Effect of Boiling on Chemicals, Phytochemicals and Nutritional Composition of Solanum nigrum L. Leaves Harvested in Abidjan (Côte d’Ivoire)

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Authors’ contributions

This work was carried out in collaboration among all authors. Author DKD designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors NKK and DYN managed the analyses of the study. Author GNA managed the literature. All authors read and approved the final manuscript.

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ABSTRACT

Aims: In tropical Africa, leafy vegetables are traditionally cooked and eaten as a relish together with a starchy staple food. The current study aimed to evaluate the influence of boiling on bioactive, proximate and antinutrients compounds in Solanum nigrum leaves.

Methodology: The leaves were subjected to boiling in pressure cooker for 10, 15 and 20 min and proximate composition, minerals, nutritive and anti-nutritional components were determined according to standard methods for nutritional guidelines.

Results: The result of the study revealed that longer time of boiling (higher than 10 min) caused negative impact by reducing nutritive value but positive impact by reducing anti-nutrients. The
1. INTRODUCTION

The medicinal properties of plants have been investigated in the recent scientific developments throughout the world, due to their potency against several diseases, minor side effects and economic viability. Several compounds widely distributed among plants have been reported to exert multiple biological effects [1,2]. Traditional vegetables are valuable sources of nutrients [3], with some having important medicinal properties [4]. Leafy vegetables represent high quality nutritional sources, for the poor segment of the population especially where malnutrition is wide spread [5]. It was reported that an estimated amount of 6376 useful spontaneous African plants of which 397 are vegetables, about twenty (20) species are widely consumed and cultivated by Ivorians populations [6]. The consumption of these leafy vegetables is linked to the region and ethno-botanical. Studies have stated that most people in Northern Côte d’Ivoire consume indigenous green leafy vegetables [7]. Solanum nigrum L. is important aspects of medicinal plant resources for treatment of primary health care. The leaves of Solanum nigrum L. have a potential source of useful drugs due to the presence of phytochemicals and can be utilized for the treatment of many diseases and also be exploited for use in the pharmaceutical [7]. Before consumption Solanum nigrum post-harvest processing that can affect their nutrient contents [8]. Although most vegetables are cooked prior consumption, evidence is emerging that in vivo bioavailability of many bioactive compounds enhanced as vegetables are cooked. Fresh raw plants can be prepared in various ways to eat dish. Besides, cooking gives final product pleasanter sensory characteristics, as well as being more digestible and microbiologically safer to eat [9]. Feed processing is a versatile high temperature process that may alter the nutritional value of feeds [10]. The feed process involves water, heat, and mechanical stress, all of which can impact on vitamin and carotenoid stability [11]. Traditionally, boiling, squeeze-washing, and sun-drying are practiced, and generally result from the destruction of some nutrients [12,13]. The cooking method not only affects the nutritional composition of the food, but also the level of available bioactive compound [9]. However, both positive and negative effects have been reported depending upon differences in process conditions and morphological and nutritional characteristics of vegetable species [11,14]. But there is a lack of scientific data with regard to the effect of cooking methods on their physicochemical and nutritive characteristics [15]. Moreover, a real interest for consumers is emerged since epidemiological studies have linked the dietary habits and the prevalence of certain diseases such as cancers, obesity and cardiovascular diseases [16,17]. These leafy vegetables form a part of the diets and incomes of rural and urban households by providing adequate amounts of protein, many vitamins, dietary fiber and other important nutrients which are usually in short supply in daily diets [18]. In the present study, the effect of boiling of phytochemical contents and total antioxidant capacities of Solanum nigrum was investigated.

2. MATERIALS AND METHODS

2.1 Materials

Solanum nigrum L. was previously authenticated by National Floristic Center (University Felix Houphouët-Boigny, Abidjan-Côte d’Ivoire).
leaves were collected fresh and at maturity from cultivated farmlands located at Dabou (latitude 5°19'14" North longitude: 4°22'59"West) in the District of Abidjan.

2.2 Methods

2.2.1 Preparation of raw and cooked leaves.

Leaves were put into clean and dry sample containers and transferred to the Laboratory of Food Biochemical and Tropical Products Technology (Nangui Abrogoua University, Abidjan, Côte d’Ivoire) [19] and were cooked after cleaning and washing with water [20].

2.2.2 Boiling of leaves

The boiling of leaves was carried out as described previously [21]. One (1) L of water was brought to boil in 2-L beaker. The beaker was covered to prevent water loss to evaporation. Five hundred grams of Solanum nigrum leave were boiled separately for 0, 10, 15 and 20 min. After cooking, the vegetables were drained, cool for a few minutes at room temperature homogenized, and, be oven-dried at 40°C for 72 h, grounded into flour and stored in polyethylene bags for biochemical analyzes.

2.2.3 Proximate analysis

The methods used for sample treatment and analysis (ash, protein, lipids) were carried following standard procedures recommended by AOAC [22]. Ash was determined by gravimetric of incinerated sample, in muffle at 550°C, total nitrogen was determined according to the Kjeldahl method and converted into protein, using factor 6.25; total lipids were extracted by the Soxhlet technique with hot solvent (hexane) and afterwards were determined by gravimetry. The total carbohydrate content was calculated by using the equation: 100-(moisture + protein + lipid +ash) [23]. Total crude fiber was determined according method described by Prosky et al. using sulfuric acid [24]. The caloric value of samples was calculated using the Atwater conversion factor [25].

2.2.4 Mineral analysis

Minerals were determined by American Association of Cereal Chemists method [26]. Glass and polypropylene materials were soaked in concentrated nitric acid (specific gravity = 1.41 g. cm⁻³) for 15 min and then rinsed 3 times for distilled deionized water before use. Mineral component comprising magnesium (Mg), iron (Fe), calcium (Ca), Zinc (Zn) and phosphorus (P) were determined by atomic absorption spectrophotometry (Unicam Analytical system, Model 919, Cambridge, UK). All samples were previously subjected to dry digestion at 550°C. To dissolve the ashes, 3 mL of concentrated HCl (specific gravity = 1.19 g.cm⁻³) were added, the vessel was covered with a watch glass and gently hat (~70°C) for 3.5 h, leaving about 1 mL of liquid at the end of heating. The solution was then transferred to a 10 mL volumetric flask and brought to the volume with water. The solution was then used for individual mineral determination using spectrophotometer and flame photometer. These measurements were carried out in triplicates, for two measurements per analysis. All minerals were expressed in mg/100 g dry weight basis.

2.2.5 Anti-nutritionals compound

The method described by Day and Underwood [27] was used to determine the oxalate content of defatted leaves flour of S. nigrum extracts. This method consists of extracting the total oxalates (insoluble and soluble) followed by titrimetric analysis. Briefly, one gram of dried powder and 75 mL of 1.5 N H₂SO₄ was added in 100 mL conical flask. The mixture was carefully shaken on a mechanical shaker for 1 h. Then, the solution was filtered using Whatman No.1 filter paper. A 25 mL of filtrate was titrated hot (80–90°C) with freshly prepared 0.1 N KMnO₄ solution until the colour of the solution become pink persisted for 30 seconds. The results were expressed as mg/100 g dry weight.

2.2.6 Phytochemicals compounds

The phenolic compounds were extracted following the procedure described by N'Dri, et al. [28] and polyphenols content (expressed as gallic acid equivalent, mg/100 g dry weight) was determined by colorimetry, using the Folin Ciocalteu’s method described by Singleton, et al. [29]. Vitamin C contained in analyzed samples was determined by titration using the method described by Pongracz, et al. [30]. The tannins assay was performed according to the method described by Bainbridge, et al. [31] using vanillin reagent. The total flavonoids content was evaluated using the method reported by Meda, et al. [32]. Antioxidant activity assay was carried out using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) spectrophotometric method [33].
3. RESULTS AND DISCUSSION

3.1 Results

3.1.1 Proximate composition

The Proximate composition of cooked and uncooked leaves of *S. nigrum* is shown in Table 1. The ash in raw leaves (17.96±0.03%) was significantly higher (P = .05) than those of boiled. The ash content after 10 and 20 min of boiling decreased, it ranged from 12.37±0.12% to 9.30±0.42%. The lipid content was 3.22±0.06% in uncooked leaves and ranged from 5.09±0.10% to 6.36±0, 06 in boiled at 10 min and boiled at 20 min. However, the lipids content increased significantly (P = .05) in leaves after boiling. Total crude fibers contents were significantly different (P = .05) in all samples with the values of 27.62±0.00, 39.41±0.00, 41.39±0.10 and 49.99±1.26% respectively for uncooked, boiled at 10, 15 and 20 min. The proteins content of raw and boiled samples falls between 30.33±0.39 and 34.42±0.49%. Boiled at 20 min have presented significantly lower (P = .05) amounts of protein compared to the raw and the other boiled samples. However, the protein content of those others processed samples (boiled at 15 and 20 min) was significantly from boiling (16.78±0.52 to 04.02±0.79%). However, the amount of sugar varies significantly (P = .05) from the boiling higher (P = .05) than those of boiled at 10 min and raw leaves. The amount of carbohydrates of leaves was decreasing method. Calorific energy decreasing during boiling from and 233.78±0.10 to 194.01±4.43 kcal/100 g during boiling.

3.1.2 Mineral composition

The results of the evaluation of total minerals in raw and boiled leaves are shown in Table 2. Minerals content decreased significantly (P = .05) compared to fresh forms after the boiling. Calcium content in uncooked leaves (1687.65 ± 78 mg / 100 g) was significantly lower (P = .05) than those of boiled. The Magnesium content was 1008.22 ± 20.72 mg / 100 g in uncooked leaves and decreasing from 662.99 ± 27.23 to 572.78 ± 5.36 mg / 100 g by increasing the degree of boiling. The Phosphorus content after 15 min of boiling decreased significantly (P = .05) compared to the fresh leaves and ranged from 423.71±15.12 to 405.24±10 mg / 100 g (boiled at 20 min). The Iron content after 10 min of boiling decreased significantly (P = .05) compared to the fresh leaves and ranged from 33.48±0.93 mg / 100 g to 44.50±1.23 mg / 100 g (boiled at 10 min). The Zinc contents were significantly different (P = .05) in all samples with the values of 25.14±1.80 mg / 100 g, 18.90 ± 0.10 mg / 100 g, 17.07 ± 0.02 mg / 100 g and 11.12 ± 0.08 mg / 100 g respectively for uncooked, boiled at 10 min, 15 min and 20 min. The computed molar ratios of oxalate/calcium were lower than 2.5. All the samples analyzed Na / K molar ratio less than 1.

3.1.3 Phytochemical properties

The Phytochemical properties composition of the boiled and uncooked leaves was presented in Table 3. The phytochemical properties parameters generally differ significantly (p < 0.05) from boiled to fresh leaves. Vitamin C contents after 10 min of boiling decreased significantly (P = .05) compared with the fresh leaves and ranged from 11.23 ± 0.00% to 9.90±0.00% (boiled at 20 min). Total polyphenols content was decreased significantly (P = .05) from all boiled samples with the values of 240.98±3.00, 125.41±0.92 and 86.11±0.5 mg/100 g respectively for uncooked, boiled at 10, 15 and 20 min. The tannins content after 10 min of boiling decreased significantly (P = .05) compared to the fresh leaves and ranged from 157.45±1.38 to 73.82±1.22 mg / 100 g (boiled at 20 min). The Flavonoids content was 16.46±0.34 mg / 100 uncooked leaves and which recorded 10.19±0.46 mg / 100 (boiled at 20 min) and 14.03±0.21 mg / 100 g (boiled at 10 min). However, the tannins content decreased significantly (P = .05) in leaves after boiling. The antioxidant activity was decreased significantly (P = .05) from all boiled samples and ranged from 95.05±0.50% to 76.34±0.92%.

3.1.4 Principal component analysis.

Principal component analysis (PCA) performed on proximate, minerals and phytochemical of
cooked and uncooked leaves is illustrated in Fig. 1. This analysis showed two axes (axis 1 and 2) explaining the essential variability revealed by the nine (09) proximate, and phytochemical variables. The first and the second PCs described 97.79 and 06.10% of the variance respectively. Leaves from Solanum nigrum cooked during 10 min (SN10) and uncooked were located at the left of the score plot, while those cooked during 15 min (SN15) and 20 min (SN20) had positive score in the first principal component (PC1) (Fig. 1). Solanum nigrum fresh leaves (SN00) and SN 20 had a large positive component (PC2) (Fig. 1). The projection on the score, whereas SN 15 and SN10 had a negative score in PC2 (Fig. 1). The projection on the first and the second PCs variables. The first and the second PCs explaining the essential variability revealed by those cooked during 15 min (SN15) and 20 min (SN20) had positive score in the first principal component (PC1) (Fig. 1). Solanum nigrum fresh leaves (SN00) and SN 20 had a large positive component (PC2) (Fig. 1). The projection on the data revealed that SN00 and SN 20 were located at the left of the score plot, whereas SN 15 and SN10 had a negative score in PC2 (Fig. 1). The projection on the components CP1 and CP2 showed that SN00 and SN 10 were characterized by Vitamin C, Polyphenols, Antioxidant activity, ash, Proteins, oxalate and Phytic acid contents. SN 15 and SN 20 were characterized by high Crude fibers and lipid contents (Fig. 2).

### 3.2 Discussion

Food processing may affect the functionality and nutritional quality of the food products [34]. Also, it leads to beneficial transformations, compound degradation, or nutrient loss [35]. The effect of boiling on proximate composition of Solanum nigrum leaves is given in Table 1. The physicochemical parameters generally differed significantly \((P = .05)\) according to boiling time. The ash content of fresh Solanum nigrum \((17.96 \pm 0.03\%)\) is significantly higher than those of Corchorus olitorus and Hibiscus sabdariffa which are 7.00±0.00% and 9.14±0.02% respectively [22]. These high values show that Solanum nigrum leaves are higher in mineral content. After boiling, the reduction of ash content up to 28.73% in boiled at 10 min to 52.22% in boiled at 20 min may be due to leaching of mineral compounds into the boiling water [36].

### Table 1. Proximate composition of Solanum nigrum leaves during boiling

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Times of cooking</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Raw</td>
</tr>
<tr>
<td>Ash (%)</td>
<td>17.96±0.03(^a)</td>
</tr>
<tr>
<td>Crude fibres (%)</td>
<td>27.62±0.00(^b)</td>
</tr>
<tr>
<td>Lipids (%)</td>
<td>0.02±0.00</td>
</tr>
<tr>
<td>Proteins (%)</td>
<td>34.42±0.49(^c)</td>
</tr>
<tr>
<td>Carbohydrates (%)</td>
<td>16.78±0.52(^e)</td>
</tr>
<tr>
<td>Calorific energy (kcal/100 g)</td>
<td>233.78±0.10(^f)</td>
</tr>
</tbody>
</table>

*Data are represented as Means ± SD (n = 3). Means in the lines with no common superscript differ significantly \((P = .05)\)*

### Table 2. Mineral composition of Solanum nigrum leaves during boiling

<table>
<thead>
<tr>
<th>Minerals (mg/100)</th>
<th>Times of cooking</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Raw</td>
</tr>
<tr>
<td>Calcium (Ca)</td>
<td>1687.65±78(^a)</td>
</tr>
<tr>
<td>Magnesium (Mg)</td>
<td>1008.22±20.72(^a)</td>
</tr>
<tr>
<td>Phosphorus (P)</td>
<td>459.39±24.28(^a)</td>
</tr>
<tr>
<td>Iron (Fe)</td>
<td>55.07±2.07(^a)</td>
</tr>
<tr>
<td>Na/K</td>
<td>0.02±0.00</td>
</tr>
<tr>
<td>Oxalates/Ca</td>
<td>0.13±0.01</td>
</tr>
</tbody>
</table>

*Data are represented as Means ± SD (n = 3). Means in the lines with no common superscript differ significantly \((P = .05)\)*

### Table 3. Nutritive and antioxidant properties of Solanum nigrum leaves during boiling

<table>
<thead>
<tr>
<th>Minerals (mg/100 g)</th>
<th>Times of cooking</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Raw</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>33.51±01(^a)</td>
</tr>
<tr>
<td>Polyphenols</td>
<td>240.98±3.00(^a)</td>
</tr>
<tr>
<td>Tannins</td>
<td>157.45±2.73(^a)</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>16.46±0.34(^a)</td>
</tr>
<tr>
<td>Antioxidant activity (%)</td>
<td>98.21±2.00(^a)</td>
</tr>
</tbody>
</table>

*Data are represented as Means ± SD (n = 3). Means in the lines with no common superscript differ significantly \((P = .05)\)*
The fiber content in boiled leaves increased significantly \((P = .05)\) compared those of the raw leaves as mentioned by Slavin, et al. [37] and Lintas, et al. [38] where boiling caused decrease soluble fiber and increase insoluble fiber content. Indeed, increased temperature during cooking leads to breakage of weak bonds between polysaccharides and the cleavage of glycosidic linkages, which may result in solubilization of the low molecular weight carbohydrate from the thermal degradation [39]. Therefore, the adequate intake of leafy may be advantageous since high fibers content of foods help in digestion, prevention of colon and breast cancer and in the treatment of diseases such as obesity, diabetes and gastrointestinal disorders [38]. Fat content of boiled samples was significantly \((P = .05)\) higher than uncooked samples. This increased in fat content may be attributed to the increase activity of lipolytic enzymes which produced more free fatty acid [40]. However, high contents of lipid in human diet are principal sources of obesity and other related diseases. Dietary fat composition can interfere in the development of obesity due to the specific roles of some fatty acids that have different metabolic activities, which can alter both fat oxidations and deposition rates, resulting from changes in body weight and/or composition [41]. Talking about the protein content, a significant decrease \((P = .05)\) was registered for boiling at 10 min, 15 min, 20 min compared for uncooked leaves. This reduced protein content could be attributed to the severity of thermal process during boiling for leads to protein degradation [42]. The total carbohydrate (Table 1) showed that the amount of sugar varies significantly \((P = 0.05)\) from processing method. The amount of carbohydrate of boiled leaves, significantly \((p<0.05)\) decreased during boiling compared to the control. As previously mentioned, this can be related to the Maillard reaction, as carbohydrates (reducing sugar) are also substrates of no enzymatic browning [43]. The estimated calorific values \((194.01–233.78 \text{ kcal/100 g})\) of the cooked leafy vegetables \((10 \text{ min})\) compared favorably to \(248.8 – 307.1 \text{ kcal/100 g}\) reported on some Ivorian vegetables [44]. Thus, the calorific values agree with general observation that vegetables have low energy values due to their low crude fat and relatively high level of moisture [45]. This justifies that cooked leafy vegetables are usually eaten as a relish together with a starchy staple food, usually in the form of porridge [46]. The effect of boiling on mineral composition of \(Solanum nigrum\) leaves is given in Table 2. The residual

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**Fig. 1. Sample plot of principal components 1 and 2 of leaves from raw and cooked of \(Solanum nigrum\) leaves**

**SN 00:** *Solanum nigrum fresh*; **SN 10:** *Solanum nigrum leaves cooked in water during 10 min*; **SN 15:** *Solanum nigrum leaves cooked in water during 15 min*; **SN 20:** *Solanum nigrum leaves cooked in water during 20 min*
Contents of minerals after 10 min of cooking were: calcium (1386.56–1079.83 mg/100 g), phosphorus (508.88–405.24 mg/100 g), Magnesium (662.99–572.78 mg/100 g) and Iron (44.50–33.48 mg/100 g). These reductions may be due to leaching of the mineral compound into the boiling water [47]. With regard to these values, the consumption of 10 min boiled leaves of Solanum nigrum could cover all the standard mineral requirements for human. Indeed, these standard requirements are: Calcium (1000 mg/day); Magnesium (400 mg/day) and Iron (8 mg/day) [48]. The presence of these minerals in African leafy vegetables is also beneficial, due to their direct relationship with hypertension in humans. This may be the reason why the plant is used to prevent and control high blood pressure [49]. They are implicated in several body functions such as enzymatic reactions, energy production, transmission of nerve impulses, and multiple biological reactions [50]. Magnesium is known to prevent cardiomyopathy, muscle degeneration, growth retardation, alopecia, dermatitis, immunologic dysfunction, gonadal atrophy, impaired spermatogenesis, congenital malformations and bleeding disorders [51]. Iron plays numerous biochemical roles to the body, including oxygen binding of haemoglobin and acting as an important catalytic center in many enzymes as the cytochrome oxidase. Thus, the selected leaves of this study could be recommended to diets of reducing anaemia which affects more than one million people worldwide [52]. The species analyzed in this study showed appreciable levels of potassium. Sodium and potassium are involved in membrane and cellular exchange, thus contribution to the regulation of plasma volume, acid-base balance and muscle contraction. In addition, the sodium/potassium ratio for all fresh leafy vegetables studied being less than one, this would mean that they could use in diet of

Fig. 2. Circle of correlation of proximate, minerals, phytochemical properties of leaves from fresh and cooked of leaves of Solanum nigrum on axis 1 and 2

Vit c: Total vitamin C, Pol: Total polyphenols, Phy: phytic acid, AA: antioxidant activity, Cen: Total ash, Fib: Total fibers, Pro: Crude protein; Lip: Crude fat, Oxa: Total oxalate
The effect of cooking time on oxalates contents is depicted in Table 2. Levels losses at 10 min and 20 min for oxalates (140.87-91.64 mg / 100 g) could be advantageous for improving the health status of boiled leafy vegetables consumers. Indeed, oxalate is anti-nutrients which chelate divalent cations such as calcium, magnesium, zinc and iron, thereby reducing their bioavailability [54]. Therefore, cooking of leafy vegetables appears as a detoxification procedure by removing these anti-nutritional factors [55]. To predict the bioavailability of calcium, the calculated oxalates/Ca ratios molar ratios in the cooked and uncooked leaves were below the critical level of 2.5 known to impair calcium bioavailability [56]. The effect of boiling on nutritive and antioxidant properties of Solanum nigrum leaves is given in Table 3. A significant reduction vitamin C (11.23 mg / 100 g) was highlighted at 10 min during boiling processing. These values support the results obtained for other studies which indicate losses up to 66% in cooked vegetables [57]. With regard to the drastic decrease of vitamin C during cooking, consumption of cooked leafy vegetables may be supplemented with other sources of vitamin C such as tropical fruits to cover the daily need for humans (40 mg/day) as recommended by food agriculture organization [58]. The predominant polyphenols of Solanum nigrum leaves were tannins followed by flavonoids. The less negative effect of boiling on total polyphenols notably tannins (47.95–53.12%) and flavonoids (14.76–38.09%) can be attributed to the water-soluble compounds leaching into the cooking water as well as the breakdown of these compounds during cooking [59,60]. The decrease of total polyphenols contents (47.96-64.27%) could be the consequence of a destruction of the cellular structure during cooking. The cells swell and explode in the presence of an excess of water during the cooking then releasing their contents [61]. Indeed, the losses of polyphenols contents decrease of the polyphenolic compounds content and overall antioxidant activity could be due to leaching or heat ability of specific flavonoids [62,63]. DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging ability of raw and boiled leaves samples are shown in Fig. 1. In comparison to raw leaves of all boiled leaves, studied showed significantly (P<.05) lower free radicals scavenging ability. When vegetables are submitted to cooking processes, in one hand, variations appear in their antioxidant activity or scavenger capacity [64]. On the other hand, the DPPH assay and total antioxidant capacity assay of leafy vegetable extracts are correlated with the total phenolic content [65]. The principal component analysis illustratively divided the uncooked, cooked at 10 min and cooked at 15 min, cooked at 20 min according to the assessed parameters. Only those cooked at 10 min had good contents.

4. CONCLUSION

This study indicate that boiling time has determined effect on the levels of phytochemicals, bioactive components and antioxidant capacities of Solanum nigrum leaves. Boiling caused the loss of nutrients, this might be due to leaching during heat application. It was not a significant reduction of nutrient anti-nutrients content of leaves after boiling. This is probably because the antinutrients are heating labile. Nevertheless, the losses in anti-nutrients (oxalates) might have asserted a beneficial effect on bioavailability of minerals like iron and calcium. Thus, the study suggests time of domestic cooking must be less than 10 min for the studied leafy vegetables in order to the nutritional requirement and to the food security of Ivorian population.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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