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Laccase-Aided Antioxidant Activity Assay and Antioxidant Activity of Selected Thai Vegetables

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Abstract: This study aimed to develop the enzymatic preparation of $ABTS^{\bullet+}$ radical cation using laccase from *Trametes versicolor*. The result revealed that $ABTS^{\bullet+}$ preparation with the aid of the enzyme was reasonable easy, quick and high yield. The radical yields were controlled by concentration of ABTS substrate and enzyme amount. These $ABTS^{\bullet+}$ radicals were used for antioxidant activity assay of 31 Thai vegetables. The dried sample powders extracted by ethyl alcohol showed as high TEAC values as in *Spondias pinnata*, *Eugenia grata* and *Polygonum odoratum*. TEAC values determined using $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ radical decolourisation methods were strongly consistency and correlate with total phenolic content in the samples. The quick and easy of the method as well as stability of the radical and reliable of the antioxidant capacity including environmental friendly suggest it to be a potential method for a total antioxidant activity measurement in biological samples.

Key words: Enzymatic, ABTS, antioxidant, TEAC, total phenolic content, biological sample

INTRODUCTION

Fungal laccases (*p*-diphenol: dioxygen oxidoreductases, EC 1.10.3.2) are oxidoreductases which contain four Cu-atoms in an active centre. These enzymes catalyze the four-electron reduction of dioxygen to water concomitantly through the oxidation of substrate molecules (Baldrian, 2006; Couto and Toca-Herrera, 2007). Laccases have broad substrate specificity, ranging from various phenolic compounds to aromatic compounds. Laccases are industrially interesting enzymes with several application potentials in detergents, pulp bleaching, adhesives, fibre functionalization, detoxification, denim bleaching, textile dye decolourisation and baking (Couto and Toca-Herrera, 2006) as well as biosensors (Shleev *et al*., 2006) and biofuel cells (Chen *et al*., 2001; Zheng *et al*., 2008).

Antioxidants have attracted considerable attention over the past two decades, because of their great potential for combating Reactive Oxidative Species (ROS) that is ubiquitous in nature. In current scientific literature, antioxidant is among the most frequently occurring terms. One can find numerous papers associated with antioxidants. There are several of the commonly used methods for *in vitro* determination of antioxidant capacity. Those methods based on biological oxidants or specific ROS including peroxyl radical ($ROO^{\bullet}$), superoxide radical anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^{\bullet}$), hypochlorous acid (HOCl) and singlet oxygen ($^1O_2$). Another method based on non-biological assays including scavenging of 2,2-azinobis-(3-ethyl benzothiazoline-6-sulphonate) (ABTS) radical cation (TEAC assay), scavenging of 2,2-diphenyl-1-picrylhydrazyl radical ($DPPH^{\bullet}$ assay), ferric reducing antioxidant power (FRAP assay), Folin-Ciocalteu reducing capacity (FC assay), electrochemical total reducing capacity (Magalhaes *et al*., 2008). One most popular method for antioxidant screening and assay is TEAC assay. In this method, $ABTS^{\bullet+}$ radical cation is reduced by antioxidants, causing absorbance decrease at 414 or 734 nm, which first developed by Miller *et al*. (1993). It was based on the activation of metmyoglobin, acting as peroxidase, with H_2O_2 to generate ferrylmyoglobin radical, which then reacted with ABTS to form the $ABTS^{\bullet+}$ radical cation. In that method, the tested sample is added before the $ABTS^{\bullet+}$ generation and the sample having antioxidant activity is one that reduce or inhibit the $ABTS^{\bullet+}$ radicals formation. The method cost is still very expensive and has a major disadvantage as some antioxidants can react with H_2O_2 and/or with derivated oxidizing species that inhibit the $ABTS^{\bullet+}$ radical formation (Re *et al*., 1999). To prevent the interference of antioxidant compounds with radical formation, a post-addition assay or decolourisation approach was proposed.

$ABTS^{\bullet+}$ radical normally chemically generated by an oxidizing agent, potassium persulfate (Re *et al*., 1999) or by enzymatic reaction using metmyoglobin (Miller *et al*. (1993) or horseradish peroxidase (Cano *et al*., 1998). In

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this study, we interested to generate $ABTS^{\bullet+}$ radical cation by another oxidoreductase enzyme namely laccase. Laccase study is being conducted in our laboratory a few years ago. We accidental triggered that this enzyme might be able to use for antioxidant determination. When laccases catalyze the oxidation of its substrates, corresponding free radicals are generated as a product. Substrate, such as ABTS is normally used for laccase activity determination and $ABTS^{\bullet+}$ radical could be observed. Therefore, in this research we aimed to generate $ABTS^{\bullet+}$ radical cation by laccase from *Trametes versicolor* and assess the use of the radical for determination of total antioxidant activity of selected thai vegetables compared with another common method, $DPPH^{\bullet}$ radical decolourisation. Free radicals generation with enzymatic reactions is considerably more environmental friendly.

MATERIALS AND METHODS

Chemicals: Glacial acetic acid, HCl, sodium acetate, sodium carbonate, sodium citrate and Folin-ciocalteu reagent were purchased from BDH, England. ABTS was obtained from Sigma, Germany. 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was purchased from Aldrich, Germany. Gallic acid was a product of Fluka, Switzerland.

Laccase activity assay: The laccase from *Trametes versicolor* was purchased from Fluka, Germany (specific activity 0.53 U mg^{-1}) and was used without further purification. The enzyme assay was based on the oxidation of ABTS as previous described (Khammuang and Sarnthima, 2007).

$ABTS^{\bullet+}$ radical preparation by laccase: The $ABTS^{\bullet+}$ cation radical solution was prepared by incubating 1.0 mM ABTS with 0.02 U mL^{-1} laccase (*Trametes versicolor*) in distilled water or 0.1 M sodium acetate buffer (pH 4.5) at room temperature (approximately 25°C) for 10 min. The radical solution (about 4 absorbance units at 734 nm) was filtered through a Nanosep 10 K Omega (PALL Life Sciences) to remove the enzyme. The $ABTS^{\bullet+}$ radical filtrate was diluted with distilled water or ethyl alcohol to the initial absorbance about 0.7 and studied for its stabilization or using for antioxidant activity determination.

Preparation of standard curve of trolox for TEAC assay: Trolox, a water-soluble form of vitamin E was prepared as a 2.0 mM stock solution. The standard curve was performed using various amounts of Trolox (0-0.2 μmole) reacted with 1 mL of $ABTS^{\bullet+}$ radical at room temperature. The decrease in absorbance at 734 nm was monitored for 2 min by a UV-visible spectrophotometer (Perkin Elmer, Lamda25, USA). The inhibition percentage of the radical was calculated according to the equation (Eq. 1).

$$\text{Inhibition (\%)} = OD_i\text{-}OD_f/OD_i \times 100 \quad (1)$$

where, OD_i is initial absorbance of $ABTS^{\bullet+}$ radicals and OD_f is a final absorbance after 2 min of incubation. The standard curve was a plot of inhibition percentages and micromole of Trolox.

Preparation of standard curve of gallic acid for total phenol content determination: Gallic acid at various concentrations (0-0.01 mg) was used as a standard phenolic compound for total phenolic content determination. The experiments were performed according to the adapted method of Singleton and Rossi (1965).

Antioxidant activity analysis of selected Thai vegetable samples: The various fresh vegetables were obtained from local markets in Maha Sarakham province during July 2007 to December 2007. The samples were washed by tap water, rinsed with distilled water, left to shred out of water at room temperature. Some of each sample was left air-dried. The extraction of dried samples was followed by the method of Kulys and Bratkovskaja (2007). After filtration through sheet cloth and centrifugation, the ethyl alcohol extract was subjected to antioxidant activity assay. All experiments were performed in the Protein and Enzyme Technology Research Unit, Faculty of Science, Mahasarakham University.

RESULTS AND DISCUSSION

$ABTS^{\bullet+}$ radical preparation: The objective of this study was to generate $ABTS^{\bullet+}$ radical by the oxidation reaction of ABTS by laccase from *T. versicolor* comparison to those prepared by potassium persulfate. The results showed that the $ABTS^{\bullet+}$ radical prepared by the latter was very much lower in absorbance unit when reaction time and same starting concentration of ABTS compared with those prepared with the aid of laccase. The absorbance spectrum from both reactions was similar as shown in Fig. 1. $ABTS^{\bullet+}$ radicals could be generated by the enzyme concentration manner as shown in Fig. 2. We could use the enzyme as low activity (0.02 U mL^{-1}) and the reaction finished within 10 min at room temperature. The spectrum of $ABTS^{\bullet+}$ radical cation prepared by laccase in our study was unsurprisingly similar to those generated by potassium persulfate from Re *et al*. (1999).

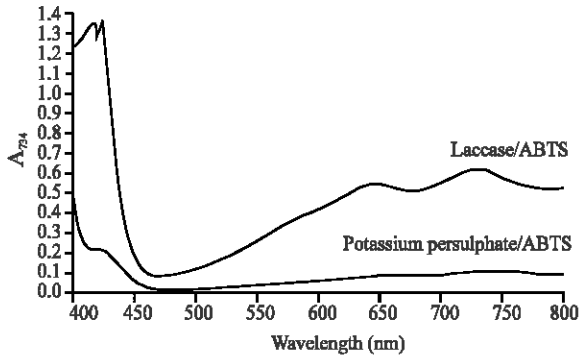

Fig. 1: ABTS$^{\bullet+}$radical cation spectrum after reaction was reached to 16 h comparison between prepared with the aids of *Tramates versicolor* laccase and with potassium persulfate (4.9 mM) at room temperature (approximately 25°C). ABTS final concentration in both reactions was 1.0 mM. Laccase used in the reaction was 0.02 U mL^{-1} and was diluted 10 times with distilled water before scanning. Reaction prepared by potassium persulfate was non-diluted

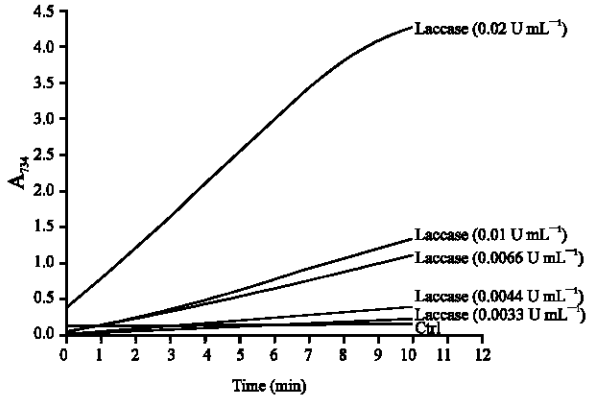

Fig. 2: Influence of enzyme concentration on ABTS radical formation. Reactions were prepared using distilled water in a quart cuvette at room temperature (~ 25°C). Concentration of ABTS in each reaction was 1.0 mM. The reaction was initiated by the adding of laccase. Control reaction (Ctrl) was a solution of 1.0 mM ABTS without the enzyme

Major advantage of using laccase over potassium persulfate is quick, high yield and environmental friendly. The laccase-prepared ABTS$^{\bullet+}$ radical (about 4 absorbance units at 734 nm, 1 mL) were investigated for its stability after dilution with distilled water or ethyl alcohol. The radicals were well stable after dilution with either distilled water or ethyl alcohol at least 30 min (data not shown). ABTS$^{\bullet+}$ radical decolourisation assay could be done within a minute as shown in Fig. 3. This result was

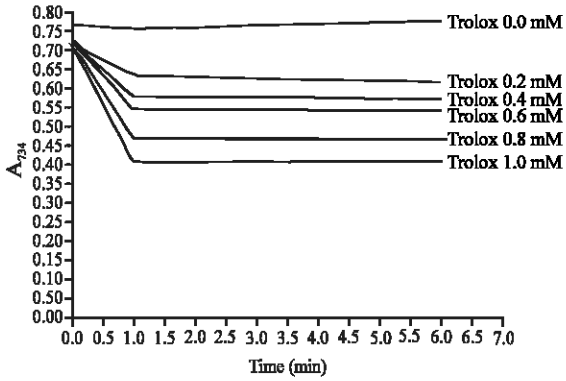

Fig. 3: Absorbance monitoring of ABTS$^{\bullet+}$ radicals scavenging reactions of standard Trolox solution in various concentrations (from 0-1.0 mM). The initial absorbance in each experiment was around 0.7 absorbance unit at 734 nm. Reactions were performed in a quart cuvette at room temperature and initiated by the adding of standard Trolox solution

accordance with the previous report (Re *et al*., 1999). The decrease absorbance at 2 min was chosen for calculating the inhibition percentage. The results suggest that ABTS$^{\bullet+}$ radical preparation with the laccase enzyme is reasonably quick and easy. This might be suitable for antioxidant activity determination of biological samples.

More environmental friendly of enzymatic radical generation is one important advantage over chemical synthesis. This also supports the idea of green chemistry of the application of laccases (Riva, 2006). The radical generated by the enzymatic catalysis and used for antioxidant activity determination has been reported by horseradish peroxidase (HRP) catalyzed oxidation of luminol by hydrogen peroxide (Minioti and Georgiou, 2008). Another example of using HRP and hydrogen peroxide to oxidize ABTS has been reported as the application of interdigitated array microelectrodes as electrochemical sensors (Milardovic *et al*., 2007). Using laccases in the enzymatic method of antioxidant activity measurement can be used practically if the application of other peroxidases is unfavourable. Radical formation by laccase has been firstly reported by Kulys and Bratkovskaja (2007). In their report, recombinant laccase *Polyporus pinsitus* (rPpL) and *Myceliophthora thermophila* (rMtL) with several high reactive laccase substrates including ABTS, 2-phenoxazin-10-yl-ethanol (PET), 3-phenoxazin-10-yl-propane-1-sulfonic acid (PPSA) and 3-phenoxazin-10-yl-propionic acid (PPA) have been used. They showed that the method permits to measure sub-micromole concentration of an antioxidant and

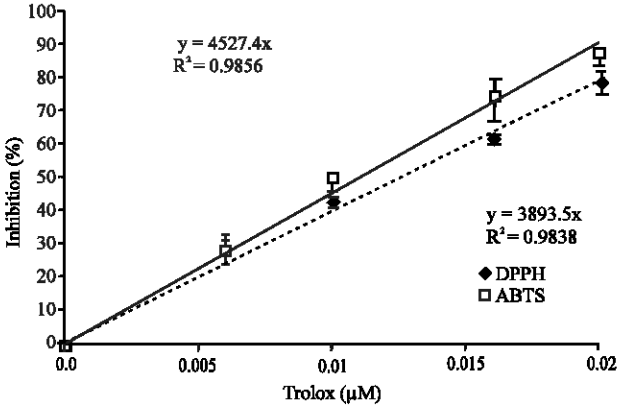

Fig. 4: Inhibition percentage by standard Trolox determined by using $ABTS^{\bullet+}$ radical prepared by the laccase reaction and determined by using $DPPH^{\bullet}$ radicals. Data were averaged from a triplicate experiment

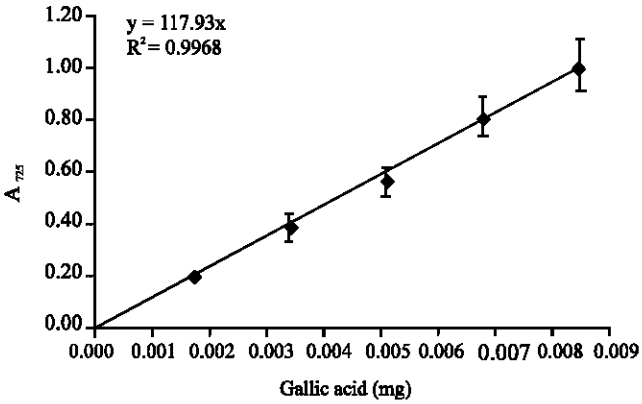

Fig. 5: Concentration-response curve for the absorbance at 725 nm for total phenolic content as a function of concentration of standard Gallic acid solution. (separately prepared stock standard solutions ± SD)

suggested that the optimization of the method may significantly increase the sensitivity. We here show the support results with available substrate ABTS. Antioxidant activity of tested vegetable samples as reported in term of TEAC values by laccase-prepared $ABTS^{\bullet+}$ assay was also in the sub-micromole concentration. This reveals a reasonable good sensitivity of the method. Moreover, the $ABTS^{\bullet+}$ radical cations were relative stable after diluted with distilled water or ethyl alcohol. This characteristic suggests the possibility of using these radicals for measurement of the total antioxidant activity in biological samples of both aqueous and ethyl alcoholic extracts.

Antioxidant activity analysis of selected Thai vegetables: Standard curve of Trolox for TEAC assay by $ABTS^{\bullet+}$ radical and $DPPH^{\bullet}$ radical scavenging methods revealed very similar in their slopes and allowed measuring an antioxidant in sub-micromole concentration (0-0.02 µmole Trolox equivalent) as shown in Fig. 4, whereas a standard curve of gallic acid for total phenol content was linear in the range of 0-0.01 mg mL^{-1} as shown in Fig. 5. According to both standard curves of Trolox and gallic acid, inhibition percentage of 31 Thai vegetable extracts were calculated in terms of trolox equivalent antioxidant capacity (TEAC; µmole Trolox equivalent/mg dried weight of sample) by $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ radicals assays and total phenolic content as mg gallic acid equivalent (mg GAE/g dried weight of sample).

When laccase-aided generated $ABTS^{\bullet+}$ was using for antioxidant activity determination in biological samples, the results were as shown in Table 1. Among 31 plant samples tested, interesting high TEAC values were obtained from *Eugenia grata* (0.335), *Polygonum odoratum* (0.251) and *Spondias pinnata* (0.220), respectively. Present results from $DPPH^{\bullet}$ assay was relative low compared to those from $ABTS^{\bullet+}$ assay. This is in agreement with the study of Stratil *et al.* (2007). They suggested that the $DPPH^{\bullet}$ method gives several times lower values for extracts than TEAC. This phenomenon could be explained by a relatively higher stability of the $DPPH^{\bullet}$ radical which may result in significantly lower reactivity. Due to all assays for the assessment of phenolic compounds and antioxidant capacity are based on redox properties, there should have some correlation between content of phenolic compounds and antioxidant capacity as measured by each method. It was found that the TEAC values determined using $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ radical decolourisation method were strongly consistency as shown in Fig. 6A (with r = 0.85). Thaipong *et al.* (2006) have observed high correlation results for the antioxidant activity measured in methanol extract of guava fruit extracts as determined by ABTS, DPPH, FRAP and ORAC assays. The result was in the same trends as reported by Stratil *et al.* (2007). In this study, antioxidant activity resulted in less correlate with total phenolic content in the samples Fig. 6B (with r = 0.67). A good correlation was found between TEAC and FC reducing capacity (R>0.9) for red wines, herbal and tea infusions and beers (Magalhaes *et al.*, 2007). TEAC values correlated well with results found by elimination of $DPPH^{\bullet}$ and both values revealed a linear relationship with the concentration of phenolics obtained with the Folin-

Table 1: Total antioxidant activity and total phenolic contents of 31 selected thai vegetable extracts

Vegetables	Local name	Part of plant tested	TEAC of ABTS[a]	TEAC of DPPH[b]	Total phenol (GAE)[c]
Eugenia grata	Pak-mek	Leaves	++++	+++	+++
Azadirachta indica A. Juss.	Sa-dao	Flowers	+	+	++
Cassia siamea Lamk.	Khee-lek	Shoots	+	++	++
Cassia siamea Lamk.	Khee-lek	Flowers	+	+	++
Polygonum odoratum Lour	Pak-praew	Leaves	++	+++	+++
Ocimum basilicum Linn.	Ho-ra-pa	Leaves	+	++	+
Ocimum sanctum Linn.	Kra-prao	Leaves	+	+	++
Solanum torvum Sw.	Ma-khue-puang	Fruits	+	+	+
Spondias pinnata Kurz.	Ma-kok	Fruits (ripen)	++	++++	+++++
Brassica sp.	Pak-kard-hin	Leaves	+	+	+
Mentha cordifolia Opizex Fresen	Sa-ra-nae	Leaves	+	++	++
Cajanus cajan (L.) Mill. sp.	Tua-hae	Fruits	+	+	+
Coccinia grandis (Linn.) Voigt	Tum-lueng	Shoots	+	+	++
Basella alba Linn.	Pak-plang	Shoots/leaves	+	+	+
Colubrina asiatica Brongn.	Pak-kan-trong	Leaves	+	+	+
Anethum graveolens Linn.	Pak-chee	Shoots	+	+	+
Acaciapennata (L.) Willd. subsp. Insuavis Nielsen	Cha-om	Shoots	+	+	+
Brassica campestris	Pak-kard-krajon	Leaves	+	+	+
Lactuca sativa Linn.	Pak-salad	Leaves	+	+	+
Amaranthus lividus Linn.	Pak-kan-kom	Shoots	+	+	+
Impomoea aquatica Forsk.	Pak-bung	Shoots/leaves	+	+	+
Momordica charantia Linn.	Pak-sai	Leaves	+	+	+
Sesbania grandiflora	Dok-kae-khao	Flowers	+	+	+
Eryngium foetidum Linn.	Pak-chee-pha-rang	Leaves	+	+	+
Allium ascalonicum	Dok-hom	Flowers	+	+	+
Limnophila aromatica (Lomk.) Merr.	Pak-kha-yaeng	Leaves/shoots	+	+	++
Piper samentosum Roxb.	Cha-plu	Leaves	+	+	+
Sesbania grandiflora	Dok-kae-daeng	Flowers	+	+	+
Tiliacora triandra Diels.	Ya-nang	Leaves	+	+	+
Psophocarpus tetragonolobus	Tua-pooh	Fruits	+	+	+
Carica papaya Linn.	Ma-la-kor	Fruits (young)	+	+	+

[a]++++ (TEAC ≥ 0.4); +++ (TEAC 0.39-0.3); ++ (TEAC 0.29-0.2); + (TEAC 0.19-0.001) μmole Trolox/mg dried weight, [b]++++ (TEAC ≥0.1); +++ (TEAC 0.099-0.05); ++ (TEAC 0.049-0.01); + (TEAC 0.009-0.001) μmole Trolox/mg dried weight, [c]+++++ (GAE≥2); ++++ (GAE 1.5-1.99); +++ (GAE 1-1.499); ++ (GAE 0.5-0.999); + (GAE 0.001-0.499) mg gallic acid/g dried weight, All analysis were averaged from triplicate

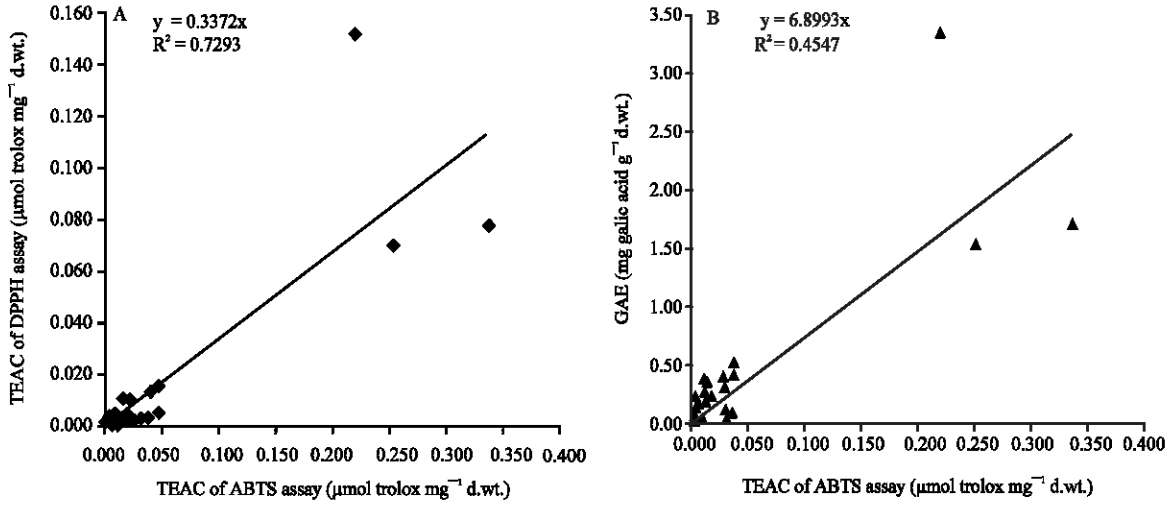

Fig. 6: Correlation between TEAC values in μmole Trolox/mg dried weight determined by $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ radical assays (A) and correlation between total phenol content (GAE) in mg gallic acid equivalent/g dried weight and TEAC value in μmole Trolox/mg dried weight (B) of 31 selected Thai vegetables. Each data were averaged from triplicate

Ciocalteu phenol test (Zalibera *et al*., 2008) . In this study, due to relatively less correlation between total phenolic content and antioxidant activity, it might possible that there are some phenolic compounds other than antioxidant substances present in the plant extracts. Meanwhile, antioxidant substances in these samples might be other chemical groups apart from phenolic compounds. Antioxidant compounds in those three vegetables are interesting to identify and further deeply characterize. That is because they might be vegetables of choice for thai people and the rest as great functional vegetables and fruits.

CONCLUSION

Preparation of $ABTS^{\bullet+}$ radical using the oxidation reaction of ABTS by *T. versicolor* laccase was a quick and easy procedure. The radicals were stable after diluted with either distilled water or ethyl alcohol and were suitable for antioxidant activity determination in dried-vegetable samples. The results of scavenging activity are reliable when compared to those values determined by $DPPH^{\bullet}$ method. The benefits of the radicals; quick, easy and environmental friendly preparation, as well as good stability, suggest it should be very useful to a total radical scavenging capacity screening of various kinds of samples including biological sources.

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