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# A comparative study on extraction processes of *Stevia rebaudiana* leaves with emphasis on antioxidant, cytotoxic and nitric oxide inhibition activities

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ABSTRACT

In this paper, the extraction conditions yielding the highest steviol glycosides from the leaves of *Stevia rebaudiana* were determined using microwave (MAE) and ultrasonically assisted extraction (UAE) and biological activities of the extracts along with analyses of the residue from the extraction process were investigated. Under optimum conditions, 21.21 mg glycosides/g dried leaf in MAE and 14.90 mg glycosides/g dried leaf in UAE were quantified by HPLC analyses. After extraction, total chlorophyll, carotenoid contents and total dietary fibers were quantified as 15.14 mg/100 g, 2.93 mg/100 g and 6.5% in the raffinate phase. The total phenols were determined as 80.13 and 86.47 mg gallic acid/g extract, whereas the total flavonoids were 111.16 and 126.70 mg quercetin/g extract and DPPH radical scavenging activities were 91.39 and 92.40%, respectively. The extracts exhibited no cytotoxicity against healthy cell line and macrophage RAW 264.7 cells. The $IC_{50}$ values were 68 μg/ml and >100 μg/ml for MAE and UAE. Overall, obtained results suggest that stevia extracts and its residue can be utilized holistically on industrial scale.


## 1. Introduction

Recently, the demand for natural sweeteners fulfilling safety requirements with acceptable taste is increasing due to growing health concern over the safety of some chemical sweeteners. Moreover, a frequent metallic aftertaste of synthetic sweeteners does not provide the realistic taste of sugar. Some types of chemical sweeteners are associated with the potential risk of cancer of bladder when they are used heavily. Therefore, uses of low-calorie/non-calorie sweeteners as additives are encouraged with increasing health consciousness (Abou-Arab et al., 2010; Brahmachari et al., 2011; Rao et al., 2012). Consequently, low-calorie natural sweeteners are urgently required as substitutes for table sugar.

*Stevia rebaudiana* Bertoni is a perennial shrub of the Asteraceae family native to certain regions of South America (Chaturvedula et al., 2011; Puri et al., 2012; Lemus-Mondaca et al., 2012). The leaves accumulate a mixture of at least eight different steviol glycoside diterpenes (Wallin, 2007). Stevioside and rebaudioside A are the major steviol glycosides found in stevia which can accumulate up to 20% of dry leaf weight in some strains, and are approximately 250–300 times sweeter than sucrose, respectively (Chaturvedula et al., 2011; Lemus-Mondaca et al., 2012). Other diterpene glycosides present in lower concentrations are steviolbioside, rebaudioside B, C, D, F, dulcoside A and rubusoside (Woelwer-Rieck et al., 2010; Yadav and Guleria, 2012).

Toxicological studies have shown that stevioside does not have mutagenic, teratogenic or carcinogenic effects and no allergic reactions have been observed when it is used as a sweetener (Abou-Arab et al., 2010). Moreover, genetic toxicological studies have shown that steviol glycosides do not pose a risk of genetic damage following human consumption (Brusick, 2008). In addition, a number of studies suggested that stevioside may also offer therapeutic benefits beyond sweetness such as antioxidant, anti-diabetic, anti-hyperglycemic, insulinotropic, glucagonostatic, anti-hypertensive, anti-diarrheal, diuretic, anti-cariogenic, anti-viral, anti-microbial, anti-inflammatory, immunomodulatory and chemopreventative activities (Chatsudthipong and Muanprasat, 2009; Puri et al., 2012; Yadav and Guleria, 2012; Boonkaewwana and Burodom, 2013; Shivanna et al., 2013; Thiyagarajan and Venkatachalam, 2012).

Extracts of *S. rebaudiana* leaves induce effects on cardiovascular and renal systems and affects hypertension and hyperglycemia. Since, these activities may be correlated with the presence of antioxidant compounds, extracts of *S. rebaudiana* leaves were evaluated for their total phenols, flavonoid contents and total

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antioxidant activity (Madan et al., 2010). Phenolic compounds are very crucial in the plants for plant growth development and defense against infection and injury. These compounds in injured plants have an important impact on the oxidative stability and microbial safety (Abou-Arab and Abu-Salem, 2010). Many of the biologically active substances found in plants, including phenolic compounds such as flavonoids, phenolics are known to possess potential antioxidant properties (Shukla et al., 2011). The most efficient way to eliminate and decrease the action of free radicals which cause the oxidative stress is antioxidative defense mechanisms. Antioxidants have free radical chain reaction breaking properties (Veeru et al., 2009) and possess the ability to neutralize free radicals and prevent oxidative damage caused by free radicals. They can interfere with the oxidation process, chelating catalytic metals and also act as oxygen scavengers (Lemus-Mondaca et al., 2012). Antioxidant-based drug formulations can be used for the prevention and treatment of complex diseases like atherosclerosis, stroke, diabetes, Alzheimer's disease and cancer (Ramya et al., 2014).

Biologically active substances found in plants also exhibit effects on macrophages which play central role in inflammatory diseases and produce many pro-inflammatory cytokines, such as interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and interleukin-1β (IL-1β). Macrophages also produce inflammatory mediators, including nitric oxide (NO) and prostaglandin E2 ($PGE_2$) (Cho et al., 2013; Lu et al., 2012). Among them, the proinflammatory cytokines TNF-R, interleukin (IL)-1α, and the reactive free radical nitric oxide (NO) synthesized by inducible NO synthase (iNOS) are the important inflammatory mediators reported to be involved in the development of a number of inflammatory diseases (Boonkaewwan et al., 2006). Therefore, inhibition of these pro-inflammatory mediators may be an effective strategy for the treatment of inflammatory diseases.

The aim of this study was to optimize the operating parameters in microwave-assisted extraction (MAE) and ultrasonically-assisted extraction (UAE) of *S. rebaudiana*. The biological (total phenolic, flavonoid contents and DPPH free radical scavenging activities), cytotoxic and nitric oxide inhibition activities of the extracts were investigated in order to interpret the results from the perspective of human welfare. Moreover, the raffinate phase after extraction was characterized to determine the utilization possibilities in industry with a holistic engineering approach.

## 2. Materials and methods

### 2.1. Plant material

Dried *S. rebaudiana* leaves were supplied by Ocal Organic Agriculture in Manisa, Turkey. Prior to the extraction processes, the plant material was ground into fine powder using a Waring laboratory scale blender and sifted using a 30 MESH-sieve (590 μm, average). Powdered plant material was then packed in plastic bags and stored at −20 °C.

### 2.2. Reagents

Rebaudioside A (98%) and stevioside and were purchased from Extrasynthese (France). HPLC grade methanol, ethanol and acetonitrile were from Merck (Darmstadt, Germany). Folin–Ciocalteu's reagent, 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH), sodium carbonate, aluminum chloride hexahydrate and potassium acetate were obtained from Sigma. All other chemicals were of analytical-grade purity. Nanopure water was from an in-house nanopure water system (Sartorius Arium 611, Sartorius-Stedim, Goettingen, Germany).

### 2.3. Extraction processes

#### 2.3.1. Microwave-assisted extraction

About 2 g of dried aerial parts of *S. rebaudiana* was transferred into a vessel containing ethanol by using microwave equipment (Sineo Microwave Chemistry Technology Co., Shanghai). The microwave extraction experiments were performed according to a 4 × 3 full factorial design. Temperature (40, 50, 90 °C), time (5, 30, 45 min), solid/liquid ratio (1:5, 1:10, 1:15 g/ml) and power (300, 400, 500 W) were the independent variables. The set process parameters were automatically controlled throughout the extraction process. At the end of the extraction time, the system was allowed to cool down to room temperature for an additional 5 min prior to opening the vessels. The extract obtained was concentrated to dryness at 55 °C in rotary vacuum evaporator (Hahnvapor RS2005V-N) and subsequently stored at +4 °C until biological activity assays.

#### 2.3.2. Ultrasonically-assisted extraction

About 2 g of dried aerial parts of *S. rebaudiana* was transferred into a centrifuge tube containing ethanol by using ultrasonic bath (Everest Ultrasonic, Istanbul, Turkey). The independent variables were temperature (40, 50, 90 °C), time (30, 60, 90 min), solid/liquid ratio (1:5, 1:10, 1:15 g/ml) and power (50, 75, 100%). The obtained extract was concentrated to dryness at 55 °C in rotary vacuum evaporator (Hahnvapor RS2005V-N) and subsequently stored at +4 °C biological activity assays.

### 2.4. HPLC analysis of the extracts

#### 2.4.1. Sample preparation

About 5 mg of extracts were dissolved in 5 ml HPLC grade methanol and filtered through 0.45 μm nylon membrane (SRP 15, Machery Nagel; Duren, Germany) filters prior to injection.

#### 2.4.2. HPLC-UV conditions

HPLC-UV analyses were performed on a HPLC equipped with Thermo Scientific (America) SCL-10A VP control unit, LC-10 AT VP pump, DGU-14A degasser, SPD-10Avp UV dedector and Rhynodyne manual injector as described in Erkucuk et al. (2009). A SUPELCO $5NH_2$-MS (4.6 mm × 150 mm × 5 μm; Sigma–Aldrich) column was used. The mobile phase comprised water (A) and acetonitrile (B). Isocritic elution was started with 20A/80B, the composition was changed to 20A/80B in 20 min. The column was equilibrated for 10 min with the initial conditions prior to the next injection. The detection wavelength, the flow rate and the sample injection volume were set to 210 nm, 1 ml/min, respectively and the column temperature was ambient (25–27 °C).

#### 2.4.3. Calibration

The following concentrations were prepared by diluting stock solutions with methanol: 1075, 537.5, 268.75, 134.375, 67.1875, 33.5938 and 16.7968 μg/ml for rebaudioside A; 1175, 587.5, 293.75, 146.875, 73.43, 36.7 and 18.35 μg/ml for stevioside. The analysis was carried out as triplicates and linear calibration curve was set off by average of obtained values by using Thermo Scientific Surveyer Chromquest 4.2 software.

#### 2.4.4. Experimental design

Response surface methodology (RSM) is a collection of mathematical and statistical techniques in connection with the fit of a polynomial equation to the experimental data, which describes the behavior of a data set and used as a tool to optimize the process parameters to (Bezerra et al., 2008). In the scope of RSM, the Box–Behnken is a technique which provides (i) estimation of the parameters of the model, (ii) building of sequential designs, (iii)