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RESEARCH ARTICLE

Optimization of Plant Factory for Sourcing Natural Antioxidants: A Paradigm Shift

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Abstract

This research work was carried out to evaluate the antioxidant activities of two medicinal plants; Fluted pumpkin (*Telfairia occidentalis*) and Roselle (*Hibiscus sabdariffa*). This was done using different assays such as 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging, total antioxidant capacity, reducing power and hydroxyl (OH) radical scavenging assay. Total phenolic and flavonoid contents were estimated. Result revealed that TPC was significantly higher in *H. sabdariffa* extract (7.90µGAE/mg) than in *T. occidentalis* extract (5.62µgGAE/mg) while *T. occidentalis* extract had higher TFC (12.88µgRE/mg) than in *H. sabdariffa* (5.38 µgRE/mg). Our result also indicated that there were significant differences ($P < 0.05$) in the free radical scavenging efficacy of the herbal extracts and the standards used though the hydroxyl radical scavenging capacity of *T. occidentalis* extract performed better even to gallic acid standard. However, comparing the extracts, *H. sabdariffa* extract was better for DPPH scavenging ability, total antioxidant capacity and reducing power ability. These effects were concentration dependent. Explicitly, the consumption of *H. sabdiraffa* and *T. occidentalis*, especially their extracts could significantly reduce several pathological conditions linked to oxidative stresses going by the *in vitro* assays.

Introduction

Several activities of man generate free radicals either endogenously or exogenously (Abheri et al., 2010) and these undoubtedly result in serious pathological effects such as cancer, cardiovascular disorder, liver diseases, ischemia, anaemia, asthma, arthritis, inflammation, neuro-degeneration, Parkinson diseases, monogolism, ageing process etc. (Lee et al.,2001; Kumar etal.,2007; Kottaimuth, 2008; Tripathy et al.,2010). These pathological state , aforementioned occurs due to the fact that there is imbalance of the oxidative stress orchestrated by the generated free radicals via the production of reactive oxygen species (ROS) and the capacity of the biological system to neutralize the free radicals (Hadi et al.,2007). The free radicals that could be generated are superoxide anion(O_2^-), hydrogen perioxide (H_2O_2), hydroxyl radical (OH), nitric oxide (NO), and organic hydroperoxide (ROOH) which are chemically unstable atoms owing to the lone pair of electrons in their outer shells (Vilasrao et al., 2010; Aluko et al.,2013).

According to Oboh (2006) these are enzymatic antioxidant (catalase, superoxide dismutase, glutathione reductase) and non enzymatic (vitamin C & E, β-carotene, uric acid) whose function is to quench and neutralize damaging effect of these free radicals. These notwithstanding, there are synthetic antioxidants that are taken to enhance the capacity of the inherently antioxidant to scavenge free radical such as terbutyhydroxyl toluene(IBHCL), butylated Hydroxyl Anisole (BHA), Butylated Hydroxyl toluene (BHT), etc. Interestingly, in the last three decades, there is a paradigm shift as it pertains to sourcing natural antioxidants from plant origin (Dehghan et al., 2007; Kai-Wei et al., 2009),

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especially the indictment of these synthetic antioxidants are being harmful (Kukic et al.,2006; Oyetoyo, 2009; Rao et al., 2010; Vijayakumar et al., 2012;).

Obviously, medicinal plants elicit pharmacological and therapeutic potential because of the inherent bioactive compounds such as polyphenolic compounds, flavonoids, proanthocyanidius, flavoids, saponins, alkaloids, tannins(Ikpeme et al., 2012) and some of these metabolites have been implicated to possess antioxidant properties (Middleton et al.,2000; Oboh and Rocha, 2007; Uijayakumar et al.,2012; Aluko et al.,2013; Uyoh et al., 2013). *Telfairia occidentalis* has been reported to be highly nutritive with superb medicinal values (Dina *et al.,* 2006; Oboh *et al.,* 2006; Nwanna and Oboh, 2007; Kayode *et al.,* 2010; Kayode and Kayode, 2011; Anokwuru *et al.,* 2011). Iweala and Obidoa (2009) reported that *T. occidentalis* contains high level of phenols, favonoids, ascorbic acid, vitamins and other important macronutrients. On the other hand, studies have revealed that *Hibiscus sabdariffa* commonly known as roselle has medicinal importance including antioxidant potential (Obi *et al.,* 2005; Mowuogwu and George, 2008; Mordi *et al*., 2011). Some of the phytochemical inherent of the plant are flavonoid, phenols, ascorbic acid, glycolic acid, hibiscetine, sabdaretine, gossypetine, vitamins, etc (Yadong *et al.,* 2005).

T. occidentalis and *H. sabdarifta* are two plants that indigenous people of Nigeria consume on a daily basis, thus the need to be screen for antioxidant capacity. This current research is expedient owing to the fact that plant resource needed to be commercially developed and optimized as sources of natural antioxidant reservoir, especially plant that are naturally consumed such as *T. occidentalis* and *H. sabdariffa*

Materials and methods

Plant materials and extraction

Fresh leaves of *T. occiddentalis* and calyces of *H. sabdariffa* were purchased from Watt Market, Calabar. The plants were properly identified and authenticated at the herbarium of the Department of Botany, University of Calabar. The leaves and calyces were freed from dirt, air dried at room temperature for one week and then finely milled separately using a blender (Model: 5KSB655CCS0). The ground samples were extracted by soaking in 100ml of methanol for 3 days with intermittent shaking. The filtrates were then filtered into separate containers using Wattman No.1 filter paper and concentrated in a water bath at 45^0C. 0.3g of each concentrated extract was accurately weighed and dissolved in 100ml of methanol :water (90 :10 v/v) to a sample stock solution of 3000µg/ml and stored in the refrigerator from where the working solutions of each extract were prepared subsequently as desired by appropriate dilutions. DPPH (1,1-diphenyl-2-picry hydrazyl) radical and rutin were purchased from Sigma Aldrich Chemical Company, USA while others were procured from Kem Light Laboratories pvt. Ltd., Mumbai, India and these chemicals were of analytic grade.

Determination of total phenol content (TPC)

The total phenolic content of *T. occidentalis* and *H. sabdariffa* extracts were determined using the Folin-Ciocalteau method (Duarte *et al*., 2006). One hundred microlitre (100µl) of Folin-Ciocalteau reagent and 6ml of distilled water were added into a test tube containing 0.5ml of each of the extracts. Each test tube was shaken and 2ml of 15% sodium carbonate ($NaCO_3$) was added and then shaken once again for 30 seconds. Finally, the solution was brought up to 10ml by adding distilled water. After 1.5hr incubation at room temperature, the absorbance at 750nm was read using a spectrophotometer (LABTECH UV/VIS Spectrophotometer, India). Gallic acid monohydrate, a standard phenol in different concentration (0, 30, 60, 90, 120µg/ml) was used to prepare a standard reference curve. The total phenolic content of the extracts were expressed as Gallic Acid Equivalents (GAE).

Determination of total flavonoid content (TFC)

The method of Dewanto *et al*. (2002) was adopted to assay total flavonoid content of *T. occidentalis* and *H. sabdariffa* extracts. 1ml of each extract (containing 0.1µg/ml) was diluted with 4ml of distilled water in a 10ml volumetric flask. Initially, 0.3ml of 15% sodium nitrite ($NaNO_2$) solution was added to each volumetric flask, at 5mins, 0.3ml of 10% aluminium chloride ($AlCl_3$) was added; and at 6min, 2ml of 1.0M sodium hydroxide (NaOH) was added to each reaction flask and properly mixed. Absorbance of the reaction mixture was read at 510nm after 30mins. The total flavonoid content (TFC) of the two extracts was expressed as Rutin Equivalents (RE).

2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

The ability of the extracts to scavenge DPPH radical was determined according to the method of Mensor *et al*. (2001). Four different concentrations (30, 60, 90, 120µg/ml) of the extract were prepared in methanol. 2.5ml solution of the extract or reference standard was added to 1ml of 0.3mM of freshly prepared DPPH solution in methanol and allowed to

react in the dark at room temperature for 30mins. The absorbance of the resulting mixture were measured at 518nm using a spectrophotometer. Methanol (1ml) and each extract or standard solution (2.5ml) was used as a blank. 1ml of 0.3mM DPPH solution and 2.5ml of methanol was used as the negative control. Solution of gallic acid and ascorbic acid were used as a reference standard and positive control. Percentage scavenging (antioxidant) activity of DPPH radical was determined using the formula:

% scavenging activity = 100 – [(As-Ab/Ac) × 100]

Where As= Absorbance of sample; Ab= Absorbance of blank; Ac= Absorbance of control

Total antioxidant capacity

The total antioxidant capacity was carried out using the phosphate-molybdenum assay (Jayaprakasha *et al*., 2002). 300µl different concentrations (30, 60, 90, 120µg/ml) of the extract and standard (ascorbic acid and rutin) were mixed with 3ml of reagent solution containing, 0.6 mM H_2SO_4, 28mM sodium phosphate and 4mM ammonium molybdate in test tubes. The test tubes were capped with aluminium foil and incubated in a boiling water bath at 95°C for 90 minutes. A blank was constituted with 3ml of reagent solution and 300µl of distilled water and incubated under the same condition as the rest of the samples. The reaction mixtures where then allowed to cool to room temperature and absorbance was measured at 695nm against the blank.

Reducing power assay

Different concentrations (30-120µg/ml) of the samples were prepared and 1ml of each solution was mixed with 2.5ml of phosphate buffer (0.2M, pH 6.8) and 2.5ml of potassium ferricyanide. The mixture was then incubated in a water bath at 50°C for 20mins. To this mixture, 2.5ml of 10% trichloroacetic acid was added and then centrifuged (Rolofix 32, Germany) at 3000rpm for 10mins. The upper layer of the solution (2.5ml) was mixed with 2.5ml of distilled water and 0.5ml of 0.1% ferric chloride was added. Absorbance of the pert Prussian blue solution formed was measured at 700nm. Increasing absorbance value indicates increasing reducing power (Sheetal *et al.*, 2007).

Hydroxyl (OH) radical scavenging assay

This was determined by the Fenton reaction using the ortho-phenanthroline method as modified by Nagulendran *et al*. (2007). 4 ml of sodium phosphate buffer (0.2M, pH 7.4), 1.5ml of 5mM orthophenanthroline (1, 10 phenanthroline) in ethanol and Iml of 7.5mM Iron (II) sulphate were mixed immediately, in 1ml of the extracts and references at different concentrations (0.5 – 2.5mg/ml). 1.5ml of distilled water and 1ml of 1% hydrogen peroxide were added to the mixture solution in sequence. After incubating at 37°C for 60 min, the change of reaction mixture in absorbance, caused by the colour change of Fe-orthophenanthroline was measured at 510 nm. Hydroxyl radicals scavenging activity was evaluated as:

Scavenging Activity (%) = $[(A_s – A_o/A_b – A_o)] \times 100$

Where A_s = absorbance of reaction mixture with sample; A_o = Absorbance of damage control; A_b = Absorbance of blank

Data collection and analysis

The experiment was laid out in a completely randomized design using a 4 x 4 factorial layout. Data collected were analyzed using analysis of variance (ANOVA) and significant means separated using Least Significant Difference (LSD) test. However, total phenolic and flavonoid contents were analyzed using the student's t-test.

Results

Total phenolic content (TPC) and total flavonoids content (TFC) in the two plant extracts

Our result revealed that TPC was significantly higher in *H. sabdariffa* extract (7.90µGAE/mg) than in *T. occidentalis* extract (5.62µgGAE/mg) while *T. occidentalis* extract had higher TFC (12.88µgRE/mg) than in *H. sabdariffa* (5.38 µgRE/mg) (Fig. 1).

Extracts effect of DPPH, total antioxidant capacity, reducing power and hydroxyl radical scavenging activities of *T. occidentalis, H. sabdariffa* and the standard controls

Our result indicate that there were significant differences ($P < 0.05$) in the free radical scavenging efficacy of the herbal extracts and the standards used though the hydroxyl radical scavenging capacity of *T. occidentalis* extract performed better even to gallic acid standard. However, comparing the extracts, *H. sabdariffa* extract was better for DPPH scavenging ability, total antioxidant capacity and reducing power ability (Table 1).

Fig. 1: Total phenolic and flavonoid contents in *T. occidentalis* and *H. sabdariffa* extracts

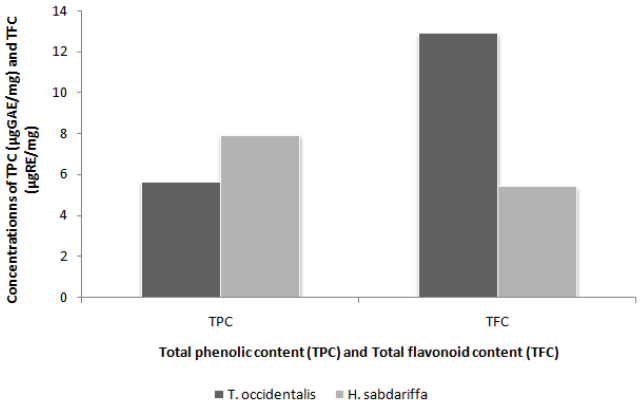

Table 2: Effect of concentration on the scavenging properties of the extracts and the standards

Concentration of extract (µg/ml)	DPPH scavenging activity (%)	Total antioxidant capacity	Reducing power
T. occidentalis extract			
30	10.60j±0.01	0.018p±0.001	0.051l±0.001
60	11.91i±0.02	0.069l±0.001	0.057k±0.002
90	7.84k±0.01	0.091j±0.003	0.058k±0.001
120	14.49h±0.03	0.108g±0.002	0.073j±0.001
H. sabdariffa extract			
30	63.62g±0.23	0.025o±0.001	0.051l±0.001
60	71.55f±0.34	0.082k±0.002	0.075j±0.002
90	78.89e±1.02	0.093i±0.001	0.099i±0.001
120	86.47d±0.69	0.096h±0.003	0.117h±0.002
Gallic acid/rutin			
30	94.47c±0.84	0.059m±0.002	1.111g±0.004
60	95.14b±0.79	0.329f±0.001	1.230f±0.003
90	95.29b±1.23	0.355e±0.002	1.230f±0.001
120	95.26b±2.12	0.753c±0.001	1.265c±0.005
Ascorbic acid			
30	97.05a±0.03	0.049n±0.001	1.251d±0.002
60	96.82a±0.54	0.696d±0.003	1.240e±0.003
90	96.75a±0.56	0.800b±0.002	1.277b±0.004
120	97.12a±2.34	1.028a±0.002	1.288a±0.001

Means with different superscripts within each column differ significantly (P<0.05) from each other

Fig. 2: Effect of concentration on the hydroxyl radical scavenging capacity of the herbal extracts and the standards.

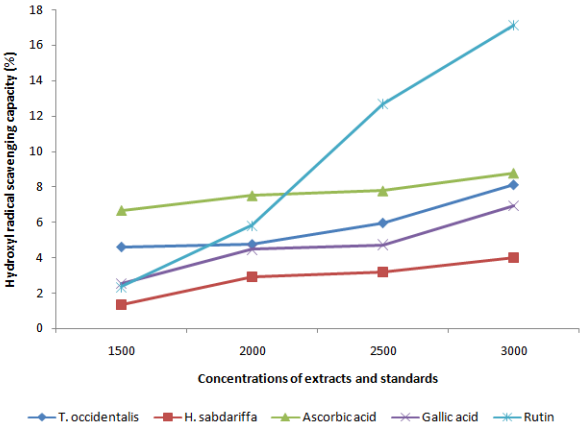

Table 1: DPPH radical scavenging, total antioxidant capacity, reducing power and hydroxyl radical scavenging of *T. occidentalis, H. Sabdariffa* and the standard controls

	Extracts		Standards		
Parameters	*T. occidentalis*	*H. sabdariffa*	Gallic acid	Ascorbic acid	Rutin
DPPH scavenging (%)	$11.21^{d} \pm 0.55$	$75.13^{c} \pm 1.95$	$95.04^{b} \pm 0.08$	$96.99^{a} \pm 0.03$	-
Total antioxidant capacity	$0.072^{d} \pm 0.01$	$0.074^{c} \pm 0.01$	-	$0.643^{a} \pm 0.08$	$0.369^{b} \pm 0.06$
Reducing power	$0.060^{d} \pm 0.002$	$0.086^{c} \pm 0.01$	-	$1.264^{a} \pm 0.004$	$1.209^{b} \pm 0.014$
Hydroxyl radical scavenging (%)	$5.840^{c} \pm 0.30$	$2.839^{e} \pm 0.20$	$4.656^{d} \pm 0.40$	$7.676^{b} \pm 0.20$	$9.488^{a} \pm 1.30$

Means with different superscripts within each row differ significantly ($P<0.05$) from each other. (-) = The particular standard was not used in the assay.

Concentration effect of DPPH, total antioxidant capacity, reducing power and hydroxyl radical scavenging activities of *T. occidentalis, H. sabdariffa* and the standard controls

Results from these assays showed that when the concentration of the extracts and the standards increase, the capacity to scavenge free radicals also increases. However, for DPPH free radical scavenging ability, *T. occidentalis* extracts performed poorly while extract of *H. sabdariffa* comparatively with the standards was good. Additionally, *T. occidentalis* extract had better total antioxidant capacity (0.108) than *H. sabdariffa* extract (0.096) at the concentration of 120µg/ml in comparison with standards at the concentration of 30µg/ml. On the contrary, the reducing power of *H. sabdariffa* extract was significantly higher ($P < 0.05$) (0.117) than *T. occidentalis* extract (0.073) at the concentration of 120 µg/ml (Table 2). In hydroxyl radical assay, extract of *T. occidentalis* performed better than *H. sabdariffa* extract, gallic acid standard while ascorbic acid was a bit higher, especially at the highest concentration of 3000 µg/ml (Fig. 2).

Discussion

The optimization of medicinal plants for pharmaceutical and therapeutic purposes, including free radical scavenging is informed undoubtedly by the reported safety, efficacy and economy (Ikpeme et al., 2011; 2012). According to Ikpeme et al. (2012) the current global trend in the utilization of medicinal plants may have obviously led to a paradigm shift in sourcing and screening medicinal plants for their ability to scavenge free radicals produced in the human systems (Dehghan et al., 2007; Kai-Wei et al., 2009; Modi et al., 2010). This shift is orchestrated by the reports of Middleton et al. (2000), Oboh and Rocha (2007), Vijayakumar et al. (2012), Aluko et al. (2013) regarding the toxic and harmful effects of synthetic antioxidants.

Our result on total phenolic and total flavonoids contents showed that *H. sabdiraffa* extract had higher TPC and the extract of *T. occidentalis* while *T. occidentalis* extract had more TFC than *H. sabdiraffa* extract. Polyphenolic content and flavonoids have been reported to elicit antioxidant properties (Miliauskas et al., 2004; Melo et al., 2005; Nickavar et al., 2007; Oyedemi et al., 2010; Basma et al., 2011; Uyoh et al., 2013). It is therefore wise to speculate that *H. sabdiraffa* and *T. occidentalis* extracts might possess antioxidant properties. This is what this current research is poised to investigate using *in vitro* assays.

Different assays were carried out to determine the potency of the two extracts scavenging free radicals using standards as reference points. It revealed that *H. sabdiraffa* extract possess high antioxidant activity using DPPH and reducing power assays while *T. occidentalis* was better in total antioxidant capacity and hydroxyl radical scavenging assays. Though the standards used were better in all the assays, the highest concentrations of the herbal extracts used were better than the lowest concentrations of the standards. Comparing the two herbals, the observed differentials are obviously linked to the levels of TPC and TFC. One might be tempted to assume that TPC enhances the scavenging of DPPH and reducing power of the extract while TFC acts more on the hydroxyl radical scavenging and increasing the total oxidative capacity of the extract. However, beyond this, the nature and the variants of these compounds are very crucial in their functions. Though polyphenolic compounds and flavonoids have been chiefly implicated in free radical scavenging activities, it will not be a disconnect to postulate that

there may also be direct or indirect synergism between the other bioactive mixture inherently in these plants in their free radical scavenging power.

According to Duh et al. (1999) the reducing capacity of a compound is an indication of its potential antioxidant activity due to the presence of reductants. This suggests that *H. sabdiraffa* might possess a significant level of reductants. Hydroxyl radicals have been implicated to cause oxidative damage of DN, proteins and lipids (Spencer et al., 1994), which is as a result of the interactions between metal ions such as ferrous or copper with hydrogen peroxide. This means that *T. occidentalis* extract could be a candidate for combating the oxidative stress caused by hydroxyl radicals.

Dean and Davies (1993) observed that free radicals such as hydroxyl radicals, superoxide anion radical, etc. attack the unsaturated fatty acids in the biomembranes resulting in membrane lipid peroxidation. This is a condition where there is a decrease in membrane fluidity, loss of enzymes and receptor activity and damage to membrane proteins, leading to cell inactivation (Kottaimuthu, 2008). The implication of our result implicitly is that extracts from *H. sabdiraffa* and *T. occidentalis* could increase membrane fluidity, enzymes and receptor, thus averting the damage to membrane proteins. It is also probable that these extracts might impede ROS-mediated injuries due to lipid peroxidation, oxidative DNA damage and protein oxidation (Bahramikia et al., 2009; Sfahlan et al., 2009).

It is interesting to mention that free radicals generated during normal metabolic processes can be scavenged by dietary antioxidants assimilated in the liver through the hepatic portal vein (Youdim et al., 2002; Mazza et al., 2003). This perfectly fit in the context of this present research. Our results point to the fact that the two plant extracts have different capacities in their oxidative stress amelioration and more importantly specific to the type of oxidative stress initiated. This is thrillingly informative as this is an indication that to achieve holistic and perfect free radical scavenging activity, more than one herbal combination might be necessary. It should be stressed that the beauty in sourcing medicinal plants, especially vegetables, fruits and spices with inherent capacity to scavenge free radicals in the human system is the fact that they are consumed in our everyday meal.

Aiyegoro et al. (2010) documented that plants are reservoirs for free radical scavenging molecules such as vitamins, terpenoids, phenolic acids, flavonoids and other metabolites, which are basically rich in antioxidant activities. It is quite interesting to inform that these plant metabolites can be genetically manipulated to increase their yield. Additionally, the DNA copy or gene(s) responsible for the expression of these metabolites could be cloned and inserted into other edible crops for ease of consumption by end users. This implies that it might be unnecessary to go over the counter medicine stores to buy synthetic drugs to this respect.

Conclusion

Explicitly, the consumption of *H. sabdiraffa* and *T. occidentalis*, especially their extracts could significantly reduce several pathological conditions linked to oxidative stresses going by the *in vitro* assays. We are still mindful of the fact that there is a seeming controversy that herbals with antioxidant properties *in vitro* might not elicit same *in vivo*. Interestingly, our research team is currently verifying this claim using rat model.

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