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Identification of Pigment and its Antioxidant Activity of Several Species of Indonesian Dragon Fruit

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Dragon fruit has been known as source of betalain pigment. The aims of this study were to identify pigment compound through spectral and TLC analyses and to evaluate its antioxidant activity by DPPH IC_{50} and FRAP methods on peel and flesh extracts of four Indonesian dragon fruit species or varieties. The identified pigment in new variety of dragon fruit (the orange and yellow peels) was not reported yet. Dragon fruit extract pigments showed a good stability in pH range of 3-10. Spectral and TLC analyses showed that two dragon fruits varieties (the red peels) contained betalains and the other varieties (the orange and yellow peels) consisted anthocyanins. They were characterized by the spectra peaks ranged from λ_{max} 480-490 nm (betaxantin), 530-540 nm (betacyanin) and 580-582 nm (anthocyanin). TLC analysis yielded R_f values ranged from 0.43-0.52 with red-spot chromatogram identical to the betacyanin pigment, while R_f values ranged from 0.51-0.69 and 0.85 with orange, yellow and pink-spot chromatograms were identified as anthocyanins. Betaxantin in dragon fruit extract was in the range of 12.44-92.64 mg indicaxantin Eq/g and betacyanin ranged from 37.50-204.55 mg betanin Eq/g. Dragon fruit extracts had antioxidant activity by DPPH method with IC_{50} concentration ranged from 0.95-1.97 mg/g extract and could reduce Fe^{3+} at concentration of 0.02-0.05 mgAAE/g extract. The ratio of pigment (betaxanthin : betacyanin) values ranged from 0.33-0.53 indicated that the proportion of betacyanin was more dominant in influencing the antioxidant activity of dragon fruit extract while evaluated using correlation analysis with DPPH IC_{50} and FRAP antioxidant activity values.

Keywords: Anthocyanins, Antioxidant, Betalains, Dragon fruit, Pigment.

INTRODUCTION

Dragon fruit plant is a type of cactus vines that has fruit, both flesh and peel has a bright colour. There are several species of dragon fruit plants grown in Indonesia, namely red-peel white-flesh (*Hylocereus undatus*), red peel-red flesh (*Hylocereus polyrhizus*), red-peel super-red flesh (*Hylocereus costaricensis*), orange-peel pink-flesh and yellow-peel white-flesh (*Selenicereus megalanthus*) [1]. Currently, dragon fruit is consumed as fresh fruit or juice. In addition, the use of dragon fruit as food colouring ingredients such as butter, dairy products, dried noodles have been carried out [2-4]. Several studies have shown that dragon fruit is one of the sources of betalains pigment [5-7].

Betalain is a water-soluble pigment that gives colour to flowers and fruits. Betalain is divided into two groups namely betacyanin which responsible to purplish red colour and betaxanthin which responsible to yellow-orange colour [8]. By-products from vegetables and fruits such as peels and seeds have potential as an antioxidants. Several previous studies have shown that red-peel red-flesh dragon fruit is rich in polyphenols and antioxidants, and even the peel exhibits higher antioxidant activity than the flesh [9,10]. However, the peel that occupies 33 % of the fruit part is generally discarded [11]. Hence, the utilization of dragon fruit peels become very important, as they have positive benefits for health aspects.

Natural pigment is generally correlated with antioxidant properties as reported by Wu *et al.* [9] that the higher pigment content, the higher antioxidant activity. The literature on the identification and antioxidant activity of dragon fruit pigment extract is still limited to red dragon fruit peels. To the best of our knowledge, there have been no study on pigment contents and antioxidant activity of other dragon fruit species.

Therefore, the purpose of this study was to identify pigments and measure antioxidant activity and to evaluate the relationship between pigment content and antioxidant activity of peels and flesh of dragon fruit in some species. This studies may provide information on the identity and characteristics of functional food ingredients compounds.

EXPERIMENTAL

Four species of dragon fruits were studied, namely red-peel red-flesh (*Hylocereus polyrhizus*), red-peel white-flesh (*Hylocereus undatus*), yellow-peel white-flesh (*Selenicereus megalanthus*) and orange-peel pink-flesh. The dragon fruit was obtained from dragon fruit farm in Pasir Bentik village, Cibadak, Sukabumi, West Java province and Sabila *Farm*, Sleman, Special Region of Yogyakarta.

Preparation and sample extraction: Dragon fruit extraction was done based on Marpaung [12] and Harivaindaran *et al*. [5] method with slight modification. The extraction were begun with selection of a fresh and mature dragon fruit, green scales and smooth thorns were removed from dragon fruit peels. Dragon fruit then washed and peeled, the peels and flesh were separated. Dragon fruit peel and flesh were cut into 16 parts. Then, 10 g of peels or dragon fruit flesh were put into high density polyethylene plastic then blanched for 6 min at 100 ºC.

The flesh and the peel of the dragon fruit inside the plastic were immediately cooled under running water. Then were blended for 2 min at medium speed using distilled water at pH 5 with temperature 60 °C. Furthermore, the pulp of the flesh and the peel of the dragon fruit were centrifuged for 15 min at a rate of 3000 rpm at 4 °C and filtered using Whatman filter paper No.1. The pectin of the filtrate was then precipitated. Precipitation was done by dissolving 1 part of extract with 2 parts of ethanol 96 % (v/v) then centrifuged at 3000 rpm for 15 min at 4 °C. The supernatant obtained was evaporated using a rotary vacuum evaporator until the volume of the extract reached one quarter of the total volume before evaporated. The extract was centrifuged for 15 min at a rate of 12000 rpm at 4 °C. Then the extract was ready for analysis.

Measurement of maximum wavelength spectrum and absorbance of dragon fruit extracts: Determination of pH 1-14 solution was conducted based on the report of Marpaung *et al.* [12]. A total of 1 mL of extract was mixed with 9 mL of distilled water at pH 1-14. Colour spectrum measurements were performed using a UV-visible spectrophotometer (Hitachi U-2900, Japan) at a wavelength of 350-700 nm.

Identification of pigment compounds using thin layer chromatography: The separation of pigments in dragon fruit extracts was done by using preparative thin layer chromatography based on the method of Lotfi *et al*. [13] with modifications. The activated silica gel G 60 F_{254} powder was heated for 3 h at 105 °C, then mixed with two parts of the volume of distilled water until the texture was slurry. The silica gel slurry was poured immediately over the surface of the 20 cm × 20 cm glass plate used a Heidelberg Despise spreader tool with a thickness of 0.5 mm until the surface became flat. The silica gel plate was dried in the oven at 105 °C. The TLC plate was done and ready to use for fractionation process.

TLC eluent was prepared by mixing methanol and acetic acid with a ratio of 60:40 (v/v). Eluent was conditioned into the TLC chamber until it reached 0.5 cm high, then the chamber was kept in closed condition for 1 h until the chamber saturated by the eluen. The following TLC plate conditions were used:

- Lower edge: 2 cm from lower side
- Upper edge: 1 cm from upper side
- Tack distance: 15 cm
- Distance between sample: 2 cm
- Total sample apllied: 6

The extract was taken with a capillary pipe up to ± 2 cm, then spotted on the TLC plate until the extract was perfectly absorbed and the surface of the plate has dried. The TLC plate was then inserted into the chamber for the elution process. The elution process was stopped when the eluent has reached the upper edge of the plate. Then the plate was removed quickly and dried. Plates were observed under UV light with wavelengths of 254 and 366 nm (short and long waves). Separation results were formed, in the form of spots and irregular areas marked by a pencil. Then the R_f value was calculated by the formula:

$$R_f = \frac{\text{Distance traveled by solute (cm)}}{\text{Distance traveled by solvent (cm)}}$$

To obtain a pigment separation result, the spots formed and marked were then scraped with a spatula and fed into the test tube. The sample chromatogram powder was dissolved using pure ethanol and centrifuged for 15 min at a rate of 3000 rpm at 4 °C. The supernatant obtained was blown with N_2 to dry, then the fractions were weighted.

Determination of pigment betalain content: The measurement of pigment content was performed based on Cai *et al.* [14] and Cejudo-Bastante *et al*. [11] methods with slight modifications. Absorbance of peel and flesh of dragon fruit extract was measured used UV-visible spectrophotometer at 480 nm (λ_{max} betaxantin) and 538 nm (λ_{max} betacyanin) wavelengths. The pigment content was determined by the following equation:

$$\text{Pigment content (mg/g)} = \frac{A \times MW \times D \times V \times 1000}{(\varepsilon \times L \times W)}$$

$$\text{Pigment ratio} = \frac{\text{Betaxanthin content}}{\text{Betacyanin content}}$$

where, A = absorbance of samples at each wavelength; MW = molecular weight of pigment (betanin = 550 g/mol; indicaxantin = 308 g/mol) [6,15]; D = dilution factor; V = final volume of the extract (mL); ε = molar extinction coefficient of betanin (60.000 L M^{-1} cm^{-1}) and indicaxantin (48.000 L M^{-1} cm^{-1}); L = path length (1 cm); W = dried weight of the sample (g).

Analysis of antioxidant activity by DPPH IC_{50} method: Antioxidant analysis was performed using DPPH IC_{50} method based on Wu *et al*. [16] with slight modifications. Five series of concentrations of peel and flesh of dragon fruit extracts were prepared, concentration range for peel extracts between 0.5-3.0 mg/mL and flesh extracts between 2.0-7.0 mg/mL. A 1.5 mL of sample solution was poured into the test tube and then 1.5 mL of 0.15 mM DPPH reagent in 95% ethanol was added

then homogenized using vortex. The solution was incubated at ambient temperature inside the dark room for 30 min. The solution was measured immediately with UV-visible spectrophotometer at wavelength of 517 nm. As a control, the sample solution was replaced with distilled water and mixed with DPPH reagent, subsequently treated the same as sample solution. Percentage of free radical inhibition activity was calculated by the formula:

$$\text{Inhibition } (\%) = \frac{(A - B)}{A} \times 100$$

where, A = Absorbance of control solution; B = Absorbance of sample solution

The percentage of inhibition obtained was then plotted into a linear regression curve, with the equation y = ax + b. Where the x axis was the concentration and the y axis was the percent of inhibition. The value of IC_{50} (inhibition concentration 50) was obtained by entering the number 50 into the equation, as y.

Analysis of antioxidant activity by ferric reducing antioxidant power (FRAP): Antioxidant analysis was performed using FRAP method from Vitchitphan *et al*. [17] with slight modifications. The FRAP reagent was prepared by mixing 10 volumes of acetate buffer pH 3.6 with a concentration of 1.0 mol/L, added 1 volume of 10 mmol/L of TPTZ (2,4,6-tripyridyl-S-triazine) in 40 mmol/L HCl, and 1 volume 20 mmol/L $FeCl_3^+$. A 200 µL sample solution and 2 mL of FRAP reagent was mixed, then was incubated for 30 min at 37 °C. Furthermore, the absorbance was measured at a wavelength of 593 nm. Ascorbic acid was used as standard with a concentration range of 0.001-0.035 mg/mL. The antioxidant effectiveness was calculated referred to the regression equation of the ascorbic acid standard curve based on known concentrations. The FRAP results were expressed in milligrams equivalent to ascorbic acid per gram (mgAAE/g extract).

Statistical analysis: Complete randomized design with four replicates in duplicate (except on spectrum measurement and pigment identification using TLC performed twice) was used as research design. Data of spectral observation and pigment identification using TLC were presented descriptively. One way analysis of variance was conducted to see the difference in antioxidant activity in each group of peel and flesh of dragon fruit extracts. Duncan Multiple Range Test (DMRT) with α = 5 % by SPSS 22.0 software was used to identify difference between sample. The data of parameters such as mean and standard deviation, and correlation regression analysis was calculated with Microsoft Excel 2010 software.

RESULTS AND DISCUSSION

Identification of dragon fruit pigment compounds: The result of spectral observation (Fig. 1) can be used as one of the predictors of pigment on dragon fruit extract. The shape of the spectrum of dragon fruit extract in Fig. 1(a) showed the

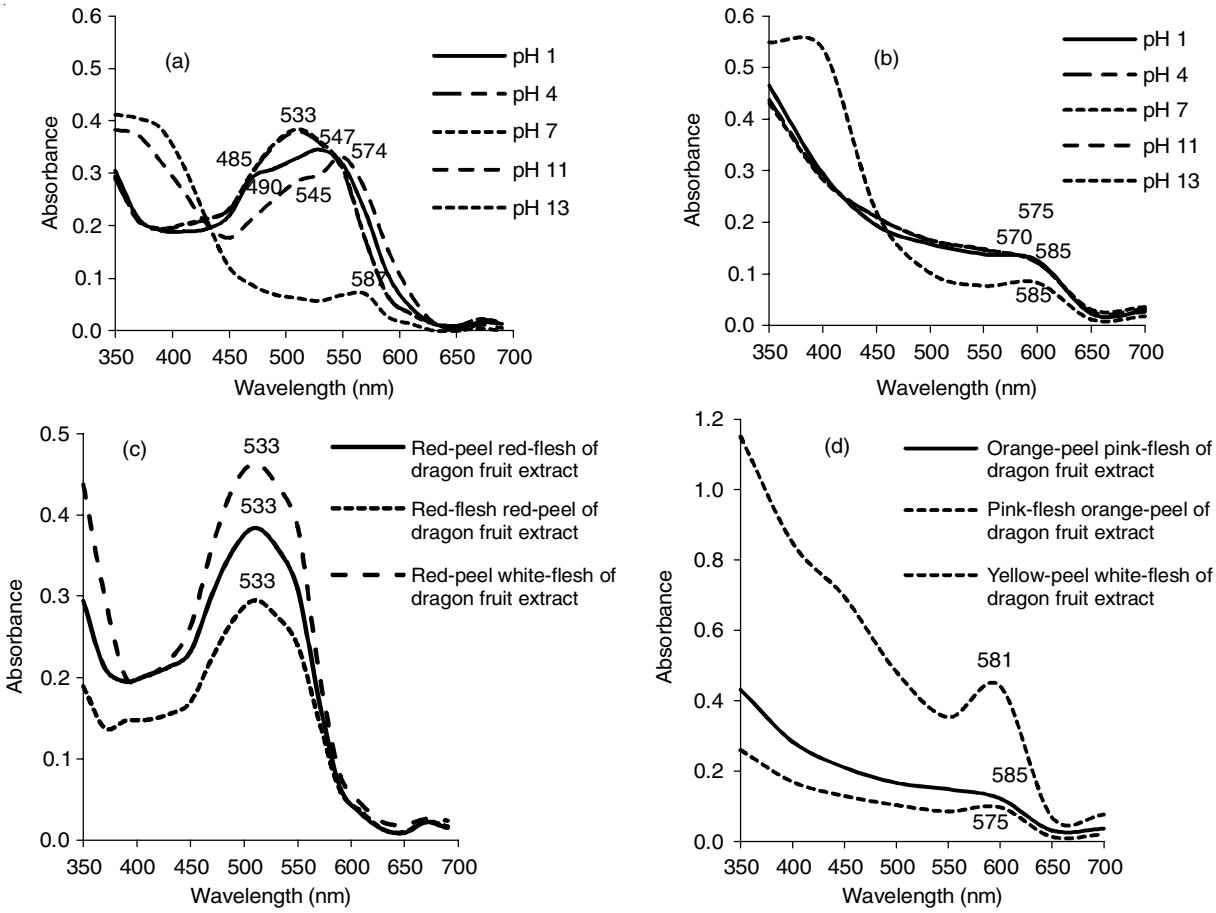

Fig. 1. Spectrum of dragon fruit extracts at various pH; (a) spectrum of red-peel red-flesh extract; (b) spectrum of orange-peel pink-flesh extract; (c) spectrum of red-peel red-flesh extract, red-peel white-peel extract and red-flesh red-peel extract at pH 7; (d) spectrum of orange-peel pink-flesh extract, yellow-peel white-flesh extract and pink-flesh orange-peel extract at pH 7

presence of two peaks in the maximum wavelength range (λ_{max} nm) 547 nm (pH 1), 533 nm (pH 4 and 7), 574 nm (pH 11), 587 nm (pH 13), and appears on the spectrum with λ_{max} 490 nm (pH 1), 485 nm (pH 4 and 7), 545 nm (pH 11). The spectrum pattern of dragon fruit extract is identical to the spectral pattern generated by red turnip extract which contains betacyanin pigment with λ_{max} 536 nm and betaxantin with λ_{max} 484 nm [18].

Shifting spectral peaks of dragon fruit extract found in pH 1 and 11, whereas the spectral pattern changes occurred at pH 13. pH 4 and 7 represents the spectrum on the pH range of 3-10 because it has the same spectrum pattern and there is no shift in λ_{max}. It indicated the occurrence of degradation by acidic conditions under pH 3 and bases above pH 10 [19]. Furthermore, the spectrum results [Fig. 1(b)] shown peak formation at λ_{max} 575 nm (pH 1), 570 nm (pH 4 and 7), 585 (pH 11 and 13). This was similar to the shape of the spectrum of purple sweet potato that has been identified contains anthocyanin pigment and has a peak at λ_{max} 610 nm [20]. Hence, it can be expected that the orange-peel pink-flesh extract contains anthocyanin. The spectral observations were also carried out at pH 7 (neutral) conditions and showed that in each extract, both in Fig. 1(c) and 1(d) did not change the spectrum pattern, only differed in the absorbance level.

Based on the results of pigment separation using TLC, some spot colour chromatogram with various R_f values was obtained. The pigment identification was obtained based on similarity between R_f value of sample with literature (Table-1). The existence of pigment betacyanin allegedly found in red-peel red-flesh extract, red-flesh red-peel extract as well as red-peel white-flesh extract. These refers to the results of Suganyadevi *et al*. [21] and Viloria-Matos *et al*. [22] that the main fraction with red colour and has R_f value in the range of 0.43-0.52 was betacyanin pigment. While the yellow fraction of red-peel red-flesh and red-peel white-flesh extracts, the pigment fractions have not been identified [22] but suspected as betaxanthin pigment referring to the results of spectral form observations. Different fraction have been shown by the extract of orange-peel pink-flesh and pink flesh orange peel. Both have been suspected to contain anthocyanin pigment because it has an orange, yellow and pink colour with an R_f value close to 0.32-0.62 and 0.93 [23,24]. Meanwhile, the pigment fractions of yellow-peel white-flesh extract have not been identified further because of unavailable information, but allegedly has anthocyanin pigment based on identification in the form of spectrum extract.

TABLE-1
IDENTIFICATION OF DRAGON FRUIT PIGMENT COMPOUNDS WITH TLC

Dragon fruit extract	Spot color chromatogram	R_f	Pigment identification	Ref.
Red-peel red-flesh	Purplish red	0.45	Betacyanin	[21]
	Yellow	0.76	Unknown	[22]
Red-flesh red-peel	Purplish red	0.43	Betacyanin	[21] [22]
Red-peel white-flesh	Purplish red	0.52	Betacyanin	[21]
	Yellow	0.79	Unknown	[22]
Orange-peel pink-flesh	Orange	0.51	Anthocyanins	[24]
	Yellow	0.69	Anthocyanins	[24]
Pink-flesh orange-peel	Pink	0.85	Anthocyanins	[23,24]
Yellow-peel white-flesh	Yellow	0.57	Unknown	*

*Literature not available

Betalain content of dragon fruit extracts: Table-2 demonstrated the results of betaxanthin (BX) and betacyanin (BC) pigments in dragon fruit extracts. Levels of betaxanthin pigment in two dragon fruit species are in the range 12.44-92.64 mg indicaxantin/g. Associated levels of betacyanin pigment in the present study were in the range of 37,50-204,55 mg betanin Eq/g. This difference in pigment content is influenced by the type, condition and level of maturity during harvesting [25]. In addition, pigment ratio data showed that the value of pigment ratio is below 1. This is illustrated if the proportion of both pigments is not equal and more dominated by the proportion of betacyanin. Further pigment ratio data was used to see its association with antioxidant activity.

TABLE-2
CONTENT AND RATIO OF BETAXANTHIN-BETACYANIN PIGMENTS OF DRAGON FRUIT EXTRACT

Dragon fruit	Pigment content (mg/g)		Pigment ratio (BX:BC)
	Betaxanthin (BX)	Betacyanin (BC)	
Red-peel red-flesh	92.64 ± 2.16	173.33 ± 0.93	0.53
Red-flesh red-peel	12.44 ± 0.51	37.50 ± 1.80	0.33
Red-peel white-flesh	89.05 ± 1.39	204.55 ± 0.71	0.44
White-flesh red-peel	*	*	*

*not be measured

Antioxidant activity of dragon fruit extracts: Based on Fig. 2(a), the highest antioxidant activity in the dragon fruit peel extract is produced by the extract of red-peel white-flesh with significant difference compared to the other three peel extracts. The inhibitory value of IC_{50} radical of DPPH in dragon fruit peel extracts was observed to be in the range of 0.95-1.97 mg/g extract. Significant results were also obtained in the dragon fruit flesh extract, where the highest antioxidant activity was obtained from red-flesh red-peel extract. The inhibitory value of IC_{50} radical of DPPH in dragon fruit flesh extract was observed to be in the IC_{50} value range of 3.77 to 11.83 mg/g extract. Further analysis of antioxidant activity of dragon fruit peel extract by FRAP method in Fig. 2(b), showed that the extract of red-peel white-flesh had the highest FRAP value of 0.05 mgAAE/g extract and showed a significant difference compared to the three peel extracts of dragon fruit. Furthermore, red-flesh red-peel dragon fruit extract has the highest FRAP value of 0.04 mg/g extract and significantly different than the other three flesh extracts of dragon fruit. The two methods of antioxidant activity analysis showed that dragon fruit peel extract has higher antioxidant activity than dragon fruit flesh extract.

Referring to the results of an analysis of the antioxidant activity of DPPH IC_{50} (Fig. 2a) and FRAP (Fig. 2b) it can be seen that two red dragon fruit species (red and white flesh) have higher antioxidant activity when compared to two other species of orange and yellow peel dragon fuit. This is related to the identification of the four species of dragon fruit. Predictably, if betalains pigment (betacyanin and betaxanthin) have

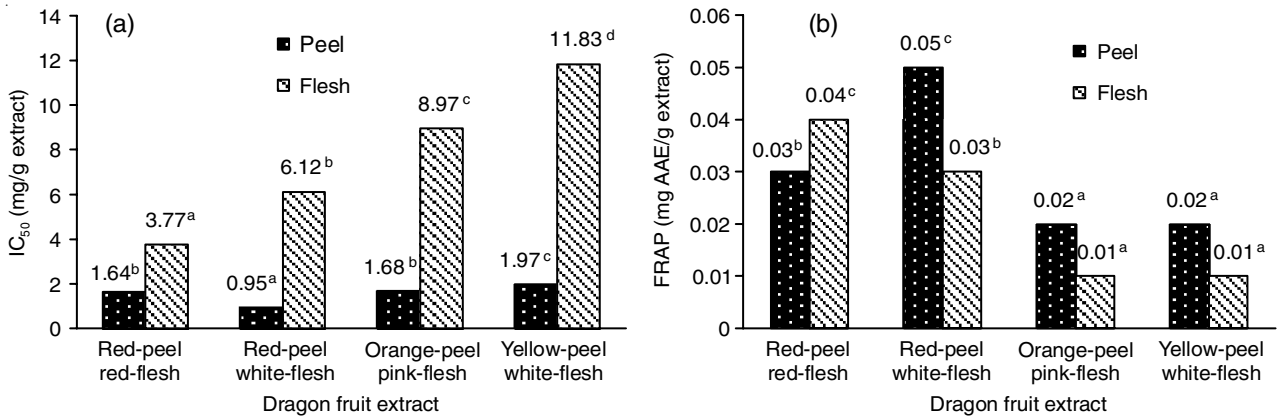

Fig. 2. Antioxidant activity of dragon fruit extracts; (a) IC_{50} values of dragon fruit extracts; (b) FRAP values of dragon fruit extracts; The numbers on the same pattern followed by the same letter were not significantly different at the 5 % test level

higher antioxidant activity than anthocyanin in four dragon fruit species.

From the results of correlation regression analysis on both antioxidant activity analysis methods have obtained r value of -0.62, the value can be indicated if both methods of antioxidant analysis have moderate correlation level and inversely (Fig. 3a) [26]. This relationship showed that if IC_{50} value is lower then FRAP value is higher. A high correlation value is generated both in betacyanin (Fig. 3b) and betaxanthin pigments with DPPH IC_{50} (graphs not shown), with r values of -1 and -0.96, respectively. This is reinforced by the result of correlation regression analysis on graph (Fig. 3c) which has shown that there was a strong correlation between the value of DPPH IC_{50} with pigment ratio having r value of -0.73. In other words, the pigment level affected DPPH radical scavenger activity, because if the pigment level was higher then the value of DPPH IC_{50} was lower and antioxidant activity increased. On the other side, correlation regression analysis between each pigment (betacyanin and betaxanthin) with FRAP value showed that each pigment has no correlation with FRAP value (data not shown). However, correlation regression analysis between pigment ratio with FRAP value (Fig. 3d) showed that the pigment ratio has a negative strong correlation with FRAP value (r = -0.74). Based on this studies, pigment is one important factor that affected the antioxidant activity.

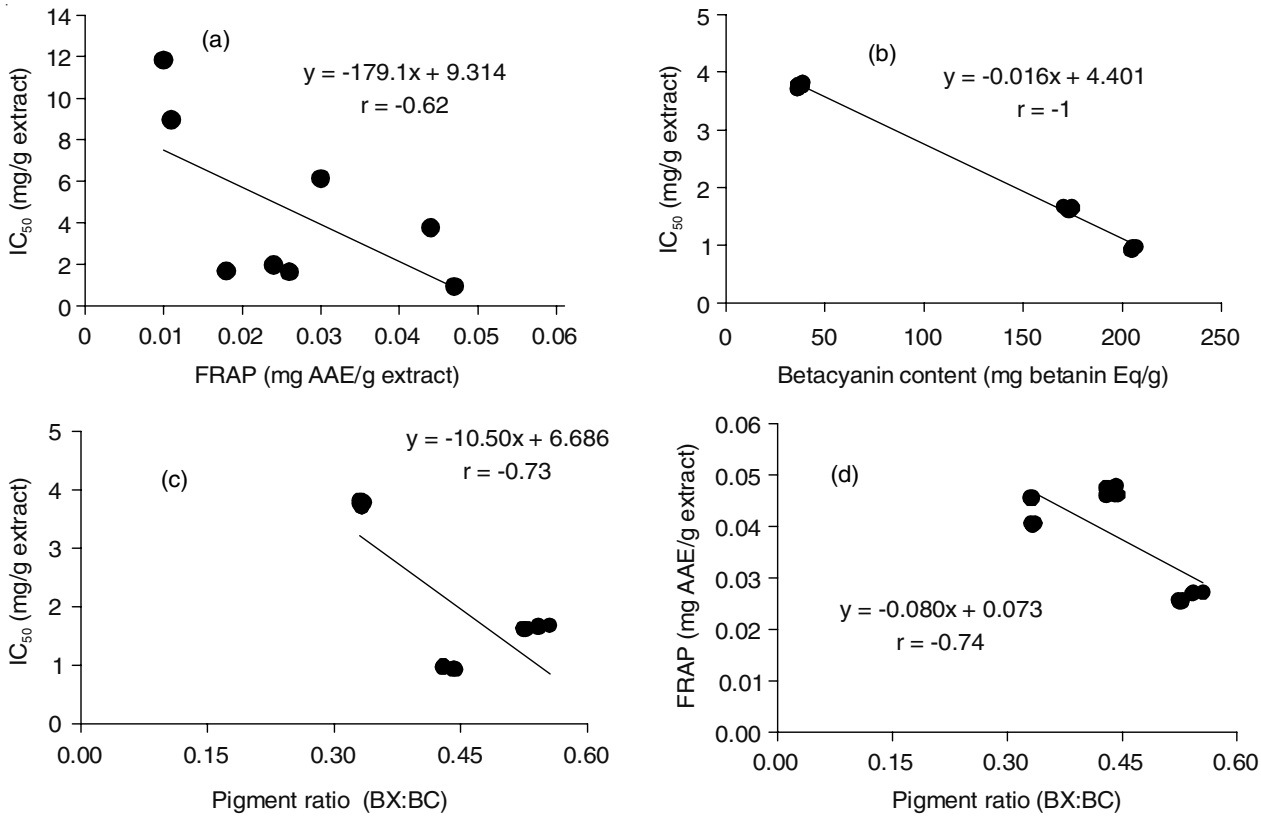

Fig. 3. Correlation graph between antioxidant activity analysis method with pigments; (a) correlation graph between IC_{50} values and FRAP values; (b) correlation graph between IC_{50} values and betacyanin pigment content; (c) correlation graph between IC_{50} values and pigment ratio; (d) correlation graph between FRAP values and pigment ratio

Conclusion

Based on identification using spectrum and TLC, two species of dragon fruits (red peels) have been suspected containing betacyanin and betaxanthin pigments, whereas other two species (orange and yellow peels) containing anthocyanin pigments. The highest levels of betacyanin and betaxanthin were observed in red-peel white-flesh extracts and red-peel red-flesh extracts. The highest antioxidant activity in the dragon fruit peels was obtained from the extract of red-peel white-flesh dragon fruit, while in the dragon fruit flesh was produced by red-flesh red-peel extract. There has been a correlation between the levels of betalain pigments (betacyanin and betaxanthin) with the antioxidant activity of DPPH IC_{50} method. But there has no correlation with FRAP antioxidant activity. There has been a correlation between pigment ratio with antioxidant activity of DPPH IC_{50} method and FRAP method.

The identification of dragon fruit pigments using complete instrument facilities should be studied extensively to reveal the identity and structure of the pigment. Determination of anthocyanin pigment levels is also needed to confirm its association with antioxidant activity in two species of dragon fruit (orange and yellow peels) which was not performed in this study.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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