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Review Article

Effects of Cooking Time on Some Antinutrients Contents and *in vitro* Digestibility of Leaves Proteins of *Gnetum* spp

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Abstract: G*netum* spp are creepy lianas well known by people around Guinea Gulf most notably for their edible leaves. With the aim at contributing to nutritional valorization of these leafy vegetables, we studied the effects of cooking time on some antinutrients contents and on proteins digestibility. Phytates, phenolic compounds, tannins, oxalates and proteins were extracted and evaluated by conventional methods. *In vitro* digestibility of proteins was estimated using pepsin and pancreatin. Results showed that Phytates contents in *G. africanum* leaves were 240.23±14.25 against 90,68±3,09 mg/100g (DM) for *G. buchholzianum*. After two hours of cooking, values decreased significantly (P< 0.05) to 208.84±1,57 and 82.146±5.134 mg/100g (DM) respectively. In the same time, contents of phenolic compounds decreased from 507.20±21.53 to 245.56±3.04 and from 460.37±3.09 to 230.83±13.87mg/100g (DM) for the two species. Tannins contents were 298.09±13.70 and 222.73±13.90 mg/100g DM for *G. africanum* and *G. Buchholzianum*. Retention rates after 2 hours of cooking were 34.55% and 46.28% respectively. Oxalates contents varied between 5.10±0.07 and 6.76±0.14 mg/100 DM with retention rates ranging from 22.85% to 26.50% during the same cooking time. Proteins contents (16.04±0.05 and 18.14±0.16% g DM) decreased significantly (P< 0.05) by half while *in vitro* digestibility of proteins doubled after 2 hours of cooking. This study shows that *Gnetum* spp vegetables have high contents of some antinutrients which important amounts are easily remove with long cooking time. This treatment improves digestibility of theirs proteins.

Keywords: *Gnetum* spp, Vegetables, Antinutriments, Protein Digestibility

1. Introduction

G. africanum and *G. buchholzianum* are non woody plants which belong to *Gnetacea* family [1]. They are about 10 m lenght and grow mainly in forest of medium altitude. *Gnetum* spp is found in many countries of Gulf of Guinea, notably in Nigeria, Cameroon, Gabon, Democratic Republic of Congo, Central African Republic and Angola [2]. In Central Africa and particularly in Cameroon, leaves of *Gnetum* spp are used as a vegetable for soups and stews commonly called *eru* or *okok* no matter on whether they are prepared in the english-speaking or french-speaking region of the Country. *Gnetum spp* is a good source of protein and is rich in essential

and non-essential amino acids. It also has appreciable levels of unsaturated fatty acids, vitamine and some minerals. It also contents antinutrients capable of reducing bioavailability of proteins and minerals [3, 4].

Food antinutrients belong to different chemical groups with various effects. Some antinutrients like enzymes inhibitors, lectines, polyphenols and phytates have ubiquitous character. Others like cyanogens are much more specific and are found only in some plants or group of plants [5]. Phytates are mainly chelating agents. During digestion, they form complex compounds with minerals. They can also form complexes with protein either directly by establishing ionic bonds or indirectly through a cation such as calcium [6]. Polyphenols are antioxidants found in many fruits and vegetables. They are involved in cancers prevention, cardiovascular diseases and other degenerative diseases linked to oxidative stress in humans [7]. They covalently bind to oxidized lysine residues of proteins and decrease their nutritional quality [8]. Tannins are polyphenolic compounds which play important role in plants protection against predators like herbivores and insects. They have the property of combining with dietary proteins to form insoluble complexes. In monogastric animals, tannins reduce nitrogen retention and consequently reduce growth rate and food efficiency [9]. Oxalic acid and its salts appear as end products of metabolism in many plant tissues. High levels of oxalic acid in food can reduce bioavailability of calcium and cause stones in the urinary tract. Interactions between proteins and oxalates are formed via divalent ions such as calcium and induce protein denaturation because of resulting complex formed [10].

Leafy vegetables cooking result in destruction of germs or parasites, the removal of toxic substances and the improvement of nutritional quality. These phenomenons depend on leaf texture, temperature and cooking time [11]. *Gnetum* spp vegetables are tough and have low water content of less than 40% [12, 4]. In order to soften these tough leaves and facilitate digestion, they are usually cut into tiny pieces by housewives and water cooked for more than 1 hour. Some nutrients and antinutrients are lost when foods are cooked [11]. Reduction of antinutrients content in a food, especially protein food, could improve their nutritive value. The extent of antinutrients reduction is not known by local people as far as *Gnetum* spp leaves are concerned. Similarly, determining the effect of prolonged cooking on protein levels and digestibility could lead to a better nutritional use of these leafy vegetables knowing that the changes occurring in food from preparation to table is essential not only for scientific research, but also for the consumer, who can make decisions about how to prepare and cook a selected number of healthy legumes and vegetables. In order to contribute to the improvement of nutritional value of *G. africanum* and *G. buchholzianum* leaves, we studied effects of cooking time on levels of some antinutrients (phytates, crude phenolics compounds, tannins and oxalates) and on the *in vitro* digestibility of proteins of these leaves.

2. Materials and Methods

2.1. Samples

G. *Africanum* and *G. buchholzianum* leaves were harvested on the domestication plots of CENDEP (Centre for Nursery Development and Eru Propagation) of Limbe, Republic of Cameroon (longitude: 4°0'46" N, latitude: 9°13'13" E) or directly obtained from farmers on their way back from farms. Leaves were directed to laboratory of Biochemistry, Faculty of Science, University of Douala where they were identified, authenticated, separated from wastes, washed with tap water, sliced into small pieces (less than 0.2 cm) and cooked into deionized water. For cooking procedure, samples were divided into 6 batches according to the following cooking time: 0, 30, 60, 90,120 and 150 min.

Cooked and uncooked samples were separated, shade dried for three week with frequent turning to avoid fungal growth. Later on, they were transferred into an oven where they were dried for 24 hours at 45°C and then finely ground to obtain a powder using an electric blender (Scientz-11L). Samples were then frozen (-18°C) in labeled polystyrene container for subsequent analysis.

2.2. Antinutrients Extraction and Quantification

2.2.1. Phytates

Phytates were extracted and quantified by Vaintraud *et al.* (1986) method [13]. In this extraction, 20 g of powder were mixed with HCl 3.5%, shook for 1 hour and centrifuged at 6000 rpm for 30 min. The supernatant was recovered, treated with Wade reagent and subjected to the determination of the phytates by spectrophotometry at 500 nm.

2.2.2. Total phenolic Compounds

Phenolic compounds were extracted with 70% ethanol (0.5 g of sample was mixed with 25 mL ethanol) and then assayed by spectrophotometer (UV-Vis-1600 PC) at 725 nm using the Folin-Ciocalteu reagent [14].

2.2.3. Tannins

Tannins were determined using acidified vanillin and (+) cathechine as standard [15]. Two grams of sample were mixed with 30 mL acetone 80%. The mixture was stirred for 15 min and filtered under pressure. Acetone was separated using a rotavapor (Buchi, R124) and then mixed with freshly prepared 4% vanillin in ethanol. The mixture was stirred, treated with concentrated HCl and quantified by spectrophotometry at 500 nm.

2.2.4. Soluble and Total Oxalates

Soluble oxalates were extracted with distilled water in a boiling water bath for 1 hour. Total oxalates were extracted in a mixture of distilled water-HCl 6N (1/19: V/V) also in a bath boiling for 1 hour. Extracted oxalates were assayed after oxidation with potassium permanganate (0.098N) and hot sulfuric acid (1N) [16].

2.3. Crude Proteins

Total nitrogen was determined after mineralization of the

samples according to the method of Kjeldahl [17] and assayed according to the colorimetric technic of Devani *et al*. (1989) [18].

2.4. In vitro Digestibility of Crude Proteins

The *in vitro* digestibility of crude proteins of *Gnetum* spp expressed against cooking time was estimated by the modified method of Marrion *et al*. (2005) [19] using pepsin and pancreatin successively. Undigested nitrogen was mineralized and then assayed by the Kjeldhal method [20]. Results were expressed as percentage of digested protein by the following relation (1):

$$\text{Digested proteins (\%)} = \frac{total\ proteins - non\ digested\ proteins}{Total\ proteins} \times 100 \quad (1)$$

2.5. Statistical Analysis

Each value is presented as mean ± standard deviation for three replicates. Statistical analyses were carried out using Statistica version 6.0 sofware. Values were compared using Duncan test. Statistical significance was attained when a p-value was less than 0.05.

3. Results

3.1. Phytates

Table 1 shows that phytates contents of *G. africanum* and *G. buchholzianum* were respectively 240.23 ± 14.25 and 208.84 ± 1.57 mg /100 g DM. Cooking in water has reducing effects on phytates contents which for both *Gnetum* species were dropped to 90.68 ± 3.09 and 82.15 ± 5.13 mg /100 g DM respectively after 150 min. Respective retention rates for the two species were 37.74% and 39.33%.

Table 1. *Variation of phytates levels of Gnetum spp leaves with cooking time.*

Species	Cooking time (min)	Phytates levels (mg /100g DM)	% Phytates retained
G. africanum	0	240.23±14.25^{a}	100
	30	184.65±11.61^{b}	76.86
	60	131.37±1.57^{c}	54.68
	90	117.07±5.22^{d}	48.73
	120	113.64±22.31^{d}	47.30
	150	90.68±3.09^{e}	37.74
G. buchholzianum	0	208.84±1.57^{f}	100
	30	160.86±4.17^{b}	77.02
	60	142.29±1.04^{c}	68.13
	90	120.82±9.85^{c}	57.85
	120	101.27±4.13^{e}	48.49
	150	82.15±5.13^{g}	39.33

Values on the same column assigned to the same superscript letter are not significantly different at the threshold 0.05%. DM= dried Matter

3.2. Phenolic Compounds

Table 2 shows changes in levels of total phenolic compounds with cooking time. In fresh leaves, contents ranged between 507.19 ± 21.53 and 460.37 ± 3.09 mg/100g DM, for G. *africanum* and *G. buchholzianum* respectively.

Table 2. *Variation of total phenolic compounds contents of Gnetum spp leaves with cooking time.*

Species	Cooking time (min)	Total phenolic compounds levels (mg /100g DM)	%Total phenolic compounds retained
G. africanum	0	507.19±21.53^{a}	100
	30	323.78±2.85^{b}	63.83
	60	305.54±16.46^{c}	60.24
	90	287.13±0.51^{d}	56.61
	120	267.98±1.78^{d}	52.66
	150	245.56±3.04^{e}	48.41
G. buchholzianum	0	460.37±3.09^{g}	100
	30	343.88±19.50^{b}	74.69
	60	301.10±7.14^{c}	65.40
	90	288.17±1.78^{d}	62.59
	120	250.84±8.12^{e}	54.48
	150	230.83±13.87^{f}	50.14

Values on the same column assigned to the same superscript letter are not significantly different at the threshold 0.05%. DM= dried Matter

3.3. Tannins

Tannins contents are shown in Table 3. Fresh leaves of *G. africanum* have significant (P< 0.05) high value (298.1 ± 13.75 mg/100 DW) compared to those of *G. buchholzianum* (222,73±13,9 mg / 100g DM). Decrease in tannins levels was more fast than those of phytates (Table 1) and phenolic compounds (Table 2). In only 30 min, retention rate of tannins were less than 50% (41.65%) for *G. africanum* leaves. Losses were less important for *G. buchholzianum.*

Table 3. *Variation of tannins contents of Gnetum spp leaves with cooking time.*

Species	Cooking temps (min)	Tannins (mg /100g DM)	% Tannins retained
G. africanum	0	298.09±13.70^{a}	100
	30	124.17±6.10^{b}	41.65
	60	121.65±27.20^{b}	40.80
	90	112.23±1.28^{c}	37.64
	120	103.01±3.57^{c}	34.55
	150	89.61±0.50^{d}	30.06
G. buchholzianum	0	222.73±13.9^{e}	100
	30	141.78±16.7^{f}	63.65
	60	115.59±1.27^{c}	51.89
	90	110.83±0.25^{c}	49.75
	120	103.10±6.11^{e}	46.28
	150	91.07±17.91^{d}	40.88

Values on the same column assigned to the same superscript letter are not significantly different at the threshold 0.05%. DM= Dried Matter

3.4. Oxalates

Mean value of oxalate contents of leaves of *Gnetum* spp as obtained from table 4 was 58.86 mg/100g DM. Oxalate retention was poor (only 38.31%) for *G. buchholzianum* after 30 min of cooking and value felt to about 19% in 2 hours for both species.

Table 4. *Variation of oxalates contents of Gnetum spp leaves with cooking time.*

Species	Cooking time (min)	Oxalates (mg /100g DM)	% Oxalates retained
G. africanum	0	50.10±0.71[a]	100
	30	31.72±1.40[b]	63.31
	60	31.11±0.72[b]	62.09
	90	22.43±0.75[c]	44.77
	120	11.45±0.01[d]	22.85
	150	9.40±0.72[d]	18.76
G. buchholzianum	0	67.62±0.14[e]	100
	30	25.91±0.64[c]	38.31
	60	24.43±0.77[c]	36.12
	90	20.55±0.83[c]	30.32
	120	17.92±0.90[c]	26.50
	150	12.64±0.67[d]	18.69

Values on the same column assigned to the same superscript letter are not significantly different at the threshold 0.05%. DM= Dried Matter

3.5. Contents in Crude Proteins and Digestibility of Leaf Proteins of Gnetum spp

Table 5 shows crude protein content of the two leafy vegetables. Values were significantly higher ($P<0.05$) for *G. buchholzianum* (18.14 ± 0.16g / 100g DM) than for *G. africanum* (16.04 ± 0.05g / 100g DM). Cooking in water led to a significant decrease in protein levels beyond 30 min. Retention rate of proteins was less than 50% after 2 hours

Table 5. *Variation of proteins levels and in vitro digestibility of leaf proteins of Gnetum spp with cooking time.*

Species	Cooking time (min)	Proteins levels (mg/100g DM)	% Proteins retained	*in vitro* digestibility (%)
G. africanum	0	16.04±0.05[a]	100	32.68±0.00[a]
	30	14.92±0.14[b]	93.01	42.71±0.00[b]
	60	12.94±0.02[c]	80.67	48.04±0.01[c]
	90	11.12±0.02[c]	69.32	57.30±0.02[d]
	120	9.74±0.06[d]	60.72	66.06±0.02[e]
	150	7.98±0.04[e]	49.75	63.62±0.00[f]
G. buchholzianum	0	18.14±0.16[f]	100	31.00±0.02[a]
	30	16.49±0.02[a]	90.90	41.68±0.02[b]
	60	13.91±0.05[b]	76.68	47.11±0.01[c]
	90	12.41±0.02[c]	68.41	56.62±0.00[d]
	120	10.77±0.03[d]	59. 37	62.97±0.01[e]
	150	8.56±0.06[e]	47.18	60.00±0.02[f]

Values on the same column assigned to the same superscript letter are not significantly different at the threshold 0.05%. DM= Dried Matter

4. Discussion

Similar values of phytates contents were obtained by Ekop (2007) [21] in seeds of *G. africanum* (238.5mg /100g DM) and by Ayodeji and Fasuyi (2005) [22] in leaves of *Manihot esulenta* (249.1mg/100g). But values were high when compared to those of *Amaranthus hybridus* [23] and *Solanum nigrum* [24] which are other leafy vegetables commonly consumed locally. Boiling reduces phytates content of vegetables as was found in previous studies [25, 26] but as shown in table 1, this effect increases with cooking time. It has been found that phytates are heat-resistant and not as easily degraded by boiling. But a longer cooking time often results in greater reduction of antinutrients [27]. Our samples were cut into small pieces and this could have favored loss of antinutrients and others substances. Also, at an appropriate temperature, stimulation of phytases results in hydrolysis of phytates [5].

Contents in phenolic compounds were lower than the 7.5g/100g (DM) found in *Manihot esculenta* [22] and higher than the 13.17mg/100g (DM) of *Solanum nigrum* [24] and 0.35mg/100g (DM) of *Amaranthus hybridus* [23]. There was almost 50% loss of phenolic compounds after 2h cooking (table 2). Processing, particularly under high thermal and pressure conditions, influence phenolic compounds by disrupting cell wall matrix of foods, followed by the release of insoluble-bound phenolics. Predominant polyphenols in fresh leaves are water soluble polyphenols which have number of hydrophobic functional groups. Release of leaf polyphenol is increased by slicing, heating and cooking time [28, 25]. But in our study, phenolic compounds had the highest percentage of retention which was more than 50% after 2 hours cooking (table 2).

Tannins contents were higher than those of *Amaranthus hybridus* (0.49 mg/100g DM) and *Solanum nigrum* (0.19 mg/100). Ekop *et al*. (2007) found a content of 100.74 mg/100g in seeds of *G. africanum* harvested in Nigeria [21]. Leaf morphology and stage of development are some of the variation factors of tannins levels. Smaller leaves may produce more tannin in water and nutrients-poor soils [29]. Leaves of *G. africanum* are thin and grow mainly in forest fallows; those of *G. buchholzianum* are large and grow in evergreen forests [12]. Tannins are water soluble polyphenol compounds having wide prevalence in plant. Hydrolyzable tannins yield various water-soluble products, such as gallic acid, protocatechic acid and sugars and this process is accelerated by heat [30].

Mean value of oxalates (58.86 mg/100 DM) were lower compare to that found in *Solanum nigrum* leaves (75.65±0.04 mg/100 DM) [24]. High content of this antinutrient was found in *G. africanum* seeds (209 mg/100 mg) [21]. Boiling has markedly reduced soluble oxalate content by 36-81% and this process seems to be more important in *G. africanum* leaves (table 4). These leaves are probably rich in soluble oxalates which have the property of being easily dissolved in water [31]. Approximately 75% of all kidney stones are composed primarily of calcium oxalate, and hyperoxaluria is a primary risk factor for this disorder [32]. Water cooking *Gnetum* spp leaves that significantly reduce soluble oxalate may be an effective strategy to prevent kidney stones in individuals consuming these vegetables.

Results of crude proteins corroborate those of others authors [12, 3, 33]. Thermal treatments above 45°C produce denaturation of proteins and lead to loss of particle structure in favor of more compact structures [34]. In this work, leaves were cut into thin strips (less than 0,2cm thick) as do housewives all over the region. This treatment, combined with the effect of heat and time, can lead to significant loss of nutrients by broken cells.

Mean value of *in vitro* digestibility of leaf proteins of *Gnetum* spp as shown in table 5 is about 31.84% which corresponds to about 5.11% and 5.77% of digested crude proteins respectively for *G Africanum* and *G. buchholzianum.* Unlike protein levels that decline, *in vitro* digestibility increases with cooking time from 32.68% to 63.62% for *G. africanum* and from 31.00 to 62.97% for *G. buchholzianum* in 2 hours. Digestibility also depends on the nature (structure) of protein and occurrence of antinutritional factors [35]. The increase in protein digestibility with cooking time could be explained by the distorsion of cellular structures and denaturation of proteins which makes them more easily accessible to proteases [36] and decreases action of certain antinutrients such as phenolic compounds, phytates and tannins [37]. These antinutrients have the property of forming complexes with proteins and proteolytic enzymes thus reducing digestibility [38]. Drying and grinding may also have induced changes of protein structures and digestibility in our sample. Introducing a third enzyme (a peptidase for example) could have increased digestibility knowing that small peptides can escape action of pepsin and pancreatin that have been used in the measurement of the *in vitro* digestibility. Nevertheless, these methods do not take into account interactions at the intestinal mucosa. *In vivo* assays and precisely true ileal digestibility measurements were found to be more appropriate in evaluating protein digestibility, but they are difficult to perform in healthy humans [34].

Gnetum spp vegetables are rich in fiber and antinutrients which can also hamper proteins digestibility in rats [30]. Their water contents is low (about 32%) [33]. Cutting into small pieces and long cooking time are frequently used by housewives to soften these vegetable because of their toughness. Long cooking time has resulted in a significant decrease in protein levels and the following antinutrients: oxalates, tanins, phytates and total phenolic compounds. Lewu *et al*. (2009) also found significant drop in antinutrients content as consequence of boiling of *Colocasia esculenta* leaves (16 – 78% drop in oxalates, 28 – 61% in tannins level and 17 – 41% reduction in phytates after 5 min of boiling) [39]. If loss of proteins probably favored by cutting is deplorable, that of phytates, phenolic compounds and oxalates could be considered as useful because those substances impede assimilation of useful nutrients or even have toxic effects when ingested in excessive amounts. Reduction or elimination of these antinutrients is necessary to prevent poisoning and to improve the biological utilization of vegetables. However, these substances play major role in adaptation strategies because they allow plants to better ensure access to nutrients and also protect them against predators [5]. Cooking foods also has nutritional benefits by making number of nutrients available for digestion [11]. As shown in this study, removing antinutrients by water cooking promotes digestibility of proteins in *Gnetum* spp leafy vegetables.

5. Conclusion

G. africanum and *G. buchholzianum* vegetables are potential source of protein. They have appreciable amounts of phenolic compounds, phytates and oxalates which are significantly removed by water cooking. Amount of antinutrients released increases with cooking time and this process which depends on the type of antinutrient was greater with oxalates. Increasing cooking time reduced proteins contents but improves *in vitro* digestibility of proteins of *Gnetum* spp.

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