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Assay of Total Polyphenols, Flavonoids, and Proanthocyanidins and Determination of the Antioxidant Activity of Different Extracts of *Merremia tridentata* Leaves

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Abstract: The aqueous and organic solvent extracts of *Merremia tridentata* leaves are studied. Extraction conditions are optimized: an extraction time of 20 minutes, a temperature of 80°C and a plant material/water mixture of 1% give the best yield of polyphenol. The amount of polyphenols, flavonoids, and proanthocyanins of each organic solvent extract was investigated. The results obtained show that the aqueous extract is richer in phenolic compounds and in flavonoids with, respectively, a Total Polyphenolic Content (TPC) of (29.823±0.56 mg EQAG/g) and a Total Flavonoid Content (TFC) of 10.18±0.32 mg EQ/g. For the proanthocyanidins, it can be noted that all the extracts have substantially equal values varying between 0.6377-0.6906 mg/g). Antioxidant activities are measured using two different methods (DPPH· and CUPRAC). The aqueous extract have the highest values of (77.67±0.98 mg/g) and (96.37±2.02 mg/g), respectively. The study of the antioxidant activities of the extracts do not show a correlation between the amount of polyphenols and the IC50. The ethanolic and methanolic extracts which are although less rich in polyphenol, seem more effective with IC50 estimated at 0.108 mg/mL and 0.110 mg/mL respectively, while the aqueous extract, which is richer in polyphenol records an IC50 of 0.283 mg/mL.

Keywords: *Merremia tridentata*, Extraction, Antioxidant, Polyphenols, Flavonoids, Proanthocyanidins

1. Introduction

The physiological reactions lead to oxidation phenomena. Oxygen, which is responsible of the cellular respiration passes through very toxic intermediates called reactive oxygen species (ROS). ROS species are free radicals derivatized from oxygen as superoxide ($O_2^{\cdot-}$), hydroxyl ($OH^{\cdot}$), peroxyl ($ROO^{\cdot}$), peroxynitrite ($ONOO^{-}$) and nitric oxide ($NO^{\cdot}$) as reported in the literature [1-4]. Excess free radicals are often responsible for irreversible damage [5]. These free radicals have a useful role to play in protecting cells from bacteria and viruses, but they can also be harmful and cause oxidative stress.

Merremia tridentata is a plant widely used in traditional medicine where the leaves and roots are used to treat various diseases such as ulcers, diabetes, high blood pressure, arthritis, skin infections, inflammation, among others. Bibliographic research has shown that few studies are devoted to this plant in the African continent [6-8]. It is with this in mind that we have proposed to carry out an in-depth chemical study on the plant, in order to have reliable information. The principal aim of this study is to optimize the extraction of polyphenols in the leaves of *Merremia tridentata.* Secondary, the different categories of molecules of interest (polyphenols, flavonoids or proanthocyanidin) are quantified and the antioxidant activities of the leaves of *Merremia tridentata* are studied using different solvent.

2. Experimentation

2.1. Plant Material

Leaves of *Merremia tridentata* were harvested in

September 2021 from a field near the town of Ndoffane, which is located in the Kaolack region in central-western Senegal. This area bordered by the Gambia straddles, the southern area Sahelian zone, and the northern Sudanian zone with geographical coordinates 14°00′00″ north, 16°00′00″ west.

Samples were rinsed with water and distillate water and were air-dried for 7 days. The dried leaves were finely ground using a grinder and stored at 4°C.

2.2. Extraction Process

The method of Vuong et al. [9] is used as the extraction method. In this study, we were interested in the optimization of three parameters: the temperature, the duration, and the ratio (leaf powder/volume of solvent). Indeed, a mass of 0.5 g of the sample is introduced into 50 mL of distilled water and subjected to different temperatures (50°C, 60°C, 70°C, 80°C and 90°C) for 20 minutes and maintained in a stirring water bath. The noted optimal temperature is now used for optimization of the extraction time. The mass of 0.5 g of *Merremia tridentata* leaf powder was introduced into 50 mL of distilled water at the optimum temperature found previously with variable extraction times (10, 20, 30, 40.50, and 60 min). The same procedure is used to optimize the solvent mass-volume ratio, fixing the optimum time and the optimum temperature. The optimal conditions found at the end of these experiments will be used for the rest of the work.

2.3. Determination of Total Phenolic Content (TPC)

The test was carried out according to the method of Mohdaly *et al*. [10] with some modifications. A final 200 µL sample was mixed with 150 µL of Folin-Ciocalteu reagent, 600 µL of 20% Na_2CO_3 and 2.32 mL of distilled water. After 30 minutes of incubation in the dark at room temperature, the absorbance was read at 760 nm with a Perkin-Elmer UV/Visible Lambda 365 spectrophotometer. Gallic acid (GA) was used as a standard. The results were expressed in mg GAE/g.

2.4. Determination of Total Flavonoid Content (TFC)

The method used is that described by Gurnani *et al*. [11]. 2.5 mL of sample were mixed with 2.5 mL of an ethanolic solution of $AlCl_3$. After 1 h of incubation in the dark at room temperature, the absorbance was read at 425 nm with a Perkin-Elmer Lambda 365 UV/Visible spectrophotometer. Quercetin (Q) was used as a standard. The results were expressed in mg QE/g.

2.5. Determination of Total Proanthocyanidin Content

The proanthocyanidin contents were determined according to the method of Li *et al*. [12] with slight modifications. 500 µL of diluted sample, 3 mL of 4% vanillin are mixed before adding 1.5 mL of concentrated HCl. This mixture is incubated at room temperature for 15 minutes before reading the absorbance at 500 nm. Catechin (C) was used as standard, and the results are expressed in mg CE/g.

2.6. Antioxidant Capacity

2.6.1. Determination of Total Antioxidant Capacity, Antioxidant Activity and IC50 with DPPH• Method

DPPH• is an unstable free radical, by accepting an electron or a hydrogen radical, it becomes a stable molecule. The evaluation of the antioxidant activity by the DPPH• radical test and the determination of the inhibitory concentration IC50 at 50%, are carried out using a UV spectrophotometer. The reported method [13, 14] is used with slight modifications. Indeed, 200 µL of this solution are mixed with 3.8 mL of a methanolic solution of the radical DPPH• (0.1014 mM). After 30 minutes of incubation in the dark at ambient temperature, the absorbance was read at 517 nm. Afterwards, an adequate dilution was made to determine the IC50. The DPPH• radical scavenging activity was calculated using the following formula:

$$Scanvenging\ activity = \frac{A_c - A_S}{A_c} \times 100$$

A_c = Absorbance Control (DPPH containing no antioxidant).

A_s = Absorbance of the sample (DPPH in the presence of the antioxidant).

2.6.2. Determination of Antioxidant Capacity: CUPRAC Method

The antioxidant capacity was determined according to the method described by Apak *et al*. [15]. A 10^{-2} M copper chloride solution, an ammonium acetate buffer pH 7 and a 7.5 mM ethanol solution of neocuproine are prepared. 1.1 mL of a solution of the sample is mixed with 1 mL of $CuCl_2$ solution, 1 mL of ammonium acetate and 1 mL of neocuproine solution. The mixture is incubated for one hour in the dark before reading the absorbance at 450 nm. At the same time, a range of TROLOX is prepared under the same conditions from a stock solution of TROLOX at 1 mM.

3. Results and Discussion

3.1. Extraction Optimization

Figure 1A shows the variation of polyphenol extraction and the scavenging activity according to the temperature, the duration, and the ratio. As observed in the Figure 1A these parameters have an influence on the yield of the extraction of polyphenols from the leaves of *Merremia tridentata* and on the scavenging capacity of the sample. Indeed, the yield of extraction of polyphenol as well as the antioxidant capacity increase gradually as functions of the temperature to reach maxima at 80°C. The yield of the extraction of polyphenol and scavenging capacity decrease when the temperature raises from 80°C to 100°C. The value of 80°C will be retained as the optimal temperature for the process. This result may be linked to thermally induced decomposition as reported in several studies by Vuong et al. [9] in the case of Carica papaya, by Pinelo et al., [16] for grape pomace, by Ballard et al., [17] for peanut skins and by Alu'datt et al. [18] for olive seeds.

Furthermore, the extractability of the polyphenols was affected by the time contact at 80°C as shown in Figure 1B. When the times increases from 10 min to 40 min the polyphenol yield and the antioxidant activity increase. The yield of polyphenol extraction reaches a maximum at 40 minutes. Between 40 min and 50 min the yield of polyphenol extraction decreases very slightly. Longer extraction time resulted in the decrease of the yield of polyphenol extraction. The antioxidant activity continues to increase and reaches a maximum at 50 min. The difference between the two times is probably due to the type of polyphenol extracted after 40 min. Given the best yield of polyphenols at 40 min, we retained this value as the optimum time of extraction.

Figure 1C shows the influence of ratio vegetal material on water at the optimal temperature of 80°C and optimal time of 40 min extraction time. The best results on the yield of the polyphenol extraction and antioxidant activity were obtained for the 1% ratio. In fact, when the ratio plant/water increases from 1% to 10%, the values of the yield of polyphenol extraction and the antioxidant activity decrease drastically. These results are in accordance with the observations of Gertenbach et *al.* [19] which shows that the lower the plant/water ratio, the higher the extraction rate.

Indeed, it has been reported that a strong dilution accelerates the transfer of polyphenolic compounds located inside the foliar particles to the solvent because of the concentration gradient between the inside of the surface of the foliar particles. Additionally, the presence of hydroxyl groups on polyphenols make them hydrophilic, they are consequently extracted by water. For this present study, the extractions are carried out at a temperature of 80°C for 40 minutes with a vegetal matter/water ratio of 1/100, to quantify the total phenolic compounds, the total flavonoids and the proanthocyanidins.

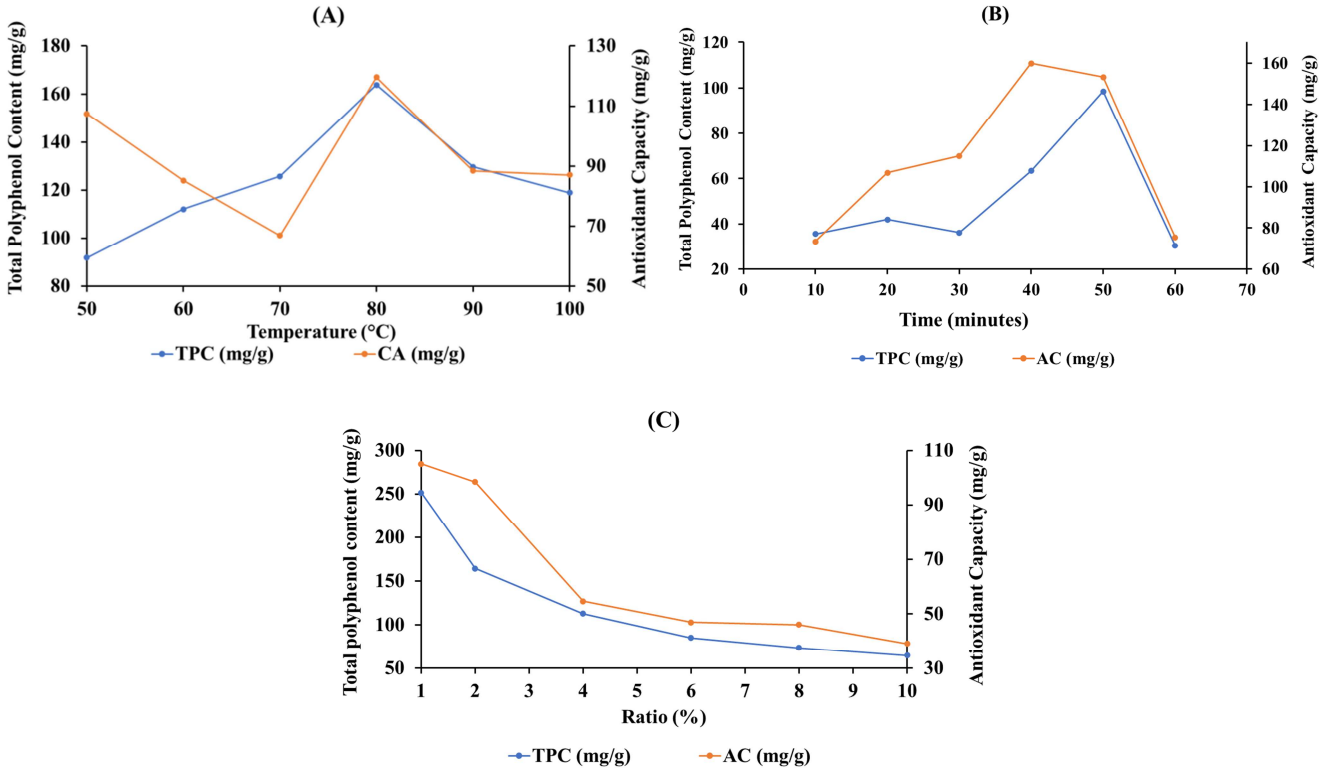

Figure 1. *Optimization of temperature, time, and ratio of the aqueous extraction of leaves of Merremia tridentata: (A) Duration; (B) Temperature; (C) leaves/water ratio.*

3.2. Content of Total Polyphenols and Total Flavonoids of Merremia tridentata

The results of the quantitative determination of the polyphenol and flavonoid contents of the leaves of *Merremia tridentata* are recorded in Table 1.

The leaves are respectively macerated in ethanol, methanol, hexane, and ethyl acetate. Leaves extraction was also done in hot water to yield a decoction. Decoction seems to be the best method for extracting total polyphenols. In fact, the aqueous extracts record the highest total polyphenol contents (29.83±0.56 mg EQ AG/g), followed by the ethanolic and methanolic extracts which give contents estimated at 22.30 ± 0.07 mg EQ AG/g and 14.43 ± 0.07 mg EQ/g, respectively. Since ethyl acetate and hexane are less polar solvents, they do not make it possible to extract a large quantity of polyphenols which are polar compounds. Ethyl acetate and hexane extracts give very low polyphenol contents evaluated, respectively, at 0.06± 0.01 mg EQ AG/g and 0.10± 0.01 mg EQ AG/g (Table 1). In fact, the TPC was significantly affected by solvent polarity since, polyphenols are polar compounds, they are more easily extracted by polar solvents [9].

Table 1. *Total polyphenol and total flavonoid contents of leaves of Merremia tridenta.*

Extraction solvent	TPC (mg Eq AG/g)	TFC (mg Eq Q/g)
Water	29.83± 0.56	4.63± 0.32
Methanol	14.43± 0.07	3.30 ±0.02
Ethanol	22.30 ± 0.07	3.54± 0.01
Ethyl acetate	0.06 ± 0.01	0.23 ± 0.00
Hexane	0.10 ± 0.01	0.13± 0.00

The total flavonoid contents of *Merremia tridentata* leaves extracted from different solvents (Table 1) show that the aqueous extract records the highest content 4.63 ± 0.32 mg EQ/g, followed by ethanolic and methanolic extracts which give similar contents, estimated respectively at 3.54 ± 0.01 mg EQ/g and 3.30 ± 0.02 mg EQ/g. Ethyl acetate and hexane extracts give the lowest TFC value evaluated, respectively, at 0.23±0.00 mg EQ/g and 0.13 ± 0.00 mg EQ/g. For the organic solvents, flavonoids are better extracted by ethanol and methanol. The comparable yields of TFC in water and organic solvents such as methanol and ethanol can be explained by the lower dipole moments of flavonoids comparatively to dipole moments of polyphenols.

3.3. Total Proanthocyanidin Content

Proanthocyanidin have very low dipole moments. Their extraction by polar solvent gave low yield. The proanthocyanidin contents reported in Table 2 are comparable (0.64 – 0.69 mg/g) for water solvent as well as for methanol, ethanol, and ethyl acetate solvents.

Table 2. *Proanthocyanidin content of Merremia tridentata leaves.*

Solvents extraction	Proanthocyanidin content (mg eq EQ/g)
Water	0.69 ± 0.03
Methanol	0.66 ± 0.02
Ethanol	0.69 ±0.04
Ethyl acetate	0.64 ± 0.05
Hexane	n/a

3.4. Antioxidant Capacity and IC50

Polyphenol compounds are known for its capacity to trap free radicals. Their antioxidant activity and IC50 were carried out for the aqueous and the organic solvents extract of leaves of *Merremia tridentata.* The antioxidant activity of these extract were evaluated by the DPPH· radical and the CUPRAC methods. The impact of antioxidants on the DPPH· radical results in their ability to give it a hydrogen radical. This reduction capacity is determined by the decrease in absorbance at 517 nm, which is induced by the antioxidant compounds. This is observed by the color change from purple for DPPH· to yellow for DPPH—H.

Table 3. *Antioxidant capacity (DPPH· and CUPRAC) and CI50 of the different extracts of Merremia tridentata leaves.*

Solvents	DPPH ·			CUPRAC
	mg TrE/g	IC50 (mg/mL)	% Inb max	mg TrE/g
Aqueous	77.67±0.98	0.283	77.76	96.37±2.02
Ethanol	30.10±0.74	0.108	71.08	83.87±0.40
Methanol	14.09±1.34	0.11	72.80	91.48±1.56
Ethyl acetate	n/a	n/a	n/a	0.75±0.03
Hexane	n/a	n/a	n/a	0.89±0.02

DPPH· radical makes it possible to evaluate three parameters: the antioxidant capacity, the kinetics and the IC50, which is the concentration of the sample tested, necessary to reduce the DPPH· radical concentration by 50%. The kinetics was studied to see the evolution of the radical DPPH· and the antioxidant solution over time. The results are recorded in Figure 2. For the first 5 minutes, the aqueous extract exhibits a higher percentage of inhibition (34.3%) than the ethanolic (28.5%) and methanolic (30.8%) extracts. After 5 minutes, the percentage of inhibition of the aqueous extract increases progressively and greatly exceeds the ethanolic and methanolic extracts, which themselves begin to be superimposable. In addition, at 240 minutes, the percentage of inhibition is 77.76% for the aqueous extract, while the ethanolic and methanolic extract, respectively, record a percentage of inhibition of 72.80% and 71.08%. The results of the antioxidant capacities of the various extracts are determined by the DPPH· method and recorded in Figure 3. The aqueous extract has the highest antioxidant capacity estimated with 77.67±0.98 mg TrE/g, followed by the ethanolic and methanolic extract with antioxidant capacity values of 30.1012±0.7412 mg TrE/g and 14.0882 ±1.3363 mg TrE/g, respectively (Table 3). The ethyl acetate extract records the lowest antioxidant capacity evaluated at 1.6821±1.0888 mg TrE/g, while the antioxidant content of the hexane extract is not determined. The ethanolic and methanolic extracts give very similar IC50 evaluated respectively at 0.108 and 0.110 mg/mL, while the aqueous extract has an IC50 of 0.283 mg/mL (Table 3). The IC50 of the hexane and ethyl acetate extracts are not determined. The IC50 of the ethanolic extract are better than that of the aqueous extract, which, however, has the highest polyphenol content. The CUPRAC method allows to determine the antioxidant capacity by reducing the Cu^{2+} ion to Cu^{+} ion. The results of the variation of the antioxidant capacity of the different extracts produced with the CUPRAC method are reported in Figure 4. This method consider phenolic compounds as well as compounds other than those of polyphenolic types. The aqueous extract has the highest antioxidant capacity with a value of 96.3683±2.0182 mg TrE/g. Ethanolic and methanolic extracts present antioxidant capacities values of 83.8727±0.4032 and 91.4836±1.5612 mg TrE/g, respectively (Table 3). The antioxidant activity values obtained from CUPRAC method are comparable [97—83 mg

TRE/g] unlike those obtained with DPPH· which are different from each other [78—14 mg TRE/g]. This can be explained by the fact that DPPH· considers polyphenolic compounds while CUPRAC considers both polyphenols and other compounds. Indeed, DPPH· is involved in a mechanism of H-abstraction [20] while CUPRAC is involved in an oxidation-reduction mechanism [21].

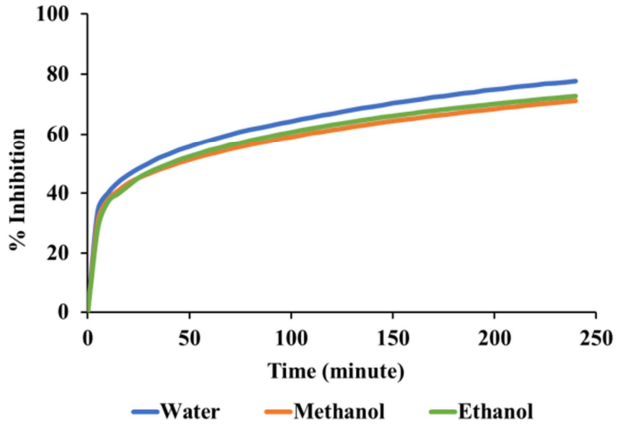

Figure 2. *The evolution of the percentage of inhibition of the different extracts over time.*

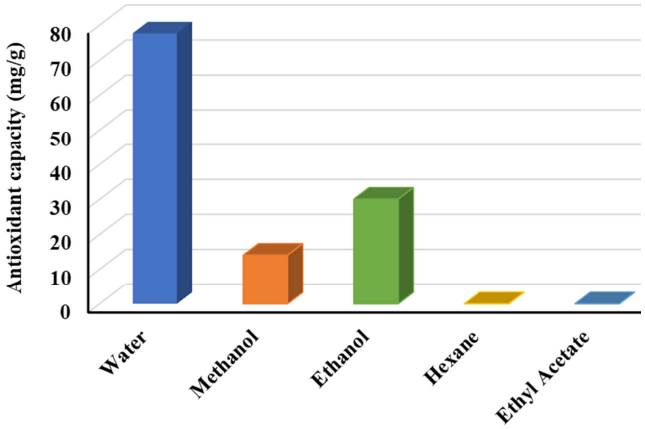

Figure 3. *Comparative histogram of the DPPH· antioxidant capacity of the different Meremia leaf extracts.*

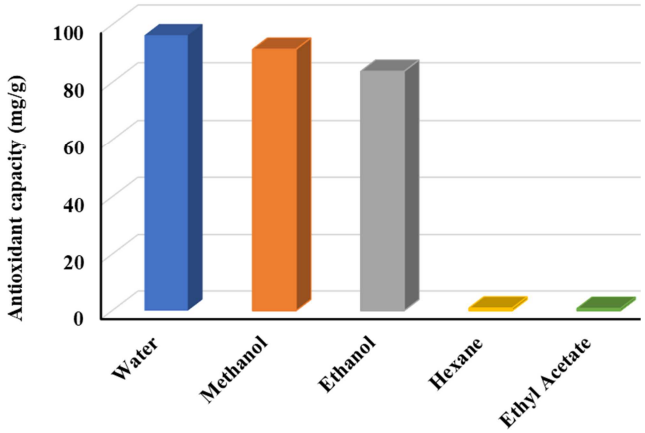

Figure 4. *Comparative histogram of the antioxidant power of the different extracts of Meremia tridentata leaves by the CUPRAC method.*

4. Conclusion

Merremia tridentata is a plant, which exists throughout the Senegalese territory. It is known for its therapeutic use. This is how a comparative study was carried out on the different extracts of *Merremia tridentata* through quantitative analyzes and by evaluating the antioxidant activities. This study revealed that the aqueous extract recorded the highest contents of polyphenols and flavonoids. Despite its high polyphenol content, the aqueous extract does not present the best IC50 (0.283 mg/mL) when DPPH· is used. The ethanolic and methanolic extract constitute the most effective extracts with IC50 values of 0.108 and 0.110 mg/mL. When the antioxidant activities are determined by the CUPRAC method, comparable values are observed for the aqueous, ethanolic and methanolic extracts. The mechanism of CUPRAC involves both polyphenolic compounds and other non-polyphenolic compounds. Considering the antioxidant activity of the leaf extracts of this plant, it is a need to conduct a detailed phytochemical study, to quantify and identify the compounds of interest.

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