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Research Article

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Secondary and Primary Metabolite Contents in the Branches of *Rhipsalis baccifera* (Cactaceae)

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Abstract The purpose of this article is to determine the primary and secondary metabolite content in the twigs of *Rhipsalis baccifera*. Four solvents were used to extract polyphenols including three solvent's for medium polarity 70/30 (v/v) methanol-water, 70/30 (v/v) acetone-water, 70/30 (v/v) ethanol-water and a very polar solvent (water). The results of the analysis show that the polyphenols contents in the methanolic, acetonic, ethanolic and aqueous extract are respectively: 76.66 ± 1.04 mg per 100 g of DM; 48.44 ± 0.65 mg per 100 g of MS; 34.42 ± 0.68 mg per 100 g of DM and 2.52 ± 0.31 mg per 100 g of DM, and their flavonoid contents in the same row of solvent are respectively: 1.31 ± 0.02 mg per 100 g of DM; 1.08 ± 0.01 mg per 100 g of DM; 0.94 ± 0.01 mg per 100 g of DM and 0.64 ± 0.05 mg per 100 g of DM. The polysaccharide extraction yield is 18.12 %. Methanol is the best polyphenols extraction solvent with very high polyphenols and flavonoid content. This part of the studied plant is rich in polysaccharides but low in polyphenols and flavonoids. And that of a plant rich in primary metabolite is low in secondary metabolite. This secondary metabolite can be concentrated in the other part of the plant.

Keywords twigs, crawling, tannin, epiphyte, succulent

1. Introduction

Rhipsalis baccifera is an epiphytic and succulent plant of the Cactaceae family. It is one of the very rare cactus species found in Africa, the eastern half of Madagascar, Sri Lanka, India and Nepal **[1]**. The stems are green, drooping, cylindrical, multi-branch, leafless and without thorns. They produce small star-shaped white flowers. Their fruits are like round berries which turn from green to white as they ripen.

In Madagascar, this plant is traditionally used to treat several diseases like toothache, broken bones, scorpion stings, stomach and abdominal pains, warts, impotence, snake bites, syphilis, and sexually transmitted disease, by mixing with many herbal teas. It is also used to treat skin irritation and hair loss. Other than the therapeutic use, it is also highly sought after as an ornamental plant.

The main objective of this paper is to determine the content of polyphenols and flavonoids in the methanolic, acetonic, ethanolic and aqueous solvent, and polysaccharide in the *Rhipsalis baccifera* branch. The obtained results make it possible to know the content of polyphenols and polysaccharides in this plant.

2. Materials and Methods

2.1. Plant material Collection

This study was carried out on the twigs (stems) of *Rhipsalis baccifera* harvested at Joffre Ville in northern Madagascar on May 27, 2015. The twigs were dried, according to the model of traditional healers, in the open

air, at room temperature for 2 weeks, then in the oven at 40 °C for five days and pulverized into a fine powder using a mixer of the type: Retsch SM 2000.

2.2. Extraction of polyphenols

In this study, four solvents were used to extract polyphenols from *Rhipsalis baccifera*. The mixtures of solvents used included methanol and water (methanolic) of proportion 70/30 (v/v), acetone and water (acetonic) of 70/30 (v/v), ethanol and water (ethanolic) of 70/30 (v/v) with polarity as intermediate and very polar water.

To extract the polyphenols from *Rhipsalis baccifera* stem, we used the maceration method at room temperature according to the protocol described by Romani *et al*. [2] by making some modifications on the amount of plant mash, the amount of solvent and the extraction time: a quantity of 15 g of ground material was macerated at room temperature for 5 hours in 120 ml of solvent (ethanolic, acetonic, methanolic), and 180 ml of water, after filtration on a tissue and the filtrates are centrifuged for 20 minutes at 4000 rpm at room temperature, then filtered on N°1 filter paper and stored at room temperature 4 °C until use.

2.3. Determination of extraction yield of polyphénols

The extraction yield of polyphenols was determined after evaporation of the solvent; it is expressed as a percentage relative to the initial mass of the plant subjected to extraction according to the formula (1) described by Falleh *et al*. [3].

$$R\ (\%) = 100 \times \frac{Mext}{Mech} \qquad (1)$$

With: R is the yield in %; M_{ext} is the mass of the extract after evaporation in g; M_{ech} is the mass of the vegetable dry matter in g.

2.4. Phytochemical screening

Phytochemical screening is a method used to detect previously the presence or the absence of the tested molecules in a plant part studied. These tests are generally based on precipitation and/or coloring reactions.

-Detection of flavonoids

The flavonoid detection reaction consists in treating 5 ml of the ethanolic extract with 1 ml of concentrated HCl and 30 mg of Zn powder. The presence of flavonoids is highlighted if a red or red-orange color with Zn develops after a few minutes [4-5].

-Detection of tannins

The presence of the tannins is demonstrated by adding 1 ml of the extract, 2 ml of water and a few drops of diluted $FeCl_3$ solution. The tannins are revealed by the appearance of a blue-black (Gallic tannin), blue-green (Catechic tannin) coloration [6].

-Detection of polysaccharides

To detect the presence of polysaccharides, 1 ml of aqueous extract is added in 3 ml of 96 % ethanol. The presence of precipitate on the aqueous extract indicates the existence of polysaccharides.

2.5. Determination of total polyphénols

The polyphenols produced by plants as secondary metabolites constitute a wide range of chemical molecules, whose chemical nature and content are extremely variable from one species to another. Several analytical methods can be used for the quantification of total polyphénols. Folin-Ciocalteu reagent analysis is the most widely used [7].

-Reagent preparation

-Phosphotungstic acid solution: 75 ml of distilled water, 8 ml of phosphoric acid and 10 g of sodium tungstate. The reaction mixture is refluxed for 3 hours. After cooling, the solution is supplemented to 100 ml with distilled water. This solution will be stored at 4 °C while waiting for use.

-Sodium carbonate solution decahydrate at 15 % in distilled water.

-Preparation of standard solutions

The stock solution of catechin was prepared at 200 mg/l in distilled water. The catechin is diluted in the extraction solvent (methanol, ethanol, acetone and water) to complete 500 µl of the solution, after stirring, 100 µl of the phosphotungstic acid solution ($H_3PW_{12}O_{40}$) and 4.4 ml of 15 % sodium carbonate solution were added to the assembly. After 5 minutes of vortexing, the absorbance was determined at 710 nm by a UV spectrophotometer (UV-1600PC).

The standard range is performed with a catechin solution of 0 to 500 mg/l.

-Preparation of the sample solution

The sample solution diluted ten times (10) in the extraction solvent (methanol (70 %), ethanol (70 %), acetone (70 %) and water) was added 100 µl of the solution of phosphotungstic acid and 4.4 ml of the 15 % sodium carbonate solution. After 5 min of vortexing, the absorbance was determined at 710 nm by a UV spectrophotometer (UV-1600PC).

-Results processing

From the equation of the straight line of the calibration curve and the absorbance obtained by the range of standards, the polyphenols concentration was calculated taking into account the dilution factor. The results are expressed in mg catechol equivalent per 100 g of dry matter.

2.6. Determination of total flavonoïdes

The determination of aluminum trichloride ($AlCl_3$) forms a very stable complex with the hydroxide (OH) groups of the phenols. This yellow complex absorbs visible light at a wavelength of 425 nm. Phenols are estimated by UV spectroscopy, of which quercetin is used as a standard at a wavelength $\lambda = 425$ nm [8].

-Reagent preparation

A solution of aluminum chloride ($AlCl_3$) is made at 2 % in methanol. The weighing is carried out with a paper mask; the solubilization is carried out under the hood.

-Preparation of the standard solution

Quercetin solution concentrations of 0 and 20 µg/ml were prepared.

-Sample preparation

For the samples, a 0.1 % solution in the solvent is made. The solution is then filtered on paper to obtain a solution of plant shredded material.

2.7. Thin layer chromatography of polyphénols

Thin layer chromatography was used to know and to verify the number of photochemical presented in the phytochemical screen. The result is to check if many of the tasks appear on the chromatographic plate.

-Preparation

The quantity 2 g of ground material was dissolved in 10 ml of methanol. The mixture was heated in the water bath at 65 °C for 10 minutes. Filtration was carried out after cooling. An amount of 2.5 mg of control (quercetin) was dissolved in 10 ml of methanol. The mobile phase is the mixture of formic acid, glacial acetic acid, water and ethyl acetate in the volume ratio 7.5V / 7.5V / 18V / 67V.

-Revelation of deposits

After the 8 cm run, the plate was dried in the open air and then heated in the oven at 100-105 °C for 3 min. The solution of 10 g/l aminoethanol diphenylborate in methanol was sprayed into the hot plate at the outlet of the oven. The drying of the plate was done under the cold air current. The well-dried plate was sprayed with a

solution of macrogol (400 g/l) in methanol. Examination of the plate with ultraviolet light 365 nm was carried out after drying in the open air.

2.8. Polysaccharide extraction

The dried stems of *Rhipsalis baccifera* were crushed and pretreated with absolute ethanol for 5 hours at room temperature and gentle stirring, refluxing for 3 hours and maceration at 4 °C for 48 hours; and filtered through a tissue to remove ethanol-soluble compounds such as polyphenols, single monosaccharides and amino acids and chlorophylls [9]. The residue of the pretreated crushed material was dried in the dark, ventilated in the open air and at room temperature for 48 hours [10].

The ground matter thus dried was macerated in 2 volumes of cold distilled water for 3 hours at room temperature and with gentle stirring and maceration at 4 °C, overnight. The mixture was filtered through a sieve of 100 µm porosity and on muslin fabric. The polysaccharides of the concentrate were precipitated to three absolute ethanol volumes and then macerated at 4 °C, for 1 hour. After filtration on a muslin tissue, the precipitates were dried in the dark and the crude extract of water-soluble polysaccharide is obtained.

2.9. Polysaccharide assay

-Determination of neutral oses

Most colorimetric methods are based on one of the following two properties of sugars: their reducing power or the formation in strong acids of furfural-type compounds that react easily to give colored drifts. The methods are classified into several types corresponding to the estimate of total sugars, hexoses or pentoses.

Under the action of concentrated mineral acids (sulfuric acid) when heated, the hexoses and pentoses of the medium undergo a strong internal dehydration, followed by a cyclization resulting in the formation of furfural and 5-hydroxymethylfurfural derivatives, reacting with resorcinol, or m-dihydroxybenzene. The formation of a yellow-brown complex makes it possible to follow the concentration of neutral sugars in the sample by reading the absorbance at 450 nm [11].

A quantity of 100 µl of the polysaccharide solution to be dosed at concentrations ranging from 0 to 1.4 mg/ml is introduced into glass tubes. The volume is completed at 1000 µl using distilled water. Two hundred micro liters (200 µl) of the resorcinol solution and 1 ml of 80 % sulfuric acid are added. The tubes are vortexes and then placed in a water bath at 90 °C for 30 minutes. After cooling, they are placed in the dark for 30 minutes, and then the absorbance of each solution is read at 450 nm (development of an orange color).

-Determination of acidic oses

The amount of uronic acid is determined by the colorimetric assay [12], modified by Filisetti-Cozzi & Carpita. [13].

In acidic medium, the polysaccharides are hydrolyzed to galacturonic acids and converted into furfuric derivatives; these derivatives are condensed with meta-hydroxydiphenyl, to form colored complexes whose absorbance at 520 nm is proportional to the amount of uronic acids. In a test tube, 1 ml of sodium tetraborate in concentrated sulfuric acid (0.95 g of sodium tetraborate in 2 ml of water and made up to 100 ml with concentrated sulfuric acid) is added to 200 µl of a solution of monosaccharides The mixture is brought to 100 °C in a water bath for 5 minutes. The reaction is stopped by immersing the tubes in an ice bath. 20 µl of mHDP reagent (0.15 g mHDP in 500 mg of NaOH and 100 ml of water) are added, the reaction medium gives a stable pink color for 30 minutes, after vortexing, the absorbance is then measured at 520 nm. A standard range is achieved with galacturonic acid solutions between 0 - 0.09 mg /ml. A range is made with a galacturonic acid stock solution.

3. Results

-Phytochemical screening of extracts

The phytochemical screening results of Rhipsalis baccifera are shown in Table 1

Table 1: Phytochemical screening results

Tests	Results
Flavonoid	+
Catechin tannin	+
Gallic tannin	-
Polysaccharide	+ + +

With: + + +: Strongly present; + +: Moderately present; +: Weakly present; - : Absent

The results of the phytochemical tests show that *Rhipsalis baccifera* contains traces of flavonoids and catechin tannin and that the polysaccharide is strongly present.

-Polyphenols extraction yields, total polyphenols and total flavonoid contents

The results of total polyphenols extraction yields, total polyphenols and total flavonoid contents are summarized in Table 2.

Table 2: Extraction yields of polyphenols, polyphenols and flavonoid contents

Measured parameters	Methanol extract	Acetonic extract	Ethanol extract	Aqueous extract
Extraction yield (%)	1.79 ± 0.25	1.68 ± 0.13	1.78 ± 0.41	9.47 ± 0.74
Polyphenols content (mg/100 g Cat eq of DM)	76.66 ± 1.04	48.44 ± 0.66	34.42 ± 0.68	2.52 ± 0.31
Flavonoids content (mg/100 g Quer eq of DM)	1.31 ± 0.02	1.08 ± 0.01	0.94 ± 0.01	0.64 ± 0.05

Cat eq: Catechin equivalent; DM: Dry matter; Quer eq: quercetin equivalent. Each result represents the mean ± standard deviation of 3 independent determinations (n = 3). The values obtained have a significant difference ($p < 0.05$).

-Thin layer chromatography of flavonoïdes

After the revelation on 365 nm wavelength ultraviolet light, the spots on the plate shown below are obtained.

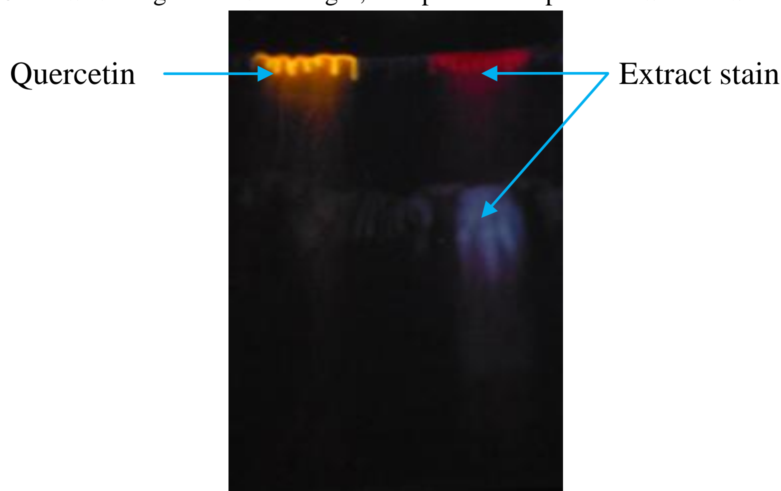

Figure 1: Thin layer chromatography

On the plate, we observe the appearance of two well separated spots of different colors (brick red and white). The appearance of two spots shows the existence of two types of polyphenols in the methanolic extract of *Rhipsalis baccifera.* This verifies the result of phytochemical screening in Table 1. The polyphenols extracted from *Rhipsalis baccifera* contain flavonoid and catechin tannin.

-Extraction yield of polysaccharide

The water-soluble polysaccharide extraction yield of *Rhipsalis baccifera* is equal to 18.12 %.

-Rate of neutral oses and acid oses

The neutral and acidic polysaccharide contents of *Rhipsalis baccifera* are summarized in Table 3.

Table 3: Content of neutral and acidic oses

Measured parameters	Content (% by mass)
Neutral oses	78.32 %
Acid oses	6.54 %

The polysaccharide of *Rhipsalis baccifera* contains more neutral oses than the acidic oses.

4. Discussion

The results of polyphenols extraction yield are similar to the work done by Souhila *et al*. [14] on the artichoke stem (*Cynara scolymus* L.) by the maceration extraction process. Water is the best extraction solvent on the artichoke stem followed by methanol (70 %). The aqueous extract obtained in the branches of *Rhipsalis Baccifera* is very viscous texture so it contains a huge amount of water-soluble polysaccharide. This explains the increase in this aqueous extraction yield.

The results of the *Rhipsalis baccifera* polyphenols assay are comparable with the results of the work done by Souhila *et al.* [14] for the artichoke (*Cynara scolymus* L.). The methanolic extract contains more total polyphenols (2659 ± 2.02 mg gallic acid equivalent per 100 g dry matter) and the aqueous extract contains very low polyphenols (801 ± 1.70 mg equivalent of gallic acid per 100 g of dry matter). For the artichoke (*Cynara scolymus* L.), after the methanolic extract, the ethanolic extract contains more polyphenols (2636 ± 0.81 mg of gallic acid equivalent per 100 g of dry matter) than the acetone extract (2522 ± 1.08 mg gallic acid equivalent per 100 g dry matter) therefore different from *Rhipsalis baccifera*. The difference can be explained by the method of extraction of polyphenols used, the control and the range of standard used and the varietal difference of the plants studied.

The results of the flavonoid assay are different from the work obtained by Bourgou *et al*. [15] of *Euphorbia helioscopia*. The ethanol extract (70 % ethanol) contains more flavonoid followed by the aqueous extract, the acetone extract (70 % acetone) and the methanolic extract (70 % methanol) is the lowest amount. The two plants are different origins, families and extraction methods which explain the differences in flavonoid content.

The water-soluble polysaccharide extraction yield of *Rhipsalis baccifera* is lower than the yield of the crude polysaccharide extract of *Cereus triangularis* (cactaceae) (24 %) [16] and greater than the yield of the crude extract of leaf polysaccharide of *Malva parviflora* L. (Malvaceae) (1.46 %) [17]. The difference can be explained by varietal difference, extraction method, location and harvest period and plant part studied.

The level of neutral polysaccharide oses of *Rhipsalis baccifera* is inferior to the results obtained by Petera *et al*. [16] *Cereus triangularis* polysaccharide (cactaceae) rate 85 % neutral oses. And that the rate of *Cereus triangularis* acid oses between 2-3.5 % [16] is lower than *Rhipsalis baccifera*. These differences can be explained by two plants of the same family but different genera.

5. Conclusion

In conclusion, the branch of *Rhipsalis baccifera* is rich in polysaccharide and low in polyphenols and flavonoid. Methanol is the best solvent for extracting polyphenols and flavonoid. The polysaccharide extracted from *Rhipsalis baccifera* branch contains more neutral oses than acidic oses. The obtained result can conclude that a part of plant rich in primary metabolite is low in secondary metabolite. This secondary metabolite is therefore concentrated in the other part of this plant.

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