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Phenological Variation in the *In-vitro* Antioxidant Properties and α-Amylase Inhibitory Activity of *Tetrapleura tetraptera* Pod

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ABSTRACT
Variation in the *in-vitro* antioxidant properties and α-amylase inhibitory activity of *Tetrapleura tetraptera* pod was assessed at two phenologic stages of the fruit development, that is, the mature green (MG) and ripe brown (RB) stages. RB pod (RBP) had significantly higher ($P < 0.05$) antioxidant phenolic phytochemicals - total phenol, tannin and total flavonoid - levels than the MG pod (MGP). Conversely, MGP had significantly higher ($P < 0.05$) total carotenoid and vitamin C contents than RBP. Methanolic extracts of both MGP and RBP had appreciable trolox equivalent antioxidant capacity (TEAC), and were effective in reducing Fe^{3+} to Fe^{2+}. However, RBP extract had significantly higher ($P < 0.05$) TEAC and ferric reducing antioxidant power (FRAP) than MGP extract. Furthermore, both MGP and RBP extracts scavenged 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical in a dose-dependent manner, with the half maximal inhibitory concentration (IC_{50}) of RBP (0.11 mg/ml) being lower than that of MGP (0.16 mg/ml). Both extracts also displayed potent inhibitory activity against porcine pancreatic α-amylase in a dose-dependent manner, having IC_{50} values of 0.33 mg/ml and 0.46 mg/ml for RBP and MGP respectively. Therefore, *T. tetraptera* pod is more potent in scavenging free radicals and inhibiting pancreatic α-amylase *in vitro* when fully ripe than when green, and thus could be more effective in alleviating oxidative stress and managing postprandial hyperglycemia in type 2 diabetes at the fully ripe stage than at the mature green stage.

Keywords: *Tetrapleura tetraptera*; Antioxidant activities; Alpha-amylase; Free radicals; Hyperglycemia.

INTRODUCTION

Reactive oxygen species and free radicals formed during oxidation have been reported to contribute to diseases such as cancer, diabetes, cardiovascular diseases and ageing. [1] Postprandial hyperglycemia is the main risk factor for the development of non-insulin dependent diabetes mellitus. [2] Hence, control of postprandial blood glucose level plays key role in treatment and decrease progression of diabetes mellitus. [3] Research has shown that polyphenols can inhibit α-amylase, and therefore have antidiabetic activity. [4]

Medicinal plants contain phytochemicals with antioxidation potential which are responsible for their therapeutic effects. [5] In addition to antioxidative properties, these bioactive compounds have been shown to have other possible health benefits such as, anticarcinogenic, antihypertensive, antimutagenic, antidiabetic and antimicrobial activities. [6-7] Natural antioxidants from plants have the ability to protect the body against oxidative damage [8], by scavenging the free radicals and inhibiting peroxidation and other radical mediated processes.

Tetrapleura tetraptera is a deciduous plant belonging to the mimosaceae family. It is generally found in the lowland forest of tropical Africa. The fruits consist of a fleshy pulp with small, brownish-black seeds. The fruits are green when tender and dark brown when fully ripe, and possess both nutritional and medicinal values. [9] In Nigeria, the tree begins flowering towards the end of February and is over in early April. The indehiscent pods are ripe from September to December, during which it is deciduous. [10]

The dry fruit has a pleasant aroma and hence used to spice as a seasoning spices in the Southern part of Nigeria. [11-12] In West Africa this plant is used in ethnomedicine for the treatment of several ailments such as diabetes mellitus, hypertension, arthritis, asthma, epilepsy, schistomiasis, and for prevention of post-partum contraction. [13] Due to its

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antibiotic property, it has been reported that *T. tetraptera* has the potential for providing an alternative drug therapy. [14]

Phenology, defined as study of periodically occurring phenomena in plants in relation to the climate and changes of seasons, is among the physiologic factors that strongly influence the bioactive components (phytochemicals) from herbal drugs and their contents. [15-16] Three environmental factors (photoperiod, temperature and moisture) have been generally identified to trigger phenological progress of the plant. [17] Knowledge of the influence of phenological variations on the medicinal properties of *T. tetraptera* pod is of central importance not only for its ethnomedicinal uses, but also for its potential pharmaceutical applications. Therefore, the objective of this study is to evaluate the influence of phenology on the *in-vitro* antioxidant properties and α-amylase inhibitory activities of *T. tetraptera* pod at two different stages of the fruit development.

Fig. 1a: Mature green *T. tetraptera* Pod

Fig. 1b: Ripe brown *T. tetraptera* pod

MATERIALS AND METHODS

Samples collection and preparation

Triplicate samples of *T. tetraptera* fruit were collected at Idi-Ose village in Ibadan, Nigeria, at two phenologic stages of the fruit development, and were authenticated at the Department of Botany, University of Ibadan, Nigeria. The first stage was at the MG stage (seven months after fruiting, September, 2012), while the second was at the FRB stage (nine months after fruiting, November, 2012). The pods which were manually separated from seeds were later oven-dried at 50^{o}C to a constant moisture content of about 5%. The dried MG and RB pod samples (MGP and RBP, respectively) were subsequently milled to a fine particle size (0.5 mm) packed in air-tight plastic vials and stored at 4°C until analysis.

All the chemicals used for analysis were of analytical grade.

Preparation of methanolic extract

Methanolic extract of the pod samples was prepared following the method of Chan *et al.* [18], by adding 25 ml of methanol to 0.5g of sample contained in a 50 ml centrifuge tube, and shaking continuously for 1 h at room temperature. The mixture was centrifuged at 3,000 rpm for 10 min, and then the supernatant (subsequently referred to as methanolic extract) was collected and store at -4^{o}C for further analysis.

Determination of total phenol content

The total phenol content of the methanolic extracts was determined according to the Folin–Ciocalteu method reported by Chan *et al.* [18] Briefly, 300 µl of extract was dispensed into test tube (in triplicates). To this was added 1.5 ml of Folin–Ciocalteu reagent (diluted 10 times with distilled water), followed by 1.2 ml of Na_2CO_3 solution (7.5% w/v). The reaction mixture was shaken, allowed to stand for 30 min at room temperature before the absorbance was measured at 765 nm against a blank prepared by dispensing 300µl of distilled instead of sample extract. TPC was expressed as gallic acid equivalent (GAE) in mg/g material.

Determination of tannin content

Tannin content of samples was determined according to the method of Padmaja [19] as follows. Sample (0.1g) was extracted with 5 ml of acidified methanol (1% HCl in methanol) at room temperature for 15 minutes. The mixture was centrifuged at 3,000rpm for 20minutes. 0.1 ml of the supernatant was added to 7.5 ml of distilled water, followed by 0.5 ml of Folin-Denis reagent, 1 ml of 35% sodium carbonate solution and 0.9 ml of distilled water. The mixture was shaken, kept at room temperature for 30 min and absorbance was measured at 760 nm. Blank was prepared with water instead of the sample. Tannin content, expressed as tannic acid equivalent (TAE) in mg/g material, was subsequently calculated.

Determination of total flavonoid content

Total flavonoid content was determined using aluminum chloride method as reported by Kale *et al.* [20]. 0.5 ml of methanolic extract was dispensed into test tube, followed by 1.5 ml of methanol, 0.1 ml of aluminum chloride (10%), 0.1 ml of 1M potassium acetate and 2.8 ml of distilled water. The reaction mixture was shaken, allowed to stand at room temperature for 30 minutes, before absorbance was read at 514 nm. TFC was expressed as quercetin equivalent (QE) in mg/g material.

Determination of total carotenoid content

Total carotenoid content of samples was determined according the method of Rodriguez-Amaya. [21] Briefly, 0.2 – 0.3g of sample was exhaustively extracted using cold aecetone in a mortar with small amount (0.5g) of celite. Thereafter, the acetone extract was partitioned with 10 ml of petroleum ether in a 250 ml separatory funnel. The mixture was washed repeatedly with distilled water until acetone was completely washed and drained off. Then the total carotenoid was separated from other pigments in the ether extract by passing it through an alumina (Activity III) column, and eluted with petroleum ether to a final volume of 25 ml. The absorbance of the extract was read at 450nm. To avoid the degradation of the total carotenoid, analysis was carried out under subdued light and the extract was collected into a vial wrapped with aluminum foil.

Determination of vitamin C content

The vitamin C content of the aqueous extract of samples was determined using the method reported by Benderitter *et al.* [22] Briefly, to 300 µl of sample aqueous extract 100 µl of 13.3% trichloroacetic, 100 µl of distilled water and 75 µl DNPH reagent (2 g dinitrophenyl hydrazine, 230 mg thiourea and 270 mg $CuSO_4.5H_2O$ in 100 ml of 5M H_2SO_4) were added. The reaction mixture was subsequently incubated for 3 h at 37°C, after which 0.5 ml of 65% H_2SO_4 (v/v) was added to it and the absorbance was measured at 520 nm. Vitamin C content of the sample was subsequently calculated from a calibration curve prepared with ascorbic acid standard.

Estimation of DPPH free-radical-scavenging ability

The free-radical-scavenging ability of the methanolic extracts against 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical was estimated as described by Cervato *et al.* [23] with slight modification. Briefly, appropriate dilutions of the extracts (0.024 to 0.096 mg/ml) totaling 1 ml was mixed with 3 ml of 60µM methanolic solution of DPPH radicals; the mixture was left in the dark for 30 min before the absorbance was taken at 517 nm. The decrease in absorbance of $DPPH^{\cdot}$ on addition of test samples in relation to the control was used to calculate the percentage inhibition (%Inh.) following the equation:

$\%Inhibition = [(A517_{control} - A517_{sample}) \div A517_{control}] \times 100.$

The IC_{50} was calculated from the dose-inhibition linear regression curve of each extract.

Estimation of ABTS* radical-scavenging ability

The 2,2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) radical (ABTS*) scavenging ability of methanolic extract of samples were determined according to the method described by Sellappan and Akoh. [24] The ABTS* radical was generated by incubating equal volume of a 7 mM ABTS aqueous solution with $K_2S_2O_8$ (2.45 mM) in the dark for 16 h at room temperature and adjusting the absorbance at 734 nm to 0.7 ± 0.02 with 95% ethanol. Then 0.2 ml appropriate dilution of the extract was added to 2.0 ml ABTS* solution and the absorbance was measured at 734 nm after 15 min. The trolox equivalent antioxidant capacity (TEAC) was subsequently calculated.

Determination of ferric reducing antioxidant power (FRAP)

The reducing property of the methanolic extracts was determined by assessing the ability of the extract to reduce $FeCl_3$ solution as described by Oyaizu. [25] A 2.5 ml aliquot was mixed with 2.5 ml of 200mM sodium phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min. and then 2.5 ml of 10% trichloroacetic acid was added. This mixture was centrifuged at 650 rpm for 10 min. Then 5 ml supernatant was mixed with an equal volume of water and 1 ml of 0.1% ferric chloride. The absorbance was measured at 700 nm. The ferric reducing antioxidant property was subsequently calculated.

Alpha-amylase inhibition assay

Alpha-amylase inhibition assay was conducted following the protocol described by Kwon *et al*. [26] Appropriate dilutions (0.024 to 0.096 mg/ml) of methanolic extract amounting to 500µl, and 500µl of 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M sodium chloride) containing α-amylase solution (0.5 mg/ml) were incubated at 37°C for 10 min. After pre-incubation, 500µl of 1% starch solution in 0.02 M sodium phosphate buffer was added. The reaction mixture was then incubated at 37°C for 15 min and the reaction was terminated with 1.0 ml of DNS acid color reagent (1% 3, 5-dinitrosalicylic acid and 12% sodium potassium tartrate in 0.4 M NaOH). The reaction mixture was then incubated in a boiling water bath for 5 min, cooled to room temperature, and diluted with 10 ml distilled water. The absorbance was measured at 540 nm.

Percentage α-amylase inhibition was calculated as using the formula:

$\%Inhibition = [(A540_{control} - A540_{sample}) \div A540_{control}] \times 100.$

The IC_{50} was calculated from the dose-inhibition linear regression curve of each extract.

Statistical Analysis

All assays were performed in three independent experiments and expressed as mean ± standard deviation (SD). Analysis of variance (ANOVA) was done by SPSS statistical software package version 17. The P values less than 0.05 were considered as statistically significant.

Table 1: Phenological variation in the antioxidant phytochemicals composition of *T. tetraptera* pod on dry weight basis.

Phytochemical	MGP	RBP
Total phenol (mg/g)	27.48 ± 0.16^b	42.18 ± 0.16^a
Tannin (mg/g)	32.54 ± 0.22^b	46.99 ± 0.17^a
Total flavonoid (mg/g)	0.18 ± 0.01^b	0.44 ± 0.02^a
Total carotenoid (µg/g)	5.03 ± 0.09^a	1.31 ± 0.04^b
Vitamin C (mg/100g)	3.91 ± 0.06^a	2.86 ± 0.05^b

Data represent the mean ± standard deviation of triplicate readings; values with the different lowercase superscript letter along the same row are significantly different (P >0.05).

Table 2: Phenological variation in the antioxidant activities and α-amylase IC_{50} of methanolic extracts of *T. tetraptera* pod.

Antioxidant activity	MGP	RBP
TEAC (mM TE/g)	4.47 ± 0.02^b	4.82 ± 0.03^a
FRAP (µg GAE/g)	49.84 ± 0.43^b	77.96 ± 0.85^a
DPPH IC_{50} (mg/ml)	0.16	0.11
α-Amylase IC_{50} (mg/ml)	0.46	0.33

Data for FRAP and TEAC represent the mean ± standard deviation of triplicate readings; values with different lowercase superscript letter along the same row are significantly different (P >0.05).

RESULTS AND DISCUSSION

Fruits are known to undergo several changes in flavor, texture and color due to qualitative and quantitative variation in the composition of phytochemicals during the course of maturity and development. [27] The antioxidant phytochemicals results of *T. tetraptera* pod (on dry weight basis) at two different phenological stages of the fruit development (MGP and RBP) are presented in table 1. The results revealed that RBP had significantly higher ($P < 0.05$) total phenol, tannin and total flavonoid contents than MGP. This may be attributed to longer exposure of the RBP to sunlight (October to November) during which the pod became fully ripe and turned brown. According to Duenas et al. [28], phenolic compounds are synthesized in response to light. Jaakola *et al.* [29] also reported that the expression of the flavonoid biosynthetic genes is activated by enhanced light. While polyphenols are prominently known to exhibit antioxidant activity through a variety of mechanisms, including free radical scavenging, lipid peroxidation and chelating of metal ions [30], they also have many other biological activities, such as anti-histamine [31], anti-inflammatory and anticarcinogenic [32], antibacterial [33], and antiviral activities. [34]

In contrast to the phenolics results, MGP had significantly higher ($P < 0.05$) total carotenoid content than RBP. This may be due to the abundance of chlorophyll in the MGP as indicated by its greenness, than in the RBP. Gitelson *et al.* [35] established a linear relationship between chlorophyll and carotenoids contents in higher plant leaves. In *T. tetraptera* fruit, the transition to ripening is accompanied by de-greening of the pod in which the photosynthetically active chloroplasts are differentiated to chromoplasts, resulting in its characteristic brown colour. Carotenoids are lipid-soluble antioxidant, which have been reported to have health benefits such as pro-vitamin A activity [36], antioxidant [37], anticancer [38], and antiobesity effects. [39]

Similarly, MGP had significantly higher ($P < 0.05$) vitamin C content than RBP. This decrease could be attributed to the susceptibility of Vitamin C to oxidative destruction during ripening. [40]

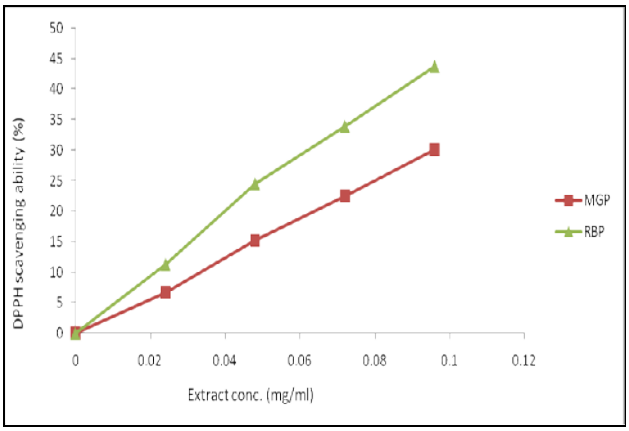

Fig. 2a: Concentration-response curve for inhibition of DPPH free-radical by MGP and RBP methanolic extracts.

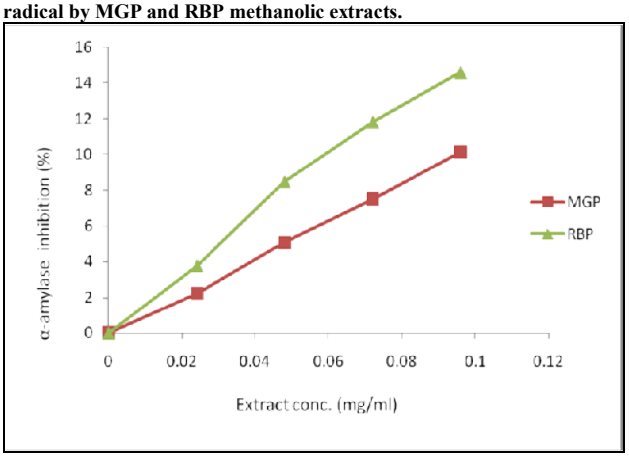

Fig. 2b: Concentration-response curve for inhibition of porcine pancreatic α-amylase activity by MGP and RBP methanolic extracts.

Vitamin C has powerful antioxidant properties and indirectly contributes to several key oxidative and reductive enzyme systems. It also has the ability to regenerate other biologically important antioxidants, such as glutathione and vitamin E, into their reduced state. [41]

Table 2 presents the antioxidant activities and porcine pancreatic α-amylase IC_{50} of methanolic extracts of MGP and RBP. Antioxidants are substances which counteract free radicals, thus preventing oxidative damage to biomolecules such as lipids, proteins and DNA. [42-43] The ABTS* scavenging ability reported as the Trolox equivalent antioxidant capacity (TEAC) showed that RBP had a significantly higher ($P < 0.05$) scavenging ability than MGP. ABTS assay involves electron transfer process [32] and is based on the discolouration of ABTS by antioxidant compounds, thus reflecting the amount of ABTS radicals that are scavenged within a fixed time period in relation to that of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox).

Similarly, the reducing power (from Fe^{3+} to Fe^{2+}) of MGP and RBP reported as gallic acid equivalents, GAE (table 2) revealed that RBP had a significantly higher ($P < 0.05$) reducing power than MGP. FRAP assay is based on electron-transfer reactions, in which a ferric salt, potassium ferricyanide is used as an oxidant. The reaction mechanism involves the reduction of ferric 2,4,6-tripyridyl-*s*-triazine (TPTZ) to the coloured ferrous form. [44]

Free radical scavenging abilities of MGP and RBP methanolic extracts were further tested by the DPPH method at different concentrations (0.024 to 0.096 mg/ml). Both extracts exhibited very high DPPH radical-scavenging activity in a dose-dependent manner as depicted by their concentration-response curves (Figure 2a). Antioxidant reacts with DPPH, a stable free radical with characteristic deep purple colour in solution, converting it to α,α-diphenyl-β-picryl hydrazine. On accepting proton from antioxidants, DPPH solution loses its characteristic deep purple, leading to absorption decrease (λmax 515–517 nm). So the degree of discoloration is an indication of the scavenging potentials of the antioxidant extract. Relative to MGP with IC_{50} of 0.16 mg/ml, RBP had IC_{50} of 0.11 mg/ml. Since IC_{50} is a measure of inhibitory concentration, a lower IC_{50} value is a reflection of greater antioxidant activity of the sample. [45]

MGP and RBP methanolic extracts displayed potent inhibitory activity against porcine pancreatic α-amylase in a dose-dependent manner *in-vitro* (Figure 2b). The IC_{50} values showed that RBP (IC_{50} = 0.33 mg/ml) has a higher potency to inhibit the activity of porcine pancreatic α-amylase than MGP (IC_{50} = 0.46 mg/ml). The higher potency of RBP over MGP in inhibiting porcine pancreatic α-amylase may be attributed to its higher phenolic levels (total phenol, tannin and total flavonoid), which possibly were also responsible for the higher antioxidant activities observed in RBP. This finding is in agreement with earlier report of positive correlations between total phenolic content and antioxidant activity, and between antioxidant activity and α-amylase inhibition activity of plant extracts. [46] Tiwari and Rao [47] reported that polyphenols inhibit alpha-amylase, sucrase, as well as the action of sodium glucose-transporter 1 (SGLUT-1) of the intestinal brush border, hence their antidiabetic action.

Inhibition of gastrointestinal carbohydrate hydrolyzing enzymes such as the salivary and pancreatic α-amylase (EC 3.2.1.1), can significantly decrease the postprandial increase of blood glucose after a mixed carbohydrate diet and therefore is an important strategy in the management of postprandial blood glucose level in type 2 diabetic patients and borderline patients. [48]

In conclusion, the results of this study confirmed that there were variations in the *in-vitro* antioxidant properties and α-amylase inhibitory activities of *T. tetraptera* pod at the two phenologic stages of the fruit development that were studied. The predominance of phenolic antioxidant phytochemicals (total phenol, tannin and total flavonoid) in RBP than in MGP conferred it with higher *in vitro* antioxidant activities

and α-amylase inhibitory activity than MGP. Hence, for a more effective use of *T. tetraptera* pod for the management of oxidative stress and postprandial hyperglycemia in type 2 diabetes, it is recommended that the pod should be used at the ripe brown stage.

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