

Antioxidant Ability, Total phenolic and Flavonoid contents of Leaf extract of *Stevia rebaudiana* Bertoni Cultivated in Morocco

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Abstract— Leaf extract of *Stevia rebaudiana* promotes effects on certain physiological systems such as the cardiovascular and renal and it also influences hypertension and hyperglycemia. The objective of the current study is to evaluate the contribution made through the free radical scavenging capability of leaves of *S. rebaudiana* cultivated in different areas in Morocco. The leaves extracts of *S. rebaudiana* were evaluated for their total phenols, flavonoids content and total antioxidant capacity. Total phenols and flavonoids were quantified using standard procedures and total antioxidant activity of aqueous, ethanolic and methanolic extracts of *S. rebaudiana* leaves was assessed by 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay. The total phenolic compounds ranged from 37.13 to 67.85, 27.56 to 47.77 and 25.39 to 43.45 mg/g dry weight, expressed as gallic acid equivalents to different solvents in aqueous, ethanolic and methanolic extracts, respectively. The flavonoids content varied from 33.31 to 50.04, 19.87 to 33.86 and 18.92 to 27.03 mg/g dry weight, expressed as rutin equivalents to different solvents in aqueous, ethanolic and methanolic extracts, respectively. Total antioxidant activity ranged from 78.08 to 69.01%, 66.42 to 59.56% and 68.53 to 61.90% equivalent to different solvents in aqueous, ethanolic and methanolic extracts of stevia leaves, respectively. The result showed that aqueous extraction was the most efficient, exhibiting the highest antioxidant capacity, followed by ethanol and methanol, respectively. The obtained results clearly indicates that *S. rebaudiana* leaves cultivated in Morocco had an important value of utilization and development as a natural antioxidant.

Index Terms— *Stevia rebaudiana*, Antioxidant activity, Total phenolic, Flavonoids, DPPH, Morocco.

1 INTRODUCTION

Active oxygen free radicals have been implicated as causative agents of cancer, atherosclerosis, cerebral and cardiac ischemia, Parkinson's disease, gastrointestinal disturbances and aging [1]. To remedy these problems, approximately 80% of the world populations depend exclusively on plants for their health and healing [2]. Recently there has been an upsurge of interest in the therapeutic potentials of plants, as antioxidants in reducing free radical induced tissue injury. Although several synthetic antioxidants, such as butylatedhydroxyanisole (BHA) and butylatedhydroxytoluene (BHT), are commercially available, but are quite unsafe and their toxicity is a problem of concern [3]. Hence, strong restrictions have been placed on their application and there is a trend to substitute them with naturally occurring antioxidants. Natural plant-based antioxidants especially phenolics and flavonoids have been exploited commercially either as antioxidant additives or as nutritional supplements [4]. Also many other plant species have been investigated searching new antioxidants [5]. Herbs are used in many domains, including medicine, nutrition, flavouring, beverages, dyeing, repellents, fragrances, cosmetics [6].

Therefore, natural antioxidants are known to exhibit a wide range of biological effects, including antibacterial, antiviral, anti-inflammatory, anti-allergic, antithrombotic and vasodilatory activity. In fact, a fundamental property considered important for life is antioxidant activity and this property may give rise to anticarcinogenicity, anti-mutagenicity, and anti-aging activity, among others [7]-[8].

Recently, there is an increased interest in *S. rebaudiana* Bertoni leaves as a good source of non-caloric sweeteners known as steviol glycosides, with a great potential in food industry as a strategy to reduce sugar consumption [9].

Stevia sweetener extractives have been suggested to exert beneficial effects on human health, including antihypertensive [10], antihyperglycemic, non-cariogenic, anti human rotavirus activities, glucose metabolism and renal function [11]. Moreover, stevia leaves have a high amount of phenolic compounds, vitamin C, carotenoids, chlorophylls [12]. And also, stevia leaf which is a good source of carbohydrates, protein, crude fiber, minerals, that promotes wellness and reduces the risk of certain diseases [13]. This plant is a perfect answer to the needs of consumers, combining the qualities of a sweetener, but also constituting a source of many substances with a nutritional effect on the human organism [14].

The leaves of this green plant, which is particularly very popular in Japan where it has been widely used as a sweetener over 35 years, have a delicious and refreshing taste that can be 30 times sweeter than sugar. In 2006, the World Health Organization (WHO) performed a thorough evaluation of recent experimental studies of stevioside and steviolins conducted on animals and humans, and concluded that "stevioside and

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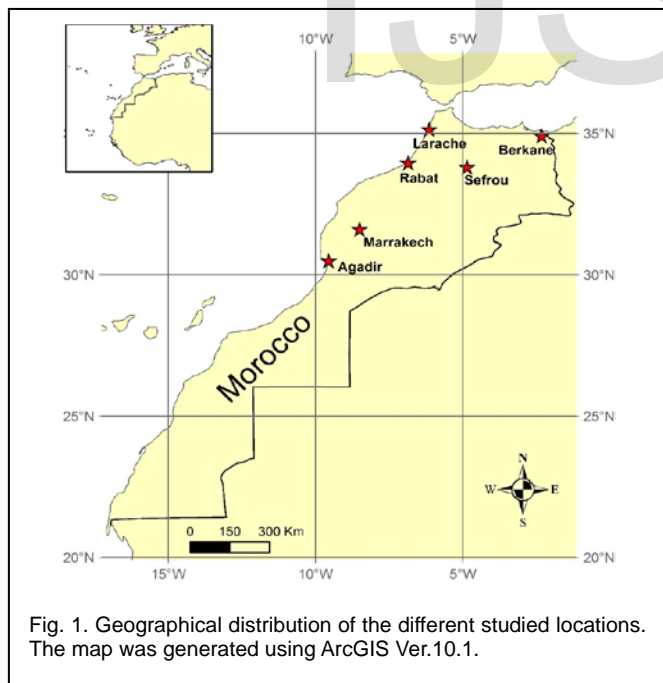
rebaudioside A are not genotoxic in vitro or in vivo and that the genotoxicity of steviol and some of its oxidative derivatives in vitro is not expressed in vivo" [15].

Stevia is a newly cultivated crop in Morocco that is known for its multiple benefits for human health. However, despite its multiple benefits, stevia extract use is limited. The antioxidant capacity of *S. rebaudiana* cultivated in Morocco has not been evaluated before. In this study, we evaluated for the first time, the antioxidant activity of *S. rebaudiana* plants cultivated in different regions in Morocco. The content of total phenolic and flavonoids were also determined. The present investigation evaluated three different solvents for their relative capacity to extract total phenolic and total flavonoid components of the leaves of *S. rebaudiana*: aqueous, ethanol and methanol, with the aim of classification and identification of the similarity and differences between the cultivars bioactive compounds.

2 MATERIALS AND METHODS

2.1 Plant material

S. rebaudiana plants were reproduced by seedlings in the greenhouse during February 2017, and transplanted in the experimental field on late April, in six areas in Morocco (Agadir, Berkane, Larache, Marrakech, Rabat and, Sefrou). These regions (Fig.1) are ecologically different. The stevia plants that reached the maximum growth stage (mature stage before flowering) were harvested in August by cutting the plant at 5-10 cm from the soil level. The brown and yellow leaves were removed. The plants were dried at 40 °C for 48 h



2.2 Preparation of the extract

The solvents and the chemicals used were of analytical grade, aqueous, methanol and ethanol were used as solvent for extraction of antioxidants compounds. 2,2-diphenyl-1-picrylhydrazyl (DPPH), sodium carbonate (Na_2CO_3), Folin-

Ciocalteu, gallic acid, aluminiumtrichlorid (AlCl_3) and rutine.

The air-dried leaves of *S. rebaudiana* (10 g) were powdered and then extracted with 100 ml of methanol, ethanol and aqueous by maceration process. The crude extract was filtered and evaporated under reduced pressure to give a viscous mass. The extract was stored at 4 °C for further use [16].

2.3 Determination of total phenolic content

Total soluble phenolic amount in the leaf extract of *S. rebaudiana* was measured spectrophotometrically by Folin-Ciocalteu method based on a colorimetric oxidation/ reduction reaction of phenols [17]. This method was employed to evaluate the phenolic content of the samples. A standard curve must first be plotted using gallic acid as a standard. The amount of total phenolic compounds was measured using the method of (Ghanta et al., 2007) [18]. 2 ml of 2% Na_2CO_3 was added to 10 μl of extract, and the samples were incubated at 25 °C for 2 minutes. Then, 100 μl of 50% Folin-ciocalteu's phenol reagent was added to mixture, and the contents were mixed thoroughly. The final mixture was shaken and then incubated for 30 min in the dark at room temperature. The absorbance of all samples was measured at 760 nm and the results are expressed in mg of gallic acid per g of the dry weight of plant.

2.4 Flavonoids Contents

The Total total flavonoids contents were determined according to colorimetric method described by Ordonez et al., (2006) [19]. Using a method based on the formation of a complex flavonoid-aluminium, having the absorptivity maximum at 430 nm. Rutin was used to make the calibration curve. 1 ml of diluted sample was separately mixed with 1 ml of aluminium trichlorid AlCl_3 (2%). After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 430 nm and the flavonoids content was expressed in mg per g of rutin equivalent ($\text{mg rutin g}^{-1} \text{ dw}$).

2.5 Determination of DPPH (1,1-diphenyl 2-picryl hydrazyl) radical scavenging activity

For determination of the antioxidant activity of stevia extracts, the stable, DPPH radical was used by Sun & Ho method [20]. An aliquot 0.5 ml of DPPH solution was diluted in 4.5 ml of methanol and 10 μl of extract was added. A control without extract was also maintained. The mixture was shaken vigorously and allowed to stand for 45 minutes in the dark and the absorbance was measured at 515 nm. The DPPH radical is commonly used for the assessment of antioxidant activity in vitro and it is foreigner to biological systems. It's a very stable organic free radical with deep violet color which gives absorp-tion maxima within 515-528 nm range [21].

DPPH radical scavenging activity was expressed as the inhibition percentage of free radical by the sample and was calculated using the following formula:

$$\% \text{ Inhibition} = (A_0 - A_t / A_0) \times 100$$

Where A_0 was the absorbance of the control (blank without extract) and A_t was the absorbance in the presence of the extract. All the tests were performed in triplicate and the graph

was plotted with the mean values.

The synthetic antioxidants butylhydroxyanisole (BHA) and butylhydroxytoluene (BHT) prepared in ethanol (0.1 ml of each) were allowed to react with 3.9 ml of the DPPH solution and vortex and then the absorbance was measured at 515 nm after 45 min.

2.6 Statistical Analysis

Statistical analyses were conducted using SPSS (Statistical Program for Social sciences) version 23.0. All analyses were performed in triplicate (n=3) and data reported as means ± standard deviation (SD) using one-way analysis of variance (ANOVA). Significance of the F-test was estimated at P<0.05. A Duncan multiple range test was used to determine specific differences between means.

3 RESULTS AND DISCUSSION

The present study was carried out to evaluate the in vitro antioxidant potential of different organics extracts of *S. rebaudiana* (Leaves) cultivated in Morocco. Three solvents were used for the extraction of *S. rebaudiana*. For each sample, DPPH free radical scavenging, reducing power, total phenolics and total flavonoids concentration were determined. The results are presented in Figures 2, 3, 4 and 5.

3.1 Antioxidant activity of *Stevia rebaudiana* extracts

DPPH method is widely reported for screening of antioxidants and for determining comparative antioxidant effectiveness [21]. DPPH is a stable free radical in room temperature and accepts an electron or hydrogen radical to become a stable diamagnetic molecule [22]. Positive DPPH test suggests that the samples were free radical scavengers (Fig.2). The yield of aqueous extracts of the stevia's leaves in the six regions were found to be ranging between 69.01 and 78.08% in Sefrou and Agadir regions respectively. These levels are higher to those reported by Tadhani et al. (2007) [23]. Also for ethanolic extracts, the data shows that they varied widely from one area to another and ranged from 59.56 to 66.42% in Sefrou and Agadir cultivars respectively. From a comparative point of view, the values found in this study are generally equal to those reported by Ahmad et al. (2010) [24] and Shukla et al. (2009) [25]. Concerning methanolic extracts, the mean values content varies from 61.90 to 68.53% in Sefrou and Agadir regions respectively. Comparing our results about methanolic extracts of *S. rebaudiana* leaves with those found by other authors, our values are generally higher than those reported by Tadhani et al. (2007) [23]. On the other hand, these values are lower than those presented by Ahmad et al. (2010) [24]. Agadir had the highest total phenolics and flavonoids contents and the strongest scavenging abilities to DPPH followed by Larache, Rabat, Marrakech, Berkane and Sefrou, respectively.

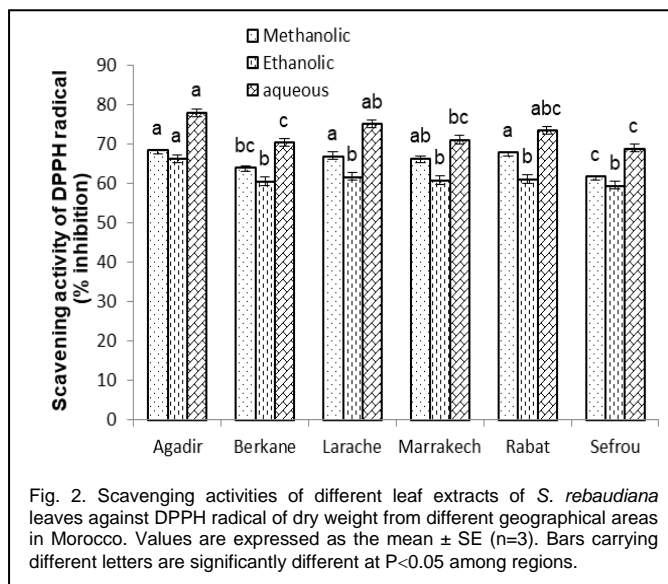


Fig. 2. Scavenging activities of different leaf extracts of *S. rebaudiana* leaves against DPPH radical of dry weight from different geographical areas in Morocco. Values are expressed as the mean ± SE (n=3). Bars carrying different letters are significantly different at P<0.05 among regions.

Percent inhibition of DPPH radical with aqueous extracts of stevia leaves was significantly (P<0.05) different from ethanolic and methanolic ones. In fact, Aqueous extracts showed a significantly (P<0.05) higher percent inhibition of DPPH radical compared to ethanolic and methanolic extracts of stevia leaves. Their antioxidant activity is mainly due to their redox properties, hydrogen donors and singlet oxygen quenchers, which can play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides [26]. As the concentration of phenolic compounds or degree of hydroxylation of the phenolic compounds increases, their DPPH radical scavenging activity also increases, thus correlating directly to the extent of antioxidant efficacy of a typical plant material [27].

Recently, it has been reported that Rebaudioside A had significant relationship with the total antioxidant capacity as total phenolics [28]-[29].

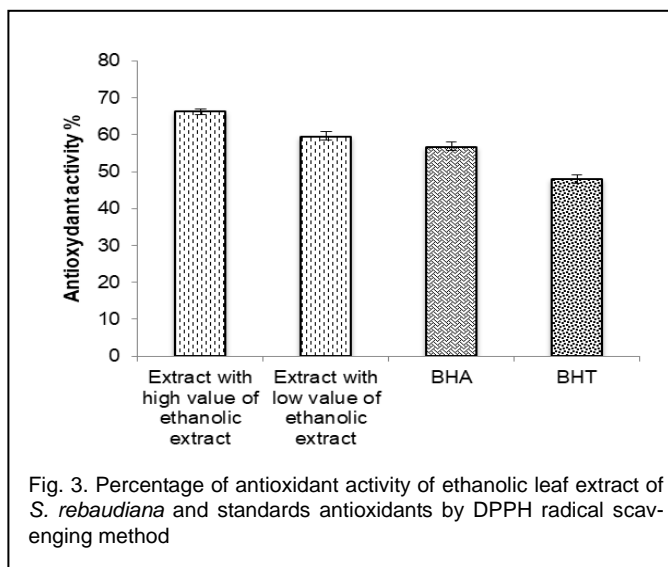


Fig. 3. Percentage of antioxidant activity of ethanolic leaf extract of *S. rebaudiana* and standards antioxidants by DPPH radical scavenging method

Compared with the two standards reference BHA and BHT, DPPH radical-scavenging abilities of the different cultivars of stevia are more important (Fig.3). The extracts demonstrated a concentration dependent scavenging activity by quenching DPPH radicals: DPPH radical scavenging activities of different cultivars increased with increased content. The scavenging activities of the extracts were significantly higher ($P < 0.05$) than that of BHT and BHA, showing the values 47.89% and 56.70% respectively. Many synthetic antioxidants such as butylated hydroxyl anisole (BHA) and butylated hydroxyl toluene (BHT) are very effective and are used for industrial processing but they possess potential health risk and toxic properties to human health and should be replaced with natural antioxidants [30].

3.2 Total phenolic and flavonoids contents

Phenolic compounds are frequently found in both edible and non-edible plants, and have been reported to have multiple biological effects, including antioxidant activity. Contents of flavonoid and other phenolic substance have been suggested to play a preventive role in the development of cancer and heart diseases [31].

The total phenolic compounds and flavonoids content presented in the different extracts of the *S. rebaudiana* leaves are shown in Fig.4 and 5. Total phenolic compound contents measured with different extracts of samples belonging to different geographic region of Morocco was found to be ranging from 37.13 to 67.85 mg gallic acid per gram dw of stevia's leaves on dry weight, of aqueous extracts in this order Agadir > Larache > Rabat > Marrakech > Sefrou > Berkane. However the data of total phenolic contents in the ethanolic and methanolic extracts, shows that they varies widely from one area to another and ranges from 27.56 to 47.77 mg/g, 25.39 to 43.45 mg/g dw. Total phenolic contents in stevia leaves from various regions showed significant difference. Agadir owned the highest value in total phenolic content compared to the other six Moroccan cultivars.

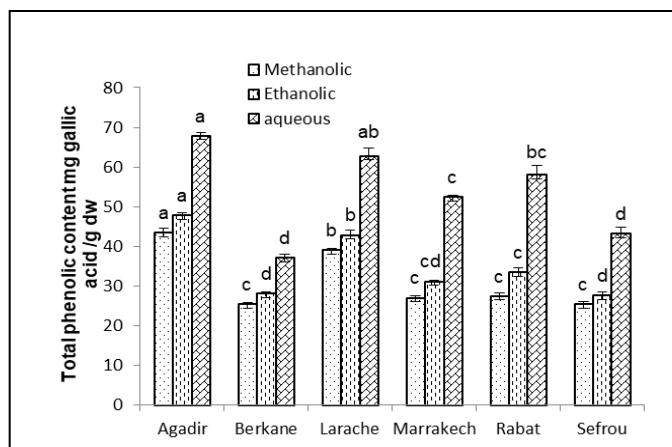


Fig. 4. Total phenolic compounds content of *S. rebaudiana* leaves is equivalent to gallic acid of dry weight from different geographical areas in Morocco. Values are expressed as the mean \pm SE (n=3). Bars carrying different letters are significantly different at $P < 0.05$ among regions.

Flavonoids contents varied from 33.31 to 50.04 mg rutin/g, 19.87 to 33.86 mg/g and 18.92 to 27.03 mg/g dw, respectively for aqueous, ethanolic and methanolic extracts of stevia leaves (Fig.5). These result of the content of total phenolic compounds and flavonoids suggest that aqueous extracts are the highest compared to ethanolic and methanolic extracts. The amount of total flavonoids taken from different samples of *S. rebaudiana* leaves were in the following order of: Agadir > Larache > Rabat > Marrakech > Berkane > Sefrou. The total contents of flavonoids in the leaves of *S. rebaudiana* showed significant differences from one area to another.

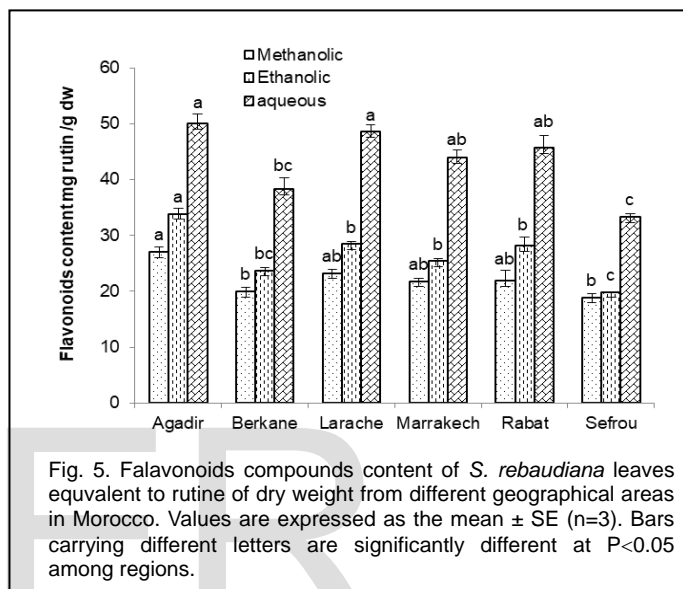


Fig. 5. Flavonoids compounds content of *S. rebaudiana* leaves equivalent to rutin of dry weight from different geographical areas in Morocco. Values are expressed as the mean \pm SE (n=3). Bars carrying different letters are significantly different at $P < 0.05$ among regions.

The polyphenolic compounds have inhibitory effects on mutagenesis and carcinogenesis in humans when ingested up to 1 g daily from a diet rich in fruits and vegetables [32]. Phenolic compounds from plants are known to be good natural antioxidants. However, the activity of synthetic antioxidants was often observed to be higher than that of natural antioxidants [33].

In this study, the total phenolic and flavonoids extracted from *S. rebaudiana* by aqueous were found to be higher than those reported by Kim et al. (2011) [34] and Shukla et al. (2012) [16]. And also, for methanolic extracts, our results were equal or higher than those found by Tadhani et al. (2007) [23], which suggested its potential use as a natural source of phenolics and flavonoids. Furthermore, our ethanolic extracts values are lower than those found by Zeng et al. (2013) [29]. These significant differences between results may be likely due to environments and could also have an impact on the total leaf phenolics content. The leaves of *S. rebaudiana* plants have grown under conditions of Morocco. These results suggest that the higher levels of antioxidant activity were due to the presence of phenolic and flavonoid components. The phenolic compounds may contribute directly to the antioxidative action [35], and they are suggested to play a preventive role in the development of cancer and heart diseases [31]. The interest in phenolics is in increase in the food industry because these compounds delay the oxidative degradation of lipids, thereby

improving the quality and the nutritional value of the food [36]. Moreover, stevia has numerous therapeutic values in the treatment of patients with diabetes-related obesity, hypertension or cardiac disease, antioxidant, antimicrobial and antifungal activity, for which sweetening properties have been identified. And also, many studies assessing the in vitro potential of leaf extract of *S. rebaudiana* indicates that it has a significant potential for use as a natural antioxidant [37].

4 CONCLUSION

This study researched the contents of bioactive compounds and the antioxidants activities of six cultivars from different areas in Morocco. A large variability in these contents was observed among the different cultivars. We demonstrated that different leaf extract of *S. rebaudiana* contained higher levels of total phenolic compounds and was capable of inhibiting or reducing free radicals to terminate the radical chain reaction, and acting as a reducing agent. Stevia extracts have stabilization efficiency comparable to commonly employed synthetic antioxidants BHT and BHA at their legal limit; Therefore, they can be recommended as a potent source of natural antioxidants with consequent health benefits and uses for food preservation as well as pharmaceutical and natural plant based products owing to its high antioxidant activity.

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