EFFECT OF BLANCHING ON ANTIOXIDANT ACTIVITY, TOTAL FLAVONOIDS AND PHENOLICS OF TWO UNDERUTILISED TRADITIONAL GREEN LEAFY VEGETABLES IN NIGERIA: *PIPER UMBELLATUM* AND *SOLANUM MACROCARPON*

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ABSTRACT

The effect of blanching on the antioxidant activity, total flavonoid, and phenolic composition in the edible portions of Piper umbellatum and Solanum macrocarpon leaves was investigated. Edible portions of each vegetable were divided into two 450g groups: one portion was blanched for 5 minutes, while the other was unblanched. Antioxidant activity was measured using the DPPH assay, total flavonoids and total phenols were determined using aluminum chloride and Folin–Ciocalteu colorimetric methods, respectively. Blanching led to a marginal increase in the total phenolic content of S. macrocarpon leaves (0.06% (p>0.05)), from 159.3 ± 0.30 to 159.4 ± 0.29 GAE/100g. In contrast, total phenolic content in P. umbellatum was significantly reduced by 93.5% (p<0.00), from 158.5 ± 0.25 to 10.20 ± 0.33 GAE/100g. Total flavonoid content also decreased in both vegetables: a 26.4% loss (p<0.00) in S. macrocarpon (258.3 ± 6.24 to 190.0 ± 7.07 mg/100g) and a 4% loss in P. umbellatum (163.3 ± 6.24 to 156.70 ± 6.24 mg/100g). DPPH radical-scavenging activity modestly but significantly increased (p<0.05) in blanched samples: 1.6% for S. macrocarpon (61.4 ± 0.34 to 62.5 ± 0.09% inhibition) and 6.3% for P. umbellatum (59.5 ± 0.17 to 63.23 ± 0.25% inhibition). Blanching did not affect the phenolic content of S. macrocarpon but caused significant loss in P. umbellatum; it decreased the flavonoid content of the two vegetables but increased their antioxidant activity. Other factors responsible for the mixed trend observed need further investigation.

Keywords: ***food processing, indigenous vegetables, phytochemicals***

INTRODUCTION

Traditional green leafy vegetables (TGLVs) are important components of human diets that provide a variety of nutrients, ascorbic acid, β-carotene, minerals, and dietary fiber (Amin and Cheah, 2003; Adefegha and Oboh, 2011; Ejoh *et al*., 2019). They have also been reported to be rich sources of health-promoting non-nutrient bioactive phytochemicals (Oboh, 2005; Shahidi *et al*., 2011; Palermo *et al.,* 2014).

Evidence from several epidemiological studies showed that diets rich in vegetables and fruits have been associated with a reduced risk of several chronic diseases, in particular cancers, cardiovascular diseases, and type 2 diabetes (Steffen *et al*., 2003; Palermo *et al*., 2014). This has been attributed to the presence of the beneficial phytochemicals found in them (Prior and Cao, 2000; Jiménez-Monreal *et al*., 2009). Phytochemicals, which include phenols, flavonoids, and antioxidants, protect the human body from free radicals that cause degenerative diseases because of their antioxidant activity (Prior and Cao, 2000; Palermo *et al*., 2014)

Solanum macrocarpon, also known as the African eggplant, is a traditional leafy vegetable popularly called *'igbagba' or 'igbo'* among the Yoruba in south-west Nigeria (Oboh *et al*., 2005). The plant is characterized by its leaves, fruits, seeds, and flowers, which are variously used in soup preparation (particularly leaves and fruits) and for medicinal purposes (Oboh *et al.*, 2005; Dougnon *et. al*., 2012**;** Ejoh and Samuel, 2016). *Piper umbellatum,* also called cow foot leaf in English, and *ebe-urumhihen* among the *Esan* cultural group in Edo State in south-south Nigeria, is widely acknowledged as a medicinal plant in parts of Africa, Central and South America, and South East Asia (Roersch, 2010). *P. umbellatum* is characterized by its very broad leaves, unique scent, and it gives a distinct flavor when used in food preparation (Ndukwu and Ben-Nwadibia, 2005). For example, the leaves are a major ingredient in the preparation of a cultural delicacy, 'black soup' (Ejei-Okeke *et al*., 2017), popular among the *Esan* peoples of Edo State, Nigeria.

However, the health-promoting benefits of vegetables are affected by the kind of processing they undergo, particularly domestic cooking (Palermo *et al*., 2014). Hence, domestic cooking processes such as boiling, blanching, steaming, and microwaving, among others, need to be investigated because they are expected to affect nutrient content, activity, and bioavailability of the bioactive compounds found in traditional green leafy vegetables (Amin *et al.*, 2006; Adefegha and Oboh, 2011; Ejoh *et al*., 2019; Wireko-Manu *et al*., 2020). This is particularly important in Nigeria, where most traditional green leafy vegetables are usually cooked using various methods, especially blanching before consumption. Blanching is a process whereby the vegetables are heated in hot water or steam in order to inactivate enzyme activity, improve the texture and palatability (Wen *et al*., 2010; Shaldini *et al*., 2011).

The objective of this study was therefore to ascertain the effect of blanching on the antioxidant activity and phenol content of these two underutilised traditional green leafy vegetables in Nigeria.

MATERIALS AND METHODS

Sample collection

Fresh vegetable samples of *Solanum macrocarpon (*eggplant leaves*)*, *Piper umbellatum* (cow foot leaf) were purchased from two local markets in Ibadan metropolis (*Olorunda* local market and *Ojoo* market), Nigeria. Authentication of the vegetables was carried out at the Herbarium unit of the Department of Botany, University of Ibadan, Nigeria. All chemicals used for analysis were of analytical grade.

Sample preparation

The fresh vegetable samples were destalked, and the edible (leaves and tender stem) and inedible portions (hard stem and wilted leaves) were separated; the inedible portion was discarded. The edible portion of the vegetables was washed under tap water at room temperature to remove dirt and sand particles and rinsed with distilled water thereafter. The vegetables were drained using a stainless sieve under atmospheric conditions to remove water used for rinsing. Samples of each variety of vegetable were further divided into two equal portions, with each portion weighing approximately 450 g. For each variety of the vegetable, one portion was blanched while the other was left unblanched for further analysis. The unblanched portion of the vegetables was oven-dried at 60-70 ºC for about 20 h.

Blanching procedure

Approximately 450 ml of distilled water was brought to a boil (100˚C), and 450 g each of the edible portions of *P. umbellatum* and *S.*

macrocarpon were chopped and added to the hot water separately. The vegetable was allowed to stand in the hot water for 5 minutes. During the blanching process, the vegetables were softly agitated to ensure that all the leaves had uniform contact with the hot water. The vegetables were then drained using a stainless sieve. This blanching procedure was done in triplicate.

Extraction procedure

Approximately 2 g of pulverised unblanched and blanched samples of each of the vegetables were measured separately into 50 ml centrifuge tubes, and 20 ml of 70% ethanol (v/v) was added to each of the samples in the centrifuge tubes. Each of the samples was vortexed for one minute and then centrifuged at 500×*g* for 10 min at room temperature. After centrifugation, the supernatants were filtered using Whatman No. 42 filter paper. The filtrates were stored in the refrigerator at 4 ^{0}C before analysis. All the extracts were prepared in triplicate.

Preliminary qualitative phytochemical screening of vegetable sample extracts

The sample extracts were qualitatively evaluated for the presence of alkaloids, tannins, glycosides, saponins, steroids, resins, cardiac glycosides, carbohydrates, phlobatannins, and anthraquinones using standard methods described by Debiyi and Sofowora (1978) and Haebone (1993).

Alkaloids (Meyer's test)

About 1 g of the vegetable sample extract was acidified with 1% HCl, and the solution was allowed to stand for 2 min. Afterwards, a few drops of Dragendoff's reagents were added to the crude extract and mixed thoroughly. Formation of orange brown precipitate showed that alkaloids were present.

Tannins (Gallatin test)

About 1 g of the vegetable sample extract was stirred with water and filtered. A dirty-green precipitate, on addition of a few drops of 5% ferric chloride ($FeCl_3$) to the test extract, was taken as an indication of the presence of tannins.

Glycosides

Modified Born-Trager's test was employed to test for glycosides. About 5ml of dilute H_2SO_4 was added to about 1 g of sample extracts, and the mixture was heated in boiling water for 15 min. Fehling solutions A and B were then added, and the resulting mixture was heated to boiling. A brick red precipitate indicated the presence of glycosides.

Saponins

The froth test for saponin was used to determine the presence of saponins. About 1 g of the vegetable sample extract was dissolved in 5 mL of distilled water and mixed thoroughly. Frothing, which persisted on warming, indicated saponins.

Steroids (Salowoski test)

Chloroform and concentrated H_2SO_4 (0.2 ml) were added to 0.1 g of the vegetable sample extract to form a lower layer. A reddish brown colour at the interface between the layers indicates the deoxy-sugar characteristic of Cardolides, which indicates the presence of steroids.

Flavonoids (Shinoda's test)

About 1g of the vegetable sample extract was dissolved in dilute NaOH. A yellow solution that turns colourless on the addition of dilute HCl indicates flavonoids.

Phenols

About 1g of the vegetable sample extract was first extracted with ethyl acetate and then filtered with Whatman No. 42 filter paper. The development of blue, black, or brown coloration upon the addition of ferric chloride reagent to the filtrate indicated the presence of phenol.

Carbohydrates (Molish test)
A few drops of molish reagent were added to 2 ml of vegetable sample extract in a test tube. One (1) ml of concentrated H_2SO_4 was then allowed to flow down the side of the inclined test tube so that the acid forms a layer beneath the aqueous solution without mixing it. A reddish brown colour solution indicated a positive test.

Anthraquinones (Born-Trager's test)
To 1g of vegetable sample extract, 4mL of benzene was added and shaken together, and then filtered. 2 mL of 10% ammonia solution was added to the filtrate and shaken together. The presence of pink coloration in ammoniacal solution (lower phase) showed the presence of anthraquinones.

Resins
About 1 g of the vegetable sample extract was mixed with distilled water and filtered. Copper acetate solution (1 ml) was added, shaken vigorously, and allowed to separate. A green-colour solution was evidence of the presence of resins.

Cardiac glycosides
About 1 g of the vegetable sample extract was dissolved in 2 ml of glacial acetic acid containing one drop of ferric chloride solution. This was then underplayed with 1 mL concentrated sulphuric acid. A brown ring at the interface indicated the presence of deoxy-sugar characteristics of cardiac glycosides.

Phlobatannins
A few grams of vegetable sample extract was boiled with 1% aqueous HCL; the presence of deposit of a red precipitate was indicative of the presence of phlobatannins.

Determination of total phenol content of the vegetable samples
Total phenolic content of the extracts was quantified using the Folin-Ciocalteu colorimetric method as described by McDonald *et al*. (2001). 2 ml of the extract was mixed with 0.5 ml of Folin-Ciocalteu reagents and was allowed to stand at 40 ºC for 5 min. Approximately 1.5 ml of 20% Na_2CO_3 solution was added to the mixture and incubated for 30 min at 22ºC. The absorbance was measured at 765nm using a UV-visible spectrophotometer (Spectrum Lab 725s, China). Total phenol amount was quantified by calibrating the curve obtained from measuring the absorbance of a known concentration of Gallic acid Equivalent standard (15-300ng/ml; R^2=0.9985). The concentration was expressed as mg of Gallic acid equivalents (GAE) per 100g dry weight of samples.

Determination of total flavonoid content of the vegetable samples
Total Flavonoid content of the sample extract was determined using the aluminum colorimetric method as described by Chang *et al*. (2002). An aliquot (1 ml) of the extract was added to 0.3 ml of 5% $NaNO_3$ in a 10 ml volumetric flask and allowed to stand for 5 min. Thereafter, 0.3 ml of 10% $AlCl_3$ was pipetted into the volumetric flask, and the reaction was allowed to take place for one minute. This was followed by pipetting 2 ml of NaOH (1M) into the flask, and the volume was made up to 10 ml with distilled water. The flask content was further mixed thoroughly, and optical density was measured at 510 nm using a UV-Vis Spectrophotometer (Spectrum Lab 725s, China) in triplicate. To get the standard calibration curve, different concentrations were prepared from the stock solution of quercetin standard (1 mg/ml) using serial dilution. Quercetin standard calibration curve was constructed, and the concentration of total flavonoids in each sample was determined and expressed as milligrams quercetin equivalent per gram of dry weight of the sample.
All analyses were done in triplicate.

Determination of free radical scavenging activity of vegetable samples using 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay

Free radical scavenging activity was determined using the DPPH assay as described by Waterhouse *et al*. (2002). Different dilution of the extract was prepared in methanol and ascorbic acid (10mg/ml) and were used as standard solutions. 0.002% DPPH was prepared in methanol, and the solution was added to 0.2ml of the sample extract. The resultant solution was thoroughly mixed and thereafter incubated for 20 min at room temperature. Optical density (OD) was measured at 517 nm using a UV-visible spectrophotometer (Spectrum Lab 725s, China). Methanol (1ml) with DPPH solution (0.002%, 2.8 mL) was used as a blank. OD was recorded, and the percentage inhibition (i.e., percentage of remaining DPPH) of the sample was recorded after 5 min for individual experiments using the formula below. The concentration at which 50% of the remaining DPPH concentration is calculated from the standard DPPH graph to know the absorbance.

Percentage inhibition of DPPH Activity or Radical Scavenging Capacity (RSC) % = (1-A_{sample}/A_{blank}) X 100% where A_{blank} is Absorbance for control, while A_{sample} is Absorbance for test sample

Data analysis

Data were analysed using IBM SPSS version 20.0. Mean values obtained were compared using a paired sample t-test at $p<0.05$. The results were expressed as means and standard deviation (SD) of three replicates.

RESULTS AND DISCUSSION

Qualitative Phytochemical Screening of S. macrocarpon and P. umbellatum

Table 1 shows the results of the qualitative screening of the vegetable samples for the presence of phytochemical compounds. Unblanched sample of *S. macrocarpon* showed the presence of tannin, flavonoids, alkaloid, saponin, reducing sugar, and phenols, while the blanched sample showed the presence of the same compounds except resins.

Table 1. Phytochemical compounds present in unblanched and blanched samples of *Piper umbellatum* and *Solanum macrocarpon*

Phytochemical compound	***S. macrocarpon* (African eggplant)**		***P. umbellatum* (Cow foot leaf)**	
	Unblanched	**Blanched**	**Unblanched**	**Blanched**
Saponin	+	+	+	+
Flavonoids	+	+	+	+
Tannins	+	+	-	-
Alkaloids	+	+	+	+
Reducing sugar	+	+	+	+
Steroids	-	-	-	-
Cardiac glycosides	-	-	-	-
Resins	-	+	+	-
Phenols	+	+	-	+
Phlobatanins	-	-	-	-
Glycosides	-	-	-	-
Anthraquinones	-	-	-	-

(+) represents present while **(–)** represents absent

Dougnon *et al*. (2012) found similar phytochemicals in *S. macrocarpon* leaves except for flavonoids, which were not detected in their sample. For the *P. umbellatum*, saponin, flavonoids, alkaloids, and reducing sugar were present in both unblanched and blanched samples. Resin was present in only the unblanched sample, and phenols were present in only the blanched sample. However, tannins and steroids were absent in the *P. umbellatum* sample screened. This was contrary to the findings of Nwazuoke *et al*. (2013), whose results showed a very high amount of steroids (95%) and the presence of tannins, in addition to similar compounds, in our study. Mensah *et al*. (2013) also observed the presence of flavonoids, saponins, alkaloids, tannins, and phlobatannins in *P. umbellatum* leaves, but phlobatannins were absent in the samples of the present study.

The variations observed in the composition of phytochemical compounds could be attributed to varietal, genetic, and environmental factors (Shaldini *et al*, 2011). Phytochemical compounds are plant secondary metabolites known to contribute to the characteristic taste, odour, flavour, and colour of plants (Akula and Ravishankar, 2011). The presence of these phytochemical compounds and secondary metabolites are indications for their usefulness in varying degrees in orthodox and folk medicine and nutrition as reported by many studies as well as their contribution to the overall antioxidant capacity of the leafy vegetables (Prior and Cao, 2000; Oboh *et al.*, 2005; Roersch, 2010; Akula and Ravishankar, 2011; Mensah *et al*., 2013). For example, flavonoids have been known for a long time to have antiallergic, anti-inflammatory, antiviral, antiproliferative, and anticarcinogenic activities (Prior and Cao, 2000). They have also been reported to be the largest group of plant polyphenols responsible for most of the colour and flavor-forming compounds in fruits and vegetables (Palermo *et al*., 2014). Furthermore, ethnopharmacological and ethnobotanical studies have also shown that the uses of many of these plants for medicinal purposes is due to the presence of the plant secondary metabolites like saponins and alkaloids known to exhibit antimalarial, antimicrobial activity, antioxidant activity, modulation of detoxification enzymes, stimulation of the immune system, decrease of platelet aggregation and modulation of hormone metabolism and anticancer property, among others (Saxena *et al*., 2013; Bizuayehu *et al*., 2019; Uzor, 2020).

Effect of blanching on total phenolic content in S. macrocarpon and P. umbellatum

The total phenolic contents of unblanched and blanched leaves of *S. macrocarpon and P. umbellatum* are presented in Table 2.

Table 2. Total phenolic and total flavonoid content of unblanched and blanched samples of *Solanum macrocarpon* and *Piper umbellatum*

Leafy vegetable	Total phenols (GAE/100g)		% loss/gain	Total flavonoids (mg/100g Quercetin equivalent)		% loss
	unblanched	blanched		unblanched	blanched	
African eggplant	159.3 ± 0.30	159.4 ± 0.29	+0.06	258.3 ± 6.24	190.0 ± 7.07**	-26.4
Cow foot leaf	158.5 ± 0.25	10.20 ± 0.33**	-93.5	163.3 ± 6.24	156.70 ± 6.24	-4.0

Values are means and SD of triplicate measurements. ** values are significantly different at p>0.01

Phenolic values obtained for unblanched *S. macrocarpon and P. umbellatum* were within the range that had been reported for similar traditional green leafy vegetables in Nigeria (Adefegha and Oboh, 2011). However, the values obtained were lower than those reported by Ramos (2012). There was a mixed trend with respect to the phenolic content of the blanched vegetables. Blanching did not seem to have any significant effect on the total phenolic content of *S. macrocarpon*; the results indicated a slight increase (+0.06% $p<0.05$). This is similar to the findings of Oboh (2005), in which there was no change in the total phenol content of *Amaranthus cruentus* and *Vernonia amydalina* leaves after blanching. On the contrary, there was a significant reduction (93.5%) in total phenolic content after cow foot leaves were blanched ($p<0.00$). Similar findings were reported by Amin *et al.* (2006), who reported significant decreases of up to 51% in total phenolic content in four Amaranthus species due to blanching for 10 minutes. Cow foot leaves, compared with African eggplant leaves, are soft-textured. Being a soft-textured vegetable, it was chopped and further softened by the blanching process.

Literature sources indicate post-harvest food processing factors involving mechanical (e.g., cutting) and/ or thermal (e.g., domestic cooking) affect the level of phytochemicals, like phenols in vegetables and fruits (Tiwari and Cummins, 2013). A linear increase in blanching time decreases phytochemical levels in vegetables and fruits (Tiwari and Cunmins, 2013). But in some cases, conventional cooking, such as steaming and boiling, has brought about an increase in the total phenolic content of some vegetables. For instance, Adefegha and Oboh (2011) observed an 18 – 85% increase in phenolic content of some tropical green leafy vegetables, which were chopped and cooked by steaming for 10 minutes in 200 mL of distilled water. They suggested that the percent gain in the total phenol content after cooking could have been due to the breakdown of tough cell walls and release of phenolic compounds trapped in the fibre of green leafy vegetables. Oboh (2005) also observed up to a 2-fold increase in phenolic content after blanching of some green leafy vegetables found in Nigeria and attributed the increment to a possible breakdown of tannins to simple phenols during the blanching process.

The findings of the present study are in disagreement with the previous studies highlighted above, but in agreement with the mixed trend observed by Wen *et al*. (2010) in raw and blanched vegetables. Depending on the type of cell matrix, which was further disrupted and exposed by the mechanical process of chopping the vegetable (Ruiz-Rodriguez *et al*., 2008), the amount of water soluble phenolics (Xu and Chang, 2008) in cow foot leaves leaching into the surrounding water coupled with the heating process, that can cause degradation of the total phenolic content (Tiwari and Cunmins, 2013; Tian *et al*., 2016) are hypothetical factors that cannot be ignored in the contrasting findings with previous studies.

Effect of blanching on total flavonoid content in samples of S. macrocarpon (African eggplant) leaves and P. umbellatum (Cow foot leaf).

Blanching of the vegetables resulted in a reduction in total flavonoids in the studied vegetables (Table 2). The greater loss was observed in blanched *S. macrocarpon* leaves (26.4%), which was statistically significant ($p<0.001$) compared to 4% loss of total flavonoids in *P. umbellatum*.

As already shown for total phenol content, a general decrease of total flavonoids could be due to their leaching into the water used for blanching. For example, Mazzeo *et al*. (2011) observed significant reductions in two major flavonoids after boiling (10 minutes) and steaming (20 mins) of spinach. Similarly, different cooking treatments resulted in significant losses of anthocyanins (a flavonoid) in purple-fleshed potatoes, with the greatest losses due to frying (57.0%) and the least due to steaming (7.4%). The authors attributed the observed losses to a combined effect of thermal degradation and soaking of the potato strips in water. Flavonoids seem to decrease when subjected to thermal processing in water due to leaching and degradation (Ruiz-Rodriguez *et al*., 2008). Contrary to our observation in the present study, Adefeigha and Oboh (2011) observed a gain in flavonoid content between 8.7 – 57% in tropical green leafy vegetables.

Effect of blanching on total antioxidant activity in samples of African eggplant leaves and Cow foot leaf.

Figure 1 shows the effect of blanching on the DPPH radical-scavenging activity of African eggplant and cow foot leaves, respectively.

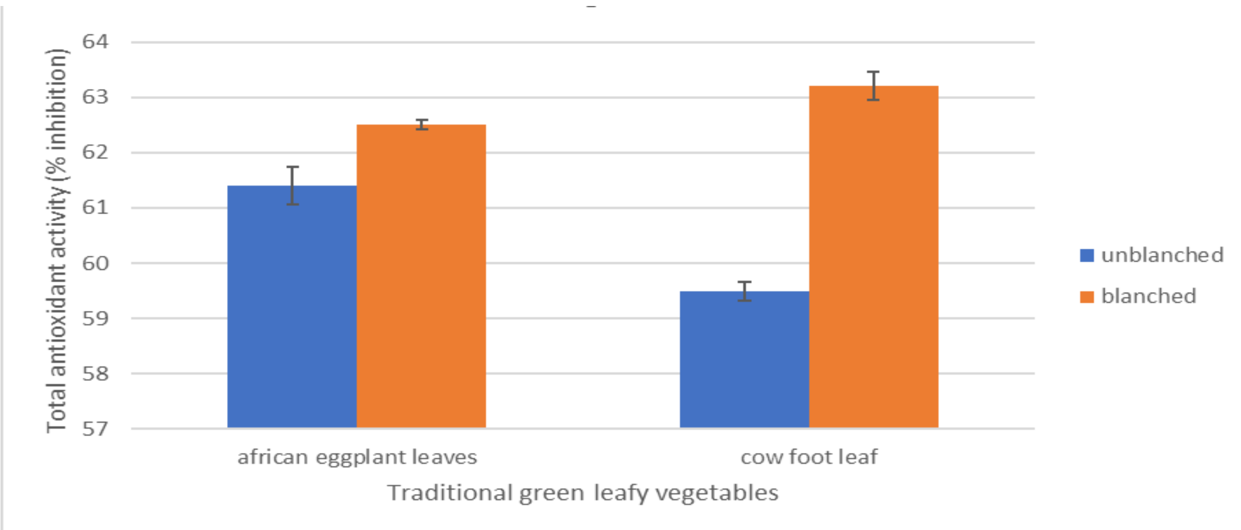

Fig 1: Total antioxidant activity (% inhibition) in *S. macrocarpon and P. umbellatum leaves*

Interestingly, despite the significant decrease in the phenolic content and flavonoid content of cow foot leaves and African eggplant leaves, respectively, after blanching, one would have expected a decrease in the antioxidant activity. However, compared with the DPPH radical-scavenging activity of the unblanched (raw) vegetables, a modest but significant increase in antioxidant activity was observed after the vegetables were blanched: 1.6% and 6.3% increase for African eggplant and cow foot leaves, respectively.

In the literature, there are mixed findings regarding the effect of blanching and other cooking methods on the antioxidant capacity of vegetables. While some previous studies have reported an increase in antioxidant capacity (Adefegha and Oboh, 2001; Oboh, 2005; Mazzeo *et al*., 2011), like our study has, others have shown otherwise (a reduction or no change at all) (Amin *et al*., 2006; Xu and Chang, 2008; Wen *et al*., 2010; Mazzeo *et al*., 2011; Tian *et al*., 2016). Previous studies have also reported that phenolic acids, flavonoids, β-carotene, ascorbic acid, among others, but especially phenolic compounds (Wen *et al*, 2010; Tian *et al*., 2016) are phytochemicals able to contribute to high antioxidant activity. The present study, therefore, suggests the possibility that the presence of other antioxidant compounds could have contributed to the increase in antioxidant activity of the blanched vegetables, despite the reducing effect of blanching on the total phenols and flavonoids.

Whether a loss or gain occurs in antioxidant activity/ scavenging capacity when vegetables are subjected to cooking procedures would depend on a number of variables. It was observed from previous studies that the type of cooking method (Oboh, 2005; Jim´Enez-Monreal, *et al*., 2009; Tian *et al*., 2016), cooking duration (time) and temperature (Amin *et al*., 2006; Xu and Chang, 2008; Mazzeo *et al*., 2011), extraction methods/ medium (Wen *et al*., 2010), nature of the food matrices, nature of the antioxidant compounds (Salawu *et al*., 2011; Tiwari *et al*., 2013), the method (assay system) used to measure the antioxidant scavenging activity (Jiménez-Monreal *et al*., 2009) among other variables, are some important factors that influence the variation in antioxidant activity/ scavenging capacity of vegetables.

CONCLUSION

There was a mixed trend with respect to the phenolic and flavonoid contents and antioxidant activity as a result of blanching the green leafy vegetables. Blanching did not affect the phenolic content of *S. macrocarpon* but caused a loss in *P. umbellatum*. This cooking method decreased the flavonoid content of the two vegetables but increased their antioxidant activity. It is necessary to further investigate other factors that could be responsible for the mixed trend observed in the traditional green leafy vegetables, such as the food matrices and the class of phenolic and flavonoid compounds present

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