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Phenolic Profiles of Four Processed Tropical Green Leafy Vegetables Commonly Used as Food

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The phenolic profiles are presented of four tropical green leafy vegetables (*Ocimum gratissimum*, *Vernonia amygdalina*, *Corchorus olitorius* and *Manihot utilissima*) commonly used as food, after application of traditional treatments, such as boiling and abrasion. The HPLC/DAD/MS technique was mainly used to carry out this study. Preliminary evaluation of the antioxidant properties of the vegetables was also performed using the DPPH *in vitro* test. For the first time, seasonal variations in the phenolic content of the four investigated vegetables were highlighted. Of the four plants, all showed only quantitative differences, except for *Ocimum graticimum*, in which cichoric acid, previously detected as one of the main constituents of this vegetable collected in November (dry season), was absent in the sample harvested in March. The phenolic constituents are chemically unmodified after a strong heating process, such as the traditional blanching (about 15 minutes) applied by Nigerian people prior to consuming these vegetables. Nevertheless, these typical preparations showed a consistent decrease in the total phenolic compounds with respect to the raw material, particularly for *Corchorus olitorius* (from 42.3 to 5.56 mg/g dried leaves) and *Vernonia amygdalina* (from 40.2 to 4.4 mg/g dried leaves). As expected, when the blanching treatment is reduced to a few minutes, as for *Manihot utilissima* leaves, the cooked vegetable maintained almost unaltered its original phenolic content (around 10 mg/g dried leaves). The unique exception is the blanched *Ocimum gratissimum* sample that showed a consistent increment of the total phenols, particularly of rosmarinic acid (from 6.1 to 29.8 mg/g dried leaves) with respect to the unprocessed vegetable.

Keywords: *Ocimum gratissimum*, *Vernonia amygdalina*, *Corchorus olitorius*, *Manihot utilissima*, phenolic compounds, traditional preparations, HPLC/DAD/MS.

Most of the compositional aspects of vegetables commonly used in the Western diet are well known, but scant data are available on endemic plants from African regions. The present study attempts to improve knowledge of the phenolic content of the processed leaves of four vegetables commonly used for food and medicinal purposes in Nigeria.

Ocimum gratissimum L. (Og) (Labiateae), *Manihot utilissima* Pohl. (Mu) (Euphorbiaceae), *Vernonia amygdalina* L. (Va) (Compositae), and *Corchorus olitorius* L. (Co) (Tiliaceae) are considered in the present work. Among them, Og, Va and Co are mainly consumed as fresh or pot vegetables. The tuber of Mu is the part mainly eaten, but the young leaves are gaining acceptance also as a pot vegetable.

Ocimum gratissimum, African basil, and known as "efinrin" by Nigerian people, is usually collected from May to October. With regards to its composition, the principal data are related to its flavonoid composition [1], but mainly related to plants grown in the UK [2]. The authors found that the profile of the main flavonoids was similar in all accessions belonging to the same species and they showed vicenin-2, luteolin 7-*O*-glucoside, quercetin 3-*O*-glucoside and quercetin 3-*O*-rutinoside as constituents of the leaves, together with xanthomicrol, cirsimaritin, and kaempferol-3-*O*-rutinoside. For medicinal properties, in south-western Nigeria, Og is mainly known for its antimicrobial activities against bacteria causing diarrhea [3].

Manihot utilissima or cassava, known by the local population as "ewe ege", is harvested throughout the year. This vegetable is widespread in Nigeria, which is the world's largest producer. Though less popular in the Nigerian diet compared with other vegetables, the dietary acceptance of Mu leaves has been increasing within local populations [4].

Vernonia amigdalina leaves, known as "ewuro" by the local population, are harvested throughout the year and are characterized by an intense bitterness. Previous reports on the composition of this plant highlighted the presence of luteolin, luteolin 7-*O*-β-glucoside and luteolin 7-*O*-β-glucuronide as main flavonoids, together with several dicaffeoyl derivatives [5]. Also, some saponins and sesquiterpene lactones have been reported in the leaves [6]. Due to its documented antimalarial [7-9], antimicrobial [10-11] and anticancer properties [12], Va is probably one of the most used medicinal plants in Nigeria.

Corchorus olitorius or "Tossa Jute", is quite popular for its leaves, which are usually collected from May to December and used as a pot herb. Jute leaves, also known as Jew's Mallow, are popular in West Africa and the Yoruba people of Nigeria call it "ewedu". The leaves are made into a common mucilaginous soup or sauce in some West African cooking traditions. *C. olitorius* leaves contain kaempferol glycosides, rutin and isoquercitrin [13], together with chlorogenic acid and several dicaffeoyl derivatives of quinic acid [5]. With regard to its use as a medicinal plant, this vegetable is mainly known in Nigeria for its laxative activity and as a blood purifier [14].

The present study was mainly focused on comparing the phenolic profile of the four vegetables obtained in different seasons to determine the qualitative and quantitative content of these compounds in the processed vegetables after application of traditional treatments, and to evaluate some antioxidant properties by using the DPPH *in vitro* test.

Phenolic distribution in the unprocessed leaves. To recover all the main phenolic constituents, the dried leaves were extracted with ethanol/acidic water (7:3) at room temperature, and the extracts were then analyzed using a previously optimized chromatographic method [5]. The identification of the components of the extract was carried out by comparison with our previous results [5], with the help of their retention times, and UV-Vis and MS spectra. When necessary, the use of standard reference compounds and/or laboratory extracts helped to complete this identification.

Our previous findings, which highlighted for the first time the phenolic composition of these vegetables, took into account samples collected in Nigeria during the dry season (November), while in this study we analyzed plants collected in March. Given that these vegetables are consumed throughout the year by the local population, it is of interest to evaluate if seasonality affects the phenolic composition of the vegetables.

Regarding Co, its chromatographic profile at 330 nm is similar to that obtained for the sample collected in November, even if an increment of the ratio between the more polar monocaffeoyl quinic derivative (Co1) and the 1,5 dicaffeoyl quinic acid (Co 5) is observed. It is reasonable to hypothesize that these two molecules are biosynthetically correlated and that different climatic conditions can modulate their production in the plant.

The dominant compound detected in the Va sample was 1,5 dicaffeoyl quinic acid (Va 10), differently from the findings observed for the leaves collected in November. At the same time, almost all the flavonoids, both luteolin and apigenin glycosides, were minor constituents.

Among the four plants, the chemical profile of Og is the most complex due to the co-presence of metabolites belonging to different chemical classes (Table 1d). Major differences were observed between the leaves collected in the two seasons. In the March sample, together with a consistent amount of rosmarinic acid and the presence of the polar glycoside, vicenin 2 or apigenin 6,8-di-*C*-glucoside, a high concentration of the metoxyflavone, nevadensin, has again been highlighted [5]. Nevertheless, it is worth noting that cichoric acid, one of the main phenolic constituents previously detected and estimated at nearly 2.5 mg/g in the dried leaves collected in November [5], was completely absent in this sample. In the light of this finding, it can be said that, differently from the other phenolic constituents, the biosynthesis of this dicaffeoyl tartaric acid in African basil is particularly sensitive to seasonal variation. Contrary to the HPLC profiles observed at 330 nm for Va and Og, that for Mu seemed to be unaffected by the time of harvest. In fact, the relative ratios between the four main

Table 1a

Compounds of *Corcorus olitorius*	Raw	Blanched
Co1 - caffeoyl quinic derivative	14.8±0.4	2.8±0.03
Co2 - chlorogenic acid	0.2±0.02	0.07±0.00
Co3+4 - hyperoside+isoquercitrin	2.1±0.06	0.3±0.004
Co5 1, 5 -dicaffeoyl quinic acid	23.2±0.6	2.0±0.03
Co6 - dicaffeoyl quinic acid	0.1±0.004	0.002±0.002
Co7 - dicaffeoyl derivative	1.7±0.03	0.5±0.004
Co8 - quercetin derivative	0.1±0.009	nd

Table 1b

Compounds of *Vernonia amigdalina*	Raw	Blanched	Abrasion
Va1+2 - caffeoyl quinic and chlorogenic acids	2.3±0.3	0.4±0.02	0.5±0.04
Va4 - rutin	0.2±0.04	nd	nd
Va5 - luteolin 7-O-glu	2.7±0.3	0.3±0.02	0.4±0.02
Va7 - flavonoid	0.3±0.03	0.1±0.009	0.005±0.006
Va9 - luteolin 7-O-glucuronide	4.9±0.5	0.7±0.02	0.7±0.04
Va10 -1,5 dicaffeoyl quinic acid	23.47±3.0	1.4±0.2	6.9±1.5
Va11 - dicaffeoyl quinic acid	2.6±0.1	0.3±0.02	0.5±0.08
Va12 - dicaffeoyl quinic acid	2.5±0.4	0.4±0.03	1.3±0.5
Va13 - apigenin-O-glucuronide	0.7±0.02	0.4±0.02	0.2±0.02
Va14 - luteolin	0.2±0.03	0.1±0.01	0.6±0.06
Va15 - flavonoid	0.1±0.02	0.1±0.01	0.02±0.004
Va16 - flavonoid	0.09±0.01	0.04±0.004	0.3±0.01
Va17 - flavonoid	0.06±0.01	0.1±0.001	0.0015±0.0

Table 1c

Compounds of *Manihot utilissima*	Raw	Blanched
Mu1 - rutin	5.1±0.2	5.8±1.0
Mu2 - kaempferol 4'-O-rut	0.7±0.05	0.8±0.2
Mu3 - kaempferol 3'-O-rut	2.5±0.1	2.8±0.5
Mu5 - amentoflavone	0.9±0.008	1.1±0.2

Table 1d

Compounds of *Ocimum graticimum*	Raw	Blanched	Abrasion
Og1 - vicenin-2	1.5±0.002	1.0±0.06	0.9±0.06
Og2 - caffeic acid	0.5±0.01	0.5±0.03	0.3±0.007
Og3 - rutin	0.2±0.003	0.1±0.001	0.02±0.001
Og4 - luteolin-7-O-glucoside	0.3±0.01	0.2±0.02	nd
Og5 - kaempferol 3'-O-rutinoside	0.04±0.006	0.03±0.0	nd
Og6 - rosmarinic acid	6.1±0.6	29.8±1.7	1.0±0.2
Og10 - cirsimaritin	0.7±0.05	0.6±0.01	0.5±0.02
Og11 - nevadensin	5.8±0.4	5.0±0.3	4.6±0.2

Table 1a-d: Phenolic compounds in processed and unprocessed leaves. All the data are a mean of three different determinations and are expressed as mg/g (SD) dried leaves.

constituents (Mu1-Mu4) was unaltered when compared with those observed for the material collected in rainy season [5].

Evaluation of the phenolic content. In Nigeria several green vegetables, among them Va, Mu, Co and Og, are usually blanched before consumption using either hot water or steam. Often indigenous people apply an abrasion treatment to the fresh leaves to remove part of the juice in order to reduce the bitterness and/or acidity of the plant. Among these selected Nigerian plants, this latter treatment is traditionally applied only to Va and Og and, therefore, in this study manually squeezed leaves

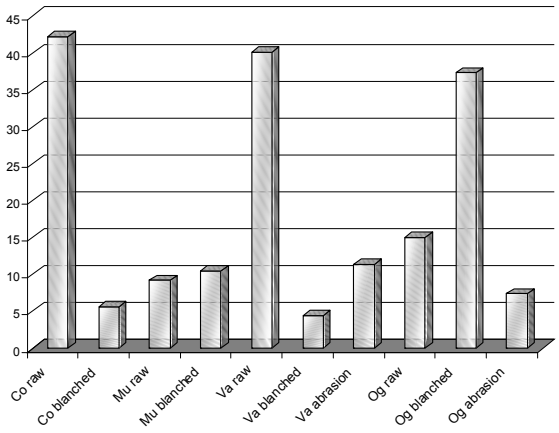

Figure 1: Comparison of the total phenolic content in all the samples expressed as mg/g dried leaves.

Table 2: DPPH radical scavenging activity.

	DPPH results from hydroalchoolic extracts		
	0.05 mg/mL	**0.1 mg/mL**	**0.15 mg/mL**
Og	82.9 ±1.0	88.0 ± 0.4	88.9 ± 1.0
Va	55.9 ± 3.2	65.7 ±1.9	79.0 ±0.4
Mu	53.1 ±1.4	78.4 ±0.2	81.1±1.8
Co	84.2 ±0.1	79.2± 2.2	88.9 ±1.0

were also analyzed. The quantitative distribution of the different phenolic compounds in the processed and unprocessed leaf extracts is summarized in Tables 1a-d and in the histogram of Figure 1.

Comparison of the chromatographic profiles of Co obtained from the blanched and raw leaves showed an inversion of the relative contents of the Co1 and Co5 compounds. Moreover, a consistent decrease of the total phenolic amount (from 42.3 to 5.6 mg/g) in the processed leaves was observed (Table 1a and Figure 1). The greater amount of soluble fiber of this plant [15,16] is removed during the blanching process producing a gel that can entrap part of the more soluble phenols released from the leaves.

A dramatic decrease in the total phenolic content was observed for Va after blanching and abrasion, suggesting that these compounds are mainly localized in the juice and consequently they are almost completely removed from the vegetable after these treatments.

With regard to Mu, as confirmed by the quantitative data (Table 1c), very few changes were observed between the raw and blanched leaves. Effectively the young Mu leaves, differently from the other plants, are traditionally blanched only for a few minutes. This short time of boiling is not efficacious in extracting the phenolic fraction from the leaves and consequently the blanched vegetable, used as food by the local population, remains a good source of phenolic compounds.

As highlighted for the unprocessed leaves, the total absence of cichoric acid in the Og extracts after blanching and abrasion was confirmed. Moreover, a peculiar behaviour was revealed for the rosmarinic acid that increased consistently (from 6.1 to 29.7 mg/g) in the blanched sample (Table 1d).

Antioxidant activity by DPPH test. One of the aims of this study was to make a preliminary evaluation of the antioxidant potency in terms of free radical scavenging of the phenolic fractions obtained from the leafy vegetables of these plants. Most of the antioxidant activities of vegetables and fruits have been established to be related to phenolic compounds [17]. Free radical scavenging is one of the known mechanisms by which antioxidants inhibit lipid peroxidation [18,19]. The DPPH radical scavenging activity has also been used extensively for screening antioxidants from fruits and vegetables [20].

This activity was measured for the extracts from unprocessed leaves at three different concentrations. A summary of the results expressed as % DPPH radical scavenging activity is reported in Table 2. Overall, all the tested vegetable materials showed a relatively high inhibition at the highest tested concentration and this activity could be attributed to the presence of flavonoids and cinnamoyl derivatives in the hydroalcoholic extracts. The results from Va and Mu seem to be dose dependent. Taking into account the lower concentration, the best results in terms of antioxidant potency were obtained for Og and Co, but no differences among the extracts were highlighted with the higher concentrations.

Experimental

Materials: The vegetables were harvested from the teaching and research farm of the Federal University of Technology, Akure, Nigeria in March 2008 and voucher specimens were deposited at the Department of Biochemistry, Federal University of Technology, Akure, Nigeria and the Department of Pharmaceutical Science, University of Florence, Italy. About 500 g of fresh green leafy vegetables, *Vernonia amygdalina* (Va), *Corchorous olitorius* (Co), *Ocimum graticimum* (Og) and *Manihot utilissima* (Mu), were rinsed in water, and the edible portions separated. The edible portions were chopped

into small pieces (300 g) and divided into two (Mu and Co) or three portions (Va and Og). The first portion of the chopped vegetables served as the unprocessed sample, the second portion was blanched in 300 mL boiling water for 15 min (Va, Og and Co) and 5 min for Mu, while the third portion, with the aid of small quantity of water, was manually squeezed by hand (abrasion) to remove the juice. The blanched and squeezed portions were subsequently drained of water.

All samples were dried in an air oven at 30°C prior to analysis and treated as described in the next experimental section.

The standards used to confirm the chemical structure of some compounds (Table 1a-d) were purchased from Extrasynthese (Geney-France); rutin was from Sigma-Aldrich (St. Louis, MO-USA).

Extraction methods for HPLC/DAD analysis of the processed samples: (1 g each) was extracted with stirring for 2 h in 40 mL (20 mL x 2) of ethanol/water 7:3 (v/v) with water acidified with formic acid (pH 2.5). The samples were centrifuged (4,400 rpm for 10 min) and the supernatant was centrifuged again (12,000 rpm for 8 min) to obtain a clear solution directly analyzed by HPLC/DAD.

HPLC/DAD/MS analysis: Analyses were performed using a HP 1100 liquid chromatograph equipped with HP DAD and 1100 MS detectors; the interface was a HP 1100 MSD API-electro spray. All the instruments were from Agilent Technology (Palo Alto, CA, USA). The MS analyses were carried out in negative mode with a fragmentor range between 80-150 V. *Method 1*. A C12 column, 150 × 4 mm (4μm) Synergi Max (Phenomenex-Torrance CA) maintained at 30°C and equipped with a 10 × 4 mm pre-column of the same phase was used with a flow rate of 0.4 mL min^{-1}. The eluents were H_2O acidified to pH 3.2 with formic acid (A) and acetonitrile (B). The following linear solvent gradient was applied: from 95 % A to 85% A in 5 min, to 75% A in 8 min and a plateau of 10 min, to 55% A in 12 min and a plateau of 5 min, to 10% A in 3 min, and a final plateau of 2 min to wash the column. The total time of analysis was 45 min.

Quantitative determination: Chlorogenic acid, rutin and luteolin 7-*O*-glucoside were used for the quantitative evaluation. Three five-point calibration curves were prepared as follows: chlorogenic acid at 330 nm (range 0.038-0.3 mg/mL and r^2 of 0.9996) was used to evaluate all the cinnamoyl compounds; luteolin 7-*O*-glucoside at 330 nm (range 0.11-0.88 mg/mL and r^2 of 0.9999) was selected to evaluate all the luteolin and apigenin derivatives, together with nevadensin; rutin at 350 nm (range 0.13-1.02 mg/mL and r^2 of 0.9999) was used to quantify all the derivatives of quercetin and kaempherol. All the quantitative data were obtained in triplicate.

DPPH assay: A dried sample (1 g each) was extracted, by stirring for 2 h, with 40 mL (20 mL x 2) of either ethanol or ethanol/water 7:3 (v/v), with water acidified with formic acid (pH 2.5). The samples were filtered, concentrated to dryness and redissolved in 96% ethanol. The clear solutions were directly analyzed by HPLC/DAD and 3 concentrations (0.05, 0.1 and 0.15 mg/mL) used for the DPPH assay. The radical scavenging activity of ethanol and ethanol/water extracts was carried out as follows: 2 mL of each extract was mixed with 1mL of 0.125 mM DPPH ethanol solution. After shaking the mixture, the absorbance was measured at 517 nm after 5 min of incubation. Radical scavenging activity is expressed as the inhibition percentage.

Conclusions: For the first time, seasonal variation in the phenolic content of the four investigated vegetables was highlighted. Within the four plants, almost all showed only quantitative differences, with the exception of *Ocimum graticimum.* In fact, cichoric acid, previously detected as one of the main constituents of this vegetable collected in November (dry season), was completely absent in the sample harvested in March. The phenolic constituents are chemically unmodified after a strong heating process such as the traditional blanching (about 15 minutes) applied by Nigerian people prior to consuming these vegetables. Nevertheless, these typical preparations showed a consistent decrease in the total phenolic compounds with respect to the raw material, particularly *Corchorus olitorius* (from 42.3 to 5.56 mg/g dried leaves) and for *Vernonia amygdalina* (from 40.2 to 4.4 mg/g dried leaves). As expected, when the blanched treatment is reduced to a few minutes, as for *Manihot utilissima* leaves, the cooked vegetable maintained almost unaltered its original phenolic content (around 10 mg/g dried leaves). The unique exception is the blanched *Ocimum gratissimum* sample that showed a consistent increment in total phenols, particularly of rosmarinic acid (from 6.1 to 29.8 mg/g dried leaves) in comparison with the unprocessed vegetable.

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