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Phytochemistry, Antioxidant Activity and Heavy Metal Content on Precooked and Raw of *Celosia argentea* L., *Launaea taraxacifolia* Wild. and *Ocimum gratissimum* L., Three Leafy Vegetables Eaten in Southern Benin

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Abstract

This study aimed to estimate and compare the antioxidant potential as well as the levels of heavy metal contamination of Celosia argentea (Amaranthaceae), Launaea taraxacifolia (Asteracea) and Ocimum gratissimum (Lamiaceae) collected in four municipalities of South-Benin by taking into accounts the treatment prior to consumption (precooked and raw). A low extraction yield was obtained during the extraction of leafy vegetables subjected to precooking. The phytochemical screening of Launaea taraxacifolia revealed the presence of triterpenes, steroids, flavonoids and tannins. The phytochemical analysis of Ocimum gratissimum revealed the presence of triterpenes, the steroids, tannins, anthraquinones, coumarins and alkaloids. The analysis of Celosia argentea revealed the presence of triterpenes, steroids, flavonoids, tannins, anthraquinones and alkaloids. The antioxidant activity of the various extracts was found to be dose-dependent. The extracts showed significant antioxidant activities with IC_{50} ranging from 4.03 to 11.42 µg/ml. Ocimum gratissimum had the strongest antioxidant power followed by Celosia argentea and Launaea taraxacifolia respectively. Pre-cooking of leafy vegetables would have an effect on antioxidant activity, which resulted in a slight increase in the DPPH inhibition power of pre-cooked vegetables compared to raw one. The analysis of heavy metals showed the presence of arsenic, mercury and manganese in the different plant extracts. The highest levels were observed in leafy vegetables collect at Cotonou (Houeyiho) followed by those from Sèmè-Podji but all below the thresholds defined by the World Health Organization. The precooking promoted a decrease of the quantity in heavy metals by leaching, causing low concentration in metals of vegetables precooked in contrast with raw ones.

Keywords: Leafy vegetable, Precooking, Phytochemistry, Antioxidant activity, Heavy metals

1. Introduction

Over the last decades, population growth and increasing urbanization have contributed significantly to the devastating consequences on human and environmental health. The use of pesticides in the agricultural sector to increase yields has led to the onset of certain diseases such as Parkinson's disease, endocrine and reproductive disorders as well as certain cancers (Mostafalou et al., 2013). Furthermore, emissions from fossil fuel combustions, industrial processes or means of transport contaminate the environment with harmful toxins (Olowu et al., 2015). Among these toxic molecules are heavy metals, metals or metalloids characterized by a relatively high density of 5 g/cm3 or above (Miquel, 2001). These metals are bio-cumulative and continued exposure can lead to several acute (nausea, abdominal pains, diarrheas, dyspnea, fever etc.) and chronic health conditions (hyperkeratosis, arrhythmias, peripheral neuropathies, leucopenia, cancerogenic, metabolic and endocrine disorders) (Khan et al., 2013). Hence, intoxication by heavy metals is one of the major threats to food safety and quality.

Leafy vegetables, cultivated or wild edible plant species (CTA, 2004), are some of the main ingredients used in traditional African cuisine (Maundu et al., 1993). These vegetables are low in calories and rich in nutrients (vitamins, minerals and fibers) hence their consumption lowers the risks of obesity, diabetes and cardiovascular diseases (Boeing et al., 2012; WHO, 2015). In addition to their nutritional value, several of these

leafy vegetables possess a strong antioxidant potential that helps the body fight the destructive effects of reactive oxygen species (ROS) (Kim et al., 2013). Depending on their texture, leafy vegetables are precooked or eaten raw.

However, poor agricultural practice through the misuse of inputs and the establishment of market gardening centres in urban areas could lead to food safety problems. Ten leafy vegetables are commonly cultivated and consumed in the southern Benin regions (Dansi et al., 2008). Among these leafy vegetables, include *Celosia argentea* L. (Amaranthacée), Launaea taraxacifolia Wild. (Asteraceae) and *Ocimum gratissimum* L. (Lamiaceae). In order to measure the impact of the consumption of these leafy vegetables on the health and well-being of the populations, this study was initiated evaluate and compare the antioxidant potential as well as the levels of EMT contamination of these vegetables harvested in four municipalities in South Benin, taken into account the cooking method (precooked and uncooked). The objective of this study was to investigate the phytochemistry and antiradical potential as well as the levels of EMT contamination, especially mercury (Hg), arsenic (As), and Manganese (Mn) of uncooked (raw) and precooked of leafy vegetables.

2. Material and Methods

2.1 Sampling

Leafy vegetables of Celosia argentea L. (Amarantacée), Launaea taraxacifolia Wild., (Asteracea) and Ocimum gratissimum L. (Lamiacée) were collected in June, 2017 at the stage of maturity for the consumption of one site (truck farmer) by municipality particularly in Cotonou, Pobè, Sèmè-Podji, and Zè. These species were authenticated in the National herbarium of the University of Abomey-Calavi (UAC) under the respective codes of identifications AA6732 / HNB, AA6733 / HNB, AA6734 / HNB. Samples were then washed, dried in the temperature of laboratory (T = 22 ± 3 °C) then reduce powder by means of an electric crusher (MARLEX Electroline Excella).

2.2 Extraction of Plant Material

The powders of each sample were divided into two batches. The first batch was pre-cooked and then subjected to ethanol extraction. The second batch is subject only to ethanol extraction.

Pre-cooking: 50 g of each sample were heated in 500 ml of distilled water using a heating cap

(Electromantle MA Solid Stirrer) at 90 °C during 15 min boiling. The mixture obtained was carefully filtered through a Whatman filter paper (Qualitative Circles 150 mm Cat No. 1001 150). The residues resulting from obtained following the filtration were then dried.

Ethanolic extraction: 50 g of each sample (raw and precooked) were subjected to maceration, overnight, with mechanical stirring in 300 ml of ethanol. The mixture obtained after maceration was filtered on Whatman paper (Qualitative Circles 150 mm Cat No. 1001 150). Two successive extractions with the sonicator (Bioblock Scientific, Vibra-cell 75,115) for 15 min were then carried out on the residues obtained after filtration. The ethanolic filtrates obtained were concentrated under reduced pressure using a rotavapor (Buchi Rotavapor RII). Extracts were collected in glass pills and stored at 4 °C. In total Twenty-four (24) extracts were obtained. The extraction yields were calculated according to the formula below:

$$\text{Yield (\%)} = \frac{\text{mass of extract obtained}}{\text{mass of plant material}} \text{x100}$$

2.3 Phytochemical Screening of Extracts

Tube staining tests (Ould et al., 2017) were used to identified the chemical groups contained in the leafy vegetables studied. Alkaloids, anthraquinones, coumarins, flavonoids, saponins, tannins, triterpenes and steroids are the chemical groups sought.

2.4 Evaluation of the Antiradical Activity of the Extracts

The antiradical activity of the extracts was evaluated according to the method used by Amoussa et al. (2016). This method is based on the principle of reducing the unstable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH). The tests were carried out on 96-well microplates and the extracts were prepared in analytical methanol. A range of eight (8) concentrations obtained by successive dilution from the stock solution, ranging from 1000 µg / ml to 7.8 µg / ml was tested by adding DPPH (2%). Ascorbic acid and quercetin were used as a positive control and the negative control consisted only of methanolic solution and DPPH. The plates are then incubated in the dark and at room temperature. After 20 min of incubation, the optical densities (OD) were measured at 492 nm with the ELISA plate reader (Rayto-R6500). The tests were performed in triplicate. The percentage inhibition (PI%) of the DPPH radical by the extracts was calculated according to the formula:

$$\text{PI (\%)} = \frac{\text{OD White} - \text{OD Sample}}{\text{OD White}} \text{x100}$$

Sample concentrations for which 50% of the free radicals are inhibited (IC50) were also determined from the curves representing the percent inhibition of the DPPH radical as a function of the extract concentrations.

2.5 Evaluation of the Heavy Metals Content of the Extracts

The determination of heavy metals was carried out according to the method used by Lagnika et al. (2016) using the Metalyser HM 3000. This apparatus is based on cathodic and anodic re-dissolution voltammetry using disc electrodes. A first step in the technique consists of concentrating the metals (ions) in solution on the surface of the electrode (electroplating) and then the second step, stripping (stripping) this metal electrode (electrodissolution) by analyzing the potential. The current observed during the pickling step is related to the amount of metal in the solution. In this study, he measured the concentration of metals such as arsenic, mercury and manganese. The analysis consists of adding a sample volume of 70 ml prepared at 1 mg / ml with deionized water followed by buffers specific to the metal to be assayed.

3. Results and Discussion

In Africa leafy vegetables have been cultivated and consumed for generations and are appreciated not only because of their nutritional values but also because they contained several groups of chemical families known for their biological or antioxidant activities against reactive oxygen species that are responsible for aging diseases. These leafy vegetables according to their texture are eaten either precooked or uncooked beforehand. It is then normal to measure the impact of this cooking method on the antioxidant properties of the leaves but also against the heavy metals from agricultural chemical inputs and exhaust gases from industries and means of transport.

3.1 Yield of Extractions

The analysis of the data in Table 1 showed that the extraction yields of pre-cooked vegetables were much lower than those which did not undergo any treatment. On the other hand, the highest extraction yields were found in the samples collected at Cotonou (Houeyiho) and Sèmè-Podji. The low yield obtained in precooked vegetables could be attributed to thermal treatment applied to said vegetables prior to the actual ethanol extraction. These results could also be justified by the high polarity of water (9.0) compared to ethanol (5.2) one (Bourgou et al., 2016). Thus, pre-cooking would have led to a first extraction before that of ethanol. These results reinforce the trend observed in a previous study where a decrease in the content of essential elements such as vitamins and minerals were observed in leafy vegetables after pre-cooking (Vodouhe et al., 2012).

3.2 Phytochemical Screening

Phytochemical screening of the studied leafy vegetables was evaluated in order to identify the major phytochemicals and results were displayed in Table 2. Phytochemical screening of the ethanolic extract of *Launaea taraxacifolia* revealed the presence of triterpenes and steroids, flavonoids and tannins. However, saponosides, anthraquinones, coumarins and alkaloids are absent. In the case of *Ocimum gratissimum*, phytochemical analysis revealed the presence of triterpenes and steroids, tannins, coumarin anthraquinones and alkaloids without flavonoids and saponosides. The extract of *Celosia argentea* was presented the presence of triterpenes, steroids, flavonoids, tannins, anthraquinones and alkaloids with the absence of saponosides and coumarins.

Qualitatively, the identified phytochemical compounds in the raw leafy vegetables were also identified in the in the cooked sample. As expected with respect to quantitative point of view, the raw samples generally contained higher amount of the identified phytochemicals in contrast with the corresponding cooked samples. These different families of secondary metabolites found are known for their therapeutic properties (antimicrobial, anti-inflammatory, antihypertensive, etc) (Ogundare and al., 2006; Gulcin et al., 2010) and low-dose antioxidants (Olowu et al., 2010). Our results are consistent with those obtained by Koukoui et al. (2015), Karthiyayini et al. (2015), Ganiyu (2006) for Launaea taraxacifolia, Celosia argentea and Ocimum gratissimum respectively. The variability of secondary metabolites from one region to another may depend on several factors, including climatic and soil conditions (temperature, sun exposure, drought, and salinity) (Hougton and Amala. 1998).

Table 1. Extraction yields

Yield (%)					
Samples	Treatment	Cotonou	Sèmè	Pobè	Zè
L.t	nc	8.50	14.44	8.38	5.20
	c	4.34	4.49	1.36	1.81
O.g	nc	9.97	8.76	8.41	4.88
	c	4.49	5.04	6.07	0.86
C.a	nc	872	10.02	5.45	4.40
	c	3.38	1.80	1.55	1.07

L.t: Launaea taraxacifolia, O.g: Ocimum gratissimum, C.a: Celosia argentea c: cooked, nc: uncooked.

3.3 Antioxidant Activities of Different Extracts

The inhibition of the DPPH radical as a function of the concentration of the extracts is presented in figures 1, 2 and 3. The analysis of these figures respectively describing the inhibition of the DPPH radical by the extracts of Launaea taraxacifolia, Celosia argentea and Ocimum gratissimum harvested in Cotonou (Houeyiho), Sèmè-Podji, Pobè and Zè shows that the antiradical activity of the extracts is dose-dependent, varies according to the extraction method and the collection site. In general, precooked leaf vegetable extracts had the highest percentages of DPPH radical inhibition compared to those without any treatment. These results are verified by the low IC50 values obtained for precooked vegetables (Table 3) but all of them were superior to the positive controls (4.46 and 2.03 µg / ml respectively for ascorbic acid and quercetin). On the other hand, vegetable extracts from the Cotonou (Houeyiho) and Sèmè-Podji and Pobè sites showed much better activity. Our results are in agreement with the work of Bourgou et al. (2016), who reported on the effect of the solvent and the extraction method on the content of phenolic compounds and the antioxidant potential of Euphorbia helioscopia. These authors have shown that the antiradical activity of Euphorbia helioscopia is significantly influenced by the nature of the solvent and the type of extraction technique. These data showed that the precooking of leafy vegetables optimizes the antioxidant power of these vegetables. The best activities observed in the leafy vegetables of Cotonou (Houeyiho) then Sèmè-Podji and Pobè could be due to the environmental stress suffered by the species of these localities. In fact, the secondary metabolites of plants are secreted under stress conditions (exposure to the sun, soil condition, microorganisms, pests, volatile molecules) or in relation to cultural practices (use of fungicides, pesticides and synthetic insecticides) (Arimura et al., 2004, Kaliche & Djemoui, 2014). The great antiradical power of the extracts is due to the presence of phenolic compounds such as flavonoids,

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tannins and coumarins. The antiradical capacities of these phenolic compounds have been previously demonstrated (Sharma et al., 2014).

Table 2. Phytochemical analysis of extracts

Wanted Families	Samples	Houeyiho		Sèmè		Pobè		Zè	
		nc	c	nc	c	nc	c	nc	c
Triterpenes & Stéroids	L.t	+	+	++	++	+	+	+	+
	O.g	++	++	++	++	++	++	+	+
	C.a	+	+	++	-	+	+	+	+
Flavonoids	L.t	+	+	+	+	+	+	+	+
	O.g	-	-	-	-	-	-	-	-
	C.a	+	+	+	+	+	-	+	+
Tanins	L.t	+	+	+	+	+	+	+	+
	O.g	++	++	++	++	++	++	++	++
	C.a	++	++	++	++	++	++	++	++
Saponosides	L.t	-	-	-	-	-	-	-	-
	O.g	-	-	+	-	-	-	-	-
	C.a	-	-	-	-	-	-	-	-
Anthraquinones	L.t	-	-	-	-	-	-	-	-
	O.g	++	++	++	+	+	+	+	+
	C.a	+	+	-	-	+	+	+	+
Coumarines	L.t	-	-	-	-	-	-	-	-
	O.g	+	+	+	+	+	+	+	+
	C.a	-	-	-	-	-	-	-	-
Alcaloids	L.t	-	-	-	-	-	-	-	-
	O.g	+	+	+	+	+	+	+	+
	C.a	+	-	-	-	+	-	+	-

Families Wanted (+): presence, (++): strong presence, (-): absence, L.t: *Launaea taraxacifolia*, O.g: *Ocimum gratissimum*, C.a: *Celosia argentea*, c: cooked, nc: uncooked

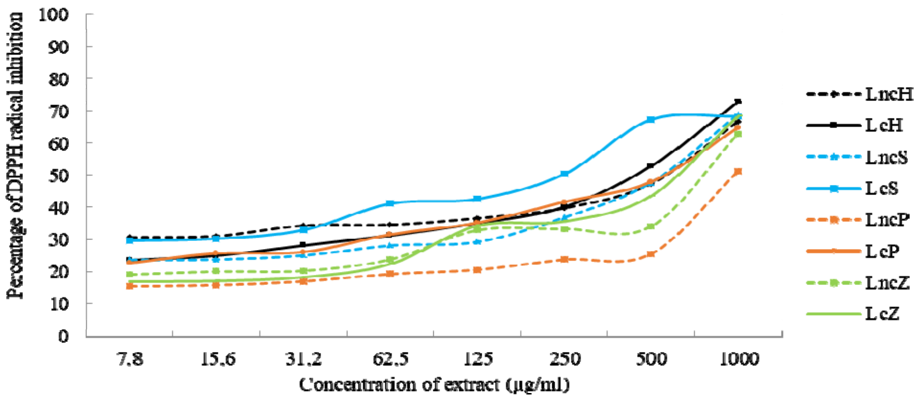

Figure 1. Inhibition of the radical DPPH by the extracts of *Launaea taraxacifolia* Wild.

LncH: *Launaea taraxacifolia* uncooked of Houeyiho (Cotonou), LcH: *Launaea taraxacifolia* cooked of Houeyiho (Cotonou), LncP: *Launaea taraxacifolia* uncooked of Pobè, LcP: *Launaea taraxacifolia* cooked of Pobè, LncS: *Launaea taraxacifolia* uncooked of Sèmè-Podji, LcS: *Launaea taraxacifolia* cooked of Sèmè-Podji, LncZ: *Launaea taraxacifolia* uncooked of Zè, LcZ: *Launaea taraxacifolia* cooked of Zè

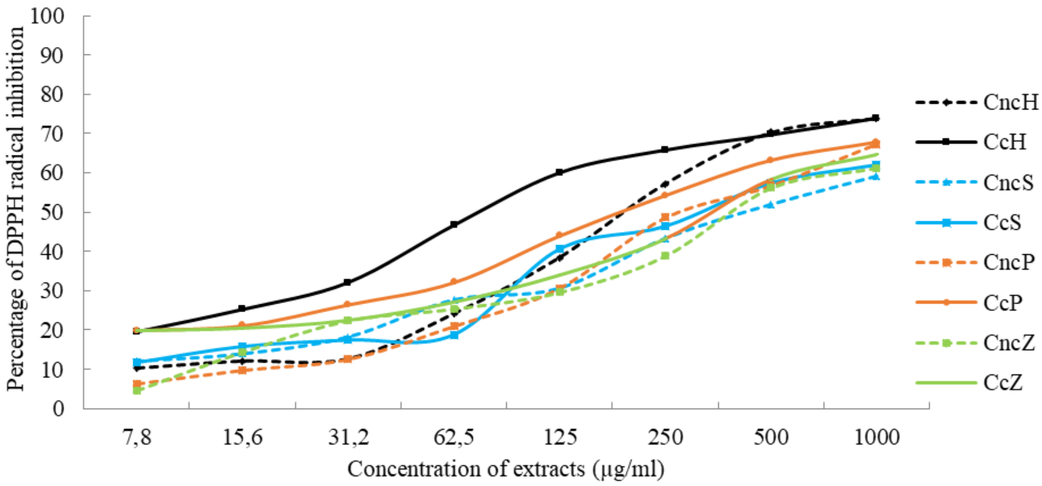

Figure 2. Inhibition of the DPPH radical by the extracts of *Celosia argentea L.*

CncH: *Célosia argentea* uncooked of Houeyiho (Cotonou), CcH: *Célosia argentea* cooked of Houeyiho (Cotonou), CncP: *Célosia argentea* uncooked of Pobè, CcP: *Célosia argentea* cooked of Pobè, CncS: *Célosia argentea* uncooked of Sèmè-Podji, CcS: *Célosia argentea* cooked of Sèmè-Podji, CncZ: *Célosia argentea* uncooked of Zè, CcZ: *Célosia argentea* cooked of Zè

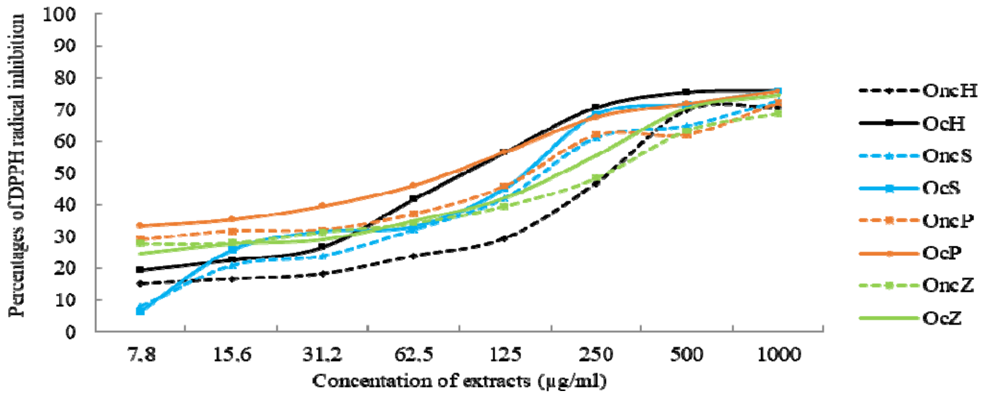

Figure 3. Inhibition of the DPPH radical by extracts of *Ocimum gratissimum* L.

OncH: *Ocimum gratissimum* uncooked of Houeyiho (Cotonou), OcH: *Ocimum gratissimum* cooked of Houeyiho (Cotonou), OncP: *Ocimum gratissimum* uncooked of Pobè, OcP: *Ocimum gratissimum* cooked of Pobè, OncS: *Ocimum gratissimum* uncooked of Sèmè-Podji, OcS: Ocimum gratissimum cooked of Sèmè-Podji OncZ: *Ocimum gratissimum* uncooked of Zè, OcZ: *Ocimum gratissimum* cooked of Zè

3.4 Dosage of Heavy Metals

Table 4 presents the results of evaluation of heavy metals in the extracts of the samples studied. Thus, considering these two sites, the analysis of extracts of Launaea taraxacifolia indicated the presence of arsenic and mercury in Cotonou (Houeyiho), whereas mercury and manganese were found in extracts of Sèmè-Podji. The analysis of the extracts of Ocimum gratissimum indicated the presence of mercury in Cotonou (Houeyiho) whereas the mercury and the manganese were found in the extracts of Ocimum gratissimum of Sèmè-Podji. For Celosia argentea extracts, the presence of arsenic and manganese at the Cotonou (Houeyiho) site was noted, while at the Sèmè-Podji site, arsenic, mercury and manganese were found. However, none of these extracts contained metals above the threshold defined by the WHO (As $\leq$ 1 ppm, Hg $\leq$ 0.1 ppm, Mn $\leq$ 300 ppm). The extracts of Pobè and Zè contained only traces or no heavy metals compared to those of Cotonou (Houeyiho) and Sèmè-Podji. The determination of the metals showed that the highest quantities were found at the Cotonou (Houeyiho) site followed by the Sèmè-Podji site. The presence of heavy metals in plants is influenced by various factors including, among others, plant species, soil type, climate, cultural practices, environment (exhaust gas from transport, industrial activity...) (Yahia et al., 2014). The distribution of heavy metals takes place in two ways: 1) by the aerial where these elements present on the surface of organs (leaves, stems) penetrate into the stomata in the form of particles, gaseous compounds or dissolved in rainwater or irrigation, 2) they can also be absorbed by the roots as a particle in solution in the soil and then stored or transported from the place of absorption to another organ (Chidikofan, 2010). The location of Cotonou (Houeyiho) and Sèmé cultivation sites situated in the urban zone, unlike those of Pobè and Zè, which are rural coupled with farming practices (use of fertilizers and chemical pesticides) could justify their high heavy metals (Yehouenou et al., 2010; Agueh et al., 2015)). In addition, for the assays performed, there was a slight decrease in the metal concentration of the boiled vegetable extracts compared with untreated vegetable extracts (raw). This would be related to leaching of heavy metals in solution during precooking (Bounit et al., 2005). In the end, the quantity of metals found in the extracts of leafy vegetables being below the thresholds set by the WHO, one could thus say that the consumption of these vegetables does not present a visible health risk.

However, previous studies have shown liver and kidney damage in mice exposed to leafy vegetable extracts containing simultaneously small amounts of different heavy metals even below the WHO-defined threshold of concentration (Cobbina et al., 2015; Lagnika et al., 2016). Previous studies have also reported hepatocellular, renal, and brain damage in all three organs (liver, kidney, and brain) in mice previously exposed to low-dose Pb + Hg + Cd (lead, mercury, cadmium) Pb + Hg + As + Cd (lead, mercury, arsenic, cadmium) (Tchounwou et al., 2012; Jadhav et al., 2006; Lin et al., 2016). This results in synergistic toxicity among these heavy metals. These heavy metals have the capacity to induce oxidative stress either by initiating reactive reactions of reactive oxygen species or by interfering with the mechanisms of antioxidant defence. The hydrogen peroxide (H2O2) obtained by disproportionation reaction from the superoxide radical (O2-) can cross the cell membrane and then generate the most powerful reactive species: the hydroxyl radical (OH·) by interaction with heavy metals such as copper, chromium, vanadium and iron. This oxidation reaction, called the Fenton reaction, leads to the formation of two molecules of the hydroxyl radical from a molecule of hydrogen peroxide (Gardès-Albert et al. 2003).

Table 3. Inhibitory concentration at 50% of the extracts

			IC_{50} (µg /ml)		
Samples	Treatment	Cotonou	Sèmè	Pobè	Zè
L.t	Nc	6.87	7.08	11.42	8.29
	C	6.32	5.27	6.91	7.21
O.g	Nc	6.04	5.49	5.04	5.71
	C	4.65	5.03	4.03	5.18
C.a	Nc	5.69	7.01	6.47	6.48
	C	4.64	6.54	5.65	5.04

L.t: *Launaea taraxacifolia,* **O.g**: *Ocimum gratissimum,* **C.a**: *Celosia argentea*, **c**: cooked, **nc**: uncooked

4. Conclusions

The leafy vegetable species studied showed interesting antioxidant powers against the DPPH radical with IC50s of 4.03 and 11.42 µg / ml with increasing activity as follows: *O. gratissimum> C. argentea> L. taraxacifolia*. The data has also demonstrated that precooking led to the optimization of the antioxidant power. This strong activity is due to the presence of several groups phytochemicals (triterpenes and steroids, flavonoids, tannins, alkaloids, saponosides, anthraquinones, coumarines) present in the studied vegetables. The evaluation of heavy metals revealed the presence of arsenic, mercury and manganese. The highest levels were observed in leafy vegetables harvested in Cotonou (Houeyiho) followed by those in Sèmè-Podji but all below the thresholds set by WHO. The leafy vegetables of Zè and Pobè showed a very weak absence or quantity of metals compared to those of Houeyiho and Sèmè-Podji. Pre-cooking would favor a small decrease in the amount of heavy metals, which resulted in a low metal concentration of precooked vegetables compared to those without any treatment. From this work, it appears that there is an interest in precooking vegetables. As a recommendation, given the public's lack of knowledge about the health risks of heavy metals, particularly in the Republic of Benin, it would be important to promote widely disseminated information on the toxic effects of these to allow all professionals and consumers to make informed choice.

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