Antioxidant properties of cell wall polysaccharides of *Stevia rebaudiana* leaves

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**Comments**
In the present manuscript authors have appropriately described the free radical quenching potential, reducing properties, and chelating ability as well as membrane and DNA protective activities of *S. rebaudiana* leaf polysaccharides. Results have been interpreted suitably with the discussion part. *S. rebaudiana* was found to be a promising antioxidant agent. Hence phytochemicals present in the plant part have potential for developing drug lead compounds against oxidative stress.

1. Introduction

Free radicals are products of cellular metabolism and transition–metal ions, and they seem to play an important role in causing bio–macromolecule damage in *vivo* [1]. Increasing oxidative stress and disorders in energy metabolism may lead to mutations and eventually to many severe diseases. Antioxidants may play an essential role in protecting the body from various oxidative damages that are linked to obesity, diabetes, cancer, cardiovascular diseases and neurodegenerative ones, including Parkinson’s and Alzheimer’s disease [2,3]. The etiology of these several health disorders implicates free radicals or reactive oxygen species generated by normal physiological processes and various exogenous factors. Reactive oxygen exogenous can cause oxidative stress and thereby initiate peroxidation of...
membrane lipids, causing damage to a wide range of other biological molecules including DNA, lipids and proteins[4]. The extent of damage caused by free radicals might be mitigated by supplementation with one or more antioxidants, hence the search for new sources of natural antioxidants is currently become the major interest to scientists. Natural antioxidants such as polysaccharides, originating from plants, are safe therapeutic substances providing protection to humans against infections, degenerative and metabolic diseases[5,6].

Non–cellulosic cell wall polysaccharides of higher plants represent a potential source of pharmacologically active polysaccharides. In traditional medicine, extracts of polysaccharide–containing plants are widely used for the treatment of skin problems, epithelial wounds and mucous membrane irritations[6,7]. Moreover, several reports showed that polysaccharides from different plants could be responsible for the effects associated with the healing of wounds. Some of them have an influence on the immune system and are often called immunomodulators when the complement system is involved[8,9].

Stevia rebaudiana Bertoni (S. rebaudiana) is a well–known native South American sweet herb. Stevioside, a diterpene glycoside, is an abundant component in the leaf. It has become well–known for its intense sweetness, and is used as a non–caloric sweetener in several countries. In addition, the crude extract and stevioside from leaves have beneficial effects on human health, including antihypertensive[11], anti–hyperglycemic[12], anti–inflammatory, anti–cancer[13], antiviral[14] and antioxidant[15]. However, no reports are available on the antioxidant activity of the cell wall polysaccharides of S. rebaudiana. Therefore, the present investigation was undertaken to examine the total phenolic and the protein contents, and the antioxidant activities of pectin and hemicellulosic wall polymers. For this, CWM (6.86 g) was subjected to a sequential extraction regime using 50 mmol/L ethylene diamine tetra acetic acid (EDTA), pH 6.5; 0.05 mol/L KOH and 4 mol/L KOH (Figure 1). The pH of alkaline extraction was adjusted to 4.5 with acetic acid. Each fraction was filtrated and dialysed against distilled water, except the EDTA one, which was dialysed against 1 mol/L NaCl to exchange the chloride anion before lyophilisation[16].

2. Materials and methods

2.1. Plant material and preliminary treatment

S. rebaudiana was grown at the Biotechnology Centre–Nkolbisson (Yaounde, Cameroon) and the leaves were collected in February, 2012. The identification of the voucher specimens was confirmed by the National Herbarium of Cameroon. The leaves were chopped into small pieces and dried under shade in the laboratory for further use. The air-dried leaves were then powdered.

2.2. Extraction and isolation of polysaccharide–enriched fractions

The leaf powder (14 g) was suspended in boiling ethanol (85%) for 30 min in order to inactivate any existing enzymes. The residue recovered after centrifugation (4000 r/min) was subjected to a series of extractions to remove the lipids, polyphenols, and other low–molecular weight metabolites as previously described by Boudjeko et al[10]. It was then extracted overnight at room temperature with 100 mL of 90% dimethylsulfoxide, 24 h with methanol–chloroform (1:1; v/v), 24 h with methanol–acetone (1:1; v/v), and finally with acetone–water (4:1; v/v). The residue was air–dried at 40 °C and the cell wall material (CWM) obtained was used for the extraction of pectic and hemicellulosic wall polymers. For this, CWM (6.86 g) was subjected to a sequential extraction regime using 50 mmol/L ethylene diamine tetra acetic acid (EDTA), pH 6.5; 0.05 mol/L KOH and 4 mol/L KOH (Figure 1). The pH of alkaline extraction was adjusted to 4.5 with acetic acid. Each fraction was filtrated and dialysed against distilled water, except the EDTA one, which was dialysed against 1 mol/L NaCl to exchange the chloride anion before lyophilisation[16].

2.3. Determination of total protein and phenolic content

The protein content of the polysaccharide fractions of S. rebaudiana was measured by the Bradford method using bovine serum albumin (BSA) as the standard[17]. The protein content was expressed in µg of BSA equivalent.
The phenolic content of the leaf polysaccharide fractions was determined spectrophotometrically at 760 nm according to the method of Nguimbou et al. using the Folin–Ciocalteu reagent[18]. Phenolic contents were expressed in µg equivalent of Ferulic acid per mg of CWM. A volume 75 µL of the polysaccharide fraction at 4 mg/mL was diluted with 675 µL of distilled water. Then 75 µL of Folin–Ciocalteu reagent at 0.2 mol/L was added and mixed thoroughly and incubated for 3 min. This was followed by the addition of 750 µL of 20% sodium carbonate and the mixture was allowed to stand for 1 h at room temperature. The absorbance of the blue colour that developed was measured at 760 nm.

2.4. Antioxidant activities

2.4.1. Determination of DPPH (1, 1-diphenyl-2-picryl hydrazyl) free radical scavenging activity

The free radical scavenging activity of the polysaccharide fractions was measured in terms of their hydrogen donating or radical scavenging ability using the DPPH radical[19]. For the assay, 500 µL of the fraction at different concentrations 100–500 µg/mL were introduced into test tubes and 500 µL of the freshly prepared solution of 400 μmol/L DPPH in methanol were then added. The mixture was stirred and left in the dark at 37 °C for 30 min. The absorbance was measured at 517 nm using a UV–1605 Shimadzu spectrophotometer and gallic acid and catechin were used as the positive controls. A low absorbance of the reaction mixture indicated high free radical scavenging activity. The DPPH radical scavenging effect was calculated as “percentage of inhibition” according to the following formula: percentage of inhibition (%)=\[1-(A_{0}/A_{1})\] where A0 is the absorbance of the control reaction and A1 is the absorbance in the presence of the sample.

2.4.2. Antioxidant activities by ferric reducing antioxidant power (FRAP) assay

The FRAP reagent containing 1 volume of 10 mmol/L TPTZ (2, 4, 6-tripyridyl-s-triazine) solution in 40 mmol/L HCl, 1 volumes of 20 mmol/L FeCl3 and 10 volume of 0.3 mol/L acetic buffer (pH 3.6) was prepared freshly. Aliquots of 100 µL extract (100 µg/mL) were mixed with 500 µL of FRAP reagent and the absorbance of the reaction mixture was measured spectrophotometrically at 593 nm after incubation at 37 °C for 30 min in the dark using a UV–1605 Shimadzu spectrophotometer[20]. Gallic acid was used as the standard. The final result expressed that the concentration of antioxidant has a ferric reducing ability and was determined by µg of gallic acid equivalent (GAE).

2.4.3. Chelating ability of ferrous ions

The method of Dinis et al. was used to determine the ferrous ion chelating ability[21]. Various concentrations 100–500 µg/mL of sample solution (1 mL) were mixed with FeCl3 (2 mmol/L, 100 µL) and left in dark for 1 min at room temperature. The mixture was added to 200 µL of 5 mmol/L ferrozine. After 10 min at room temperature, the absorbance of the mixture was read at 562 nm against a blank (distilled water) using a UV–1605 Shimadzu spectrophotometer. EDTA was used as the standard and the ability of all samples to chelate ferrous ion was calculated using the following equation: Chelating ability (%)=\[1-(A_{0}/A_{1})\] where A0 is the absorbance of the control reaction and A1 is the absorbance in the presence of the sample.

2.4.4. Inhibition of lipid peroxidation by thiobarbituric acid-reactive substances method

2.4.4.1. Animal material

Investigations on animals were conducted in accordance with the internationally accepted principles for laboratory animal use and care as found in the United States guidelines [United States National Institutes for Health Publication No. 85–23 revised in 1985]. The animals were maintained under standard laboratory conditions with a 12 h light and dark cycle, free access to standard laboratory rat food and tap water, temperature of (24±1) °C and humidity of (55±10)%.

2.4.4.2. Lipid peroxidation assay

One millilitre of liver homogenate (10%, w/v) was added to 100 mL of the tested fraction at different concentrations 100–500 µg/mL. Lipid peroxidation was initiated by adding 50 µL of 500 µmol/L FeCl3 and 50 µL of 500 µmol/L H2O2. After 1 h of incubation at room temperature, 1 mL of trichloro acetic acid (15%, w/v) and 1 mL of TBA (0.67%, w/v) were added and the mixture was heated in a water bath at 65 °C for 15 min and centrifuged at 3000 g for 10 min. The homogenates were prepared at a concentration of 10% the resulting supernatants stored at −20 °C.

2.4.5. Protective effect on human DNA

2.4.5.1. Human material

Human DNA used in this work came from the bank of Laboratory of Biochemistry, Immunology and Biotechnology, Faculty of Medicine and Biomedical Sciences, University of
2.4.5.2. Assay for protective effect on human DNA

The degree of damage induced on human DNA was evaluated by the difference in migration patterns on agarose gel[23]. The reaction was conducted in a total volume of 30 µL containing 2 µL of tris-buffer 50 mmol/L, pH 7.4; 5 µL of human DNA and 5 µL of polysaccharide fraction prepared at different concentrations (62.5–1000 µg/mL). Then, 12 µL of 30% H2O2 and 6 µL of 500 µmol/L FeCl2 were added and incubated at 37 °C for 30 min. The reaction mixture was then homogenised with 6 µL of gel loading dye, loaded into a 1% agarose gel and run at 100 V for 15 min in a submarine gel electrophoretic apparatus (Mupid eXu). The DNA was finally visualized and photographed.

2.5. Statistical analyses

The data was subjected to statistical analysis to verify and evaluate the differences between the antioxidant activities of the three leaf polysaccharide fractions. All data is reported as mean±SEM for three independent samples (n=3). One-way analysis of variance (ANOVA) was used and the least significant difference (LSD) at P<0.05 was calculated using the SPSS 18.0 version for Windows package. The graphical representations were designed in Microsoft Excel 2007.

3. Results

3.1. Total protein and phenolic compounds content

The pre-treatment of leaf powder with organic solvent yielded a CWM from which cell wall polysaccharides were extracted. Three fractions were obtained, namely, pectin EDTA (FPE), pectin 0.05 mol/L KOH (FPK) and hemicellulose (FH). The fractions accounted for 319.24, 77.26 and 517.49 mg per gram of cell wall, respectively for FPE, FPK and FH. The FPK fraction represented the lowest quantity of cell wall polysaccharides in S. rebaudiana leaves.

3.2. Inhibition of DPPH radical

The DPPH free radical, a stable radical with purple color and a maximum absorption at 517 nm, has been widely used as a tool to evaluate the free radical scavenging activities of antioxidants[24]. When DPPH encounters antioxidant scavengers, its purple color fades rapidly because it is changed to the non–radical form DPPH–H. On the basis of this principle, the scavenging effects of the three polysaccharide leaf fractions on the DPPH radical were measured and the results are shown in Figure 4.
concentration of 400 µg/mL, the scavenging activities were 27.70%, 59.91% and 23.20% for FPE, FPK and FH, respectively. The scavenging activities of the positive controls (gallic acid and catechin) were 98.52% and 93.72% at the 100 µg/mL concentration. Compared to the polysaccharides, gallic acid and catechin were more effective for scavenging DPPH. The results suggest that all the polysaccharides carry out scavenging activities on DPPH in a concentration-dependent manner, and especially FPK (low alkali fraction) had a much stronger antioxidant activity.

3.3. Antioxidant activities by FRAP assay and ferrous ion chelating ability

3.3.1. Reducing power of polysaccharide fractions

The reducing power of FPE, FPK and FH determined at 700 nm are depicted in Figure 5. As shown in Figure 5, all the polysaccharide fractions had a reducing power. At 100 µg/mL, the reducing powers were (33.79 ± 3.46) µg GAE, (64.11 ± 1.28) µg GAE and (36.32 ± 2.75) µg GAE for FPE, FPK and FH, respectively. FPK, with its highest reducing power, showed that there is a direct correlation between antioxidant activities and the reducing capacity of certain plant extracts[24].

3.3.2. Ferrous ion chelating ability

At 100–500 µg/mL, the chelating ability was (34.37±0.13)%–(35.19±0.19)% for FPE, (36.30±0.21)%–(94.51±0.09)% for FPK and (1.26±0.36)%–(2.8±0.2)% for FH (Figure 6). Only FPK had a dose dependent activity. When the concentration was higher than 300 µg/mL, the metal chelating activity was the highest for the three different polysaccharide fractions. However, as shown in Figure 6, the chelating ability of all the samples was weaker when compared to that of EDTA [(99.48±0.05)% at 100 µg/mL].

3.3.3. Inhibition of lipid peroxidation

*S. rebaudiana* leaf polysaccharide fractions inhibited the amount of MDA generated (and thus lipid peroxidation) in liver homogenate (Figure 7). Thus, the decrease in the MDA level in the presence of the leaf polysaccharide fractions indicated the role of the fraction as an antioxidant. The FPK fraction showed the highest inhibition percentage [(35.92±1.8)% and (48.58±0.06)% at 300 and 500 µg/mL, respectively] compared to the FPE and FH fractions. However, these activities were lower than those of the standard catechin [(47.28±0.17)% and (72.59±0.67)% at 100 and 500 µg/mL, respectively].

3.3.4. Protective effect of polysaccharides on DNA damage

The gel pattern of human DNA exposed to the polysaccharide leaf fractions and to radical hydroxide (OH) generated in vitro by the reaction of H2O2/FeCl2 is presented in Figure 8. DNA migration assay is a sensitive biomarker of the DNA damage[25]. From the migration pattern, it can be deduced that OH−-damaged human DNA lead to the shredding and caused the absence of specific band in H2O2+FeCl2 treated DNA (Lane 3). The three polysaccharides leaf fractions of *S. rebaudiana* reduced the DNA damage induced by H2O2/FeCl2 to human DNA caused the presence of specific band in Lanes 3 to 7. Visually, this protection is not significantly dose dependent. *S. rebaudiana* leaf fractions, by themselves, did not cause any DNA damage at the concentration of 1 mg/mL as evidenced by the intact DNA band (Lane 8). The striking observation that could be made was that the extent of DNA damage induced by H2O2/FeCl2 was completely reverted by
the leaf polysaccharides fractions at concentrations of 62.5, 125, 250, 500 and 1000 µg/mL (Lane 3 to 7).

4. Discussion

Natural polysaccharides are not isolated molecules in plants, animals or microorganisms but are associated with other compounds such as proteins and lipids[10]. Moreover, some plant polysaccharides contain important amounts of polyphenolic compounds with antioxidant activity[2,5]. Phenolic compounds have been reported to have multiple biological effects including antioxidant activity. These polyphenols have both hydrophobic aromatic rings and hydrophilic hydroxyl groups with the ability to bind to polysaccharides and proteins at several sites on the cell wall surface[26].

At 100 µg/mL, the scavenging activity of FPK (the most effective polysaccharide fraction) was 35.68% which was higher than that of the same plant aqueous leaf extract (10.15%) found by Kim et al[27]. The concentrations required for IC50 of DPPH radicals was 330.12 µg/mL for FPK whereas it was found to be 752.6 µg and 541.3 µg of sample for the water extracts of Stevia leaves and callus[28]. However, Schulka et al. found that 200 µg/mL of aqueous leaf extract of Stevia exhibited 72.37% inhibition and its IC50 value was found to be 83.45 µg/mL[29].

It had been previously reported that there was a direct correlation between the antioxidant activities and the reducing power of certain polysaccharide fractions. The reducing power of a compound may serve as a significant indicator of its potential antioxidant activity[10]. A measurement of the reducing power may directly reflect the production capacity of the electron donor. Among the three fractions of polysaccharides, FPK showed an obviously higher ferric reducing ability than that of FPE and FH. It suggested that the reductone–associated and hydroxide groups of Stevia polysaccharides can act as electron donors and can react with free radicals to convert them into more stable products[30]. The presence of reductants such as antioxidant substances in the antioxidant samples causes the reduction of ferric tripyridyltriazine [Fe(III)–TPTZ] complex to ferrous tripyridyltriazine [Fe(II)–TPTZ] at low pH.

Metal chelating capacity is important since it reduces the concentration of transition metals that may act as catalysts to generate the first few radicals and initiate the radical–mediated oxidative chain reactions in biological systems. Ion–chelating agents may also inhibit the Fenton reaction and hydroperoxide decomposition. Fe2+ ion is the most powerful pro–oxidant among various species of metal ions, being able to form complexes with ferrozine. Earlier reports have revealed that Fe2+ accelerates lipid peroxidation by breaking down hydrogen and lipid peroxides formed by the Fenton free radical reaction. Therefore, the chelating effect on ferrous ion is a widely used method to evaluate antioxidant activity. Our data on the chelating ability of the fractions revealed that the polysaccharides demonstrated an effective capacity for iron bindings, which suggesting that their action as antioxidants may also be related to this capacity.

Lipids in the membrane are continuously subjected to the action of free oxygen radicals that initiate membrane lipid peroxidation. The oxidant induced subtraction of a hydrogen atom from an unsaturated fatty acyl chain of membrane lipids initiates the process of LPO, which propagates as a chain reaction. Lipid aldehydes (like malondialdehyde) generated during the breakdown of lipid superoxides are especially dangerous for the organism. These aldehydes which have a longer half–life (a few minutes) can easily migrate over a considerable distance and can react with
other molecules far away from the site of their origin\[^{31}\].

DNA contains reactive group in its bases that are highly susceptible to free radical attack. \(\text{HO}_2/\text{FeCl}_3\) generates \(\text{OH}^-\) radicals which cause oxidative damage. \(\text{OH}^-\) bounded to DNA leads to stranded breakage, deoxysugar fragmentation and base modification. Moreover, \(\text{HO}_2\) and associated free radical can induce DNA mutations\[^{32}\]. Many studies have reported the protection against oxidative DNA damage by herbal extracts and formulations\[^{33,34}\]. For instance, the degraded polysaccharides from *Poria cocos sclerotium* can protect the native SC-DNA against UV irradiation and \(\text{HO}_2\)^\[^{33}\]. Furthermore, water polysaccharides from rhizomes of *Panax japonicas* conferred the protection on \(\text{HO}_2\), and UV--induced damage of \(\text{p}IC333\) plasmid DNA\[^{34}\]. The results of the present study are also in agreement with the above reports. These findings support the use of the cell wall polysaccharides leaves of *S. rebaudiana* to protect DNA against oxidative damage and indicate a correlation with the antioxidant potential of the polysaccharides.

The researches of natural polysaccharides have attracted a lot of attention because of their abundance in resources and non--toxicity. This article mainly related to the isolation of the antioxidant activities polysaccharides from *S. rebaudiana* leaves. According to the results stated above, it could be concluded that cell wall polysaccharides of *S. rebaudiana* leaves bear a potent antioxidant activity. Their constituents scavenge free radicals and exert a protective effect on cellular macromolecules against oxidative damage. The analysis revealed major differences in the antioxidant activity in the three polysaccharides fractions, the FPK showed better antioxidant activity and it might be attributed to the presence of higher amount of proteins and polyphenolic compounds. They might be equally beneficial to human antioxidant protection system against oxidative damage. What’s more, it should be planned for further investigation to know more about the polysaccharides of *S. rebaudiana*, the relationship of structures and various activities.

**Conflict of interest statement**

We declare that we have no conflict of interest.

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**Comments**

**Background**

Free radicals lead to oxidative stress, which has been implicated in many diseases. Antioxidants counteract the action of free radicals by inhibiting their formation, mitigating their activity or scavenging them. Therefore, there is a need of new and potent antioxidants of natural origin. Hence, the manuscript deals with the relevant issues.

**Research frontiers**

The present work has reported the *in vitro* antioxidant capacity of cell wall polysaccharides of *S. rebaudiana* leaves by measuring their radical scavenging activity, reducing power, metal ion chelating ability and lipoprotective efficacy. The protection of DNA damage has also been shown. In addition, the protein and phenolic contents have also been determined. Screening of natural phytochemicals for bioactivity is required for the development of drug leads.

**Related reports**

The variety of secondary metabolites is produced in the plant kingdom. Many of them possess medicinal attributes. There are many reports showing effectiveness of herbs in treating various disorders that arise from the oxidative stress. The folklore medicine has been shown to produce antioxidant activity.

**Innovations and breakthroughs**

The usage of cell wall polysaccharides of *S. rebaudiana* as a source of antioxidants offers a further scope for the use of this plant in addition to its other biological properties. FPK fraction deserves special mention because of its medicinal potential.

**Applications**

It has been found that antioxidant therapy has been being used in the treatment of many degenerative diseases including cancer, diabetes, cardiovascular diseases and aging. Current research has provided an insight for the utilization of *S. rebaudiana* as potential antioxidant. Further researches are required for characterization of cell wall components as well as the combinations with proteins and polyphenols related with the bioactivity.

**Peer review**

In the present manuscript, the authors have appropriately described the free radical quenching potential, reducing properties, chelating ability as well as membrane and DNA protective activities of *S. rebaudiana* leaf polysaccharides. Results have been interpreted suitably with the discussion part. *S. rebaudiana* was found to be a promising antioxidant agent. Hence, phytochemicals presented in the plant part have potential for developing drug in lead compounds against oxidative stress.
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