

Extracts of Thai Indigenous Vegetables as Rancid Inhibitor in a Model System

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ABSTRACT

The antioxidant property of twenty five vegetables extracted with ethanolic and water was determined by monitoring their capacities to scavenge the stable free- radicals DPPH. Oxidative rancidity in oil-in-water emulsion model was evaluated by ferric thiocyanate (FTC) method. Total phenolic content was also determined by Folin-Ciocalteu method. The ethanolic extracts were found to exhibit a higher phenolic content as well as DPPH radical scavenging activities than the water extracts. However, the data indicated that both ethanolic and water extracts had dramatically antioxidant activity determined by FTC method. Sixteen and eighteen plants of ethanolic and water extracts, respectively, performed greater rancid inhibition than synthetic antioxidant (BHA, 10 ppm). This study found no relationship between antioxidant activities through the DPPH radicals scavenging or through lipid radicals scavenging.

Key words: vegetables, antioxidant capacity, free radical scavenging activity, total phenolics, rancidity

INTRODUCTION

Synthetic antioxidants have been applied for decreasing lipid oxidation during storage of processed food products. The use of chemical additives has raised questions regarding food safety and toxicity (Chang *et al.*, 1977). Many research works have been directed toward safe antioxidants with high antioxidative activity from natural sources. The antioxidant properties of herbs and spices, cinnamon, turmeric, clove, black pepper, nutmeg, dry ginger, rosemary, sage and paprika were continuously reported (Chang *et al.*, 1977; Nakatani *et al.*, 1986; Kikuzaki and Nakatani., 1993; Tomaino *et al.*, 2005). Many plant extracts were revealed on antioxidation efficiency when applied in oils, fats and fat containing foods, meat

products (Karpińska *et al.*, 2001). For example, rosemary and sage prolong the induction period in chicken fat and show antioxidant activity comparable with butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) (Bracco *et al.*, 1981).

For Thai local vegetables, study on antioxidant activity was reported regarding to the potency of over 100 varieties by using β -carotene bleaching method ((Nakahara and Trakoontivakorn, 1999; Trakoontivakorn and Saksitpitak, 2000; Na Thalang *et al.*, 2001). More than fifty varieties were reported to contain antioxidant more than 100 mg BHA equivalent in 100g fresh weight. With their high potential and lack in information for rancid inhibition, the present study was aimed to evaluate 25 Thai vegetables for

anti-rancidity in a model system. Extract solvents, water and ethanol, were taken into a consideration. Total phenolic content was examined to find a relationship between phenolic content and antioxidant activity of crude extracts from both solvents.

Presenting antioxidant activity of plant crude extract was expressed in many aspects. These methods revealed differently on mechanisms of antioxidant defense system, i.e., inhibition of lipid peroxidation, reduction of lipid peroxy radicals or scavenging of oxygen and hydroxyl radicals (Tsushida *et al.*, 1994; Velioglu *et al.*, 1998; Kähkönen *et al.*, 1999; Pulido *et al.*, 2000). Stable free radicals, frequently applied to study natural antioxidant efficiency were 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), DPPH or *N,N*-Dimethyl-*p*-phenylenediamine dihydrochloride (DMPD). Koleva *et al.* (2002) recommended using DPPH due to being simple, rapid, convenient and independent of sample polarity.

Antioxidant capacity was another perspective to present antioxidant activity, applying different methodologies such as TEAC (Trolox equivalent antioxidant capacity, Cook *et al.*, 1998), β -carotene bleaching method expressed as BHA content (Tsushida *et al.*, 1994), stable radical ABTS or DPPH expressed as vitamin C (Kim *et al.*, 2002). These antioxidant capacity methods were developed in an attempt to have a meaningful interpretation relating to health. In this study, rancid inhibition was concerned, therefore, scavengers of DPPH were stated in equivalent to BHA content.

MATERIALS AND METHODS

Sample preparation

Twenty five Thai indigenous vegetables were either purchased from local markets or collected from nature (Table 1). Edible portions of vegetable were weighed in 10 g/plastic bags and

kept at -20 °C until extracting.

Vegetable extraction

Individual vegetable was extracted with 2 different solvents, 95% aqueous ethanol and distilled water. The 10 g of frozen vegetables were homogenized (Ultra Turrax) in 40 ml 95% aqueous ethanol or distilled water at room temperature for 1 min. and centrifuged at 10000 g for 10 min. The residue was re-extracted with either 95% aqueous ethanol or distilled water and extracts were pooled and made to 100 ml. The extracts were stored in capped bottles and kept at -20°C until further use for antioxidant capacity and total phenolics determinations and rancid inhibition test.

DPPH Radical scavenging activity and antioxidant capacity

DPPH scavenging activity was determined using a modified method of Onichi *et al.* (1994). The free radical scavenging activity of vegetable extracts were tested, indicated as bleaching of the stable 1,1 -diphenyl-2-picrylhydrazyl radical (DPPH). A diluted extract of the right concentration to possess not more than 60% scavenging activity (%SA), 0.15 ml, was added to 0.9 ml of 0.1 mM DPPH dissolved in 95% ethanolic solution. The mixture was vortexed and allowed to stand at room temperature. After 20 min., the absorbance was recorded at 517 nm. 95% aqueous ethanol was used as a control. Percentage of DPPH scavenging activity (%SA) was calculated from this equation $(C-X)100/C$, where C = absorbance of control and X = absorbance of extract.

In order to express antioxidant activity of plant extract to an easily understood manner, antioxidant capacity as mg *t*-butylated hydroxyanisole equivalent (BHAE) /g fresh vegetable was introduced. A standard curve of *t*-butylated hydroxyanisole (BHA) was obtained from DPPH %SA (x) plotted against various BHA concentrations (y). Prepared concentrations of BHA solution were 0.1, 0.25, 0.5, 1.0, 2.5 and 5.0

Table 1 Antioxidant capacity (AC), reported as BHA equivalent (BHAe), and total phenolic (TP) content, reported as gallic acid equivalent (GAE), of vegetables.

| Botanical name | Local name | Tested part | Place and time of collection | Dry matter (%) | Ethanol extract | | Water extract | | Ratio of AC, EtOH/water |
|--------------------------------|------------------------|-------------|------------------------------|----------------|-------------------|------------------|-------------------|------------------|-------------------------|
| | | | | | AC (mg BHAe/g FW) | TP (mg GAE/g FW) | AC (mg BHAe/g FW) | TP (mg GAE/g FW) | |
| <i>Aganosma marginata</i> | Saton | Leaf | Ubon Ratchathani, March | 31.16 | 131.72±14.38 | 42.16±5.49 | 17.73±3.15 | 18.46±0.06 | 7.4 |
| <i>Anacardium occidentale</i> | Mamuang himaphan Nieng | Leaf | Surat Thani, March | 23.06 | 176.48±32.07 | 51.33±4.42 | 68.25±9.86 | 30.08±10.67 | 2.6 |
| <i>Archidendron jiringa</i> | Kradon nam | Fruit | Surat Thani, March | 28.86 | 15.09±7.64 | 6.71±3.84 | 4.78±3.50 | 3.53±1.37 | 3.2 |
| <i>Barringtonia acutangula</i> | | Leaf | Ubon Ratchathani, March | 19.61 | 31.85±0.81 | 11.86±0.11 | 19.75±1.76 | 7.12±0.64 | 1.6 |
| <i>Careya sphaerica</i> | Kradon bog | Leaf | Sakon Nakorn, February | 28.15 | 174.18±14.67 | 54.02±9.45 | 34.63±7.43 | 17.68±1.14 | 5.0 |
| <i>Centella asiatica</i> | Baibua bog | Leaf | Surat Thani, March | 8.72 | 1.24±0.15 | 1.18±0.22 | 0.14±0.01 | 0.46±0.05 | 8.7 |
| <i>Colubrina asiatica</i> | Gan tong | Leaf | Lampang, April | 21.94 | 4.06±0.09 | 4.94±0.69 | 1.05±0.23 | 3.86±0.54 | 3.9 |
| <i>Cratogeomys formosum</i> | Tew | Flower | Sakon Nakorn, February | 18.74 | 53.91±0.97 | 33.77±3.54 | 31.04±6.71 | 16.35±1.95 | 1.7 |
| <i>Cymbopogon citratus</i> | Ta kai | Stem | Bangkok, May | 17.18 | 1.95±0.03 | 1.25±0.04 | 0.51±0.26 | 0.36±0.02 | 3.8 |
| <i>Dregea volubilis</i> | Huan | Leaf | Lampang, April | 14.26 | 3.39±0.07 | 3.39±0.08 | 1.56±0.73 | 1.95±0.05 | 2.2 |
| <i>Eugenia grata</i> | Sa meg | Leaf | Sakon Nakorn, February | 17.60 | 88.72±0.95 | 25.34±4.96 | 46.77±9.91 | 11.27±0.91 | 1.9 |
| <i>Feroniella lucida</i> | Ma sung | Flower | Sakon Nakorn, February | 24.32 | 25.34±0.34 | 34.51±1.14 | 4.78±0.59 | 12.87±2.20 | 5.3 |
| <i>Glinus oppositifolius</i> | Khee khom | Whole | Sakon Nakorn, February | 8.38 | 0.03±0.00 | 0.62±0.03 | 0.03±0.03 | 0.38±0.00 | 1.0 |
| <i>Glochidion wallichianum</i> | Mun pu | Leaf | Surat Thani, March | 25.48 | 167.22±19.06 | 49.06±7.32 | 33.86±4.15 | 11.43±0.11 | 4.9 |
| <i>Gnetum gnemon</i> | Lieng | Leaf | Surat Thani, March | 20.14 | 1.20±0.07 | 1.76±0.10 | 0.18±0.06 | 1.74±0.27 | 6.7 |
| <i>Gynemina inodorum</i> | Chengda | Leaf | Lampang, April | 16.76 | 1.77±0.08 | 3.61±0.15 | 1.51±0.88 | 3.60±0.42 | 1.2 |
| <i>Ipomoea aquatica</i> | Phak bung thai | Leaf, stem | Bangkok, May | 8.22 | 9.56±0.02 | 5.74±0.66 | 1.56±0.05 | 1.91±0.08 | 6.1 |
| <i>Lasta spinosa</i> | Phak nham | Leaf, stem | Sakon Nakorn, February | 8.16 | 1.65±0.07 | 1.11±0.08 | 0.45±0.02 | 0.73±0.09 | 3.7 |
| <i>Leucaena leucocephala</i> | Kratin | Leaf, stem | Bangkok, May | 16.33 | 12.82±0.15 | 7.28±0.37 | 4.60±1.83 | 13.13±0.30 | 2.8 |
| <i>Limnophila aromatica</i> | Phak ka yheng | Leaf, stem | Ubon Ratchathani, March | 9.76 | 10.48±0.97 | 6.51±0.29 | 0.34±0.15 | 0.97±0.05 | 31.2 |
| <i>Mangifera indica</i> | Ma muang | Leaf | Lampang, April | 22.47 | 114.36±8.39 | 40.22±4.27 | 37.51±8.06 | 12.51±1.89 | 3.0 |
| <i>Micromelum minutum</i> | Mhui | Leaf | Surat Thani, March | 25.18 | 24.76±2.13 | 12.64±0.49 | 6.13±0.56 | 12.55±3.26 | 4.0 |
| <i>Parkia speciosa</i> | Sator | Leaf | Surat Thani, March | 26.26 | 23.44±3.75 | 11.73±0.30 | 15.08±3.11 | 5.95±0.34 | 1.6 |
| <i>Passiflora foetida</i> | Ka tok rok | Leaf | Ubon Ratchathani, March | 19.19 | 7.82±2.80 | 5.02±0.47 | 2.55±0.59 | 2.59±0.50 | 3.1 |
| <i>Spirogyra sp.</i> | Tao | Filaments | Sakon Nakorn, February | 3.90 | 14.71±2.17 | 4.08±0.06 | 6.74±0.09 | 1.70±0.11 | 2.2 |

mg/100 ml 95% ethanol. The regression line was $y = 0.0832x - 0.0469$.

Determination of total phenolic contents

Total phenolics were determined using the Folin-Ciocalteu reagent, adopted from Singleton and Rossi (1965). Two millilitres of suitable diluted vegetable extracts was transferred and reacted with 10 ml of Folin-Ciocalteu reagent (previously diluted 10 fold with distilled water) in 25 ml volumetric flask. After 30 sec. and before 8 min., 8 ml of 7.5% of sodium carbonate was added and mixed, and the contents of the flask made to volume with distilled water. Solutions were heated in a 40°C water bath for 30 min. The color was developed and absorbance measured at 765 nm. The standard curve was prepared using 0, 0.5, 1.0 and 1.5 ml of gallate stock solution (8 mg/100ml) in 25 ml volumetric flask. The regression line between absorbance (y) and gallic acid content (x) was $y = 0.0046x + 0.0163$. The results were expressed as mg gallic acid equivalent / g of fresh vegetable.

Antioxidative assay in model system

The lipid oxidation was monitored by ferric thiocyanate (FTC) method described by Kikuzaki and Nakatani (1993). A 20 ml experimental mixture made of 4 ml of an ethanolic extract (0.4 g fresh vegetable), 4.1 ml of 2.51% linoleic acid in 99.5% ethanol, 8 ml of 0.05 M phosphate buffer (pH 7.0) and 3.9 ml of water was placed in a plastic bottle (25 mm diameter, 60 mm height) with a screw cap. For water extract, an assay mixture was prepared the same as above except 3.9 ml of water was replaced by 99.5% ethanol. The mixtures were placed in an oven at 40°C in the dark. FTC was carried out by adding 0.1 ml of incubated mixture, 9.7 ml of 75% ethanol and 0.1 ml of 30% ammonium thiocyanate into a test tube. Precisely 3 min after addition of 0.1 ml of 0.02M ferrous chloride in 3.5% hydrochloric acid to the reaction mixture, the absorbance of red color was measured

at 500 nm. Antioxidative assay was carried out with 7 days interval until the absorbance of the tested mixture reached maximum or 142 days. BHA at concentration of 1.0 mg/100 ml and 5.0 mg/100 ml were used as a control.

RESULTS AND DISCUSSION

Antioxidant capacity

Within twenty five vegetables, ethanolic extract was found to obtain more antioxidant capacity than water extract (Table 1). The result agreed with Kaur and Kapoor (2000) who applied β -carotene bleaching test. Ratio of antioxidant capacity of components soluble in ethanol to water of 25 tested vegetables varied in a range of 31.2 to 1.0, with mean 4.7 and median of 3.2. *Limonophila aromatica* was the one that contained antioxidants most susceptible to dissolve in ethanol. And it was also revealed that antioxidant capacity of ethanolic extracts was in different order from that of water extracts, however, not dramatically.

A large variation in the antioxidant capacity was observed in ethanolic extracts, ranging from as high as 176.48 mg BHAЕ /g fresh weight of *Anacardium occidentale* to as low as 0.03 mg BHAЕ /g fresh weight of *Glinus oppositifolius*. The ranking of five vegetables possessing high antioxidant capacity through ethanol extraction was *Anacardium occidentale*, *Careya sphaerica*, *Glochidion wallichianum*, *Aganosma marginata* and *Mangifera indica*. The result agreed to Trakoontivakorn and Saksitpitak (2000) who reported that methanolic extracts of *Anacardium occidentale*, *Careya sphaerica*, *Glochidion wallichianum* and *Mangifera indica* contained great amount of antioxidants analyzed by β -carotene bleaching method. The results of water extract were found to have antioxidant capacities between 0.03 - 68.25 mg BHAЕ /g fresh vegetable. The five greatest antioxidant capacities were *Anacardium occidentale*, *Eugenia grata*, *Mangifera indica*, *Careya sphaerica* and *Glochidion wallichianum*.

Total phenolic content

The results of phenolic analysis of twenty five vegetables are exhibited in Table 1. The phenolic contents of the vegetable in ethanol extraction varied from 0.62 mg GAE/g fresh weight of *Glinus oppositifolius* to 54.02 mg GAE/g fresh weight of *Careya sphaerica*. The ethanolic extractable phenolic compounds greater than 25 mg GAE/g fresh vegetable were found in *Anacardium occidentale*, *Glochidion wallichianum*, *Aganosma marginata*, *Mangifera indica*, *Feroniella lucida*, *Cratoxylum formosum* and *Eugenia grata*. In water extraction, the total phenolic contents was found highest in *Anacardium occidentale*, 30.08 mg GAE/g fresh weight, followed by *Aganosma marginata*, *Careya sphaerica*, *Cratoxylum formosum* and *Leucaena leucocephala* as 18.46, 17.68, 16.35 and 13.13 mg GAE/g fresh weight, respectively. The present study demonstrated that total phenolics in tested Thai vegetables generally were greater than those of Western herbs, 0.23 -17.51 mg GAE/g fresh weight, reported by Zheng and Wang (2001).

Phenolic compounds were found to be generally susceptible to dissolve more in ethanol than in water. An exception was observed in *Leucaena leucocephala* where extractable phenolic compounds were almost double in water extract than in ethanolic extract. *Micromelum minutum*, *Gymnema inodorum* and *Gnetum gnemon* possessed similar phenolic contents in both extract media. A study reported on phenolic components extracting ability that ethanol was less effective than acetone when applied in Greek oregano and summer savory. However, acetone extracts exhibited incredible low in DPPH scavenging activity (Exarchou *et al.*, 2002). With this detection, therefore, an extract medium should be taking into account in order to obtain antioxidative compounds.

Phenolic contents existed in these twenty five Thai indigenous vegetables were considered as moderate to high. Converting vegetable weight into dry weight basis, ethanolic extractable phenolic

compounds ranged from 7.28 mg GAE/g dry weight in *Cymbopogon citratus* to 222.59 mg GAE/g dry weight in *Anacardium occidentale*. Comparing to the result of Kähkönen *et al.* (1999), the selected cereals and vegetables contained greatly lower amount of phenolics, 0.2 – 6.6 mg GAE/ g dry weight and in moderate level in herb extracts, 9.1 – 23.1 mg GAE/g dry weight.

A relationship between phenolic content and antioxidant activity was extensively investigated, and both positive and negative correlations were demonstrated. Velioglu *et al.* (1998), Rapisarda *et al.* (1999), Zheng and Wang (2001), and many other research groups stated that there was a positive correlation. In the mean time, a few evidences of no significant correlation were confronted (Heinonen *et al.*, 1998; Kähkönen *et al.*, 1999). In this study, the regression analysis was done separately on the extracting medium, ethanol and water. The results revealed that both ethanolic ($R^2 = 0.8893$) and water extracts ($R^2 = 0.6601$) held a positive linear relationship between phenolic content and antioxidant capacity as displayed in Figure 1. The degree of correlation coefficient pronounced greatly in ethanolic extracts ($R = 0.9430$) and lesser in water extracts ($R = 0.8125$).

Antioxidative effect in model system

The oxidation of oil-in-water emulsion was monitored by the ferric thiocyanate (FTC) method. The FTC method was used to measure the amount of peroxide in initial stages of lipid oxidation. Peroxide oxidizes ferrous iron to the ferric state resulted in the formation of a red thiocyanate complex. The determined values at 500 nm with low absorbance indicated a high ability to delay rancidity. On the contrary, if high absorbance values and sharp increasing curve were noted, an uncontrollable lipid oxidation stage was confronted. In this study, extracts from 25 vegetables were analyzed and illustrated separately by the extracting media (Figure 2).

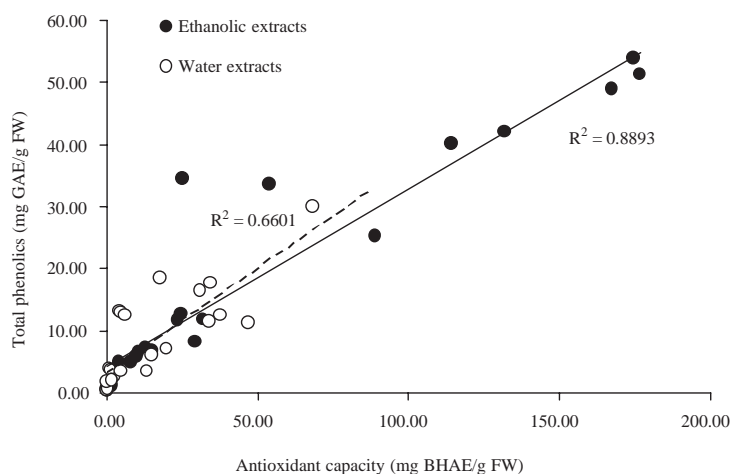


Figure 1 Relationship between total phenolic content and antioxidant capacity of 25 vegetables, extracted by ethanol and water.

Within 142 days of rancid inhibiting evaluation, only 9 vegetable ethanol extracts were lost or deteriorated in their abilities indicated by reaching an absorbance of 0.4 (Figure 2a). They were *Cratoxylum formosum*, *Aganosma marginata*, *Anacardium occidentale*, *Glinus oppositifolius*, *Feroniella lucida*, *Micromelum minutum*, *Careya sphaerica*, *Spirogyra* sp. and *Gnetum gnemon*. Absorbance of the control, no antioxidant in the system, reached absorbance of 0.4 after day 7. Seven out of 9 vegetables and 0.04 mg BHA reached absorbance of 0.4 within 60 days. From the whole experiment, only the extracts from *Glinus oppositifolius*, *Spirogyra* sp., *Gnetum gnemon*, 0.04 mg BHA and 0.2 mg BHA displayed a sharp raise curve similar to the control. A sharp changing in absorbance is a common oxidation pattern monitored by FTC method as shown in other studies (Kikuzaki and Nakatani, 1993; Chen and Ho, 1997). The indicator used to monitor the generation of rancid odor from oil oxidation during incubation was the absorbance of 0.4 by FTC as reported by Chen and Ho (1997). Extract of *Anacardium occidentale* and *Careya sphaerica* could retain absorbance values around 0.4 indicating potential antioxidant plants, and there

were other 16 vegetables performed better than 0.2 mg BHA. The plant extracts that exhibited substantial ability in rancid inhibition were *Gymnema inodorum*, *Dregea volubilis*, *Cymbopogon citratus*, *Colubrina asiatica*, *Passiflora foetida*, *Lasia spinosa* and *Centella asiatica*.

Water extracts illustrated a good result as rancid inhibitors as well (Figure 2b). The plants that possessed this property were *Leucaena leucocephala*, *Gymnema inodorum*, *Dregea volubilis*, *Archidendron jiringa*, *Ipomoea aquatica*, *Passiflora foetida* and *Spirogyra* sp.. Water extracts of plant that could not inhibit rancidity were *Glinus oppositifolius*, *Cratoxylum formosum*, *Aganosma marginata*, *Gnetum gnemon*, *Lasia spinosa*, *Cymbopogon citratus*, *Limonophila aromatica* and *Centella asiatica*.

It was interesting to point out that some plants performed oppositely in controlling rancidity in this model system. Water extract of *Spirogyra* sp. contained effective antioxidants but not in ethanolic extract. Whiles, ethanolic extracts of *Centella asiatica*, *Lasia spinosa* and *Cymbopogon citratus* were effective antioxidant, they became ineffective when extracted with water. The plants

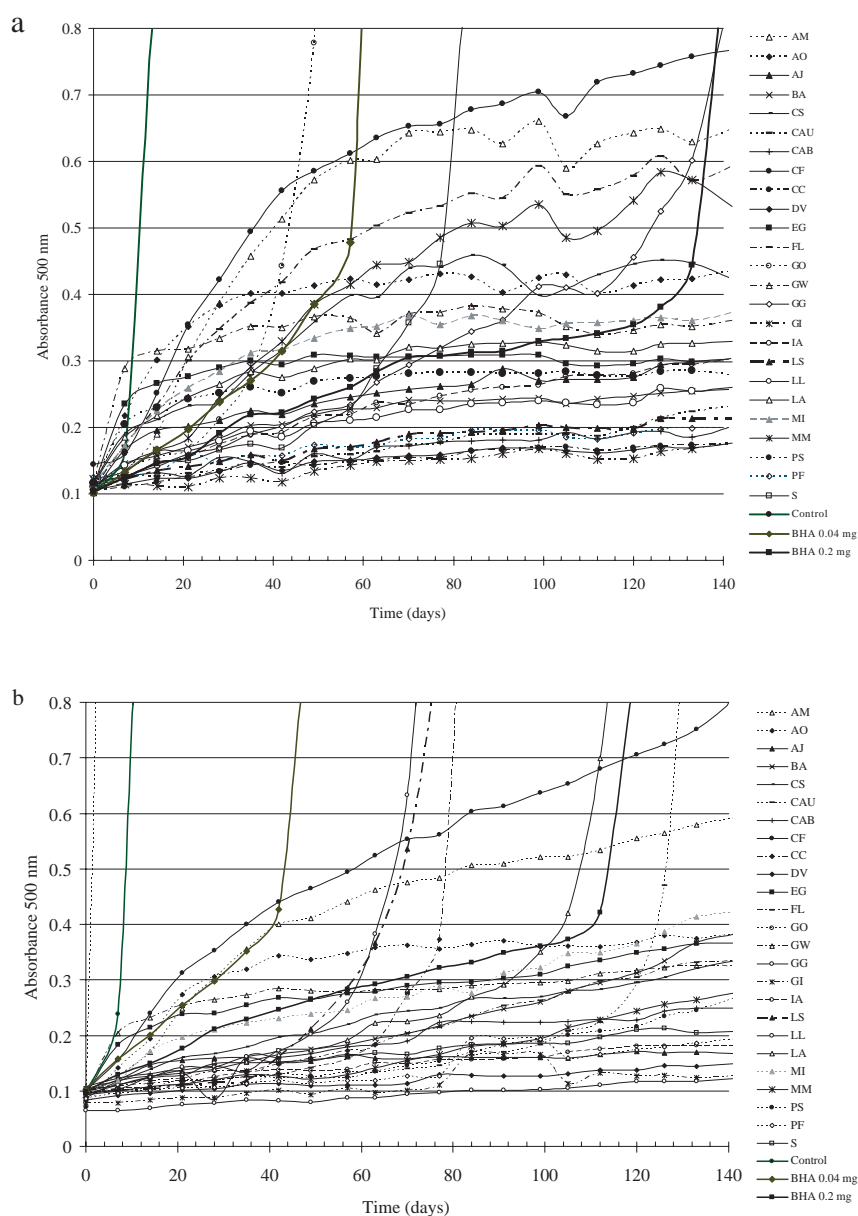


Figure 2 Oxidation of oil-in-water emulsion monitored by the ferric thiocyanate method (a) ethanol extraction (b) water extraction. *Aganosma marginata* (AM), *Anacardium occidentale* (AO), *Archidendron jiringa* (AJ), *Barringtonia acutangula* (BA), *Careya sphaerica* (CS), *Centella asiatica* (CAU), *Colubrina asiatica* (CAB), *Cratoxylum formosum* (CF), *Cymbopogon citrates* (CC), *Dregea volubilis* (DV), *Eugenia grata* (EG), *Feroniella lucida* (FL), *Glinus oppositifolius* (GO), *Glochidion wallichianum* (GW), *Gnetum gnemon* (GG), *Gymnema inodorum* (GI), *Ipomoea aquatica* (IA), *Lasia spinosa* (LS), *Leucaena leucocephala* (LL), *Limonophila aromatica* (LA), *Mangifera indica* (MI), *Micromelum minutum* (MM), *Parkia speciosa* (PS), *Passiflora foetida* (PF), *Spirogyra* sp. (S)

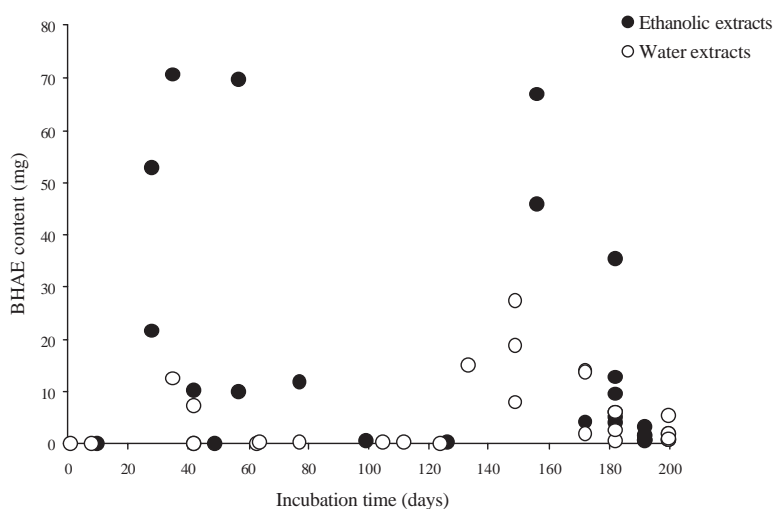


Figure 3 Relationship between BHA content obtained from antioxidant capacity and incubation time to reach an absorbance of 0.4 by ferric thiocyanate method of 25 vegetables, extracted by ethanol and water.

that displayed as a good antioxidant sources when either extracted with water or ethanol were *Passiflora foetida*, *Dregea volubilis* and *Gymnema inodorum*.

Results from the model system experiment revealed that plant extracts good in scavenging DPPH radical was not always good in scavenging lipid radicals. A relationship between BHA content and incubation time (reached absorbance of 0.4) was investigated. BHA content of tested vegetables calculated from antioxidant capacity which used DPPH scavenging method as a tool. The regression values of ethanolic extract and water extract were 0.093 and 0.0122, respectively (Figure 3).

CONCLUSION

It was apparent from the study that plant extracted by different solvents scavenged DPPH radical and lipid radicals differently. Water extracts of some vegetables were efficient comparable to their ethanolic extracts for oxidative rancidity inhibition. And the model of scavenging DPPH

radical could not be used to predict lipid oxidative inhibition. This study also found that extractable antioxidants from 0.4 g of some vegetables demonstrated stronger rancid inhibition than the synthetic antioxidant, 10 ppm BHA. It thus constitutes an interesting source for use as natural protecting agent to prevent oxidative deterioration of food.

ACKNOWLEDGMENTS

Kasetsart University Research and Development Institute (KURDI) financially supported this research.

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