

Antioxidant Activities of Steviol Glycosides from Moroccan Cultivated *Stevia Rebaudiana Bertoni* Leaves: An In Vitro Study

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Abstract. This study explores the antioxidant potential of three steviol glycosides isolated from *Stevia Rebaudiana Bertoni* leaves acclimatized in Morocco. Pure compounds were isolated and characterized by column chromatography, and their antioxidant activities were assessed using Ferric Reducing Antioxidant Power (FRAP) and Total Antioxidant Capacity (TAC) tests. The process of soxhlet extraction followed by column purification revealed three steviol glycosides with the following yields: steviolbioside (0.26%), rebaudioside-A (0.63%), and stevioside which exhibited the highest abundance (1.47%). Antioxidant activity tests showed that the compounds exhibited remarkable antioxidant properties, particularly in TAC test of 69.54, 72.32, and 51.6 mg AAE/1gDM for stevioside, steviolbioside, and rebaudioside-A respectively.

1. Introduction

Stevia Rebaudiana Bertoni, a perennial herb from the Asteraceae family, originates in Paraguay and Brazil. Its leaves have long been used as a natural sweetener by the indigenous Guarani Indians for numerous centuries [1]. While there is a diverse array of approximately 150 *stevia* species documented, it is noteworthy that *Stevia Rebaudiana Bertoni* stands out as the sole variety renowned for its remarkable sweetening attributes [1]. Presently, this botanical specimen holds global significance due to the utilization of its leaves as a non-nutritive highly potent sweetening agent. It is primarily used in China, Korea, Japan, and several South American regions. [2].

The leaves of *Stevia Rebaudiana Bertoni* are abundant in steviol glycosides, including rebaudioside-A, rebaudioside-B, rebaudioside-C, rebaudioside-F, dulcoside-A, steviolbioside, and stevioside. These compounds are responsible for the plant's distinctive sweetness. [3]. Beyond glycosides, the plant leaves also harbor phenolic compounds, which encompass, alkaloids, tannins, flavonoids, and other bioactive substances. These constituents not only exhibit antioxidant and antibacterial properties, but also contribute to a range of health-promoting attributes [4, 5, 6]. Compounds with antioxidant properties play a crucial role in inhibition of lipid oxidation, reduction of rancidity, retardation of the

formation of harmful byproducts, preservation of nutritional integrity, and extension of the shelf life of food products [7]. Moreover, they may prove beneficial in combating diseases linked to oxidative stress [8].

Numerous conventional extraction methods including maceration and soxhlet find extensive application in extracting bioactive compounds from plant materials. Despite their drawbacks of prolonged extraction times, substantial solvent usage, and energy intensity, these methods are still viable for commercial use because they are cost-effective, simple, and produce high-quality results [9]. This attribute makes them occasionally utilized by researchers, which would be the selection for this study, as the soxhlet technique.

Aqueous extracts derived from the plant leaves offer advantageous impacts on human well-being, encompassing hypoglycemic effects [10], hypotensive effects [11], in addition to serving as a source of antioxidants [12]. Moreover, ethanol proves to be an appropriate solvent for extracting polyphenols and is widely used as a safe extraction solvent for products intended for the food processing industry [13]. Given the attendance of diverse bioactive compounds and the multitude of biological advantages offered by *Stevia* leaves [14-16], it becomes crucial to employ appropriate, secure, and efficient solvents for steviol glycosides extraction with potential antioxidant capabilities.

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Therefore, this study aims to evaluate the antioxidant potential of steviol glycosides extracted from *Stevia* leaves by employing soxhlet extraction and solvents comprised of distilled water, ethanol, and ethanol/distilled water (80/20).

2. Material and method

2.1 Extraction and separation of steviol glycosides

Steviol glycosides were obtained following a method based on Jaitak's procedure (2008), with slight modifications involving the use of two alternative solvents. To summarize, 10 grams of powdered dried plant leaves were placed in a soxhlet apparatus and subjected to extraction for 12 hours using a combination of ethanol/distilled water (80/20), ethanol, and distilled water. Subsequently, the resulting extracts were concentrated under reduced pressure at a temperature of $50 \pm 3^\circ\text{C}$. The dried residues were reconstituted in butanol, with the addition of anhydrous Na_2SO_4 , and once again concentrated under reduced pressure at $50 \pm 3^\circ\text{C}$, resulting in the isolation of total steviol glycosides extracts [17].

The collected extracts were valued based on their yields. Steviol glycosides were successfully isolated solely from the extract with the highest yield. This particular extract underwent column chromatography using silica gel and a gradient elution process through a $\text{CHCl}_3/\text{MeOH}$ mixture, gradually increasing the proportion of methanol (5% to 30%) in chloroform. This chromatographic procedure led to the separation of four distinct fractions, designated as (I) to (IV). Fraction (III) was subjected to a secondary chromatographic process on silica gel, employing a gradient elution from 5% to 20% MeOH in chloroform. This procedure resulted in the isolation of pure steviolbioside, which demonstrated a melting point in the range of $188\text{--}192^\circ\text{C}$. Similarly, Fraction (IV) underwent a repeat chromatography on silica gel, utilizing a gradient elution from 5% to 30% MeOH in chloroform. This process led to the isolation of pure stevioside, characterized by a melting point falling between $196\text{--}198^\circ\text{C}$, as well as rebaudioside-A, with a melting point range of $242\text{--}244^\circ\text{C}$ [17].

2.2 Antioxidant activity evaluation

2.2.1 FRAP activity and total antioxidant role (TAC)

Different concentrations of the extract (0.1 to 0.5 mg/mL) were mixed with phosphate buffer and 1% potassium ferricyanide, then incubated at 50°C for 20 minutes. After cooling, 2.5 mL of 10% trichloroacetic acid was added, and the mixture was centrifuged at 3000 rpm for 10 minutes. The supernatant was combined with 2.5 mL of distilled water and 0.5 mL of 0.1% ferric chloride. Ascorbic acid was the standard, and absorbance was recorded at 700 nm using a Shimadzu UV-1600 PC UV spectrophotometer [18].

For the Total Antioxidant Capacity (TAC) assay, 100 μL of the extract was mixed with 900 μL of a reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). Absorbance was

measured at 695 nm, with a blank of 100 μL methanol and 900 μL reagent as a reference [19].

Antioxidant activity values were standardized and presented as mg ascorbic acid equivalents per one gram of dry matter (mg AAE/gDM).

3. Discussion of results

3.1 Extraction and separation of steviol glycosides

The three different solvents resulted in varying extraction yields. The findings demonstrated that the aqueous extract contained the majority of steviol glycosides, boasting an extraction yield of 9.51%. Figure 1 illustrates the extraction yields achieved through each experimental condition.

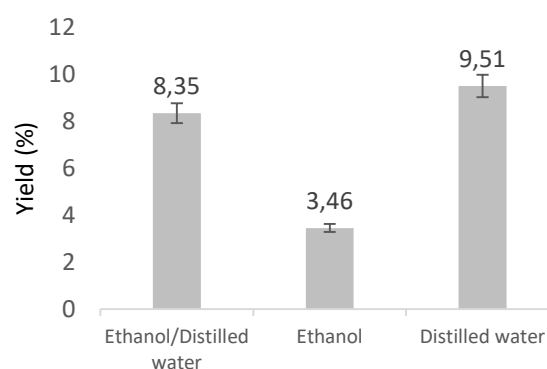


Fig. 1. Extraction yields for the three solvents.

As depicted in Figure 1, the aqueous extract notably dominated in terms of yield, accounting for 9.51% of total steviol glycosides. This observation can be attributed to the principle of polarity, as bioactive compounds with varying polarities may dissolve differently in a given solvent [20]. Generally, polar solvents are employed to extract highly polar compounds such as polyphenols from plant matrices [21]. This preference for polar solvents can elucidate the superior performance of the aqueous extract compared to the other two, given that steviol glycosides are characterized as highly polar compounds [22].

As previously outlined in section 2, the decision was made to select the major steviol glycosides extract based on its yield. Therefore, the aqueous extract was subjected to the column chromatography process afterwards, resulting in the isolation of stevioside, steviolbioside, and rebaudioside-A. The respective yields of these compounds are presented in Figure 2.

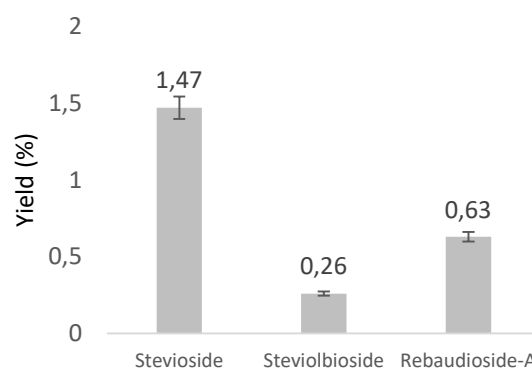


Fig. 2. Yields of stevioside, steviolbioside, and rebaudioside-A obtained after column chromatography process.

Figure 2 clearly illustrates that among the three analyzed steviol glycosides, stevioside is the most predominant compound, with a content of 1.47%.

Alternatively, Jaitak (2008) developed a chromatographic method to simultaneously quantify three steviol glycosides—steviolbioside, stevioside, and rebaudioside-A—in plant leaves. The calibration curves obtained using this method demonstrated linearity within the following ranges: 160-960 ng/spot for steviolbioside, 1-6 µg/spot for stevioside, and 0.5-3 µg/spot for rebaudioside-A. The correlation coefficients were notably high, between 0.998 and 0.999. These findings align with our results, indicating that steviolbioside is the predominant compound isolated among all the steviol glycosides extracted [17]. Additionally, Rao A (2012) conducted research on the extraction of steviol glycosides from the plant leaves using a pressurized hot water extractor. Subsequently, glycosides were subjected to purification and concentration through ultra (UF) and nano (NF) filtration membranes. The outcomes were consistent with ours, revealing that rebaudioside-A was obtained in relatively lower quantities (0.2g per 100g) in comparison to stevioside (9.05g per 100g). This supports the notion that stevioside is more abundant in *Stevia Rebaudiana Bertoni* leaves compared to rebaudioside-A [23].

3.2 Evaluation of the antioxidant activity

3.2.1 Ferric reducing antioxidant power (FRAP) and Total antioxidant activity (TAC)

Ferric reducing antioxidant power and Total Antioxidant Capacity procedures have been conducted as described in section 2. Figures 3a and 3b illustrate the antioxidant activity results, presented as optical density versus concentration.

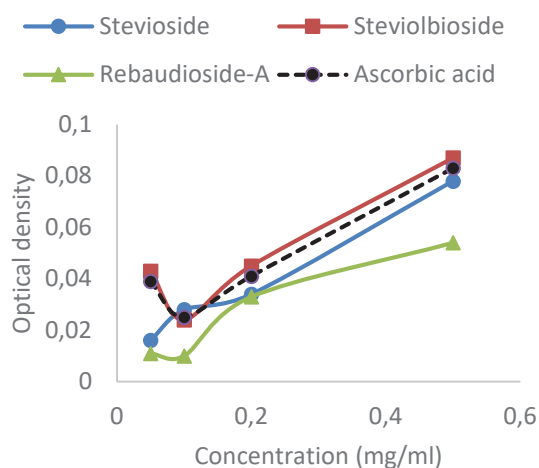


Fig. 3a. FRAP test of the aqueous extract compared to ascorbic acid.

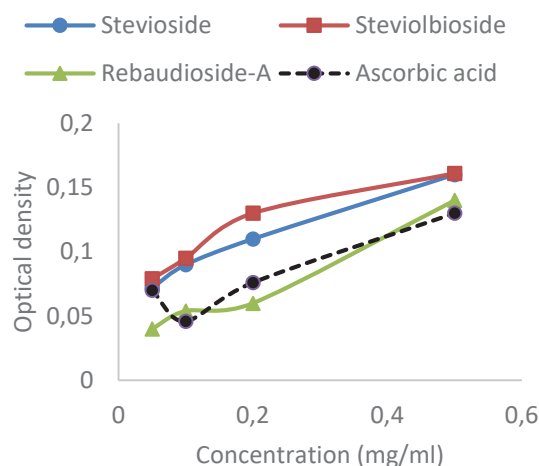


Fig. 3b. CAT test of the aqueous extract compared to ascorbic acid.

The data in the figures clearly show that steviolbioside exhibited the highest antioxidant capacity in both tests and at all concentrations, even exceeding that of ascorbic acid. The differences in antioxidant activity between rebaudioside-A and stevioside are due to their varying abilities to reduce Mo (VI) to Mo (V). Stevioside, at all concentrations, effectively reduced Mo (VI) and neutralized free radicals via electron transfer. Rebaudioside-A only did so at 0.1 mg/mL and 0.5 mg/mL. Neither compound reduced iron from Fe (III)-TPTZ to Fe (II)-TPTZ in the FRAP reagent, indicating they lack electron-donating properties. Thus, their reducing power and electron transfer abilities are not significant mechanisms as antioxidants [08, 24, 25].

An additional explanation for the inability of stevioside and rebaudioside-A to reduce ferric iron to ferrous iron may stem from the absence of other potential antioxidants typically found alongside steviol glycosides. Total extracts of steviol glycosides often contain a diverse array of bioactive compounds, such as tannins, flavonoids, and polyphenols. These compounds likely collaborate synergistically, amplifying the overall antioxidant capacity of the extract. In contrast, isolated steviol glycosides lack this synergistic effect and may exhibit limited reactivity towards specific radicals. This nuanced interplay between the various constituents underscores the importance of considering the complex composition of natural extracts in elucidating their antioxidant properties. In a study by López et al. (2016), the antioxidant activity of *Stevia Rebaudiana* ethanolic extract (SREE) and isolated stevioside was evaluated in terms of their ability to scavenge free radicals, including superoxide radicals and DPPH. The results revealed that *Stevia Rebaudiana* ethanolic extract (SREE) exhibited dose-dependent scavenging of both DPPH and superoxide radicals. In contrast, stevioside demonstrated no radical scavenging activity or DPPH scavenging capacity, with its activity not surpassing that of the reference ascorbic acid. These findings indicate that *Stevia Rebaudiana* ethanolic extract (SREE) has stronger antioxidant properties than stevioside, suggesting a preference for bioactive stevia extracts. [26].

Still, there are multiple factors that could account for the lower optical density values observed at a concentration of 0.1mg/ml in both tests. Concentration-dependent effects are commonly observed, where the efficacy of steviol glycosides as antioxidants may not follow a linear trend with increasing concentration. At lower concentrations, they may exhibit heightened reactivity towards ferric ions, resulting in an apparent increase in optical density. However, as concentrations rise, saturation effects or interactions between the compounds could occur, leading to a possible decrease in observed activity. In addition, some compounds may display pro-oxidant properties at higher concentrations, counteracting their antioxidant effects and contributing to fluctuations in observed results.

FRAP and CAT tests at 0.5 mg/mL measured ascorbic acid equivalents per gram of dry matter (Figure 4).

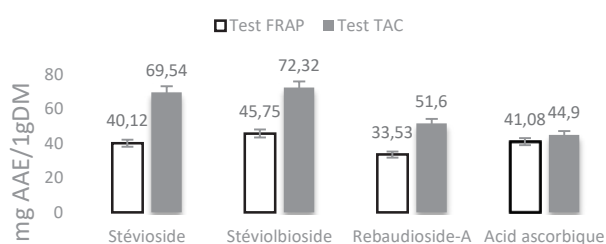


Fig. 4. FRAP and CAT test of the aqueous extract at 0.5mg/mL compared to ascorbic acid

Figure 4 clearly indicates that the CAT test yields optimal values compared to the FRAP test. However, the three steviol glycosides consistently exhibit the same order of antioxidant activity. Steviolbioside emerges as the most potent antioxidant, followed by stevioside, with rebaudioside-A showing the lowest antioxidant values.

4. Conclusion

This research focuses on the extraction yield and antioxidant potential of three steviol glycosides from *Stevia Rebaudiana Bertoni* leaves acclimatized in Morocco. Both extraction technique and solvent choice impact yield and antioxidant activity. Stevioside dominates in yield, while steviolbioside excels in antioxidant properties. These findings suggest the potential of steviol glycosides, particularly steviolbioside, in mitigating diseases related to oxidative stress, showcasing promise for various biological activities and applications in food and pharmaceutical industries.

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References

1. V. Jaitak, A. P. Gupta, V. K. Kaul, P. S. Ahuja, *Validated high-performance thin-layer*

2. D. J. Meta, *Alternative Sweeteners*, Br J Nutr, **2**, 191 (2001).
3. V. Srivastava, R. Chaturvedi, *An interdisciplinary approach towards sustainable and higher steviol glycoside production from in vitro cultures of Stevia Rebaudiana*, J Biotechnol, **358**, 76-91 (2022).
4. D. Bursac Kovačević, F. J. Barba, D. Granato, C. M. Galanakis, Z. Herceg, V. Dragović-Uzelac, P. Putnik, *Pressurized hot water extraction (PHWE) for the green recovery of bioactive compounds and steviol glycosides from Stevia rebaudiana Bertoni leaves*, Food Chem, **254**, 150–157 (2018).
5. U. H. Zaidan, N. I. Mohamad Zen, N. A. Amran, S. Shamsi, S. S. A. Gani, *Biochemical evaluation of phenolic compounds and steviol glycoside from Stevia rebaudiana extracts associated with in vitro antidiabetic potential*, Biocatal Agric Biotechnol, **18**, 101049 (2019).
6. A. Amnay, T. Ouriagli, S. M. Raoui, F. Errachidi, F. O. Chahdi, B. Bennani, Y. Kandri Rodi, R. Chabir, *Antioxidant and Anti-Inflammatory Activities of Saponins Extracted from Marrubium vulgare L. Collected in Fez-Meknes Region in Morocco*, Trop J Nat Prod Res, **8**, 3613–3619 (2023).
7. J. OrtizViedma, N. Romero, L. Puente, K. Burgos, M. Toro, L. Ramirez, S.P. Aubourg, *Antioxidant and antimicrobial effects of stevia (Stevia rebaudiana Bert.) extracts during preservation of refrigerated salmon paste*, Eur J Lipid Sci Technol, **10**, 1600467 (2017).
8. T. Ouriagli, A. Amnay, S. M. Raoui, F. Errachidi, F. O. Chahdi, R. Chabir, *Alkaloids from Marrubium vulgare L.: Antioxidant and Anti-Inflammatory Activities as a Function of Extraction Methods*, Trop J Nat Prod Res, **7**, 3411–3420 (2023).
9. M. Waseem, Y. Majeed, T. Nadeem, L. H. Naqvi, M. A. Khalid, M. M. Sajjad, J. M. Lorenzo, *Conventional and advanced extraction methods of some bioactive compounds with health benefits of food and plant waste: A comprehensive review*, Food Front, **4**, 1681-1701 (2023).
10. P. B. Jeppesen, S. Gregersen, K. K. Alstrup, K. Hermansen, *Stevioside induces antihyperglycaemic, insulinotropic and glucagonostatic effects in vivo: studies in the diabetic goto-Kakizaki (GK) rats*, Phytomed. **9**, 9-14 (2002).
11. P. Chan, B. Tomlinson, Y. J. Chen, J. C. Liu, M. H. Hsieh, J. T. Cheng, *A double-blind placebo-controlled study of the effectiveness and tolerability of oral stevioside in human hypertension*, British J Clin Pharmacol, **3**, 215-220 (2001).
12. Y. Xi, T. Yamaguchi, M. Sato, M. Takeuchi, *Antioxidant mechanism of Stevia rebaudiana extract and antioxidant activity of inorganic salts*, JJSFST, **45**, 317–322 (1998).

13. E. Nakilcioglu-Tas, S. Otles, *Influence of extraction solvents on the polyphenol contents, compositions, and antioxidant capacities of fig (Ficus carica L.) seeds*, *An Acad Bras Cienc*, **1** (2021).
14. U. H. Zaidan, S. N. Hamid, M. F. M. Yusof, S. Ahmad, S. S. A. Gani, S. Shamsi, *Chemical evaluation and antioxidant properties of extracts and essential oil from Stevia rebaudiana leaves*, *Malays Appl Biol*, **2**, 15-23 (2018).
15. U. H. Zaidan, N. I. M. Zen, N. A. Amran, S. Shamsi, S. S. A. Gani, *Biochemical evaluation of phenolic compounds and steviol glycoside from Stevia rebaudiana extracts associated with in vitro antidiabetic potential*, *Biocatal Agric Biotechnol*, **18** (2019).
16. M. Papaefthimiou, P. I. Kontou, P. G. Bagos, G. G. Braliou, *Antioxidant activity of leaf extracts from Stevia rebaudiana Bertoni exerts attenuating effect on diseased experimental rats: a systematic review and meta-analysis*, *Nutrients*, **15**, 3325 (2023).
17. V. Jaitak, A. P. Gupta, V. K. Kaul, P. S. Ahuja, *Validated high-performance thin-layer chromatography method for steviol glycosides in Stevia rebaudiana*, *J Pharm Biomed Anal*, **4-5**, 790-794 (2008).
18. A. E. Motto, P. Lawson-Evi, B. Bakoma, K. Eklugadegbeku, K. Aklikokou, *Antihyperlipidemic and antioxidant properties of hydro-alcoholic extracts from Anogeissus leiocarpus (Combretaceae)*, *Heliyon*, **4**, 6648 (2021).
19. S. Mouffouk, S. Gómez-Ruiz, M. Benkhaled, S. Carralero, H. Haba. *Phytochemical Composition, Antioxidant and Antibacterial Activities of Crude Extracts from the Species Euphorbia Atlantica Coss*, *Pharm Chem J*, **9**, 831-837 (2019).
20. L. Li, S. Chiroma, T. Hashim, S. Adam, M. Mohd Moklas, Z. Yusuf, S. Rahman, *Antioxidant and anti-inflammatory properties of Erythroxylum cuneatum alkaloid leaf extract*, *Heliyon*, **6**, 04141 (2019).
21. O. U. Amaeze, G. A. Ayoola, M. O. Sofidiya, A. A. Adepoju-Bello, A. O. Adegoke, H. A. B. Coker, *Evaluation of antioxidant activity of Tetracarpidium conophorum (Müll. Arg) Hutch & Dalziel leaves*, *Oxid Med Cell Longev*, **2011**, 1-7 (2011).
22. N. R. Mohamad, S. S. Abdul Gani, R. Abdul Wahab, W. Darham, *Natural antioxidant from Hibiscus sabdariffa extract: Assessments on extraction yield, antioxidant capacity and total polyphenol content of different polarities of solvent extracts*, *J Smart Sci Tech*, **2**, 52-60 (2022).
23. E. Gill-Martin, T. Forbes-Hernandez, A. Romero, D. Cienciosi, F. Giampieri, M. Battino, *Influence of the extraction method on the recovery of bioactive phenolic compounds from food industry by-products*, *Food Chem*, **378**, 131918 (2022).
24. T. Zhang, Q. Peng, Y. Xia, Y. Zhang, K. Myint, J. Wu. *Steviol glycosides, an edible sweet surfactant that can modulate the interfacial and emulsifying properties of soy protein isolate solution*, *J Food Eng*, **289**, 110264 (2021).
25. A. B. Rao, G.R. Reddy, P. Ernala, S. Sridhar, Y. V. L. Ravikumar, *An improvised process of isolation, purification of steviosides from Stevia rebaudiana Bertoni leaves and its biological activity*, *Int J Food Sci Tech*, **12**, 2554-2560 (2012).
26. V. López, S. Pérez, A. Vinuesa, C. Zorzetto, O. Abian, *Stevia rebaudiana ethanolic extract exerts better antioxidant properties and antiproliferative effects in tumour cells than its diterpene glycoside stevioside*, *Food & function*, **4**, 2107-2113 (2016).