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PHARMACOGNOSIE

Phytochemistry and Antioxydante Activity of *Stevia rebaudiana*

Étude phytochimique et évaluation de l'activité antioxydante de *Stevia rebaudiana*

S. Kharchouf · A. Bouchador · A. Drioiche · Z. Khiya · F. El Hilali · T. Zair

Abstract *Stevia rebaudiana* is a perennial shrub belonging to the Asteraceae family. Recently, it has been introduced to Morocco from its native Paraguay; its molecules have a sweetening power that is about 300 times sweeter than sucrose. This characteristic makes of this plant a considerable natural noncalorific sweetener in case of hypoglycemic or low carbohydrate diet. This important interest has prompted us to make further studies on this plant. The objective of this work is to show the value of *Stevia rebaudiana* grown in the Larache region of Morocco by the chemical characterization and evaluation of the antioxidant activity of its extracts. A phytochemical screening was carried out to highlight the qualitative composition of secondary metabolites. This analysis showed the presence of flavonoids, tannins, oses and holosides, sterols, triterpenes, and free anthraquinones. However, the absence of alkaloids and reducing compounds has been observed. In addition, extraction of the total polyphenols was carried out by maceration using a 70% methanol–water mixture. The yield is of the order of 28.6%. Subsequently, fractionation of the crude extract was carried out by successively using three organic solvents of different polarities: chloroform, ethyl acetate, and *n*-butanol. Polyphenol dosage with Folin-Ciocalteu's reagent showed that ethyl acetate fraction is richer in phenolic compounds (26.4%) than the other fractions. Flavonoids dosage with aluminum trichloride showed the richness of this plant in these compounds. The antioxidant activity of different fractions was evaluated by 2,2-diphenyl-1-picrylhydrazyl-free radical scavenging method and ferric reducing/antioxidant power method; the values of the 50% inhibitory concentrations (IC_{50}) were determined graphically. It is equal to 0.32 mg/ml for the ethyl acetate fraction, compared to 0.08 mg/ml for the ascorbic acid used as a reference. In this study, we have shown that *Stevia rebaudiana* is very rich in phenolic compounds and possesses a very important antioxidant power.

Keywords *Stevia rebaudiana* · Phytochemical screening · Polyphenols · Antioxidant activity · DPPH · FRAP

Résumé *Stevia rebaudiana* est un arbrisseau appartenant à la famille des Astéracées. Récemment, elle a été introduite au Maroc à partir de son pays originaire le Paraguay, ses molécules possèdent un pouvoir sucrant qui est environ 300 fois plus sucré que le saccharose. Cette caractéristique fait de cette plante un édulcorant naturel non calorique en cas de régime à teneur réduite en glucides. Cet intérêt important nous a incités à faire d'autres études sur cette plante. L'objectif du présent travail est la valorisation de *Stevia rebaudiana* cultivée dans la région de Larache au Maroc par la caractérisation chimique et l'évaluation de l'activité antioxydante de ses extraits. Un criblage phytochimique a été réalisé pour mettre en évidence la composition qualitative en métabolites secondaires. Cette analyse a montré la présence des flavonoïdes, des tanins, des oses et holosides, des stérols, des triterpènes et des anthraquinones libres. Mais il a été observé l'absence des alcaloïdes et des composés réducteurs. De plus, l'extraction des polyphénols totaux a été effectuée par macération en utilisant un mélange (méthanol–eau) à 70 %. Le rendement est de l'ordre de 28,6 %. Ensuite, le fractionnement de l'extrait brut a été réalisé en utilisant successivement trois solvants organiques de polarités différentes : le chloroforme, l'acétate d'éthyle et le *n*-butanol. Le dosage des polyphénols par la méthode de Folin-Ciocalteu a montré que la fraction d'acétate d'éthyle est la plus riche en composés phénoliques (26,4 %). Le dosage des flavonoïdes par le trichlorure d'aluminium a montré la richesse de cette plante en ces composés. L'activité antioxydante des différentes fractions a été évaluée par la

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méthode de piégeage du radical libre DPPH$^{\bullet}$ (2,2-diphenyl-1-picrylhydrazyl) et la méthode de FRAP, les valeurs des concentrations inhibitrices à 50 % (CI_{50}) ont été déterminées graphiquement. Elle est égale à 0,320 mg/ml pour la fraction d'acétate d'éthyle, contre 0,41 mg/ml pour l'acide ascorbique utilisé comme référence. Dans cette étude, nous avons montré que *Stevia rebaudiana* est très riche en composés phénoliques et possède un pouvoir antioxydant très important.

Mots clés *Stevia rebaudiana* · Criblage phytochimique · Polyphénols · Activité antioxydante · DPPH · FRAP

Introduction

Stevia is a plant of the family Asteraceae, which is a very large family containing about 10% of the total number of flowering plants. It includes about 950 genera and more than 20,000 species. The origin of the name "*Stevia*" goes back to the 16th Century by the Spanish botanist Pedro Jaime Esteve. In everyday language, the plant is commonly referred to as "*Stevia*", "water hemp," or "sugar plant". Subsequently, this plant became the genus name for a group of plants possessing a natural sweetening power.

Stevia has a number of advantages over other sweeteners. Indeed, the extract of *Stevia rebaudiana* increased glucose tolerance [1,2]. The plant enjoys a dual positive effect by acting as an antihyperglycemic and it contains blood pressure-lowering substances; it has effects that may have therapeutic potential in the treatment of type 2 diabetes and the metabolic syndrome [3–5].

Most data from the literature describe steviol glycosides as the main source of beneficial properties of *Stevia*. In addition to steviol glycosides, *S. rebaudiana* leaves also contain a number of other natural ingredients with potentially significant biological activity [6–8].

The objective of this study consists, initially, of a phytochemical screening of *S. rebaudiana*. Subsequently, its content of phenolic compounds is determined and its antioxidant activity is evaluated by two methods: the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method and the ferric reducing/antioxidant power (FRAP) method.

Material and Methods

Preparation of Vegetable Materials

The aerial part of *S. rebaudiana* was harvested in Larache region of Morocco in 2015 at the time of flowering. Afterward, the vegetable material was dried in the open air, in the shade and at room temperature later and then stored for various uses. The identification of the plant species was carried out at the Rabat Scientific Institute in the floristics laboratory.

Phytochemical Study

Phytochemical Screening

This study consists of detecting the different families of compounds existing in the plant by qualitative characterization reactions. These reactions were based on precipitation or staining reactions by reagents specific to each family of compounds.

Detection of alkaloids was carried out by the precipitation of salts and revelation with the Mayer and Dragendorff reagent. The characterization of catechic tannins was realized by isoamyl alcohol and hydrochloric acid .The gallic tannins were detected by adding the Stiasny reagent, sodium acetate, and ferric chloride. Cyanidin reaction allowed the detection of free flavonoids. For the detection of sterols and triterpenes, acetic anhydride and concentrated sulfuric acid were used. Diluted hydrochloric alcohol, magnesium chips, and isoamyl alcohol were used to identify flavonoids. Chloroform, diluted ammonia, and hydrochloric acid allowed the detection of quinone substances. The characterization of mucilage was reassured by the addition of absolute ethanol to the aqueous decoction. The tetrahydrocannabinols is detected by adding KOH at 5% in alcohol. Oses and holosides were highlighted by means of concentrated sulfuric acid and a saturated solution of thymol in ethanol. Finally, saponins were characterized by their foaming power in aqueous solution by measuring the index of foam.

Extraction of Polyphenols

30 g of the plant powder was cold macerated in 300 ml of a methalonic solution (70%) for 48 hours. Extraction was performed three times. The filtrates were combined and the solvent was evaporated to dryness under reduced pressure at 50 °C by means of a rotary evaporator.

Fractioning

The hydromethanolic extract was successively treated with three organic solvents of different polarities: chloroform, ethyl acetate, and *n*-butanol. Four fractions were recovered: the hydrometanol fraction (F_0), the ethyl acetate fraction (F_1), the butanol fraction (F_2), and the aqueous fraction (F_3). The various extracts were stored at 4 °C until their use.

Determination of Total Phenols

The determination of total polyphenols by the Folin–Ciocalteu reagent has been described since 1965 [9]. The reagent

consists of a mixture of phosphotungstic acid ($H_3PW_{12}O_{40}$) and phosphomolybdic acid ($H_3PMo_{12}O_{40}$). It is reduced, during the oxidation of the phenols, to a mixture of blue oxides of tungsten and molybdenum [10].

100 µl of each extract (the extracts of each fraction (F_0, F_1, F_2, F_3) is introduced into a 100 ml volumetric flask and then 1.5 ml of the Folin–Ciocalteu reagent diluted 10 times and 1.5 ml of 7.5% sodium carbonate were added. The flasks were stirred and supplemented with distilled water and then stored for 30 min at ambient temperature. The absorbance was measured at 765 nm against a blank using a spectrophotometer. A calibration curve was carried out in parallel under the same operating conditions using gallic acid as a positive control. The results obtained were expressed in milligrams (mg) equivalent of gallic acid per gram of the dry plant material (mg GAE/g).

Determination of Flavonoids

Quantification of flavonoids was performed with aluminum trichloride [11]. The latter forms with the flavonoids a yellow complex, which absorbs in the visible at 433 nm.

100 µl of each extract was mixed with 100 µl of aluminum trichloride ($AlCl_3$) at 10% (w/v), followed by 20 µl of distilled water, and the mixture is supplemented to 50 ml with pure methanol. After 30 min of incubation at room temperature, the absorbance was determined at 433 nm against a blank. A calibration curve is carried out in parallel under the same operating conditions using quercetin as a positive control. The flavonoid content of the different fractions was expressed in milligrams equivalent of quercetin per gram of the dry plant material (mg EQ/g).

Evaluation of Antioxidant Activity

• Free Radical-Scavenging Activity

The method applied to measure the antioxidant activity was the free radical scavenging by using $DPPH^{\bullet}$.

$DPPH^{\bullet}$ is a purplish stable free radical, which absorbs at 515–518 nm [12,13]. In the presence of antiradical compound, the $DPPH^{\bullet}$ radical is reduced and changes color by turning yellow. The absorbances measured at 517 nm are used to calculate the percentage inhibition of the $DPPH^{\bullet}$ radical, which is proportional to the antiradical power of the sample [14].

This method is based on measuring the ability of antioxidants to scavenge the radical DPPH.

200 µl of each ethanolic solution of the extracts of each fraction at different concentrations (0.08, 0.16, 0.32, 0.48, 0.64, 0.80, 0.96, 1.12, 1.28, 1.44 mg/ml) was added to 2.8 ml of the ethanolic solution of DPPH (0.024 g/l). At the same time, a negative control was prepared by mixing 200 µl of the ethanol with 2.8 ml of the ethanolic solution of DPPH. The reading of the absorbance was made against a white at 515 nm after 30 min incubation in the dark and at ambient temperature. The positive control was represented by a solution of a standard antioxidant ascorbic acid whose absorbance was measured under the same conditions as the samples and for each concentration. The test was repeated three times. The ability of extract to scavenge DPPH free radical was calculated using the following equation:

Scavenging activity% = [(Abs control − Abs test) / Abs control] × 100

With

Abs control: absorbance of control

Abs test: absorbance of the test performed

IC_{50} or inhibiting concentration 50% is the concentration of the test sample required to reduce 50% of radical $DPPH^{\bullet}$.

IC_{50} is graphically calculated by linear regressions.

• Ferric Reducing/Antioxidant Power Assay

The reducing power of iron (Fe^{3+}) in the extracts is determined according to the method described by Oyaizu [15].

A volume of each extract at different concentrations was mixed with 2.5 ml of a 0.2 M phosphate buffer solution (pH = 6.6) and 2.5 ml of a solution of potassium ferricyanide $K_3Fe(CN)_6$ at 1%. The whole was incubated in a water bath at 50 °C for 20 min. Then 2.5 ml of 10% trichloroacetic acid was added to stop the reaction and the tubes are centrifuged at 3000 rpm for 10 min. 2.5 ml of supernatant was combined with 2.5 ml of distilled water and 0.5 ml of a 0.1% aqueous solution of $FeCl_3$.

The absorbance of the reaction medium was read at 700 nm against a similarly prepared white, replacing the extract with distilled water. The positive control is represented by a solution of a standard antioxidant ascorbic acid, the absorbance of which was measured under the same conditions as the samples.

An increase in absorbance corresponds to an increase in the reducing power of the extracts tested [9].

Results and Discussion

Phytochemical Screening

The experimental results of the phytochemical tests carried out on the powder of the plant mentioned in Table 1 show the presence of tannins, flavonoids, oses, and holosids, sterols and triterpènes, and combined anthracenics. On the other hand, Table 1 displays a complete absence of alkaloids, saponosides, coumarins, carotenoids, free anthracenics, and tetrahydrocannabinols.

Table 1 Results of phytochemical screening of *Stevia* extracts

Chemical group	Reagents/Reaction	Results
Alkaloids	Valse–Mayer reagent Dragendorff reagent	−
Gallic tannins	Reagent Stiasny	++
Catechic tannins	HCl	+
Flavonoids	Cyanidin with Mg	++
Saponosids	Foam Index (FI)	−
Coumarins	Fluorescence reaction	−
Carotenoids	Color reaction	−
Sterols and triterpenes	Libermann-Burchard reaction	++
Free anthracenics	Borntrger reaction	−
Combined anthracenics	Color reaction	++
Oses and holosids	Color reaction	++
Mucilage	Precipitation reaction	+
Tetrahydrocannabinols	Color reaction	−

(−): negative test; (+): slightly present; (++): strongly present

Phenol Total and Flavonoid Content

The determination of the total phenol and flavonoid contents in the different fractions was made using separately the colorimetric methods (Folin–Ciocalteux and aluminum trichloride ($AlCl_3$)). The total phenol content estimated by the Folin–Ciocalteux method for each extract was reported in mg equivalent gallic acid/g of the dry plant material (Figs 1, 2).

The results showed that the extract of the acetate-ethyl fraction has a high total phenol content (26.4 ± 0.038 mg GAE/g) relative to the other fractions (Table 2). The flavonoid content determined by the aluminum trichloride method for each extract was reported in milligrams equivalent of quercetin/g of the dry plant material (mg EQ/g). The results also indicated that the ethyl acetate fraction has high flavonoid content (38.8 ± 0.098 mg GAE/g).

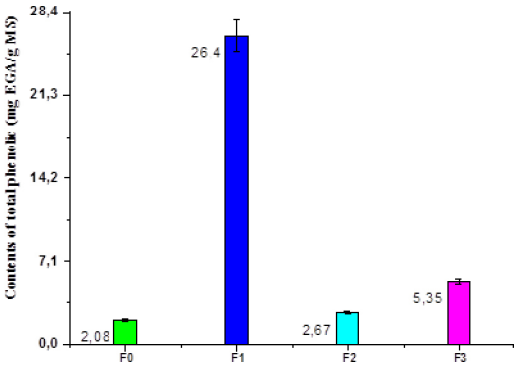

Fig. 1 Total phenol contents of the extracts of the various fractions

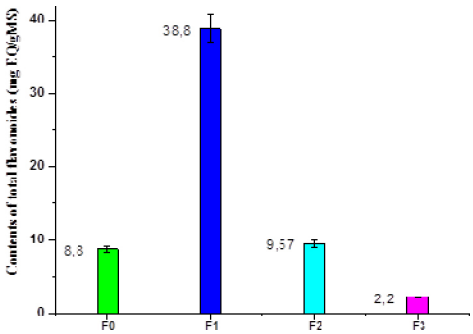

Fig. 2 Flavonoid contents of the extracts of the various fractions

A study was carried out on ethanolic extracts from the leaves of three varieties of *S. rebaudiana*, which showed that the highest content of polyphenols was 125.33 ± 2.52 mg GAE/g extract. And the highest content of flavonoids was 59.70 ± 2.41 mg EQ/g of extract [16]. Other similar studies have been carried out using other extraction solvents [17,18].

Antioxidant Activity

Free Radical-Scavenging Activity

The antioxidant activity of the various fractions of *S. rebaudiana* and of the standard antioxidant (ascorbic acid) with respect to the $DPPH^{\bullet}$ was evaluated using a spectrophotometer following the reduction of this radical, which is accompanied by its passage from the violet color ($DPPH^{\bullet}$) to the yellow color (DPPH-H) measurable at 515 nm. This reduction in capacity is determined by a decrease in absorbance induced by antiradical substances [19].

The results of the antioxidant power of the extracts from different fractions studied are shown in Fig. 3.

The IC_{50} values determined in mg/ml, expressing the effective concentration of the various antioxidant extracts of *S. rebaudiana* required for trapping and reducing 50% moles of $DPPH^{\bullet}$ dissolved in ethanol, are summarized in Table 3.

The results obtained showed that the extracts of *S. rebaudiana* present a very important antioxidant power that is superior to that obtained by ascorbic acid for the crude extract and the ethyl acetate fraction. This justified the importance of consuming *Stevia* as an antioxidant in addition to its use as sugar that is calorie free.

We also noted that from a concentration greater than 1.0 mg/ml, the antioxidant power becomes constant for the various extracts as well as for the ascorbic acid. These results are extremely important and require studies of the in vivo antioxidant activity in order to exploit it in the agri-food sector.

Table 2 Yield (%) and total phenol and flavonoid contents of the extracts of the various fractions				
	Crude extract (F_0)	**Ethyl acetate fraction (F_1)**	**Butanol fraction (F_2)**	**Aqueous fraction (F_3)**
Extraction yield (%)	28.60	1.02	13.90	7.06
Total phenol content (mg EAG/g)	2.08 ± 0.01	26.40 ± 0.04	2.67 ± 0.03	5.35 ± 0.01
Flavonoid content (mg EQ/g)	8.80 ± 0.07	38.80 ± 0.09	9.57 ± 0.14	2.20 ± 0.03
The values are the average of three repetitions ± standard deviation				

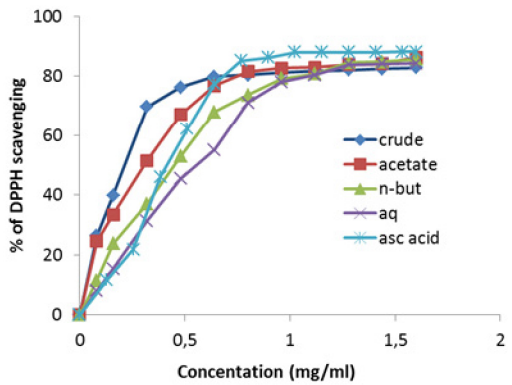

Fig. 3 Percentages of inhibition of DPPH• as a function of the concentrations of the fractions

The results obtained in this study clearly indicate that the leaves of *S. rebaudiana* from Larache have a significant potential to be used as a natural antioxidant. These results are similar to those of other researchers [20,21].

Frap Iron Reduction Test

It is a method of measuring the plasma's ability to reduce ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}) [22]. Therefore, Fe^{2+} can be evaluated by measuring and monitoring the increase in the blue color density in the reaction medium at 700 nm [23,24]. The reducing power of the various fractions studied is probably due to the presence of the hydroxyl group in the phenolic compounds, which can serve as an electron donor. Therefore, antioxidants are considered reductants and inactivators of oxidants [25].

Some previous studies have also shown that the reducing power of a compound can serve as a significant indicator of its potential antioxidant activity [26,27].

In order to compare the antioxidant activity of the extracts of the four fractions obtained from the plant by this method, we calculated EC_{50}, which is defined as the concentration necessary to reduce 50% of the iron. The results obtained are illustrated in Table 4. We noted that the capacity to reduce iron is variable between the different fractions studied, it is much higher in the crude extract (EC_{50} = 0.47 ± 0.028 mg/ml) followed by the aqueous fraction and ethyl acetate, with an EC_{50} = 0.605 ± 0.007 mg/ml and EC_{50} = 0.609 ± 0.013 mg/ml, respectively). Therefore, we can deduce that all fractions have the capacity to reduce iron but are less than that of ascorbic acid (EC_{50} = 0.07 ± 0.025 mg/ml).

Figure 4 also shows that although ascorbic acid has a capacity to reduce iron higher than those of the various extracts, from a certain concentration (> 2 mg/ml) the three extracts (crude, butanolic, and aqueous) are more antioxidant than ascorbic acid and these results can be explained by the fact that our extracts contain a mixture of molecules

Table 3 Reduction Concentrations of 50% of DPPH•					
	Crude extract (F_0)	**Ethyl acetate fraction (F_1)**	**Butanol fraction (F_2)**	**Aqueous fraction (F_3)**	**Ascorbic acid**
IC_{50} ± type **Ecart**	0.21 ± 0.01	0.32 ± 0.06	0.45 ± 0.02	0.550 ± 0.002	0.41 ± 0.01

Table 4 Antioxidant test result expressing the effective concentration 50% in mg/ml					
	Crude extract (F_0)	**Ethyl acetate fraction (F_1)**	**Butanol fraction (F_2)**	**Aqueous fraction (F_3)**	**Ascorbic acid**
EC50 ± type **Ecart**	0.470 ± 0.028	0.609 ± 0.013	0.69 ± 0.014	0.605 ± 0.007	0.070 ± 0.025

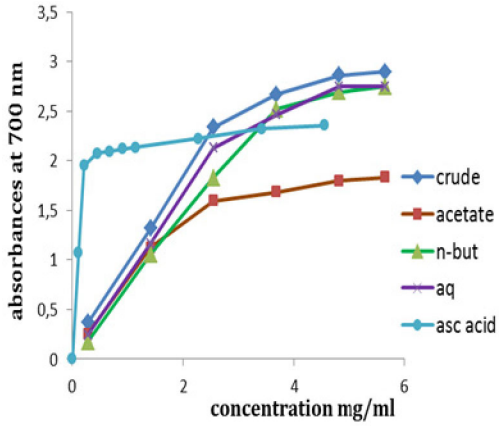

Fig. 4 Reductive power of the extracts of *S. rebaudiana* and of the ascorbic acid

in a small amount having an appreciable antioxidant power (even higher than that of ascorbic acid), which requires a certain amount concentration to reduce iron.

Other studies have shown that the conditions of cultivation of *S. rebaudiana* have a great influence on the chemical composition of the plant as well as the conservation conditions that can influence certain bioactive compounds present in this plant [28,29].

Conclusion

The present study concluded that *S. rebaudiana* is rich in secondary metabolites, particularly polyphenols and flavonoids.

The study of the antioxidant activity of the extracts resulting from the species *S. rebaudiana* according to the method of the reduction of the iron and that of the trapping of the free radical DPPH$^{\bullet}$ showed that the various extracts are endowed with an antioxidant activity.

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