ORIGINAL ARTICLE

Antioxidant capacity and phenolic content of holy basil

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Abstract

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The antioxidant capacity of white and red holy basil leaves (*Ocimum sanctum* Linn., white and red varieties) from fresh markets in Chiang Mai was estimated by three different methods; ferric reducing antioxidant power (FRAP) assay, improved ABTS radical cation decolorization assay, and DPPH free radical scavenging activity; together with their total phenolic contents. Water and 95% ethanol ratios of extraction solvents were also studied, and it was found that 57 and 76% ethanol solvents were more suitable in this study. All three antioxidant capacity assays showed that red holy basil was higher in antioxidant capacity than white holy basil ($p<0.05$). ABTS values were higher than FRAP and DPPH values, respectively, because of their different mechanisms, but their significant correlation ($p<0.01$) expressed their similar trends. In addition, correlation between results of all antioxidant capacity and total phenolic content was found ($p<0.01$). ABTS values were highly correlated into the results of total phenolic content than were FRAP and DPPH values.

Key words : holy basil, antioxidant capacity, phenolic content

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ความสามารถในการเป็นสารต้านออกซิเดชันและปริมาณสารประกอบฟีนอลของกะเพรา

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ความสามารถในการเป็นสารต้านออกซิเดชันของใบกะเพราขาวและกะเพราแดง (*Ocimum sanctum* Linn., white and red varieties) ที่วางจำหน่ายในตลาดสดในเขตจังหวัดเชียงใหม่ถูกนำมาศึกษาโดยใช้วิธีการที่แตกต่างกัน 3 วิธี คือ Ferric reducing antioxidant power (FRAP) assay, Improved ABTS radical cation decolorization assay และ DPPH free radical scavenging activity ร่วมกับการวิเคราะห์ปริมาณสารประกอบฟีนอลทั้งหมดโดยใช้สารละลายผสมระหว่างน้ำและเอทานอล (95%) ในอัตราส่วนที่แตกต่างกันเป็นตัวทำละลายในการสกัด ผลการทดลองพบว่าสารละลายเอทานอล 57 และ 76% เป็นตัวทำละลายที่เหมาะสมในการสกัด ผลการวิเคราะห์ความสามารถในการเป็นสารต้านออกซิเดชันทั้ง 3 วิธี พบว่า กะเพราแดงมีความสามารถในการต้านออกซิเดชันมากกว่ากะเพราขาว ($p<0.05$) และค่าความสามารถในการเป็นสารต้านออกซิเดชันที่วิเคราะห์ด้วยวิธี ABTS มีค่ามากกว่าการวิเคราะห์ด้วยวิธี FRAP และ DPPH ตามลำดับ เนื่องจากความแตกต่างของปฏิกิริยาที่ใช้ในการวิเคราะห์ แต่ยังคงพบความสัมพันธ์ระหว่างผลการวิเคราะห์ของทั้งสามวิธี ($p<0.01$) แสดงว่าผลของทั้งสามวิธีมีแนวโน้มไปในทางเดียวกัน นอกจากนี้ยังพบความสัมพันธ์ระหว่างความสามารถในการเป็นสารต้านออกซิเดชันและปริมาณสารประกอบฟีนอลทั้งหมด ($p<0.01$) โดยค่าที่วิเคราะห์ด้วยวิธี ABTS มีความสัมพันธ์กับผลการวิเคราะห์ปริมาณสารประกอบฟีนอลทั้งหมดมากกว่าการวิเคราะห์ด้วยวิธี FRAP และ DPPH ตามลำดับ

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Holy basil, sacred basil, or Krapao (*Ocimum sanctum* Linn. or *Ocimum tenuiflorum* Linn.) is a tropical, much branched, annual herb, up to 18 inches tall. There are two kinds of holy basil: the more exuberantly flavored red holy basil (red or purple variety) has dark green leaves with reddish purple stems and a purplish cast on the younger leaves, while the milder white (white or green variety) has medium-green leaves with very light green, almost white, stems. In Thailand, this basil is used in simple stir-fries, and together with garlic, fresh chilies and fish sauce. It imparts a wonderful flavor to any meat or seafood, and this dish is named "Pad Krapao", a very easy and popular Thai recipe. Holy basil has a strong anise-like, slightly musky and lemony taste with a camphoraceous aroma. The dominant aroma component in holy basil is eugenol. This herb also has been used by Asians in traditional medicine. It is used for most stomach disorders, cramps, diarrhea, headaches, whooping cough and head colds (Uhl, 2000).

In order to establish the therapeutic uses of holy basil in modern medicine, in the last few decades several Indian scientists and researchers have studied the pharmacological effects of steam distilled, petroleum ether and benzene extracts of various parts of this plant and eugenol on the immune system, reproductive system, central nervous system, cardiovascular system, gastric system, urinary system and blood biochemistry and have described the therapeutic significance of holy basil in the management of various ailments. These pharmacological studies have established a scientific basis for therapeutic uses of holy basil (Prakash and Gupta, 2005).

Antioxidant activity of components in holy basil is one of major causes of its pharmacological actions. Phenolic compounds in holy basil extracts including eugenol, cirsilineol, isothymusin, isothymonin, rosmarinic acid (Kelm *et al.*, 2000), orientin, and vicenin (Vrinda and Uma Devi, 2001) have been proved as good antioxidant compounds and zinc, an antioxidant mineral, has been found

to be significantly high in holy basil (Samudralwar and Garg, 1996). However, there is a great deal of diversity in the composition of holy basil cultivated in different localities (Kicel *et al.*, 2005).

The antioxidant capacity of holy basil was reported by Juliani and Simon (2002). It was 297 μmol Trolox equivalent per gram of dry weight for Trolox equivalent antioxidant activity (TEAC) with ABTS assay, and 420 μmol ascorbic acid equivalent per gram of dry weight for ferric reducing antioxidant power (FRAP) assay. They also reported total phenolic content of holy basil was equal to 51.1 mg gallic acid equivalent per gram dry weight. Juntachote and Berghofer (2005) showed that ethanolic extracts of holy basil showed good heat stability (80°C, 1 h), and had high antioxidative stability at neutral and acidic pH, exhibited strong superoxide anion scavenging, Fe^{2+} chelating activity and reducing power, and also acted as lipoxygenase inhibitors.

Main objectives of this study were to estimate the antioxidant capacity of white and red holy basil available from fresh markets in Chiang Mai by three different methods including FRAP assay, improved ABTS radical cation decolorization assay, and DPPH free radical scavenging activity. Effects of water-ethanol ratios of extraction solvents were studied, and total phenolic contents of extracts were also estimated.

Materials and Methods

1. Chemicals

Trolox (6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid) [Aldrich], TPTZ (2,4,6-tripyridyl-s-triazine), DPPH (2,2-diphenyl-1-picrylhydrazyl) [Sigma], ABTS (2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)), Folin-Ciocalteu phenol reagent, ferric chloride, ferrous sulphate, gallic acid, glacial acetic acid, hydrochloric acid, sodium acetate, potassium persulphate, sodium carbonate, vitamin C [Fluka] were of analytical grade.

2. Sample extraction

White and red holy basil were harvested at 30-35 days after planting and then every 2 weeks for 7-8 months. Fresh white and red holy basil were bought from fresh markets in the morning. Leaves of white and red holy basil (85.9% and 86.4% moisture content, respectively) were blended using a blender. Two grams of each blended sample were transferred into a 25 cm x 150 cm tube, and 10 ml of a solvent was added. Deionized water and 95% ethanol in various ratios (100:0, 80:20, 60:40, 40:60, 20:80, and 0:100) were used to prepare six different solvents (0, 19, 38, 57, 76 and 95% ethanol). The extraction was done by using a vortex mixer for 60 s. The mixture was filtered by Whatman No 1 and the filtrate was used for FRAP, ABTS, DPPH, and total phenolic content assays (Modified from Leong and Shui, 2002). Spectronic 20D+ spectrophotometer of Milton Roy was used for all assays.

3. Ferric reducing antioxidant power (FRAP) assay

The FRAP, a method for measuring total reducing power of electron donating substances, was assessed according to Benzie and Strain (1999). Briefly, 6 ml of working FRAP reagent (0.1 M acetate buffer:0.02 M $FeCl_3$:0.01 M TPTZ = 10:1:1) prepared daily was mixed with 20 μl of extract sample. The absorbance at 593 nm was recorded after a 30-min incubation at 37°C. FRAP values were obtained by comparing with standard curves created by Trolox (0-35 μg) and vitamin C (0-15 μg), and reported as mg Trolox and vitamin C equivalent per gram of dry weight.

4. ABTS radical cation decolorization assay

The method, based on the ability of antioxidant molecules to quench the long-lived ABTS radical cation ($ABTS^+$), of Re *et al.* (1999) was modified. The $ABTS^+$ was produced by reacting 7 mM ABTS stock solution with 2.45 mM postassium persulphate (final concentration) and allowing the mixture to stand in the dark at room temperature for 12-16 hours before use. The $ABTS^+$ solution was diluted with deionized water and 95 % ethanol (1:1) to an absorbance of 0.70 (±0.02) at 734 nm. Twenty μl of the extract was

mixed with 6 ml of diluted $ABTS^+$ solution. The decrease of absorbance was recorded at 1, 5, 10, and 30 min after mixing. Trolox (0-30 μg) and vitamin C (0-20 μg) were used as standards, and results were reported as mg Trolox and vitamin C equivalent per gram of dry weight. In addition, the concentration of a selected extract was varied to find out the effective concentration at 50% inhibition (EC50).

5. DPPH free radical scavenging activity

The method of Brand-Williams *et al.* (1995), based on the reduction of DPPH radical solution in the presence of hydrogen donating antioxidants, was used with some modifications. 0.8 mM DPPH radical solution in 95% ethanol was prepared. Four hundred μl of the extract was diluted to 5.4 ml using deionized water and 95% ethanol (1:1) before 0.6 ml DPPH solution was added, and shaken vigorously. The decrease of absorbance was recorded at 1, 5, 10, and 30 min after mixing. Trolox (0-50 μg) and vitamin C (0-40 μg) were used as standards, and results were reported as mg Trolox and vitamin C equivalent per gram of dry weight. The concentration of a selected extract was varied to find out the effective concentration at 50% inhibition (EC_{50}).

6. Total phenolic content (TPC)

The Folin-ciocalteau micro method of Waterhouse (n.d.) was used. Sixty μl of the extract was diluted with deionized water to 4.8 ml, and 300 μl Folin-ciocalteau reagent was added and shaken. After 8 min, 900 μl of a 20% sodium carbonate was added with mixing. Leave the solution at 40°C for 30 min before reading the absorbance at 765 nm. Gallic acid (0-50 μg) was used as a standard, and results were reported as mg gallic equivalent per gram of dry weight.

Values of FRAP, ABTS, DPPH, and TPC (mg standard equivalent per gram of dry weight)

= [(SA - BA) / (Slope)] * [10 / U] / [2] [1 - MC] [1,000]

When:

SA = Sample absorbance for FRAP value and TPC or absorbance decrease of sample for ABTS and DPPH values

BA = Blank (no extract) absorbance for FRAP value and TPC or absorbance decrease of blank for ABTS and DPPH values (extract was substituted by deionized water for blank)

Slope = Slope of standard curve

[10 / U] = Total volume of extract (10 ml) / Used volume of extract (ml)

[2] = Weight of used sample (g)

MC = % moisture content of sample / 100

[1,000] = Factor for changing μg to mg.

7. Statistical analysis

The experiment was repeated three times and was conducted on separate lots of materials. A randomized complete block design (RCBD) was used and the separate lot served as the blocking variable. Mean comparisons was performed by Duncan's new multiple range test (DMRT). The bivariate correlations between all antioxidant capacity assays and total phenolic contents were analyzed.

Results and Discussion

1. Ferric reducing antioxidant power (FRAP) assay

FRAP values of red holy basil were higher than those of white holy basil, and a significant difference between extraction solvents was found ($p<0.05$) for both white and red varieties as shown in Table 1. The 76% ethanol extract yielded the highest value for white holy basil, whilst 57 and 76% ethanol extracts had highest values for red holy basil. Results of extraction solvents in this study agreed with two previous works of Juliani and Simon (2002), and Juntachote and Berghofer (2005), in which 80% ethanol and the mixture of water and ethanol (1:3) were used as extraction solvents for holy basil, respectively. However, the

Table 1. Ferric reducing antioxidant power (FRAP) assay of white and red holy basil when extraction solvents were varied[1].

Sample	mg Trolox equivalent/ g DW[2]	mg vitamin C equivalent / g DW
White holy basil		
Water	$12.11^{f} \pm 0.29$	$4.78^{f} \pm 0.12$
19% Ethanol	$19.80^{e} \pm 0.55$	$7.82^{e} \pm 0.22$
38% Ethanol	$24.94^{d} \pm 1.49$	$9.85^{d} \pm 0.59$
57% Ethanol	$35.77^{b} \pm 1.05$	$14.13^{b} \pm 0.41$
76% Ethanol	$40.46^{a} \pm 2.16$	$15.98^{a} \pm 0.85$
95% Ethanol	$29.22^{c} \pm 1.27$	$11.54^{c} \pm 0.50$
Red holy basil		
Water	$14.63^{e} \pm 0.25$	$5.92^{e} \pm 0.21$
19% Ethanol	$27.87^{d} \pm 0.79$	$11.27^{d} \pm 0.18$
38% Ethanol	$45.90^{c} \pm 3.40$	$18.55^{c} \pm 0.94$
57% Ethanol	$61.11^{ab} \pm 10.23$	$24.61^{ab} \pm 3.18$
76% Ethanol	$65.82^{a} \pm 1.86$	$26.62^{a} \pm 0.46$
95% Ethanol	$54.30^{bc} \pm 9.44$	$22.00^{bc} \pm 4.10$

[1] Each value represents the mean and standard deviation from three lots; Means with different superscripts for each sample are significantly different ($p \leq 0.05$).
[2] DW = Dry weight.

highest FRAP value of red holy basil in this study (26.62 mg or 151 μmol vitamin C (ascorbic acid) equivalent per gram of dry weight) was lower than the FRAP value of holy basil (420 μmol ascorbic acid equivalent per gram of dry weight) reported by Juliani and Simon (2002).

2. ABTS radical cation decolorization assay

The absorbance at 734 nm of all extracts and standards (Trolox and ascorbic acid) decreased rapidly within the first minute. Absorbance decreases of extracts and blank (no extract) are shown in Figure 1. Significant differences between extraction solvents were found ($p<0.05$). Ethanol mixture extracts produced more absorbance decrease than the water extract. For white holy basil, 38, 57, and 76% ethanol extracts showed the significantly highest absorbance decrement, but only 57% ethanol extract did in the case of red holy basil. ABTS values of white holy basil were lower than those of red holy basil, and highest ABTS values (57% ethanol extract) are shown in Table 2, together with EC_{50} at 1 min. Compared to the previous work of Juliani and Simon (2002), the highest TEAC value of red holy basil in this recent work (65.86 mg or 263 μmol Trolox equivalent per gram of dry weight) was a bit lower than their result (297 μmol Trolox equivalent per gram of dry weight).

3. DPPH free radical scavenging activity

The reaction of DPPH assay was completed within the first minute for almost all solvents (Figure 2) and both standards (Trolox and ascorbic acid). Significant differences between extraction solvents were also found ($p<0.05$). Both 57 and 76% ethanol extract had more absorbance decrement for both white and red holy basil. DPPH values of white holy basil were lower than those of red holy basil, and their highest DPPH values together with EC_{50} at 1 min of the 57% ethanol extract are shown in Table 3. The EC_{50} of red holy basil in this study (333 μg/ml) was close to the value of 300 μg/ml reported by Juntachote and Berghofer (2005).

Differences of results from these three antioxidant capacity assays were found. The results of ABTS assay were higher than ones of FRAP

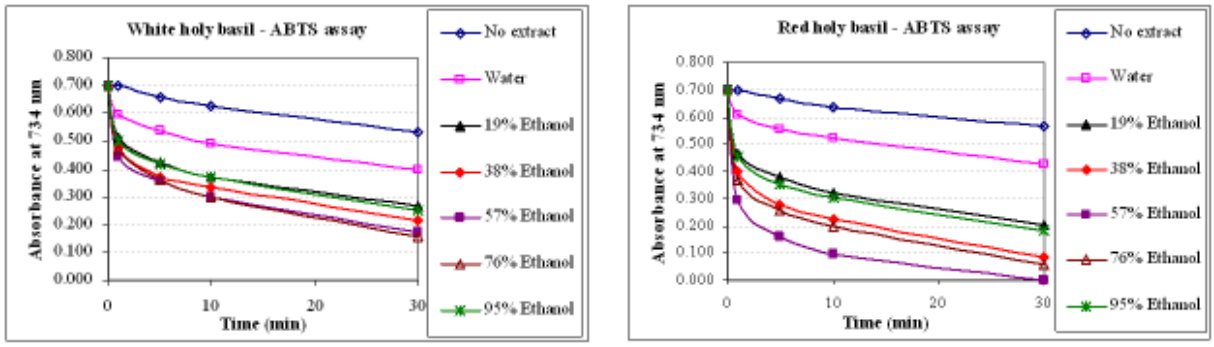

Figure 1. ABTS assay of white (a) and red (b) holy basil when extraction solvents were varied.

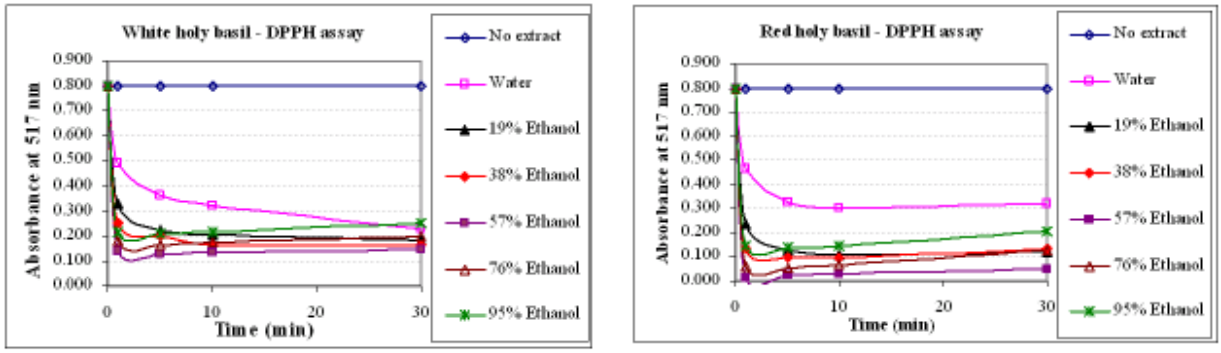

Figure 2. DPPH assay of white (a) and red (b) holy basil when extraction solvents were varied.

Table 2. Highest ABTS values and EC_{50} at 1 min of white and red holy basil (57% ethanol extract).

Sample[1]	White holy basil	Red holy basil
mg Trolox equivalent /g DW[2]	47.27 [b] ± 2.16	65.86 [a] ± 5.51
mg vitamin C equivalent / g DW	31.17 [b] ± 1.42	43.43 [a] ± 3.63
EC_{50}[3] at 1 min (µg/ml)	137 [b] ± 12	90 [a] ± 5

[1] **Samples with different superscripts are significantly different ($p \leq 0.05$).**
[2] **DW = Dry weight.**
[3] **EC_{50} means an extract concentration at 50% inhibition and is calculated under the condition used in this experiment (the reaction time was used at 1 min because reactions of both standards were completed within 1 min).**

and DPPH assays, respectively. This might be explained by the unique mechanism of each method. FRAP assay is a method for measuring total reducing power of electron donating substances, whilst ABTS and DPPH assays are methods for measuring the ability of antioxidant molecules to quench ABTS and DPPH free radicals, respectively. But ABTS and DPPH free radicals

Table 3. Highest DPPH values and EC_{50} at 1 min of white and red holy basil (57% ethanol extract).

Sample[1]	White holy basil	Red holy basil
mg Trolox equivalent /g DW[2]	5.41 b ± 0.04	6.23 a ± 0.19
mg vitamin C equivalent /g DW	4.59 b ± 0.03	5.28 a ± 0.16
EC_{50}[3] at 1 min (μg/ml)	554 b ± 17	333 a ± 13

[1] **Samples with different superscripts are significantly different ($p<0.05$).**
[2] **DW = Dry weight.**
[3] **EC_{50} means an extract concentration at 50% inhibition and is calculated under the condition used in this experiment (the reaction time was used at 1 min because almost all of reactions were completed within 1 min).**

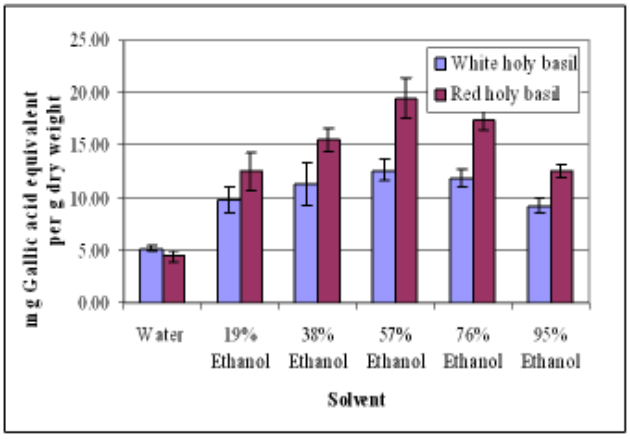

Figure 3. Total phenolic content estimation of white and red holy basil when extraction solvents were varied.

are different. To explain why DPPH values were lower than ABTS values., Wang *et al.* (1998) showed that some compounds which have $ABTS^{+}$ scavenging activity may not show DPPH scavenging activity, and Arts *et al.* (2004) found that some products of $ABTS^{+}$ scavenging reaction may have a higher antioxidant capacity and can continually react with $ABTS^{+}$.

However, these results showed the ability to reduce free radicals and reducing power of holy basil extracts which may stop the free radical initiation or retard free radical chain reaction in the propagation of the oxidation mechanism.

4. Total phenolic content (TPC)

TPC of both white and red holy basil were estimated and are shown in Figure 3. Significant differences between extraction solvents were found ($p<0.05$). Extracts of 38, 57, and 76%ethanol had higher values for white holy basil, while only 57% ethanol extract yielded the highest value for red holy basil. This is because most polyphenols in plants are water-insoluble (Yanagida, n.d.), and the ethanol mixture could help to dissolve low or non polar compounds (Wikipedia, n.d.).

The highest TPC of white and red holy basil were 12.60±1.02 and 19.46±1.97 mg gallic acid equivalent per gram of dry weight, respectively. The highest value of red holy basil in this recent study (19.46 mg gallic acid equivalent per gram of dry weight) was lower than the value (51.1 mg gallic acid equivalent per gram of dry weight)

Table 4. Bivariate correlation of results from three antioxidant capacity assays and total phenolic contents of white and red holy basil.

		Phenolic content	FRAP assay	ABTS assay	DPPH assay
Phenolic content	Pearson correlation	1	0.864	0.964	0.742
	Sig. (2-tailed)	.	0.000	0.000	0.000
FRAP assay	Pearson correlation		1	0.853	0.581
	Sig. (2-tailed)			0.000	0.001
ABTS assay	Pearson correlation			1	0.689
	Sig. (2-tailed)				0.000
DPPH assay	Pearson correlation				1
	Sig. (2-tailed)				.

reported by Juliani and Simon (2002). These results were not doubtful because phenolic compounds in plant foods are largely influenced by genetic factors and environmental conditions (Bravo, 1998). The difference in phenolic content could affect the antioxidant capacity of plants, because many phenolic compounds in plants are good sources of natural antioxidants (Ho, 1992; Amiot *et al*., 1997).

The result of bivariate correlations in Table 4 shows a good agreement between results of all antioxidant capacity and TPC assays. These might be explained by some compounds in holy basil such as eugenol, cirsilineol, isothymusin, isothymonin, rosmarinic acid (Kelm *et al*., 2000), orientin, and vicenin (Vrinda and Uma Devi, 2001) having been identified as good antioxidants. Although, different values were found from each antioxidant capacity assay, they still showed similar trends as evidenced from their significant correlation ($p<0.01$) in Table 4. But the degree of linear association between the results of TPC and ABTS assays ($r = 0.964$) was higher than those of TPC and FRAP assays ($r = 0.864$), and TPC and DPPH assays ($r = 0.742$).

Conclusion

Both extraction solvent and analytical method affected the estimation of antioxidant capacity. In this study, 57 and 76% ethanol were more suitable solvents to use for antioxidant capacity estimation of white and red holy basil, and all results showed that white and red holy basil could be sources of natural antioxidants and phenolic compounds, but the red one was better. ABTS assay produced higher values than FRAP and DPPH assays, and ABTS assay seemed to be a better method for expressing the antioxidant capacity of phenolic compounds in holy basil.

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