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RESEARCH ARTICLE

# ANTIOXIDANT CAPACITY OF THE GREEN LEAFY VEGETABLES USING OXYGEN RADICAL ANTIOXIDANT CAPACITY (ORAC), 2,2'-AZINO-BIS (3-ETHYLBENZOTHIAZOLINE-6-SULPHONIC ACID(ABTS) AND 2,2-DIPHENYL-1-PICRYLHYDRAZYL (DPPH) ASSAYS

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**ABSTRACT**

Antioxidants are believed to play a very important role in the body defence system against reactive oxygen species (ROS), the harmful by-products that are generated during normal aerobic cell respiration. The objective of this study was to determine the antioxidant capacity in green leafy vegetables using ORAC, ABTS, and DPPH assays of different polyphenol fractions (free phenolic, alkaline hydrolysate, acidic hydrolysate). The antioxidant capacity of the identified free and bound phenolic acid content was measured using different assays including ORAC, ABTS, and DPPH assay (end-point assay and kinetic assay). Only hydrophilic antioxidant activities of all selected samples were examined using ORAC assay. Strong correlations were observed in acidic and alkaline hydrolysate fractions ($p<0.01$) as determined by ORAC and ABTS assays, respectively. In the free phenolic acid extracts, the *O. basilicum* (Sweet basil) ranked first, had highest antioxidant capacities of 521804±4243 µmol TE/100g DW, 329.8±0.4mg TE/g DW and 9.0±1.8 µg GAE/g DW as determined by ORAC, ABTS and DPPH, respectively. . The *A. occidentale* (Cashew shoot) in the alkaline hydrolysate extract showed a greatest antioxidant capacity in all three assays: 889126±7193 µmol TE/100g DW, 466.5±7.9 mg TE/g DW and 3.5±0.4 µg GAE/g DW as measured by ORAC, ABTS and DPPH, separately. While, in acidic hydrolysate, the *A. occidentale* (Cashew shoot) extract also dominated the antioxidant capacity with (560504±5785 µmol TE/100g DW, 387±0.7 mg TE/g DW and 5.9±0.5 µg GAE/g DW) as determined by ORAC, ABTS and DPPH assays, respectively. The acidic and basic hydrolysis yielded higher antioxidant capacities in the present study. It suggests that hydrolysis with alkaline and acidic play significant roles in liberating more phenolic acids and flavonoids and generating high antioxidant capacity in the extracts.

**KEYWORDS**

antioxidant, bound phenolic acids, ORAC, DPPH, ABTS

## 1. INTRODUCTION

Recently, there has been increasing interest in antioxidant activity of phytochemicals or bioactives present in the diet [1]. Antioxidants are believed to play a very important role in the body defence system against reactive oxygen species (ROS), which are harmful by-products generated during normal aerobic cell respiration [2]. Some functional foods and vegetables are important sources of exogenous antioxidants. There is also an indication in the literature that the antioxidant activity of plant materials correlated well with the content of their phenolic acid compounds [3,4,5]

The chemical reactions in antioxidant capacity assays are basically classified into two groups: methods based on single electron transfer (ET) and methods based on hydrogen atom transfer (HAT) [6]. The ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) or TEAC (Trolox equivalent antioxidant capacity), and DPPH (2,2-diphenyl-1-picrylhydrazyl) are categorised as ET-based assays. Whereas, the Oxygen Radical Antioxidant Capacity (ORAC) and Total Reactive Antioxidant Potential (TRAP) are categorised as HAT-based assays. The ET-based assays measure the capacity of antioxidants in the reduction of an oxidant (synthetic free radical), which changes in colour when reduced. Being known as decolorization assays, the measurements are made based on the degree of the colour changes that are correlated with the sample's antioxidant concentration. The HAT-based assays correlate a competitive scheme, in which antioxidants and substrates compete for thermally generated peroxyl radicals through the decomposition of azo-compounds.

There are three categories to express the results according to the

researchers based on the measurements at a fixed-end point compared to the standard (eg: ABTS/TEAC, FRAP and ORAC); results expressed considering the lag-phase (eg: LDL-oxidation, TRAP) and results based on kinetic parameters (e.g: DPPH) [7]. In this study, the ORAC and ABTS assay results are expressed as fixed-end points, whereas the DPPH assay is determined based on two different measurements: fixed end-point and kinetic assay.

## 2. MATERIALS AND METHODS

### 2.1 Sample collection

All vegetable samples were purchased in Pasar Borong Seri Kembangan (wet market), Selangor, Malaysia and were processed in the food laboratory at University Putra Malaysia, Selangor. Quarantine permit was obtained from AQIS (Australian Quarantine and Inspection Service).

### 2.2 Sample preparation

All vegetables were washed under running tap water several times to remove all dust, soil and sand. The excess water was drained off in the sieving basket. The vegetable samples were weighed before and after removing the inedible part to calculate the percentage of edible portion. An inedible part of each vegetable such as blemished leaves, root and peel were removed. The vegetables were then chopped into small pieces and divided into 2 portions; one portion of moisture analysis and another portion was stored in the freezer bag and stored at -20$^{o}$C freezer for 24 hours prior to freeze-drying. Some of the vegetables that cannot be eaten raw (banana flower, cassava shoots) were subjected to cooking such as boiling before storing in the freezer. For cooking the vegetables 10 cups of deionized water were poured into a pot and brought to boil until temperature reached 100$^{o}$C. About 100 g of the vegetable sample was added and cooked without lid for 60 seconds. The cooked vegetable was placed in the sieving basket to remove all excess water.

### 2.3 Freeze-drying of samples

The frozen vegetable samples were spread out in the aluminium foil on a freeze-drier tray and straight away placed in the freeze dryer. The dried vegetable samples were taken out from the freeze-dryer after three days of drying. Freeze drying conditions as follows: Vacuum pressure 63 Pa and temperature 30$^{o}$C (Freeze Dryer ALPHA 2-4 LD, Germany). The freeze-dried samples were then blended into fine powder using the electric blender (Kenwood Stainless Steel Blender AT339, Tokyo, Japan). The powdered samples were kept in a vacuum-sealed plastic and stored at -20$^{o}$C freezer until analysis.

### 2.4 Antioxidant assays

There are many methods to measure antioxidant activity in foods. However, these methods require special equipment and technical skills for the analysis. These analytical methods measure the radical scavenging activity of antioxidants against free radicals like the 2,2-diphenyl-2-pycrylhydrazyl (DPPH) radical and ABTS (2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid). Four methods were used in this study to determine antioxidant scavenging activity in vegetable extracts as follows: -

### 2.5 Oxygen-radical absorbance capacity assay (ORAC)

The ORAC method is based on the inhibition of the peroxyl-radical-induced oxidation initiated by thermal decomposition of azo-compounds, like 2,2′-azobis (2-amidino-propane) dihydrochloride (AAPH).

#### *2.5.1 Sample preparation*

Selected freeze-dried vegetable samples were prepared and extracted for polyphenol fractions (phenolic acids and flavonoids). The extracts were diluted 1:250. All dilutions were made with phosphate buffer (75 mM, pH 7.2).

#### *2.5.2 Chemicals*

A 75 mM phosphate buffer pH 7.2, 5 μM/ml Trolox solution (6-Hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid) was prepared. The trolox standard solution was made freshly in ranges of concentration (10, 25, 50, 75, 100 μM with phosphate buffer), 140 mM AAPH 2, 2‘-azobis (2-amidino-propane), Randomly Methylated â-Cyclodextrin (RMCD) (Trappsol) pharmacy grade TRMB-P, 700 μM Fluorescein (FL). Polar-Star Optima microplate reader/ BMG Labtech equipped with Optima software version 2.20 with fluorescein polarization mode and simultaneous dual emission. FluoSTAR Optima settings: incubator was set to 37.3$^{0}$C, pump 1 primed with 70nM Fluorescein, pump 2 primed with 140mM AAPH and kinetic windows (used to control automatic reagent injection).

### 2.6 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assay

The improved technique for the generation of ABTS$^{\cdot+}$ described in the researchers involves the direct production of blue-green ABTS$^{\cdot+}$ chromophore through the reaction between ABTS and potassium persulfat [8]. The absorption is measured at wavelength 734nm. The pre-formed radical mono cation of 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS$^{*+}$) is generated by oxidation of ABTS with potassium persulfate and is reduced in the presence of such hydrogen-donating antioxidants.

#### *2.6.1 Chemicals*

ABTS solution [2, 2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid 98%], 1.5 ml of 8.17 mM $K_2S_2O_8$ potassium persulphate solution, 5mM/ml Trolox solution (6-Hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid), the Trolox standard solution was made freshly in a range of concentrations (100, 250, 500, 750, 1000 μM) with phosphate buffer, PS-40 clear 96 well microtitre plates untreated (part # 655101).

#### *2.6.2 Titre plate preparation and assay protocol*

The extracts were prepared in 1:15 dilution with phosphate buffer as indicated earlier. Trolox standard solutions were run each time in all plates in the concentration ranges of 100, 250, 500, 750, and 1000 μM. Different concentrations were prepared from the stock solution (5 mM) with phosphate buffer. 190 μL ABTS reagent was pipetted manually onto 10 μL samples (1:15 dilution) in each well. Plates were sealed with clear adhesive plate seals to avoid external oxidation. The seals were removed just before the reading. The absorbance was measured at 734 nm after 5 min from the reaction in the spectrophotometer. The antioxidant capacity of selected samples was determined by scavenging blue-green ABTS radical anions and was expressed on a dry weight basis as μmol/g Trolox equivalent (TE).

### 2.7 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay

A rapid, simple and inexpensive method to measure the antioxidant capacity of food involves the use of free radical, 2,2-‘-diphenyl-1-picrylhydrazyl (DPPH). In this study this method was slightly modified. This assay is widely used to test the ability of compounds to act as free radical scavengers or hydrogen donors, and to evaluate antioxidant activity of foods. A measure of total antioxidant capacity helps understand the functional properties of foods. The colour changed from purple to yellow as the molar absorptivity of the DPPH radical at 517nm reduces when the odd electron of DPPH radical becomes paired with hydrogen from a free radical scavenging antioxidant to form the reduced DPPH-H. The resulting decolourization is stoichiometric with respect to the number of electrons captured.

#### *2.7.1 Chemicals*

Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), DPPH reagent (2, 2‘-diphenyl-1-picrylhydrazyl), PS- 40 clear 96 well microtitreplates untreated (part # 655101), clear adhesive plate seals (100 seals/case) were obtained from Lab Advantage Cat# LAC36194,

Molecular devices: Spectramax M2 microtitreplates reader, fluorescence absorbance multi-detection readers with dual-mode cuvette port.

### *2.7.2 Titre plate preparation and assay protocol*

Gallic acid standard solutions (10, 25, 50, 75, 100 µM) were run each time in all plates in the linear range of concentrations prepared from 500 µM stock solution with phosphate buffer. 40 µL sample extract (1:15 dilution) was manually pipetted followed by 160 µL DPPH reagent into each well and allowed to react for 30 min. Plates were sealed with clear adhesive seals to protect samples from external oxidation; the seals were removed before measurement. The absorbance was measured at 517 nm in the spectrophotometer.

## 3. RESULTS AND DISCUSSION

Determination of antioxidant capacity of vegetables in this study was performed with freeze-dried powders. Freeze drying process could minimize the losses in antioxidant capacity of plant extracts [9]. The methanol hydrophilic extracts of free, alkaline, and acidic hydrolysates were tested for their antioxidant capacity using ORAC, ABTS and DPPH assay (end-point and kinetic). The results clearly varied according to the assay used. In most cases, plant extracts with high concentration of phenolic acids showed high antioxidant activity [10]. The results are divided into three categories according to the type of phenolic acid fraction (free phenolic, alkaline hydrolysate and acidic hydrolysate). The DPPH kinetic assay is discussed in each fraction after data of fixed-end points is explained.

### 3.1 Antioxidant capacity in free phenolic acid fractions

The higher the Trolox Equivalent (TE) value of the ORAC and ABTS assay the higher the antioxidants in the sample. However, in the DPPH assay, the shorter time taken to reduce the DPPH shows higher antioxidant capacity in extracts. The ABTS, and ORAC assays are common assays that measure antioxidant capacity of hydrophilic compounds [9]. The antioxidant capacities of free phenolic acid fractions determined by ORAC, ABTS and DPPH assays are compared and expressed as µmol of TE per 100 grams dry weight, mg TE per gram dry weight and µg GAE per gram dry weight, respectively as shown in Figure 1.

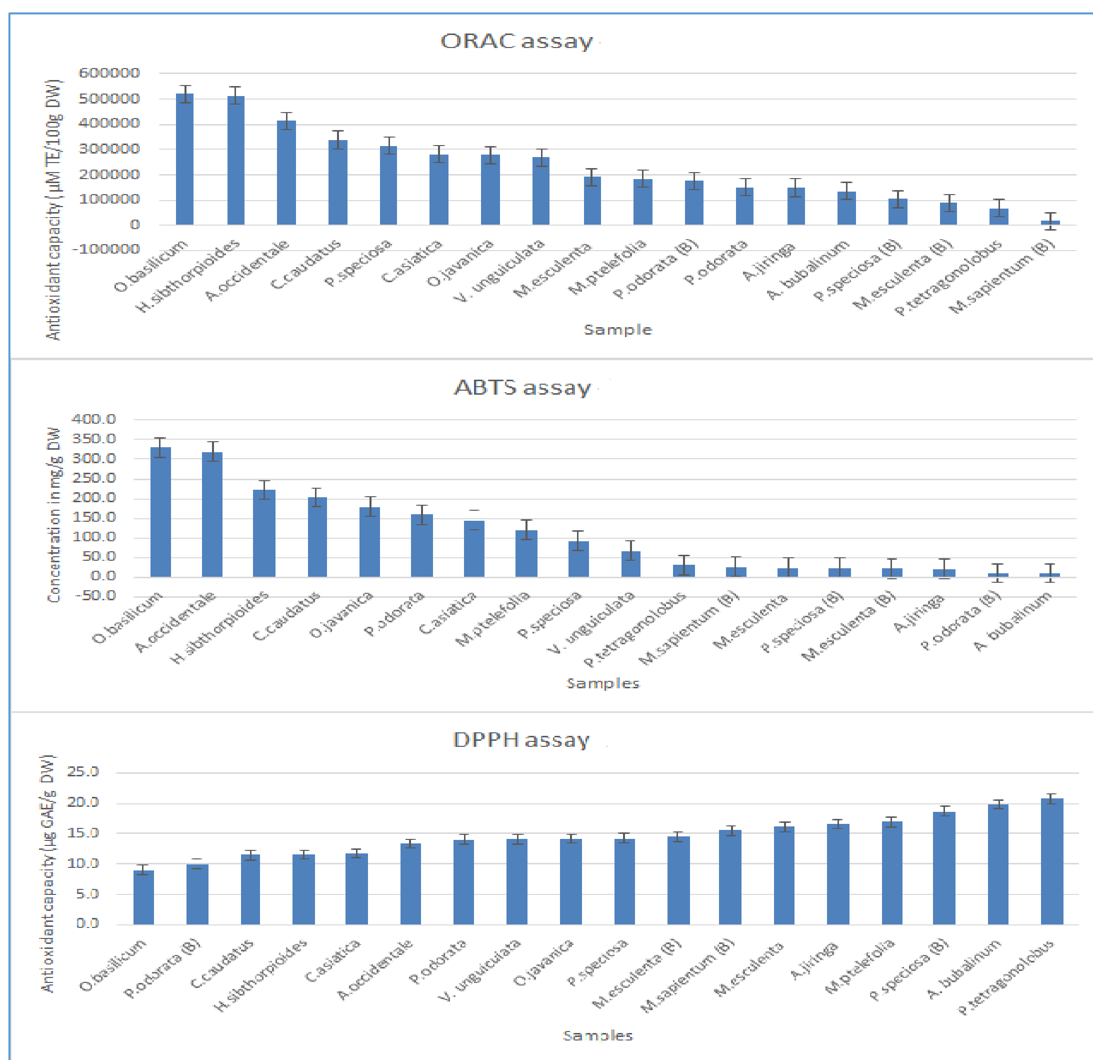


**Figure 1:** Antioxidant capacities of free polyphenols (phenolic acids and flavonoids) fraction using ORAC, ABTS and DPPH assays.

In the free phenolic acid extracts, the *O. basilicum* (Sweet basil) ranked first, had highest antioxidant capacities of 521804±4243 µmol TE/100g DW, 329.8±0.4mg TE/g DW and 9.0±1.8 µg GAE/g DW as determined by ORAC, ABTS and DPPH, respectively. The *H. sibthorpiodes* (L. pennywort) and *A. occidentale* (Cashew shoot) ranked 2nd and 3rd to have high antioxidant capacities as evaluated by ORAC and ABTS assay. However, in the DPPH assay, both were ranked 4th and 6th *H. sibthorpiodes* (L.pennywort) and *A. occidentale* (Cashew shoot), respectively). In contrast, the lowest antioxidant capacity was seen in non-leafy vegetables such as *M. sapientum* (Banana flower) (17231±689 µmol/100g DW), *A. bubalinum* (Kerdas) (9.6±2.5mg TE/g DW) and *P. tetragonolobus* (Four-angled bean) (20.8±1.9 µg GAE/g DW) as determined by ORAC, ABTS and DPPH assays, respectively. Earlier studies have indicated lower antioxidant capacity in non-leafy vegetables and higher capacities in leafy vegetables [10].

In the ORAC assay, the mean hydrophilic antioxidant capacity in free fractions ranged from 17231-521804 µmol TE in 100g dry weight. To make the comparison to the USDA ORAC (2010) data easier, the ORAC data were converted to µmol Trolox equivalent per 100 g dry weight according

to the USDA ORAC (2010) database which lists the ORAC value for foods and spices. This study is the first to show the antioxidant capacities of the traditional Malaysian vegetables**.** According to the USDA ORAC (2010) database, the highest value was found in cloves which is categorized as a spice with 314446 μmol TE in 100g dry weight, followed by cinnamon and oregano (267536 μmol TE in 100g dry weight and 200129 μmol TE in 100g dry weight, respectively).

### 3.2 Antioxidant capacity in alkaline hydrolysate extract

The hydrolysis of the polyphenols (phenolic acids and flavonoids) were done to liberate the bound phenolic acids and flavonoids in the vegetable extracts. Antioxidant activities of alkaline hydrolysate extracts obtained using ABTS, DPPH, and ORAC assays were measured in triplicate to test the reproducibility of the assays. The comparison of the extracts is shown in Figure 3. The *A. occidentale* (Cashew shoot) showed a greatest antioxidant capacity in all three assays: 889126±7193 μmol TE/100g DW, 466.5±7.9 mg TE/g DW and 3.5±0.4 μg GAE/g DW as measured by ORAC, ABTS and DPPH, separately. The lowest ranking of antioxidant capacities were determined in *M. sapientum* (Banana flower) (28870±1155 μmol TE/100g DW and 20.5±1.7 μg GAE/g DW, in ORAC and DPPH, respectively) and *A. bubalinum* (Kerdas) (7.2±0.7 mg TE/g DW) as evaluated by ABTS.

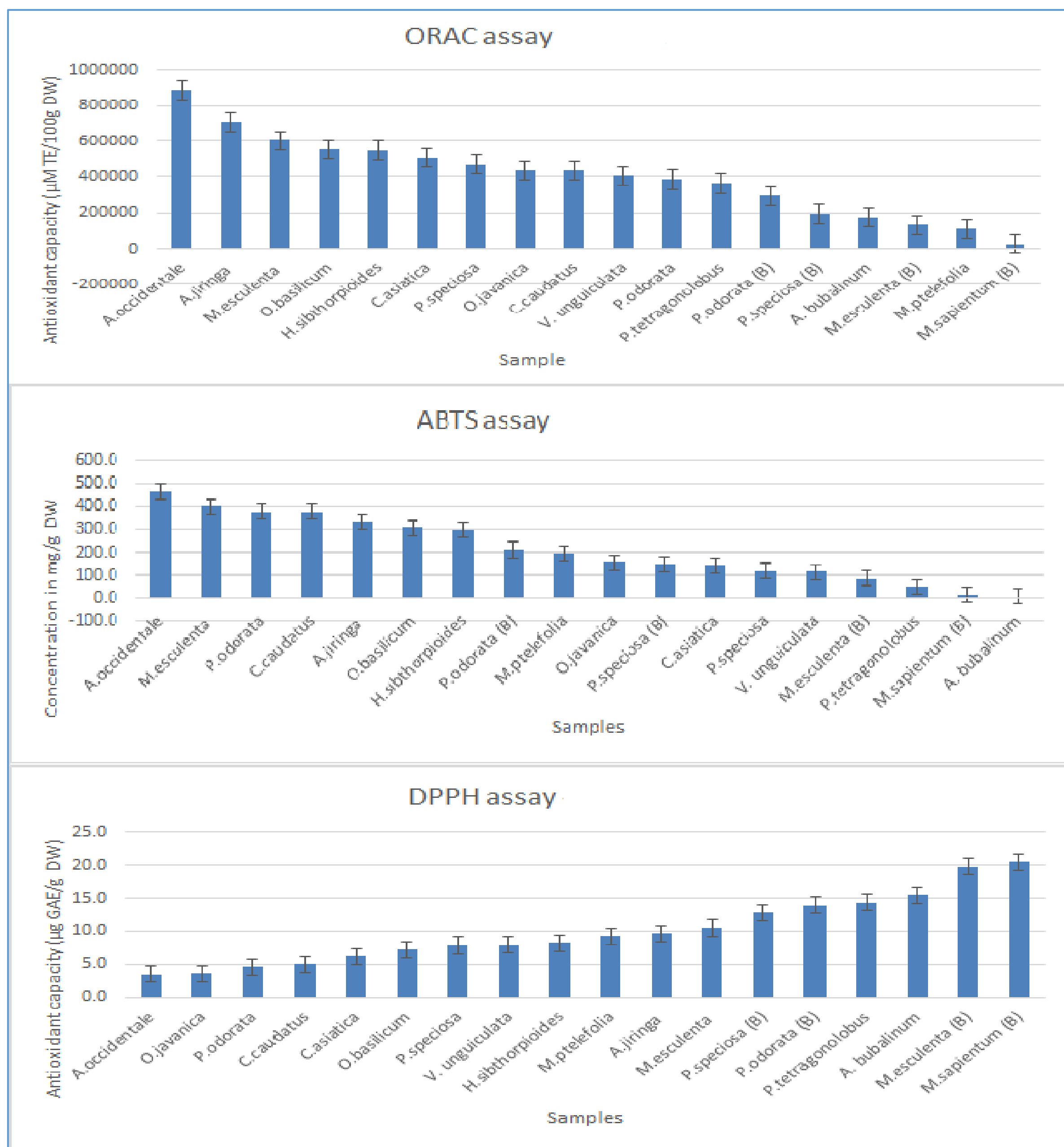


**Figure 2:** Antioxidant capacities of alkaline hydrolysate of the vegetables using ORAC, ABTS and DPPH assays.

### 3.3 Antioxidant capacity in acidic hydrolysates

The comparison of the antioxidant capacity of acidic hydrolysate extracts using all three assays are shown in Figure 4. It can be clearly seen that *A. occidentale* (Cashew shoot) dominated the antioxidant capacity (560504±5785 μmol TE/100g DW, 387±0.7 mg TE/g DW and 5.9±0.5 μg GAE/g DW) as determined by ORAC, ABTS and DPPH assays, respectively. There was a significant positive correlation between antioxidant capacity obtained in acidic hydrolysate extracts and alkaline hydrolysate extracts ($r=0.804$, $p<0.05$). The pattern of the ranking in ORAC and ABTS assays showed a significant correlation ($p<0.05$). Therefore, a ranking leader of the antioxidant capacity of the extracts was the same in both assays particularly for *O. basilicum* (Sweet basil), *P. odorata* (Laksa leaf) *(F), H. sibthorpiodes* (L.pennywort), *O. javanica* (Water dropwort) *C. asiatica* (Pennywort) and *C. caudatus* (Ulam raja) extracts*.* These indicated that those vegetables also had high potential as radical scavengers. The lowest antioxidant capacities were observed in *P. tetragonolobus* (Four angled bean) (6.8 ±0.4 mg TE/g DW and 21.3±2.0 μg GAE/g DW, in ABTS and DPPH assay, respectively) and *M. sapientum* (Banana flower) (11084±443 μmol TE/100g DW in ORAC assay).

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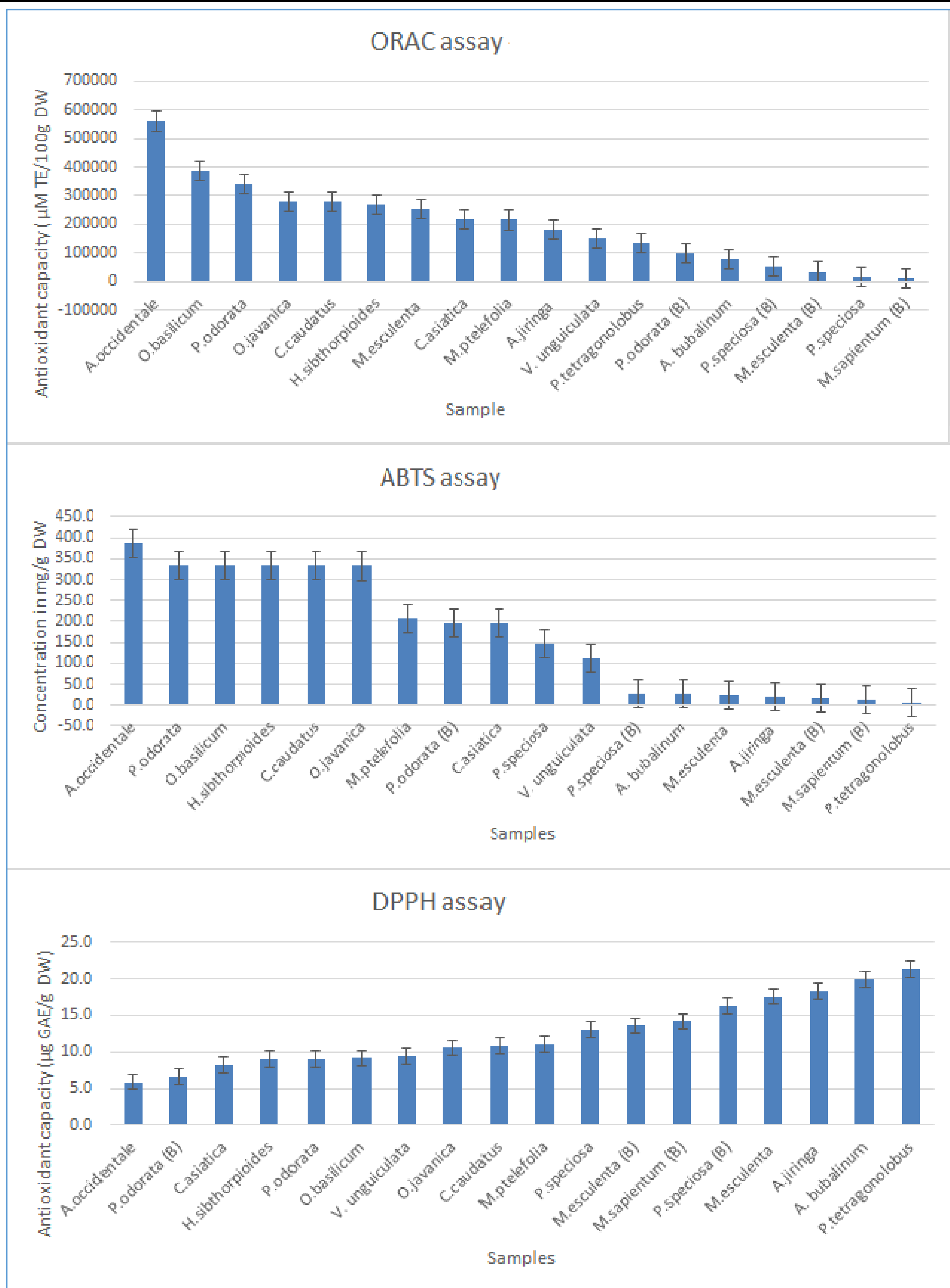


**Figure 3:** Antioxidant capacities of acidic hydrolysate of the vegetables using ORAC, ABTS and DPPH assays.

### 3.4 Correlation between antioxidant capacities using ORAC and ABTS assays in free, alkaline and acidic fractions

The ORAC and ABTS assays gave comparable antioxidant activities measured in methanolic extracts of 18 selected plant extracts. There were significantly positive correlations observed ($r=0.80$, $p<0.01$) between the assays used and the type of polyphenol fractions (Table 1). Another study on sorghum by Awika and co-workers also found that ABTS assay value positively correlated with the ORAC assay and the phenol contents of the sorghums significantly correlated with their antioxidant activities measured by both methods (ORAC and ABTS) [11]. This indicates that the ABTS method, which is more cost effective and simpler, was confirmed to have similar predictive power as an ORAC antioxidant activity. However, there is a need to standardize this method to allow for data comparisons across laboratories.

**Table 1:** Bivariate correlations between assays used (correlation coefficient=r)

| | | Free ORAC | Free ABTS |
|---|---|---|---|
| Free ORAC | Pearson Correlation | 1 | .817** |
| | Sig. (2-tailed) | | .000 |
| Free ABTS | Pearson Correlation | .817** | 1 |
| | Sig. (2-tailed) | .000 | |
| | | Alkaline ORAC | Alkaline ABTS |
| Alkaline ORAC | Pearson Correlation | 1 | .732** |
| | Sig. (2-tailed) | | .001 |
| Alkaline ABTS | Pearson Correlation | .732** | 1 |
| | Sig. (2-tailed) | .001 | |
| | | Acidic ORAC | Acidic ABTS |
| Acidic ORAC | Pearson Correlation | 1 | .777** |
| | Sig. (2-tailed) | | .000 |
| Acidic ABTS | Pearson Correlation | .777** | 1 |
| | Sig. (2-tailed) | .000 | |

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

The correlation coefficients between the three fractions (free, alkaline and acidic hydrolysates) using the assays as determined are shown in Table 2. Strong correlations were observed in acidic and alkaline hydrolysate fractions ($p<0.01$) as determined by ORAC and ABTS assays, respectively. It suggests that hydrolysis plays a significant role in liberating more phenolic acids and flavonoids and generating high antioxidant capacity in the extracts.

**Table 2:** Correlation coefficients between different fractions using ABTS and ORAC assays

| | | ORAC free | ORAC alkaline | ORAC acidic |
|---|---|---|---|---|
| ORAC free | Pearson Correlation | 1 | 0.398 | 0.199 |
| | Sig. (2-tailed) | | 0.102 | 0.428 |
| ORAC alkaline | Pearson Correlation | 0.398 | 1 | .723** |
| | Sig. (2-tailed) | 0.102 | | 0.001 |
| ORAC acidic | Pearson Correlation | 0.199 | .723** | 1 |
| | Sig. (2-tailed) | 0.428 | 0.001 | |
| | | ABTS Free | ABTS alkaline | ABTS acidic |
| ABTS free | Pearson Correlation | 1 | -0.265 | -0.095 |
| | Sig. (2-tailed) | | 0.289 | 0.707 |
| ABTS alkaline | Pearson Correlation | -0.265 | 1 | .596** |
| | Sig. (2-tailed) | 0.289 | | 0.009 |
| ABTS acidic | Pearson Correlation | -0.095 | .596** | 1 |
| | Sig. (2-tailed) | 0.707 | 0.009 | |

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

## 4. CONCLUSION

*O. basilicum* (Sweet basil) and *A. occidentale* (Cashew shoot) had the highest antioxidant capacities in free, alkaline and acidic fractions, respectively. The other vegetable extracts such as *H. sibthorpiodes* (L.pennywort), *C. caudatus* (Ulam raja) and *O. javanica* (Water dropwort) also demonstrated a high potential for antioxidant capacities as determined in all three assays. The acidic and basic hydrolysis yielded higher antioxidant capacities in the present study.

## 5. IMPLICATION

It suggests that hydrolysis with alkaline and acidic play significant roles in liberating more phenolic acids and flavonoids and generating high antioxidant capacity in the extracts.

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