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Antioxidant analysis of different parts of *Carica papaya*

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Abstract

This study was conducted to compare the total antioxidant activity (TAA), total phenolic content (TPC) and total flavonoid content (TFC) from the different parts of papaya tree including their ripe and unripe fruit, seeds and the young leaves. Two methods namely DPPH radical scavenging activity and β-carotene bleaching assay were used to determine the TAA, whereas TPC was determined by Folin-Ciocalteu's method while TFC by aluminium trichloride ($AlCl_3$). For these purposes, methanolic extracts (80%) were prepared. The results showed that the highest antioxidant activity through β-carotene bleaching assay was observed in unripe fruit (90.67 ± 0.29%) followed by young leave, ripe fruit and the seed. In other hand, young leaves exhibited a significant higher scavenging effect compared to others and the dose required in reducing the absorbance of DPPH control solution by 50% (EC_{50}) was calculated at 1.0 ± 0.08mg/ml. The EC_{50} values were 4.3 ± 0.01mg/ml, 6.5 ± 0.01mg/ml and 7.8 ± 0.06mg/ml for unripe fruit, ripe fruit and seeds respectively. Interestingly, both TPC and TFC also showed that young leaves had the highest antioxidant content (424.89 ± 0.22mg GAE/ 100 g dry weight and 333.14 ± 1.03mg rutin equivalent/ 100 g dry weight, respectively). Statistically, Pearson correlation showed there were positive correlations between TPC and TFC with antioxidant activity assayed by DPPH radical scavenging assay (r=0.846 and r=0.873, respectively). However there was no correlation between TPC and TFC with β-carotene bleaching activity. In brief, taken into account all the parameters measured, antioxidants were highly remarkable in the sequence of young leaves > unripe fruit > ripe fruit > seed. Nevertheless, further investigation for isolation and identification of the phytoconstituents responsible for antioxidant activity is desirable.

Introduction

Carica papaya (*C. papaya*) belongs to the family of *Caricaceae*, and several species of *Caricaceae* have been used as medication against a variety of diseases (Mello *et al*., 2008). It was originally derived from the southern part of Mexico, *C. papaya* is a constant plant and it is presently distributed over the whole tropical area. All parts of the papaya plant can be used as medicine; the fruit flesh, flowers, seeds and the flowers. Many scientific investigations have been conducted to evaluate the biological activities of various part of *C. papaya* including their fruits, shoots, leaves, rinds, seeds, roots or latex.

The major groups of phytochemicals that have been suggested as a natural source of antioxidants may contribute to the total antioxidant activity of plant materials including polyphenols, carotenoid and traditional antioxidant vitamins such as vitamin C and E. Antioxidant is any substance that when present at low concentration compared to those of an oxidisable substrate significantly delays or prevents oxidation of that substrate (Halliwell *et al*., 1995). Antioxidant functions are associated with decreased DNA damage, diminished lipid peroxidation, maintained immune function and inhibited malignant transformation of cells (Gropper *et al*., 2009). Several studies showed that phenolic compounds are the major bioactive phytochemicals with human health benefits (Cao *et al*., 1996). In fact, many authors have reported a direct relationship between total phenolic content and antioxidant activity in numerous seeds, fruits and vegetables (Yang *et al*., 2009).

These present findings can contribute to the increasing database for the medicinal plant or could be used as antioxidant in food and medicinal preparations. Thus, the aim of the study was to determine the total antioxidant activity (TAA), total phenolic content (TPC) and total flavonoid content (TFC) from the different parts of papaya tree including their ripe and unripe fruit, seeds and the young leaves. Two methods namely DPPH radical scavenging

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activity and β-carotene bleaching assay were used to determine the antioxidant activity to evaluate the relationship with the TPC and TFC. For these purposes, methanolic extracts (80%) were prepared and TPC were determined by Folin-Ciocalteu's method while TFC by aluminium trichloride ($AlCl_3$) method.

Materials and Method

Plant materials

Samples of the ripe, unripe, seeds, and the young leaves were obtained from Taman Pertanian Universiti Putra Malaysia (UPM), Serdang, Selangor, Malaysia.

Chemicals

β-carotene, linoleic acid, Tween 20, α-tocopherol, gallic acid, 2,2-diphenyl-2-picrylhydrazyl (DPPH) and rutin were purchased from Sigma chemical Co. (St. Louis, MO, USA). Folin-Ciocalteu reagent, sodium bicarbonate, aluminum chloride and methanol were purchased from Merck (Darmstadt, Germany), ascorbic acid (Fluka, Switzerland) and chloroform was from Fisher Scientific (Loughborough, UK).

Preparation of sample extracts

Before analysis, each of the samples was immediately washed several times with tap water, followed by rinsed it with deionized water to ensure that all contaminants were removed. The samples were then individually prepared where the edible portion (ripe and unripe) were all diced or cut into small pieces prior to packing and stored at -80°C for three consecutive days. Subsequently they were lyophilised in a freeze-dryer (Virtis route, Gardiner, New York). The lyophilized samples were ground to a fine powder and packed in air tight containers before stored in -20°C until required for further analyses.

The ground samples were extracted with 80% aqueous methanol (w/v, 1:25) at 200 rpm for 2 hour at ambient temperature with continuous stirring in a dark bottle using an orbital shaker (Heidolph Unimax 1010, Shcwabach, Germany). The mixture was filtered through a filter paper (Whatman No. 4). The obtained solutions were then used for TAA and TFC content.

Determination of antioxidant activity

β-carotene bleaching assay

The antioxidant capacity of each of the sample extracts was estimated by the β-carotene bleaching method following the procedure described by Velioglu *et al*. (1998) with modifications. One milliliter of β-carotene (0.2mg/ml chloroform), linoleic acid (0.02 ml) and Tween 20 (0.2 ml) were added to 0.2ml of sample extracts, standard (α-tocopherol) and control (80% methanol). Thereafter, chloroform was evaporated to dryness under vacuum using rotary evaporator. After evaporation, 100 ml of deionized water was added into the mixture and shaken vigorously until emulsion was obtained. Two milliliters of aliquots of the emulsions were pipetted into the test tubes and immediately placed in water bath at 45°C for 2 hours. The absorbance was read at 20 min interval at 470 nm, using a UV-visible spectrophotometer (Secomam, Anthelie Advanced 5) at initial time (t=0). Degradation rate (*dr*) of the sample was calculated according to the first order kinetics as described by Al-Saikhan *et al*. (1995):

$$dr \text{ of sample} = (\ln [A_0/A_t]) / t$$

where: ln = natural log; A_o = initial absorbance at time 0; A_t = absorbance at 20 min of incubation; t = 120 min and *dr* = degradation rate. Antioxidant activity (AA) was expressed as percent of inhibition relative to the control by using the equation:

$$AA\% = ([dr \text{ control} - dr \text{ sample}] / dr \text{ control}) \times 100$$

Free radical scavenging assay

Effect of the sample extracts on DPPH radical was measured by using a slightly modified method previously described by Tang *et al*. (2002). Amount of 200 µl of the sample extract (0.62 – 4.96 mg/ml) or ascorbic acid (0.04 – 1.28 mg/ml) were added to 1 ml of 0.1 mM DPPH in 80% methanol. The mixture was shaken vigorously and left to stand in dark room for 30 min at room temperature. Absorbance of the solution was measured spectrophotometrically at 517 nm with deionized water as blank. The capability of sample to scavenge the DPPH radical was calculated according to the equation as follows:

$$\text{Scavenging effect (\%)} = 1 - \frac{\text{Absorbance of sample at 517nm}}{\text{Absorbance of control at 517nm}} \times 100$$

Total phenolic content

Total phenolic content was estimated according to the Folin Ciocalteu method following the modified procedure described by Singleton and Rossi (1965). Each freeze-dried sample (200 mg) was extracted with 2 ml of 80% methanol at room temperature for 2hours by using an orbital shaker at 200 rpm. The mixture was then centrifuged at 1000 rpm for 15 min. An aliquot (200 µl) of the supernatant was mixed with 1.5 ml of Folin-Ciocalteu reagent (previously diluted 10 fold with distilled water) and allow standing at room temperature. Following 5 min, 1.5 ml of 6% (w/v) sodium bicarbonate solution was added to

the mixture. Following 90 min, the absorbance was read spectrophotometrically at 725 nm. The standard calibration (0.01 – 0.05 mg/ml) curve of Gallic acid in 80% methanol curve was plotted. Results were expressed as small cap Gallic acid equivalents (GAE) in mg per 100 g sample extracts.

Total flavonoid content

Total flavonoid content was determined by the aluminum chloride colometric assay according to method described by Meda *et al*. (2005). Five milliliters of 2% aluminum trichloride ($AlCl_3$) in methanol was mixed with the same volume of the extract solution at the concentration of 0.4 mg/ml. Following 10 min, the absorbance was taken against a blank that consist of the same solution but without the $AlCl_3$ at 415 nm using UV-spectrophotometer. Total flavonoid content is expressed as mg of rutin equivalents/ 100 g of sample extract.

Statistical analysis

Results were expressed as mean ± standard deviation of three determinations. Independent T-test and one way analysis of variance (ANOVA) combined with Bonforroni's post-hoc comparison were used to determine the differences of means among the samples. Pearson correlation test was used to assess the correlation between TAA and TPC. A significant difference was considered at the level of $p<0.05$.

Results

Beta-carotene bleaching assay

The comparable β-carotene bleaching rates of the control, α-tocopherol (standard) and methanolic extracts of different part of papaya fruit are shown in Figure 1. The β-carotene bleaching method is one of the most frequently applied methods for determining the total antioxidant property of the extracts. In the β-carotene bleaching assay, linoleic acid produces hydroperoxides as free radicals during incubation at 50°C and attacks the β-carotene molecules that cause reduction in the absorbance at 470 nm. Beta-carotene in the systems undergoes rapid discoloration in the absence of antioxidant and vice versa in its presence. The presence of different antioxidants can delay the extent of β-carotene bleaching by neutralizing the linoleate free radical and other free radicals formed in the system (Jayaprakash *et al*., 2003). Thus, the degradation rate of β-carotene–linoleate depends on the antioxidant activity of the extracts.

The result showed the control had a substantial and rapid oxidation of β-carotene. Accordingly, the absorbance decreased rapidly in samples without antioxidant, while the sample extracts with the presence of antioxidant retained their color and also absorbance for a longer time.

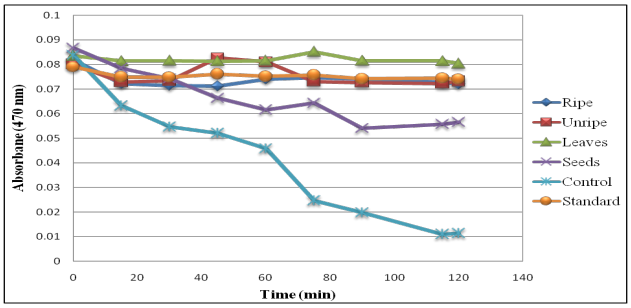

Figure 1. Degradation rate of different parts of papaya extracts assayed by β-carotene bleaching assay. Values are expressed as mean ± standard deviation (n=3). α-tocopherol was used as the standard

Table 1. Means of antioxidant activity of selected samples and standard assayed by β-carotene linoleate bleaching

Papaya plant	Antioxidant activity (%)
Ripe	88.12 ± 0.41 [b]
Unripe	90.67 ± 0.29 [b]
Seed	58.97 ± 1.08 [c]
Leaves	90.01 ± 0.44 [b]
Standard	96.73 ± 0.08 [a]

Values are expressed as mean ± standard deviation (n=3). Different letters indicate there are significant differences ($p>0.05$)

Table 1 shows the mean antioxidant activity based on the β-carotene bleaching rate of the extracts of different parts of the papaya plant (ripe, unripe, young leaves and seed). The extract with the lowest β-carotene degradation rate exhibit the highest antioxidant activity. All extracts had lower antioxidant activities than had standard (α-tocopherol). The highest antioxidant activity among the samples was observed in unripe fruit whereas seed had the lowest antioxidant activity. Result showed that there was considerably variation in the antioxidant activities where it ranges from the lowest of 58% to the highest of 91% where the orders of the antioxidant activity are as follow: α-tocopherol > unripe fruit > young leaves > ripe fruit > seed.

Reactive scavenging activity

The idea of a single measurement of total antioxidant capacity is insufficient. There is various antioxidant activity methods have been used to evaluate and compare the antioxidant activity of foods. Therefore, in this study, radical scavenging activity was determined for the selected parts of papaya plant. Being a stable free radical, the DPPH assay is a simple and rapid method frequently used to evaluate the ability of antioxidants to scavenge free radicals. It gives reliable information concerning the antioxidant ability of the tested compounds to act as free radical scavengers or hydrogen donors (Huang *et al*., 2005).

The odd electron in DPPH free radicals gives a strong absorption maximum at 517 nm (Azizah

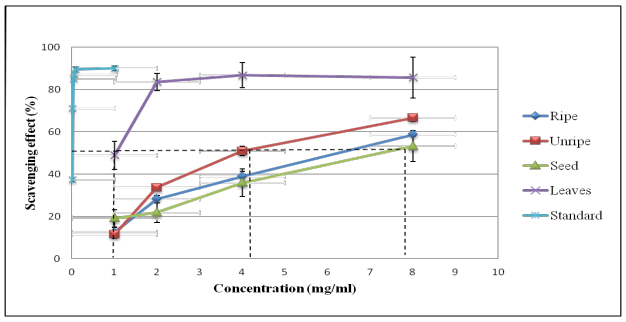

Figure 2. Scavenging effect of different parts of *C. papaya* extracts on DPPH radicals. Values are expressed as mean ± standard deviation (n=3). Ascorbic acid was used as the standard

Table 2. Means of EC_{50} of DPPH radical scavenging activities of selected samples

Papaya plant	EC_{50} (mg/ml)
Ripe	6.5 ± 0.01 ^{c}
Unripe	4.3 ± 0.01 ^{b}
Seed	1.0 ± 0.08 ^{a}
Leaves	7.8 ± 0.06 ^{d}

Values are expressed as mean ± standard deviation (n=3). Different letters in columns indicate there are significant differences (p>0.05)

Table 3. Means of total phenolic content (TPC) of selected samples

Papaya plant	(mg GAE/100g dry weight)
Ripe	272.66 ± 1.53 ^{c}
Unripe	339.91 ± 9.40 ^{b}
Seed	30.32 ± 6.90 ^{d}
Leaves	424.89 ± 0.22 ^{a}

Values are expressed as mean ± standard deviation (n=3). Different letters in columns indicate there are significant differences (p>0.05)

Table 4. Means of total flavonoid content (TFC) of selected samples

Papaya plant	(mg GAE/100g dry weight)
Ripe	92.95 ± 7.12 ^{a}
Unripe	53.44 ± 6.63 ^{b}
Seed	59.54 ± 12.23 ^{b}
Leaves	333.14 ± 11.02 ^{c}

Values are expressed as mean ± standard deviation (n=3). Different letters in columns indicate there are significant differences (p>0.05)

et al., 1999). When DPPH free radicals becomes paired with hydrogen from a free radical scavenging antioxidant, its purple color fades rapidly to yellow to form reduced DPPH-H (Yamagushi *et al*., 1998). The resulting decolorization is stoichiometric with respect to number of electrons captured.

There were reductions in the concentration of DPPH due to the scavenging activity of the antioxidant found in the sample extracts. At the concentration of 8mg/ml, the scavenging effects of methanol extract of selected parts of the papaya plant and standard decreased in the order: ascorbic acid > young leaves > unripe fruit > ripe fruit > seed (Figure 2). The young leaves exhibited a significant higher ($p<0.05$) scavenging effect compared to others and this was in agreement with Runnie *et al*. (2004), where the finding suggested that the methanolic leaves extract demonstrated vasodilatory and antioxidant effects, both implicated in the reduction of cardiovascular disease.

As shown in Figure 2, all the sample extracts exhibited significant dose dependent inhibition of DPPH activity that rapidly increase from 1 to 4mg/ml. Scavenging effect increases as the concentration of the sample increased until reached a plateau at 4 mg/ml. Table 2 shows the dose of young leaves extract that required in reducing the absorbance of DPPH control solution by 50% (EC_{50}) was calculated at 1.0 ± 0.08 mg/ml. The EC_{50} values were 6.5 ± 0.01 mg/ml, 4.3 ± 0.01 mg/ml and 7.8 ± 0.06 mg/ml for ripe fruit, unripe fruit and seeds respectively. This showed that the young leaves exhibit a strong scavenging activity and it has been reported that phytochemicals especially plant phenolics constitute a major group of compounds that act as primary antioxidant (Hatano *et al*., 1989). Their protection mechanisms are through the reaction with the oxygen radicals, superoxide anion radicals and lipid peroxyl radicals.

Total phenolic content and total flavonoid content

Phenolic compounds are widely distributed in plants (Li *et al*., 2006), which have gained greatly attention, due to their antioxidant activities and free radical-scavenging abilities, which potentially have beneficial implications for human health (Govindarajan *et al*., 2007). The TPC was determined in comparison with standard gallic acid and the results were expressed in terms of mg gallic aid equivalent (GAE)/ 100 g dry sample.

This study showed that the selected parts of the papaya plant varied significantly. It ranged from 30.32 ± 6.90 to 424.89 ± 0.22 mg GAE/ 100 g dry weight (Table 3). The TPC was observed in the selected papaya plant as: young leaves > unripe > ripe > seed. The result also indicates that the young leaves contained high phenolic content that may provide good sources of dietary antioxidant. For this reason, it is obvious that TPC present in the samples have strong effects against the scavenging activity rather than discoloration of β-carotene. However, Khamsah *et al*. (2006) found that the radicals scavenging activity is not only due to the phenolic content itself, but with other various antioxidant compounds. They respond differently depending on the number of phenolic groups that they have (Singleton and Rossi, 1965). More to the point, TPC does not incorporate necessarily to all the antioxidants that may present in the extracts. Therefore, sometimes there is a vague correlation between TPC and antioxidant activity of several plant species (Tawaha *et al*., 2007).

Other than that, TFC of the extracts in terms of rutin equivalent/ 100 g dry weight (standard curve

equation: y = 3.021x + 0.0831, R^2 = 0.9975) were between 53.44 ± 6.64 and 333.14 ± 1.03mg rutin equivalent/ 100 g dry weight as shown in Table 4. In recent years, studies have shown that papaya fruit contains not only vitamins and other nutrients but also contains biologically flavonoids (Wang *et al*., 2008).

Correlations

Previous study reported that antioxidant activity of plant material is very well correlated with the content of phenolic compounds (Velioglu *et al*., 1998). Contribution of phenolic compounds is one of the mechanisms of the overall antioxidant activities. This mainly due to their redox properties involve in the plant material. Generally, the mechanisms of phenolic compounds for antioxidant activity are neutralizing lipid free radicals and preventing decomposition of hydroperoxides into free radicals (Li *et al*., 2006).

Pearson correlation showed there was a positive correlation relationship between phenolic content and antioxidant activity assayed by DPPH radical scavenging assay (r=0.846). Conversely, no correlation was found for antioxidant activity assayed by β-carotene bleaching assay with phenolic content. This was in agreement with Motalleb *et al*. (2005), where they also did not find any relationship between antioxidant activity and phenolic content in *B. vulgaris* fruit extract.

The same pattern was observed in the relationship of TFC with the total antioxidant activity. A direct correlation between radical scavenging activity and TFC of the samples was successful to demonstrate by linear regression analysis (r=0.873). It is known that flavonoid with a certain structure and particularly hydroxyl position in the molecule can act as proton donating and show radical scavenging activity (Hou *et al*., 2003). However there was no correlation between total flavonoid content and β-carotene bleaching activity.

Conclusion

The study clearly indicates that it is vital to measure the antioxidant activity using various radicals and oxidation systems and to take both phenolic content and antioxidant activity into account while evaluating the antioxidant potential of plant extracts. The results obtained in this work have considerable value with respect to the antioxidant activities of the selected parts of the *C. papaya* plant. In brief, by taken into account all the parameters measured, antioxidants were highly remarkable in the sequence of young leaves > unripe fruit > ripe fruit > seed. Moreover, there is a strong positive correlation between TPC and TFC with free radical scavenging activity, thus showing its promising potential to be exploited as primary antioxidant. The correlations also support that the mechanism of action of the extracts for the antioxidant activity may be identical, being related with the content in phenols and flavonoid compounds, and their free-radical scavenging activity. Nevertheless, further investigation for isolation and identification of the phytoconstituents responsible for antioxidant activity is desirable.

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