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EFFECT OF DEOXALATION ON IN-VITRO ANTIOXIDANT ACTIVITY AND INHIBITION OF FERRIC INDUCED LIPID PEROXIDATION OF BEETROOT JUICE

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Abstract

There is an increased interest in the biological activity of red beetroot (*Beta vulgaris*) and its potential utility as a health promoting and disease preventing functional food. However, beetroot juice was known to be rich in oxalate which can also be a precursor to kidney stone formation. Hence, beetroot juice and its deoxalated form were investigated in this work to determine the effect of deoxalation as well as the contributory effect of the oxalate in the juice. *In-vitro* antioxidant assays (as typified by reducing potential, DPPH radical scavenging, Hydroxyl radical scavenging and total antioxidant capacities) as well as total phenolic, total flavonoid and lipid peroxidation inhibition were evaluated. The whole beetroot was shown to be the best result in enhancing the antioxidant status. As it was evident that deoxalation caused a decrease in the antioxidant potential of beetroot. These results indicated that oxalate, despite being the anti-nutrient in beetrrot, have a synergistic activity it confers on the total antioxidant activity of beetroot. It is thus presented that deoxalated beetroot juice, as the safest form of edible beetroot, a potential nutraceutic resource and may be used as a functional juice.

Keywords: Beetroot, deoxalation, antioxidant, lipid peroxidation, nutraceutic

Introduction

Beetroot (*Beta vulgaris*) is a crop belonging to the *Chenopodiaceae* family having a bright crimson colour and it is well known for its juice and medicinal properties (Kumar, 2015). Beetroot is also one of the few vegetables that contain a group of highly bioactive pigments known as betalains (Lee *et al.*, 2005; Vulić *et al.*, 2014). The betalain family are categorised as either betacyanin pigments (red-violet) or betaxanthin pigments (yellow-orange) (Clifford *et al.*, 2015**)**. A number of in vitro investigations had reported betalain pigments in particular to protect cellular components from oxidative injury (Kanner *et al.*, 2001; Reddy *et al.*, 2005; Tesoriere *et al.*, 2008). The major anti-nutrient that had hindered its exploitation of beetroot on a wide scale is its oxalic acid content (Natesh *et al.*, 2017) which takes up calcium to form calcium oxalate – a precursor for kidney stone; this informed the idea of an oxalate-free beet juice.

Oxalate is a strong dicarboxylic organic acid ($C_2O_4H_2$) that is produced endogenously and consumed in dietary sources. Although, according to Fargue *et al.,* 2018, the complete pathways that lead to endogenous oxalate synthesis are virtually still unknown, current research is being conducted to better understand the complete process of oxalate production. It is widely agreed that oxalate is

produced endogenously in the liver as the metabolic end product of various amino acids, ascorbate, glycine, and glyoxal (Fargue *et al.,* 2018). Oxalate, when in the insoluble salts form, it can pass through the intestines to be excreted in the feaces or degraded by oxalate-degrading bacteria known as *Oxalobacter formigenes*. However, when in the form of soluble salt, it is believed to be readily absorbed in the digestive tract by passive diffusion in the small intestine and colon (Savage *et al.,* 2000); or it may bind to divalent mineral cations such as calcium and magnesium, which causes the minerals to be unavailable for absorption and use by the body (Savage *et al.,* 2000)

This study is therefore targeted at assessing the effect of deoxalation of beetroot juice on the functional, antioxidant and inhibition of ferric induced lipid peroxidation activities.

Materials and Methods

Chemicals

All chemical used were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and solvents were of the highest analytical grade.

Materials

Beetroot (*Beta vulgaris*) were sourced from the local market in Akure and was identified in the School of Agriculture and Agricultural Technology, Federal University of Technology, Akure, Ondo State, Nigeria

Preparation of Extract

Beetroots were washed in running water, cut into pieces. The juice was extracted using an extractor and its oxalate content precipitated by a method described by Bong *et al.*, 2017.

In-Vitro Assays

Total Phenol Content Determination

The total phenolic content of extract was determined using the Folin-Ciocalteu's method of Singleton *et al.,* (1999) as modified by Zilic *et al.*, (2012). 0.1 ml of the juices (100 µg/ml) was rapidly mixed with 0.1 ml of Folin Ciocalteu reagent, followed by the addition of 0.3 ml sodium carbonate (7.5%, w/v) solution. The mixture was incubated in the dark for 30 min. The absorbance of the blue colour was read at 760 nm after 30 min on a spectrophotometer. The total phenolic content was extrapolated from a standard curve using gallic acid (graded concentration, 50 – 250 µg/ml) as a standard. The amount of total phenolics was expressed as gallic acid equivalent (GAE) mg GAE/g sample through the calibration curve of gallic acid.

Total Flavonoid Content Determination

The total flavonoid concentration was determined spectrophotometrically based on the procedure of (Marinova *et al.*, 2005). 0.5 ml of the juices and standard (quercetin) at different concentrations (100 µg/ml) were taken in test tubes dissolved in methanol followed by the addition of 0.1 ml of 10% aluminum chloride solution. 0.1 ml of 1 M sodium acetate solution was added to the mixtures in the test tubes. Furthermore, each reaction test tube was then immediately diluted with 2.8 ml of distilled water and mixed to incubate for 30 min at room temperature to complete reaction. The absorbance of pink colored solution was noted at 415 nm using a spectrophotometer against blank methanol. Total Flavonoid Concentration (TFC) of the extract was expressed as quercetin equivalents (QE).

Total Antioxidant Capacity of the Extract

The total antioxidant capacity was determined by the ABTS test described by Re *et al.* (1999).

The ABTS* radical cation was pre-generated by mixing equal volumes 7 mM ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and incubating for 12–16 h in the dark at room temperature until the reaction was complete and the absorbance was stable. The absorbance of the ABTS* solution was equilibrated to 0.70 by diluting

with water at room temperature. The ABTS* solution (1 ml) was mixed with 10 µl of the test sample dissolved in distilled water (40, 80 and 100 µg/ml final concentration) or trolox standard dissolved in deionized water (40, 80 and 100 µg/ml final concentration). The absorbance was measured at 734 nm after 6 min. All experiments were carried out in replicates. The trolox equivalent antioxidant capacity was subsequently calculated.

DPPH Radical Scavenging Capacity of the Extract

The DPPH radical scavenging activity was determined as described by Singh, (2002). The juice (40, 80 and 100 µg/ml final concentration) or reference compound, ascorbic acid (40, 80 and 100 µg/ml µg/ml final concentration) was added to a methanol solution of DPPH (0.03 mM). The mixture was shaken and left to stand at room temperature for 30 minutes. The absorbance of the resulting solution was measured spectrophotometrically at 517 nm. The radical scavenging activity was calculated as percentage of DPPH discolouration as given below:

$$\% \text{ Inhibition} = \left[\frac{Abs_{control} - Abs_{ext}}{Abs_{control}}\right] X\ 100$$

Where: $Abs_{control}$ = Absorbance without extract

Abs_{ext} = Absorbance with extract

Reducing Potential of the Extract

The Fe^{3+}- reducing power of the extract was determined by the method of Oyaizu (1986) with a slight modification. Different concentrations (40, 80 and 100 µg/ml) of the extract (0.5 ml) or the positive control, ascorbic acid (40, 80 and 100 µg/ml) were mixed with 0.5 ml phosphate buffer (0.2 M, pH 6.6) and 0.5 ml potassium hexacyanoferrate (0.1%), followed by incubation at 50°C in a water bath for 20 min. After incubation, 0.5 ml of TCA (10%) was added to terminate the reaction and the reaction mixture was centrifuged at 3000 g for 5 min. The upper portion of the solution (1 ml) was mixed with 1 ml distilled water, and 0.1 ml $FeCl_3$ solution (1%) was added. The reaction mixture was left for 10 min at room temperature and the absorbance was measured at 700 nm against distilled water blank and the reducing potential of the extract was subsequently calculated using ascorbic acid as standard.

Hydroxyl radical scavenging activity

The scavenging ability of hydroxyl radicals is measured by the method of Kunchandy and Rao (1990). The reaction mixture (1.0 mL) consist of 100 µL of 2-deoxy-ribose (28 mM in 20 mM KH_2PO_4-KOH buffer, pH 7.4), 500 µL of the extract, 200 µL EDTA (1.04 mM) and 200µM $FeCl_3$ (1:1 v/v), 100 µL of H_2O_2 (1.0 mM) and 100 µL ascorbic acid (1.0 mM) which is incubated at 37 ^{0}C for 1 h. One milliliter of thiobarbituric acid (1%) and 1.0 mL of trichloroacetic acid (2.8%) are added and incubated at 100 ^{0}C for 20 min. After cooling, absorbance is measured at 532 nm, against a blank sample.

$$\%\ OH\cdot \text{ radical scavenging activity} = \left[\frac{Abs_{control} - Abs_{ext}}{Abs_{control}}\right] X\ 100$$

Where: $Abs_{control}$ = Absorbance without extract

Abs_{ext} = Absorbance with extract

Iron Chelating Activity.

Metal chelating activity was determined according to a method applied by Dinis et al. (1994).

This process and solution medium included 0.05mL $FeCl_2.4H_2O$ (2mM) and 0.35mL distilled water; then the solution including BT root samples at 40, 80, 100 g/mL concentrations and 0.2mL lyophilized water was added and the last volume was completed

to the 4mL by ethanol. Then, the solution of ferrozine (0.2 mL, 5mM) was added to the reaction medium, and it was stirred strongly with vortex. After reaction mixtures were incubated at room temperature for 10 minutes, the absorbance was determined at 562 nm against the blank occurring with ethanol solution. As control, solution contents formed without BT root extract was used.

$$\% \text{ Chelating activity} = \left[\frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{ext}}}{\text{Abs}_{\text{control}}}\right] X\ 100$$

Where: $Abs_{control}$=Absorbance without extract

Abs_{ext} = Absorbance with extract

Lipid Peroxidation Assay (Thiobarbituric acid (TBA) method)

***Preparation of tissue homogenates*:** The rats (12 weeks old and weighing between 220 and 240 g) were decapitated under mild diethyl ether anaesthesia and the whole brain rapidly isolated and placed on ice and weighed. This tissue was subsequently homogenized in cold saline (1:10, w/v) with about 10 up and down strokes at approximately 1200 rpm in a Teflon glass homogenizer. The homogenate was centrifuged for 10 mins at 3000 × g to yield a pellet that was discarded, and a low speed supernatant (SI) which was kept for lipid peroxidation assay (Belle *et al.*, 2004).

Lipid peroxidation and TBA reactions: The lipid peroxidation assay was carried out using the modified method of Ohkawa *et al.* (1979). Briefly, 100 µL SI fraction was mixed with a reaction mixture containing 30 µL of 0.1M Tris-HCl buffer (pH 7.4), the extract (0 - 100 µL) and 30 µL of 250 µM freshly prepared FeSO4. The volume was made up to 300 µL by water before incubation at 37 °C for 1 h. The colour reaction was developed by adding 300 µL of 8.1% sodium dodecyl sulphate (SDS) to the reaction mixture containing SI; this was subsequently followed by the addition of 600 µL acetic acid/HCl (pH 3.4) mixture and 600 µL of 0.8% thiobarbituric acid (TBA). This mixture was incubated at 100 °C for 1 h. Thiobarbituric acid reactive species (TBARS) produced were measured at 532 nm in the UV-visible spectrophotometer and the absorbance was compared with that of standard curve using malondialdehyde (MDA).

Statistical analysis.

The results of the three replicates were pooled and expressed as mean ± standard deviation. Standard deviations were calculated using spread sheet software (Microsoft Excel©, version 2013). Analysis of variance (ANOVA) was performed using Statistical Analysis System proprietary software (SAS, 2002). Duncan's multiple range test procedure as described in the SAS software was used for mean separations. Significance was accepted at P = 0.05. IC50 (extract concentration causing 50% inhibition of antioxidant activity) was calculated using the linear regression analysis.

Results

***In-vitro* Antioxidant Activity**

The *in-vitro* antioxidant activities of the juices were assessed by Total Antioxidant Activity, DPPH Radical Scavenging Activities, Metal Chelating Activities, Hydroxyl Radical Scavenging Activities, Reducing Power Activities, Total Flavonoid, Total Phenolic Content and Lipid Peroxidation Inhibition.

Table 1 showed the Total Phenolic and Flavonoid content of the juices. It was observed that there was no significant difference between beetroot and deoxalated beetroot phenolic (30.56 and 31.83 mg/g GAE respectively) and flavonoid (23.89 and 24.44 mg/g QE) contents. The total antioxidant capacities (TAC) of the Beetroot and Deoxalated Beetroot juices were 48.38 and 32.42 mg/g Ascorbic Acid Equivalent (AAE) respectively (Fig. 1). This result revealed that

the Beetroot juice had the highest antioxidant activity compared to the deoxalated beetroot. There was a significant decrease ($P<0.05$) in the TAC of deoxalated beetroot compared to oxalate rich beetroot.

DPPH Radical Scavenging Activities (Fig. 2) showed the concentration-dependent proton-donating property of the juices. At 100µg/ml, the variation is significantly different ($P<0.05$); ascorbic acid (24.47%) exhibited the highest percentage scavenging activity than Beetroot (22.01%) and its deoxalated form. The activity of Beetroot (19.96 and 21.19%) is significantly higher ($P<0.05$) than the standard compound (ascorbic acid) (16.88 and 18.66%) at 40µg/ml and 80µg/ml respectively.

Fig. 3 showed the iron reducing potential of the juices at three varying concentration (40, 80, 100µg/ml). It was observed that the reducing potential of the juices were significantly lower ($P<0.05$) than that of the reference standard (ascorbic acid). The result from reducing power showed a significant decrease by deoxalation with beetroot juice (0.274) having the highest absorbance than the deoxalated beetroot juice (0.232).

The Hydroxyl Radical Scavenging activity (Fig. 4) represents the percentage hydroxyl ion scavenging activities of the juice at different concentrations (40, 80, 100µg/ml), and the result revealed that activities for the juice are concentration dependent. The standard (Mannitol) showed a significant difference ($P<0.05$) to the samples with the deoxalated beetroot (48.16 and 52.29) and whole beetroot juice (38.53 and 44.49) juice showing a significant yet concentration dependent activities at concentrations 80 and 100µg/ml respectively.

The Metal (Iron) chelating activities of the juice at different concentrations were represented in Fig. 5. The Chelating activity of the juices was found to be concentration dependent. The reference compound (EDTA) at the concentrations 40µg/ml and 80µg/ml was found to have a significantly higher ($P<0.05$) value (23.67 and 26.24% respectively) than the test samples. There was a significant decrease ($P<0.05$) between the oxalate rich beetroot (18.73%) and deoxalated beetroot (14.92%).

The lipid peroxidation inhibition activity as shown in Fig. 6 revealed that activities for the juice are concentration dependent. The lipid peroxidation inhibition activity for all the sample were shown to be more potent than the standard at concentrations 80 and 100µg/ml with beetroot juice showing to be the best. It was observed that there was no significant difference ($P<0.05$) between beetroot and deoxalated beetroot (58.88 and 58.32 % respectively).

Table 1: Total Phenolic and Flavonoid Contents of the juices

	Total phenol (mg/g GAE)	**Total Flavonoid (mg/g QE)**
Beetroot	30.56±2.000[a]	23.89±1.19 [a]
Deoxalated Beetroot	31.83±2.042[a]	24.44±1.22 [a]

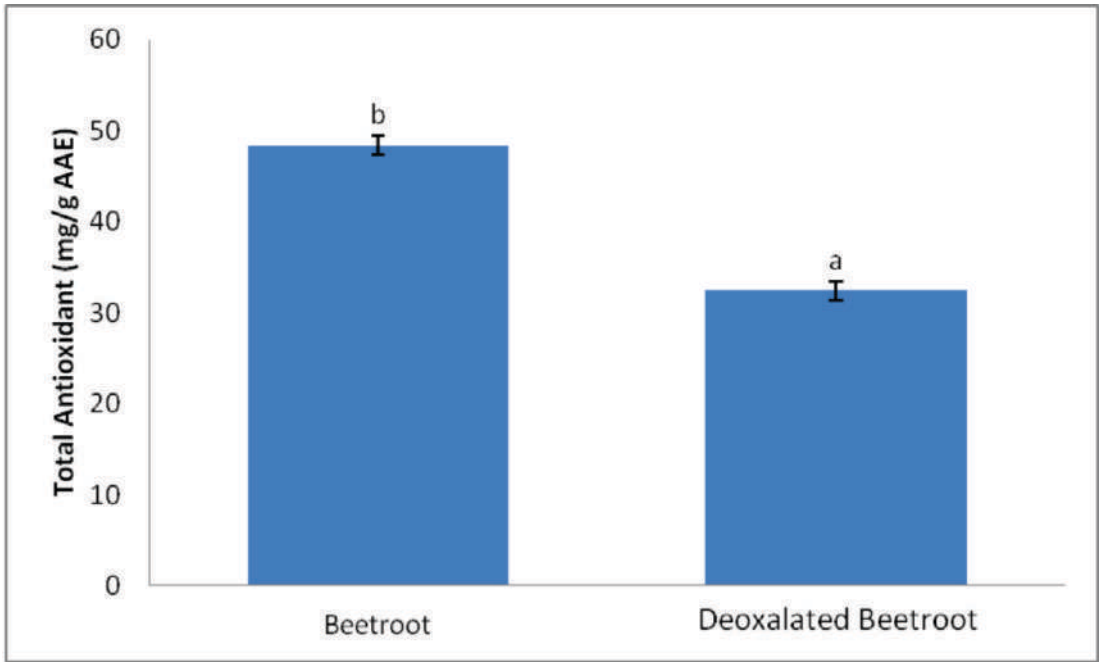

Figure 1: Total antioxidant content (mg/g AAE) of the juices. Each value represent mean. Values with different superscript are significantly different (P<0.05).

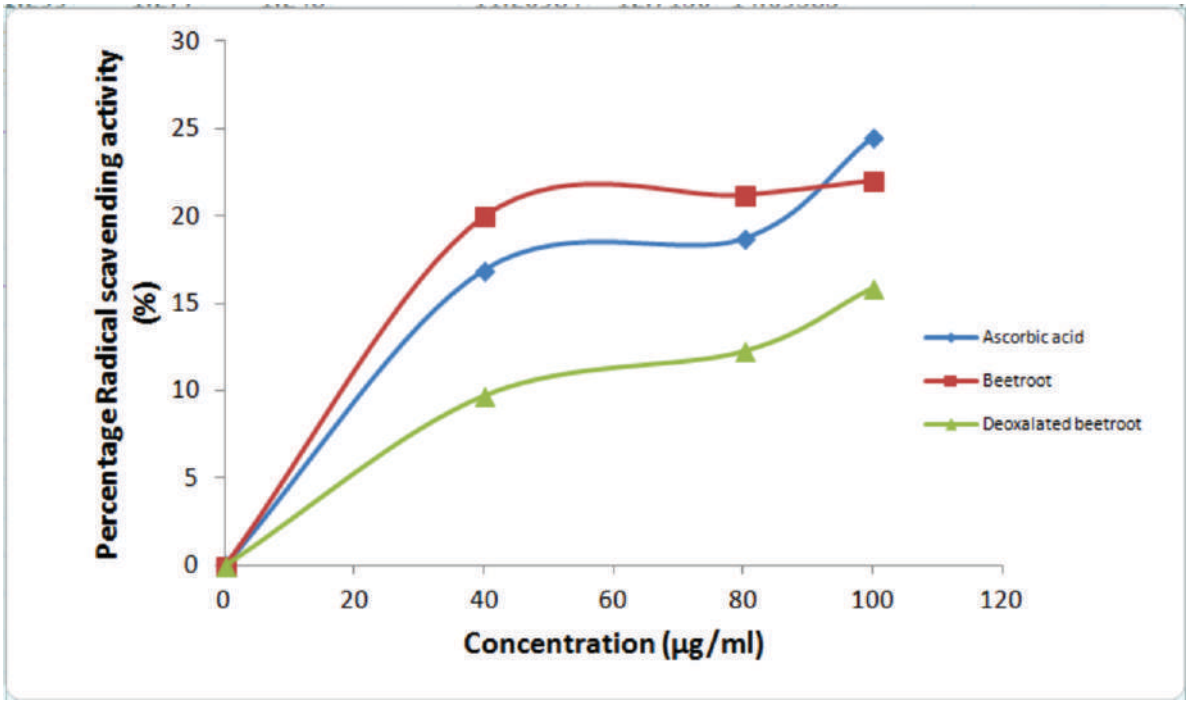

Figure 2: DPPH radical scavenging activity of the juices.

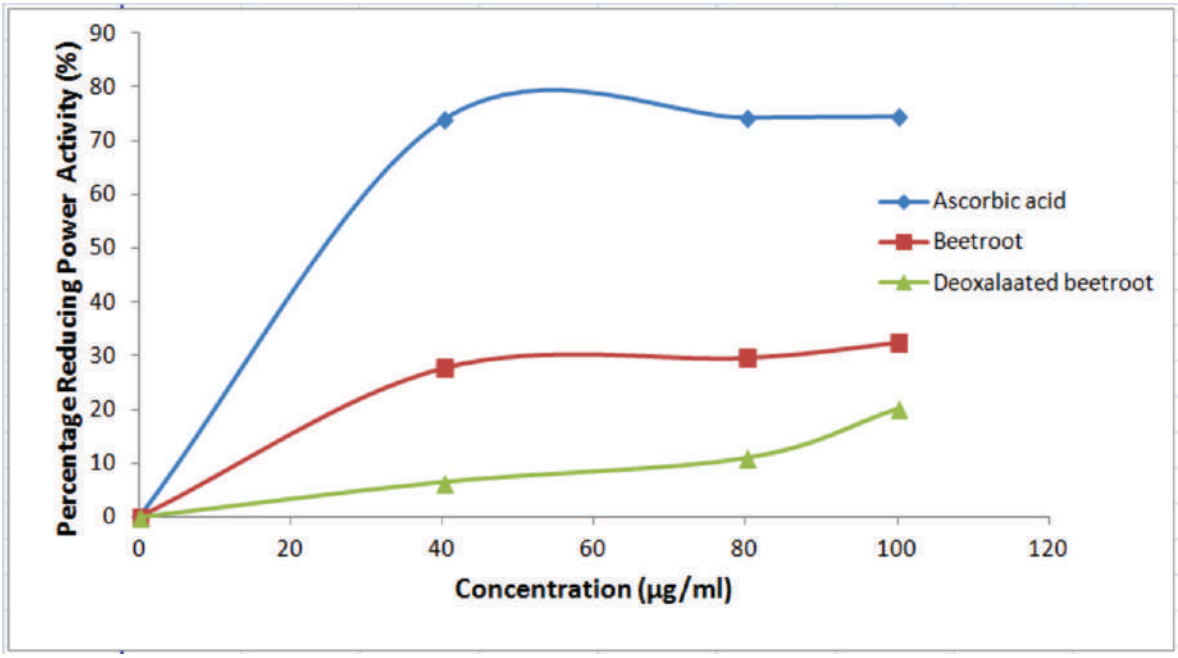

Figure 3: Reducing power activity of the juices.

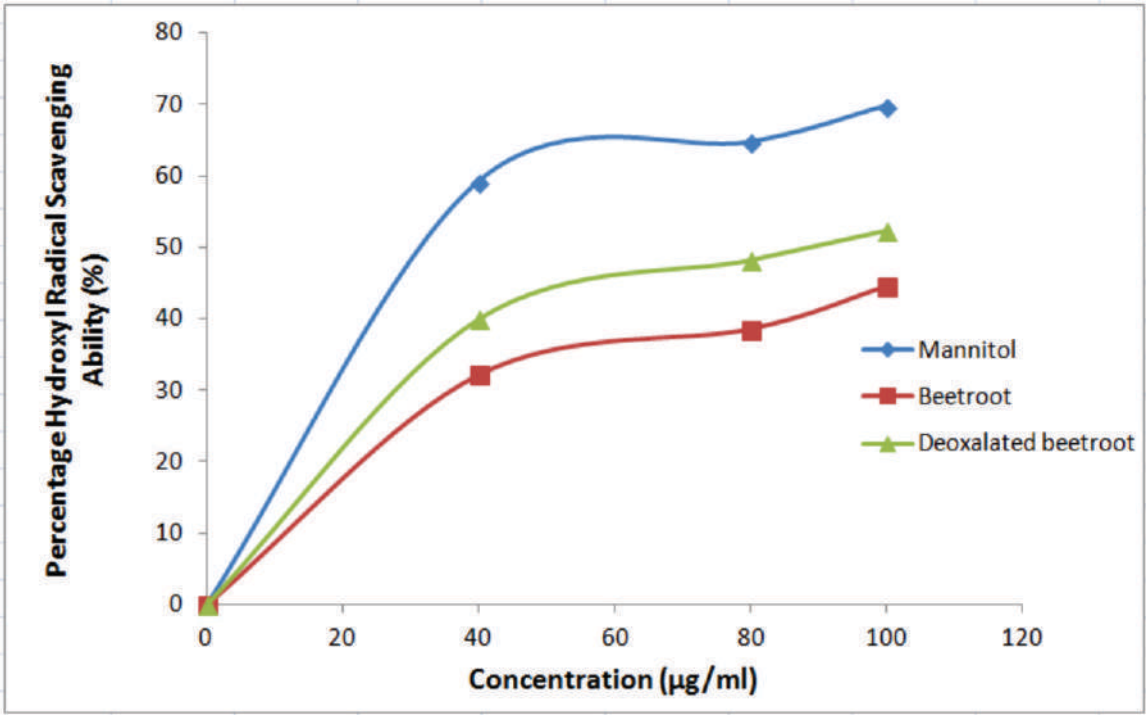

Figure 4: Hydroxyl radical scavenging activity of the juices

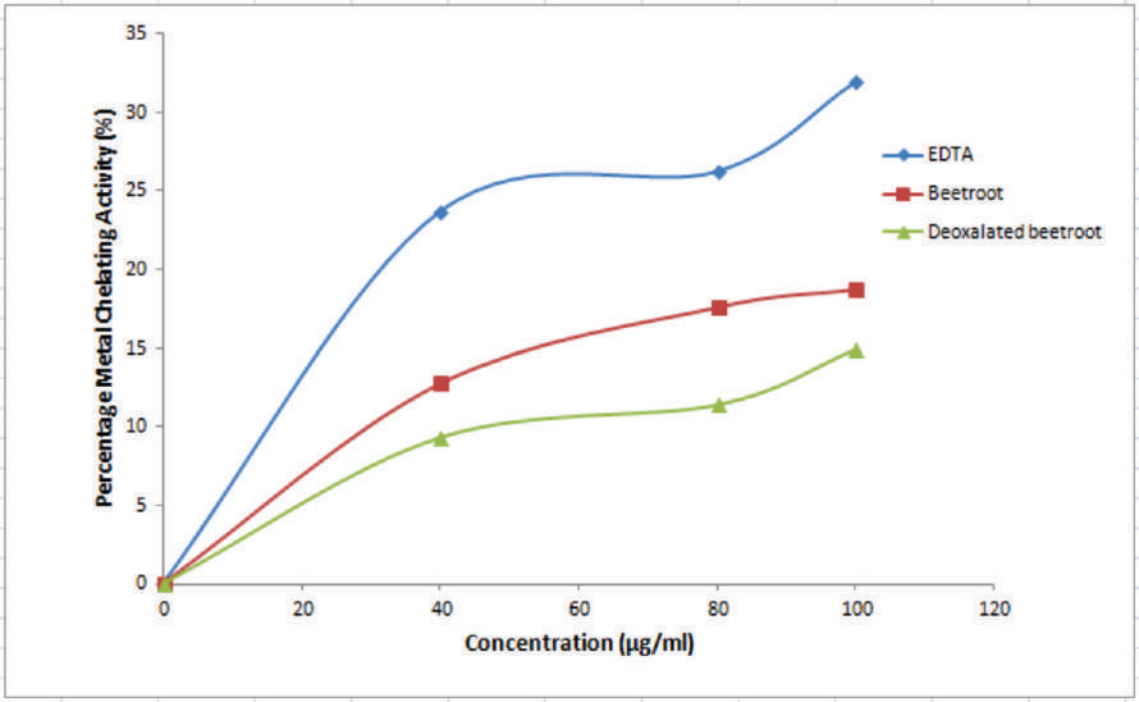

Figure 5: Metal chelating activity of the juices

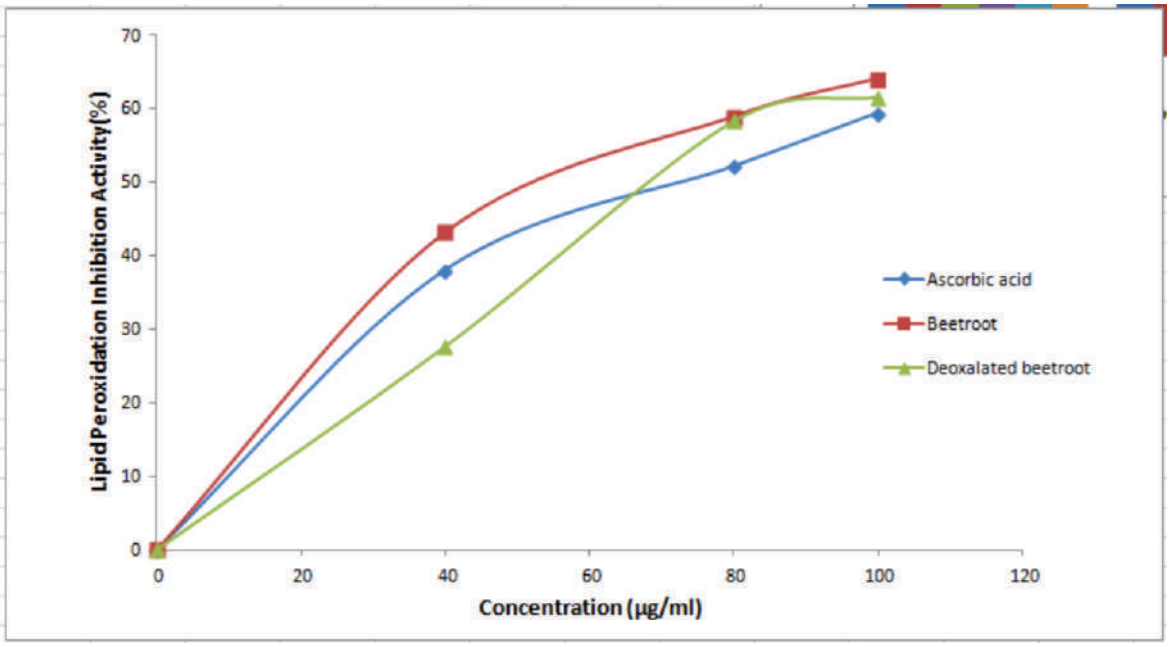

Figure 6: Lipid peroxidation Inhibition activity of the juices

Discussion

Natural sources had proved to be great therapeutic option as they reduce secondary effect usually caused by the use of synthetic drug (Ibraheem and Babatunde, 2019).

The total antioxidant capacity confirmed the various works that had been previously done on beetroot being a very rich source of many bioactive components. This is due to the presence of nitrogen pigments called betalains (Kaur and Kapoor 2002), it however revealed that there might be a synergestic mode of operation between the components and oxalic acid as precipitation of oxalate caused a decrease total antioxidant capacity.

The concentration of Diphenylpicrylhydrazyl (free radical) converted into Diphenylpicrylhydrazine (non-radical) is due to the hydrogen donating ability of the test sample (Alam *et al.*, 2012). All the test samples including the standard (ascorbic acid) scavenged DPPH radical in a concentration dependent manner. This was in line with the work of Gangwar *et al.* (2014) who reported an increased DPPH radical scavenging with increase extract concentration. A study by Wootton-Beard and Colleagues (2011) suggests that a key mechanism by which beetroot juice exerts its antioxidant effects is by scavenging radical species. They found that two commercially available beetroot juices inhibited *in vitro* radical formation in the 2,2-diphenyl-1-picrylhydrazyl (DPPH).

The Metal (iron) chelating property of the juices at different concentrations assessed revealed that the juices were found to be comparable to the reference compound (EDTA) at all the concentrations significantly. The ability of these juices to chelate these reactive metal irons was an indication of its antioxidant activity which defined the explanation for the protection of biomolecules such as nucleic acid, proteins and lipid against oxidative damage. Though the whole beetroot juice proved to be the most potent in chelating iron.

Hydroxyl radical is one of the potent reactive oxygen species in the biological system that reacts with polyunsaturated fatty acid moieties of cell membrane phospholipids and causes damage to cell (Alam *et al.*, 2012). Reaction of hydrogen peroxide (H_2O_2) with low valence forms of the transition metal ions (Fe^{2+} and Cu^{2+}) lead to the formation of OH^- (Fenton reaction) or species of comparable reactivity. The hydroxyl radical, under a physiological condition, are quite reactive and react with any type of biological molecule in living cells, such as sugar, amino acids, phospholipids and nucleobases (the components of nucleic acids) (Phaniencha *et al.*, 2015) leading to their oxidation. The juices were able to inhibit the action of the free radicals generated as a reaction of $FeCl_3$ and H_2O_2. The result reveals that the deoxalated beetroot juice possessed the highest scavenging ability. The standard (Mannitol) was markedly significant than any of the test samples.

The presence of reductants (i.e. antioxidants) causes the reduction of the ferric–ferricyanide complex to the ferrous–ferricyanide complex of Perl's Prussian blue (Vulic *et al.*, 2014). The result revealed that a concentration dependent reducing potential with the standard (ascorbic acid) showed to be the most potent. Beetroot juice was shown to have the highest reducing potential amongst the test samples. If not reduced, Fe^{2+} can participate in the Fenton reaction, generating a highly reactive hydroxyl radical ($Fe^{2+}+H_2O_2 \rightarrow Fe^{3+}+ OH+OH^-$). Also, the superoxide radical participates in the Haber-Weiss reaction ($O_2^- + H_2O_2 \rightarrow O_2 + OH+OH^-$) which combine a Fenton reaction and the reduction of Fe^{3+} by superoxide, yielding Fe^{2+} and oxygen (Liochev and Fridovich, 2002). The ability of the juice to reduce this

deleterious free iron, by preventing the formation of hydroxyl radical indicated antioxidant activities of these extracts.

Flavonoids are natural polyphenols of plant origin. They have antioxidant, anti-inflammatory and anticarcinogenic properties (Olumese and Oboh, 2016). There was only a slight decrease in flavonoid content of the beetroot and its deoxalated form. Noted also was that the precipitation of the oxalated had no significant effect on the ferric reducing potential.

A previous investigation showed that extract obtained from different beetroot varieties contained high amounts of bioactive phenolics and betalains that possessed antioxidant and antiproliferative activities in MRC5 and MCF-7 cell lines (Vulic´ *et al.*, 2014).

Free radicals in both enzymatic and nonenzymatic reactions are most easily produced. These radicals lead to lipid peroxidation and depend on the breakdown of the structure of membrane (Babagil *et al.*, 2018). H_2O_2 is rapidly decomposed into oxygen and water and this may produce hydroxyl radicals (OH^-) that can initiate lipid peroxidation and cause DNA damage in the body. Adetuyi *et al.* (2016) implicated an increased iron as a cause for reactive oxygen species (ROS) formation which facilitates lipid peroxidation through Fenton reaction. However, the juice was shown to inhibit the resultant lipid peroxidation. The lipid peroxidation inhibition ability of the juices revealed a concentration dependent activity and the samples inhibited lipid peroxidation efficiently alongside the standard (ascorbic acid) at the highest concentration used in the assay.

Conclusion

This study has provided compelling evidence that beetroot ingestion offers beneficial physiological effects that may translate to improved antioxidant status. However, deoxalation was shown to reduce its potential. Thus, a synergy in the antioxidant capacity of beetroot and its oxalic acid content is evident. Deoxalated beetroot juice was shown to remain a potent therapeutic agent despite the slight decrease observed in the activity. It is therefore established that an oxalate free beetroot juice presents a way out of the vulnerability to kidney stone and hence a regular consumption of deoxalated beetroot juice can help in the prevention of kidney stone and management of oxidative stress while it is important to seek the protocol to boost its antioxidant potential.

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