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Aqueous extracts of Paraguayan Moringa (*Moringa oleifera* Lam.), as a source of direct and indirect antioxidants for nutraceutical applications

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Abstract
Antioxidant-rich food and supplements are crucial to counteract free radicals that cause oxidative stress, which is responsible for the development of several diseases. *Moringa oleifera* Lam. is a plant with antioxidant properties mainly attributed to its phenolic compounds and, recently, to its isothiocyanates, deriving from the hydrolysis of glucosinolates. Although this species is native to Asia, its cultivation in Paraguay can generate a differential composition in its secondary metabolites, by the warm and humid environment where it is grown. In this study, we compared the total phenolic content, the direct antioxidant activity (DPPH and ABTS cationic radical assays) and the glucomoringin content (main glucosinolate of *M. oleifera* and precursor of the bioactive isothiocyanate) of aqueous extracts of different parts (leaves, seeds, bark and branches) of *M. oleifera*, grown in Paraguay. Two different drying treatments (freeze-drying and oven drying, 40°C) were performed on the vegetal samples before extraction. We could observe that branches of *M. oleifera* (in both drying treatments) are a reservoir of antioxidants that can be exploited for the preparation of extracts for nutraceutical applications, together with the commonly used oven-dried leaves and freeze-dried seeds.

Keywords: *Moringa oleifera* Lam., aqueous extracts, total phenolic content, antioxidant activity, glucosinolate

1. Introduction

Free radicals are atom or molecules containing one or more unpaired electrons, making them very unstable and reactive [1]. Their reactivity relies on the need to acquire electrons or hydrogen atoms from donor molecules. Free radicals are constantly produced during normal physiological metabolism in tissue, both from oxygen (reactive oxygen species, ROS) and nitrogen (reactive nitrogen species, RNS). In normal conditions they can be counteracted by the antioxidant defence system of the body, represented by endogenous enzymes and exogenous compounds with antioxidant properties introduced from the diet [2]. There is a delicate balance between the quantity of ROS and RNS generated in the body and the antioxidants needed to scavenge them. In the field of human nutrition, an excess of free radical production or a lack of antioxidant intake can shift the balance toward an excessive presence of ROS and RNS, and cause oxidative stress in the body [2]. In this condition, free radicals will damage cell macromolecules like DNA, proteins, lipids and lipoproteins, and such damages are among the factors responsible for diseases like cancer, cardiovascular disease, diabetes, stroke, liver cirrhosis, atherosclerosis, aging and Alzheimer's disease [3]. Consequently, consumption of foods rich in antioxidants is crucial and recommended in several studies, to enhance the variety of free radical scavengers inside the body, and it was even suggested that through the development of dietary supplement of nutraceutical antioxidants, it is possible to exert positive physiological and pharmacological effects on specific human diseases [4].

Antioxidants can be classified as direct or indirect, according to the mechanism that they use to scavenge free radicals.

Direct antioxidants are redox active, short-lived, small molecules that directly scavenge reactive oxygen and/or nitrogen species. On the other hand, indirect antioxidants induce a variety of phase II xenobiotic metabolizing enzymes (i.e. NAD (P) H quinone reductase), resulting in a higher antioxidant capacity and a long-lasting protective effect compared to direct antioxidants. As a matter of fact, both mechanisms work together to protect the body from free radical damages [5].

Antioxidants may be derived from various sources, as plants, animals and organic synthesis. Nevertheless, synthetic antioxidants might exert toxic side effects at higher concentrations than their permitted limits and, on the other side, animal-derived antioxidants face ethical issues [4]. Consequently, plants actually represent the most promising reservoir of health-protective antioxidants and they have already been shown to be an important source of compounds able to counteract free radicals, because of their antioxidant activity [3].

Moringa oleifera Lam. is a plant native to India, and is now cultivated in several tropical and subtropical areas of the world. It belongs to the family Moringaceae, together with

twelve other species of the genus. All parts of the plant are edible and have long been consumed by humans. This plant is known for its nutritional properties, mainly attributed to the leaves, and also, for its wide range of claimed medicinal properties such as antimicrobial, antiasthmatic, antioxidant, hepatoprotective, cancer protective, spasmolytic, hypotensive, cholesterol lowering and hypoglycemic [4].

The antioxidant properties of *M. oleifera* have long been attributed to the phenolic compounds present in the plant, especially in the leaves. Polyphenols, like flavonoids and phenolic acids directly eliminates free radicals in the body, by breaking the propagation chain reactions of free radicals, as it is widely known. Nevertheless, the indirect antioxidant activity of the isothiocyanates (ITCs) from *M. oleifera* has recently been demonstrated [5].

In general, ITCs are compounds derived from the bio-precursors glucosinolates. Glucosinolates are compounds typical of plants belonging to the order Brassicales (to which is linked the order Capparales that includes *M. oleifera*) and they are constituted by a backbone of glucose that is attached to sulfonated aldoxime, with a variable side chain. Inside plant cells, glucosinolates are stored separately from myrosinase, which is a thioglucosidase responsible for the hydrolysis of the glucose moiety, followed by a rearrangement of the aglycone, which causes the formation of the ITCs. In intact plant tissues, the concentration of ITCs is very low or undetectable, but upon mechanical rupture or damage of the fresh tissues, myrosinase gains contact with glucosinolates and ITCs are formed. Furthermore, it has been reported that the consumption of glucosinolates with diet can result in the conversion to ITCs, operated by the gut microbiota [5].

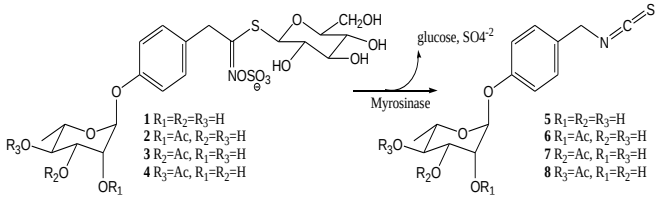


Fig 1: Structures of the *Moringa oleifera* Lam. glucosinolates and their corresponding isothiocyanates.

In particular, the principal glucosinolate of *M. oleifera* is 4-(α-L-rhamnopyranosyloxy) benzyl glucosinolate (glucomoringin) (1 Fig.1). Glucomoringin is present in all parts of the plant in different concentrations [6]. Furthermore, in some parts of the plant, three acetyl isomers of glucomoringin (2, 3, 4 Fig.1) were detected as well. Both the ITC generated from glucomoringin (5 Fig.1) and the ITC generated from the isomer of glucomoringin acetylated in the position 4' of the rhamnose moiety (8 Fig.1) proved to have an indirect antioxidant capacity comparable to that of sulforaphane. Sulforaphane is the characteristic ITC of Broccoli that is able to induce phase II metabolizing enzymes, thus contributing to the scavenging of free radicals, through an indirect mechanism of action [5].

As far as we know, there is little to no data available *on M. oleifera* from Paraguay, and very poor literature is available on the preparation of water extracts, even though water is a "green", biocompatible solvent, thus suitable for the preparation of extracts for human consumption. Moreover, water-soluble extracts are easier to use for formulation purposes [7]. So, the aim of this study was to compare the total phenolic content (TPC), the direct antioxidant activity (DPPH and ABTS cationic radical assays) and the glucomoringin content (as a precursor of the bioactive, isothiocyanate) of aqueous extracts of different parts (leaves, seeds, bark and branches) of *M. oleifera*, grown in Paraguay.

To the end of checking the effect of the drying treatment of the vegetal material in the final composition of the extracts, each part of the plant was subjected to two different drying treatments: oven-drying at 40°C and freeze-drying.

The final aim was to identify the most suitable extracts to be used for the development of a nutraceutical antioxidant supplement. This supplement could exploit the direct antioxidant activity of the polyphenols and/or the indirect antioxidant activity of the isothiocyanate obtained from the conversion of glucomoringin by the gut microbiota.

2. Materials and Methods

2.1 Plant material and drying

Fresh samples of leaves, branches, bark and seeds of *Moringa oleifera* Lam., "purple" variety, were collected in Moringa Guaraní S.A. (YGUA), located in Compañía Colonia Piraretá, Pirate Bay District, Cordillera Department, Paraguay. All samples were collected carefully, without breaking the vegetal tissues.

A specimen of *Moringa oleifera* Lam. "purple" variety was deposited at the Herbarium FCQ at Botany Department of the Faculty of Chemical Sciences of the National University of Asunción, Campus Universitario, 1144 San Lorenzo, Paraguay (Herbarium codes: Y. González and C. Povolo 139, 140).

Each plant sample was divided in two aliquots: one was oven-dried at 40 °C (Matsui, PO-200, Matsui FFG. Co. Ltd, Japan.), while the other was freeze-dried (Freezone 4.5 Lab Conco Corp. USA, collector temperature -45°C, vacuum 0,050 mBar). Samples were dried until complete removal of water, then were pulverized with a mixer and stored at -20°C. In general, freeze-drying is a technique that can preserve the original phytochemical content of a vegetal tissue, because it operates at low temperatures. It is noteworthy, however, that drying in the oven is a less time consuming and cheaper drying method.

Seeds of *Moringa oleifera* Lam., "green" variety, air-dried in the dark, were provided by Moringa Guaraní S.A. (YGUA), located in Compañía Colonia Piraretá, Piribebuy District, Cordillera Department, Paraguay.

They were pulverized with a mixer and stored at room temperature.

2.2 Extracts preparation

Aqueous extracts of *M. oleifera* were prepared according to a method previously reported in literature [8], with modifications. Briefly, the powders were extracted with deionized and distilled water, at 80°C, for 10 minutes, with a powder/water ratio of 1/12, under stirring. After that, the extracts were filtered through quantitative filter paper (MN 640, Macherey-Nagel, Ø 125 mm), and the residue was extracted again twice with deionized and distilled water, at 80°C, for 5 minutes, with a powder/water ratio of 1/4. After each extraction step, the extracts were filtered and at the end of the whole process, they were unified and centrifuged at 12000g, 25°C, for 10 minutes (Heal Force Neofuge 15R centrifuge). Potassium sorbate (0.1%) was then added as a preservative and they were stored at -20°C, inside vials.

2.3 Total polyphenol content (TPC) quantification

The total polyphenol content in the aqueous extracts of *M. oleifera* was determined by UV-visible spectrophotometry, using the Folin-Ciocalteu method, with modifications [9]. In this method, the Folin-Ciocalteu reagent (a mixture of phosphomolybdate and phosphotungstate) reacts at a basic pH with phenolic compounds, producing tungsten (W_8O_{23}) and molybdenum oxides (Mo_8O_{23}) with a blue colour. Briefly, 1.25 ml of Folin-Ciocalteu reagent (previously diluted 1:10 v/v with water) were added to 250µl of the aqueous extracts and the mixture was left at room temperature for 10 minutes. After that, 1ml of Na_2CO_3 (7,5% p/v) was added and the mixture was left in a water bath at 30°C for 30 minutes, in the dark. At the end, the absorbance of the blue metal oxides was measured in a spectrophotometer at 650 nm. A blank sample was prepared using water instead of aqueous extracts. Gallic acid was used as reference standard for the preparation of a calibration curve. TPC was expressed in mg gallic acid equivalents (GAE) per 100 ml of aqueous extract. Mean and standard deviations (n=3) were calculated.

2.4 Determination of extract antioxidant properties

The antioxidant capacity of the extracts was measured by the following in vitro assays.

2.4.1 Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

DPPH radical scavenging activity was measured using a method described in literature, with modifications [10]. Briefly, 1ml of DPPH solution in methanol (60 µM) was mixed with 1 ml of each extract, in five different dilutions. The mixture was incubated for 30 minutes at room temperature, in the dark and the decreasing in absorbance was measured at 514 nm. A mixture of methanol and DPPH solution was used as a blank of reactant. Moreover, a standard of ascorbic acid was used as a positive control to compare its antioxidant activity with the antioxidant activity of each extract. Results were expressed as Inhibitory Concentration 50 (IC50) in mg/ml. Mean and standard deviation (n=5) were calculated.

The percentage of DPPH inhibition was calculated as follows:

$$\text{Inhibition percentage (\%)}: \frac{(Abs\ DPPH - Abs\ sample) * 100}{Abs\ DPPH} \quad (1)$$

Where Abs DPPH is the absorbance of the blank of reactant and Abs sample is the absorbance of the solution when the sample extract has been added at a particular concentration. The Inhibitory Concentration 50 (IC50) value (mg/ml) is the inhibitory concentration at which the DPPH radical was scavenged at 50% and calculated by interpolation from the data.

2.4.2 Azino-bis (3-etilbenzothiazoline-6-sulphonic acid) (ABTS•+) radical scavenging activity

ABTS•+ cationic radical scavenging activity was measured using a method reported in literature, with modifications [11]. Briefly, the cationic radical ABTS•+ was produced by adding 7ml of aqueous potassium persulfate ($K_2S_2O_8$)2,45mM to 7ml of ABTS 7mM. This mixture was kept in the dark, at room temperature for almost 20 hours, before use. The mixture was then diluted with ethanol until a final absorbance of 0,700 (±0, 02) at 730 nm. After that, four different dilutions of the aqueous extracts were mixed with the solution of the cationic radical in a water bath at 35°C for 30 minutes. A blank was used with water in the place of the extracts. After 30 minutes, the absorbance was measured at 730 nm. Moreover, a standard of TROLOX was used as a positive control to compare its antioxidant activity with the activity of each extract. Results were expressed as (IC50) in mg/ml. Mean and standard deviation (n=4) were calculated. Calculations were performed using the following formula:

$$\text{Inhibition percentage (\%)}: \frac{(Abs\ ABTS\cdot+) - (Abs\ sample) * 100}{Abs\ ABTS\cdot+} \quad (2)$$

Where Abs ABTS·+ is the absorbance of the blank of reactant and Abs sample is the absorbance of the solution when the sample extract has been added at a particular concentration. The Inhibitory Concentration 50 (IC50) value (mg/ml) is the inhibitory concentration at which the ABTS·+ radical was scavenged at 50% and calculated by interpolation from the data.

2.5 Quantification of 4-(α-L-rhamnopyranosyloxy) benzyl glucosinolate (glucomoringin)

2.5.1 Extraction, isolation and characterization of glucomoringin

To isolate glucomoringin from *M. oleifera* seeds, in order to use it as external standard for HPLC analysis, an intact glucosinolate extraction method was used, with modifications [8].

20 g powdered *M. oleifera* seeds ("green" variety) were heated at 80°C in 240 ml 70% MeOH for 10 minutes. After centrifugation at 3000 rpm for 10 min, the pellet was re-extracted twice with 80 ml 70% MeOH for 5 min. Combined supernatants were dried in a rotary evaporator at 45°C up to a final volume of 15 ml.

After that, the solution was transferred to a DEAE Sephadex A-25 column that was equilibrated with MilliQ, and the column was washed with 4 volumes of 70% MeOH, followed by 2 volumes of MilliQ, according to the method of De Graaf *et al.* [12] The elution was performed according to a procedure reported by Förster *et al.* [8] with modifications. Briefly, glucomoringin was slowly eluted with 2 volumes of 0.5 M potassium sulphate and collected into an equal volume of absolute ethanol (99.9%).

After centrifugation at 3000 rpm for 10 min, the supernatants were dried in a rotary evaporator at 45°C. Dried residues were re-dissolved in three successive volumes of MeOH, that were equals to 1/25 volume of absolute ethanol added in the previous step. Solutions were centrifuged for 10 min at 3000 rpm. Absolute ethanol (99.9%) was added to the supernatants and solvents were evaporated to dryness on a rotary-evaporator at 45°C.

The identity of glucomoringin was confirmed by mass spectrometry (Mariner ESI-TOF, negative mode) and NMR spectroscopy (Bruker 400 MHz NMR). The purity was confirmed by inspection of the ^{1}H NMR for traces of other glucosinolates. An additional purity estimate (100% of total HPLC area at 223 nm) was obtained from HPLC of intact glucosinolates under the conditions of Förster *et al.* [8].

2.5.2 Chromatographic conditions and liquid chromatography apparatus

Glucomoringin determination in aqueous extracts was performed according to the method reported by Förster *et al.* [8] with modifications. Briefly, a 400 µl sample was diluted

to a volume of 800 µl with deionized and distilled water. 200µl of zinc acetate 0.4M were added and the mixture was incubated for 30 min at room temperature. After that, it was centrifuged at 16000g, 25 °C for 10 min (Heal Force Neofuge 15R centrifuge). Supernatants were analysed on a Shimadzu prominence HPLC system. A 10 µl sample was injected (SIL-20A auto sampler) on a 4.6 x 250 mm 300SB-C18 column (Zorbax, 5µm, Agilent). The separation was achieved using the following gradient program: 0-2 min: 0-1%B, 2-20 min: 1-50%B, 20-24 min: 50-100%B, 24-26 min: 100%B, 26-33 min: 100-0%B, 33-38 min: 0%B. Mobile phase composition was: eluent A: 100% 0.1M ammonium acetate, eluent B: 40% acetonitrile/ 0.1M ammonium acetate. Detection was at 223 nm using a photodiode array detector (SPD-M20A). Temperature of the column was set at 25°C (CTO-10A Column oven). Compounds were identified from their retention time and UV spectra. Glucomoringin was quantified against an external standard of glucomoringin potassium salt. Results of the quantitative analysis of glucomoringin were expressed as µmol concentration of the anion per 100 ml extract.

2.6 Statistical analysis

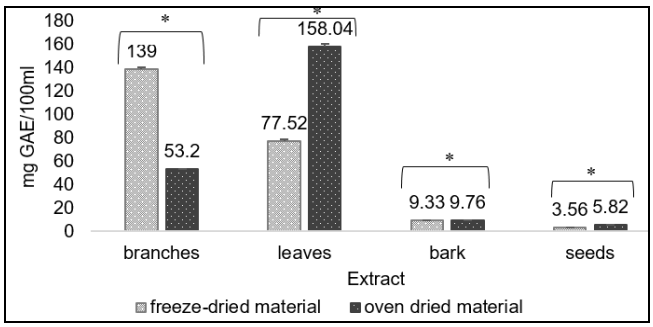
Statistical analysis was performed with Graphpad Prism 5.0 (GraphPad Software Inc., CA, USA). With the aim of assessing significant differences between drying treatments, a one-tailed Student's t-test was performed, with a 95% confidence interval. In the case of the extracts, an ANOVA analysis (p<0, 05) with 95% confidence interval was performed and also a Tukey's post hoc test. To evaluate the correlation between the total antioxidant capacity and the total polyphenol content, a Pearson's analysis was performed.

3. Results & Discussion

3.1 Total polyphenol content (TPC)

Phenolic compounds can directly contribute to the antioxidant activity of an extract [13]. For this reason, we decided to investigate the polyphenol content in each aqueous extract.

Figure 2 shows the TPC expressed as mg GAE/100 ml of each aqueous extract of different parts of *M. oleifera* obtained. In the oven-dried treatment, the TPC values varied from 5, 82 ± 0, 14 mg GAE/100mL (seeds) to 158, 04 ± 2, 89 mg GAE/100mL, (leaves). In the freeze-drying treatment, values varied from 3,56 ± 0,05 mg GAE/100mL (seeds) to 139 ± 2 mg GAE/100mL (branches).



Results are expressed as mg GAE/100ml extract with mean ± SD (n=3). (*) indicates significative difference among means (t-Student, p<0, 05).

Fig 2: Total polyphenol content of aqueous extracts of different parts of *Moringa oleifera* Lam., dried by oven or freeze-dried.

The analysis of the results (Fig.2) showed that amongst the extracts obtained from oven-dried matrices, the one obtained from the oven-dried leaves has the higher content of polyphenols (158,04±2,89 mg GAE/100 ml), while among those obtained from the freeze-dried samples, the extract from branches has the higher total polyphenol content (139±2 mg GAE/100 ml).

In both treatments, bark and seeds showed the lowest amount of total polyphenols, with values ranging from 3, 56±0, 05 mg GAE/100 ml to 9, 76±0, 11 mg GAE/ 100 ml.

In general, it was observed a significant difference in TPC among extracts of the same part of the plant (t-Student, p<0,05), where extracts obtained from oven-dried material showed a higher content in polyphenols than extracts obtained from freeze-dried material. Branches represent the only exception: in this case, the extract from the freeze-dried aliquot of the sample had the highest content in total polyphenol. This situation might be due to the presence of thermosensitive compounds in branches that could be lost because of the oven drying treatment. In all the other samples, the higher TPC value found for extracts of the oven-dried series may be due to an increasing permeability of the vegetal tissue, that is subjected to ruptures when dried in an oven at mild temperatures, according to Sriwichai *et al.* [14].

A significant difference in total polyphenol content was observed among extracts of different parts of the plant, that were dried by the same treatment (ANOVA, p<0, 05).

Up to now, there are no studies comparing the total phenolic content of aqueous extracts of different part of *M. oleifera* cultivated in Paraguay, that were dried with two different methods (oven 40 °C and freeze-drying).

3.2. Extract antioxidant capacity

The total antioxidant capacity of the extracts was measured by two spectrophotometric assays, based on the capacity of antioxidant compounds (i.e. polyphenols and other antioxidants) to donate a hydrogen atom to a free radical, which has a specific colour when it is in the oxidized form, but then loses the colour after reduction. In both assays, a standard compound is used as reference to make comparisons with the antioxidant activity of the aqueous extracts. Results are expressed as Inhibitory Concentration 50 (IC50) in mg/ml, which is the concentration of the extract that is necessary to reduce the activity of the radical at 50%. The lower is the IC50 value of an extract, the higher is its antioxidant activity, also compared to the standard reference. Both these spectrophotometric assays measure a direct antioxidant activity.

3.2.1 Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

Table 1 shows the results of the radical scavenging activity analysis, by DPPH radical. Among the extracts obtained from oven-dried material, the aqueous extract from leaves has the higher radical scavenging capacity (0, 16±0, 04 mg/ml). In the series obtained from freeze-dried material, the extract of branches presented the highest total antioxidant capacity (0, 20±0, 03 mg/ml), with an IC50 value very close to the oven-dried leaves extract. Values of IC50 ranged from 0,16±0,04 mg/ml (leaves extract) to 0,50±0,08 mg/ml (bark extract) in the oven-dried series, and from 0,20±0,03 mg/ml (branches extract) to 12,50±0,32 mg/ml (bark extract) in the freeze-dried series. It is noteworthy that the IC50 value found by us for *M. oleifera* leaves extract (prepared with the oven-dried leaves) is comparable to the IC50 value reported for an aqueous

extract of the same tissue, That was cultivated in South-Africa and subjected to air-drying [3]. This indicates similar radical scavenging power for Paraguayan leaves compared to South-African leaves and could be due to the similar latitude where *M. oleifera* was cultivated.

Nevertheless, both the leaves extract and the branches extract showed a higher IC50 value than the reference (ascorbic acid, IC50 of 0, 0042 mg/ml), thus indicating a more moderate total antioxidant activity than the reference standard.

Table 1: DPPH radical scavenging activity of aqueous extracts of *M. oleifera* Lam.

	T1	T2
Extract	**IC_{50} mg/ml**	**IC_{50} mg/ml**
leaves	0.16 ± 0.04	0.54 ± 0.03
Bark	0.50 ± 0.08	12.50 ± 0.32
branches	0.41 ± 0.02	0.20 ± 0.03
seeds	*	*

IC_{50} ascorbic acid: 0, 0042 mg/ml

Results are expressed as half-maximum inhibitory concentration (IC50) in mg per ml, with mean ± SD (n=5). T1 indicates the drying with oven at 40°C of the vegetal material. T2 indicates the freeze-drying treatment of the vegetal material.

(*) indicates the impossibility to measure the IC50 value, due to the method used.

3.2.2 Azino-bis (3-etilbenzothiazoline-6-sulphonic acid) (ABTS•+) radical scavenging activity

Table 2 showed the results of the radical scavenging activity analysis, by ABTS·+. Among those extracts obtained from oven-dried material, the aqueous extract from leaves has the higher radical scavenging capacity, expressed as IC50 (0, 80±0, 03 mg/ml). In the series of freeze-dried extracted material, the extract from branches presented the highest total antioxidant capacity (0, 71±0, 03 mg/ml). The IC50 values ranged from 0,80±0,03 mg/ml (leaves extract) to 17,51±0,40 mg/ml (seed extract) in the oven-dried series, and from 0,71±0,03 mg/ml (branches extract) to 20,93±0,38 mg/ml (seed extract) in the freeze-dried series.

In this assay, the reference standard used was TROLOX, a hydrosoluble analogue of alpha-tocopherol. Its IC50 value was 0,028 mg/ml. Also in this assay, the lowest IC50 values observed were higher than the IC50 value of the reference.

Table 2: ABTS·+radical scavenging activity of aqueous extracts of *M. oleifera* Lam.

	T1	T2
Extract	**IC50 mg/ml**	**IC50 mg/ml**
leaves	0.80 ± 0.03	1.22 ± 0.03
bark	0.81 ± 0.05	6.86 ± 0.03
branches	1.40 ± 0.09	0.71 ± 0.03
seeds	17.51 ± 0.40	20.93 ± 0.38

IC50 TROLOX: 0,028 mg/ml

Results are expressed as half-maximum inhibitory concentration (IC50) in mg per ml, with mean ± SD (n=4). T1 indicates the drying with oven at 40°C of the vegetal material. T2 indicates the freeze-drying treatment of the vegetal material.

Moyo *et al.* [3] reported the IC50 value for an aqueous extract of *M. oleifera* leaves, cultivated in South-Africa and air-dried (0, 69 mg/ml). That value is comparable with the value that was found for our aqueous extract of *M. oleifera* leaves, cultivated in Paraguay and dried in an oven at 40°C (0, 80±0, 03 mg/ml).

In the statistical analysis of the results, it was observed a significant difference between the two drying treatments for the same part of the plant in all extracts (t-Student, p<0, 05). As previously seen with TPC, the extracts obtained from oven-dried material have the highest total antioxidant activity, with the only exception of branches extracts. There is significant difference in the antioxidant activity of extracts of different parts of the plant, considering the same drying treatment (ANOVA, p<0, 05).

3.3 Correlation analysis between total polyphenol content and extract antioxidant capacity

With data reported in Table 3, it is possible to observe that the antioxidant capacity of aqueous extracts is higher when measured by DPPH assay than ABTS·+assay. This may happen because different test and solvent systems could affect comparisons of antioxidant activities [3]. This also means that no correlation between the two assays was observed. Nevertheless, both of them highlight that the leaves extract is the best one in terms of radical scavenging capacity, in the case of an oven drying treatment of the vegetal material, and that branches extract is the best one in the case of a freeze-drying treatment. On the other hand, the activities of the extracts vary according to differences in their chemical composition.

A positive correlation was observed, between total polyphenol content and antioxidant capacity of the extracts, by a Pearson's correlation analysis (rABTS=0, 9119, rDPPH=0, 8667, p<0,0001). This means that, in general, the higher polyphenol extraction yield corresponds to the higher antioxidant activity, due to the hydrogen atom donating ability of the phenolic compounds, as previously reported in literature [16]. In fact, in the series of oven-dried matrices, the aqueous leaves extract has the highest TPC value and the best radical scavenging capacity. In the series of freeze-dried matrices, the aqueous branches extract has the highest TPC value and the best antioxidant activity, too. The only exception is represented by bark; despite its low polyphenol content in both treatments, the extracts have a good antioxidant activity, probably due to the presence of other, non-phenolic, antioxidants. Vyas, [4] also reported a high antioxidant activity for bark extracts of *M. oleifera*, in spite of its low TPC value.

According to Vyas *et al.* [4] a possible explanation of the high polyphenol content, and so antioxidant activity of leaf extract, is that the leaf has a longer life span in comparison with other plant parts, and, being the site of energy production, it faces a high magnitude of oxidative damage. Therefore, they require greater production of antioxidants in their capacity to act as a shield.

Table 3: Summary table of results of total polyphenol content and antioxidant capacity of aqueous extracts of *M. oleifera* Lam. T1 indicates the drying with oven at 40°C of the vegetal material. T2 indicates the freeze-drying treatment of the vegetal material. TPC indicates the total polyphenol content, expressed as mg GAE/g dry weight.

TPC (mgGAE/ g dry weight)	T1		Extract	TPC (mg GAE/ g dry weight)	T2	
	Antioxidant capacity				Antioxidant capacity	
	ABTS·+	DPPH			ABTS·+	DPPH
	IC_{50} (mg/mL)	sIC_{50} (mg/mL)			IC_{50} (mg/mL)	IC_{50} (mg/mL)
20.61 ± 0.38	0.80 ± 0.03	0.16 ± 0.04	leaves	11.01 ± 0.18	1.22 ± 0.03	0.54 ± 0.03
1.33 ± 0.01	0.81 ± 0.05	0.50 ± 0.08	Bark	1.33 ± 0.03	6.86 ± 0.03	12.5 ± 0.3
7.31 ± 0.05	1.40 ± 0.09	0.41 ± 0.02	branches	13.39 ± 0.15	0.71 ± 0.03	0.20 ± 0.03
0.83 ± 0.02	17.51 ± 0.40	*	seeds	0.53 ± 0.01	20.9 ± 0.4	*

Results of ABTS·+ and DPPH assays are expressed as IC50 (mg/ml). (*) indicates the impossibility to measure the IC50 value, due to the method used.

3.4 Quantification of 4-(α-L-rhamnopyranosyloxy) benzyl glucosinolate (glucomoringin)

The analysis of the results (Table 4) showed that the seed extract has the highest concentration of glucomoringin (6,841±0,097 µmol/100 ml) among the extracts obtained from freeze-dried materials, with values ranging from 4,10±0,25 µmol/100 ml (leaves extract) to 6,841±0,097 µmol/100 ml (seeds extract). Among the extracts obtained from the oven-dried matrices, branches extract showed the highest concentration of glucomoringin (6, 13±0,18 µmol/100 ml), with the values ranging from 4,46±0,12 µmol/100 ml (seeds extract) to 6,13±0,18 µmol/100 ml. As reported by Bennett *et al*. [6], in the case of a freeze-drying treatment of different tissues of *M. oleifera*, seeds represent the tissue with the higher concentration of glucomoringin. It was possible to observe that there is a significant difference in glucomoringin concentration of extracts obtained from freeze-dried matrices ($p<0, 05$), with the exception of extracts from leaves and branches. Bennett *et al*. [6] reported lower glucomoringin content in branches compared to leaves, in the case of a freeze-drying treatment of the plant samples, but they only analysed the outer bark of branches instead of the entire branch, as has been done in this study.

A t-Student test ($p<0.05$) was performed to compare glucomoringin concentration in extracts of the same part of the plant, subjected to different drying treatments. In the case of leaves, there is no difference in drying the tissue by oven or freeze-drying, in terms of final glucomoringin content of the extract. In all the other cases, it was observed a significant difference among treatments.

It was observed also that in the case of branches, the oven-dried material gives the extract with the higher concentration of glucomoringin, while the opposite happens for seeds and bark. A possible explanation is that branches contain, as leaves, the acetylated isomers of glucomoringin. The higher temperature of the oven compared to a freeze-drying might promote the conversion of the isomers into glucomoringin itself, by losing the acetyl group. In fact, the conversion reaction depends on temperature as reported by Förster *et al*. [8] Even more, Sriwichai *et al*. [14] report an increase in the releasable nutrients of *M. oleifera* subjected to processing, especially drying by oven at mild temperatures. On the other hand, seeds and bark do not seem to be affected much by the difference in the drying treatment and, as far as we know, it was not detected any acetyl isomers in those tissues. Therefore, the effect that prevails is a loss of glucomoringin in the oven-dried material, according to the fact that it is a thermosensitive compound [8].

Table 4: 4-(α-L-rhamnopyranosyloxy) benzylglucosinolate (glucomoringin) content of aqueous extracts of different parts of *Moringa oleifera* Lam., dried by oven or freeze-dried. Results are expressed as µmol of glucomoringin per 100 ml of extract, with mean ± SD (n=3).

	Oven-drying	Freeze-drying
Extract	GMG (µmol/100 ml)	GMG (µmol/100 ml)
Leaves	4.61 ± 0.37	4.10 ± 0.25
Bark	5.152 ± 0.084	5.58 ± 0.16
Branches	6.13 ± 0.18	4.43 ± 0.16
Seeds	4.46 ± 0.12	6.841 ± 0.097

4. Conclusions

In the case of the freeze-drying treatment of the vegetal material, the aqueous extract obtained from seeds represent the best source of glucomoringin that is the precursor of the isothiocyanate, which has indirect antioxidant activity. On the other hand, the extract of branches represents a very good source of polyphenols as direct antioxidants.

In the case of an oven-dried treatment (mild temperature, 40°C), the highest amount of polyphenols can be found in the extract from leaves, with a TPC value comparable with that of freeze-dried branches. Even more, the extract obtained from branches represent in this case the best source of glucomoringin.

The major finding of this study is that branches of *M. oleifera* grown in Paraguay are a reservoir of antioxidant compounds that is still little known and unexploited, but that could be used for the preparation of extracts for nutraceutical applications, together with oven-dried leaves and freeze-dried seeds. Even in the case of exclusively drying in the oven, branches retain a good concentration of polyphenols, so that the extract has a TPC value that is a third of the highest TPC value, observed in the oven-dried series (leaves extract). In conclusion, the selected extracts could be used alone or in combination for the development of a nutraceutical antioxidant supplement.

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6. Conflict of interest

The authors report no conflict of interest.

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