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Study on chemical composition and biological activities of essential oil and extracts from *Stevia rebaudiana* Bertoni leaves

François N. Muanda [a,*], Rachid Soulimani [b], Babakar Diop [a], Amadou Dicko [a]

[a] Chemistry Laboratory and Methodology for Environment, Paul Verlaine University, 1, Bd Arago Technopole 2000, 57078 Metz, France
[b] Neurotoxicology Alimentary and Bioactivity Laboratory, University P. Verlaine-Metz, BP 4102, 57040 Metz, France

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ABSTRACT

Essential oil (EO), water extract (WE), and methanol–water (MWE) (50/50 v:v) were prepared from *Stevia rebaudiana* Bertoni leaves. Their chemicals compounds, antioxidant, anti-inflammation and antimicrobial activities were evaluated. The EO was analyzed by gas chromatography/mass spectrometry. The WE, and MWE compounds were identified by RP-HPLC. In EO, carvacrol, caryophyllene, caryophyllene oxide, spathulenol, cardinol, α-pinene, limonene, isopinocarveol and ibuprofen were identified as major compounds. Furthermore, in the WE and MWE, the major compounds were, quercetin dihydrate, protocatechuic acid and quercetin glucosyl. These results show that *S. rebaudiana* EO and extracts possess high antioxidant, anti-inflammation and antimicrobial properties. The antimicrobial and antifungal activities of the extracts (EO, WE, MWE) were tested on *Staphylococcus aureus*; *Bacillus subtillis*; *Escherichia coli*; *Pseudomonas aeruginosa*; *Aspergillus niger* and *Candida albicans*, the lowest activity was founded on the EO extract.

1. Introduction

EO and extracts obtained from many plants have recently gained popularity and scientific interest. Many plants have been used for the different purposes, such as food, drugs and perfumery. Researchers are interested in biologically active compounds isolate from plant species for the elimination of pathogenic microorganisms because of the resistance that microorganisms have built against antibiotics (Tepe, Daferera, Sokmen, Sokmen, & Polissiou, 2005). Since ancient times, EO is used as therapeutic agents. For example, lavender and chamomile oils are used for insomnia patients. Digestive problems are treated with peppermint oil, rosemary oil, and coriander oil. Muscle aches and pain are treated with German chamomile oil and eucalyptus oil. Chamomile oil, celery oil, juniper oil, and coriander oil are used for their anti-inflammation benefits. Moreover, some of them have been scientifically proven to possess medicinal activities including anti-inflammatory, antiviral, antitumor, anti-hyperglycemic and anticarcinogenic activities. In addition, discovery of the antioxidant activity is reported in various EO, including rosemary, lavender, eucalyptus, clove oregano, and origanum glandulosum. Consequently, antioxidant activity of EO has gained considerable attention among researchers (Balunas & Kinghorn, 2005; Wei & Shibamoto, 2007).

In recent years, the prevention of cancer and cardiovascular diseases is associated with the ingestion of fresh fruits, vegetables or teas rich in natural antioxidants (Choi, Noh, Cho, Kim, & kim, 2006; Tadhani, Patel, & Subhash, 2007). The protective effects of plant products are due to the presence of several components which have distinct mechanisms of action, some are enzymes and proteins, other are low molecular weight compounds such as vitamins, carotenoids and phenolic compounds (Tadhani et al., 2007; Zhang & Wang, 2002). The beneficial health-related effects of some phenols or their potential antioxidant properties, especially when these compounds are present in large quantities in foods, are importance to be consumers.

The antioxidant compounds present in edible plants have recently promoted as food additives because they display little or no toxic side effects (Seong, Seog, Yong, Jin, & Seung, 2004).

Stevia rebaudiana Bertoni a plant belonging to the family of Compositae, is a sweet herb native from South America. The plant is also cultivated in China and Southeast Asia (Koyama et al., 2003). Stevia sweeteners, crude extract from leaves, are used to sweeten soft drinks, soju, soy sauce, yogurt, and other foods in Japan, Korea and Brazil (Kinghorn & Kim, 2002). The dry extract from the leaves of stevia contains flavonoids, alkaloids, chlorophylls, xanthophylls, hydroxycynnamic acids (caffeic, chlorogenic, etc.), oligosaccharides, free sugars, amino acids, lipids and trace elements (Komissarenko, Derkach, Kovalyov, & Bublik, 1994).

S. rebaudiana sweetener extractives are suggested to exert beneficial effects on human health, including anti-hypertensive,

* Corresponding author. Tel.: +33 3 87 54 70 68; fax: +33 3 87 37 85 06.
E-mail address: franckmuanda@gmail.com (F. N. Muanda).

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anti-hyperglycemic and anti-human rotavirus activities (Chan et al., 2000; Jeppesen, Gregersen, Alstrupp, & Hermansen, 2002; Lee & Shibamoto, 2001).

There are few reports on the antioxidant of the stevia extracts, and to our known there is no report on the antioxidant, anti-inflammation and the antimicrobial proprieties of essential oil of the leaves.

For this purpose, besides their well known sweetening properties, the objectives of this study were: to characterize the chemical composition of EO from leaves of *S. rebaudiana* Bertoni by GC/MS, to determine by RP-HPLC and UV-spectrophotometer the phenolic compounds from WE and MWE extracts, to evaluate the antioxidant, the antimicrobial and the anti-inflammation properties of the EO and these extracts.

2. Materials and methods

2.1. Materials

Dried leaves of *S. rebaudiana* Bertoni were obtained from Nigeria, and the botanical identification was carried out by Professor Max Henry, of the Botanic and Mycology Laboratory, Nancy University France. A Voucher specimen has been kept in our Laboratory for future reference. All plant materials were dried at room temperature, powdered and sifted in a sieve (0.750 μm) before use.

All the chemicals used were analytical grade. 1,1-Diphenyl-2-picryl hydrazyl radical (DPPH), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic) (ABTS), 2,2′-azo-bis(2-amidino-propane) dihydrochloride (AAPH), gallic acid, folin–Ciocalteu's phenol reagent, aluminium chloride, catechin, *p*-coumaric acid, rutin, procatechiuc acid, vitamin C, caffeic acid, isovitexin, vitexin, chlorogenic acid, catechin, quercetin, quercetin dihydrate, quercetin-3-β-D glucosyl, epicatechin, Kuromanin chloride, cyaniding chloride were purchased from Across organics (Geel, Belgium). Sodium carbonate, sodium nitrite, chlorhydric acid, ethyl acetate sodium, sodium sulphate anhydrous, ammonium phosphate, acetonitrile, methanol, interferon-gamma (IFN-γ), polysaccharide of salmonella typhimurium (LPS), sulfanilamide, NEDA (N-(1-naphtyl) ethylenediamine dihydroxy chloride), vanillin reagent, n-hexane were obtained from Sigma and Roth (Strasbourg, France). Agar nutrient (Mueller-Hinton - agar) (Becton Dickinson, Heidelberg), paper discs, ampicillin, gentamicin, nystatin, pure cultures of the bacteria (*Staphylococcus aureus* (ATCC29213)), *Pseudomonas aeruginosa* (ATCC27853), *Escherichia coli* (ATCC25922), *Bacillus subtillis* (ATCC6059) and fungi (*Aspergillus niger* (135550/99), *Candida albicans* (ATCC90028)) were given by the I.B.I.S.E laboratory (UIT Thionville, France).

2.2. Methods

2.2.1. Sample preparations

2.2.1.1. Essential oil (EO). The EO was prepared by a previously reported method slightly modified (Tuberoso et al., 2005). Dried *S. rebaudiana* Bertoni powder leaves (500 g) were placed in a 4 L round bottom flask, and mixed with 1 L of deionised water. The powdered leaves were subjected to hydro distillation in a Clevenger-type apparatus for 5 h according to the procedure of European Pharmacopeia (Soulimani, 1992). A colourless volatile oil (yield = 3.5 ± 0.15 g/100 g dw%) with characteristic odour and sharp taste was obtained. This was dried over anhydrous sodium sulphate to remove the traces of moisture filled into a bottle and stored in a refrigerator in the dark at 4 °C until use.

2.2.1.2. Total phenolic compounds (TPC), total flavonoid compounds (TFC), total anthocyanin compounds (TAC), anti-inflammation, antimicrobial and antioxidant activities. Samples for total phenolic compounds (TPC), total flavonoid compounds (TFC), total anthocyanin compounds (TAC), anti-inflammation, antimicrobial and antioxidant assays were extracted from the powders as described by a previously reported method (El-Massry, El-Ghorab, Shaaban, & Shibamoto, 2009).

Two grams of powdered sample were extracted twice with 10 mL of cold aqueous methanol solution (50%). The two volumes were combined, made up to 20 mL, centrifuged at 1536×*g* for 20 min and transferred in small sample bottles and stored at +4 °C in the dark until analyze. Two others grams of powdered sample were extracted with di-distillate water and prepared in the same conditions as methanol–water solvent.

2.2.1.3. Condensed tannin (CT). Samples for CT were extracted from the powders as described by Villareal-Lozoya, Lombardini, & Cisneros-Zevallos (2007). One gram of powder was extracted twice with 20 mL of n-hexane while 20 min, filtered and the remaining powder was dried at 35 °C under vacuum for 2 h. The powder was stored at +4 °C until analyses.

2.2.1.4. Phenolic compounds extraction for RP-HPLC analysis. Polyphenols were extracted according to the method described by (Sharma, Gulati, Ravindranath, & Kumar, 2005) slightly modified. Two different samples were prepared with di-distillate water and methanol–water (50%).

Briefly, 0.4 g of powders were extracted with 2 × 5 mL of methanol–water (50%)/(di-distillate water) with intermittent shaking (2 min) on vortex mixer for 30 min, the sample was centrifuged at 1536×*g* for 20 min at 20 °C and the supernatant was taken into a 10 mL volumetric flask. The extract is stable for 24 h if it is stored at 4 °C.

2.3. Analysis of EO

The EO was analyzed and identified by GC/MS. An HP model 6890 GC equipped with a 30 m × 0.25 mm i.d. (D_f: 0.25 μm) DB-5 boded-phase fused-silica capillary column (Agilent, Folsom, CA) and a flame ionization detector (FID) were used. Injector and temperatures were 200 and 300 °C, respectively. The oven temperature was programmed from 35 to 250 °C at 5°C/min and held for 50 min. The linear velocity of the helium carrier gas was 30 cm/s. Injections were in the split-less mode. An HP model 6890 GC interfaced to an HP 5791A mass selective detector (GC/MS) was used for mass spectral identification of the GC components at an MS ionization voltage of 70 eV. A 30 m × 0.25 mm i.d. (D_f = 0.25 μm) DB wax bonded-phase fused-silica capillary column (Agilent, Folsom, CA) was used for GC.

The linear velocity of the helium carrier gas was 30 cm/s. The temperature of injector and detector was 250 °C. The oven temperature was programmed from 50° to 250 at 5°C/min and held for 50 min.

The percentage of each compound in the oil is determined from peak areas without correction factors account assuming that all components have coefficients of neighboring rethinking. Identification of constituents was performed by coupling an HP model 6890 GC gas to a mass spectrometer type an HP 5791A mass selective detector (GC/MS). The volume injected is 1 μl of a pure oil solution diluted to 1% in dichloromethane.

Qualitative analysis was based on the comparison of retention times and the computer mass spectra libraries using Wiley GC/MS Library and Nist, Tutore Libraries. The percentage composition was computed from the GC peak areas.

2.4. Analysis of phenolic compounds

2.4.1. Total polyphenolic compounds (TPC)

TPC content in the EO, WE and MWE was determined by the Folin–ciocalteu reaction method and the results were expressed as

(GAE/g dry weight) gallic acid equivalents. Samples (0.2 mL) were mixed with 1 mL of 10-fold-diluted Folin–Ciocalteu reagent at 0.8 mL of 7.5% sodium carbonate solution. After the mixture was allowed to stand for 30 min at room temperature, the absorbance of samples was measured at 765 nm using a spectrophotometer (UV–Vis Spectrophotometer Varian Cary 50 scan). The experiment was replicated three times.

2.4.2. Total flavonoid compounds (TFC)

The TFC contents in the EO, WE and MWE were measured according to a colorimetric assay (Bhatti, Iqbal, Chatha, & Bukhari, 2007) (UV–Vis Spectrophotometer Varian Cary 50 scan). A 250 μl of standard solution of catechin at different concentrations or appropriately diluted samples was added to 10 mL volumetric flask containing 1 mL of distillate water. At initial time, 75 μl of $NaNO_2$ (5%) was added to the flask. After 5 min, 75 μl of $AlCl_3$ (10%) was added and 6 min later 500 μl of NaOH (1N) was added to the mixture. Then, immediately the solution was diluted by adding 2.5 mL of distillate water and mixed thoroughly. Absorbance of the mixture, pink in colour, was determined at 510 nm against a blank. Total flavonoid compounds in medicinal plant were expressed as mg catechin equivalents (CE/g dry weight) (dw). Samples were analyzed in three replications.

2.4.3. Total anthocyanin compounds (TAC)

The TAC of the samples (EO, WE and MWE) were estimated using a UV-spectrophotometer (UV–Vis Spectrophotometer Varian Cary 50 scan) by the pH-differential method (Abu Bakar, Mohamed, Rahmat & Fry, 2009; Lako et al., 2007). Two buffer systems, potassium chloride buffer, pH 1.0 (0.025 M) and sodium acetate buffer, pH 4.5 (0.4 M) were used. Briefly, 400 μl of extract was mixed in 3.6 mL of corresponding buffer solutions and read against a blank at 510 and 700 nm. Absorbance (ΔA) was calculated as: $\Delta A = (A510 - A700)\ pH\ 1.0 - (A510 - A700)\ pH4.0$ monomeric anthocyanin pigment concentration in the extract was calculated and expressed as equivalent cyaniding-3-glucoside (mg/l): $\Delta A \times Mw \times D_f \times 1000/(Ma \times c)$. With ΔA: difference of absorbance, Mw: molecular weight for cyaniding-3-glucoside (449.2); D_f: the dilution factor of the samples, Ma: molar absorptivity of cyaniding-3-glucoside (26,900); and c: concentration of the buffer in mg/mL. Results were expressed as mg of Cyaniding-3-glucoside Equivalents (CgE).

2.4.4. Condensed tannin (CT)

The CT content was estimate using the method described by a previously method (Villarreal-Lozoya, Lombardini, & Cisneros-Zevallos, 2007) with some modifications. Briefly, an aliquot of 0.5 g of powder obtained after lixiviation (n-hexane) was placed in a centrifuge tube and 15 mL of 1% HCl in methanol was added to each sample. Each tube was vortexed and placed in a water bath at 35 °C with constant shaking for 20 min and vortexing every 5 min. After incubation, the tubes were centrifuged (1536×*g*) and the supernatants were extracted. Aliquots of the supernatants (100 μl) were placed in two separate assay tubes, one for the sample determination and the other for blank determination. Samples and blanks were incubated for exactly 20 min after adding 5 mL of vanillin reagent (0.5 g of reagent and 200 mL of 4% HCl methanol) to samples and 4% HCl in methanol to the blanks.

After 20 min, absorbance was read at 500 nm from each sample and blank using UV-spectrophotometer Varian Cary 50. Samples absorbance were rectified with the blank and compared against a standard curve made with catechin. Results were expressed as mg catechin equivalent (CE) of lixiviating sample. Analyses were triplicate.

Table 1
GC/MS analysis of *S. rebaudiana* essential oil.

Ref.	Rt (min)	% v/v (ml)	Name of compound
1	5.70	0.67	(E) 3,7-dimethyl−1,3,6-octatriene
2	6.87	3.75	α-pinene
3	8.31	0.46	limonene
4	10.38	1.94	propanoate
5	10.47	0.50	Z, Z-2,5-pentadecadien-1-ol
6	12.22	0.48	2(10) - pinene-3-one
7	13.20	1.26	isopinocarveol
8	17.45	1.79	ibuprofen
9	18.43	0.55	β-bourbonene
10	18.58	1.31	1-ethenyl-1-methyl-2,4-bis (1-methylethenyl)-cyclohexane
11	19.38	0.98	4,11,11-trimethyl-8-bicyclo [7.2.0] undec-4-ene
12	19.71	1.25	3,7,11-trimethyl-1,3,6,10-dodecatetraene
13	20.03	0.71	(Z)-5,9-undecatriene,7,11-dimethyl-3-methylene
14	20.19	3.61	7,11-dimethyl-3-methylene-1,6,10-dodecatriene
15	20.29	1.15	α-caryophyllene
16	20.73	0.60	5-ethyl-3-methyl-3,4-nonadien-6-yne
17	20.84	0.59	1,3-di-n-propyladamantane
18	20.90	0.53	8-cedren-13-ol
19	21.14	5.59	cardinol
20	21.30	0.94	azulene
21	21.57	0.56	α-farnesene
22	22.91	4.63	3,7,11-trimethyl-3-hydroxy-6,10-dodecadien-1-yl acetate
23	23.12	0.64	ledol
24	23.36	15.41	spathulenol
25	23.46	23.50	caryophyllene oxide
26	23.97	0.55	farnesol
27	27.67	0.51	2-propenoic acid,2-methyl-, ethenyl ester
28	27.74	0.76	N-benzenesulfonyloxy-2,2-bis (trifluromethyl)aziridine
29	27.93	67.89	carvacrol
30	29.04	3.43	6,10,14-trimethyl-2-pentadecanone
31	30.80	0.65	1-nitrosoadamantane
32	31.11	0.47	dispers Yellow 1
33	31.52	2.45	myristic acid
34	32.16	4.01	2-methyl-3-(3-methyl-but-2-enyl)-2-(4-methyl-pent-3-enyl)-isoflavone

2.5. RP-HPLC analysis

RP-HPLC analysis was performed according to the modified method described by a previously reported method (Muanda, Koné, Dicko, Soulimani, & Younos, 2009). The extracted samples (MWE, WE) were filtered through a 0.45 μm PTFE syringe tip filter before the injection. The analyses were carried out on an RP-HPLC with a Waters 600E pump coupled to a Waters 486 UV visible tunable detector and equipped with a 20 μl injection loop and an Alltech Intersil ODS (column size 4.6 mm × 150 mm; particle size, 5 μm). The flow rate was set at 1 mL/min at room temperature.

To perform this study a gradient of three mobile phases was used. Solvent A: 50 mM ammonium phosphate ($NH_4H_2PO_4$) pH 2.6 (adjusted with phosphoric acid), solvent B: (80:20 (v/v)) acetonitrile/solvent A, and solvent C: 200 mM of phosphoric acid pH 1.5 (pH adjusted with ammonium hydroxide). The solvents were filtered through a Whatman Maidstone England paper No. 3 and putted in an ultrasonic apparatus for 25 min. The gradient profile was linearly change as follows (total 60 min): 100% solvent A at 0 min, 92% A/8% B

Table 2
Phenolic compounds of EO, WE and MWE.

Extracts	TPC mg GAE/g dM	TFC mg CE/g dM	TAC mg Cg/g dM	CT mg CE/g dM
EO	1.15 ± 0.57	2.23 ± 0.80	Nd	Nd
WE	20.85 ± 0.40	20.68 ± 1.05	0.35 ± 0.01	8.15 ± 0.07
MWE	25.25 ± 0.21	23.46 ± 0.44	0.67 ± 0.09	10.20 ± 0.19

EO: Essential oil, WE: water extract, MWE: methanol– water-extract, TPC: total phenolic compounds, TFC: total flavonoid compounds, TAC: total anthocyanin compounds, CT: condensed tannins.

at 4 min, 14% B/86% C at 10 min, 16% B/84% C at 22.5 min, 25% B/75% C at 27.5 min, 80% B/20% C at 50 min, 100% solvent A at 55 min, 100% A at 60 min. After each run, the system was reconditioned for 10 min before the analysis of the next sample.

Under these conditions, 20 μl of sample were injected. Polyphenolic external standards were prepared by dissolving 2 mg/mL and used as reference. In each sample, polyphenol was identified by comparing its retention time with that of the corresponding external standard and the concentration of the polyphenolic compound was determined by comparing their peak areas. The detection was done at 280 and 320 nm. The samples were analyzed at least three replications.

2.6. Antioxidant activity analysis

The DPPH test and ABTS test were used to determine the total antioxidant capacity of the extracts (EO, WE, MWE).

2.6.1. DPPH test expressed as IP%

The DPPH radical scavenging activity was calculated according to the method previously described by Muanda et al., 2009. 1 mL of a DPPH solution (100 μM in methanol–water (50% v/v)) was mixed with 1 mL of plant extracts. The reaction mixture was incubated in the dark for 20 min, and the optical density of the different extracts (EO, WE, MWE) was recorded at 517 nm against the blank. For the control, 1 mL of the DPPH solution was mixed with 1 mL of methanol–water (50% v/v) and the absorbance of the solution was recorded after 20 min. The ability to scavenge DPPH radical was calculated using the following equation:

$$\%IP = [(A_0 - A_1)/(A_0 \times 100)]$$

Where: A_1: absorbance of the test sample and A_o: absorbance of control. Each assay was carried out in triplicate.

2.6.2. DPPH test expressed as vitamin C equivalent

To 2.90 mL of DPPH solution (100 μM in methanol–water (50% v/v)), 100 μl of the plant extract (EO, WE, MWE) was added. The mixture was shaken and stored at 20 °C in dark for 40 min. After, the decrease of the absorbance in the resulting solution was monitored at 517 nm.

A standard curve was prepared by plotting the absorbances of free radical scavenging activity of vitamin C versus its concentration. The final result was expressed as mg vitamin C equivalent antioxidant capacity in 1 g of sample (VCE). The radical solution was prepared daily.

2.6.3. ABTS test

The method used in this test is that one developed by a previously method (Muanda et al., 2009). 1.0 mM of AAPH solution was

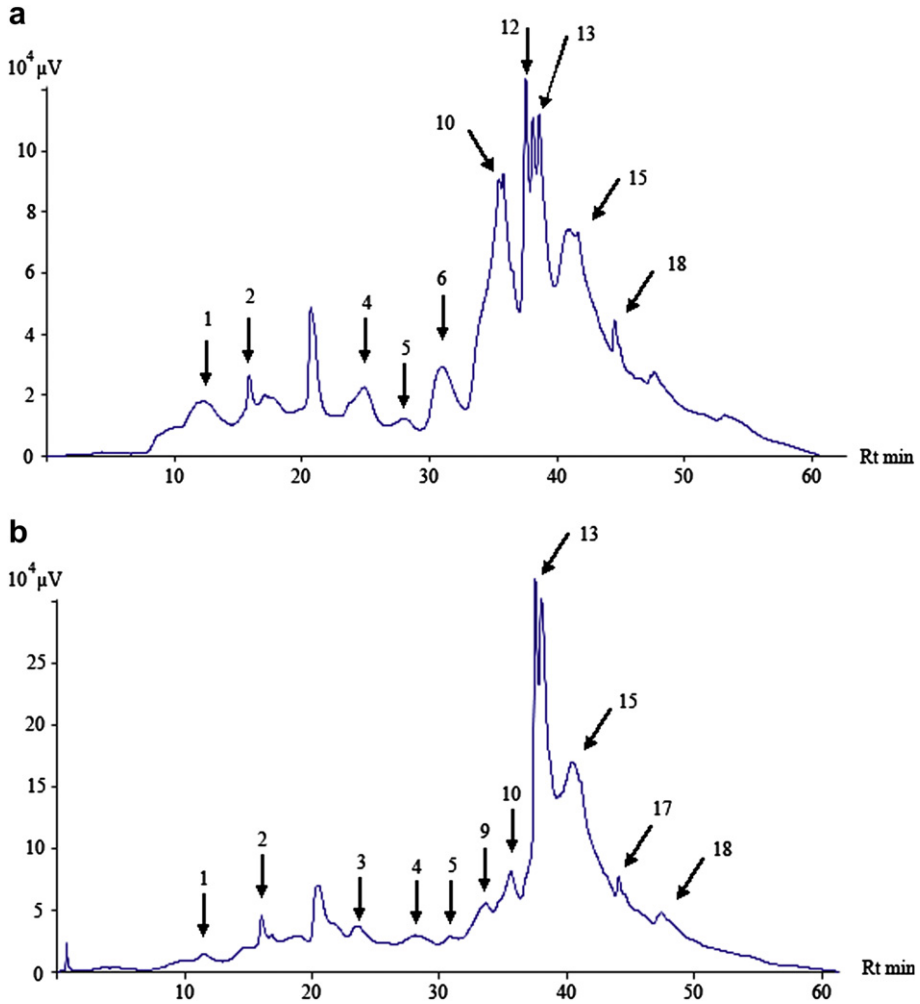

Fig. 1. a) Chromatogram HPLC analysis Water extract 280 nm, 1: Gallic acid; 2: protocatechuic acid; 4: Chlorogenic acid; 5: caffeic acid, 6: epicatechin; 12: rutin; 13: quercetin glucosyl; 15: quercetin dihydrate; 18: apigenin. (b) Chromatogram HPLC analysis Water extract 320 nm, 1: Gallic acid; 2: protocatechuic acid; 3: catechin, 4: chlorogenic acid; 5: caffeic acid; 9: *p*-coumaric acid; 13: quercetin glucosyl; 15: quercetin dihydrate; 17: quercetin 18: apigenin.

mixed with 2.5 mM of ABTS as diammonium salt in phosphate buffer saline (PBS) (solution 100 mM potassium phosphate buffered (pH 7.4) containing 150 mM NaCl). The mixture was heated in a water bath at 68 °C for 20 min. The concentration of the resulting blue–green $ABTS^{\bullet+}$ (radical cation solution) was adjusted to an absorbance of 0.65 ± 0.02 at 734 nm. The sample solution (60 μl) was added to 2.94 mL of the resulting blue–green $ABTS^{\bullet+}$. The mixture, protected from light, was incubated in a water bath at 37 °C for 20 min. A control solution (60 μl of methanol and 2.94 mL of $ABTS^{\bullet+}$). The decrease of absorbance was measured at 734 nm. The stable ABTS radical scavenging activity of the plants phenolic compounds in the extracts was expressed as mg/g dry plants powders standards compounds of VCE (vitamin C equivalent) in 20 min. All radical stock solutions were prepared fresh daily.

2.7. Anti-inflammatory activity by nitrite assay

The test used to assess the potential anti-inflammatory activity of molecules consisted of evaluating their capacity to inhibit NO production in activated macrophages (Pacheco-Sanchez, Boutin, Angers, Gosselin, & Tweddell, 2007). Released nitrite (NO_2^-) in the culture medium was measured as an indicator of NO production according to the colorimetric test based on the Griess reaction. Briefly, 1 mL of plant extract was mixed with 1 mL of Griess reagent at room temperature for 30 min. The nitrite concentration was determined by measuring the absorbance at 548 nm using a standard curve of $NaNO_2$.

The results were expressed as percentage of NO production compared to the control as follows:

$$\%\,\text{Inhibition} = 100 \times \left[NO_2^-\right]_{\text{control}} - \left[NO_2^-\right]_{\text{Ex}} / \left[NO_2^-\right]_{\text{control}}$$

With $[NO_2^-]_{control}$ is the concentration of nitrite released without addition of the extract, and $[NO_2^-]_{Ex}$ the concentration of nitrite released by the cells in presence of the plant extract.

2.8. Determination of antimicrobial activities

The following bacterial strains were employed in the screening: *Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa* and fungi: *Aspergillus niger, Candida albicans* given by IBiSE (UIT Thionville - Metz University).

2.8.1. Antimicrobial assays

The antimicrobial, the antifungal and the yeast activities were examined by the disk-diffusion method (Bauer, Kirby, Sheriss, & Turck, 1996). The bacterial cell suspension was prepared from 24 h culture and adjusted to an inoculation of 1×10^6 colony forming

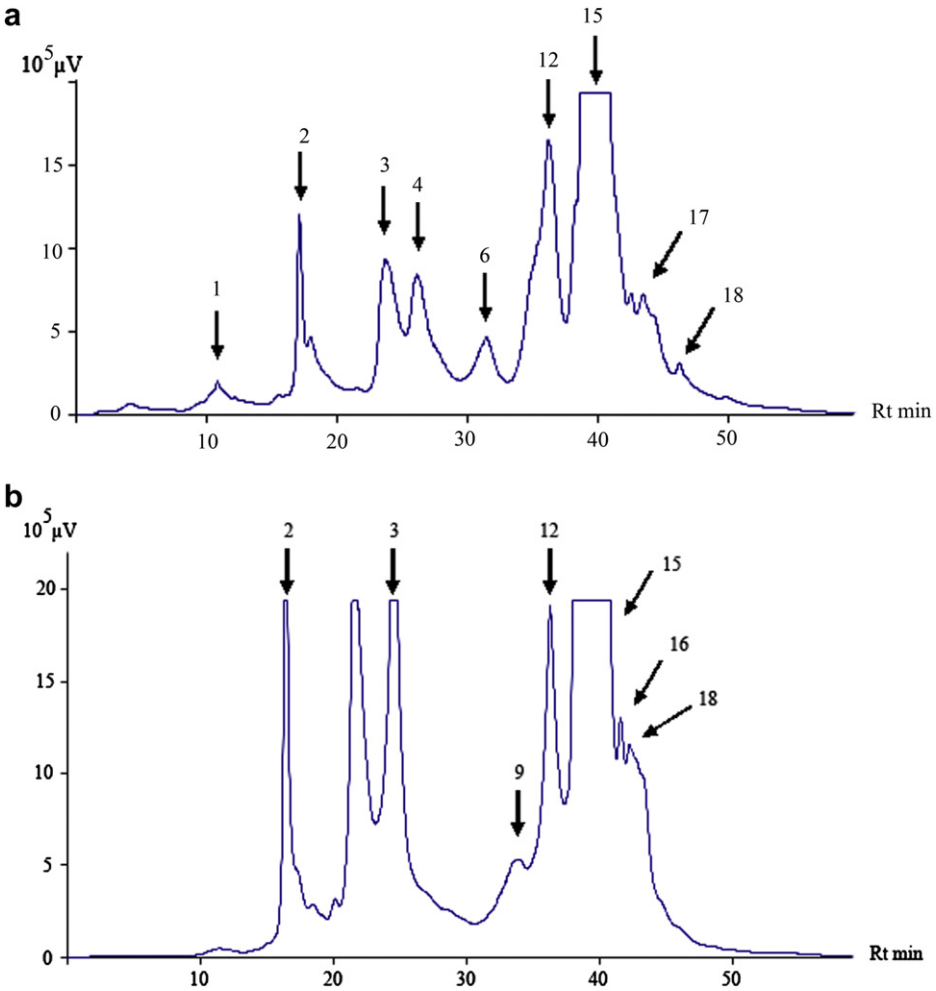

Fig. 2. a) Chromatogram HPLC analysis Methanol–water-extract 280 nm, 1: Gallic acid, 3: catechin, 4: chlorogenic acid; 6: epicatechin, 12: rutin, 15: quercetin dihydrate, 17: quercetin, 13: apigenin. (b) Chromatogram HPLC analysis Methanol–water-extract WE 320 nm, 4: Chlorogenic acid, 9: *p*-coumaric acid, 12: rutin, 15: quercetin dihydrate, 16: cinnamic acid, 17: quercetin.

units per mL. Sterile nutrient agar (Immun präparate, Berlin, D, 26 g agar/l distilled water) was inoculated with bacterial cells (100 μl of bacterial cell suspension in 25 mL medium) and poured into dishes to give a solid plate. Yeasts and hyphomycetes (1×10^6 colony forming units per mL) were inoculated into sterile Mueller-Hinton agar (Becton Dickinson, Heidelberg) according to DIN E 58940-3 for the agar disc-diffusion assay (Al-Fatimi, Wurster, Schröder & Lindequist, 2007). 10 μl of tested material (250 g/500 mL) dissolved in the same solvent of the extraction, were applied on sterile paper discs (6 mm diameter). Ampicillin, gentamicin, and nystatin were used as positive control and the solvents, water and methanol–water (50/50: v/v) as negative control.

The solvents were evaporated in a stream of air and the discs were deposited on the surface of inoculated agar plates. Plates were kept for 1 h in refrigerator to enable pre-diffusion of substances into the agar. Plates with bacteria were incubated for 24 H at 37 °C, plates with yeast for 48 h and plates with hyphomycetes for 72 h at 30 °C. The inhibition zone diameters around each disc (diameter of inhibition zone plus diameter of the disc) were measured after the incubation time. An average zone of inhibition was calculated for the three replicates. Minimal inhibitory concentrations (Mics) were determined by the agar diffusion technique as described by Rajbhandari and Schöpke (1999). The highest concentration of extract tested during the experiment was 2 mg/mL. The MIC (minimum inhibitory concentration) corresponds to the lowest concentration of the test extract (EO, WE or MWE), able to inhibit any visible microbial growth. Several concentrations of the extracts were prepared, (0.25 mL/mL, 0.2 mL/mL, 0.15 mL/mL, 0.1 mL/mL, 0.05 mL/mL, and 0.005 mL/mL) the different solutions were agitated vigorously. Approximately, 10 μl of each concentration mixture was transferred in the disk. Then the disks were transferred in the Petri dishes containing the microorganism testing. The plates were incubated for 24 h at 37 °C for bacteria, for 48 h at 30 °C for yeast, and for 72 h at 30 °C for fungus. After incubation, the number of colonies in each plate was counted. Each assay replicated three times.

2.9. Statistical analysis

Results are presented as mean ± standard Error; statistical analyses of experimental results were based on the analysis of variance method. Significant difference was statistically considered at the level of $P < 0.001$.

3. Results and discussion

3.1. Chemicals identified in Stevia rebaudiana Bertoni EO

Yield of essential oils from *S. rebaudiana* Bertoni leaves extract from water content was 3.50 ± 0.15 g/100 g dw. This value is the mean ± standard deviation ($n = 3$) ($P \leq 0.05$).

The analysis of essential oil by GC–MS is presented in Table 1. More than 33 components were identified such as carvacrol (67.80), caryophyllene oxide (23.50), spathulenol (15.41), cardinol (5.59), α-pinene (3.75), ibuprofen (1.79), isopinocarveol (1.26), caryophylle (1.15%), pinene - 3 one (0.48) dispers yellow 1(0.47), and limonene (0.46). According to the literature, many of these compounds are known for their antioxidant proprieties (pinene, carvacrol, caryophyllene) also the EO constituents of the plants varied to several factors: geographical, culture conditions, collect time, altitude, climate, different chemotype and nutritional status; all these factors can influence their composition (Kapoor, Singh, Singh, Heluani, Lampasona, & Catalan, 2009) and (Ozkan, Sagdic, Gokturk, Unal, & Albayrak, 2010).

Table 3
Compounds identified in WE, MWE and their concentrations.

Ref.	Name of compound	Rt (min)	WE (mg ml^{-1})	MWE (mg ml^{-1})
1	gallic acid	11.98 ± 0.22	0.17 ± 0.02	0.04 ± 0.01
2	protocatechuic acid	14.78 ± 0.92	1.46 ± 0.14	0.49 ± 0.16
3	catechin	23.96 ± 0.23	1.44 ± 0.02	0.97 ± 0.13
4	chlorogenic acid	25.65 ± 0.21	0.32 ± 0.09	1.03 ± 0.10
5	Caffeic acid	28.78 ± 0.38	0.08 ± 0.02	Ni
6	epicatechin	31.17 ± 0.65	0.53 ± 0.03	0.52 ± 0.08
9	*p*-coumaric acid	33.25 ± 0.36	0.05 ± 0.01	0.07 ± 0.02
12	rutin	37.02 ± 0.36	0.88 ± 0.21	1.99 ± 0.23
13	quercetin glucosyl	37.71 ± 0.18	1.19 ± 0.27	Ni
15	quercetin dihydrate	39.47 ± 0.19	1.04 ± 0.23	4.48 ± 0.38
16	cinnamic acid	41.08 ± 0.65	0.58 ± 0.14	0.63 ± 0.12
17	quercetin	42.87 ± 0.16	0.45 ± 0.11	0.27 ± 0.08
18	apigenin	44.89 ± 0.59	0.10 ± 0.02	0.19 ± 0.04

3.2. Phenolic compounds determined in EO, WE and MWE

Table 2 shows the phenolic contents of EO, WE and MWE leaves of *S. rebaudiana*. Through these results we observed that the TPC and TFC contents are almost equivalent in both WE and MWE. The TAC and CT are more important in MWE than in WE. The EO is poorly in phenolic compounds. The TPC and TFC composition in WE are similar with those found by Tadhani et al. (2007).

3.3. RP-HPLC analysis

Figs. 1 and 2 show the profiles of the injection of the extracts detected at 280 and 320 nm. The retention time and the concentration of phenolic compounds contained in these extracts are reported in the Table 3. Quantitative analysis shows that methanol–water solvent extracts more phenolic compounds than water. This is in concordance with Marwah et al. (2007) and Muanda et al. (2009) which reported that an aqueous alcohol is considered to be the best solvent for extracting phenolic compounds from plant materials. However, these results show that gallic acid (0.17 mg/mL), protocatechuic acid (1.46 mg/mL) and catechin (1.44 mg/mL) are more extracted in water than in methanol–water because these acids can be transformed into their corresponding esters in hydroalcoholic medium (Mortier, 1991). It was found that in the MWE, quercetin dihydrate (4.48 mg/mL), rutin (1.99 mg/mL) and chlorogenic acid (1.03 mg/mL) were the major compounds extracted.

3.4. Antioxidant activity analysis

The radical scavenging effect of the EO, WE and MWE determinate by the DPPH, ABTS two colouring methods and the values comparing to VCE are indicate in Table 4. To compare this activity with those of other plants described in the literature, the antioxidant activity determinate by DPPH test was expressed as IC_{50}. The IC_{50} values of *S. rebaudiana* extracts were 2.9, 5.0 and 9.26 μg/mL respectively for E.O, WE and MWE; compared to *Becium dhofarense* Sebald (IC_{50}: 11.0 μg/mL), *Ficus lutea* Vahl (IC_{50}: 11.9 μg/mL), *Cordia*

Table 4
Antioxidant activities (AOA) values of the *S. rebaudiana* leaves extract.

AOA				
Extracts	ABTS mgVCE/gdM	DPPH		
		mgVCE/gdM	% IP	Ic_{50} (μg/mL)
EO	0.22 ± 0.01	0.09 ± 0.01	77.55 ± 3.45	19.26 ± 0.35
WE	0.67 ± 0.18	0.97 ± 0.12	82.86 ± 2.04	5.00 ± 0.20
MWE	1.17 ± 0.02	1.78 ± 0.01	96.61 ± 0.90	2.90 ± 0.02

perroteetti Wight (IC_{50}: 15.2 μg/mL), *Acacia Senegal* (L.) Willd (IC_{50}: 17.8 μg/mL) (Chitindingue, Ndhlala, Chapano, Benhura, & Muchuweti, 2006), these results proved that the extracts from S. *rebaudiana* leaf possess significant antioxidant properties.

For these extracts and this oil, the medicinal benefit derived from their use may include prevention of oxidative skin damage and subsequent disease progression. Essential oils are complex mixtures and the determination of the component(s) responsible of the activity is difficult. Antioxidant activity of essential oils has often been attributed to the presence of phenolic constituents, especially thymol and/or carvacrol (Hazzit, Baaliouamer, Veríssimo, Faleiro, & Miguel, 2009). This association was confirmed in the most part of this study, but other compounds also seem to play an important role such as carvacrol, α-pinene, pinene-oxide, isopinocarveol, cardinal. The results presented here may contribute to the knowledge of the antioxidant potential of the essential oil and provide some information for its uses.

3.5. Anti-inflammatory properties of phenolic compounds

The anti-inflammatory activity of EO, WE and MWE was studied in vitro for their inhibitors effects on chemical mediators release (LPS/IFNc-induced NO production) from macrophages. Activated macrophages produce large amounts of chemical mediators that indicate inflammation.

NO, a bioactive free radical, is one of these critical mediators which is produced by inducible NO synthase (iNOS) in inflammatory macrophages when stimulated with LPS/IFNc mixture. Excessive production of NO is indicated both in chronic and acute inflammation (Muanda, Dicko, & Soulimani, 2010; Terra et al., 2007). In fact, NO production iduced by LPS/IFNc through INOs induction may reflect the degree of inflammation and provide a measure for assessing the effect of the extract/fractions on the inflammatory process.

As shown in Fig. 3, the addition of EO at different concentrations (20 μl, 50 μl, 100 μl and 150 μl) significantly reduced NO production, resulting in 19.08%, 26.67%, and 46.67% inhibition of NO production in macrophages stimulated with LPS/IFNc, respectively. As for the WE and the MWE, their use showed significant inhibitor effect varying between (28.33–62.33%) for WE and (48.11–93.33%) for MWE. These results show a significantly reduced NO production in a dose-dependent manner. Similarly to our results, it has been demonstrated in several studies that extract rich in flavonoids and proanthocyanidins inhibited NO production in RAW 264.7 macrophages (Clancy et al., 1998; Diouf, Stevanovic, & Cloutier, 2009; Ho, Hwang, Shen, & Lin, 2007; Pacheco-Sanchez et al., 2007). These results seem to indicate that phenolic compounds present in the extracts are responsible for the anti-inflammatory activity.

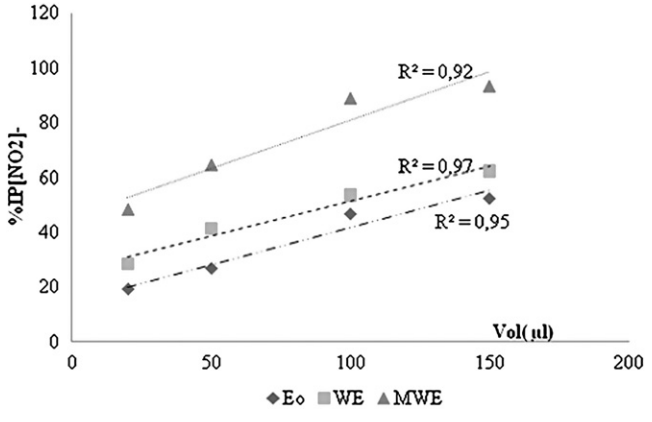

Fig. 3. Relation ship between inhibition percent of NO_2^- and the concentration (volume) of $[NO_2]^-$.

Table 5
In vitro antimicrobial activity of the essential oils and the extracts of *S. rebaudiana* Bertoni.

Samples/extract	Inhibition zones (mm)					
	E.c.	C.a.	A.n.	P.a.	B.s.	S.a.
EO	10 ± 1	15 ± 1	19 ± 3	n.a	12 ± 2	12 ± 3
WE	13 ± 1	11 ± 2	n.a	13 ± 1	12 ± 3	10 ± 1
MWE	21 ± 2	17 ± 1	16 ± 2	15 ± 2	10 ± 2	19 ± 2
Wdd(−)	0	0	0	0	0	0
MW(−)	0	0	0	0	0	0
Ap(+)	58 ± 3	n.a	n.a	45 ± 3	55 ± 3	56 ± 3
Gt(+)	55 ± 2	n.a	n.a	45 ± 2	45 ± 2	44 ± 2
Ny(+)	n.a	53 ± 1	47 ± 1	48 ± 1	47 ± 1	47 ± 1

S.a.: *Staphylococcus aureus*; B.s.: *Bacillus subtillis*; E.c.: *Escherichia coli*; P.a.; *Pseudomonas aeruginosa*; A.n.; *Aspergillus niger*; C.a.: *Candida albicans*; EO: Essential oil; WE: water extract; MWE: methanol–water-extract; Wdd(−): di-distillate water negative control, MW(−): methanol–water (50/50 v/v) negative control; Ap(+): Ampicillin positive control; Gt(+): gentamicin positive control; Ny(+): Nystatin positive control; na: not actif.

Nevertheless, in the EO, the anti-inflammatory activity is correlates positively with the radical - scavenging activity and the highest antioxidant activity was correlated with the highest total phenol content and/or the highest anti-inflammatory activity of the tested extract/fraction.

3.6. Antibacterial, anti mold and antifungal activities

The results of antibacterial screening of EO, WE and MWE against some bacteria, fungus and yeast species are summarized in Tables 5 and 6 for the inhibition zones in the agar diffusion assay and the MIC values. All the samples from *S. rebaudiana* Bertoni leaves presented an inhibitory activity against the microorganisms tested except the WE and the EO on A.n (*Aspergillus niger*) and P.a. (*Pseudomonas aeruginosa*), respectively. Comparing to others plants extract in the literature data (Al-Fatimi, Wurster, Schröder & Lindequist, 2007; Bader, Panizzi, Cioni, Flamini, & Ligustica, 2007; Wei & Shibamoto, 2007), these extracts show in the agar diffusion assay a good antibacterial activity (Table 5). WE and MWE have the most effective antimicrobial activity (Tables 5 and 6).

Meanwhile, it is observed that the EO, whose IP% value and phenolic compound contents were small, have weak antimicrobial activity with MIC values always >1000 μg/mL (Table 6). It has been reported that antibacterial activity of the extracts is due to the flavonoids, aromatic acids, terpenoids, and its esters contents (Choi et al., 2006).

In conclusion, in this study, the phytochemicals, the antioxidant and antimicrobial proprieties of *S. rebaudiana* Bertoni leaves and essential oil were investigated. The extracts WE, MWE and EO had good antioxidant, anti-inflammation and antimicrobial activities. In regard to these results, we can suggest that *S. rebaudiana* Bertoni can be a natural source of antioxidant, anti-inflammation and

Table 6
Minimal inhibitory concentrations of MWE, WE and EO.

Extract	MIC (μg/mL)					
	E.c.	C.a.	A.n.	P.a.	B.s.	S.a.
EO	>1000	>1000	>1000	–	>1000	>1000
WE	>700	>700	–	>700	>700	>700
ETWE	>500	>500	>700	>500	>500	>500
MWE	>500	>500	>700	>500	>500	>500

MIC: microbial critical concentration; Values are mean ± SD of three determinations.

antimicrobial properties. Its can also be consider as a potential source of essential oil. These results suggest that the leaves of *S. rebaudiana* Bertoni can serve as therapeutic agents or in prevention of certain inflammatory diseases associate with the increase of NO production, through their radical-scavenging capacities. It can also be used regarding to its constituents for various commodities of cosmetics, medicinal and pharmacological attributes as a supplementation in addition to its use as sugar plants. In that respect, it represents an economic alternative of great interest.

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