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ORIGINAL PAPER

Chemical composition, antioxidant, anticholinesterase, and alpha-glucosidase activity of *Stevia rebaudiana Bertoni* extracts cultivated in Algeria

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Abstract

Stevia rebaudiana Bertoni is an endemic species to Paraguay famous for its sweetening power and therapeutic potential for various diseases such as diabetes. The present work evaluates the chemical composition and antioxidant, anticholinesterase, and α-glucosidase activities of *S. rebaudiana*. The essential oil (EO) of dry Stevia leaves was analyzed by GC/MS and detected the presence of 33 components. Caryophyllene oxide (24.28%), spathulenol (12.31%) and nerolidol (11.8%), and manool oxide (7.36%) were identified as the major ones. The antioxidant activity was evaluated by four complementary methods: DPPH (2,2 diphenylpicrylhydrazyl, ABTS (2, 2'-azino-bis 3-ethylbenzthiazoline-6-sulfonic acid) free radicals scavenging, Cupric reducing antioxidant capacity (CUPRAC), and reducing power. The crude methanolic extract and its fractions showed a variable antioxidant activity and strongly correlated with the content of quantified bioactive compounds. The ethyl acetate fraction showed a very high antioxidant activity close to the tested standards, while EO was active only in the CUPRAC assy. The petrol ether and chloroform fractions showed the best butyrylcholinesterase (BChE) inhibitory activity with IC_{50} values: 123.7 ± 1.78 and 170.1 ± 0.78 µg/mL, respectively. On the other hand, EO and chloroform revealed a moderate inhibitory activity against acetylcholinesterase (AChE). The in vitro inhibitory effect of the extracts on α-glucosidase indicated that EO effectively inhibited the enzyme with an IC_{50}: 74.9 ± 6.4 µg/mL, better than the standard acarbose. The EO of *Stevia* has a significant anti-diabetic potential.

Keywords *Stevia rebaudiana* · GC/MS analysis · Essential oil · Phenolic compounds · Antioxidant activity · Enzyme inhibition

Introduction

An imbalance in the production of antioxidants by cells in the human body leads to the overproduction of free radicals, which are responsible for many diseases. Oxidative stress causes an excess of reactive oxygen species that can damage biological molecules (such as enzymes, lipids, proteins, and DNA), which can directly impact neurodegenerative diseases such as Alzheimer's disease and metabolic disorders such as diabetes [1]. The preferred therapeutic approach for controlling Alzheimer's disease is based on inhibiting acetylcholinesterase, which cleaves acetylcholine, increasing its level in the brain and improving neurotransmission [2]. The two major forms of cholinesterases are AChE and BChE, BChE or pseudocholinesterase catalyzes the hydrolysis of different types of choline and non-choline esters while acetylcholinesterase has specificity to acetylcholine and is known as the specific cholinesterase [3]. As for treating

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diabetes is based on inhibiting carbohydrate hydrolyzing enzymes, α-amylase, and α-glucosidase [4].

Synthetic drugs used in treating Alzheimer's or even diabetes, such as galanthamine, are no longer safe and may cause several adverse side effects, including diarrhea, nausea, vomiting, and abdominal disorders [5]. Therefore, much attention has been paid to the search for new bioactive molecules of natural origin. Extracts of aromatic and medicinal plants have been widely used in traditional medicine for curative purposes [6]. Natural compounds from various medicinal and aromatic plants have essential applications in the cosmetic, food, and pharmaceutical industries [7]. Therefore, scientists are increasingly interested in natural, effective, safe antimicrobials with antioxidant activity. These plants contain many chemical constituents with high antioxidant potential, including alkaloids, flavonoids, tannins, and phenolic compounds, which may be responsible for their preventive effects in various degenerative diseases, such as cancer, neurological and cardiovascular diseases [8, 9]

Stevia rebaudiana Bertoni (Fig. 1), popularly known as sweet herb, honey leaf, and candy leaf [10], is a plant that grows up to 1 m high and endowed with sessile leaves, 3–4 cm long, elongated or spatulate in shape with a blunt-tipped blade. It belongs to the family Asteraceae, native to South America, especially Paraguay and Brazil, but nowadays also cultivated in other parts of the world such as Canada, Asia, and Europe [11]. There are about 154 species of the *stevia* genus, and it is one of only two species that produce sweet glycosides; it is due to the presence of two natural sweeteners, stevioside and rebaudioside A, which are about 250 to 300 times sweeter than regular sucrose [12]. The increasing consumption of foods and beverages containing artificial sweeteners has led to severe health problems. It makes *stevia* a non-caloric substitute for sugar for blood sugar control in diabetes mellitus, obesity, hypertension, and inflammation [13]. In addition to its sweetening properties, the dry extract of *Stevia* leaves contains higher levels of vitamins, proteins, minerals, fatty acids, and secondary metabolites, including flavonoids, alkaloids, chlorophylls, xanthophylls, hydroxycinnamic acids (caffeic acid, chlorogenic acid, etc.), and essential oil (Eo), providing antioxidant, antitumor, hepatoprotective, immunomodulatory, anticariogenic, anticaries, and significant antimicrobial activity [14, 15].

Fig. 1 Leaves of *Stevia rebaudiana* Bertoni

A few authors have studied the chemical composition of *S. rebaudiana* EO [16–19]. This research aims to provide a complete and updated overview of the chemical profile (by GC/MS analysis) of the essential oil of the dried leaves of *S. rebaudiana* grown in Algeria, to quantify the phenolic compounds of the different extracts and evaluate in vitro their antioxidant properties by four complementary methods, by comparing them to reference antioxidants. In addition, we explored their ability to inhibit key enzymes associated with diabetes (α-glucosidase) and neurodegenerative diseases (acetylcholinesterase). This research should lead to a better understanding of the health benefits of this plant and its potential pharmacological and agricultural applications. Finally, it is essential to mention that this is the first study on the chemical composition and biological activity of the essential oil and extracts of *stevia* grown in Algeria.

Material and methods

Plant material

Stevia rebaudiana leaves were collected in July 2018 in Bordj el Kiffan commune (east of Algiers province, Algeria: 36° 45′ 00" N, 3° 11′ 00 E). The leaves were then separated and dried for ten days, at room temperature, in the dark, and stored in closed bags until use.

Isolation of the essential oils

The dried leaf powder (100 g) was prepared and hydrodistilled according to the method previously reported by Muanda et al. [20] in a Clevenger apparatus for 5 h. The obtained oil was then dried with anhydrous sodium sulfate (Na_2SO_4) to remove traces of water and stored in sealed glass vials at 4 °C until analysis.

The yield of essential oil was determined according to the following formula:

$$Essential\ oil\ yield\ (\%) = \left(P_h/P_p\right) \times 100$$

where P_h represents the net weight of the extracted essential oil in g and P_P represents the total weight of the treated plant in g.

Preparation of the methanol extract and fractionation

30 g of *Stevia* leaves were extracted according to the method described by Gali et al. [2] by hydroalcoholic maceration in 500 mL of methanol–water (80:20, v/v) in the dark with shaking for 24 h. After filtration, the methanol was evaporated at 40 ℃ in a rotary evaporator (BUCHI, R215). The extraction was repeated three times in succession. Part of the obtained crude residue was suspended in water and subjected to liquid/liquid extraction with solvents of different polarity: petrol ether (100 mL), chloroform (100 mL × 3), ethyl acetate (100 mL × 3), and butanol (100 mL × 3). The solvents were removed by rotary steam, and the extracts obtained extracts were stored at 4 °C until use.

GC–MS analysis

GC–MS analyzes were performed using an Agilent 7890B series GC system (Agilent, Santa Clara, CA, USA) equipped with a split-splitless injector and coupled to an Agilent MSD 5977B detector. 1 µL of a 0.01% hexane oil solution was injected. The analysis conditions were as follows: Injection mode: splitless at 300 °C; HP -5MS capillary column (Agilent, Santa Clara, CA, USA) (30 m × 0.25 mm, df = 0.25 µm); Temperature program: from 50 °C (1 min) to 300 °C (5 min) at a rate of 5 °C/min. The carrier gas was helium with a flow rate of 1.2 mL/min. Mass spectra were recorded in 70-eV electron ionization mode. The source and quadrupole temperatures were set at 230 °C and 150 °C, respectively.

The analysis was repeated three times. The components were identified by chromatographic retention indices (RI) and by comparing the recorded spectra with the calculated database (Pal 600 K ® libraries). RI values were calculated for all components according to Babushok et al. [21], by injecting a mixture of n-alkane homologous (C7-C30) under the same chromatographic conditions.

Determination of total phenolic, flavonoids, and flavonols content

The total phenolic scontent of the crude methanolic extract and its fractions were determined by the Folin-Ciocalteu method [22]. In a 96-well microplate, 20 µL of each sample was added to 100 µL of diluted Folin– Ciocalteu reagent (RCF) (1.10) and 75 µL of sodium carbonate (7.5%). After 2 h incubation in the dark at room temperature, absorbance was measured at 765 nm in a microplate reader. Phenolics content was estimated as micrograms of gallic acid equivalents per milligram of extract (µg GAE /mg).

The flavonoids content was determined by the method described by Topçu et al. [23], 50 µl of each extract/fraction was mixed with 130 µL methanol, 10 µL potassium acetate (1 M), and 10 µL aluminum nitrate (10%). After incubation for 40 min, the absorbance was measured at 415 nm in a microplate reader. Total flavonoids content was expressed as micrograms of quercetin equivalents per milligram of extract (µg QE/mg).

The flavonols content was performed as described by Kumaran et al. [24]. Briefly, 50 µl of each plant extract/fraction was mixed with 50 µL of aluminum trichloride and 150 µL of sodium acetate. After incubation for 2.5 h, the absorbance was measured at 440 nm in a microplate reader. Flavonols' content was expressed as micrograms of quercetin equivalents per milligram of extract (µg QE/mg).

Antioxidant activity

Free radical scavenging activity assay

DPPH ((2, 2-diphenyl-1-Picrylhydrazyl) radical scavenging assay was evaluated by the method of Blois [25] using a 96-well microplate. 160 µL of 1 mM L-1 DPPH solution in methanol was added to 40 µL of each extract/fraction at different concentrations (800-400-200-100-50-25–12.5 µg/mL). After 30 min of incubation, absorbance was measured at 517 nm in a microplate reader. The percentages of inhibition were calculated using the following formula:

$$\%\ Inhibition\ =\ ((Ab - Aa)/Ab)\ \times\ 100 \quad (1)$$

Ab = absorbance of the blank sample.

Aa = absorbance of the test sample.

The results were compared with the standard antioxidant BHA BHT, α-tocopherol.

ABTS scavenging activity

ABTS (2, 2'-azino-bis 3-ethylbenzthiazoline-6- sulfonic acid) radical scavenging activity was determined according to the method described by Pellegrini et al. [26]. ABTS was prepared by reacting 2 mM ABTS in water with 2.45 mM potassium persulfate ($K_2S_2O_8$) and stored in the dark at room temperature for 16 h. The solution was then diluted with distilled water to obtain an absorbance of 0.700 ± 0.025 at 734 nm. 160 µL of the ABTS solution was added to 40 µL of each extract/fraction. After 10 min of incubation, the absorbance was measured at 734 nm using a 96-well microplate reader. The percentage of inhibition was calculated according to formula (1).

Reducing power

Reducing power was determined according to the method described by Oyaizu [27]**.** 10 µL of each extract was added

to a 96-well microplate with 40 µL of phosphate buffer (Ph 6.6) and 50 µL of potassium ferricyanide solution (1%). After incubation at 50 °C for 20 min, 50 µL trichloroacetic acid (10%), 40 µL distilled water, and 20 µL $FeCl_3$ (0.1%) were then added to the mixture. The absorbance was measured at 700 nm in a microplate reader.

The results were compared to the antioxidants standards BHA, BHT, α-tocopherol, ascorbic acid, and tannic acid. The results were expressed as $A_{0.50}$.

Cupric reducing antioxidant capacity (CUPRAC)

CUPRAC was determined by the method described by Apak et al. [28]. 40 µL of each extract/fraction was mixed with 60 µL of ammonium acetate solution ($AcNH_4$), 50 µL of the neocuproine solution, and also 50 µL of ($CuCL_2$, $2H_2O$) solution in a 96 well plate. The mixture was incubated for 1 h, and then the absorbance was measured at 450 nm after 1 h of incubation. BHA, BHT, α-tocopherol, ascorbic acid, and tannic acid were used as antioxidant standards to compare the activity. The results were expressed as $A_{0.50}$, which is the concentration that produces 0.500 absorbances.

Anticholinesterase activity

The inhibitory activities of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) were evaluated by the method reported by Ellman et al. [29]. Briefly, a volume of 150 µL of sodium phosphate buffer (0.1 M, pH 8.0), 10 µL of the sample at various concentrations in methanol, and 20 µL of AChE (5.32 × 10–3 U) or BChE (6.85 × 10–3 U) solution were mixed in a 96-well microplate and incubated for 15 min at 25 °C. Then, 10 µL of 5,5′-Dithiobis (2-nitrobenzoic acid) (DTNB) (0.5 mM), 10 µL of acetylthiocholine iodide (0.71 mM), or 10 µL of butyrylthiocholine chloride (0.2 mM) were added. The absorbance was measured at 412 nm using a microplate reader. Galanthamine was used as a reference compound. The results were given as 50% inhibition concentration (IC_{50}).

The percent inhibition I (%) was determined using the following formula:

$$I(\%) = (E - S)/E \times 100$$

where E is the activity of the enzyme without the test sample, and S is the activity of the enzyme in the presence of the test sample.

α Glucosidase inhibitory activity

The α-glucosidase inhibitory assay was determined by the method described by Lordan et al. [30]. In a 96-well microplate, 50 µL of each sample was mixed with 100 µL of the enzyme solution (100 mM, pH 6.9) and 50 µL of the substrate solution (5 mM). The mixture was incubated at 37 °C for 10 min, and the absorbance was measured at 405 nm for 0 min and 30 min using a microplate reader. Acarbose was used as a positive control.

The percentage inhibition I (%) was determined using the following formula:

$$\%\ Inhibition = Absorbance\ of\ extract/Absorbance\ of\ control \times 100$$

Statistical analysis

In the present study, all tests were performed in triplicate, and the results were subjected to one-way analysis of variance (ANOVA), followed by Tukey's test using Statistica 5.0 software. The significance level was ($p < 0.05$).

Results and discussion

Essential oil yield and chemical composition

The yield of essential oil from dried leaves of *S. rebauadiana* obtained by hydrodistillation was 0.058% (w/w, dry weight). The oil obtained was light yellow with a specific odor. In comparison to literature data, the yield of EO obtained in the present study is higher than that reported by Martini et al. [31] for an accession grown in Italy (0.025%); however, it is lower than that obtained by Turko et al. [18] (0.35%) for the same species isolated by steam distillation. Thirty-three compounds were identified (Table 1), representing 99.23% of the total oil composition. The main fraction of the EO was represented by Sesquiterpenes (85.92%), with caryophyllene oxide (24.28%), spathulenol (12.31%), nerolidol (11.8%), and manool oxide (7.36%), respectively, being the main compounds. These results overlapped with that reported by Benelli et al. [16] for *Stevia* species grown in Italy, where caryophyllene oxide (20.7%) and spathulenol (14.9%) were predominant; but further reports showed a very different chemical composition**.** Muanda et al. [20] analyzed the profile of EO of this species from Nigeria and found that carvacrol (67. 80%) was identified as the main component, followed by caryophyllene oxide (23.50%), spathulenol (15.41%), cardinol (5.59), a-pinene (3.75%), ibuprofen (1.79%), isopinocarveol (1.26%), caryophyllene (1.15%). Siddique et al. [17] Studied EO from Bangladesh accessions of *S. rebaudiana* and found that a-Cadinol (2.98%), -spathulenol (2.21%), caryophyllene oxide (1.23%) were the major components. Mann et al. [18] also reported that the EO of an Indian accession was composed mainly of (E)-caryophyllene (15.9%), bicyclogermacrene (14.6%), β-pinene (12.5%), α-humulene (6.6%), and germacrene D

Table 1 Chemical composition of the essential oil of *Stevia rebaudiana* Bertoni cultivated in Algeria

Stevia rebaudiana Bertoni					
N°	Compounds	Cas number	RI [a]	RI [b]	% Composition
1	*β-Pinene*	127-91-3	975	976	Tr
2	*Linalool L*	123-35-3	1106	1094	1.4 ± 0.28
3	*γ-Pyronene*	514-95-4	1345	1339	Tr
4	*β-elemene*	515-13-9	1395	1393	1.09 ± 0.06
5	*Methyleugenol*	93-15-2	1412	1410	Tr
6	*Caryophylene*	87-44-5	1423	1419	1.028 ± 0.22
7	*α-bergamotene*	17699-05-7	1438	1438	1.84 ± 0.06
8	***β-famesene***	28973-97-9	1458	1457	**4.12 ± 0.31**
9	*aromadendrene*	489-39-4	1465	1457	0.71 ± 0.06
10	*α-elemene*	5951-67-7	1481	1469	0,68 ± 0.04
11	*α-curcumene*	644-30-4	1487	1484	0.79 ± 0.03
12	*β-lonone*	14901-07-6	1490	1488,4	1.89 ± 0.06
13	*α-selinene*	473-13-2	1500	1498	0.40 ± 0.08
14	*α-muurolene*	31983-22-9	1503	1500	Tr
15	*β-bisabolene*	495-61-4	1511	1506	0.79 ± 0.04
16	*γ-cadinene*	39029-41-9	1518	1517	1.32 ± 0.004
17	*σ-Cadinene*	483-76-1	1527	1524	1.17 ± 0.03
18	***Nerolidol***	7212-44-4	1567	1565	**11.8 ± 0.12**
19	*(7R,10S)-2,6,10-Trimethyl-7,10-epoxy-2,11-dodecadien-6-ol*	72523-45-6	1579	1561	1.85 ± 0.08
20	***Spathulenol***	77171-55-2	1585	1582	**12.31 ± 0.32**
21	***Caryophylene oxide***	1139-30-6	1589	1583	**24.28 ± 1.17**
22	*Trans-β-Elemenone*	20303-60-0	1600	1606	1.3 ± 0.08
23	*Bicyclo[5.2.0]nonane, 2-methylene-4,8,8-trimethyl-4-vinyl-*	242794-76-9	1604	–	0.93 ± 0.05
24	***humulene oxide***	19888-34-7	1615	1610	**5.44 ± 0.2**
25	***Bicyclo[4.4.0]dec-1-ene, 2-isopropyl-5-methyl-9-methylene-***	150320-52-8	1646	–	**4.79 ± 0.02**
26	***α-cadinol***	481-34-5	1660	1654	**4.51 ± 0.05**
27	*α-bisabolol*	23178-88-3	1669	1675	0.69 ± 0.046
28	*1H-Cycloprop[e]azulen-7-ol, decahydro-1,1,7-trimethyl-4-methylene-, [1ar-(1a.alp)]*	6750-60-3	1678	1624	1.48 ± 0.22
29	*4-Bromo-1-naphthalenamine*	2298-07-9	1733	–	1.75 ± 0.58
30	*Phytone*	502-69-2	1847	1847	0.72 ± 0.04
31	*5-(dimethylamino)-1,2,3,4,5-pentamethyl-1,3-cyclopentadiene*	75812-65-6	1935	–	1.21 ± 0.06

Table 1 (continued)

Stevia rebaudiana Bertoni					
N°	Compounds	Cas number	RI [a]	RI [b]	% Composition
32	*Unidentified*	–	1946	–	1.59 ± 0.02
33	***Manool oxide***	596-84-9	1998	1990	**7.36 ± 0.62**
Class of compounds					
Monoterpenes	1.40				
Sesquiterpenes	85.92				
Others	10.32				
Unidentified	1.59				
Total Identified (%)	**99.23**				

Bold values represent the different concentrations tested for the acarbose standard

Identification methods: MS, comparison of mass spectra to those of PAL 600® libraries; RI, comparison of retention indices to those reported in the literature; CAS number; RI[a], theoretical kovats indices (Pubchem and NIST); RI[b]: calculated kovats indices; Tr: traces (< 0.1%). Values are mean ± SD of three injections

(6.1%) as the main constituents. the above mentioned results, one can conclude that the EO of *S. rebaudiana* is generally dominated by the sesquiterpene fraction. Still, qualitative and quantitative variations can be observed in the composition of essential oils between accessions of different geographical origins, depending on several factors such as the part of the plant used for post-harvest treatment and the extraction technique.

The predominance of sesquiterpenes in *S. rebaudiana* may offer significant pharmaceutical interest. Caryophyllene oxide result from the oxidation of (E)-caryophyllene and has important anticancer and analgesic properties [32]. The Food and Drug Administration (FDA) has approved it as a food additive [33]. Spathulenol is a sesquiterpene that has been reported as the main volatile component of essential oils of several aromatic species [34], and presents important antioxidants, antiproliferative, antimicrobial and anti-inflammatory activities [35]. The (E)-nerolidol is sesquiterpene alcohol of several plants, fruits, and vegetables, is responsible for several biological activities (antimicrobial, antioxidant, anti-inflammatory, and antiproliferative) demonstrated by EO [35].

Total phenolics, flavonoids, and flavonols content

Figure 2 shows the total content of polyphenol compounds, flavonoids, and flavonols contained in the different extracts of *S. rebaudiana* (methanol, petrol ether, chloroform, ethyl acetate, and butanol). Total phenolic content was calculated as µg of gallic acid equivalents per milligram of dry extract. Variable contents of total phenols were found among the different extracts tested, the ethyl acetate fraction gave the highest content with 817.66 ± 3.0 (µg GAE/mg), followed by the methanolic extract 267.22 ± 1.5 (µg GAE/mg), whereas the lowest value was obtained by the petrol ether fraction (14.55 ± 1.5 µg GAE/mg). The total flavonoid and flavonol content was also determined and expressed as µg quercetin equivalents per mg dry extract (µg QE/mg). Chloroform and the butanol fraction showed the lowest flavonoid values (32.75 ± 1.73 and 42.66 ± 1.12 µg QE/mg, respectively), in comparison to the ethyl acetate fraction, which gave a significant content (440 ± 1.0 µg QE/mg); this latter followed by the methanol fraction with 162.33 ± 1.89 µg QE/mg.For flavonols, the contents varied between 28.84 ± 2.31 and 249.5 ± 0.77 µg QE/mg and decreased in the following order: ethyl acetate > chloroform > petrol ether > methanol > butanol. The results showed a wide variation in the recovery of total polyphenols, flavonoids, and flavonols content in the different *stevia* extracts. It could be due to the solubility of the phenolic compounds in the extraction solvent used [36]**.** Based on our data, the ethyl acetate extract indicates the highest quantity of bioactive compounds. These results are in accordance with previous reports, which also

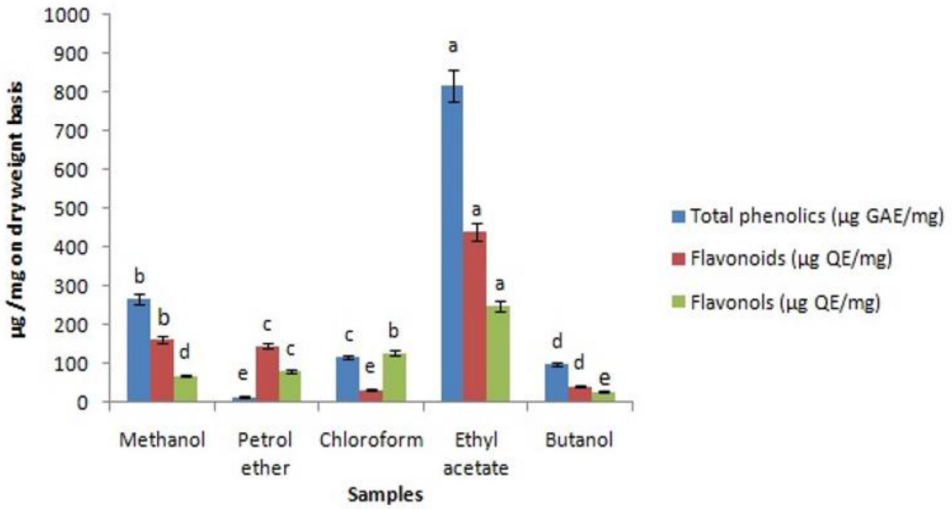

Fig. 2 Total phenolics (TPC), flavonoids (TFC), and flavonols content of crude methanol extract and its fractions. Total phenolics are expressed as µg Gallic acid equivalents/mg of extract, total flavonoids and flavonols are expressed as µg Quercetin equivalents/ mg of extract. Error bars represent mean ± SD. The values with different superscripts (a, b, c, d, or e) in the same columns are significantly different ($p < 0.05$)

revealed that with an increasing solvent polarity, the total content of phenols and flavonoids increases in the extract [37]. The contents of phenolic compounds of *S. rebaudiana* in several extracts have been documented in the literature. In a study by Zaidan et al. [38], the total phenolic content in methanol (6.96 mg GAE/g), aqueous (6.65 mg GAE/g), and ethanol (6.43 mg GAE/g) extracts, was significantly higher than in acetone extract (5.41 mg GAE/g). Another study, [39] reported content of 28.76 ± 2.94 mg GAE/100 g DW for total phenolic compounds and 88.65 ± 2.03 mg QE/g DW for total flavonoids of aqueous methanol solution 50%.

Antioxidant properties

In this work, the in vitro antioxidant capacity of the essential oil and the crude extract/fractions of the dried leaves of *S. rebaudiana* Bertoni has been evaluated by the following four tests: DPPH and ABTS radical scavenging, copper reducing antioxidant capacity (CUPRAC), and reducing power. The results are presented in Table 2 and expressed in terms of IC_{50} and $A_{0.5}$. The results of the free radical scavenging effect of DPPH show that ethyl acetate extract presented the highest antioxidant activity (IC_{50}: 7.83 ± 0.54 µg/mL), which is statistically similar to the standards used ($p > 0.05$), and followed by methanolic extract (IC_{50}: 24.87 ± 2.18 µg/mL), while essential oil manifested no activity. For the ABTS scavenging assay, the ethyl acetate extract again showed a very high radical scavenging capacity (IC_{50}: 4.50 ± 0.1 µg/mL), more potent than standard α-tocopherol (IC_{50}: 12.48 ± 0.22 µg/mL), However, no significant difference was observed ($p > 0.05$) with BHA and BHT (IC_{50}: 1.81 ± 0.10 and 1.29 ± 0.30 µg/mL, respectively), followed

Table 2 Inhibition of DPPH, ABTS, reducing power and CUPRAC of *Stevia rebeaudiana* Bertoni essential oil, crude methanol extract, and its fractions

Stevia rebeaudiana	DPPH $_{IC50\ (\mu g\ mL)}$	ABTS $_{IC50(\mu g\ mL)}$	CUPRAC $_{A0.5(\mu g/mL)}$	Reducing power $_{A0.5(\mu g/mL}$
EO	›800	›800	392.5 ± 0.71^{f}	›800
Methanol	24.87 ± 2.18^{b}	16.93 ± 1.06^{b}	24.49 ± 1.04^{b}	›200
Petrol ether	653.67 ± 3.9^{e}	266.88 ± 2.66^{e}	325.5 ± 6.36^{e}	›800
Chloroform	92.2 ± 4.69^{c}	24.8 ± 2.30^{c}	87.13 ± 0.88^{c}	123.17 ± 3.06^{d}
Ethyl acetate	7.83 ± 0.54^{a}	4.50 ± 0.1^{a}	9.34 ± 0.28^{a}	17.55 ± 2.84^{b}
Butanol	156.26 ± 2.74^{d}	105.76 ± 3.21^{d}	100.63 ± 0.88^{d}	163.5 ± 1.41^{e}
BHA^{R}	6.14 ± 0.41^{a}	1.81 ± 0.10^{a}	5.35 ± 0.71^{a}	NT
BHT^{R}	12.99 ± 0.41^{a}	1.29 ± 0.30^{a}	8.97 ± 3.94^{a}	NT
α-$Tocopherol^{R}$	$13.02 \pm 5,17^{a}$	12.48 ± 0.22^{b}	19.92 ± 1.46^{b}	34.93 ± 2.38^{c}
Ascorbic $acid^{R}$	13.94 ± 2.81^{a}	NT	12.43 ± 0.09^{a}	6.77 ± 1.15^{a}
Tannic $acid^{R}$	7.74 ± 0.19^{a}	NT	NT	5.39 ± 0.91^{a}

Results were expressed as means ± SD of three measurements

BHA butylatedhydroxyanisole, *BHT* butylatedhydroxytoluene, *NT* Not tested, *R* references compounds

The values with different superscripts (a, b, c, d, e, or f) in the same columns are significantly different ($p < 0.05$)

by methanolic extract (IC_{50}: 16.93 ± 1.06 µg/mL), while essential oil showed no activity. All *stevia* extracts revealed different copper ion reduction capacities, with $A_{0.5}$ values ranging from 9.34 ± 0.28 to 392.5 ± 0.71 µg/mL. The Cu^{2+} reduction capacity of the tested extracts decreased in the following order: ethyl acetate > methanol > chloroform > butanol > petrol ether > essential oil. The ethyl acetate fraction showed the best activity with a value of ($A_{0.5}$: 9.34 ± 0.28 µg/ mL), better than that of α-tocopherol ($A_{0.5}$: 19.92 ± 1.46 µg/ mL), and statistically similar ($p > 0.05$) to the standards BHA and BHT and ascorbic acid standards ($A_{0.5}$: 5.35 ± 0.71, 8.97 ± 3.94, and 12.43 ± 0.09 µg/mL, respectively).

Regarding the reducing power test, the data indicate that ethyl acetate extract presented activity ($A_{0.5}$: 17.55 ± 2.84 µg/mL), better than that of standard α-tocopherol ($A_{0.5}$: 34.93 ± 2.38 µg/mL), but significantly less effective than ascorbic acid and tannic acid ($p < 0.05$), and followed by butanol and Chloroform extract ($A_{0.5}$: 123.17 ± 3.06 and 163.5 ± 1.41 µg/mL respectively). Essential oil, methanol, and petrol ether showed no activity.

Methanol extract and its fractions showed a very interesting antioxidant activity by scavenging free radicals and acting as reducing agents. For all tests, the ethyl acetate fraction and the methanol extract presented the best antioxidant potential ($p > 0.05$). It could be due to the presence of a high amount of total phenols in these extracts (Fig. 2). This observation is in accordance with other studies in the literature that has reported a positive correlation between antioxidant capacity and total polyphenol content [11, 40, 41]**.** According to Ait Chaouche et al. [42], the phenolic content can be used as an important indicator of the antioxidant capacity of plants. Many papers have reported the antioxidant activity of *S. rebaudiana* performed on different extracts. Moongngarm et al. [43] reported in a study the antioxidant activity of *S. rebaudiana* leaves extracted using ohmic heating-assisted water and compared it to aqueous and methanolic extracts using DPPH assay (IC_{50} values ranged from 94.68 to 105.19 µg/mL for ohmic treatment and 241.34 and 118.41 µg/mL corresponding to aqueous and methanolic extracts respectively). Similarly, Gaweł-Bęben et al. [14] also reported the antioxidant activity of ethanolic extracts carried out by DPPH- and ABTS radicals (IC_{50} values of 4.73 ± 0.53 and 1.34 ± 0.05 µg/ mL, respectively were obtained).

Regarding *stevia* essential oil, the antioxidant activity is not well documented in the literature. The results we obtained were in contrast to the study done by Muanda et al. [20], which reported that *stevia* essential oil exerted good DPPH inhibition activity with an $IC_{50} = 19.26 \pm 0.35$ µg/mL. However, our data agree with those of previous works, which reported that the essential oil showed no activity by DPPH assay, ABTS, and that of reducing power [44, 45]. It could be explained by the fact that sesquiterpenes (major compounds of *S. Rebaudiana*) such as caryophyllene oxide and α-Humulene present a low antioxidant activity compared to that of thymol and carvacrol [46].

Anticholinesterase activity

The evaluation of the anticholinesterase activity of the essential oil and the crude extract/fractions of *Stevia* was carried out by combining two enzymes: AChE and BChE. The inhibition results were compared to the positive control, galantamine which is used in the treatment of Alzheimer's disease, and are presented in Table 3 and expressed in terms of percentage inhibition and IC_{50}. The inhibition capacity of the enzymes (AChE and BChE) of the extracts was dependent on the concentrations of the samples (100, 200 µg/mL). The percentage of inhibition increased with the extract concentration.

In the AChE assay, the essential oil and the chloroform fraction showed moderate inhibition efficiency according to the classification made by Achili et al. [47], while the crude methanolic extract, the ethyl acetate, and butanol fractions were inactive. All extracts tested presented some degree of

Table 3 Anticholinesterase activity of *Stevia rebaudiana* Bertoni essential oil (EO), crude methanol extract, and its fractions

Samples	AChE (% inhibition)			BChE (% inhibition)		
	100 (µg/mL)	200 (µg/mL)	IC_{50} (µg/mL)	100 (µg/mL)	200 (µg/mL)	IC_{50} (µg/mL)
EO	31.01 ± 1.06	48.18 ± 1.27	›200	31.31 ± 1.41	36.86 ± 4.02	›200
Methanol	Na	Na	Na	5.66 ± 1.63	31.77 ± 2.52	›200
Petrolether	Na	5.87 ± 1.47	›200	46.85 ± 1.07	69.26 ± 3.65	123.7 ± 1.78^{b}
Chloroform	23.13 ± 1.69	40.79 ± 2.29	›200	36.38 ± 8.54	54.5 ± 0.81	170.1 ± 0.78^{c}
Ethyl acetate	Na	Na	Na	Na	8.68 ± 0.85	›200
Butanol	Na	Na	Na	6.22 ± 0.51	11.62 ± 0.0	›200
Galanthamine^{R}	91,80 ± 0,2	94,77 ± 0,34	6.27 ± 1.15	73,57 ± 0,77	78.95 ± 0,58	34.75 ± 1.99^{a}

Results were expressed as means ± SD of three measurements

Na No activity, *R* References compounds

The values with different superscripts (a, b, c) in the same columns are significantly different ($p < 0.05$)

Table 4 α-glucosidase activity of *Stevia rebaudiana* Bertoni essential oil, crude methanol, andits fractions

Extracts	α-glucosidaseinhibitory assay					
	62.5 µg/mL	125 µg/mL	250 µg/mL	500 µg/mL	1000 µg/mL	IC_{50}(µg/mL)
Essential oil	38.12 ± 2.2	96.45 ± 2.09	97.92 ± 0.65	95.18 ± 1.32	98.69 ± 1.5	74.9 ± 6.4^{a}
Methanol	Na	Na	Na	42.42 ± 4.7	64.87 ± 1.71	764.6 ± 2.88^{d}
Petrol ether	29.71 ± 1.16	37.08 ± 1.7	65.87 ± 1.7	75.62 ± 0.87	92.4 ± 0.54	178.4 ± 3.22^{b}
Chloroform	Na	Na	29.79 ± 0.00	30.6 ± 1.13	62.03 ± 0.03	789.3 ± 0.02^{e}
Ethylacetate	Na	Na	Na	27.65 ± 1.65	49.81 ± 1.25	›1000
Butanol	Na	Na	17.07 ± 2.06	27.05 ± 1.16	44.69 ± 1.61	›1000
Acarbose^{R}	**78.125 µg/mL**	**156.25 µg/mL**	**312.5 µg/mL**	**625 µg/mL**	**1250 µg/mL**	**IC_{50}(µg/mL)**
	27.43 ± 2.18	38.91 ± 3.20	54. ± 1.79	67.29 ± 2.63	80.19 ± 1.66	**275.43 ± 1.59^{c}**

Bold values represent the different concentrations tested for the acarbose standard

Results were expressed as means ± SD of three measurements

Na No activity, *R* References compounds

The values with different superscripts (a, b, c, d, or e) in the same columns are significantly different ($p < 0.05$)

inhibition of BChE ($p < 0.05$). The petrol ether fraction gave the best activity (IC_{50}: 123.7 ± 1.78 µg/mL) followed by the chloroform fraction (IC_{50}: 170.1 ± 0.78 µg/mL). However, they remain significantly less effective than galantamine ($p < 0.05$). As for the essential oil, it showed a weak activity at 200 µg/mL. Based on these preliminary results, we notice that the essential oil, methanol extract, and its fractions inhibit BChE more effectively than AChE. Although this is the first report of anticholinesterase activity of *S. rebaudiana*. Some molecules that provide this inhibition could explain this activity. The petrol ether fraction showed the most potent activity ($p > 0.05$) against BChE among the other fractions despite its low content of phenolic compounds. These results suggest the implication of additional bioactive compounds like alkaloids and coumarins in the inhibition of these enzymes [2].

Stevia extracts could play a role in the treatment of Alzheimer's disease. It would be interesting to conduct further research to discover more effective compounds with minimal side effects for the management of neurodegenerative disorders [48].

α-Glucosidase inhibitory activity

Diabetes is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both [49]. Many plants have been used traditionally as a natural source in the treatment of diabetes mellitus [50]. Inhibitors of α-glucosidase are very valuable as antihyperglycemic agents because they inhibit the hydrolysis of both starch and sucrose [51], thus moderating the postprandial rise in blood sugar and minimizing the effects of food on hyperglycemia [52].

In the present research, the inhibition effect of essential oil and crude extract/fractions of *Stevia* on α-glucosidase has been explored based on the concentrations tested (62.5, 125, 250, 500, 1000 µg/mL). The results are presented in Table 4 and expressed in terms of percent inhibition and IC_{50}. Acarbose was used as a positive control. All samples showed a certain inhibition activity ($p < 0.05$) in a dose-dependent manner, which was effective in the following order: Essential oil > petrol ether > methanol > chloroform > ethyl acetate > butanol.

The essential oil and petrol ether fraction showed potent activity with a value of (IC_{50}: 74.9 ± 6.4 and 178.4 ± 3.22 µg/mL, respectively), significantly higher ($p < 0.05$) than that of the control Acarbose (IC_{50}: 275.43 ± 1.59 µg/mL). EO reached almost its maximum activity at 125 µg/mL with a percentage of inhibition (PI) of 96.45 ± 2.09%. Regarding petrol ether extract, its highest PI (92.4 ± 0.54%) was observed at the concentration of 1000 µg/ mL. Concerning ethyl acetate and butanol fractions, they showed low activity at the maximum concentration 1000 µg/mL.

As far as we know, this is the first report of the antidiabetic potential of the essential oil of the dry leaves of *S. rebaudiana*. In contrast to our results, Zaidan et al. [38] found that *Stevia* extracts into (water, methanol, ethanol, and acetone) manifested no inhibition of α-glucosidase activity. However, studies reported in the literature have shown the inhibitory effect of *stevia* leaves in various extracts. In a study reported by Ruiz-Ruiz et al. [53], it was shown that the aqueous extract of *Stevia* exerted a good dose-dependent inhibition of α-glucosidase (an IC_{50} value of 596.77 µg/mL). According to several authors, the antidiabetic effect of various plants is due to the presence of phenolic acids, phenolic diterpenes, flavonoids, steroids, triterpenoids, alkaloids, and other nitrogen compounds [54].

Thus, this study shows the inhibitory effect of *S. rebaudiana* leaf extracts on the enzyme α-glucosidase and suggests its use as a natural treatment for type 2 diabetes.

Conclusion

Our results allowed the characterization of the chemical profile of the essential oil of *Stevia*. They provided new information on the antioxidant and biological activity (anticholinesterase and α-glucosidase) of the methanolic crude extract and its fractions to search for other possible active molecules. Variability in the content of bioactive compounds and antioxidant power was observed between the different sections tested; this is probably related to the polarity of the extraction solvent used. The ethyl acetate extract showed a strong antioxidant capacity similar to the standards and sometimes even better. The essential oil showed moderate inhibition of key enzymes (AChE and BChE) and significant antidiabetic activity. Therefore, our data support the possible use of *S. rebaudiana* leaves as a potential source of dual-action molecules, which can be exploited in the food industry for preservation and shelf-life extension of raw and processed foods and also serve as a therapeutic agent to prevent some neurodegenerative and diabetic diseases. However, further studies should be conducted on this plant to understand better the mode of action of these bioactive molecules responsible for each activity.

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Declarations

Conflict of interest No potential conflict of interest was reported by the authors.

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