Introduction

Fruit ripening has always been associated with important biochemical and active compound content changes that modify quality traits (Pods and Edek, 2007; Ziosi *et al.*, 2008; Crecente-Campo *et al*., 2012; Vallone *et al*., 2013; Singh *et al*., 2015). These compounds are nutritionally and economically important, and have received much attention in recent years with the current upsurge of interest in efficacy and use of naturally derived antioxidants. Chemical compounds in fruits are a potential resource of functional food and nutraceuticals, because of their antioxidant capacity and nutritional quality. Many medicinal fruits including those of *Phaleria macrocarpa* have not been studied extensively on their functional and chemical changes in relation to fruit ripeness (Mahattanatawee *et al*., 2006; Türker and Dalar, 2013). *Phaleria macrocarpa* is a medicinal plant in tropical region that belongs to Thymelaeaceae family. It is commonly known as Mahkota Dewa, which literally means God's Crown, among people in Indonesia and Malaysia (PROSEA, 1992; Saufi, 2007). For centuries, the locals have used its fruits to counter diabetes, liver diseases, vascular problems, cancer and high blood pressure (Balick and Cox, 1996; Tate, 2002; Winarto, 2004; Harmanto, 2005; Zhang *et al*., 2006). The fruit is a drupe with very thin outer fruit skin (pericarp) enclosing the white pulp (mesocarp), which is fibrous but watery, while the hard endocarp protects the seed in the middle of the fruit. Pericarp and mesocarp, also known as fruit flesh, is used in traditional medical treatment but the seed is poisonous and must not be consumed. The fruit is round to ovoid shaped, measuring 3 to 5 cm in length. Its therapeutic effects have been reported to be related to the chemical and bio-active compounds in them (Sher, 2009; Effendy *et al*., 2011; Hendra *et al*., 2011; Kusuma *et al*., 2011).

Many fruits show significant changes in pH, total soluble solids (TSS), titratable acidity (TA), ascorbic acid (AA), polyphenol content and antioxidant activities with advancing maturity (Fu *et al*., 2011; Fawole and Opara, 2013a; Liu *et al*., 2015). The changing functional and chemical compounds in fruits with ripening progress can also be possibly related to geographical, microclimatic, agronomic and genotypic differences (Ilahy *et al*., 2011; Vandendriessche *et al.*, 2013; Mphahlele *et al.*, 2014). The current study was carried out to evaluate some functional compound changes of *P. macrocarpa* fruits during fruit ripening. Fruits collected from a single farm were used as test materials to eliminate locality and husbandry practice factors. Such study is hoped to play a role for the optimum harvest benefits, postharvest handling and industrial uses.

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Materials and Methods

Fruit collection

Fruits were freshly harvested from a mixed fruit orchard in Johor, Malaysia (N 1°31', E 103°23') in September 2013 for this study. In this farm of approximately 1 ha, *P. macrocarpa* trees occupied area of about 0.5 ha on flat terrain of mineral soil at less than 50 m elevation from sea level. Trees were planted at spacing of 3-5 m apart from one another giving approximately 280 trees within this farm. Other existing trees among *P. macrocarpa* trees within the farm were fruit trees, mainly *Durio zibethinus* trees of more than ten year-old. *Phaleria macrocarpa* trees were approximately four year-old with average plant height of 3 m and had flowered and produced fruits for the past two years. The farm is located in tropical region with rather consistent raining days throughout the year.

The full size unripe, half ripe and fully ripe fruits were studied in the experiments described below. The colour of pericarp was used as visual guide to indicate fruit ripeness (Figure 1). The fruits change from green to red upon ripening. Unripe fruits are green in colour (L^*=49.2, a^*=-7.8, b^*=29.4, C^*=30.8, hue=103.6) (Ahmed Asrity, 2016). Full size unripe fruits are usually obtained at approximately one month after fruit set. Half ripe fruits are those having green and red colour mixture, each in approximately 50% (L^*=41.1, a^*=5.7, b^*=18.9, C^*=21.0, hue=72.2) (Ahmed Asrity, 2016). Such fruits are usually found at about six weeks after fruit set. Fully ripe fruits, on the other hand, are those turn 100% red colour on the pericarp at approximately two months after fruit set (L^*=35.6, a^*=40.2, b^*=14.9, C^*=43.3, hue=21.6) (Ahmed Asrity, 2016).

Collected fruits were kept in perforated plastic bags and brought to the laboratory in air-conditioned vehicle on the same day. Travelling from the farm to laboratory took about four hours. Experimentations were carried out accordingly on the following day.

Location of laboratory

All experiments were conducted in the air-conditioned postgraduate general laboratory in Universiti Teknologi MARA, Shah Alam (N 3°4', E 101°30'). The laboratory had average temperature of 25±2°C and relative humidity of 55±5%. It housed basic equipment for postgraduate studies.

Preparation of fruit juice

For each fruit ripeness, five fresh fruits were used to prepare fruit juice for determination of pH, TSS content and TA. The fruits were first cleaned with running tap water. Then, the excessive water was drained off in a basket for 1 h.

Figure 1. Unripe (left), half ripe (center) and fully ripe (right) fruits; fruits sized 3-5 cm in length (from fruit stalk scar to base)

To prepare the fruit juice, 40 g fruit flesh was coarsely chopped and placed in a blender jug. Care was taken to avoid cutting the endocarp with seed enclosed in it. Then, 80 mL distilled water was added into the blender jug and homogenized for 1 min at high speed. The homogenized sample was vacuum filtered through a funnel with filter paper to separate the filtrate (fruit juice) and fruit residue.

Determination of pH, TSS content and TA

pH of 50 mL fruit juice was determined at 25±2°C using a pH meter after being standardized with pH 4 and pH 7 buffer solutions. TSS content of fruit juice was measured using a digital refractometer with temperature indicator. Triplicate measurements were carried out at 25±2°C and the average of the readings was used in calculation of TSS using the formula below:

TSS (%) = reading of refractometer with correction factor x dilution factor

For determination of TA expressed as % citric acid at 25±2°C, 5 mL fruit juice was placed into a clean conical flask with 2 drops of 1% phenolphthalein as indicator. The fruit juice was titrated with 0.1 N sodium hydroxide (NaOH) while swirling the conical flask constantly until the solution colour changed to pink. Triplicate measurements were also carried out and the average of the readings was used to calculate TA as below:

$$\text{TA (\% citric acid)} = \frac{\text{NaOH volume (mL) x normality of NaOH x volume of product (mL) x equivalent weight of citric acid (g) x 100}}{\text{weight of sample (g) x volume of sample for titration (mL) x 1000}}$$

Determination of ascorbic acid (AA) content

For each fruit ripeness, other five fresh fruits were used for determination of AA content using indophenol titration method (AOAC, 1990; Favell, 1998). A total of 40 g fruit flesh was cut and homogenized with 80 mL of 3% cool meta-phosphoric acid (HPO_3) at high speed for 1 min using a blender.

The suspension was filtered with a funnel and filter paper. Then, 5 mL filtrate was taken and titrated with 2.5% dye solution of 2,6-dichlorophenol-indophenol sodium at 25±2°C until pink color appeared. The volume of dye solution was recorded. To determine the dye factor, 5 mL standard solution of AA at 100 mg/L was added with 5 mL of 3% HPO_3. Then, the mixture was titrated with the dye solution at 25±2°C until pink colour appeared in the mixture. Triplicate measurements were carried out and the average of the readings was used in calculation of AA content (mg/100 g fruit flesh) as below:

$$\text{AA content (mg/100 g fruit flesh)} = \frac{\text{volume of dye (mL) x dye factor x volume of product (mL) x 100}}{\text{weight of sample (g) x volume of sample for titration (mL)}}$$

Freeze-drying of fruit flesh and preparation of fruit extract

2, 2-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activities and total phenolic content (TPC) in fruit flesh of each ripeness were determined with water and ethanolic fruit extract. For this purpose, five fresh fruits were first cleaned with running tap water and excessive water was drained off. Fruit flesh was then chopped into small pieces and subjected to freeze-drying using a freeze-dryer. After freeze-drying procedure, the freeze-dry sample was further ground into fine powder form sample. The fine powder sample was then stored in air tight glass jar at -20ºC in a freezer until DPPH free radical scavenging assay and determination of TPC within the next few days.

Preparation of fruit extract for DPPH free radical scavenging assay and TPC was carried out according to the method of Velioglu *et al.* (1998) with minor modification. An amount of 1 g freeze-dry sample was extracted with 25 mL distilled water (1:25 w/v) in capped test tube for 2 h at 50ºC on an orbital shaker at 200 rpm in a water bath. The mixture was then filtered through a funnel with filter paper. The filtrate was considered as water fruit extract for DPPH free radical scavenging assay and determination of TPC. This water extract was at a concentration of 40,000 mg/L. Ethanolic fruit extract, on the other hand, was prepared similarly using ethanol to replace water as solvent.

2, 2-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay

DPPH free radical scavenging activities of the water and ethanolic fruit extracts were estimated according to the method of Yamaguchi *et al*. (1998) and Tang *et al.* (2002) with some modifications. AA was used as the standard in this study. AA standard solutions and fruit extracts at concentrations of 0, 200, 400, 600, 800 and 1,000 mg/L were prepared. An amount of 800 µL solution was added to 4.5 mL of 0.01 mM DPPH in ethanol in a 10 mL bottle with screw cap. The mixture was shaken vigorously and left to stand for 30 min in the dark at room temperature. The solution was progressively reduced to yellow colour diphenylpicryl hydrazine with the presence of reducing agents. Absorbance of the solution was then read using UV-spectrophotometer at 517 nm and compared with the blank containing 800 µL distilled water in 4.5 mL DPPH as mentioned. Triplicate measurements were carried out and the average of the readings was used to calculate DPPH free radical scavenging activities of fruit extract as below:

$$\text{Scavenging activity (\%)} = \left[1 - \frac{\text{absorbance of fruit extract}}{\text{absorbance of control}}\right] \times 100$$

EC_{50} value of each water and ethanolic fruit extract, which is the amount of antioxidants needed to decrease the initial DPPH radical concentration by 50%, was determined from the plotted graph of scavenging activity percentage against the concentration of extract.

Total phenolic content (TPC)

TPC of water and ethanolic fruit extract was determined using Folin-Ciocalteu reagent based on the method of Taga *et al.* (1984) with some modifications, and calculated using gallic acid as the standard. With gallic acid solutions of 0, 100, 200, 300, 400 and 500 mg/L, respectively, 100 µL solution was mixed with 1 mL of 50% Folin-Ciocalteu reagent and allowed to stand at room temperature in the dark for 5 min. Then, 2 mL of 20% sodium bicarbonate (Na_2CO_3) was added to the mixture and topped up to 10 mL with distilled water. The mixture was shaken vigorously and left to stand for 2 h at room temperature in the dark. Absorbance was read at 750 nm using a spectrophotometer. Then, the absorbance against gallic acid concentration curve was plotted and the relationship between the absorbance and gallic acid concentration was determined as:

Absorbance = (0.0014 x concentration of gallic acid) + 0.003203

An amount of 25 mL water fruit extract above (40,000 mg/L) was diluted with 75 mL distilled water to obtain a suitable concentration for analysis of TPC. Then, 100 µL diluted water fruit extract was subjected to chemical reaction with 50% Folin-Ciocalteu reagent and 20% Na_2CO_3, as that carried out with

standard gallic acid solutions, followed by absorbance reading at 750 nm using the spectrophotometer. There were also triplicated measurements, and mean value was calculated. TPC of fruit extract was expressed as gallic acid equivalent (GAE) in mg/g fruit flesh extract according to the linear relationship between the absorbance and gallic acid concentration. The formula involved was:

$$\text{TPC (mg GAE/g extract)} = \frac{\text{(Absorbance of sample} - 0.003023) \times 100 \text{ mL} \times \text{dilution factor}}{0.0014 \times 1000 \text{ mL} \times \text{weight (g)}}$$

Determination of TPC of ethanolic fruit extract was also carried out, similar to that applied on the water fruit extract above.

Statistical analysis

The experimentations were all based on a completely randomized design (CRD) with three replicates. Data were subjected to analysis of variance (ANOVA) and treatment means were compared using Tukey's Honestly Significant Difference (HSD) Test. Minitab version 17 was used for statistical analysis of the data.

Results

Phaleria macrocarpa juice extracted from the fruit flesh was acidic. Unripe and half ripe fruit juice was not significantly different in pH while fully ripe fruit juice was significantly more acidic at mean pH of 5.4 (Figure 2A).

Like many other fruits, fully ripe fruit juice had significantly the highest TSS content (6.7%) while unripe fruit juice had the lowest TSS of 4.5% (Figure 2B). In contrast to pH, TA of unripe fruit juice was significantly higher while TA of half ripe and fully ripe fruit juice was lower (Figure 2C). However, TA of *P. macrocarpa* fruit juice was rather low; it was only 0.2% to 0.3%. In terms of AA content, unripe fruit juice also contained the highest amount of AA while the fully ripe fruit juice had the lowest (Figure 2D).

DPPH free radical scavenging activities of the unripe fruit extract were also the highest as compared to those in the half ripe and fully ripe fruits. For the same fruit ripeness, DPPH free radical scavenging activities of the ethanolic fruit extract, on the other hand, were higher as compared to those of the water extract. According to Songsungkan and Chanthai (2014), there was effect of organic solvents on the extraction of active compounds from plant tissues. With the least DPPH free radical scavenging activities found in the fully ripe fruit extract, its antioxidant

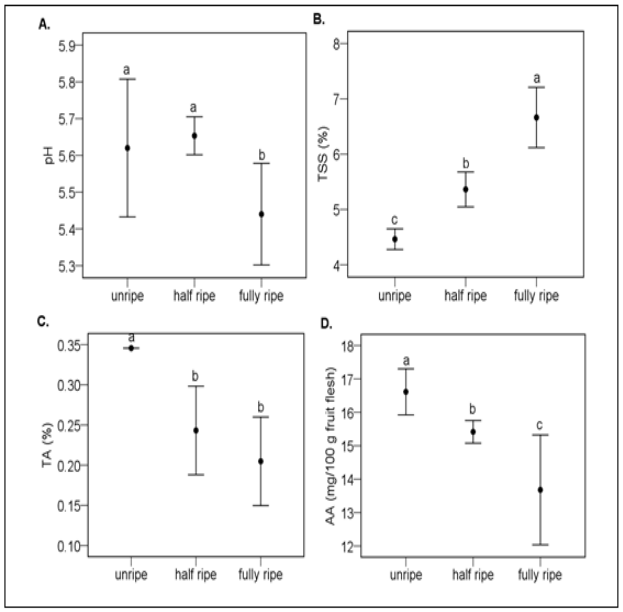

Figure 2. (A) pH, (B) TSS, (C) TA and (D) AA of fruit juice; ● indicates mean value; I indicates 95% CI for mean; means having different letters within chart are significantly different at P≤0.05.

concentration needed for decreasing the initial DPPH radical concentration by 50% (EC_{50}) was, hence, the highest, with higher value for the water extract as compared to the ethanolic extract (Figure 3). Mean EC_{50} of water extract of fully ripe fruit was 827 mg/L, as opposed to 586 mg/L with its ethanolic extract. However, ethanolic extracts of all three fruit ripeness as studied had EC_{50} ranging from 550-600 mg/L and did not differ significantly (P>0.05). This indicates that the fruits had high amount of antioxidants, with more antioxidants could be extracted from the fruits, especially the fully ripe fruits, using ethanol as the solvent.

TPC in fruit extract was also high. It was at least above 80 mg GAE/g extract of the fully ripe fruit flesh (Figure 4). The extract obtained from the unripe fruits had significantly the highest TPC while that from the fully ripe fruits was the least. In concurrent to higher AA, the highest TPC explained why the DPPH free radical scavenging activities of unripe fruit extract were the highest. TPC of the ethanolic extract of each fruit ripeness was also higher as compared to that of the water extract, respectively.

Discussion

Phaleria macrocarpa fruit flesh had low acidity. Fruit juice pH and TSS can be internal ripeness indicators. Fruit acidity decreased but soluble solids increased as fruit ripened. Similar observation of increased soluble solids was reported in a number of

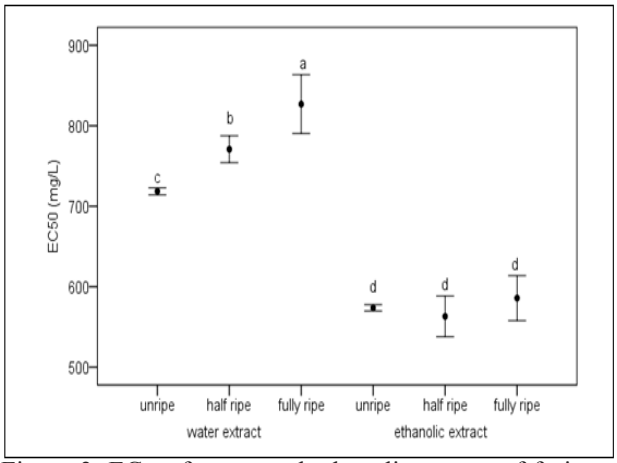

Figure 3. EC_{50} of water and ethanolic extract of fruits; ● indicates mean value; I indicates 95% CI for mean; means having different letters are significantly different at P≤0.05.

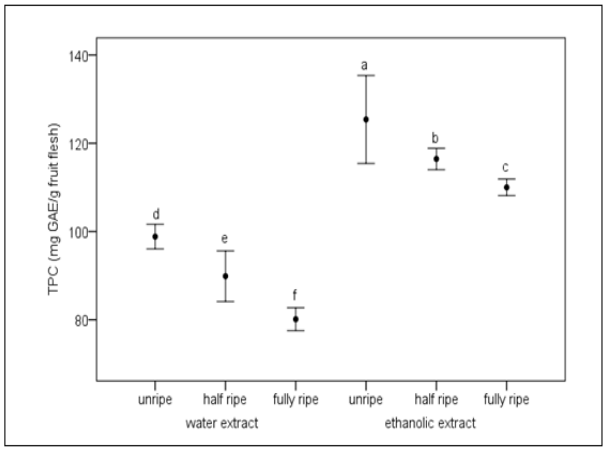

Figure 4. Mean TPC of water and ethanolic extract of fruit flesh; ● indicates mean value; I indicates 95% CI for mean; means having different letters are significantly different at P≤0.05.

ripening fruits (Jha *et al*., 2006; Kafkas *et al*., 2007; Prinsi *et al.*, 2010; Ornelas-Paz *et al*., 2013; Radunic, *et al*., 2015; Wongmetha *et al.*, 2015; Rahman *et al*., 2016). There were probably changes in carbohydrate metabolism, particularly the conversion of the starch to sucrose, as dictated in some ripening fruits (Jha *et al.*, 2006; Kafkas *et al*., 2007). In these fruits, the density of starch granules decreased as starch was converted to sugars during ripening process.

In the current study, pH of *P. macrocarpa* fruit juice decreased, indicating more acidic condition, as the fruit ripened. Such change in pH may not fully be explained by only changes of acidity and soluble solid content in fruit juice over ripening process as all these changes were found small in this study. There may be other minerals, amino acids, enzymes, glutathione, pigments, vitamins, flavonoids and phenolic compounds degraded or newly synthesized in fruit tissues and juice during ripening. All these compounds and changes may affect the overall pH and soluble solid content of the fruit and fruit juice (Ayala-Zavala *et al*., 2004; Cordenunsi *et al*., 2005).

Reduction in TA and AA is also a physiological change reported in some fruit species (Lee and Kader, 2000; Jamaludin *et al*., 2010). With progress of fruit ripening, AA in some fruits could have been converted to other compounds, thus reducing the AA content in their fully ripe fruits.

The highest amount of antioxidants was found in the unripe fruit flesh of *P. macrocarpa*; it had good amount of AA of 16.61 mg/100 g fruit, low EC_{50} of 718 mg/L and 574 mg/L with water and ethanolic extracts, respectively, and high TPC of 99 mg GAE/g extract and 125 mg GAE/g extract with water and ethanolic extracts, respectively. Nevertheless, loss of antioxidant content was significant as *P. macrocarpa* fruit ripened. Fully ripe fruit flesh had the least antioxidant content. Such phenomenon was also reported by a number of studies on some other fruit species (Nagy, 1980; Mansouri *et al.,* 2005; Kubola and Siriamornpun, 2011; Torres-Rodríguezet *et al.*, 2011; Fawole and Opara, 2013b; Nuncio-Jáuregui *et al.*, 2015). Higher antioxidant and antimicrobial contents in some unripe fruits, as compared to fully ripe fruits, were also thought to be related to their roles in protecting the developing fruits, as well as the growing seeds and embryos within them (Ighodaro, 2012; Tinrat, 2014). Despite having the lowest level of antioxidant content as compared to half ripe and unripe fruits, the amount of antioxidants in fully ripe *P. macrocarpa* fruit flesh was still considered good for the benefits of human beings. It had AA of 13.05 mg/100 g fruit flesh, EC_{50} of 827 mg/L and 586 mg/L, and high TPC of 90 mg GAE/g extract and 116 mg GAE/g extract with water and ethanolic extracts, respectively. This fruit is indeed of high value for human kind.

In view of the highest amount of antioxidants contained in full size unripe fruits, the current practice of taking the fully ripe fruits from trees should be reviewed. Instead, full size unripe fruits should be harvested for medicinal product development. The unripe and half ripe fruits were also observed to have no fruit rot or damage while most fully ripe fruits exhibited at least small part of fruit borer damage (Ahmed Asrity, 2016). Moreover, fully ripe fruits could only last three to four days before they are shed from trees. Full size unripe fruits, on the other hand, are available for about two weeks before they gradually turn half ripe.

Conclusion

Phaleria macrocarpa fruit flesh has low acidity and soluble solid content. Fruit acidity decreases but soluble solids increase as the fruit ripens. It has great amount of antioxidants and high percentage of the antioxidants is water soluble. The highest antioxidant content is found in the unripe fruit flesh; it has AA content of 16.61 mg/100 g fruit flesh, EC_{50} of 718 mg/L and 574 mg/L, and TPC of 99 mg GAE/g and 125 mg GAE/g with water and ethanolic extracts of fruit flesh, respectively. In contrast, fully ripe fruit flesh has the least antioxidant content. The findings obtained in this study suggest that fruits should be harvested at full size unripe stage, instead of fully ripe stage as currently practiced among farmers. The data are also hoped to stimulate more detailed research and safety assessment to formulate higher quality drinks and pharmaceutical products with the fruits of this medicinal plant.

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