Acantholippia salsoloides: Phytochemical Composition and Biological Potential of a Thujonic Population

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Abstract

Acantholippia salsoloides (Verbenaceae) is an aromatic plant widespread in the Andean region. The infusion (leaves and flowers) is widely used as a digestive stimulant as well as for the treatment of various diseases in traditional medicine. A. salsoloides attributes its common name “rica-rica” to the fresh and sweet fragrance of the plant. In this work, 2 different polar extracts and the essential oil of a selected rica-rica population were studied. The phenolic composition was analyzed by high-performance liquid chromatography diode array detector; the essential oil profile was determined by gas-chromatography ion-trap mass spectrometry/flame ionization detection. For all extracts, the antibacterial potential was performed by in vitro assays; the antioxidant and α-glucosidase inhibition were determined in decoction and hydroethanolic extracts. The volatile profile allowed the identification of 26 volatile compounds, β-thujone (84%) being the major one in this rica-rica population. Eighteen phenolic compounds were identified; isofurulic acid (16%-18%) and cynaroside (45%-47%) were the larger ones. In general way, the hydroethanolic extract was more active against Staphylococcus aureus and Micrococcus luteus (minimum inhibitory concentrations= 0.3-1.3 mg/mL). Both polar extracts have strong antiradical activities although decoction extract proved to be more active against DPPH• (half-maximal inhibitory concentration [IC50] =36 µg/mL) and O2•− (IC50 =28 µg/mL) while hydroethanolic extract shows higher action over α-glucosidase (IC50 =217 µg/mL). The results suggest that A. salsoloides leaves and flowers may be an interesting source of natural antioxidants, antidiabetics, or antimicrobials, and could be used in dietary supplements, medicinal products and pharmaceutical formulations.

Keywords
Acantholippia salsoloides, β-thujone, isofurulic acid, cynaroside, biological action

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Acantholippia salsoloides Griseb. (Verbenaceae) is a spiny shrub popularly known as “rica-rica”1,2; synonyms are A. hastulata Griseb., Lippia hastulata (Griseb.) Hieron., and Lippia salsoloides (Griseb.) Briq. A. salsoloides attributes its common name rica-rica to the fresh and sweet fragrance of the plant.3–5 The infusion of rica-rica (stems, leaves, and flowers) is used traditionally for the treatment of digestive infections, diarrhea, gastrointestinal bloating, dyspepsia, and to cure cold diseases.1,2,6 Rica-rica leaves and flowers are widely consumed either alone or in combination with yerba-mate, as a digestive stimulant.7,8

The essential oil (EO) composition of A. salsoloides from several locations in the Andean region has been previously investigated showing significant diversity.6,9,10 In Jujuy Province (Northwest of Argentina), the populations investigated showed different EO compositions too; in Chucbela they accumulated mainly p-cymene (52.8 %), thymol (46.8 %), or β-thujone (63.3%-65.4%).6,8 In other locations, the main components were β-thujone (70.0%-72.5%), trans-sabinol, and trans-sabiny acetate.6 Regarding the biological potential of Acantholippia, previous studies include the repellent action of A. salsoloides and A. seripholoides EOs against Aedes aegypti9,10; the antifungal, antibacterial, and
repellent effects of EOs of *A. seriphioides* and *A. deserticola* EOs,\textsuperscript{1,2,11} and the antimicrobial activity of hydroethanolic extracts (HEs) of *A. punensis* and *A. salsoloides*.\textsuperscript{7,12} The aims of this study were to investigate *A. salsoloides* regarding the volatile profile of the EO and the phenolic compounds in the HE and decoction extract (WE), in addition to the antibacterial potential of these fractions. The antiradical activity and the \(\alpha\)-glucosidase inhibition were studied too, and related to the polar extracts composition.

The yield of EO obtained from the samples by distillation was 0.9\% ± 0.2\% v/w (average of 2 extractions on dry weight basis). Comparable results were previously reported for the EO extraction in *A. seriphioides* growing in Central Argentina.\textsuperscript{13} The yields obtained with extraction procedures were 23.8\% ± 1.4\% and 21.8\% ± 1.1\% (on dry weight basis) for HE and WE, respectively (and \(p\)-value > 0.05).\textsuperscript{14,15} In the EO of rica-rica growing in Chuzalezna, 26 components were identified and quantified, representing 97.9\% of the oil (Table 1). The EO compounds present in a higher proportion were \(\beta\)-thujone (84.3\%), \(\alpha\)-thujone (2.3\%), limonene (2.7\%), and sabinene (2.0\%). These results are consistent with those obtained in previous studies.\textsuperscript{6}

The high-performance liquid chromatography diode array detector (HPLC-DAD) analysis of extracts of rica-rica allowed the determination of 21 phenolic compounds. The identified compounds comprise 6 phenolic acids and derivatives (1, 3, 4, 7, 12, and 17) and 15 flavonoids (5-11, 13-16, and 18-21) (Table 2). Both polar fractions revealed similar

### Table 1. Chemical Composition of *Acantholippia salsoloides* Essential Oil.

| No. | Component\textsuperscript{b} | RI\textsuperscript{b} | RI\textsuperscript{c} | t\textsubscript{R}\textsuperscript{d} | (%)\textsuperscript{e} |
|-----|---------------------------|----------------|----------------|----------------|----------------|
| 1   | \(\alpha\)-Thujene        | 924            | 930            | 6.3            | 0.1 ± 0.0     |
| 2   | \(\alpha\)-Pinene         | 936            | 939            | 6.6            | 0.4 ± 0.1     |
| 3   | Sabinene                  | 967            | 975            | 7.8            | 2.0 ± 0.3     |
| 4   | \(\beta\)-Pinene          | 980            | 979            | 8.4            | 1.1 ± 0.5     |
| 5   | Myrcene                   | 989            | 991            | 8.7            | 0.6 ± 0.1     |
| 6   | \(\alpha\)-Terpinene      | 1 015          | 1 017          | 9.7            | 0.1 ± 0.0     |
| 7   | \(\beta\)-Cymene          | 1 023          | 1 025          | 10.3           | 1.0 ± 0.2     |
| 8   | Limonene                  | 1 028          | 1 029          | 10.9           | 2.7 ± 0.2     |
| 9   | 1,8-Cineole               | 1 030          | 1 031          | 11.0           | 0.1 ± 0.0     |
| 10  | \(\gamma\)-Terpinene      | 1 060          | 1 060          | 12.3           | 0.4 ± 0.0     |
| 11  | cis-Thujone (\(\alpha\)-Thujone) | 1 103 | 1 102 | 12.8 | 2.3 ± 0.8 |
| 12  | trans-Thujone (\(\beta\)-Thujone) | 1 115 | 1 114 | 13.2 | 84.3 ± 1.1 |
| 13  | Sabinol-trans (OH vs IPP) | 1 145          | 1 142          | 13.4           | 0.2 ± 0.0     |
| 14  | Thujanol < neiso-3>       | 1 156          | 1 152          | 13.7           | 0.1 ± 0.0     |
| 15  | Thujanol < neo-3>         | 1 158          | 1 154          | 14.0           | tr\textsuperscript{f} |
| 16  | Sabina ketone             | 1 162          | 1 159          | 14.8           | tr            |
| 17  | Terpinen-4-ol             | 1 179          | 1 177          | 15.3           | 0.4 ± 0.0     |
| 18  | Thuj-3-en-10-al           | 1 188          | 1 184          | 15.6           | tr            |
| 19  | \(\alpha\)-Terpineol      | 1 190          | 1 189          | 16.0           | tr            |
| 20  | Cumin aldehyde            | 1 248          | 1 242          | 17.7           | 0.5 ± 0.1     |
| 21  | Carvotanacetone           | 1 255          | 1 247          | 18.2           | 1.1 ± 0.2     |
| 22  | Thujil acetate <iso-3>    | 1 274          | 1 270          | 19.0           | 0.1 ± 0.0     |
| 23  | Sabinyl acetate-trans (IPP vs acetyl) | 1 291 | 1 291 | 19.4 | 0.1 ± 0.0 |
| 24  | Cymen-7-ol < para >       | 1 295          | 1 291          | 19.5           | 0.1 ± 0.0     |
| 25  | Carvyl acetate <cis >     | 1 370          | 1 368          | 24.0           | 0.2 ± 0.0     |
| 26  | Curcumene<ar->            | 1 481          | 1 481          | 28.6           | tr            |

\(\Sigma\) 97.9

Results are expressed as relative percentage in the mixture.

\textsuperscript{a}Compounds identified by comparison of their RI (retention indices) and mass spectra with literature data, the MS library (NBS 75K, NIST98), and a spectra library built up from pure substances and components of known oils.

\textsuperscript{b}Experimental RI on HP5 MS capillary column in reference to C7-C24 n-alkanes.

\textsuperscript{c}Literature data.\textsuperscript{16-18}

\textsuperscript{d}Compounds listed in order of elution, t\textsubscript{R} (min).

\textsuperscript{e}Percentage peak area of EO components as means of 2 determinations ± standard deviation.

\textsuperscript{f}tr: traces (<0.1%).
profile but showed different compositions. With respect to flavonoids, cynaroside (luteolin-7-O-glucoside) (15) was the most abundant in HE and WE, followed mainly by several apigenin derivatives and luteolin derivatives. Regarding phenolic acids, they corresponded to ca. 51% and 56% of the phenolic contents in HE and WE, respectively. Isoferulic acid (7) was the major of both extracts (Table 2). The phenolic contents ranged between 63792.5 and 68159.0 µg/g of dried extract for HE and WE (Table 2). The recovery of phytochemicals was 1651.6 and 1641.3 µg/g of plant material from HE and WE.

The antibacterial activity of EO and polar extracts was investigated herein; minimum inhibitory concentration (MIC) and minimum lethal concentration (MLC) values were determined against 6 pathogenic bacteria (Table 3). Gram-positive strains were more sensitive to rica-rica extracts than Gram-negative ones, mainly with HE; similar results were previously reported in other studies. All extracts revealed to have bactericidal effect on S. aureus, but only polar extracts showed bactericidal action on M. luteus and Pseudomonas aeruginosa. On the other hand, EO showed bactericidal action against Salmonella typhimurium. For comparison, MIC and MLC were determined for HE against S. aureus ATCC 25923 (Table 3). Gentamicin, MIC = 0.01 mg/mL and MLC = 0.03 mg/mL, was used as control.

A. salsoloides shares with A. deserticola the identity and percentage order of the major components of EO. Previously, Sampietro et al11 attributed a good antimicrobial activity of A. deserticola EO to β-thujone and trans-sabinyl acetate.11 In another study, Lima et al. 19 reported promising activity of A. seriphioides EO against clinical bacterial strains and yeasts; this oil contained mainly thymol, p-cymene, T-terpinene and carvacrol.

Regarding polar extracts, the antifungal potential of HEs of A. salsoloides growing in Catamarca was previously screened with other extremophiles from the Argentine Puna13; rica-rica did not show the best

### Table 2. Phenolic Compounds in Acantholippia salsoloides Polar Extracts.

| Compound                              | Extract composition<sup>a,b</sup> |
|---------------------------------------|-----------------------------------|
|                                      | **HE**                           | **WE**                           |
| 1 Protocatechuic acid                 | 44.1 ± 0.4 (0.1)                  | 531.2 ± 0.4 (0.8)                |
| 2 Catechin                            | 818.0 ± 41.8 (1.3)                | 1766.0 ± 15.4 (2.6)              |
| 3 Phenolic acid derivative 1          | 303.5 ± 0.8 (0.5)                 | 863.6 ± 19.1 (1.3)               |
| 4 Phenolic acid derivative 2          | 269.8 ± 0.1 (0.4)                 | 960.5 ± 7.2 (1.4)                |
| 5 Eriodictyol-7-O-glucoside           | 392.8 ± 2.6 (0.6)                 | 177.9 ± 0.4 (0.3)                |
| 6 Luteolin-8-C-glucoside             | 678.6 ± 4.5 (1.1)                 | 888.4 ± 4.9 (1.3)                |
| 7 Isoferulic acid                     | 29746.5 ± 22.2 (46.6)             | 30553.3 ± 21.7 (44.8)            |
| 8 Apigenin-8-C-glucoside             | 1528.7 ± 16.4 (2.4)               | 293.2 ± 9.9 (0.4)                |
| 9 Luteolin-6-C-glucoside             | 221.7 ± 0.2 (0.3)                 | 232.5 ± 2.9 (0.3)                |
| 10 Luteolin-3’,7-di-O-glucoside      | 176.7 ± 0.2 (0.3)                 | 123.1 ± 1.5 (0.2)                |
| 11 Luteolin derivative (1 + 2)        | 3925.6 ± 35.1 (6.2)               | 4763.8 ± 45.0 (7.0)              |
| 12 Phenolic acid derivative 3         | 697.8 ± 4.3 (1.1)                 | 4409.1 ± 8.3 (6.5)               |
| 13 Apigenin-6-C-glucoside            | 3757.3 ± 40.3 (5.9)               | 1810.6 ± 2.2 (2.7)               |
| 14 Luteolin derivative (3 + 4 + 5)    | 4820.7 ± 19.2 (7.6)               | 4992.3 ± 13.1 (7.3)              |
| 15 Luteolin-7-O-glucoside            | 11517.9 ± 29.3 (18.1)             | 10984.8 ± 13.9 (16.1)            |
| 16 Luteolin-‘4’-O-glucoside          | 251.2 ± 0.4 (0.4)                 | 918.6 ± 2.8 (1.3)                |
| 17 Phenolic acid derivative 4         | 1520.8 ± 1.5 (2.4)                | 1068.6 ± 2.3 (1.6)               |
| 18 Luteolin derivative (6)            | 1751.8 ± 0.6 (2.7)                | 1366.1 ± 7.6 (2.0)               |
| 19 Luteolin derivative (7 + 8)        | 1116.2 ± 10.6 (1.7)               | 1304.9 ± 3.6 (1.9)               |
| 20 Luteolin                           | 112.8 ± 0.6 (0.2)                 | 102.9 ± 0.2 (0.2)                |
| 21 Apigenin                           | 140.1 ± 0.2 (0.2)                 | 45.58 ± 0.8 (0.1)                |
| Σ                                      | 63792.5                           | 68159.0                          |

HE, hydroethanolic extract; WE, decoction extract. Σ, sum of the determined phenolic compounds (mg/kg or µg/g).
<sup>a</sup>Composition of extracts as mean ± standard deviation of three assays (mg/kg or µg/g).
<sup>b</sup>Relative percentage of compounds in brackets.
performance against postharvest molds. The HEs of *A. punensis* showed promising inhibitory activity against some pathogenic bacteria; these results could be related to the use of this plant as a remedy against urinary and intestinal infections.22

The antimicrobial activity depends not only on the amount of specific compounds in EO and extracts, but also on the type of compounds in the phytocomplex.12 The antibacterial properties of *A. salsoloides* growing in Jujuy could be associated with the high content of specific phenolic compounds, terpenes and terpenoids.

The antioxidant activity of HE and WE was tested against DPPH•, superoxide (O$_2$•−), and nitric oxide (NO•) radicals. *A. salsoloides* extracts have strong scavenging activity against O$_2$•− and DPPH •. Previously Celaya et al reported half-maximal inhibitory concentration (IC$_{50}$) values ranging from 64.1 to 68.5 µg/mL against DPPH •, for water extracts of rica-rica from different populations.6 The 2 extracts studied here showed better behavior, WE being more active than HE (Table 4). For the well-known antioxidant butylated hydroxytoluene (BHT), the radical scavenging activity was comparable with that of the previous cases (Table 4). The analyzed extracts were particularly active against superoxide anion radical (Table 4). HE was slightly more active than WE against NO• (Table 4). The results obtained are comparable with the data previously reported for polar extracts of *Satureja parvifolia* and *Aphyllocladus spartioides* growing in the same region.14,15

The main phenolic compounds present in rica-rica extracts (cynaroside and isoferulic acid) are found in several medicinal plants and food matrices. These phytochemicals have demonstrated to have several beneficial effects on human health. Cynaroside among other apigenin and luteolin derivatives was previously isolated from *Salvia chloroleuca* (Lamiaceae) and *Nepeta cataria* (Lamiaceae)25–28; these flavonoids were also reported in tissues of *Elaeis guineenses* (Arecaceae) with α-glucosidase inhibitory activity, in *Agrimonia pilosa* (Rosaceae) with antioxidant and α-glucosidase inhibitory activity. Isoferulic acid is one of the main phenolic compounds extracted from *Cimicifugae* species (shengma), belonging to the Ranunculaceae family, which has a long and diverse history of medicinal use; it is the major active ingredient of *C.

### Table 3. Antibacterial Activity (MIC and MLC) of *Acantholippia salsoloides* Extracts (mg/mL) and Essential Oil (mL/mL).a

| Strain                  | HE   | WE   | EO   |
|-------------------------|------|------|------|
| *Staphylococcus aureus* ATCC 25923 | 0.6  | 5.0  | -    |
| *S. aureus* ATCC 20231  | 0.3 - 0.6 | 5.0  | 2.5  | 5.0  | 7.8  | 62.5 |
| *Micrococcus luteus* ATCC 20030   | 1.3  | 5.0  | 2.5  | 10.0 | 15.6 | >62.5 |
| *Bacillus cereus* ATCC 31      | 2.5  | 10.0 | 2.5  | >10.0| 15.6 | >62.5 |
| *Escherichia coli* ATCC 30083 | 10.0 | >10.0| 10.0 | >10.0| 31.3 | >62.5 |
| *Salmonella typhimurium* ATCC 43971 | 10.0 | >10.0| 10.0 | >10.0| 31.3 | 62.5 |
| *Pseudomonas aeruginosa* ATCC 50071 | 10.0 | 10.0 | 10.0 | 10.0 | 62.5 | >62.5 |

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*a,* MIC, minimum inhibitory concentration; MLC, minimum lethal concentration. Results are from 3 independent experiments performed in duplicate.

Strong activity is for MIC = 0.05-0.5 mg/mL, moderate activity for MIC = 0.6-1.5 mg/mL, and weak activity above 1.5 mg/mL.15

### Table 4. IC$_{50}$ (mg/mL) Values Found in the Antioxidant Activity and α-Glucosidase Assays for HE and WE.

| Sample and controls | DPPH• | NO•          | O$_2$•− | α-Glucosidase inhibition |
|---------------------|-------|--------------|---------|--------------------------|
| HE                  | 43.4 ± 0.6$^a$ | 206.0 ± 9.5$^a$ | 33.3 ± 1.1$^a$ | 216.7 ± 37.2$^a$               |
| WE                  | 35.5 ± 1.1$^b$ | 229.7 ± 13.8$^b$ | 27.5 ± 0.4$^b$ | 1690.1 ± 39.7$^b$              |
| BHT                 | 25.3 ± 1.0$^a$ | -             | -       | -                         |
| Acarbose            | -     | -            | -       | 271.7 ± 21.3$^b$            |
| Luteolin$^{23,24}$  | 14.2  | 12.4         | -       | 6.0                       |
| Cynaroside$^{25–28}$| -     | -            | -       | 8.2                       |
| Isoferulic acid$^{29,30}$ | 4.6  | -            | 13.3    | -                         |

BHT, butylated hydroxytoluene; IC$_{50}$, half-maximal inhibitory concentration; HE, hydroethanolic extract; WE, decoction extract.

$^a$,$^b$, Differences between extracts were tested for significance using the one-way analysis of variance with post hoc Tukey’s test. Differences were considered significant for p-value <0.05.
heracleifolia; the pharmacological properties of this medicinal herb include antioxidative, anti-inflammatory, antiviral, and antidiabetic properties, attributed to the high contents of isoferrulic acid in C. heracleifolia extracts.29,30

The potential of A. salsoloides HE and WE to inhibit α-glucosidase tested in vitro (Table 4). WE showed moderate action. HE performed well as α-glucosidase inhibitor, and showed inhibitory effects comparable to acarbose (Table 4). This activity may be associated with the content of isoferrulic acid and luteolin derivatives present in HE extracts.25–30 Besides the determined phenolic compounds, the presence of other metabolites in the HE that might contribute to the observed α-glucosidase inhibition cannot be ignored.31–33

In relation to the volatile fraction, a trans-thujonic population of rica-rica was studied here. Thujone is a bicyclic monoterpene ketone that occurs mainly as a mixture of alpha (cis) and beta (trans) diastereoisomers in plants such as Artemisia absinthium L. (Asteraceae), Salvia officinalis L. (Lamiaceae) (sage), Thuja occidentalis L. (Cupressaceae), among others.32–35 Thujone is commonly used as a flavoring substance in several foods and beverages32,33; cis-thujone has neurotoxic effect in mammals. Despite this, plant extracts containing the mixture are constituents of many dietary supplements and herbal medicinal products in several countries. The neurotoxic action of cis-thujone is associated with high levels of oral exposition.32–35 A. salsoloides present minor contents of this isomer; however, further investigations are required to encourage the use of rica-rica extracts in pharmaceutical and food products.34,35 The stimulating and digestive properties attributed to A. salsoloides could be related to the EO profile as well as the phenolic composition. The decoction is in correspondence with the extensive use of rica-rica for medicinal purposes and food preparations, even though the HE was more active.

Experimental

Plant Samples

Aerial parts of 12 specimens (100-200 g each one) were collected during the flowering period, April of 2013, in Chucalezna (23° 21' 39.3" S, 65° 19' 20.9" O, 2702 masl), Jujuy Province (Argentina). The plant material was identified by Professor Osvaldo Ahumada (National University of Jujuy, Argentina) and Professor Gustavo Giberti (National University of Buenos Aires, Argentina); a voucher specimen (HN1308) was deposited in the Herbarium of PRONOA (National University of Jujuy, Argentina). The plant material (leaves and flowers) was dried at room temperature for 7 days, ground to powder in a blender (mean particle size <2 mm) and stored at −20°C until required.

Preparation of Extracts

EO was obtained from 500 g dry material by steam distillation for 2.5 hours using a Clevenger-type apparatus.15 The collected oil was dried and stored at 4°C until analysis. The EO content was determined volumetrically on dried weight basis. WE was prepared boiling 5 g of dried material in 100 mL of H2O for 10 minutes.15 The resulting extracts were filtered through a Büchner funnel, frozen, and lyophilized. HE was prepared with 5 g of dried samples, and sonicated at 40°C with 100 mL of ethanol:water (70:30 v:v) for 20 minutes. The obtained extract was evaporated under reduced pressure and kept at −20°C for further analysis. Extractions were carried out in triplicate.

Analysis of the Essential Oil

EO composition was analyzed by gas chromatography mass spectrometry and gas chromatography flame ionization detector using a previously described procedure.15 The quantification of each compound was performed on the basis of their GC/FID peak areas without the use of response factor corrections.16–18

Analysis of Phenolic Compounds

WE and HE were redissolved in methanol and filtered through a 0.45 µm polytetrafluoroethylene membrane. The phenolic compounds were analyzed in an HPLC/DAD (Gilson) using the previously described procedure.15 Thirty microliters of each polar extract were analyzed with an analytical HPLC unit (Gilson), using a Spherisorb ODS2 (25.0 × 0.46 cm; 5 µm, particle size; Waters, Milford, MA) column. The chromatograms were recorded at 280 (tannins), 320 (phenolic acids), and 350 nm (flavonoids). The data were processed in Unipoint System software (Gilson Medical Electronics, Villiers-le-Bel, France). The compounds in each sample were identified by comparing their retention times and UV spectra with the library of spectra. The quantification was achieved by measuring the absorbance recorded in the chromatograms relative to external standards. This procedure was performed in triplicate. Phenolic acid derivatives were quantified as 5-O-caffeoylquinic acid; luteolin derivatives were quantified as luteolin.

Antibacterial Activity

The study included 6 bacterial strains: S. aureus ATCC 20231, B. cereus ATCC 31, M. luteus ATCC 20030, S. typhimurium ATCC 43971, E. coli ATCC 30083, and P. aeruginosa ATCC 50071. The MIC and the MLC were determined employing a previously described procedure15; S. aureus ATCC 25923 was determined as quality control. The tested concentrations were 62.5 µL/mL for the EO and 10.0 mg/mL of dry matter for polar extracts.

Antioxidant Activity

The scavenging activity against DPPH•, O2•−, and NO radicals was evaluated for WE and HE according to Celaya et al.
Three independent assays were performed for each radical in triplicate. IC$_{50}$ values represent the concentrations that caused 50% activity loss. Statistical analysis was carried out using Graph pad Prism 5 Software (San Diego, CA, USA). For the DPPH$^-$ scavenging activity, the antioxidant BHT was used as reference compound.

**α-Glucosidase Inhibitory Activity**

The effect on α-glucosidase was assessed using a previously reported procedure.15

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**Declaration of Conflicting Interests**

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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