Antioxidant Activity of Common Vegetables in Hawai'i

National Science Foundation Research Project Report

By

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1. INTRODUCTION:

Cancer is the general name of a group of more than 100 diseases in which cells in part of the body begin to grow out of the control, and which can invade adjoining parts of the body and spread to other organs. Cancer may affect people of all ages, but risk for the common variety tends to increase with age. According to the World Health Organization, 8 million people died from cancer and 14 million new cases in the world in 2012. Thus cancer constitutes the leading cause of death globally [1,2].

It has been also shown that diet rich in vegetables and fruits can significantly reduce the incidence of cancer and other chronic diseases [3,4]. The World Health Organization and Food and Agricultural Organization recommended the daily consumption of at least 400 g of fruit and vegetables for the prevention of heart disease, cancer, type-2 diabetes and obesity [5].

1.1 Background and literature review:

Diet and physical activity together with smoking are the most modifiable factors of cancer risk. That is why diet rich in fruit and vegetables has been recommended for preventing cancer [3,4]. The prevention effects of vegetables and fruit may be related to their antioxidant content. Antioxidants have the ability to destroy the free radicals by preventing them to damage biomolecules such as proteins, DNA and lipids. Moreover it has been demonstrated that dark-green leafy and brightly-colored vegetables tend to contain high antioxidants [6,7].

1.2 Research Question:

The objective of this research project is to analyze some of the leafy vegetables grown in Hawai'i for their antioxidants content. The idea behind this research is to find vegetables

loaded with antioxidants and easy to grow for the local consumption. In Hawai'i, people tend to eat some parts of the plants and discard other parts even if the latter present nutritional values. For example, in Hawai'i, the roots of cassava are consumed but the leaves are discarded despite the fact they contain a wealth of nutrients such as protein, carbohydrate and antioxidants (100 g of dried leaves contain 33 g of proteins, 35.7 g of carbohydrates, and 270 mg of vitamin C) [8]. Countries like Nigeria, Ghana, Rwanda, Vietnam, and Philippines export dried cassava leaves to USA for Asian and African communities. In Africa and Asia, rural people live on diet including bean, pumpkin, sweet potato, and cassava leaves to meet their protein and anti-oxidant daily needs since meat is very expensive and unaffordable for most family budgets. So, in order to promote some plant leaves for consumption in Hawai'i, we need to analyze them to back up our recommendation with nutritional values in antioxidants.

In this experiment, we test the antioxidant activity of common vegetables in Hawai'i to determine which vegetables exhibit high amounts of antioxidants. We also examine the variations of antioxidant activity with respect to origin and leaf color. We investigate the total phenolics content (TPC) using Folin-Denis reagent to ascertain the level of phenols present in each sample. The antioxidant capacity (AOC) was measured using ABTS. We also do a statistical analysis using Microsoft Excel.

2. METHODS:

2.1 Sample Preparation: Various Leaves of Common Vegetables:

The following vegetables were harvested from the Native Hawaiian Community Garden on the University of Hawai'i West O'ahu campus: yucca (*Manihot esculenta*: 60g FW); sweet potato (*'uala, Ipomoea batatas*: 60g FW), common taro (*kalo, Colocasia esculenta*: 70g FW),

dinosaur kale (*Brassica oleracea acephala*: 100g FW), and cranberry hibiscus (*Hibiscus acetosella*: 50g FW). Two other samples were also collected from outside the state of Hawai'i: cassava [*Manihot esculenta* (Rwanda): 185g] and sweet kale [*Brassica oleracea acephala* (South Carolina): 100g FW).

Each sample was prepared individually by weighing and grounding in a blender until the sample was finely chopped. The samples were weighed once more prior to drying (50^{o}C) for 48hr.

Table 1. Sample Identification Table. Scientific name, common name, origin of sample, and color.

Sample	Common Name	Origin	Color
Manihot esculenta	Yucca	Kapolei, Hawaii	Green
Manihot esculenta	Cassava	Rwanda, Africa	Green
Brassica oleracea	Dinosaur kale	Kapolei, Hawaii	Green
Brassica oleracea	Sweet kale	South Carolina	Green
Colocasia esculenta	Kalo (Taro)	Kapolei, Hawaii	Green
Hibiscus Acetosella	Cranberry Hibiscus	Kapolei, Hawaii	Purple
Ipomoea batatas	'Uala (sweet potato)	Kapolei, Hawaii	Green

Figure 1. Photographs of samples collected from the University of Hawai'i West 'Oahu campus. *A.) Ipomoea batatas* 'Uala (Sweet potato). B.) *Brassica oleracea acephala*, Dinosaur kale. C.) *Colocasia esculenta*, Kalo (Taro). D.) *Manihot esculenta,* Yucca. E.) *Hibiscus acetosella*, Cranberry hibiscus.

2.1.1 Extraction and Processing of Dried Samples:

The samples were weighed after drying and ground with a pestle and mortar. Then the sample was combined with 95% ethanol (1:4 w/v). The mixture was then agitated on a shaker for 24hrs. The sample was filtered and then centrifuged at 7,830rpm for 15min. After that, the sample was filtered once more and stored in a tinted flask in a refridgerator.

2.2 Total Phenolics Determination

2.2.1 Preparation of Extraction Solution:

Extraction solution (ES) was made by combining 36.5% hydrochloric acid (100mL) and methanol (300mL) [9].

2.2.3 Preparation of Folin-Denis Reagent:

A stock solution of Folin-Denis Reagent was made by combining sodium tungstate (25.0g), phosphomolybdic acid hydrate (5.0g), phosphoric acid hydrate (12.5g), and dH_2O (200mL). The Folin-Denis reagent was fluxed for 3hrs and dH_2O (300mL) was added to bring the reagent to 500mL. It was then stored in a refridgerator [10].

2.2.3 Preparation of Standard Solutions:

1,2-dihydroxybenzene (catechol) was chosen to synthesize a standard solution at concentrations of 0, 20, 40, 60, 80, and 100 mg/L. First, stock solution was made by adding 0.1g catechol into 100mL ES and stirred until the catechol was completely dissolved. This was used as a 1g/L catechol stock solution. Then, 2mL of stock solution was added into 100mL dH_2O (20mg/L). This process was repeated for the remaining standard solutions by combining 4, 6, 8, and 10mL stock solution to 100mL dH_2O respectively.

The standard solutions were prepared for total phenolic determination by adding 2mL of each of the standard solutions into 10mL Folin-Denis reagent and mixed thoroughly. Then, 30mL 7.5% sodium carbonate and 10mL dH_2O. Next, the standard solutions were stored in dark conditions for 24hr and measured at 538nm with a Lambda XLS Plus spectrophotometer after incubation.

2.2.4 Determination of Total Phenolic Content (TPC):

The extract of each sample was first prepared by diluting it to a concentration of 1:20. One mL of each extract was diluted into 19mL dH_2O. Then, 1mL of each diluted extract was added to 10mL of Folin-Denis reagent and mixed thoroughly. After 5min, 30mL of 7.5% sodium carbonate and 10mL dH_2O was then added. The solutions were stirred and kept in dark conditions for 24hrs. The samples were measured at 538nm with a Lambda XLS Plus spectrophotometer.

2.3 ABTS Assay

2.3.1 Preparation of ABTS Solution:

2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) was prepared by first making stock solution of 7mM ABTS and 2.4mM potassium persulfate (PPS). To prepare the 7mM ABTS solution: 0.96g ABTS was diluted into 250mL dH_2O. To prepare the 2.4mM PPS solution: 0.648g PPS was diluted into 1L dH_2O. Then, 100mL of 7mM ABTS solution and 100mL of PPS solution were mixed into a tinted flask and stored in dark conditions for 12hrs.

2.3.2 Calibration of ABTS/PPS Working Solution:

After incubation, the ABTS/PPS solution was calibrated to 0.950±0.001 at 734nm. Initially, ABTS/PPS solution was added in 5mL increments into 200mL EtOH and measured after each addition. Approximately, 210mL EtOH and 18.5mL ABTS/PPS solution was required to bring the solution to 0.950 absorbance at 734nm.

2.3.3 Preparation of Trolox Standard Solutions:

First, 15µM stock solution of 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) was prepared. 0.375g Trolox was added to 100mL EtOH, which produced a 15mM solution. Then 1mL of the 15mM Trolox solution was diluted into 100mL EtOH, producing a 150mM Trolox solution. Finally, 10mL of the 150mM Trolox solution was added to 100EtOH, producing a 15µM Trolox stock solution.

The standard solutions of Trolox were prepared at concentrations of 4.7, 9.4, 18.8, 37.5, 75, 150, and 300µM. First, 300µM Trolox solution was made by combining 1mL 15mM Trolox stock solution with 50mL EtOH. Then 1mL of 300µM Trolox solution was added to 2, 4, 8, 16, 32, and 64mL EtOH to give 150, 75, 37.5, 18.8, 9.4, and 4.7µM Trolox standard solutions respectively.

2.3.4 Determination of Antioxidant Content (Trolox):

Working standard solutions of Trolox were prepared by adding 9mL ABTS/PPS working solution with 1mL Trolox standard solution at the various concentrations. Each solution was measured for absorbance at 415nm within 4min after mixing to ensure an accurate reading,

since Trolox reacts immediately with ABTS/PPS solution. Water was used as a reference in this experiment.

2.3.5 Determination of Antioxidant Content (Samples):

The extract of each sample was first prepared by diluting it to a concentration of 1:20. One mL of each extract was diluted into 19mL dH_2O. Then, 9mL ABTS/PPS solution was mixed with 1mL of the sample extract and measure for absorbance at 415nm.

3. CALCULATIONS:

3.1 Total Phenols/Antioxidants in Sample

$$Total_{sample} = \frac{absorbance - y\ intercept}{slope} \times concentration \quad (1)$$

3.2 Total Phenols/Antioxidants in Extract

$$Total_{extract} = sample\ volume \times Total_{sample} \quad (2)$$

3.3 Total Phenolic/Antioxidant Content of Sample in Fresh Weight

$$TPC_{fresh\ weight} = \left(\frac{Total_{extract}\ mg\ catechol}{sample\ g\ FW}\right)\left(\frac{1000g\ FW}{1kg\ FW}\right) \quad (3)$$

$$AOC_{fresh\ weight} = \left(\frac{Total_{extract}\ \mu mol\ trolox}{sample\ g\ FW}\right)\left(\frac{1000g\ FW}{1kg\ FW}\right)\left(\frac{1\ mol\ trolox}{10^6\ \mu mol\ trolox}\right)\left(\frac{250.29g\ trolox}{1\ mol\ trolox}\right)\left(\frac{1000mg\ trolox}{1g\ trolox}\right) \quad (4)$$

4. RESULTS:

4.1 Total Phenolics Determination:

The total phenolics determination assay of catechol yielded a highly linearize curve of absorbance at 538nm that varied from 0.000 to 0.367 ($y = 0.0037x + 0.0289$; $R^2 = 0.9755$). The mean TPC of the dataset was 1589.48 mg CE/kg FW. Among the samples tested, Cassava had the highest TPC (4384.52 ± 17.18mg CE/kg FW). Yucca was the median (803.75 ± 6.88mg CE/kg FW) and had a fractional TPC in comparison to Cassava. Overall, both samples of kale exhibited low TPC. Dinosaur kale had the lowest TPC (194.16 ± 6.26mg CE/kg FW). Sweet kale was the second lowest in TPC (492.24 ± 1.40mg CE/kg FW).

4.2 ABTS Assay:

The antioxidant capacity assay of trolox yielded a highly linearized curve of absorbance at 415nm that varied from 0.017 to 1.621 ($y = -0.0054x + 1.6545$; $R^2 = 0.9964$). The mean AOC of the dataset was 641.83mg TEAC/kg FW. Cassava had the highest AOC (1420.83 ± 7.71E-06 mg TEAC/kg FW). Yucca was the median (702.33 ± 3.58E-05mg TEAC/kg FW) and had nearly half the AOC in comparison to Cassava. Again, both samples of kale exhibited low AOC. Sweet Kale had the lowest AOC (114.88 ± 4.67E-06mg TEAC/kg FW). Dinosaur kale had the second lowest AOC (183.38 ± 5.85E-06mg TEAC/kg FW).

4.3 Antioxidant Percentage of Total Phenolic Content:

The antioxidant percentage of total phenolic content varied from 94.45% to 23.34%. The mean AOC percentage of the dataset was 54.74%. Here, both samples of kale comprised both the maxima and minima of the dataset. Dinosaur kale had the highest percentage of AOC (94.45%). Sweet kale had the lowest percentage of AOC (23.34%). Cranberry hibiscus had the median percentage of AOC (56.21%). Unexpectedly, Cassava had a rather low percentage of AOC (32.41%). Yucca was comparatively higher than Cassava with respect to AOC percentage (87.38%).

4.4 Reaction Kinetics of ABTS Assay:

Cranberry Hibiscus and Cassava both exhibited a rapid reaction to the ABTS/PPS compound. This was observed in the almost immediate color change in both samples when the assay was initiated. Both Cranberry Hibiscus and Cassava changed from an intensely dark blue to a clear, light yellow hue. The other samples remained a dark color.

The variance of the rate of absorption ranged from 289.41 to -2.32 when measured after 5min and 30min. Cranberry Hibiscus exhibited the lowest variance when measured at the two time intervals (-2.32). Cassava was also stable and had the second lowest variance (12.86). Taro had the highest variance in absorption between 5min and 30min (289.41). In fact, all samples excluding Cranberry Hibiscus and Cassava demonstrated a high variance in absorption (mean variance: 156.77).

5. TABLES AND FIGURES:

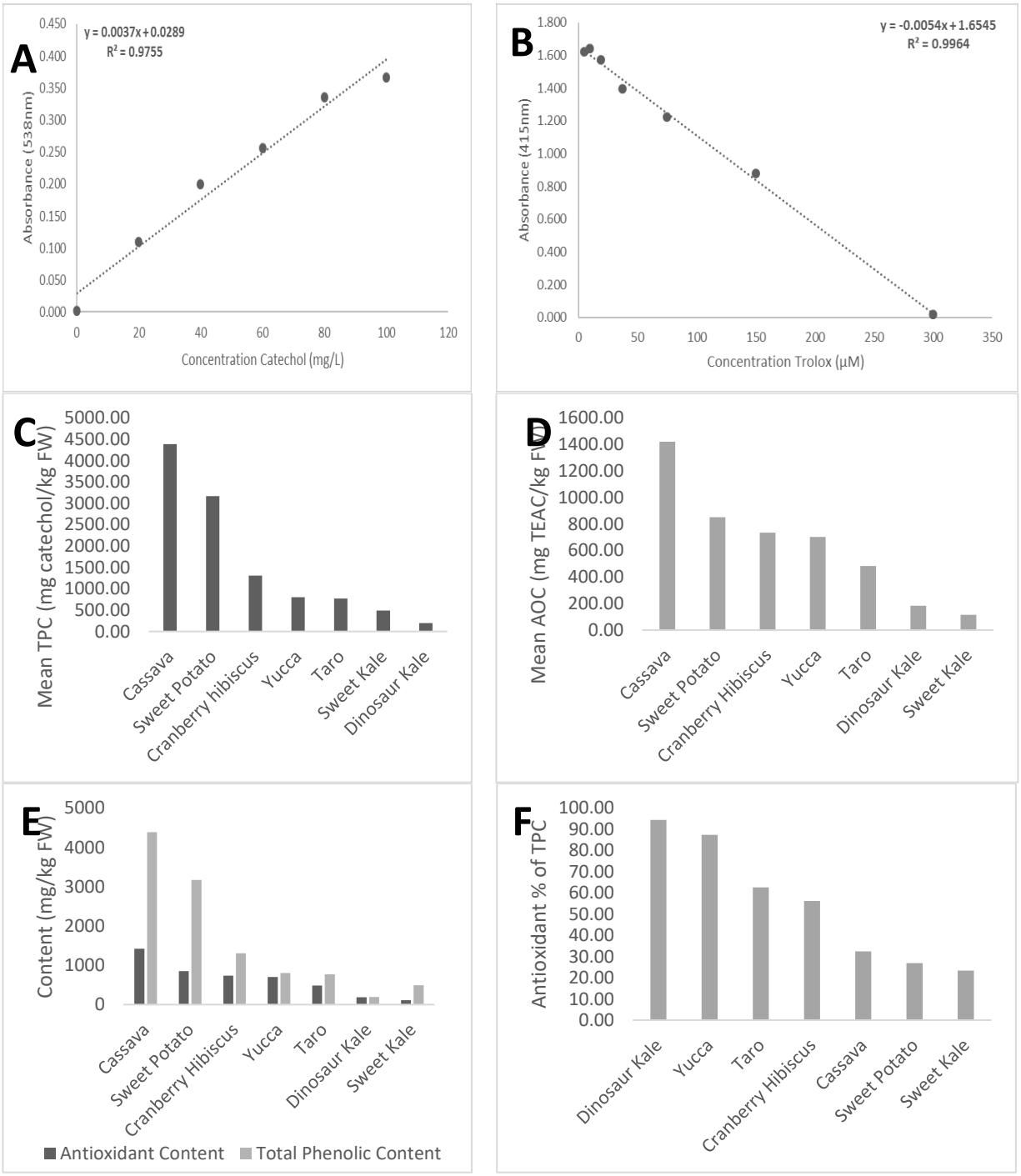

Figure 2. Graphical comparison of collected data. A.) Standard Calibration Curve of catechol measured at 538nm. B.) Standard Calibration Curve of trolox measured at 415nm. C.) Mean total phenolic content (TPC) of sample extracts measured at 538nm and reported in mg of catechol equivalents per kg of sample fresh weight. D.) Mean antioxidant content (AOC) of sample extracts measured at 415nm and reported in mg of Trolox antioxidant equivalents (TEAC) per kg of sample fresh weight. E.) Comparison of TPC and AOC. F.) Percentage of antioxidant content within the total phenolic content. TPC = total phenolic content. CE = catechol equivalents. AOC = antioxidant content. TEAC = trolox equivalents of antioxidant content. FW = fresh weight.

Table 2. Moisture content, fresh weight, dry weight, mean total phenolic content with standard deviation, mean antioxidant content with standard deviation, antioxidant percentage of total phenolic content. TPC = total phenolic content. CE = catechol equivalents. AOC = antioxidant content. TEAC = Trolox equivalents of antioxidant content. FW = fresh weight. Ranked according to moisture content from greatest to least.

Sample	Moisture Content (%)	Fresh Weight (g)	Dry Weight (g)	Mean TPC (mg CE/kg FW)	Mean AOC (mg TEAC/kg FW)	AOC Percentage of TPC (%)
Sweet Kale	97	100.00	3.00	492.24 ± 1.40	114.88 ± 4.67E-06	23.24
Dinosaur Kale	88	100.00	12.00	194.16 ± 6.26	183.38 ± 5.85E-06	94.45
Cranberry Hibiscus	86	50.00	7.00	1308.90 ± 33.16	735.71 ± 9.26E-07	56.21
Sweet Potato	83	60.00	10.00	3170.12 ± 14.48	852.45 ± 5.25E-05	26.89
Taro	83	70.00	12.00	772.68 ± 8.36	483.23 ± 5.02E-05	62.54
Cassava	73	185.00	50.00	4384.52 ± 17.18	1420.83 ± 7.71E-06	32.41
Yucca	73	60.00	16.00	803.75 ± 6.88	702.33 ± 3.58E-05	87.38

Table 3. Color change of samples at 5min and 30min after reaction with ABTS/PPS solution and corresponding absoprtion of samples at 5min and 30min after reaction with ABTS/PPS solution measured at 415nm with variance. Ranked according to variance from smallest to largest.

Sample	Color Change		Absorption (415nm)		
	5min	30min	5min	30min	Variance
Cranberry Hibiscus	Clear	Clear	738.03	735.71	-2.32
Cassava	Clear	Clear	1407.97	1420.83	12.86
Sweet Kale	Dark Blue	Dark Blue	44.84	114.88	70.03
Dinosaur Kale	Dark Blue	Dark Blue	-13.05	183.38	196.43
Yucca	Dark Blue	Dark Blue	457.19	702.33	245.14
Sweet Potato	Dark Blue	Dark Blue	566.63	852.45	285.83
Taro	Dark Blue	Dark Blue	193.81	483.23	289.41

6. DISCUSSION:

Cassava was the sample that had an absolute greater TPC and AOC. The comparatively large numbers generated by Cassava certainly appeared to be an outlier. We test the z-scores which were less than two for both TPC and AOC (1.78 and 1.76 respectively). Thus, Cassava cannot be considered an outlier among our set of samples.

Next, we consider the fact that the fresh weight and dry weight of Cassava was substantially higher than the other samples within our set. Here, the R^2 for dry weight versus TPC and AOC severally was moderate at best (0.5659 and 0.6549 respectively). Hence, dry weight, and ultimately fresh weight of the samples, were only partially attributable to the overall TPC and AOC measurements.

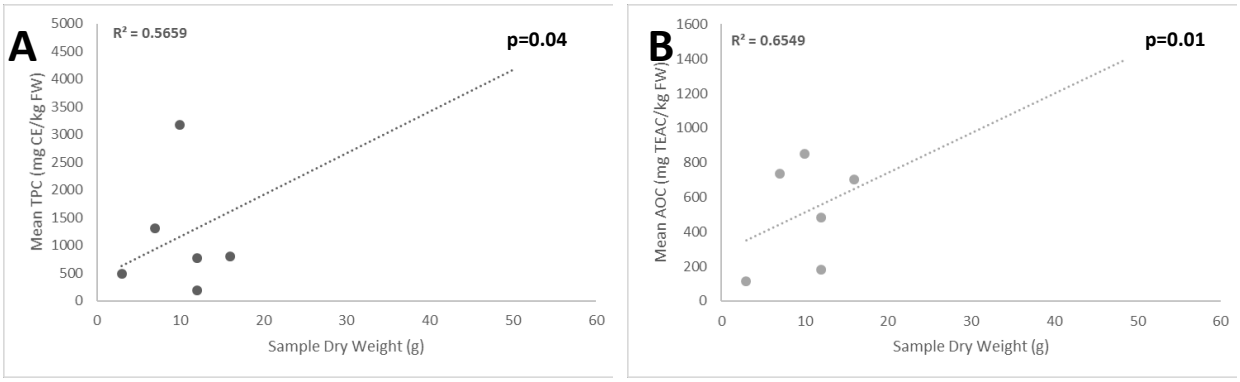

Figure 3. Graphical comparison of sample dry weight. A.) Graph of mean total phenolic content as a function of dry weight (R2=0.5659; p=0.04). B.) Graph of mean antioxidant content as a function of dry weight (R2=0.6549; p=0.01).

Despite Yucca and Cassava being the same species, there was a substantial difference in every area tested. The fact that we choose the current nomenclature should not confuse the reader, since this was done for disambiguation among the two samples which, in reality, differed only in region of origin. In all respects, both samples could have been called Cassava. The TPC between Cassava and Yucca varied by 3580.77 mg CE/kg FW and the AOC varied by 718.50 mg TEAC/kg FW. When the mean TPC and the mean AOC of both samples are compared, the error bars representing the standard deviations do not overlap. Thus, the TPC and the AOC of Cassava and Yucca exhibited no correlation despite the sameness of species.

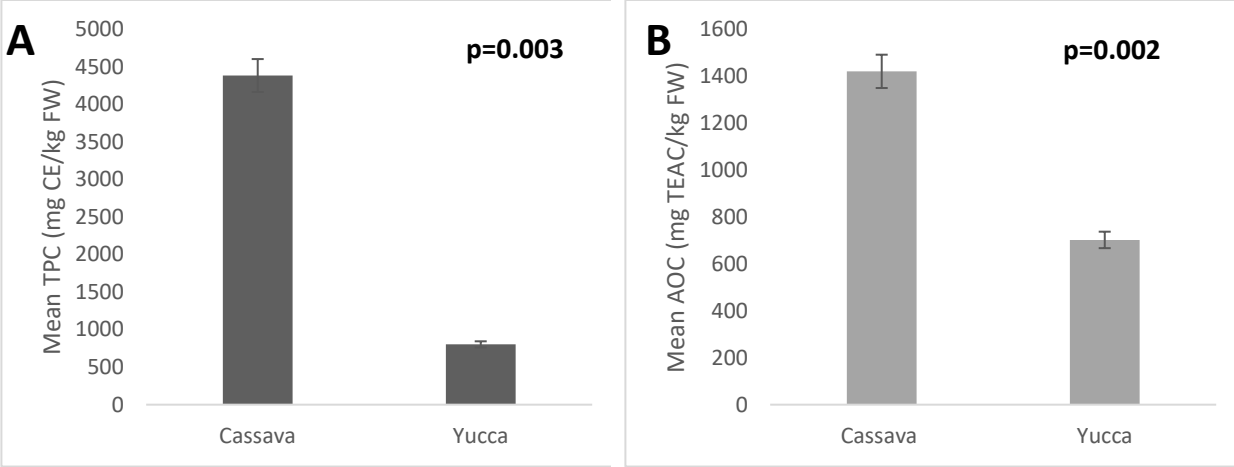

Figure 4 Comparison of the mean TPC and AOC of Cassava and Yucca. A.) The mean total phenolic content of Cassava and Yucca with standard deviation (p=0.003). B.) The mean antioxidant content of Cassava and Yucca with standard deviation (p=0.002).

Dinosaur kale and sweet kale did vary in both TPC and AOC; however their values were well within the range of one standard deviation from the mean of all sample TPC (1589.48±1571.88) and AOC (641.83±442.85). Moreover, both Dinosaur kale and sweet kale consistently ranked at the bottom two positions with respect to TPC and AOC. Thus, we do not consider their variance in TPC or AOC to be significant.

The difference we observe between dinosaur kale and sweet kale, that is supported by the data, is in the percentage of total phenols that were comprised of antioxidants. In this case, the variance was significant (71.11pp). Clearly, the data suggests that dinosaur kale has a high proportion of antioxidants despite the overall low TPC and AOC. Conversely, sweet kale has a low proportion of antioxidants and an overall low TPC and AOC.

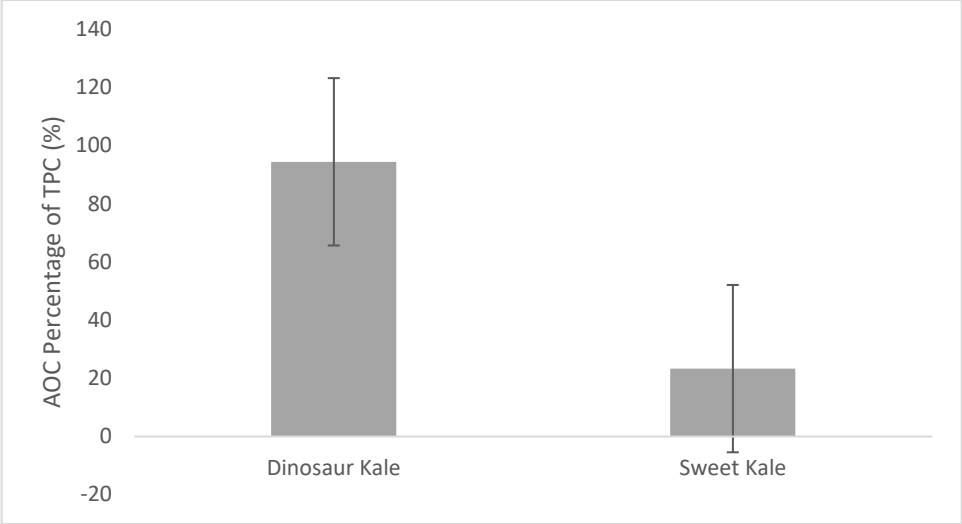

Figure 5. Graph comparing the percentage of antioxidants of dinosaur kale and sweet kale within the total phenolic content of each sample.

Careful observation of the effect of moisture content on the mean TPC and AOC appeared to show a negative relation. The sample with the highest TPC and AOC (Cassava) had the lowest moisture content (73%). Conversely, the two samples the had the lowest TPC and AOC (Sweet kale and Dinosaur Kale) had the highest moisture content (Sweet kale: 97%; Dinosaur kale: 88%). We test the correlation coefficient and determine there is a moderate, negative relation between moisture content and TPC ($r = -0.54$; $R^2=0.2942$; $p=0.04$). Furthermore, we find that there is a strong, negative correlation between moisture content and AOC ($r = -0.79$; $R^2=0.6258$; $p=0.01$).

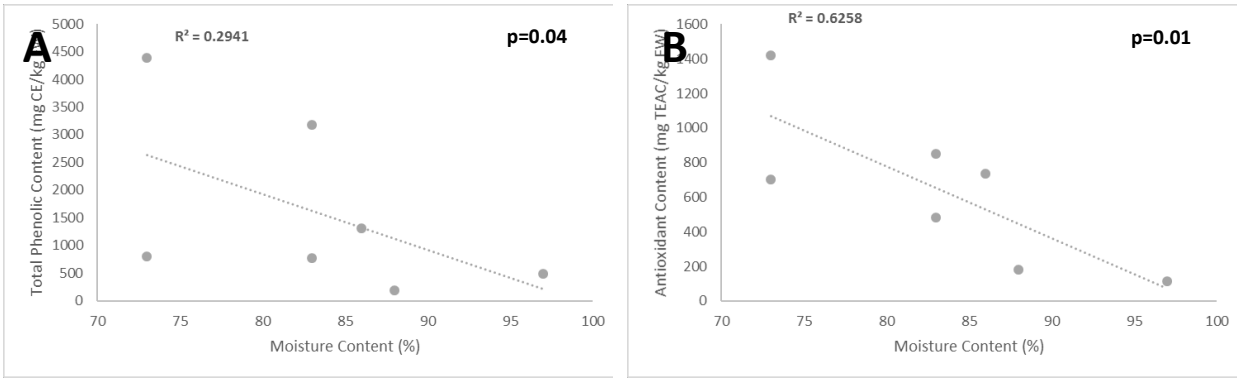

Figure 6. Comparison of the effect of moisture content on mean TPC and AOC. A.) Mean total phenolic content as a function of moisture content (R2=0.2941; p=0.04). B.) Mean antioxidant content as a function of moisture content (R2=0.6258; p=0.01).

7. CONCLUSIONS:

In conclusion, the total phenolic content and antioxidant content of five common vegetables in Hawai'i were tested and compared with vegetables from the continental United States and Rwanda, Africa. Cassava had the highest total phenolic content (TPC) and antioxidant content (AOC) of the samples surveyed. Dinosaur Kale and Sweet Kale had the lowest TPC and AOC. There were no correlation between Cassava and Yucca despite sameness of species, which implies a regional basis for the variation observed between these two samples. Additionally, the moisture content of each sample exhibited a strong, negative correlation to the AOC.

Based on the data gathered from this study: Sweet Potato, Cranberry Hibiscus, and Yucca had the highest TPC and AOC among the sample set from Hawai'i. Therefore, it follows that these vegetables should be consumed more to increase intake of dietary antioxidants and other polyphenols, such as vitamins.

8. ACKNOWLEDGMENTS:

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