



***In vitro* antioxidative and inhibitory actions of phenolic extract of some tropical green leafy vegetables on key enzymes linked to type 2 diabetes and hypertension**

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ABSTRACT

Polyphenolics found in vegetables exhibit a wide range of biological activities as a result of their antioxidant properties. This study sought to determine the antioxidative and inhibitory actions of phenolic extract of some tropical green leafy vegetables (*Telfaria occidentalis*, *Celocia argenticia*, *Occimum gratissimum*, *Structium sparejanophora*, *Corchorus olitorius* and *Talinum triangulare*) on key enzymes linked with type 2 diabetes and hypertension. Free polyphenols of these vegetables were extracted by using 80% acetone. The phenolic content, flavonoid content, ferric reducing capacity, total antioxidant capacity and lipid peroxidation in pancreas were subsequently determined. Enzyme inhibition assays of α -amylase, α -glucosidase and angiotensin I- converting enzyme were also assessed. The results of the study revealed that the free polyphenolic extract of *Corchorus olitorius* has the highest flavonoid and phenolic contents (2.9mg/100g and 1.79mg/g respectively) compared to others. All the free phenolic extracts of these vegetables demonstrated strong ABTS-free radical scavenging abilities. The ferric reducing capacity is highest in *Structium sparejanophora*. *Occimum gratissimum* has the lowest malondialdehyde produced in the lipid peroxidation of pancreas. There was a mild inhibitory activity of the vegetable extracts on α -amylase. However, *Corchorus olitorius* has the highest inhibitory activity (90.95%). All the vegetables tested showed strong inhibition against α -glucosidase and angiotensin-1-converting enzymes. However, *Corchorus olitorius* still showed the strongest inhibition.

Keywords: Polyphenolics, vegetables, α -amylase, α -glucosidase, angiotensin I- converting enzyme, diabetes, hypertension.

INTRODUCTION

Type 2 diabetes mellitus (T2DM) has been recognized as one of the most common metabolic disorders and the world prevalence of diabetes among adults (aged 20–79 years) is 6.4%, affecting 285 million adults, in 2010, and will increase to 7.7%, and 439 million adults by 2030 [1]. Hyperglycemia, a condition characterized by an abnormal postprandial increase of blood glucose level, has been linked to the onset of type 2 diabetes and associated oxidation-linked vascular complications.

Hyperglycemia is the most common feature of this disorder. Drugs involved in diabetes management include α -amylase and α -glucosidase inhibitors, which are oral anti-diabetic drugs used for diabetes mellitus type 2 and work by preventing the digestion of complex carbohydrates.[2,3].

Hence, inhibition of these enzyme systems reduces the rate of digestion of complex carbohydrates resulting in reduced glucose absorption because the carbohydrates are not broken down into glucose molecules. However, high inhibition of pancreatic α -amylase could result in the abnormal bacterial fermentation of undigested starch in the colon, and therefore mild α -amylase inhibitory activity is useful [4]. Therefore, natural inhibitors from dietary plants are useful as they have lower inhibitory activity against α -amylase and a stronger inhibitory activity against α -glucosidase and can be used as effective therapy for postprandial hyperglycaemia with minimal side effects [5].

Free radicals have been implicated in the development and complications of diabetes in a number of ways; the white blood cell production of reactive oxygen species mediates the immune destruction of the beta cells in the islets of langerhans in the pancreas [6]. Furthermore, abnormalities in transition metal metabolism are postulated to result in the establishment of diabetes [7]. Diabetes-associated hyperglycemia causes intracellular oxidative stress, which contributes to vascular dysfunction [8].

Phenolics have been shown to have high antioxidant activity and certain therapeutic properties, including antidiabetic and antihypertensive activity [5]. Fruits and vegetables are the major source of dietary polyphenolic antioxidants. Vegetables contain compounds that are valuable antioxidant and protectants, the main protective action of vegetables have been attributed to the presence of antioxidants, especially antioxidant vitamins including ascorbic acid, α -tocopherol, β -carotene and phenolics [9, 10, 11]. However, numerous studies have conclusively shown that the majority of the antioxidant activity may be from compound such as flavonoids, isoflavone, flavones, anthocyanin, catechin and isocatechin rather than vitamin C, E and β -carotene [10,11]. Several green leafy vegetables abound in tropical Africa that is utilized as either condiments or spices in human diets. These vegetables could be harvested at all stages in the process of growth, and could be fed upon in fresh, processed, or semi processed forms. . Due to the dearth of information on these vegetables vis-à-vis their therapeutic uses, It is therefore hypothesised that the arrays of nutrient and non-nutrient phytochemicals in these leafy vegetables could be harnessed in the management of type 2 diabetes and hypertension which necessitated this study.

EXPERIMENTAL SECTION

Sample collection

Fresh green leafy vegetables [*Struchium sparganophora* (Water bitterleaf), *Amaranthus cruentus* (Amaranth), *Telfairia occidentalis* (Fluted pumpkin), *Ocimum gratissimum* (Wild basil), *Talinum triangulare*(Water leaf), *Cnidioscolous aconitifolius* (Chaya) and *Vernonia amygdalina* (Bitter leaf) and *Corchorus olitorius* (Jute) leaves] were collected in various farm settlements in South-western Nigeria. They were authenticated in the Department of Crop, Soil & Pest Management of Federal University of Technology Akure, Nigeria. Subsequently the edible portions of the vegetables were separated from the inedible portion, then the edible portion was chopped into small pieces before sundrying and milling. The milled vegetables were kept in the refrigerator for subsequent analysis.

Extraction of free soluble phenols

The free soluble phenolics of vegetables were extracted out using the method reported by [9]. Briefly, 50g of the vegetables was homogenized with 80% acetone (1:2 w/v) in a chilled blender for 5 minutes. Then the samples were homogenized further for 3 minutes to obtain a thoroughly homogenized sample. The homogenates were filtered (filter paper Whatman no. 2) under vacuum. The filtrate was then evaporated using a rotary evaporator under vacuum at 45 °C until about 90% of the filtrate had been evaporated. The phenolic extracts were frozen at -40 °C until further analysis.

Determination of total phenol content

The total phenol content was determined according to the method of [12]. Briefly, appropriate dilutions of the extracts were oxidized with 2.5ml 10% Folin-Ciocalteu's reagent (v/v) and neutralized by 2.0ml of 7.5% sodium carbonate. The reaction mixture was incubated for 40minutes at 45°C and the absorbance was measured at 765nm in the spectrophotometer. The total phenol content was subsequently calculated as gallic acid equivalent.

Determination of total flavonoid content

The total flavonoid content was determined using a slightly modified method reported by [13], briefly 0.5ml of appropriately diluted sample was mixed with 0.5ml methanol, 50 μ l 10% $AlCl_3$, 50 μ l 1M Potassium acetate and 1.4ml water, and allowed to incubate at room temperature for 30min. The absorbance of the reaction mixture was

subsequently measured at 415 nm; the total flavonoid content was subsequently calculated. The non-flavonoid polyphenols were taken as the difference between the total phenol and total flavonoid content.

***In vitro* Antioxidant Studies**

2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) Radical Scavenging Ability

The ABTS* scavenging ability of the extracts were determined according to the method described by [14]. The ABTS* was generated by reacting an (7 mmol/l) ABTS aqueous solution with K₂S₂O₈ (2.45 mmol/l, final concentration) in the dark for 16 h and adjusting the Abs_{734nm} to 0.700 with ethanol. 0.2ml of appropriate dilution of the extract was added to 2.0ml ABTS* solution and the absorbance were measured at 734nm after 15mins. The trolox equivalent antioxidant capacity was subsequently calculated.

Determination of reducing property

The reducing property of the extracts was determined by assessing the ability of the extract to reduce FeCl₃ solution as described by [15]. 2.5ml aliquot was mixed with 2.5 ml 200 mM sodium phosphate buffer (pH 6.6) and 2.5 ml 1% potassium ferricyanide. The mixture was incubated at 50 °C for 20 min. and then 2.5 ml 10 % trichloroacetic acid was added. This mixture was centrifuged at 650 rpm for 10 min. 5 ml of the supernatant was mixed with an equal volume of water and 1 ml 0.1% ferric chloride. The absorbance was measured at 700 nm. The ferric reducing antioxidant property was subsequently calculated.

Lipid peroxidation assay

Preparation of Tissue Homogenates

The rats were decapitated under mild diethyl ether anaesthesia and the pancreas was 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 rev/min in a Teflon glass homogenizer. The homogenate was centrifuged for 10 min at 3000xg to yield a pellet that was discarded, and a low-speed supernatant (S1) was kept for lipid peroxidation assay [16].

Lipid Peroxidation and Thiobarbituric Acid Reactions

The lipid peroxidation assay was carried out using the modified method of [17], briefly 100µl S1 fraction was mixed with a reaction mixture containing 30µl of 0.1M pH 7.4 Tris-HCl buffer, extract (0 - 100µl) and 30µl of 250µM freshly prepared FeSO₄. The volume was made up to 300µl by water before incubation at 37°C for 1h. The colour reaction was developed by adding 300µl 8.1% SDS (Sodium dodecyl sulphate) to the reaction mixture containing S1, this was subsequently followed by the addition of 600µl of acetic acid/HCl (pH 3.4) mixture and 600µl 0.8% TBA (Thiobarbituric acid). This mixture was incubated at 100°C for 1h. TBARS (Thiobarbituric acid reactive species) produced were measured at 532nm and the absorbance was compared with that of standard curve using MDA (Malondialdehyde).

Enzyme Inhibition Assays

***α*-Amylase inhibition assay**

Appropriate dilution of the phenolic extracts (500µl) and 500 µl of 0.02M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) containing Hog pancreatic *α*-amylase (EC 3.2.1.1) (0.5mg/ml) were incubated at 25°C for 10 minutes. Then, 500µl of 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) was added to each tube. The reaction mixtures was incubated at 25°C for 10 minutes and stopped with 1.0 ml of dinitrosalicylic acid colour reagent. Thereafter, the mixture was incubated in a boiling water bath for 5 minutes, and cooled to room temperature. The reaction mixture was then diluted by adding 10 ml of distilled water, and absorbance measured at 540 nm [18].

***α*-Glucosidase inhibition assay**

Appropriate dilution of the phenolic extracts (50µl) and 100 µl of *α*-glucosidase solution (1.0 U/ml) in 0.1 M phosphate buffer (pH 6.9) was incubated at 25 °C for 10 min. Then, 50 µl of 5 mM p-nitrophenyl-*α*-D-glucopyranoside solution in 0.1 M phosphate buffer (pH 6.9) was added. The mixtures were incubated at 25 °C for 5 min, before reading the absorbance at 405 nm in the spectrophotometer [19]. The *α*-glucosidase inhibitory activity was expressed as percentage inhibition.

Angiotensin I converting enzyme (ACE) inhibition assay

The Angiotensin I- converting enzyme (ACE) inhibitory activity was measured according to the principles of [20]. ACE solution (50 μ l, 4 mU) was preincubated with each phenolic extract (50 μ l) for 15 min at 37°C. The enzymatic reaction was initiated by adding 150 μ l of 8.33 mM of the substrate Bz-Gly-His-Leu in 125 mM Tris- HCl buffer (pH 8.3) to the mixture. After incubation for 30 mins at 37°C, the reaction was arrested by adding 250 μ l of 1M HCl. The Gly-His bond was cleaved and the Bz-Gly produced by the reaction was extracted with 1.5 ml ethyl acetate. Thereafter the mixture was centrifuged to separate the ethyl acetate layer; then 1 ml of the ethyl acetate layer transferred to a clean test tube and evaporated. The residue was redissolved in distilled water and its absorbance measured at 228 nm against a series of standards. The ACE inhibitory activities will expressed as percentage inhibition.

Data Analysis

The results of the three replicates were pooled and expressed as mean \pm standard error (S.E.). Student t-test, one-way analysis of variance (ANOVA) and the least significance difference (LSD) were carried out[21]. Significance was accepted at $p \leq 0.05$.

RESULTS AND DISCUSSION

Studies on the relationship of phenolic contents, antioxidant properties and enzyme inhibitory activities of plants foods, in order to survey the nutraceutical significance of plant foods are numerous [22, 23, 24]. Therefore, the phenolic content was determined.

The total phenolic content of the vegetables was shown in figure 1. The total phenolic content varies in the vegetables. The result showed that OG and CO has the highest total phenolic contents when compared to others. However, TT has the lowest total phenolic content.

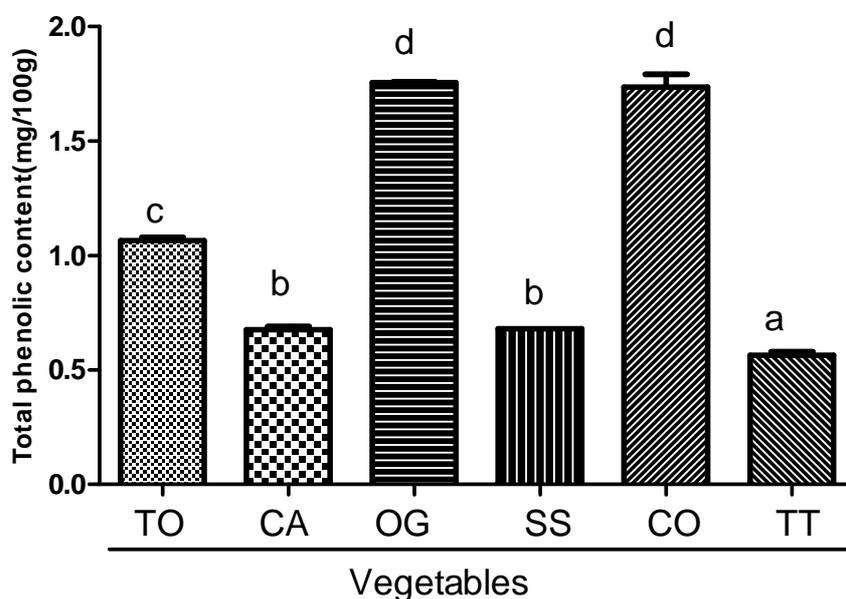


Figure 1: Total phenol contents of free phenols of some green leafy vegetables.

Bars with different letters are significantly different from the others at $P < 0.05$.

TO=*Telfaria occidentalis*, CA=*Celocia argenticum*, OG=*Occimum gratissimum*, SS=*Structium sparejanophora*, CO=*Corchorus olitorius* and TT=*Talinum triangulare*

Flavonoids are the most abundant polyphenols reported to possess antioxidant activity in plant foods [25]. The total flavonoid content was shown in figure 2. There was a significant variation in the flavonoid content of the vegetables. CO also has the highest total flavonoid content while SS has the lowest.

The total antioxidant capacity of the extracts, a function of the radical scavenging ability of the extracts, was studied using a moderately stable nitrogen-centered radical species,

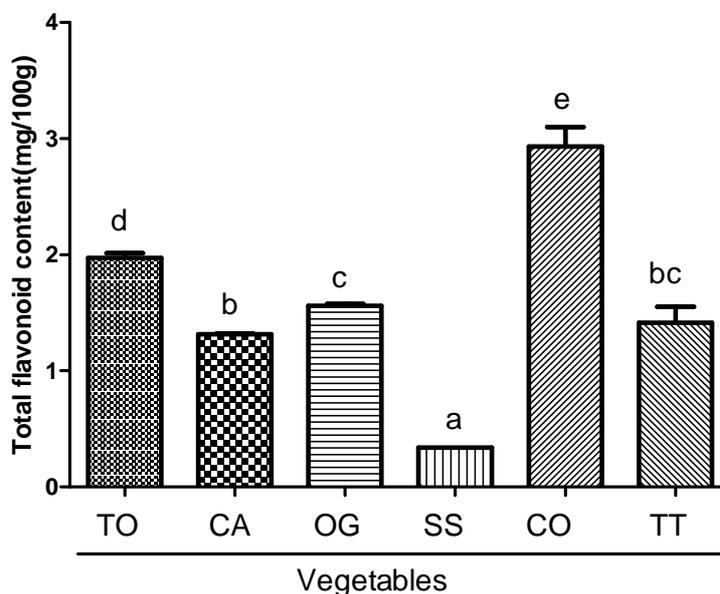


Figure 2: The total flavonoid content of some green leafy vegetables.

Bars with different letters are significantly different from the others at $P < 0.05$.

TO=*Telfaria occidentalis*, CA=*Celocia argentia*, OG=*Occimum graticimum*, SS=*Structium sparejanophora*, CO=*Corchorus olitorius* and TT=*Talinum triangulare*

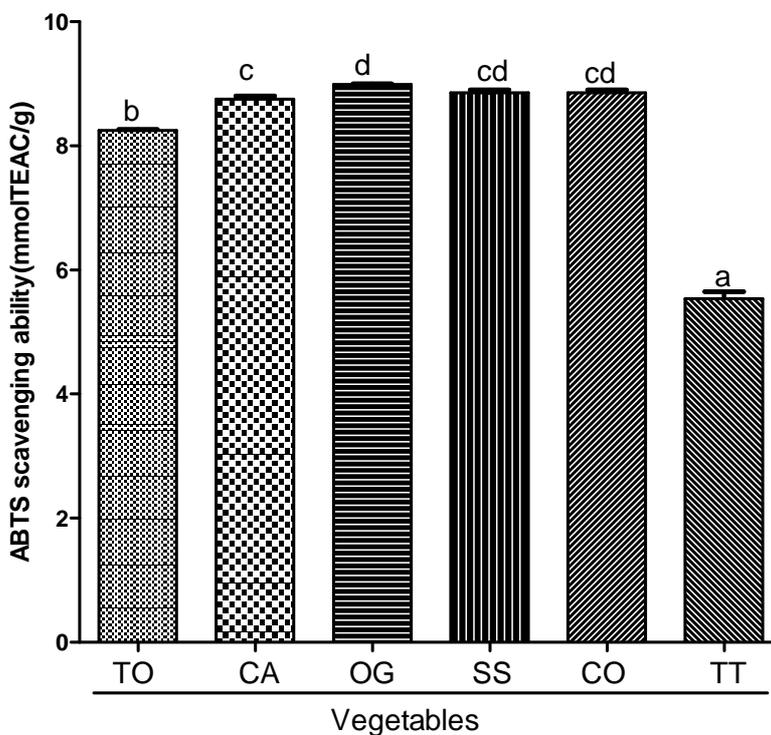


Fig.3: ABTS* scavenging ability of some green leafy vegetables.

Bars with different letters are significantly different from the others at $P < 0.05$.

TO=*Telfaria occidentalis*, CA=*Celocia argentia*, OG=*Occimum graticimum*, SS=*Structium sparejanophora*, CO=*Corchorus olitorius* and TT=*Talinum triangulare*

ABTS+[14].The result in figure 3 revealed that the free phenolic extracts of the vegetables exhibited scavenging ability on ABTS radical. However, TT showed the lowest scavenging ability.This agrees with the phenolic contents of these vegetables.

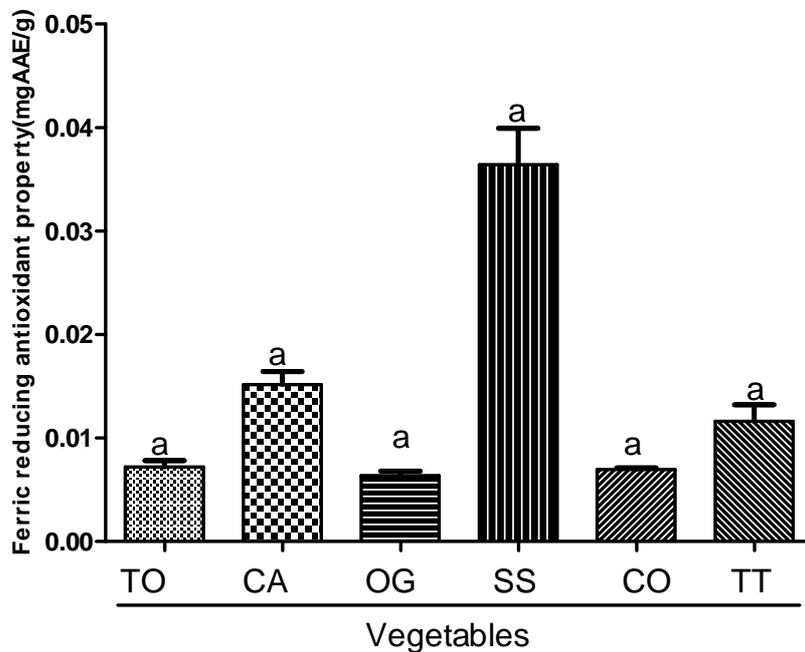


Fig.4:Ferric reducing antioxidant properties of some green leafy vegetables.

Bars with different letters are significantly different from the others at $P < 0.05$.

TO=Telfaria occidentalis, CA=Celocia argentia, OG=Occimum graticimum, SS=Structium sparejanophora,, CO=Corchorus olitorius and TT=Talinum triangulare.

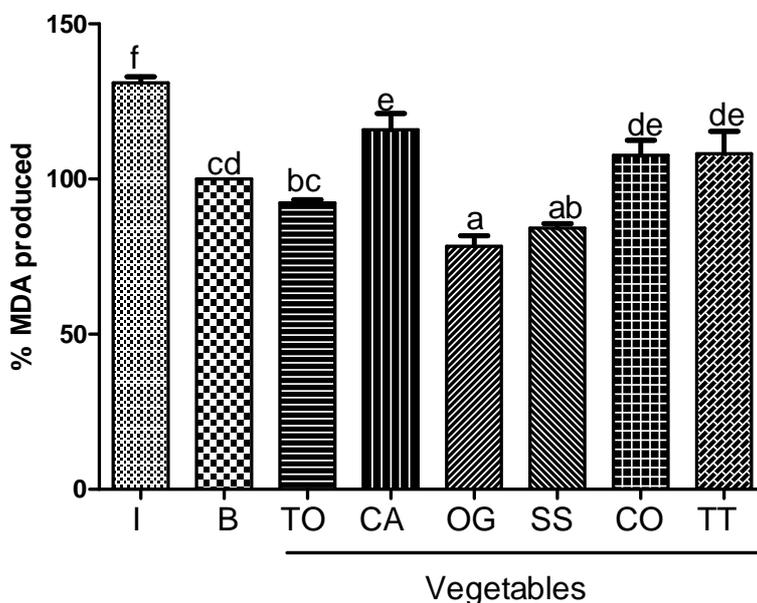


Fig.5:Inhibition of lipid peroxidation by some green leafy vegetables

Bars with different letters are significantly different from the others at $P < 0.05$.

I=Induced,B=Basal, TO=Telfaria occidentalis,CA=Celocia argentia, OG=Occimum graticimum, SS=Structium sparejanophora,, CO=Corchorus olitorius and TT=Talinum triangulare.

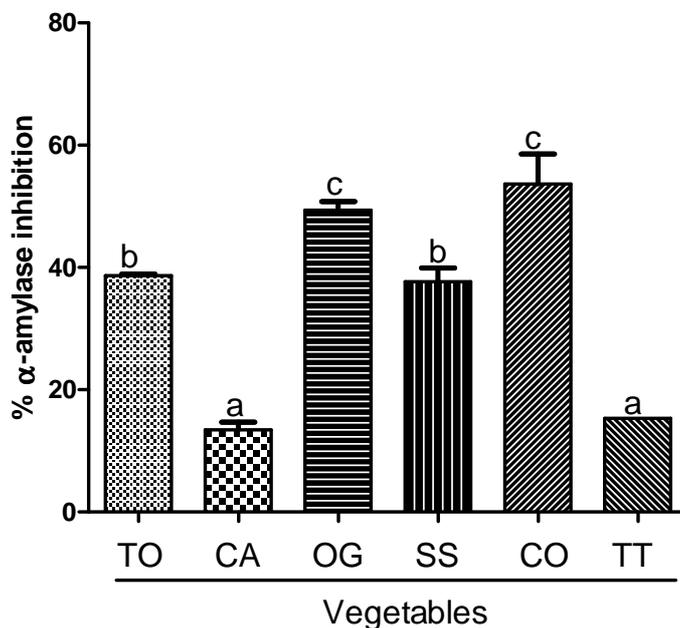


Fig.6:Inhibition of α -amylase activity by some green leafy vegetables.
 Bars with different letters are significantly different from the others at $P<0.05$.
 TO=*Telfaria occidentalis*, CA=*Celocia argentia*, OG=*Occimum gratiticum*, SS=*Structium sparejanophora*, CO=*Corchorus olitorius* and TT=*Talinum triangulare*.

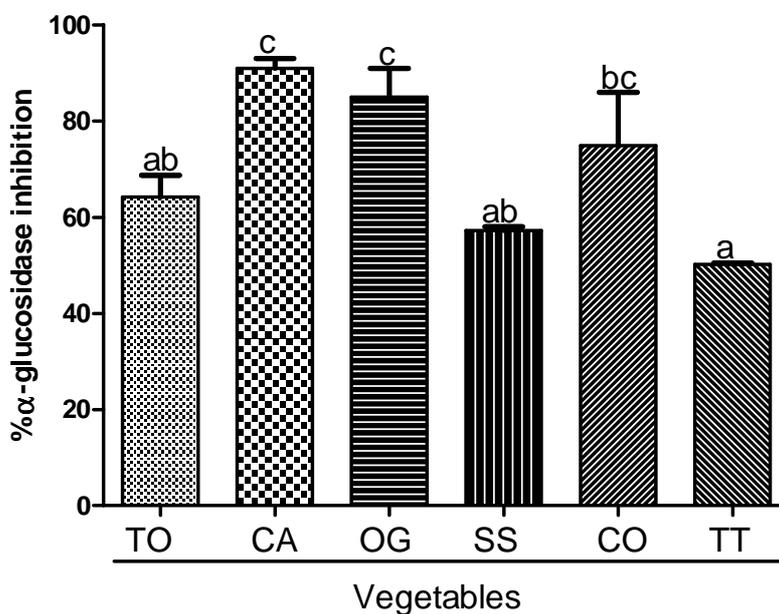


Fig.7: Inhibition of α -glucosidase activity by some green leafy vegetables.
 Bars with different letters are significantly different from the others at $P<0.05$.
 TO=*Telfaria occidentalis*, CA=*Celocia argentia*, OG=*Occimum gratiticum*, SS=*Structium sparejanophora*, CO=*Corchorus olitorius* and TT=*Talinum triangulare*.

Reducing power is a potent antioxidant defense mechanism which is based on either electron transfer or/ and hydrogen atom transfer by the antioxidant molecule[26].

Ferric reducing antioxidant properties of some green leafy vegetables was shown in figure 4. The result revealed that only SS has better ferric reducing antioxidant property while others showed less effect.

In the pancreas, Fe^{2+} accumulates in acinar cells and in the islets of Langerhans, thereby resulting in the destruction of beta-cells associated with DM[27]. The ability of the phenolic extracts to inhibit Fe^{2+} -induced lipid peroxidation in rat's pancreas was investigated. The results clearly showed that incubation of the rat pancreas in the presence of Fe^{2+} caused a significant increase ($P < 0.05$) in the MDA contents of the rat pancreas (131%) when compared with the basal pancreas homogenate (100.0%). The increased lipid peroxidation in the presence of Fe^{2+} could be attributed to the fact that Fe^{2+} can catalyze one-electron transfer reactions that generate reactive oxygen species.

The incubation of rat pancreas in presence of Fe^{2+} caused a significant increase ($P < 0.05$) in MDA content; however, all the extracts exhibited inhibitory effects on pancreatic MDA produced.

This inhibition could be attributed to the phenolic phytochemicals present in the extracts; which could have formed complexes with the $\text{Fe}(\text{II})$, thereby preventing them from catalyzing the initiation of lipid peroxidation.

Figure 5 showed the inhibition of lipid peroxidation by some green leafy vegetables. There was a mild reduction of malondialdehyde produced however, OG seems to show the highest reduction of MDA.

Inhibition of α -amylase activity by some green leafy vegetables as shown in figure 7 depicted that CO has the highest alpha amylase inhibition.

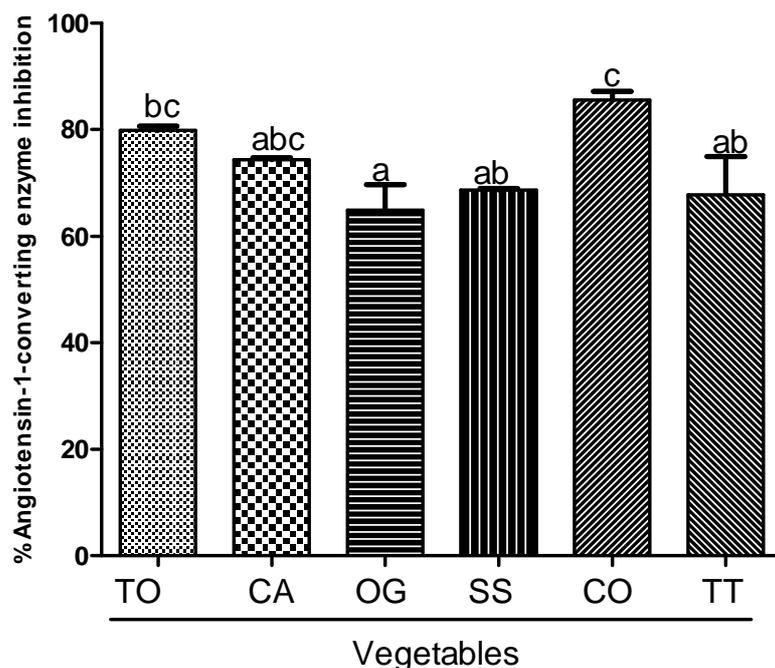


Fig.8: Inhibition of Angiotensin-1-converting enzyme activity by some green leafy vegetables.

Bars with different letters are significantly different from the others at $P < 0.05$.

TO=Telfaria occidentalis, CA=Celocia argentic, OG=Occimum gratificum, SS=Structium sparejanophora,, CO=Corchorus olitorius and TT=Talinum triangulare.

Fig.8 shows the inhibition of α -glucosidase activity by some green leafy vegetables. CA shows the highest inhibitory activity while TT shows the lowest inhibitory activity on α -glucosidase.

These results (figure 6 and figure 7) show that there is higher inhibition of alpha glucosidase than their respective alpha-amylase inhibitory activities. This result agrees

with previous works that plant phytochemicals are mild inhibitors of alpha-amylase and strong inhibitors of alpha-glucosidase activities[22,23,28]. Therefore, stronger inhibition of alpha-glucosidase activity and mild inhibition of alpha-amylase activity exhibited by the extracts may be of great nutraceutical importance, in minimizing some of the side effect (such as abdominal distention, flatulence, meteorism and possibly diarrhea) associated with the drugs (Acarbose and Voglibose), presently used for the management of diabetes.

It can therefore be suggested that the inhibition of alpha-amylase and alpha-glucosidase activities could be part of the possible mechanisms involved in the use of green leafy vegetables in therapeutic/dietary management of diabetes, by retardation of starch hydrolysis in the gastrointestinal tract since polyphenols such as flavonoid have potent alpha-glucosidase inhibitory activities[24].

As shown in figure 8, all the vegetable extracts demonstrated strong inhibition against angiotensin-1- converting enzyme. Angiotensin-I- converting enzyme (ACE) cleaves angiotensin I to produce angiotensin II, a powerful vasoconstrictor that has been identified as a major factor in hypertension [29]. As a result, ACE inhibitors have been widely developed to prevent angiotensin II production in cardiovascular diseases, and these have been utilized in clinical applications since the discovery of ACE inhibitors in snake venom[30]. Hence, this finding reveals possible antihypertensive potentials of these polyphenolic rich-extracts of these vegetables in folk medicine.

Taken together all these antioxidant potentials and inhibitions of key enzymes linked to diabetes and hypertension by the free polyphenol-rich extracts of these vegetables, our findings have shown that these vegetables could serve as sources of cheaper and safer alternatives to the synthetic drugs that are in use now for the management of oxidative stress induced diseases especially in type 2 diabetes and hypertension.

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