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Radical scavenging activity of steviol glycosides, steviol glucuronide, hydroxytyrosol, metformin, aspirin and leaf extract of *Stevia rebaudiana*

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ABSTRACT

Introduction: Reactive oxygen species (ROS), generated in many bioorganic redox processes, are the most dangerous by-products in the aerobic environment. The aim of this study was to compare the *in vitro* antioxidant activity of some chemical compounds and leaf extract of *Stevia rebaudiana* as natural antioxidant.

Methods: The radical scavenging activity of ascorbic acid, quercetine, stevioside (ST), rebaudioside A (Reb A), steviol glucuronide (SVglu), glucose, sucrose, hydroxytyrosol, metformin and aspirin were measured and expressed as the inhibitory concentration in mM giving 50% reduction of radicals (IC_{50}).

Results: Ascorbic acid, quercetine, ST, Reb A, SVglu, glucose, sucrose, hydroxytyrosol, metformin and aspirin were active •OH scavengers. Superoxide radicals were efficiently scavenged by ascorbic acid, SVglu, quercetine, hydroxytyrosol, ST, Reb A and aspirin. Quercetine, hydroxytyrosol, ascorbic acid and aspirin were active in limiting the amount of thiobarbituric acid (TBA) reactive material. Only ascorbic acid, quercetine and hydroxytyrosol showed DPPH and NO scavenging activity. Leaf extract of *S. rebaudiana* had an excellent ROS scavenging activity. Treatment of leaf extracts with PVPP and active charcoal removed a part of their scavenging activity. Results showed that sugars were not efficient ROS scavengers.

Conclusion: Radical scavenging activity of steviol derivatives, aspirin, metformin and hydroxytyrosol might explain most of beneficial pharmacological effects on ROS related diseases, such as hypertension, type 2 diabetes, atherosclerosis, inflammation and certain forms of cancers. The results obtained in this study indicate that leaf extract of *S. rebaudiana* has a great potential to use as a natural antioxidant agent.

1. Introduction

Reactive oxygen species (ROS), generated in many bioorganic redox processes, are the most dangerous by-products in the aerobic environment. Many diseases are caused by or related to ROS. Stress also induces the production of ROS[1,2] which results in cell death and tissue damage. The role of oxygen radicals has been implicated in several diseases, including cancer, diabetes and cardiovascular diseases, ageing, neural disorders and arthritis.[3]

Recently, it was shown that during evolution organisms began using ROS to control and regulate different biological processes, e.g., growth, cell cycle, hormone signalling and apoptosis.[4] In spite of this adaptation, organisms must possess ROS scavenging mechanisms to survive and to control the amount of ROS. In the body, there are different ROS scavenging enzyme systems like superoxide dismutases, catalase, glutathione peroxidase and ascorbic acid peroxidase. These, together with non-enzymatic antioxidants such as, e.g., glutathione, uric acid, ascorbic acid and tocopherol are able to scavenge and detoxify ROS in the cells very efficiently.[1]

Many plant compounds are known to be ROS scavengers or have beneficial pharmacological effects. The active ingredients of a medicinal plant are mainly its secondary metabolites, among which the phenolic fraction is an important antioxidant. *Stevia rebaudiana*

Abbreviations: CVD, cardiovascular disease; DPPH, 2,2-diphenyl-1-picrylhydrazyl; HTPA, 2-hydroxy-terephthalic acid; IC_{50}, inhibiting concentration at which 50% of the radicals are scavenged (expressed in mM); NBT, nitro blue tetrazolium; PMS, phenazolium methosulphate; PVPP, polyvinylpyrrolidone; Reb A–F, rebaudioside A–F; RNS, reactive nitrogen species; ROS, reactive oxygen species; ST, stevioside; SVglu, steviol glucuronide; SVgly, steviol glycoside; TBA, thiobarbituric acid; TPA, terephthalic acid.

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is a medicinal plant with a huge demand in pharmaceutical, food and beverage industries as a source of low calorie and high potency natural sweeteners. Steviol glycosides (SVglys) are the compounds responsible for the sweet taste of *S. rebaudiana*. Stevioside (ST) and rebaudioside A (Reb A) are the major SVglys.[5] Shukla et al[6] found significant ROS scavenging activity of crude ethanolic *Stevia* leaf extracts. Kroyer[7,8] reported that ST significantly reduced the breakdown of ascorbic acid (vitamin C) when a water solution containing both substances was heated at 80 °C for up to 4 h. In *Stevia* leaves, some compounds other than SVglys, e.g. folic acid, pyrogallol, phenolic and flavonoid, have ROS scavenging activity.[9] Among the 230 species in the genus *Stevia*, only the species *S. rebaudiana* and *S. phlebophylla* produce SVglys.[10]

Many studies showed that SVglys have different beneficial pharmacological effects, such as lowering of blood pressure, regulation of blood glucose in type 2 diabetes while enhancing the insulin sensitivity, prevention of atherosclerosis and prevention of certain forms of cancers.[11] As all these effects are induced by or related to reactive oxygen and nitrogen species, we studied the possible ROS and reactive nitrogen species (RNS) scavenging activity of SVglys and steviol glucuronide (SVglu), which occurs in peripheral blood after consumption of ST and which is excreted in urine.[12–14] Previously, it was shown that SVglys had a very potent •OH scavenging activity.[15,16] As SVglys contain glucose and other sugar units, we were interested to know if ROS scavenging is due to the sugar units and therefore some sugars were included in this research.

Metformin is an oral diabetes medicine that helps control blood sugar levels. It is surprising that type 2 diabetes patients treated with metformin seem to have a reduced cancer burden including colorectal cancer compared with patients treated with other drugs.[17] Memmott et al[18] suggested that metformin might be a good candidate for lung cancer chemoprevention. Engelman and Cantley[19] further discuss the potential mechanisms of metformin action and their implications for cancer prevention and therapy.

Aspirin is a widely prescribed drug used primarily to treat inflammation. Long term use of aspirin in human has also been reported to protect against the development of colon cancer and other digestive systems cancer, including cancer of oesophagus and stomach. Aspirin may protect biological targets from hydroxyl radical-induced cell injury by scavenging this radical.[20] Aspirin has also been shown to play a rule in prevention of atherosclerosis by protecting endothelial cells of vascular wall from damage caused by oxygen radicals.[21]

Hydroxytyrosol and other phenolic compounds from vergin olive oil are well known antioxidants delaying *in vitro* metal-induced and radical-dependent low density lipid oxidation.[6,19] Their antioxidant activity may potentiate them to prevent atherosclerosis and cardiovascular diseases.[22–24]

The aim of the present study is to compare ROS scavenging activity of some chemical and natural compounds. Ascorbic acid and quercetine were used as a positive control. SVglys and metformin help control blood sugar levels and therefore they were included in this study to compare their antioxidant activity. To study the correlation between anti-inflammatory activity of aspirin and ROS scavenging activity, aspirin was included in this study. Glucose units are part of the SVglys and therefore glucose, sucrose and trehalose, were included in this experiment to investigate if there is a correlation between SVglys antioxidant activity and their sugar units. As *S. rebaudiana* is a source of SVglys, the crude leaf extracts were included to evaluate their ROS scavenging activity.[25] Hydroxytyrosol known to have similar pharmacologic effects as SVglys, is also included in this study. The 2,2-diphenyl-1-picrylhydrazyl (DPPH), hydroxyl (•OH), superoxide and nitric oxide (NO) radicals were studied to compare the activity of ROS scavengers. ROS can directly attack membrane lipids and increase lipid peroxidation which increases malondialdehyde content (Anjum et al 2011). Therefore, thiobarbituric acid (TBA) reactive material was analysed to better understand antioxidant activity of chemical and natural ROS scavengers.

2. Materials and methods

ST and Reb A were purified and crystallized to a purity of over 99%. The purity was checked by MS and NMR as described by Struyf et al.[26] The sample of SVglu was prepared from urine, collected from volunteers who consumed 750 mg ST about 3 h before the start of the 24 h urine collection period.[13] Glucose, sucrose and trehalose were of PA quality of Merck. Metformin, aspirin and ascorbic acid were from pharmacia. Hydroxytyrosol of 98% purity came from Xian App-Chem, Biotech Co, Ltd, Xian Hi-tech Zone, China. Quercetine was from Sigma. Ascorbic acid and quercetine were used as positive controls.

2.1. •OH radical scavenging activity assay

•OH scavenging activity was measured according to a modified method of Linxiang et al[27]; Šnyrychová & Hideg[28]; Geuns & Struyf[15]; Stoyanova et al.[16] Terephthalic acid (TPA) is used as a radical scavenger which after contact with hydroxyl radicals, 2-hydroxy-terephthalic acid (HTPA) is formed as a stable end product. TPA itself is barely fluorescent, but the HTPA has a strong fluorescence. The reaction mixture (1800 μL) contained 500 μM TPA, 10 μM EDTA, 10 μM $FeSO_4$, 100 μM ascorbic acid, 600 μL scavengers solution and 100 μM H_2O_2. All solutions were made in 50 mM potassium-phosphate buffer (pH 7.2). As the 2-hydroxy-terephthalic acid (HTPA) formation progresses for several hours, and as the HTPA formed is very stable, the reaction product was measured after about 16 h at room temperature. To measure the fluorescence of the HTPA formed, an HPLC with a fluorescence detector (excitation at 315 nm, emission at 420 nm) was used. The HPLC column was substituted for by an empty tube of about 1.0 m length. The solvent used was water of HPLC quality at a flow rate of 1.0 mL/min. Each run takes about 0.3 min, allowing an injection every 2 min.

2.2. Superoxide radical scavenging activity assay

The superoxide radical scavenging assay is based on the reduction of nitro blue tetrazolium (NBT) in the presence of NADH and phenazolium methosulphate (PMS) under aerobic conditions.[29] The assay mixture consisted of 500 μL 180 μM NBT solution (prepared in 0.02 M tris buffer; pH 8), 500 μL 540 μM NADH solution (prepared in 0.02 M tris buffer; pH 8) and 600 μL scavengers solution. Then, 200 μL 135 μM PMS solution was added to the mixture. The samples were centrifuged at 13,000 rpm for 2 min. The absorbance was read at 560 nm by spectrophotometer.

2.3. MDA by TBA fluorimetric assay

A modified protocol of the TBA assay for lipid peroxidation was used.[30] All solutions were made in 50 mM potassium-phosphate buffer (pH 7.2). The reaction mixture (1800 μL) consisted of 3.6 mM 2-deoxyribose, 10 μM EDTA, 10 μM $FeSO_4$, 100 μM ascorbic acid, 600 μL plant extract and 100 μM H_2O_2. A blank was also prepared which contained everything but without 2-deoxyribose. The reaction mixture was kept at 37 °C for 1.0 h. After incubation, 200 μL was taken and 200 μL 1.0 N HCl was added, followed by 100 μL 0.35% TBA. The pH should be around 1.0. The cocktail was heated at 90 °C for 15 min. After cooling on ice, 200 μL H_2O was

added and the pink complex was extracted into BuOH (2 × 600 μL). After centrifugation of the samples, the upper phases were collected and pooled. The pink complex was extracted from the BuOH into 100 μL 4 N NaOH. After centrifugation, the BuOH was discarded. Then 80 μL of the lower phase was acidified with 2.0 N HCl in MeOH (170 μL) to avoid breakdown of the complex at pH values above 8.0. Ten μL of sample was injected into an HPLC with a fluorescence detector (excitation at 520 nm, emission at 550 nm), using an empty tube of about 1.0 m length instead of a column. The solvent used was HPLC quality water at a flow rate of 1.0 mL/min. Samples were quantified using a standard curve based on tetramethoxypropane. The results were expressed as percentage of the blank.

2.4. DPPH radical scavenging activity assay

DPPH makes stable free radicals in aqueous or ethanol solution.[31] The reaction mixture consisted of 1200 μL of 50 μM freshly prepared DPPH and 600 μL of the various scavengers (scavengers were prepared in different concentrations). After 30 min, the absorbance of samples was measured at 517 nm by a spectrophotometer.

To measure the radical scavenging of crude plant extracts, the protocol had to be adapted because after the disappearance of the red colour of the DPPH radical, the yellow pigments of the plant extract interfered with the measurement in the spectrophotometer. Therefore, the reaction mixtures were analysed by HPLC. It was also necessary to increase the DPPH stock solution to 500 μM. The samples of crude plant extracts were centrifuged before analysis in the HPLC. Twenty μL of sample was injected in the HPLC system with 1 Grace Alltima C18 column (250 × 4.6 mm ID, 5 μm particle size, UV detection at 517 nm, 10 mm path length). The samples were eluted with a isocratic solvent of AcCN : H_2O (90:10 v/v) at 1.0 mL/min flow rate for 10 min.

2.5. NO radical scavenging activity assay

NO is generated from sodium nitroprusside and measured by the Greiss reaction.[32] Six hundred μL of scavenger stock solution was added to 200 μL of sodium nitroprusside (100 mM, prepared in 50 mM phosphate buffer; pH 7.4) and incubated at 25 °C for 150 min in dark. Five hundred μL of sulfanilamide solution (prepared in 5% H_3PO_4) was added to samples and incubated at room temperature for 5–10 min in dark. Then 500 μL of NED solution was added and the samples were incubated at room temperature 5–10 min in dark. The absorbance of samples was read at 546 nm.

To measure the radical scavenging of crude plant extracts, the protocol had to be adapted and the reaction mixtures were analysed by HPLC. The samples of crude plant extract were centrifuged at 13,000 rpm for 5 min before analysis in the HPLC. Twenty μL of sample was injected in the HPLC system with 1 Grace Alltima C18 column (250 × 4.6 mm ID, 5 μm particle size, UV detection at 546 nm, 10 mm path length). The samples were eluted with a isocratic solvent of AcCN: H_2O (35:65 v/v) at 1 mL/min flow rate for 5 min.

2.6. Scavenging activity of plant extracts

Freeze-dried leaves of *S. rebaudiana* Bertoni were homogenized to a fine powder. One gram amount was 3 times extracted by boiling for 15 min with 15 mL HPLC quality water in closed Falcon tubes. The extracts were combined and were centrifuged and filtered to remove particles. Kim et al[9] suggested that *S. rebaudiana* accumulates high amounts of phenolic compounds. An alternative to the removal of phenolics from an aqueous medium is the addition of polyvinylpyrrolidone (PVPP)[33] and active charcoal.[34] Fractions of the water extracts were shaken with different amounts of PVPP (0–20% w/v of the water extract) or with active charcoal (0–20%) to remove phenols and pigments, respectively. PVPP and active charcoal were removed by centrifugation and the supernatants were filtered on DynaGard 0.2 μm ME Syringe Tip Filters. The results demonstrated that 10% of PVPP and active charcoal were the most effective amounts and clear extracts without pigments were achieved. The efficiency of higher amounts of PVPP and active charcoal in removing phenols and pigments were almost the same as 10% w/v. The phenol content of extracts after treatment with PVPP and active charcoal was measured using Folin and Ciocalteu's reagent.[35] The results showed that almost no phenols pigments were detected. Therefore, the subsequent experiments were done with 10% of PVPP and active charcoal. The extracts were stored at 4 °C for further use. The samples of plant extracts should be centrifuged before measuring their antioxidant activity by spectrophotometry or by HPLC. To measure the DPPH and NO radical scavenging of crude plant extracts, an HPLC analysis was required (see under the radicals concerned). All the results were normalised to percentage of the original ROS activity.

3. Results

3.1. Treatment of plant extracts

Table 1 shows the amounts of dry extract after freeze-drying the extracts before treatment, and after treatment with PVPP and active charcoal. Treatment of crude extracts with the insoluble PVPP (10%, w/v) mainly removed phenols and polyphenols whereas treatment with active charcoal (10% w/v) removed all pigments.

3.2. •OH radical scavenging activity

The •OH scavenging activity was measured by the areas of the fluorescence peaks of HTPA that is formed from terephthalate in the presence of •OH radicals. The obtained fluorescence values of the tested compounds were normalised to percentages of the control (100%) and plotted as log ($F^0/F-1$) against log (concentration of scavenger). This produces straight lines, proving that the HTPA inhibition, as a function of scavenger concentration, is a first order exponential function (Fig. 1). In this formula, F^0 is the fluorescence of the reaction mixture without scavenger, whereas F is the fluorescence of the mixture after the addition of scavenger.

From the log–log plots of Fig. 1, the half-inhibitory concentration of scavenger (IC_{50}•OH) can be calculated when $y = 0$ or when log ($F^0/F-1$) = 0, i.e., when the fluorescence value of F equals half the fluorescence of F^0. It follows then that log (1/0.5–1) = log (2–1) = 0 (Table 2). Table 2 gives the equations of the different log–log curves, their correlation coefficients (as r^2), IC_{50}•OH and the number of sugar units per molecule.

With $y = 0$ or (log ((F^0/F)–1) = 0), the calculation for e.g., stevioside becomes: $0 = 1.452x + 0.956$, or $x = -0.956/1.452 = -0.658$. The half-inhibitory concentration is obtained as follows: $IC_{50} = 10^{-0.658} = 0.219$ mM.

Table 1
Total dry weight obtained after extraction of 1 g and purification of crude plant extracts of *S. rebaudiana* with PVPP or active charcoal.

Plant species	Crude extract (mg)	Treatment with PVTP (mg)	Treatment with charcoal (mg)
Stevia rebaudiana	549	292	87

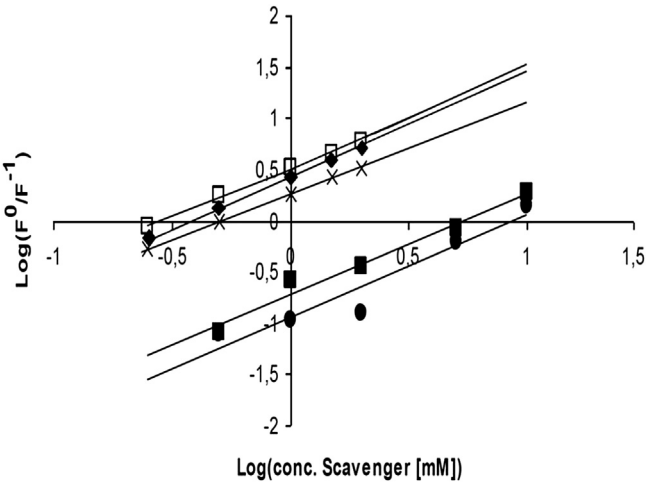

Fig. 1. Log–log plot of the inhibition of HTPA formation by different scavengers (□ ST, ◆ Reb A, × SVGlu, ● Gluc, ■ Sucr).

The results of chemical compound are presented in Table 2. ST, Reb A and SVglu have similar and excellent •OH scavenging activity as very small values for their IC_{50}•OH were found. Quercetine, one of the positive controls, had a still better •OH scavenging activity whereas the activity of ascorbic acid and hydroxytyrosol had an IC_{50}•OH of the same order of magnitude as sugars. Metformin and aspirin were also good •OH scavenging molecules. The half-inhibitory concentrations for SVgly and SVglu (average 0.225 ± 0.018 mM) are significantly smaller than those for the sugars (sucrose, glucose, trehalose; 1.377 ± 0.132). The number of sugar units attached to the steviol skeleton is given in the last column of Table 2.

Crude leaf extract of *S. rebaudiana* was a very reactive •OH scavenger and virtually destroyed all radicals (Fig. 2). Treatment of the extract with insoluble PVPP removed the phenols and moderately reduced radical scavenging from 95% to 92% (Fig. 2). However, after treatment with charcoal much of the scavenging activity was lost as about 64% of the radicals of the control were present.

3.3. Superoxide radical scavenging activity

The superoxide radical scavenging measurement was based on the reduction of nitro blue tetrazolium that was measured by spectrophotometer. The obtained values were normalised to percentages of the control (100%) and plotted as log (F^0/F−1) against log (concentration of scavenger). The positive control ascorbic acid is by far the best superoxide radical scavenging molecule, whereas quercetine had a value about 5× greater (Table 3). ST and RebA had a scavenging activity that was less than that of the positive controls. SVglu has an excellent IC_{50} value of 0.21, which is even better than that of quercetine. Glucose and sucrose had no superoxide radical scavenging activity. The radical scavenging activity of trehalose and of metformin was probably too small to be of any physiological significance. However, aspirin had an IC_{50} of 3.921 which might be enough to explain its anti-inflammatory effects. Hydroxytyrosol had an excellent superoxide scavenging activity (Table 3).

Crude extracts of *S. rebaudiana* were able to scavenge about 82% of the superoxide radicals (Fig. 2). Treatment with PVPP or charcoal reduced scavenging activity leaving about 25 and 55% of the radicals, respectively.

Table 2
•OH radical scavenging activity (Half-inhibitory concentrations, IC_{50} in mM) of the different scavengers. The obtained values of the tested compounds were normalised to percentages of the control (100%) and plotted as log (F^0/F−1) against log (concentration of scavenger).

•OH scavenger	Equation	r^2	IC_{50} in mM	Sugar units
Ascorbic acid	$y = 1.134x - 0.071$	0.953	1.154	0
Quercetine	$y = 0.678x + 0.638$	0.912	0.115	0
ST	$y = 1.452x + 0.956$	0.98	0.219	3
RebA	$y = 1.253x + 0.885$	0.983	0.196	4
SVglu	$y = 1.090x + 0.747$	0.97	0.206	1
Hydroxytyrosol	$y = 1.003x$	0.988	1	0
Glucose	$y = 1.339x - 0.260$	0.92	1.562	1
Sucrose	$y = 1.052x - 0.052$	0.969	1.119	2
Trehalose	$y = 0.879x - 0.142$	0.976	1.45	2
Metformin	$y = 0.974x + 0.015$	0.988	0.966	0
Aspirin	$y = 0.841x + 0.435$	0.934	0.305	0

3.4. TBA reactive material

The fluorescent MDA/TBA complexes were extracted and measured by fluorimetry. The area of obtained peaks were measured and normalised to percentages of the control (100%) and plotted as log (F^0/F−1) against log (concentration scavenger). Quercetine had the best activity in preventing the production of TBA reactive material, followed by hydroxytyrosol, ascorbic acid and aspirin (Table 4). The values for glucose and trehalose were extremely large, suggesting a lack of physiological function *in vivo*. The method could not be used for sucrose, probably because of interference by the fructose part of sucrose. The values obtained for SVgly were rather large and those for SVglu and metformin might be still too large to be of physiological significance (Table 4).

Crude water extracts of *S. rebaudiana* leaves reduced the production of TBA reactive material to about 32% of the control, *i.e.* 68% scavenging activity (Fig. 2). Treatment of *S. rebaudiana* extract with PVPP reduced the scavenging effect from about 68 to about 38%. Charcoal was able to further remove more of the scavenging activity from 38 to 12%.

3.5. DPPH radical scavenging activity

The obtained data from DPPH radical scavenging activity was normalised to percentages of the control (100%) and plotted as log (F^0/F−1) against log (concentration of scavenger). The positive controls and hydroxytyrosol showed a significant radical scavenging activity (Table 5). The other compounds did not show DPPH scavenging activity. DPPH scavenging activity of crude plant extracts could not be measured by spectrophotometer used for the purified compounds because the coloured products of the crude plant extracts interfered with the photometer readings. An HPLC analysis was used to study the DPPH radical scavenging of crude plant extracts as well as of hydroxytyrosol (example given in Fig. 3). Crude plant extract is a strong DPPH scavenger. To be able to measure their scavenging activity, the plant extracts had to be diluted 10 × proving the very strong DPPH radical scavenging of crude leaf extracts. Crude plant extract of *S. rebaudiana* (10 × diluted) scavenged DPPH radicals by 58% (Fig. 2). PVPP treatment removed part of the scavenging activity, whereas active charcoal was able to remove all of the scavenging activity.

3.6. NO radical scavenging activity

NO radicals generated from sodium nitroprusside were measured by the areas of the fluorescence peaks during the diazotization of nitrite with sulphanilamide and subsequent coupling with napthylethylenediamine. The obtained fluorescence values of the tested compounds were normalised to percentages of the

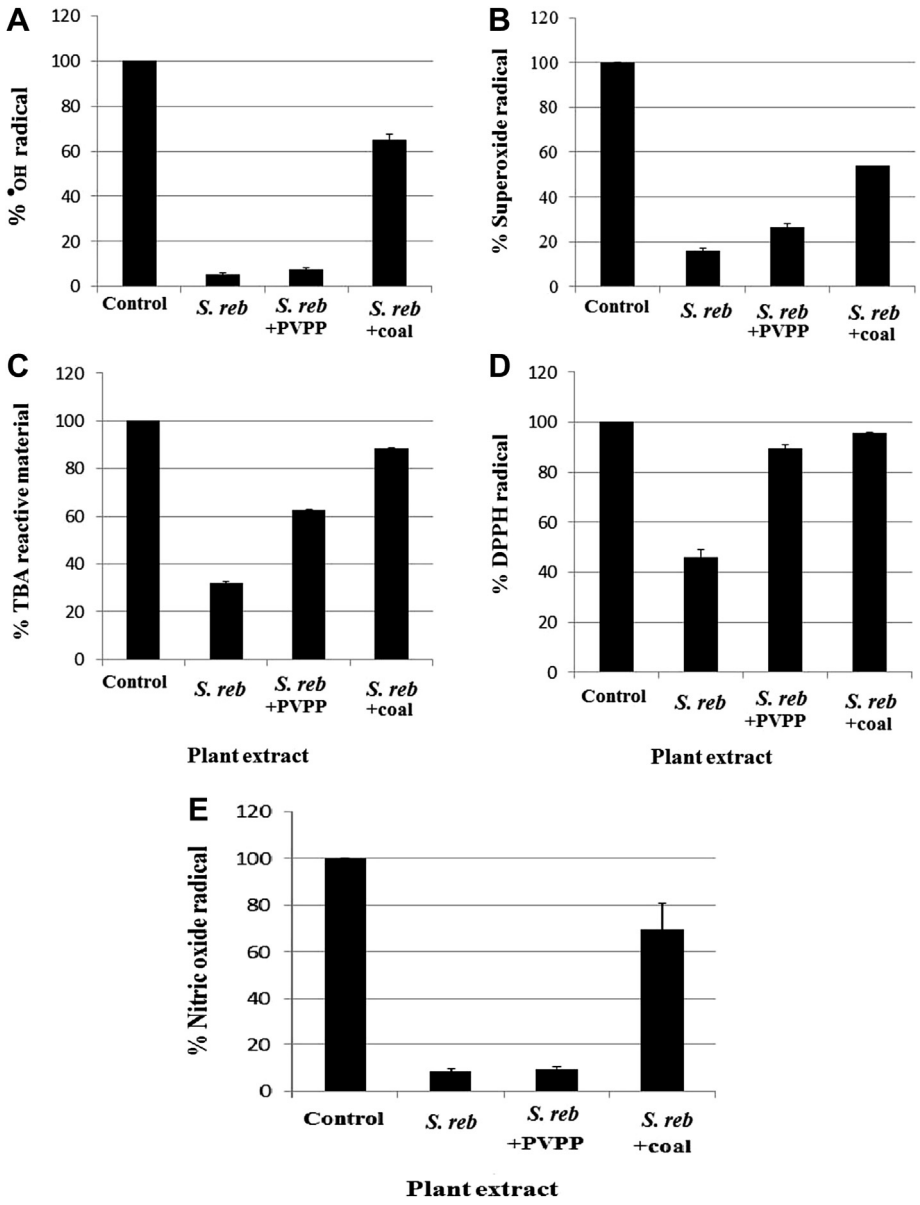

Fig. 2. Radical scavenging activity of •OH radical (A), superoxide radical (B), TBA reactive material (C), DPPH radical (D) (Note: extracts for DPPH assay were 10 times diluted) and NO radical (E) by crude extract of *S. rebaudiana* (*S. reb*) before and after purification with PVPP or charcoal. The obtained values of the tested samples were normalised to percentages of the control (100%).

control (100%) and plotted as log ($F^0/F-1$) against log (concentration of scavenger). Only the positive controls had a significant scavenging activity on NO (Table 5). The IC_{50} of hydroxytyrosol (34.3) is probably too high to be of any physiological significance. The other tested compounds showed no NO radical scavenging activity.

An HPLC analysis was used to study the NO radical scavenging of crude plant extracts as well as of hydroxytyrosol (example given in Fig. 3). Crude plant extracts had a very potent scavenging activity towards NO radicals (Fig. 2). Treatment with PVPP could remove only a small amount of activity, whereas treatment with active charcoal was able to remove about 60% for extracts of *S. rebaudiana*.

4. Discussion

The positive control quercetine is the most active •OH scavenger, followed by the group of SVglys, SVglu and aspirin, which might explain its anti-inflammatory effects. Ascorbic acid, hydroxytyrosol, metformin and sugars are less efficient •OH scavengers. In superoxide scavenging, ascorbic acid was most active, followed by SVglu, quercetine and hydroxytyrosol. SVgly and aspirin were less efficient scavengers than SVglu. Sugars and metformin could not efficiently scavenge superoxide radicals. Quercetine and hydroxytyrosol were very potent in reducing the TBA reactive material, followed by ascorbic acid. Metformin and SVglu had an intermediate activity. SVglys were less efficient than SVglu. Sugars had no significant scavenging activity. Only the positive controls and hydroxytyrosol could scavenge DPPH and NO radicals. All the other tested compounds were without activity. In general, sugars were weak radical scavengers and this is opposed to the excellent scavenging effect of inulin.[16] There is no relationship between the radical scavenging of steviol derivatives and the number of sugar units attached, or with the number of OH groups. This is in contrast to the results by Morelli et al[36] who found a strong correlation between the •OH

Table 3
Superoxide radical scavenging activity (Half-inhibitory concentrations, IC_{50} in mM) of the different scavengers. The obtained values of the tested compounds were normalised to percentages of the control (100%) and plotted as log ($F^0/F-1$) against log (concentration of scavenger).

Superoxide scavenger	Equation	r^2	IC_{50} in mM	Sugar units
Ascorbic acid	$y = 1.053x + 1.290$	0.9685	0.059	0
Quercetine	$y = 1.005x + 0.497$	0.9345	0.32	0
ST	$y = 0.447x - 0.078$	0.9205	1.491	3
RebA	$y = 0.3246x - 0.131$	0.9315	2.529	4
SYglu	$y = 0.6124x + 0.413$	0.9843	0.211	1
Hydroxytyrosol	$y = 1.873x + 0.351$	0.984	0.51	0
Glucose	No effect	–	–	1
Sucrose	No effect	–	–	2
Trehalose	$y = 0.5935x - 1.173$	0.9139	94.841	2
Metformin	$y = 0.6753x - 1.769$	0.9928	416.9	0
Aspirin	$y = 2.187x - 1.298$	0.9038	3.921	0

Table 4
IC_{50} values of TBA reactive material (Half-inhibitory concentrations, IC_{50} in mM) of the different scavengers. The obtained values of the tested compounds were normalised to percentages of the control (100%) and plotted as log ($F^0/F-1$) against log (concentration of scavenger).

Scavenger	Equation	r^2	IC_{50} in mM	Sugar units
Ascorbic acid	$y = 0.400x - 0.421$	0.911	11.3	0
Quercetine	$y = 0.893x + 0.036$	0.942	0.912	0
ST	$y = 0.234x - 0.589$	0.917	323	3
RebA	$y = 0.239x - 0.587$	0.905	288	4
SVglu	$y = 0.259x - 0.564$	0.908	149	1
Hydroxytyrosol	$y = 0.755x - 0.279$	0.976	2.34	0
Glucose	$y = 0.208x - 0.910$	0.996	23993	1
Sucrose	No effect	–	–	2
Trehalose	$y = 0.374x - 1.074$	0.999	746	2
Metformin	$y = 0.369x - 0.744$	0.945	104	0
Aspirin	$y = 0.246x - 0.336$	0.947	23.17	0

Table 5
DPPH and NO radicals scavenging activity (Half-inhibitory concentrations, IC_{50} in mM) of the different scavengers. The obtained values of the tested compounds were normalised to percentages of the control (100%) and plotted as log ($F^0/F-1$) against log (concentration of scavenger).

DPPH scavenger	Equation	r^2	IC_{50} in mM	Sugar units
Ascorbic acid	$y = 0.352x + 0.444$	0.935	0.055	0
Quercetine	$y = 1.180x - 1.346$	0.995	13.817	0
Hydroxytyrosol	$y = 4.117x + 3.3-003$	0.905	0.186	0
NO scavenger	**Equation**	r^2	IC_{50} in mM	**Sugar units**
Ascorbic acid	$y = 0.204x + 0.381$	0.97	0.013	0
Quercetine	$y = 0.922x + 1.253$	0.995	0.184	0
Hydroxytyrosol	$y = 0.720 - 1.106$	0.984	34.3	0

scavenging activity and the number of alcoholic hydroxyl groups of "true" sugars (glucose, fructose, deoxyribose, sucrose, maltose).

Crude plant extracts, especially of *S. rebaudiana*, were very potent ROS and RNS scavengers in all assays used. Even in the assays in which SVgly or SVglu were less active (superoxide, TBA reactive material) or inactive (DPPH, NO), the crude extract of *S. rebaudiana* was very active. It can be concluded from the experiments with crude plant extracts that part of the scavenging activity was due to phenol or polyphenols that could be removed by PVPP treatment. Most of the residual scavenging activity remaining after PVPP treatment could be removed by active charcoal, suggesting that still other radical scavenging compounds are present in the crude extracts. Their identity remains unknown.

SVgly and SVglu possess a high ROS (•OH) scavenging activity. So what? SVglys are not absorbed by the intestines.[37] The only possible beneficial effect of SVgly is the protection of food components against ROS, as, e.g., demonstrated in the stability study of stevioside by Kroyer[7,8] wherein the degradation of vitamin C was significantly reduced. An additional beneficial effect might be a protection against radical attack of the walls of the digestive tract. However, the most interesting result is the ROS scavenging activity of SVglu, which, after daily administration of 750 mg stevioside, occurs in the peripheral blood in concentrations up to 21.3 μg SV eq/mL or 67 μM SVglu.[14]

The pharmacological effects of ST and steviol have at least been confirmed in animal models and in isolated organ and/or cell cultures and in some human studies. These very diverse effects induced by just one substance led to an atmosphere of disbelief, certainly in the medical world, such as "too good to be true". A selection of the beneficial effects are: lowering of blood pressure in case of hypertension, without effect on persons with normal or hypotension; lowering of blood glucose in type 2 diabetes, without effects on basal glucose levels; enhancement or decrease of insulin secretion depending on the stage, enhancement of insulin sensitivity, effects on blood glucose in a glucose concentration dependent manner, reduction of inflammation, reduction of skin cancers and prevention of atherosclerosis.[11,22,38]

The ROS can damage proteins and lipids as well as the mitochondrial DNA. Mutations of mitochondrial DNA can lead to changes in the respiratory chain, which leads to a reduction of its efficiency and thus the production of still more ROS, creating a vicious circle leading to apoptosis of cells, e.g., in specific brain regions, skeletal muscles, optic nerve and liver.[4] Mutations within the mitochondrial genome accumulate with age and in common neurodegenerative diseases such as Parkinson's and Alzheimer's disease.[39] There is also a relationship between the altered levels of oxidative and nitro-oxidative stress within the cardiovascular environment and the development of cardiovascular disease (CVD).[4] Atherosclerosis is the leading cause of CVD-related mortality, accounting for 3/4 of all deaths from heart disease. Free radical mediated changes within the cardiovascular environment are most popular suspects for CVD, as exemplified by the cytotoxic and atherogenic properties of oxidised Low Density Lipoprotein cholesterol, which can be formed *via* several pathways involving oxidative and/or nitro-oxidative stress.[4] Hulsmans and Holvoet[40] described the roles of ROS and oxidised lipoproteins in the activation of inflammatory cells and inducing signalling pathways related to cell death and apoptosis. They also presented evidence that the vicious circle between oxidative stress and inflammation does not only occur in the diseased arterial wall, but also in adipose tissues. There, oxidative stress and inflammation impair adipocyte maturation resulting in defective insulin action and adipocytokine signalling. The last is associated with increased infiltration of inflammatory cells, loss of antioxidant protection and cell death in the arterial wall. Diabetes increases vascular oxidative stress, including increased $O_2^{\bullet -}$ production, lipid peroxides, isoprostanes, 3-nitrotyrosine levels and DNA damage.[4] Hyperglycaemia induces $O_2^{\bullet -}$ generation in the endothelial cells *in vitro*, most of the radicals being produced by the mitochondria. This might happen by an increase of the inner membrane proton gradient resulting from an overproduction of electron donors (NADH, $FADH_2$) by the tricarboxylic acid cycle overproducing $O_2^{\bullet -}$.

Fatty liver disease can be associated with chronic high alcohol consumption or with obesity/type 2 diabetes. The start of this disease is the accumulation of triglycerides in hepatocytes, a process called steatosis. Oxidative stress, disrupted (NO) signalling and mitochondrial dysfunction leading to ROS in the liver, are the key molecular events that accelerate or worsen steatosis and initiate progression to steatohepatitis, fibrosis and cirrhosis.[2]

NO is a free radical and is also an important cellular signalling molecule in many physiological and pathological processes and it is

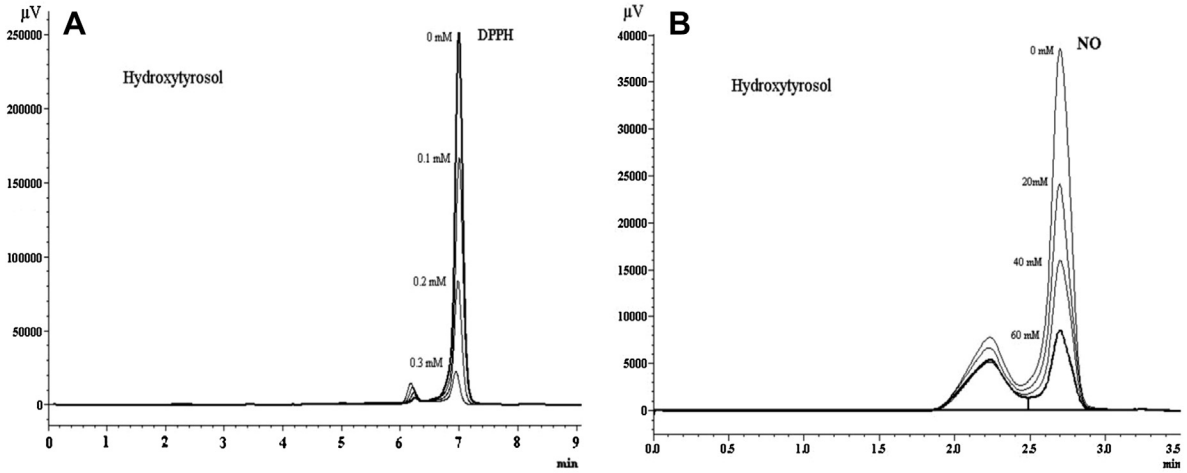

Fig. 3. Example of an overlay of HPLC trace of DPPH radical (A) for different concentrations of hydroxytyrosol (From top to bottom: 0, 0.1, 0.2, 0.3 mM) and NO radical (B) for different concentrations of hydroxytyrosol (from top to bottom: 0, 20, 40 and 60 mM).

formed by NO synthase enzyme.[41] Dessy and Ferron[42] reported that humans with atherosclerosis, diabetes or hypertension often show impaired NO pathways. Bornia et al[43] reported that the reduction of arterial tension by stevioside is dependent upon NO synthase and guanylate cyclase activities when the endothelium is intact. Tateya et al[44] concluded that endothelial NO could limit the obesity-associated inflammation and insulin resistance in hepatocytes. Kupffer cell activation during high-fat feeding would be dependent on reduced NO signalling. Kwak et al[45] showed that NO-mediated redox regulation is the mechanism of PTEN protein degradation (phosphatase and tensin homologue), which accounts for PTEN loss in neurodegeneration such as in Alzheimer's disease, in which NO plays a critical pathophysiological role. Hale et al[46] reported that NO signalling is also involved in uterine vascular remodelling during pregnancy, thus altering the matrix metalloproteinase expression as well as collagen and elastin deposition.

To explain the myriad of beneficial effects of SV, SVgly, SVglu, metformin, hydroxytyrosol and aspirin and to convince the medical world of the interesting healing and/or preventive effects of these compounds, a common trigger had to be found that was responsible for all the effects. It is known that SVglu, metformin, hydroxytyrosol and aspirin can be found in the peripheral blood at sufficient elevated concentrations to show radical scavenging *in vivo*. All these compounds have strong ROS or RNS scavenging activity and they can be transported all over the body. By their ROS scavenging, they can positively influence the above cited diseases, as these are in some way related to excess of radicals. Too much blood glucose, *e.g.*, leads to an excess of radicals that cannot be detoxified any more by the body, and which damage the insulin signalling pathway, whereas low blood glucose does not lead to excess of radicals, and hence there is no effect of SVglu. In the same way and in other processes too, the occurrence or lack of effects of stevioside might be related to the production of an excess of radicals, or lack of overproduction, respectively. It is known that by their ability to decrease oxidative stress in tissues, antioxidants can improve or prevent diseases.

5. Conclusion

Different scavengers showed different activities in the assays of this study. SVglys, SVglu, hydroxytyrosol, aspirin and metformin are active antioxidants. ROS scavenging of SVglys was not due to the number of sugar units. Combination of several scavengers might have to be advised to prevent many of the obesitas related diseases. Crude *S. rebaudiana* leaf extracts showed very potent radical scavenging activity towards both ROS and RNS. This might explain why crude leaf extracts were more efficient in the care of type 2 diabetes as shown by Ferreira et al.[25] The antioxidant activity of leaf extract cannot be totally related to SVglys. Some compounds other than phenols, SVglys, flavonoids in crude extract are effective antioxidant which could not be removed by PVPP and active charcoal. It shows that leaf extract can be used as a natural antioxidant agent.

Conflicts of interest

All authors have none to declare.

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