

Microscopic Features, Chromatographic Fingerprints and Antioxidant Property of Some Unconventional Green Leafy Vegetables Consumed in Bandundu, DR Congo

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ABSTRACT

Introduction: Nutrition is an important aspect of public health because it is linked to many significant diseases and health problems. This work is part of the promotion of traditional foods from the Democratic Republic of Congo, particularly in the province of Bandundu, by assessing the antioxidant capacity of 4 unconventional green leafy vegetables and determining their microscopic features and their chromatographic fingerprints. **Materials and Methods:** Methanolic extracts from leaves of traditional vegetables were characterized for their chemical fingerprint by TLC, HPLC-DAD and their *in vitro* antioxidant activities using ABTS, DPPH assays. **Results:** The microscopic analysis of the powder of leaves showed that each species presents characteristic elements allowing their distinction. Phytochemical screening revealed the presence of flavonoids, terpenes, steroids and quinones in all species. However, *Entada gigas* and *Psophocarpus scandens* also contain alkaloids, *Salacia pynaertii* and *Tetrorchidium congolense* also contain anthocyanins. All extracts displayed a good radical-scavenging activity at the concentration range 1 – 60 µg/mL in the following order: *S. pynaertii* > *T. congolense* > *E. gigas* > *P. scandens*. *S. pynaertii* and *T. congolense* extracts showed significant antioxidant activity compared to

quercetin, and this activity may depend on a synergic effect of several polyphenolic compounds. **Conclusion:** The high nutritive value of these traditional vegetables associated with their important antioxidant activities could contribute to diversifying diets for increased antioxidant consumption for Bandundu's population, and could provide benefits for protection against oxidative damage under different conditions including konzo.

Key words: *Entada gigas*, Konzo, *Psophocarpus scandens*, *Salacia pynaertii*, *Tetrorchidium congolense*, Traditional vegetable.

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DOI : 10.5530/pc.2017.4.23

INTRODUCTION

Nutrition is an important aspect of public health because it is linked to many significant diseases and health problems. Multiple epidemiologic studies have shown that the consumption of vegetable foods could be a public health issue. Polyphenols are the most abundant antioxidants in the diet, and consumption of foods and beverages containing polyphenols may have significant public health implications. Based on existing research, polyphenols may be useful in primary and secondary prevention, particularly concerning diseases associated with oxidative damage such as obesity, diabetes, cardiovascular disease, and cancers.¹ Finding alternative and complementary ways to reduce the redox processes might have a beneficial interest in the context of developing countries.

The Flora of Kwilu Kwango in Bandundu area (Democratic Republic of Congo) is rich in traditional foods that are mostly unexploited. For this reason, Mbemba *et al* assessed the nutritional value of some traditional foods by determining their relative amino acid composition, in order to contribute to the equilibration of the diet in the population of this area severely affected by malnutrition² and diseases such as konzo, a toxico-nutritional neurological disease in which oxidative damage plays a decisive role.³ Among traditional foods studied by Mbemba *et al*, we focus our research on four species of plants commonly used as traditional vegetables to Kenge city in Bandundu area: *Entada gigas* (L.) Fawc. & Rendle, *Psophocarpus scandens* (Endl.) Verdc, *Salacia pynaertii* De Wild, and *Tetrorchidium congolense* J.Léonard.

Entada gigas, native from Central America, Caribbean, and Africa, is

commonly known as sea heart. It is a vegetable flowering plant of the pea family, Fabaceae. Seeds of *E. gigas* have not been used by people as food but it is a very popular seed used in the preparation of traditional concoctions for curing diseases in Nigeria.⁴ Young leaves are consumed as a vegetable in DRC where it was called *Futi futi* or *Nzembo futi*.² *Psophocarpus scandens* (Fabaceae) is a vigorous, perennial vine with mauve, blue, lilac or white flowers. The primary use of *P. scandens* is as food in DRC where it was commonly called *kikalakasa*.² *Salacia pynaertii* (Celastraceae) is a liana native of Africa. The young leaves are eaten as a vegetable in DRC and it is called *Mbondi* in the local language. They are usually pounded and cooked with peanuts, caterpillars or mushrooms and local tomatoes.^{2,5} *Tetrorchidium congolense* (Euphorbiaceae) is native to tropical Africa. This plant is used medicinally in Central Africa, where the bark latex is taken with palm wine to treat diarrhea.⁶ It is called in Bandundu local language "Nkeleketete". Young leaves are consumed as a traditional vegetable in DRC.²

To our knowledge, few investigations have been performed on the phytochemical composition and the biological properties such as antioxidant capacities of these food plants from Bandundu. The present work aimed to determine the microscopic features of these vegetables and to investigate the antioxidant activities of their methanolic extracts using ABTS and DPPH assays. Thin Layer Chromatography (TLC) and High-Performance Liquid Chromatography (HPLC-DAD) were used to achieve phytochemical analysis of phenolic compounds.

MATERIALS AND METHODS

Study environment

Plants have been harvested in Kenge (S04° S1' E16° S8'), a commune in Pelende-Nord sector, Kwango district, Bandundu Province in the Democratic Republic of the Congo.

Plant materials

The leaves of *Psophocarpus scandes*, *Entada gigas*, *Salacia pynaerti* and *Tetrorchidium congolense* were collected from the territory of Kenge, Bandundu (Democratic Republic of Congo, DRC). The collected plants were identified by experts of the herbarium of Kinshasa (National Institute of Studies and Research in Agronomy, Department of Biology, Faculty of Science, University of Kinshasa, DR Congo) and Professor Lukoki. They were dried at the room temperature. Once in the laboratory, the leaves were dried at 37 °C in an oven while calculating the rate of moisture and were finely ground in a high-speed mill (Retsch ZM 100 Model) sieved at 180 µm particle size. The powdered leaves were stored in the dark at room temperature and used for solvent extraction.

Chemicals and reagents

All solvents were of analytical grade and purchased from Merck (Germany) and VWR Chemicals Prolabo (Leuven, Belgium). Caffeic acid, gallic acid, rutin, hyperoside and isoquercitrin (HPLC grade); 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), 2-aminoethyl-diphenylborat, dimethyl sulfoxide (DMSO), Folin-Ciocalteu reagent and potassium persulfate were purchased from Sigma (Bornem, Belgium). 1, 1-Diphenyl-2-picrylhydrazyl (DPPH) was purchased from Eastman Kodak (Rochester, NY, USA). Water was treated in a Milli-Q water ultra-purification system (Easy Pure Purification System) before use.

Microscopic analysis

Powder observations were made using lactic acid reagent (Steimetz reagent).^{7,8} Observations were made with a Hund WETZLAR microscope, and pictures were taken with Digital Camera CANON IXUS 165.

Preparation of extracts

Methanolic extracts were prepared by extracting successively 10 g of leaves powder with methanol HiPerSolv Chromanorm from VWR Chemicals Prolabo (Leuven, Belgium) to obtain 200 mL of percolate. Evaporation of the solvent was performed under reduced pressure (40°C) followed by a 48-72 h vacuum drying to provide the dry extracts which were weighed and kept in hermetic and dark flasks at 4 °C.

Phytochemical analysis

Thin Layer Chromatography (TLC) analysis

The phytochemical screening was performed following some standard lab techniques.⁹ Aqueous extracts at the concentration of 10 mg/mL were analyzed by TLC on normal phase Silica gel 60 F₂₅₄ plates (Merck), using a 10 µL deposit and as eluent either:

Ethyl acetate, formic acid, glacial acetic acid and water (100:11:11:26; v/v/v/v)

Toluene/ethyl acetate (9:1; v/v)

Flavonoids and phenolic acids were revealed using Natural Products-PEG reagent and observed under UV-365 nm light. Flavonoids were detected as yellow-orange fluorescent spots and phenolic acid as blue fluorescent spots.¹⁰ Chlorogenic acid, Kampferol 3-O-glucoside (astragaline), quercetin 3-O-galactoside (hyperoside), quercetin 3-O-rhamnoside (quercitrin) and quercetin 3-O-rutinoside (rutin) were used as standards.

High-Performance Liquid Chromatography (HPLC-DAD) analysis

Analytical HPLC- DAD separations were carried out on a Hypersil ODS⁺ RP18 column as described previously.¹¹ Chlorogenic acid, rutin, and isoquercitrin were used as standards.

Determination of total phenolic content

Total phenolic content of methanolic extracts (Methanol 80%) was determined according to the Folin-Ciocalteu method as described previously.¹² A calibration curve of gallic acid (0.025-0.4 mg mL⁻¹) was prepared, and phenolic contents were determined in triplicate from the linear regression equation of this curve. The results were expressed as milligrams of gallic acid (GA) equivalent per gram of dried matter.

Flavonoid content

The flavonoid content of the extracts was determined by UV-Vis spectrophotometry¹³ Results are expressed in mg equivalent of quercetin per g (mg QE/g) of dry vegetal material using the following equation $y = 0.0232x + 0.1535$ ($R^2 = 0.945$).

Tannins content

The extraction of tannins was carried out according to the adapted method used by Zhang *et al.*, 2008.¹⁴ Tannin extract contents were quantified by vanillin method using the procedure reported by Sun *et al.* 1998.¹⁵ This method is based on the ability of vanillin to react with tannins units in the presence of acid to produce a colored complex measured at 500 nm.

Determination of anthocyanins

The determination of the anthocyanin content of the extracts was performed by the procedure reported by Adedapo *et al.*¹⁶

Evaluation of radical scavenging activity

ABTS radical scavenging capacity

ABTS assay was based on the method described by Kapepula *et al.*¹⁷

DPPH radical scavenging capacity

DPPH assay was performed according to the method described previously by Floegel *et al.* (2011) with slight modifications.¹⁸ A solution of 0.004% of DPPH in 80% (v/v) methanol was prepared one hour before use. The absorbance of the solution was adjusted to 0.75 ± 0.03 at 517 nm using fresh 80% (v/v) methanol. Then 0.02 mL of standard or sample were mixed with 1.98 mL of DPPH solution and incubated for 30 min in the dark covered with aluminum foil. The decrease of absorbance was monitored at 517 nm with Spectrophotometer Hewlett-Packard 8453. The antiradical capacity analysis was performed on dichloromethane and methanolic dry extracts. Quercetin was used as positive control and ABTS^{•+}, DPPH[•] scavenging activities of extracts were expressed as IC₅₀ (IC₅₀ is the amount of antioxidant necessary to decrease the initial concentration of radical by 50%).

Statistical Analysis

Results were expressed as mean values \pm standard deviation (SD). IC₅₀ were calculated with GraphPad Prism 6.0 under application of the function "log (inhibitor) vs. normalized response-variable slope" after converting the concentrations into their decimal logarithm. One-way analysis (ANOVA) and Student's paired t-test were used to compare scavenging capacities determined by ABTS and DPPH assays and the level of statistical significance was set at $p < 0.05$, for two-sided testing.

RESULTS

Botanical microscopic characters

Powders of the leaves of the different vegetables selected were treated with Steimetz reagent and showed the following specific botanical microscopic characters. *Entada gigas* revealed fragments of parenchyma with acicular raphides of calcium oxalate, rectangular epidermal cells containing crystals, paracytic stomata. Also reticulate vessels, smooth no glandular trichome and few starch grains were observed (Figure 1).

Psophocarpus scandens leaves powder revealed abundant prisms of calcium oxalate, starch grains, fragments of lignified fibers, diacytic stomata, circular epidermal cells, spiral vessels and unicellular non glandular trichomes. *Salacia pynaertii* showed the presence of fibers containing crystals, epidermis fragments containing numerous paracytic stomata, cluster crystals (druse), spiral vessels, few starch grains and sclerotic cells (Figure 1). Leaf powder of *Tetrochirdium congolense* exhibited palisade parenchyma, anticlinal beaded cell walls, numerous anisocytic stomata, lipid droplets, unicellular nongrandular trichoma, punctuated vessels and fibers with crystals.

Chemical analysis

Phytochemical screening

The results of phytochemical screening showed that flavonoids, terpenes, steroids and quinones are present in all the species studied. However, *E. gigas* and *P. scandens* contain also alkaloids, *S. pynaertii*, and *T. congolense* contain also anthocyanins. Iridoids were present in *P. scandens* and *S. pynaertii*.

TLC and HPLC-DAD analysis have shown the presence of polyphenolic compounds in all methanolic extracts. TLC fingerprints of extracts showed the presence of glycosylated flavonoids (yellow, orange and green fluorescent spots) and phenolic acids (blue fluorescent spots) as major compounds (Figure 2). This was confirmed by HPLC-DAD analysis (Figure 3). By comparison with the retention factor, the coloration of spots, retention time and UV-spectrum of standards used, some phenolic compounds could be identified in these extracts. *E. gigas* contains,

quercetin-3-O-rutinoside and kaempferol 3-O-glucoside; *P. scandens* contains quercetin 3-O-rhamnoside; *S. pynaertii* contains kaempferol 3-O-glucoside and *T. congolense* contains kaempferol 3-O-glucoside and quercetin 3-O-rhamnoside (quercitrin). Further chromatographic and spectroscopic studies are needed to characterize the others unknown compounds.

Results from the quantitative determination of anthocyanins, flavonoids, tannins, and total phenolic content are summarized in Table 1.

Anthocyanin and tannin contents were determined as catechin equivalents in milligrams per gram of dry weight (mg CE/g DW), while total polyphenol contents were calculated as gallic acid equivalents in milligrams per gram of dry weight (mg GAE/g DW). Flavonoid content was determined as quercetin equivalents in milligrams per gram of dry weight (QE/g DW). The total phenolic contents varied significantly ($P < 0.05$) between the studied vegetables. Results showed that *S. pynaertii* is the vegetable with the highest amount of total phenolic compounds and anthocyanins. *P. scandens* had the highest amount of flavonoids. Furthermore, concentrations of tannins were also high in the studied vegetables, with higher values in *P. scandens* leaves, followed by the leaves of *T. congolense*.

Antioxidant activity

Radical scavenging activity of tested traditional food items, determined by two biochemical *in vitro* methods namely ABTS and DPPH assays is presented in Table 2 and is expressed as IC_{50} values.

IC_{50} is the amount of antioxidant necessary to decrease the initial concentration of radical by 50%. Lower IC_{50} value indicates a higher antioxidant activity. Quercetin-equivalent (QE) is calculated as IC_{50} of quercetin used as positive control divided by IC_{50} of plant extract. All the extracts had significant scavenging effects with antiradical activities connected to their ability to scavenge free radicals according to their IC_{50} and quercetin equivalent (Q-E) values. IC_{50} and Q-E values for extracts showed that *S. pynaertii* is the most active followed by *T. congolense*, *E. gigas* and *P. scandens* in ABTS assay; while in DPPH assay, *T. congolense* is the most active.

Table 1: Total anthocyanins, flavonoids, tannins and total phenolic contents of selected vegetables.

Secondary metabolites	<i>E. gigas</i>	<i>P. scandens</i>	<i>S. pynaertii</i>	<i>T. congolense</i>
Anthocyanins (mg CE/g DW)	nd	nd	47.42 ± 0,52	37,73 ± 0,25
Flavonoids (mg QE/g DW)	37,08 ± 1,02	40,78 ± 1,11	31.62 ± 2.17	21,36 ± 0,65
Tannins (mg CE/g DW)	42,56 ± 0,05	102,56 ± 0,08	nd	100,89 ± 0,08
Total phenolic content(mg GAE/g DW)	54,74 ± 0,49	64,2 ± 0,58	508.34 ± 14,42	241,54 ± 2,18

nd: not determined

Table 2: IC_{50} values ($\mu\text{g} / \text{mL}$) and Q-E of methanolic extracts of selected traditional vegetables on ABTS and DPPH assays (Mean ± SD, n = 6).

Samples	ABTS		DPPH	
	IC_{50} ($\mu\text{g} / \text{mL}$)	Q-E	IC_{50} ($\mu\text{g} / \text{mL}$)	Q-E
<i>Entada gigas</i>	35,4 ± 0,96	0.04	45,5 ± 0,97	0.07
<i>Psophocarpus scandens</i>	53,2 ± 0,97	0.03	62,81 ± 0,97	0.05
<i>Salacia pynaertii</i>	2.11 ± 0.48	0.67	9.48 ± 0.61	0.34
<i>Tetrochirdium congolense</i>	3.65 ± 1.03	0.39	7.13 ± 2.14	0.45
Quercetin	1.42 ± 0.40		3.21 ± 0.99	

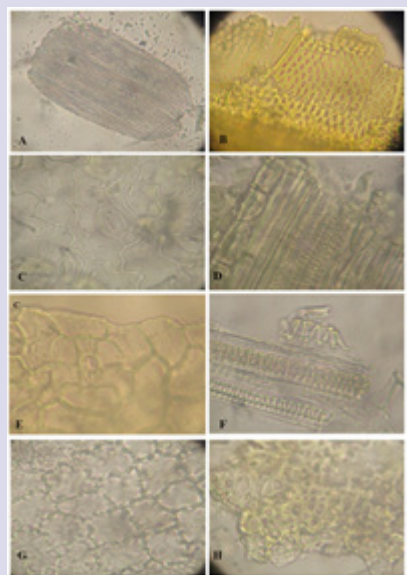


Figure 1: Raphides of calcium oxalate (A), Reticulate vessels (B) of *Entada gigas*; Diacytic stomata (C) and Spiral vessels, Prism of calcium oxalate (D) of *Psophocarpus scandens*; Paracytic stomata (E) and Spiral vessels (F) of *Salacia pynaertii* and Anticlinal beaded cell walls (G) and Punctuated vessels(H) of *Tetrochirdium congolense* at 40X.

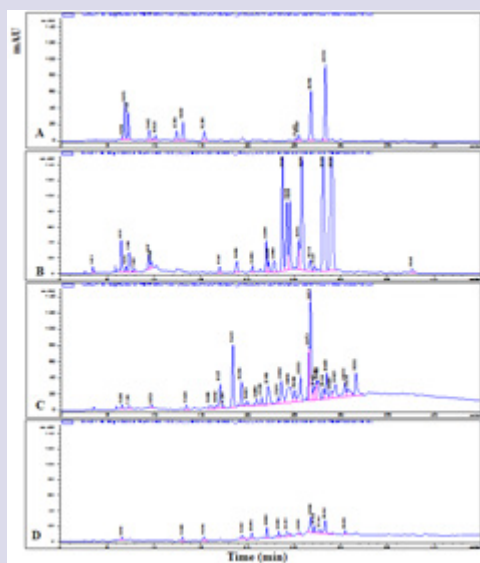


Figure 3: HPLC-DAD chromatograms of methanolic extracts from *Entada gigas* (A), *Psophocarpus scandens* (B), *Salacia pynaertii* (C) and, *Tetrochirdium congolense* (D) using a nonlinear gradient of acetonitrile (A) and 0.05% trifluoroacetic acid in ultrapure water (B): 0 min, 0:100 (A:B), 1 min, 3:97(A:B); 45 min, 40:60 (A:B); 46min, 0:100 (A:B) and 60 min, stop at 1 mL/min on Hypersil ODS column (4 mm × 250 mm) with detection at 340 nm.

DISCUSSION

Considering the microscopic examination of the four vegetables, the presence or shape of specific crystals of calcium oxalate, grain starch, epidermal cells, fibers, cuticle, stomata, and vessels allowed the identification of these four species.

There is few reports on the phytochemical screening of these Congolese traditional vegetables. However, Fankam *et al.*²⁰ Reported the presence

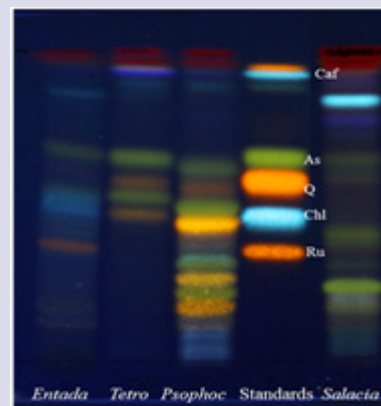


Figure 2: TLC chromatogram of methanolic extracts from *E. gigas*, *P. scandens*, *S. pynaertii* and *T. congolense* with astragalalin (As), chlorogenic acid (Chl), quercitrin(Quer) and rutin(Rut) as standards; developed with ethyl acetate/formic acid/glacial acetic acid/water (100:11:11:26; v/v/v/v) and visualized at 365 nm with Natural Products-PEG reagent. Flavonoids are detected as yellow-orange fluorescent spots and phenolic acids as blue fluorescent spots.

of alkaloids; phenols and tannins in *E. gigas* from Cameroon but flavonoids, quinones and terpenoids were not detected. Some studies showed that seeds from *E. gigas* used in the preparation of traditional concoction for curing diseases in Nigeria contained essential minerals and high amount of anti-nutrient factors such as oxalate, phytate, and tannins.^{4,21} These anti-nutrient factors are reduced through processing of the vegetables. Oxalate content of leaves of *E. gigas* is in accordance with microscopic analysis that showed abundant fragments of parenchyma with cells containing calcium oxalate crystals. Previous studies on *P. scandens*, *S. pynaertii* and *T. congolense* reported their highest protein and mineral contents.^{2,22,23} Interestingly, *S. pynaertii* constitute the richest vegetable in methionine and cysteine among all studied traditional vegetables in Bandundu.²⁴ Methionine and cysteine are sulphur amino acids, essential for the detoxification of cyanogen glycosides implicated in the occurrence of konzo. *S. pynaertii* is an unconventional green leafy vegetable consumed by populations of Popokabaka, another area severely affected by konzo.⁵

Many studies were performed to assess the nutritional value of *P. scandens*. Leaves of *P. scandens* were showed to contain lipids and proteins with high nutritive quality. One of the most important traditional use of this plant is to increase the quantity and quality of milk produced by nursing mothers.²² Our results obtained from ABTS and DPPH assays show that all the extracts possessed antioxidant properties. Our results were correlated between the two chemical methods used. The small differences observed between the two methods could be attributed to the fact that DPPH assay only detect hydrophilic antioxidants in the contrary of ABTS assay. The highest radical-scavenging capacity of *S. pynaertii* and *T. congolense* extracts is in adequacy with their higher total phenolic content.

Earlier studies have reported that the selected vegetables have a reasonable nutritive value. They are good source of essential amino acids and minerals especially for children who are exposed to many diseases.^{2,22,23} The high nutritive value of these traditional vegetables associated with their important antioxidant activities could contribute to a diversification of the diet in Bandundu's population, and could then provide benefits leading to a protection against oxidative damage under different conditions including konzo.

CONCLUSION

The microscopic analysis of powders from leaves of selected species allowed the identification of specific botanical microscopic characters. TLC and HPLC-DAD analysis indicated that phenolic compounds are major secondary metabolites of leaves from *E. gigas*, *P. scandens*, *S. pynaertii* and *T. congolense*. All extracts exhibited good antioxidant activity with *S. pynaertii* and *T. congolense* as the most actives. However, these antioxidant *in vitro* activities should be complemented in the future by *in vivo* evaluation in healthy humans. Further studies are then needed to evaluate the cellular antioxidant and the *in vivo* antioxidant activities of these traditional vegetables and particularly in their cooked forms. This could lead to the valorization of these traditional vegetables, which could be promoted as food or as food supplements with high antioxidant capacity.

ACKNOWLEDGEMENT

The authors thank Professor Lukoki, Mr. Kikufi. A of Faculty of Sciences and Mr. Landu of the National Institute for Research in Agronomics (INERA), the University of Kinshasa for identification of the plants studied. They thank also the community of Kenge of their collaboration.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

ABBREVIATION USED

ABTS: 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulphonic acid); **DPPH:** 1, 1-Diphenyl-2-picrylhydrazyl; **HPLC-DAD:** High-Performance Liquid Chromatography with Diode-Array Detection; **IC₅₀:** Half maximal Inhibitory Concentration; **TLC:** Thin Layer Chromatography.

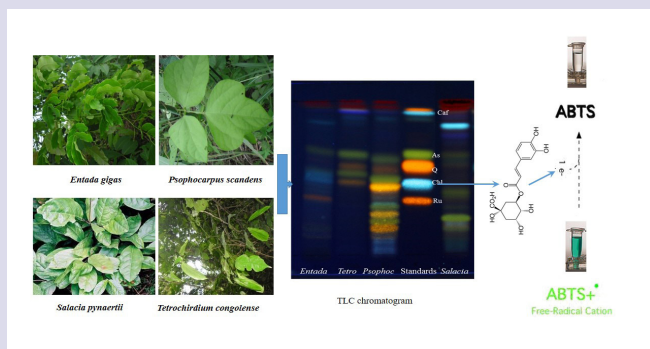
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PICTORIAL ABSTRACT

SUMMARY

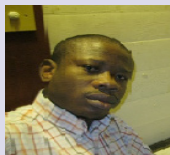


- Unconventional green leafy vegetables have a high nutritive value and important antioxidant activity.
- Salacia pynaertii* and *Tetrochidium congolense* exhibited a high antioxidant activity with IC₅₀ < 10 µg/ mL.
- Antioxidant activity would depend on a synergic effect of several polyphenolic compounds.
- Studied vegetables could provide benefits in protecting against oxidative damage under different conditions including konzo.

ABOUT AUTHORS



Ms Lydia Maganla Bahati: She is a Master student in Nutritional Epidemiology, B.Sc. in Molecular biology and searcher to the Faculty of Sciences of University of Kinshasa, Democratic Republic of Congo. Interested in adolescent girl's diet and health, her research involves phytochemistry studies into a variety of rich vegetables.



Mr Paulin Mutwale Kapepula: Ph D, Scientific staff and searcher to the Faculty of Pharmaceutical Sciences of University of Kinshasa, Democratic Republic of Congo. His research involves bioactivity and phytochemistry studies into a variety of plant species of DR Congo including mushrooms, wild tropical fruits. He works on the project "Traditional foods as putative nutraceuticals in konzo, a paralytic disease associated with oxidative stress and irreversible brain damage in sub-Saharan Africa.