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Identification of chicoric acid as a hypoglycemic agent from *Ocimum gratissimum* leaf extract in a biomonitoring in vivo study

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ABSTRACT

Ocimum gratissimum L. is popularly used to treat diabetes mellitus. The hypoglycemic activity of this medicinal species has been confirmed by in vivo studies. The present study conducted a chemical investigation of a leaf decoction (10% p/v) of *O. gratissimum* monitored by in vivo hypoglycemic activity assays. Four phenolic substances were identified: L-caftaric acid (**1**), L-chicoric acid (**2**), eugenyl-β-D-glucopyranoside (**3**) and vicenin-2 (**4**). The acute hypoglycemic activity of the *O. gratissimum* decoction fractions Og1-S (300 mg/kg), Og1-A (240 mg/kg) and Og1-B (80 mg/kg) was evaluated intraperitoneally in normal and streptozotocin-induced diabetic mice. They reduced glycemia by 63%, 76% and 60% (in 120 min), respectively, in the diabetic mice. Subfractions of Og1-A were also evaluated under the same conditions: Og1-AS (200 mg/kg) and Og1-AP (40 mg/kg) produced a decrease of only 37% and 39%, respectively. Among the major phenolic substances, only chicoric acid (**2**; 3 mg/kg) reduced significantly the glycemic levels of diabetic mice by 53%, 120 min after treatment. This is the first study describing the hypoglycemic activity of chicoric acid in an animal model of diabetes mellitus. In addition, we suggest that there may be other substances contributing to this activity. Thus, for the first time, a correlation is established between the hypoglycemic activity of *O. gratissimum* and its chemical composition.

1. Introduction

Diabetes mellitus (DM) is a group of metabolic diseases, which have hyperglycemia as a common characteristic, resulting from a defect in insulin production, insulin action or both [1,2]. Chronic hyperglycemia leads to microvascular and macrovascular complications, the major cause of premature mortality and morbidity among diabetic people [1,3].

DM represents a global health challenge due to its increasing prevalence [4]. It affected 366 million people in 2011 and this number is likely to reach 552 million by 2030 [5]. WHO predicts that in 20 years, the disease will become the 7th leading cause of death [6].

DM therapeutic options focus on glycemic control, which encompasses appropriate diet, regular exercise, and, especially, exogenous insulin administration and/or oral hypoglycemic agents [7,8]. Some issues, however, limit the effectiveness of those options, such as: failure to hinder diabetic complications and prominent side effects [9,10]. Therefore, the search for new therapeutic approaches to treat DM is the goal of multiple efforts globally.

For centuries, natural products have been used to treat diseases. They are extensively recognized as a relevant source of drugs. As reported by Newman & Cragg (2012) of the 1355 new drugs approved between 1981 and 2010, 26% were derived from natural products [11]. Additionally, 13% of them

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are synthetic drugs exhibiting a pharmacophore from a natural product.

The potential of medicinal plants and their bioactive compounds to treat DM has been extensively explored, as exemplified by the large number of reviews on this subject [10,12–15].

The genus *Ocimum* (Lamiaceae) comprises 65 aromatic species, distributed in tropical and subtropical regions worldwide [16,17]. Species belonging to this genus are popularly used in Africa and Asia for treating diabetic symptoms [14,18–20]. Some of them have had their hypoglycemic activity confirmed by in vivo studies: *Ocimum sanctum*, *Ocimum canum* and *Ocimum gratissimum* [18,21–25].

O. gratissimum L. is an arbustive species native from Africa, where it is reputed for its anti-diabetic effect [19,20]. In vivo pharmacological studies evidenced the hypoglycemic activity of the methanol and aqueous leaf extracts from this medicinal plant [23–25]. However the substances responsible for this activity remain to be identified.

The present study aims to contribute to the chemical knowledge of *O. gratissimum* and to identify compounds potentially useful in the therapy of DM.

2. Materials and methods

2.1. General experimental procedures

^{1}H and ^{13}C NMR spectra were recorded on a Varian VNMRS-500 (^{1}H: 499.77 MHz; ^{13}C: 125.68 MHz) (LAMAR - Núcleo de Pesquisa de Produtos Naturais, UFRJ). ESI mass spectra were obtained on a Waters Micro Q-TOF quadrupole time of flight mass spectrometer (Instituto de Química, UFRJ) or on a BRUKER MicroTOF-II mass spectrometer (Núcleo de Pesquisa de Produtos Naturais, UFRJ). Specific optical rotation ($[\alpha]_D^{20}$) measurements were obtained at a P-2000 Jasco polarimeter (Núcleo de Pesquisa de Produtos Naturais, UFRJ). HPLC-DAD analyses were performed on a Shimadzu liquid chromatograph LC-20AT with a diode-array wavelength SPD-M20A detector (Laboratório de Produtos Naturais, UFRJ - Campus Macaé), using a Merck reverse-phase column C-18 (5 μm, 250 mm, 2.5 mm). The mobile phase consisted of water containing 0.1% formic acid (eluent A) and acetonitrile containing 0.1% formic acid (eluent B). The samples were run for 50 min at 1 ml/min and the absorbance was monitored between 200 and 500 nm. The gradient used was: 0–10 min (100–80% A), 10–20 min (80–78% A), 20–30 min (78–75% A), 30–35 min (75–70% A), 35–40 min (70–50% A), 40–45 min (50–30% A) and 45–50 min (30–0% A). Reversed-phase chromatography was performed on RP-2 (70–230 mesh, Merck) or RP-18 (40–60 μM, Merck) silanized silica (using a water/methanol gradient) and size-exclusion chromatography on Sephadex LH-20 gel (25–100 nm, Sigma), Sephadex G-15 (40–120 μm, Sigma) or Sephadex G-10 (40–120 μm, Sigma). Eluates were monitored by TLC on silica 60 F_{254} (Merck) using *n*-butanol/acetic acid/water (BAW 8:1:1 and BAW 3:1:1), visualized under UV and revealed with cerium sulfate solution.

2.2. Plant material

O. gratissimum leaves were collected from specimens cultivated in Barra do Piraí, RJ (Brazil), in July 2010 and July 2011. The botanical species was identified by Prof. Dr. Rosana Conrado Lopes (UFRJ, Brazil), and a voucher specimen (RFA 35593) was deposited at the herbarium of the Institute of Biology (UFRJ, Brazil).

2.3. Extraction and isolation

Two batches of plant material submitted to extraction were collected in 2010 (Og1) and 2011 (Og2). Extraction and isolation procedures for these batches are described in the following sections.

2.3.1. 1st plant batch (Og1)

The Og1 leaves were triturated in a food processor before boiling with distilled water (10% w/v) for 5 min. The extract obtained by decoction was filtered and concentrated at 50 °C to approximately 1/2 of the initial volume. An aliquot corresponding to 20% (2.00 g) was separated and lyophilized for analytical and pharmacological assays. The addition of the remaining extract to ethanol (1:1) yielded a precipitate that was separated by filtration and subsequently lyophilized (2.02 g). The supernatant (Og1-S) was concentrated in a water-bath until complete evaporation of the ethanol. After the separation of a 20% (1.46 g) aliquot, Og1-S was partitioned with *n*-butanol affording an aqueous fraction (Og1-A) and a butanol fraction (Og1-B). The yield of each fraction was calculated after lyophilization (Og1-A, 4.70 g and Og1-B, 1.13 g). Og1-A was re-suspended in distilled water and precipitated with ethanol (1:1). The precipitate (Og1-AP) was separated by centrifugation and lyophilized (0.4 g). The supernatant (Og1-AS), after ethanol evaporation, was dissolved in distilled water and lyophilized. A 52% aliquot (1.3 mg) was separated and the remaining material (1.2 g) was injected into a Sephadex G-15 column (30.0 × 1.1 cm, water), giving four fractions: Og1-A-a (860 mg), Og1-A-b (131 mg), Og1-A-c (30 mg) and Og1-A-d (13 mg).

Og1-A-b was chromatographed on a Sephadex G-10 column (12 × 1.2 cm, water) and afforded three fractions: Og1-A-b1 (87 mg), Og1-A-b2 (30 mg) and Og1-A-b3 (7 mg). The fraction Og1-A-b1 was purified on a Sephadex LH-20 column (32 × 0.8 cm, water/methanol 50%), yielding compound **1**, a phenolic acid (13 mg).

Fraction Og1-A-c was chromatographed on a Sephadex LH-20 column (19 × 0.7 cm, water/methanol 50%), affording two fractions: Og1-A-c1 (10 mg) and Og1-A-c2 (15 mg). The last one, Og1-A-c2,was purified on a Sephadex LH-20 column (32 × 0.8 cm, water/methanol 50%) and gave compound **2**, a second phenolic acid (6 mg).

Fraction Og1-A-d was purified on a Sephadex LH-20 column (32 × 0.8 cm, water/methanol 50%), affording 3 mg of a flavonoid rich fraction: Og1-A-d2.

Og1-B (560 mg) was re-suspended in distilled water (12 ml) and centrifuged (3000 rpm, 10 min). The insoluble material was lyophilized (109 mg). The supernatant was partitioned with ethyl acetate, affording an aqueous fraction (Og1-BAq, 383 mg) and an acetate fraction (Og1-BAc, 67 mg). The yield of each fraction was calculated after evaporation of the organic solvent and lyophilization.

An aliquot of Og1-BAq (330 mg) after chromatography on an RP-2 column (25 × 1.1 cm, water/methanol gradient) gave four fractions: Og1-BAq-1 (water, 93 mg), Og1-BAq-2 (water, 87 mg), Og1-BAq-3 (methanol 10%, 23 mg) and Og1-

BAq-4 (methanol 30-100%, 52 mg). The flavonoid rich fraction Og1-BAq-3 originated 4 mg of a flavonoid-containing precipitate (Og1-BAq-3P), separated by centrifugation (3000 rpm, 10 min). The supernatant of this fraction was injected in a Sephadex G-10 column (20 × 0.4 cm, water), giving the flavonoid fraction Og1-BAq-3b (4.5 mg).

2.3.2. 2nd plant batch (Og2)

Leaves from batch Og2 were extracted and fractioned by similar procedures used for Og1. A 10% (w/v) decoction was prepared from triturated fresh leaves. The extract was concentrated to 1/2 of the initial volume. An aliquot of 45% (6.07 g) was separated and lyophilized, while the remaining extract was precipitated with ethanol (1:1), yielding 1.06 g of precipitate. The supernatant (Og2-S) was partitioned with *n*-butanol giving an aqueous fraction (Og2-A, 3.70 g) and a butanol fraction (Og2-B, 0.80 g).

An aliquot of Og2-B (480 mg) was re-suspended in distilled water (10 ml) and centrifuged (3000 rpm, 10 min), affording an insoluble material (129 mg). The supernatant was extracted with ethyl acetate, giving an aqueous fraction (Og2-BAq, 180 mg) and an acetate fraction (Og2-BAc, 99 mg).

Og2-BAc was injected on a Sephadex LH-20 column (25.5 × 1.3 cm, ethanol), yielding 2 fractions: Og2-BAc-1 (47 mg) and Og2-BAc-2 (9 mg). Og2-BAc-1 was chromatographed on the same column and afforded two fractions: Og2-BAc-1a (30 mg) and Og2-BAc-1b (10 mg). Og2-BAc-1a was purified on an RP-18 column (19 × 1.3 cm, water/methanol gradient), giving 5 mg of the phenolic compound **3**.

Og2-BAq was chromatographed on an RP-2 column (25 × 1.1 cm, water/methanol gradient), yielding five fractions: Og2-BAq-1 (water, 42 mg), Og2-BAq-2 (water, 22 mg), Og2-BAq-3 (methanol 10%, 38 mg), Og2-BAq-4 (methanol 10–50%, 7 mg) and Og2-BAq-5 (methanol 50–100%, 8 mg). Og2-BAq-3 was purified on a Sephadex G-10 column (12 × 1.2 cm, water) and afforded a flavonoid-enriched fraction: Og2-BAq-3b (8 mg). The chromatography of Og2-BAq-4 on an RP-18 column (21 × 0.8 cm, water/methanol gradient) originated an additional amount of compound **3** (3 mg).

The flavonoid-rich fractions Og1-BAq-3P, Og1-BAq-3b, Og1-A-d2 and Og2-BAq-3b obtained from Og1 and Og2 were grouped and injected into a Sephadex LH-20 column (32 × 0.8 cm, water/methanol 50%). From this fractionation the flavonoid compound **4** (9 mg) was obtained.

2.4. ^{1}H NMR analysis of fractions from O. gratissimum

The chemical profiles of three fractions from the decoction of *O. gratissimum* were compared by ^{1}H NMR (Og1-A, Og1-AS and Og1-AP). A 5 mg sample of each fraction was dissolved in 0.75 ml of D_2O with 0.05% of 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid sodium salt (TSP-*d*4). ^{1}H NMR spectra were acquired at 25 °C with 256 scans, 0.24 Hz/point, spectral width (SW) = 8012.8 Hz, relaxation delay (RD) = 1.0 s, and receiver gain (RG) = 30. TSP-*d*4 signal was used as a reference for integration of signals and comparison of different spectra.

2.5. Diabetes induction and treatment

Male Swiss mice (8 weeks old) were housed in a temperature-controlled room, with a 12-hour light–dark cycle, having free access to water and food. DM was induced through a single intraperitoneal (i.p.) dose (150 mg/kg) of streptozotocin (STZ) dissolved in sodium citrate (100 mM, pH 4.5) as described by Da Silva et al. (2010) [26]. Control group received only sodium citrate i.p. After 5 days, the mice that had developed hyperglycemia (≥300 mg/dl) were considered diabetic. Diabetic mice, as well as normal ones, were divided into control groups ($n = 9$) and treatment groups ($n = 3$). Normal and diabetic mice were treated with i.p. injection of human regular insulin (Humulin − 2 U/kg), Og1-S (300 mg/kg), Og1-B (80 mg/kg), Og1-A (240 mg/kg), Og1-AS (200 mg/kg), Og1-AP (40 mg/kg), compounds **1** and **2** (3 mg/kg) and compound **4** (4 mg/kg). Doses of the fractions and substances were chosen according to their approximate yield. All samples were lyophilized and stored under low temperature (−20 °C) in order to avoid degradation of possible active components. Fractions and phenolic substances were diluted in saline solution (NaCl 0.9%) for 100 μl of injection. Normal and diabetic control groups received only saline solution. Fasting glucose levels were measured from blood mice samples drawn by tail snip before ($t = 0$) and 1 and 2 h after the treatment using a glucometer (Accu-Chek_ Active − Roche). This protocol (Ref. Nr. NPPN01) was approved by the internal institutional animal use committee (CEUA − CCS/UFRJ).

2.6. Statistical analysis of data

Results were expressed as means ± SEM. Differences between two values were determined using Student's *t*-test and data were considered different at a significance level of $P < 0.05$. Analyses were performed using the Sigma Plot 11.0 software.

3. Results and discussion

3.1. Phytochemical study

The phytochemical study of the *O. gratissimum* aqueous leaf extract led to isolation of four phenolic compounds: L-caftaric acid (**1**), L-chicoric acid (**2**), eugenyl-β-D-glucopyranoside (**3**) and vicenin-2 (**4**). Phenolic acids **1** and **2** were obtained from the aqueous fraction (Og1-A), while phenylpropanoid **3** was isolated from the butanol fraction (Og2-B) and flavonoid **4** from the aqueous and butanol fractions (Og1-A, Og1-B, Og2-B). These substances are shown in Fig. 1, and have already been isolated from other vegetal sources. Their structures are in accordance with UV, MS and NMR data reported early by Mulkens & Kapetanidis (1988), Xie et al. (2003), Sobolev et al. (2005), Maier et al. (2006), Nuissier et al. (2010) and Zeng et al. (2011) [27–32]. The fractionation process used in this study is summarized in Fig. 2.

Compounds **1** and **2** are esters of caffeic and tartaric acids, respectively. Specific optical rotation ($[\alpha]_D^{20}$) value measured for caftaric acid was −14.5° ($c = 0.7$, H_2O), while for chicoric acid was −263.9° ($c = 0.5$, H_2O). These values seem compatible with L-caftaric and L-chicoric acids. Lamidey et al. (2002) reported a specific rotation of −242.7° ($[\alpha]_D^{22}$, $c = 0.89$, methanol) for L-chicoric acid, a value similar to that observed in our study [33]. Scarpati and Oriente (1958) however found a quite different value: −384.2° ($[\alpha]_D^{25}$, $c = 1.075$, methanol) using the same solvent employed by Lamidey et al. (2002) [34].

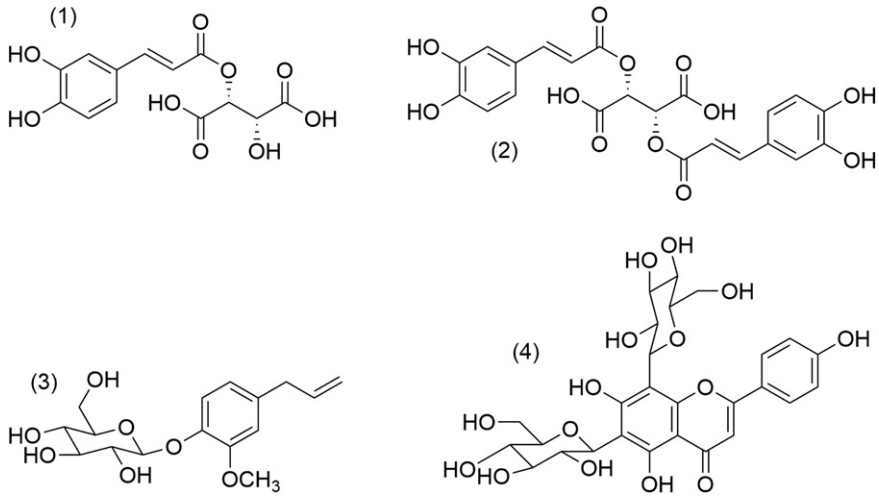

Fig. 1. Structures of isolated compounds: L-caftaric acid (**1**), L-chicoric acid (**2**), eugenyl-β-D-glucopyranoside (**3**) and vicenin-2 (**4**).

For L-caftaric acid, the reported $[\alpha]_D^{20}$ is −30.3° ($c = 2$, H_2O) [35]. It is important to note that temperature, solvent and/or concentration used in the literature are different from those used in our study, which may influence the results [36].

Ola et al. (2009) detected vicenin-2 and chicoric acid in *O. gratissimum* leaf decoction by HPLC-DAD-MS analysis [37]. However, those substances were not isolated. Chicoric acid is typically found in the family Asteraceae, although it is also reported in some species from other families, such as Lamiaceae in which the genus *Ocimum* is included [38]. It is important to point out that this is the first report on caftaric acid and eugenyl-β-D-glucopyranoside in *O. gratissimum*. The former seems to be a way to store eugenol, the major constituent of the essential oil of *O. gratissimum* cultivated in Brazil [39,40]. Phenylpropane glycosides that frequently occur in plants are cleaved to their corresponding aglycones by enzymatic processes and thus increase the quantity of essential oil in the plant [41].

3.2. Hypoglycemic activity assay of supernatant, aqueous and butanol fractions

The decoction fractionation of the leaves from *O. gratissimum* collected in 2010 (Og1) was monitored by acute hypoglycemic activity assays in streptozotocin-induced diabetic mice.

The decoction was precipitated with ethanol in order to promote an initial fractionation, and afforded a precipitate and a supernatant (Og1-S). This procedure allows the separation of the polysaccharides, proteins and salts as insoluble materials, while the micro-metabolites remain in the supernatant [42].

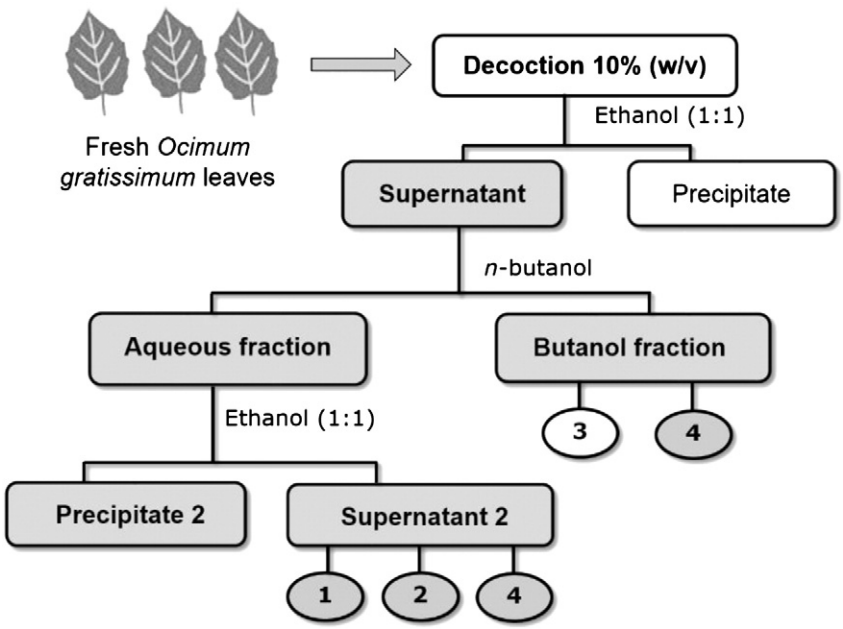

Fig. 2. Flowchart of Og fractionation. Numbers represent compounds **1**–**4**. Fractions and compounds highlighted were pharmacologically assayed.

The supernatant was partitioned with *n*-butanol, yielding a butanol fraction (Og1-B) and an aqueous fraction (Og1-A).

Initially the hypoglycemic activity of Og1-S and its fractions, Og1-A and Og1-B was evaluated. The results are shown in Fig. 3. Insulin treatment promptly decreased glycemia of either normal or diabetic mice showing that they were responsive to this hormone.

Og1-S and both fractions Og1-A and Og1-B produced significant glycemia reduction in diabetic mice, by 63%, 76% and 60%, respectively. The major fraction Og1-A was the most active. After 120 min, the glycemic levels of diabetic treated mice were not statistically different from normal untreated mice. On the other hand, all three samples produced no great effect on normal mice. The decreased blood glucose concentration observed in normal groups and untreated diabetic groups over time of treatment appears to be related to fasting.

The hypoglycemic activity of *O. gratissimum* leaf extracts has already been reported in other studies. Aguiyi et al. (2000) evaluated the effect of a methanolic extract in alloxan-induced diabetic rats. In this study the extract, which was intraperitoneally injected at a dose of 400 mg/kg, lowered the glycemic levels of normal and diabetic mice 180 min after treatment by 56% and 68%, respectively [23]. However, it is important to emphasize that the qualitative and quantitative chemical compositions of a plant extract obtained with methanol may be considerably different from that obtained with water [43,44]. In the above mentioned study, the authors only suggested the presence of saponins and tannins in the methanolic extract.

Egesie et al. (2006) evaluated the toxicity and the in vivo chronic hypoglycemic activity of a decoction from *O. gratissimum* leaves [24]. The extract orally administered at doses of 500–1500 mg/kg was not toxic and produced a reduction in the glycemic levels of streptozotocin-induced diabetic rats after 14 and 28 days. The chemical composition of the extract was not reported by the authors.

In a recent study, an aqueous *O. gratissimum* leaf extract exhibited hypoglycemic activity on type-2 diabetic rats. The extract administered orally at a dose of 200 mg/kg was able to reduce significantly the post-prandial glycemic levels of diabetic rats in comparison with the control rats, after 30, 60, 120 and 180 min [25]. Again, the chemical analyses of the extract were not performed.

3.3. Hypoglycemic activity assays of subfractions from the aqueous fraction (Og1-A)

Due to higher hypoglycemic activity and greater mass availability, Og1-A was selected for further fractionation. This fraction was precipitated with ethanol. The hypoglycemic activities of the two subfractions obtained, Og1-AS and Og1-AP, were subsequently evaluated. The results are shown in Fig. 4.

Og1-A exhibited a very high activity while both subfractions Og1-AS and Og1-AP tested in doses proportional to their yield had low activity on diabetic mice. The reduction rates observed for these fractions were 37% and 39%, respectively. These results indicate that fractionation was detrimental for the hypoglycemic activity.

In order to explain these findings two hypothesis were formulated: the substances present in both subfractions are important for the activity or the fractionation procedure produced degradation of the active substances. To investigate the second hypothesis, we tested a pool of the two subfractions in the proportion in which each is represented in the mother fraction (1:5). The results obtained are also shown in Fig. 4. The pool of fractions had higher activity on diabetic mice reducing their glucose levels by 61%. The glycemia of treated mice was not statistically different from normal mice after 120 min, as observed for Og1-A. This result suggests that the fractionation did not modify the chemical structure of bioactive substances since the activity of the pool of both subfractions was not significantly different from that observed for the mother fraction. Thus the first hypothesis seems to be the most plausible.

In order to investigate the composition of the aqueous fraction and its two subfractions, they were analyzed by HPLC-DAD and by ^{1}H NMR (Fig. 5). The major fraction Og1-AS has a composition very similar to that of Og1-A as can be seen in the ^{1}H NMR spectra (Fig. 5). The fraction and subfractions also presented similar phenolic compositions, exhibiting chicoric and caftaric acids as their major substances. The minor subfraction Og1-AP has a much lower phenolic concentration. The

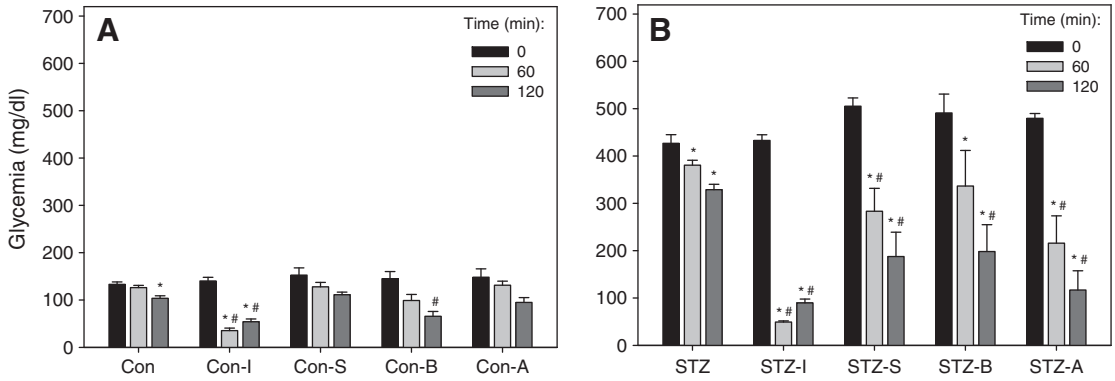

Fig. 3. Hypoglycemic activity of Og1-S, Og1-B and Og1-A. (A) Con: normal mice treated with saline ($n = 9$); Con-I: normal mice treated with insulin (2 U/kg; $n = 3$); Con-S: normal mice treated with Og1-S (300 mg/kg; $n = 3$); Con-B: normal mice treated with Og1-B (80 mg/kg; $n = 3$); Con-A: normal mice treated with Og1-A (240 mg/kg; $n = 3$). (B) STZ: diabetic mice treated with saline ($n = 9$); STZ-I: diabetic mice treated with insulin (2 U/kg; $n = 3$); STZ-S: diabetic mice treated with Og1-S (300 mg/kg; $n = 3$); STZ-B: diabetic mice treated with Og1-B (80 mg/kg; $n = 3$); STZ-A: diabetic mice treated with Og1-A (240 mg/kg; $n = 3$). Data represent the mean ± SEM. $^{*}P < 0.05$ compared to $t = 0$ (Student's *t*-test). $\#P < 0.05$ compared with control mice (Student's *t*-test).

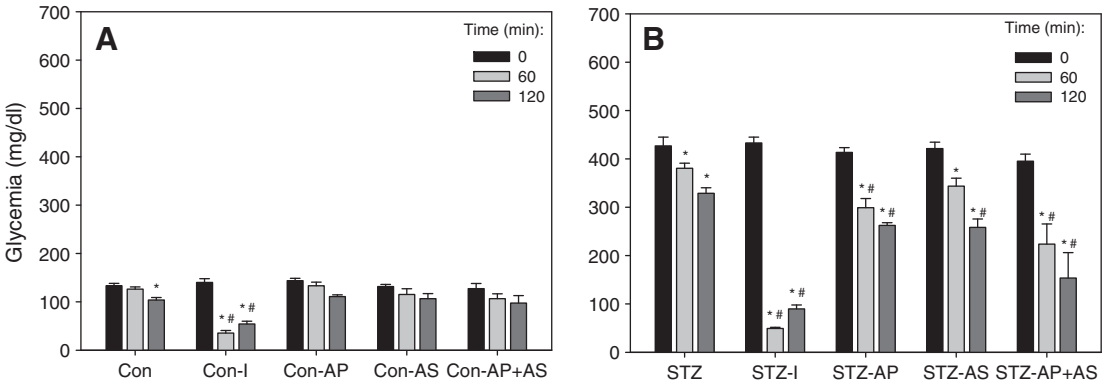

Fig. 4. Hypoglycemic activity of Og1-AP, Og1-AS and the pool of both subfractions. (A) Con: normal mice treated with saline ($n = 9$); Con-I: normal mice treated with insulin (2 U/kg; $n = 3$); Con-AP: normal mice treated with Og1-AP (40 mg/kg; $n = 3$); Con-AS: normal mice treated with Og1-AS (200 mg/kg; $n = 3$); Con-AP + AS: normal mice treated with Og1-AP and Con-AS (1:5; 240 mg/kg; $n = 3$). (B) STZ: diabetic mice treated with saline ($n = 9$); STZ-I: diabetic mice treated with insulin (2 U/kg; $n = 3$); STZ-AP: diabetic mice treated with Og1-AP (40 mg/kg; $n = 3$); STZ-AS: diabetic mice treated with Og1-AS (200 mg/kg; $n = 3$); STZ-AP + AS: diabetic mice treated with Og1-AP and Og1-AS (1:5; 240 mg/kg; $n = 3$). Data represent the mean ± SEM. *$P < 0.05$ compared to $t = 0$ (Student's t-test). #$P < 0.05$ compared with control mice (Student's t-test).

^{1}H NMR spectra showed important differences between the two subfractions. A singlet signal at 4.37 ppm compatible with the presence of tartaric acid was clearly intensified in the Og1-AP sample. This signal was three times greater in Og1-AP than in Og1-AS. We assume that this substance contributes to the hypoglycemic activity, since this activity decreases when it is separated from the mixture. There was also a pair of doublets (2.54 ppm; $J = 16.5$ Hz and 2.71 ppm; $J = 16.6$ Hz) sharper and more intense in Og1-AP, which seems to be compatible with citric acid. These signals are 35% greater in this fraction when compared to Og1-AS.

Tartaric acid is used as an excipient in many pharmaceutical formulations and shows no significant pharmacological activities. However, in spite of that, the use of tartaric acid as an excipient can increase the intestinal absorption of substances through some putative mechanisms: intermolecular interactions, glycoprotein P inhibition and enhancement of paracellular transport [45–47]. Some of these mechanisms possibly contribute in some extent to absorption through the peritoneal barrier, considering that intraperitoneally administered substances must transpose it to gain the bloodstream [48].

3.4. Hypoglycemic activity assays of compounds **1**, **2** and **4**

We also evaluated the two phenolic acids isolated from Og1-A, compounds **1** and **2** (3 mg/kg), in order to investigate their eventual role in the hypoglycemic activity observed for the fractions. Of both substances tested, only compound **2** (chicoric acid) exhibited significant hypoglycemic activity on diabetic mice (60 and 120 min after treatment). The reduction rate was 54%, as shown in Fig. 6. This result suggests that compound **2** contributes to the hypoglycemic activity observed for *O. gratissimum*. However, this phenolic acid is not likely to be the only component responsible for the hypoglycemic activity, since the isolated substance is less active than the fraction.

The flavonoid **4**, isolated from Og1-A and Og1-B, was evaluated at a dose of 4 mg/kg but did not show any hypoglycemic activity under the same conditions (data not shown). Compound **3** was not tested because it was obtained only from the second plant batch (Og2), whose fractionation was not biomonitored.

Chicoric acid was described by Tousch et al. (2008) [49] as a potential anti-diabetic agent. In this in vitro study the substance was able to increase insulin secretion from β-cells and to enhance muscle cell sensitivity to insulin. Chlorogenic acid, a monocaffeoyl ester of quinic acid, was also assayed and exhibited a similar activity [49]. A putative mechanism for the insulin-sensitizing activity of these two substances is the inhibition of protein tyrosine phosphatase 1B (PTP1B), a negative regulator of insulin sensitivity [50].

Chicoric and chlorogenic acids frequently co-occur in plant species [51–53]. Nevertheless, this substance is not reported in *O. gratissimum* and was not detected in the present study.

The anti-diabetic potential of chicoric acid was also evaluated in vivo. As described in a patent, the intraperitoneal administration of chicoric acid to normal rats at a dose of 5 mg/kg was able to potentiate the insulin response to glycemia, increasing insulin secretion [54]. Additionally, a chicoric acid-enriched extract (85–95% of purity) obtained from *Cichorium intybus* increased glucose tolerance on normal rats, at daily doses of 5, 15 and 30 mg/kg (i.p.) administered for four days. The beneficial effect produced by the extract was probably related to an insulin-sensitizing action [55].

However, it is important to note that the studies mentioned above were based on in vitro tests and on in vivo assays with normal animals. Therefore, as far as we know we have demonstrated here for the first time the hypoglycemic activity of chicoric acid in a diabetic animal model. We can find in literature many reports on in vivo hypoglycemic activity for caffeic acid esters, such as chlorogenic acid, romarinic acid and caffeic acid phenethyl ester [56–58]. These substances showed to be responsible for hypoglycemic activity of several medicinal plants [56,59,60]. Most of these studies, however, describe the effects of long-term treatments on glycemic parameters, which hinder comparison with the acute effects evaluated in our assays.

Nevertheless, studies on in vivo acute hypoglycemic activity for caffeic and chlorogenic acids were reported. Chlorogenic acid (10 mg/kg) showed significant hypoglycemic activity when orally administered to STZ-induced diabetic rats. The

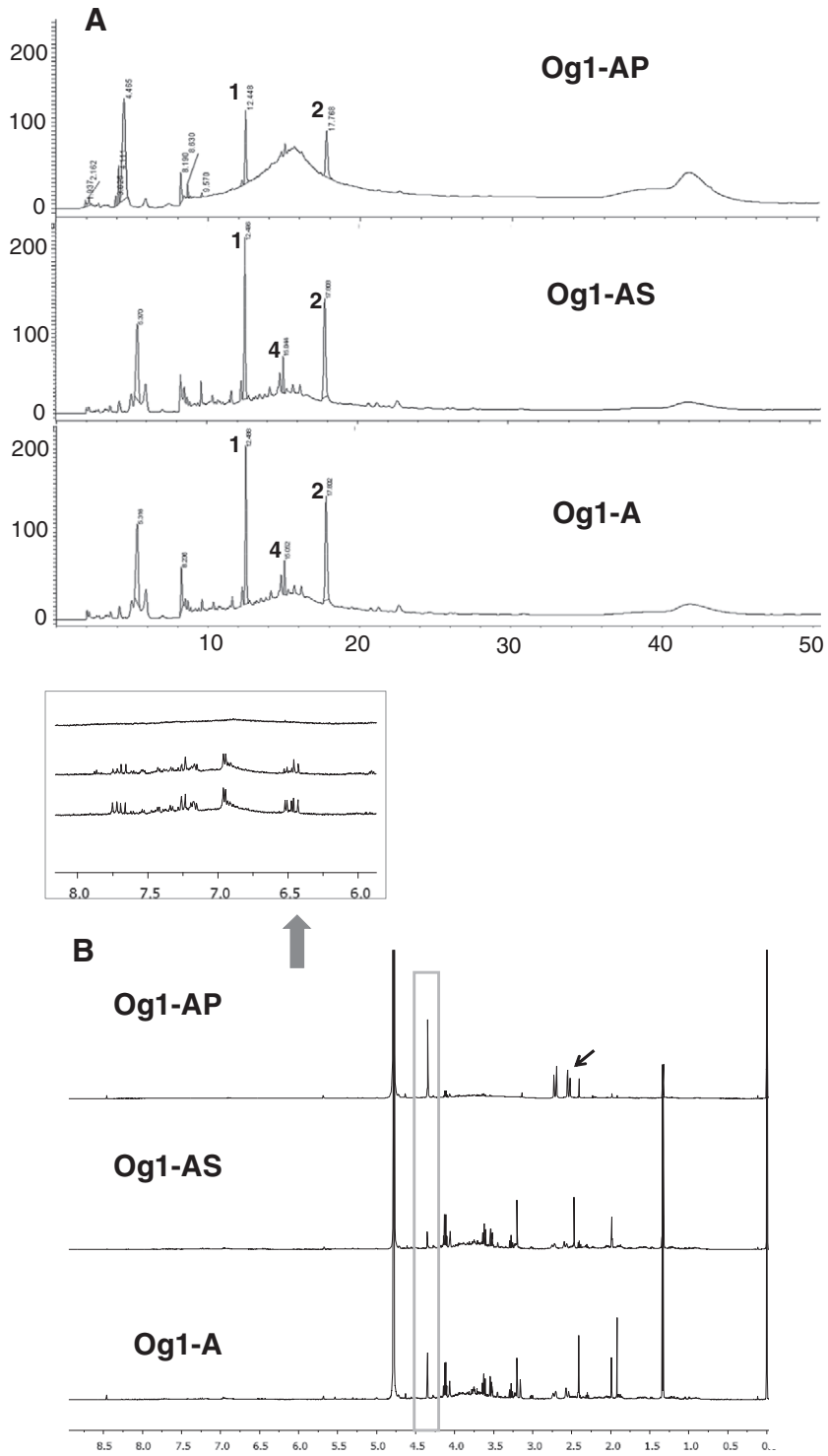

Fig. 5. Chromatograms (254 nm) of Og-A and subfractions (10 mg/ml). (A) Caftaric acid (**1**), vicenin-2 (**4**) and chicoric acid (**2**); (B) ^{1}H NMR spectra of Og-A and subfractions (6.7 mg/ml; D_2O). Singlet in 4.37 ppm is highlighted. The arrow indicates the doublets in 2.54 and 2.71 ppm. A close-up on the region of aromatic signals (6.0–8.0 ppm) is shown in a box.

reduction rate observed after 180 min was 26% [61]. Additionally, caffeic acid intravenously administered to diabetic rats (3 mg/kg) reduced glycemic levels in 24–33% after 30 min [62]. As caffeic acid is a product of caffeoyl ester metabolism, it could possibly account for the hypoglycemic activity of these substances, as suggested by Tsuda et al. (2012) [63].

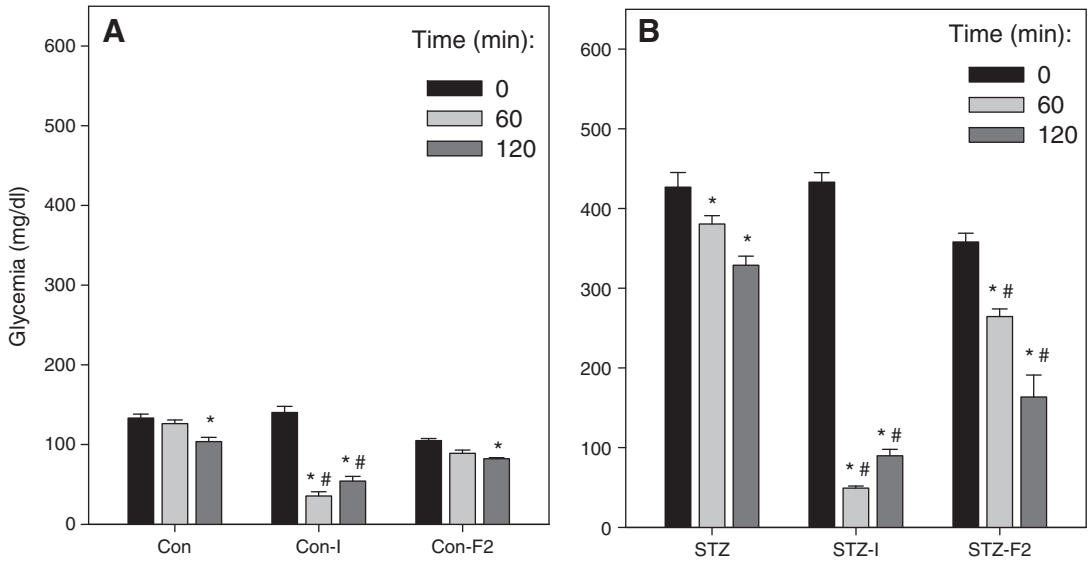

Fig. 6. Hypoglycemic activity of chicoric acid (compound **2**). (A) Con: normal mice treated with saline ($n = 9$); Con-I: normal mice treated with insulin (2 U/kg; $n = 3$); Con-F2: normal mice treated with compound **2** (3 mg/kg; $n = 3$). (B) STZ: diabetic mice treated with saline ($n = 9$); STZ-I: diabetic mice treated with insulin (2 U/kg; $n = 3$); STZ-F2: diabetic mice treated with compound **2** (3 mg/kg; $n = 3$). Data represent the mean ± SEM. $^{*}P < 0.05$ compared to $t = 0$ (Student's *t*-test). $\#P < 0.05$ compared with control mice (Student's *t*-test).

However, recent findings of Azay-Milhau et al. (2013) [55] do not corroborate this hypothesis. The authors assayed in vitro ferulic acid, caffeic acid and a chicoric acid enriched-extract and observed different pharmacological profiles for these substances. While the chicoric acid-enriched extract stimulated insulin release on pancreatic cells as expected, caffeic acid produced an opposite effect, decreasing insulin secretion from these cells. On the other hand, caffeic acid was able to reduce glycogenolysis on hepatocytes, which was not observed for chicoric acid-enriched extract.

Furthermore, in the present study the monocaffeoyl ester, caftaric acid (**1**) revealed to be inactive under the same conditions where chicoric acid was active. It is possible that both caffeoyl moieties are required for acute hypoglycemic activity in our model. The possible influence of pharmacokinetic factors that may favor the bioavailability of the dicaffeoyl over the monocaffeoyl ester should also be taken into consideration. Direct comparison with literature data on acute hypoglycemic activity of caffeic and chlorogenic acids is difficult because the animal model and administration route (i.p.) used in our study were different from those used in the previous reports above mentioned.

There is no data on chicoric acid bioavailability as far as we know. On the other hand, the bioavailability of caffeoylquinic acid esters (chlorogenic acids) in humans after coffee consumption is reported in a number of studies, yet, it is a controversial subject, as discussed by Erk et al. (2012) [64]. While in some studies a high bioavailability was observed (up to 70%) [65,66], the authors above mentioned detected much lower plasma concentrations of these substances after ingestion of different doses [64] due to their extensive metabolism. Caftaric acid, in turn, is only detectable in small amounts in plasma after wine consumption [67]. This substance is hydrolyzed by intestinal enzymes. There are evidences that chicoric acid is hydrolyzed as well, although it was not yet assayed in vivo [68]. In the present study compounds were not orally administered. A new set of in vivo experiments is necessary to investigate the effects of oral administration of fractions and compounds from *O. gratissimum* on glycemic levels in view of clarifying these issues.

4. Conclusion

In this study a correlation is established for the first time between *O. gratissimum* hypoglycemic activity and its chemical composition by means of a biomonitored fractionation. Chicoric acid, the active substance identified, is not likely to be the only compound responsible for the hypoglycemic activity. Results obtained suggested a possible interaction between chicoric acid and a substance present in the precipitate from the aqueous fraction, probably tartaric acid. This interaction is under investigation in our laboratories. From the above observations, it was possible to conclude that *O. gratissimum* is a promising source of hypoglycemic agents.

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References

[1] American Diabetes Association. Diagnosis and classification of diabetes mellitus. Position statement. Diabetes Care 2010;33:S62–9.
[2] Balasubramaniam K, Viswanathan GN, Marshall SM, Zaman AG. Increased atherothrombotic burden in patients with diabetes mellitus

and acute coronary syndrome: a review of antiplatelet therapy. Cardiol Res Pract 2012.
[3] Clarke PM, Glasziou P, Patel A, Chalmers J, Woodward M, Harrap SB, et al. Event rates, hospital utilization, and costs associated with major complications of diabetes: a multicountry comparative analysis. PLoS Med 2010;7:e1000236.
[4] Home P. The challenge of poorly controlled diabetes mellitus. Diabetes Metab 2003;275:101–9.
[5] Whiting DR, Guariguata L, Weil C, Shaw J. IDF diabetes atlas: global estimates of the prevalence of diabetes for 2011 and 2030. Diabetes Res Clin Pract 2011;94:311–21.
[6] World Health Organization. Diabetes. Fact sheet no 312 [online]. http://www.who.int/mediacentre/factsheets/fs312/en/index.html; 2012 . [accessed 26 January 2013].
[7] Ismail-Beigi F. Glycemic management of type 2 diabetes mellitus. N Engl J Med 2012;366:1319–27.
[8] Moser EG, Morris AA, Garg SK. Emerging diabetes therapies and technologies. Diabetes Res Clin Pract 2012;97:16–26.
[9] Dey L, Attele AS, Yuan CS. Alternative therapies for type 2 diabetes. Altern Med Rev 2002;7:45–58.
[10] Qi LW, Liu EH, Chu C, Peng YB, Cai HX, Li P. Anti-diabetic agents from natural products—an update from 2004 to 2009. Curr Top Med Chem 2010;10(4):434–57.
[11] Newman DJ, Cragg GM. Natural products as sources of new drugs over the 30 years from 1981 to 2010. J Nat Prod 2012;75:311–35.
[12] Ivorra MD, Payá M, Villar A. A review of natural products and plants as potential antidiabetic drugs. J Ethnopharmacol 1989;27:243–75.
[13] Marles RJ, Farnsworth NR. Antidiabetic plants and their active constituents. Phytomedicine 1995;2:137–89.
[14] Mukherjee PK, Maiti K, Mukherjee K, Houghton PJ. Leads from Indian medicinal plants with hypoglycemic potentials. J Ethnopharmacol 2006;106:1–28.
[15] Afifi-Yazar FU, Kasabri V, Abu-Dahab R. Medicinal plants from Jordan in the treatment of diabetes: traditional uses vs. in vitro and in vivo evaluations—part 2. Planta Med 2011;77:1210–20.
[16] Paton A, Harley MR, Harley MM. *Ocimum*: an overview of classification and relationships. In: Hiltunen R, Holm Y, editors. Basil: the genus *Ocimum*. Amsterdam: Harwood Academic Publishers; 1999. p. 1–38.
[17] Stevens PF. Angiosperm phylogeny website. Version 12. http://www.mobot.org/MOBOT/research/APweb/html; 2012 . [accessed 30 May, 2013].
[18] Nyarko AK, Asare-Anane H, Ofosuhene M, Addy ME, Teye K, Addo P. Aqueous extract of *Ocimum canum* decreases levels of fasting blood glucose and free radicals and increases antiatherogenic lipid levels in mice. Vascul Pharmacol 2002;39:273–9.
[19] Abo KA, Fred-Jaiyesimi AA, Jaiyesimi AE. Ethnobotanical studies of medicinal plants used in the management of diabetes mellitus in South Western Nigeria. J Ethnopharmacol 2008;115:67–71.
[20] Gbolade AA. Inventory of antidiabetic plants in selected districts of Lagos State, Nigeria. J Ethnopharmacol 2009;121:135–9.
[21] Vats V, Grover JK, Rathi SS. Evaluation of anti-hyperglycemic and hypoglycemic effect of *Trigonella foenum-graecum* Linn, *Ocimum sanctum* Linn and *Pterocarpus marsupium* Linn in normal and alloxanized diabetic rats. J Ethnopharmacol 2002;79:95–100.
[22] Chandra A, Mahdi AA, Ahmad S, Singh RK. Indian herbs result in hypoglycemic responses in streptozotocin-induced diabetic rats. Nutr Res 2007;27:161–8.
[23] Aguiyi JC, Obi CI, Gang SS, Igweh AC. Hypoglycaemic activity of *Ocimum gratissimum* in rats. Fitoterapia 2000;71:444–6.
[24] Egesie UG, Adelaiye AB, Ibu JO, Egesie OJ. Safety and hypoglycaemic properties of aqueous leaf extract of *Ocimum gratissimum* in streptozotocin induced diabetic rats. Niger J Physiol Sci 2006;21:31–5.
[25] Oguanobi NI, Chijioke CP, Ghasi SI. Effects of aqueous leaf extract of *Ocimum gratissimum* on oral glucose tolerance test in type-2 model diabetic rats. Afr J Pharm Pharmacol 2012;6:630–5.
[26] Da Silva D, Zancan P, Coelho WS, Gomez LS, Sola-Penna M. Metformin reverses hexokinase and 6-phosphofructo-1-kinase inhibition in skeletal muscle, liver and adipose tissues from streptozotocin-induced diabetic mouse. Arch Biochem Biophys 2010;496:53–60.
[27] Mulkens A, Kapetanidis I. Eugenylglucoside, a new natural phenylpropanoid heteroside from *Melissa officinalis*. J Nat Prod 1988;51:496–8.
[28] Xie C, Veitch NC, Houghton PJ. Flavone C-glycosides from *Viola yedoensis* MAKINO. Chem Pharm Bull 2003;51:1204–7.
[29] Sobolev AP, Brosio E, Gianferri R, Segre AL. Metabolic profile of lettuce leaves by high-field NMR spectra. Magn Reson Chem 2005;43:625–38.
[30] Maier T, Sanzenbacher S, Kammerer DR, Berardini N, Conrad J, Beifuss U. Isolation of hydroxycinnamoyltartaric acids from grape pomace by high-speed counter-current chromatography. J Chromatogr A 2006;1128:61–7.
[31] Nuissier G, Rezzonico B, Grignon-Dubois M. Chicoric acid from *Syringodium filiforme*. Food Chem 2010;120:783–8.
[32] Zeng Q, Chang R, Qin J, Cheng X, Zhang W, Jin H. New glycosides from *Dracocephalum tanguticum maxim*. Arch Pharm Res 2011;34:2015–20.
[33] Lamidey AM, Fernon L, Pouységu L, Delattre C, Quideau S, Pardon P. A convenient synthesis of the echinacea-derived immunostimulator and HIV-1 integrase inhibitor (−)-(2R,3R)-chicoric acid. Helv Chim Acta 2002;85:2328–34.
[34] Scarpati ML, Oriente G. Chicoric acid (dicaffeyltartic acid): its isolation from chicory (*Cichorium intybus*) and synthesis. Tetrahedron 1958;4:43–8.
[35] Singleton VL, Timberlake CF, Lea AGH. The phenolic cinnamates of white grapes and wine. J Sci Food Agric 1978;29:403–10.
[36] Wood GC. General physical methods. In: Beckett AH, Stenlake JB, editors. Practical pharmaceutical chemistry: part II. Cambridge: University Press; 1988. p. 9–51.
[37] Ola SS, Catia G, Marzia I, Francesco VF, Afolabi AA, Nadia M. HPLC/DAD/MS characterisation and analysis of flavonoids and cynnamoil derivatives in four Nigerian green-leafy vegetables. Food Chem 2009;115:1568–74.
[38] Lee J. Caffeic acid derivatives in dried Lamiaceae and *Echinacea purpurea* products. J Funct Foods 2010;2:158–62.
[39] Nakamura CV, Ishida K, Faccin LC, Filho BP, Cortez DAG, Rozental S, et al. In vitro activity of essential oil from *Ocimum gratissimum* L. against four *Candida* species. Res Microbiol 2004;155:579–86.
[40] Borges AR, Aires JR, Higino TM, de Medeiros Md, Citó AM, Lopes JA, et al. Trypanocidal and cytotoxic activities of essential oils from medicinal plants of Northeast of Brazil. Exp Parasitol 2012;132:123–8.
[41] Kurkin VA. Phenylpropanoids from medicinal plants: distribution, classification, structural analysis, and biological activity. Chem Nat Compd 2003;39:123–53.
[42] Schmourlo G, Mendonça-Filho RR, Alviano CS, Costa SS. Screening of antifungal agents using ethanol precipitation and bioautography of medicinal and food plants. J Ethnopharmacol 2005;96:563–8.
[43] Parekh J, Jadeja D, Chanda S. Efficacy of aqueous and methanol extracts of some medicinal plants for potential antibacterial activity. Turk J Biol 2005;29:203–10.
[44] Koffi E, Sea T, Dodehe Y, Soro S. Effect of solvent type on extraction of polyphenols from twenty three Ivorian plants. J Anim Plant Sci 2010;5:550–8.
[45] Takatsuka S, Kitazawa T, Morita T, Horikiri Y, Yoshino H. Enhancement of intestinal absorption of poorly absorbed hydrophilic compounds by simultaneous use of mucolytic agent and non-ionic surfactant. Eur J Pharm Biopharm 2006;62:52–8.
[46] Iida A, Tomita M, Idota Y, Takizawa Y, Hayashi M. Improvement of intestinal absorption of P-glycoprotein substrate by D-tartaric acid. Drug Metab Pharmacokinet 2006;21:424–8.
[47] Hayashi M, Sakai T, Hasegawa Y, Nishikawahara T, Tomioka H, Iida A, et al. Physiological mechanism for enhancement of paracellular drug transport. J Control Release 1999;62:141–8.
[48] Weryński A, Galach M. Membrane model of peritoneal barrier. Biocybern Biomed Eng 2008;28:3–9.
[49] Tousch D, Lajoix AD, Hosy E, Azay-Milhau J, Ferrare K, Jahannault C, et al. Chicoric acid, a new compound able to enhance insulin release and glucose uptake. Biochem Biophys Res Commun 2008;377:131–5.
[50] Baskaran SK, Goswami N, Selvaraj S, Muthusamy VS, Lakshmi BS. Molecular dynamics approach to probe the allosteric inhibition of PTP1B by chlorogenic and cichoric acid. J Chem Inf Model 2012;52:2004–12.
[51] Jaiswal R, Kiprotich J, Kuhnert N. Determination of the hydroxycinnamate profile of 12 members of the Asteraceae family. Phytochemistry 2011;72:781–90.
[52] Martynov AM, Dargaeva TD, Chuparina EV. Biologically active compounds from *Viola brachyceras* herb. Pharm Chem J 2012;46:435–7.
[53] Mai F, Glomb MA. Isolation of phenolic compounds from iceberg lettuce and impact on enzymatic browning. J Agric Food Chem 2013;61:2868–74.
[54] Andary C, Ribes G, Tousch D. Composition antidiabetique apte à stimuler la secretion d'insuline et destinée ou traitment du diabete de type 2 (diabete non insulino-dependant). FR 2904935; 2008.
[55] Azay-Milhau J, Ferrare K, Leroy J, Aubaterre J, et al. Antihyperglycemic effect of a natural chicoric acid extract of chicory (*Cichorium intybus* L.): a comparative in vitro study with the effects of caffeic and ferulic acids. J Ethnopharmacol 2013;150:755–60.
[56] Meng S, Cao J, Feng Q, Peng J, Hu Y. Roles of chlorogenic acid on regulating glucose and lipids metabolism: a review. Evid Based Complement Alternat Med 2013;2013:801457.
[57] Azevedo MF, Lima CF, Fernandes-Ferreira M, Almeida MJ, Wilson JM, Pereira-Wilson C. Rosmarinic acid, major phenolic constituent of Greek sage herbal tea, modulates rat intestinal SGLT1 levels with effects on blood glucose. Mol Nutr Food Res 2011;55:S15–25.
[58] Celik S, Erdogan S, Tuzcu M. Caffeic acid phenethyl ester (CAPE) exhibits significant potential as an antidiabetic and liver-protective agent in streptozotocin-induced diabetic rats. Pharmacol Res 2009;60:270–6.

[59] Alonso-Castro AJ, Miranda-Torres AC, González-Chávez MM, Salazar-Olivo LA. *Cecropia obtusifolia* Bertol and its active compound, chlorogenic acid, stimulate 2-NBD-glucose uptake in both insulin-sensitive and insulin-resistant 3T3 adipocytes. J Ethnopharmacol 2008;120:458–64.
[60] Hunyadi A, Martins A, Hsieh TJ, Seres A, Zupkó I. Chlorogenic acid and rutin play a major role in the in vivo anti-diabetic activity of *Morus alba* leaf extract on type II diabetic rats. PLoS One 2012;7:e50619.
[61] Andrade-Cetto A, Wiedenfeld H. Hypoglycemic effect of *Cecropia obtusifolia* on streptozotocin diabetic rats. J Ethnopharmacol 2001;78:145–9.
[62] Hsu FL, Chen YC, Cheng JT. Caffeic acid as active principle from the fruit of *Xanthium strumarium* to lower plasma glucose in diabetic rats. Planta Med 2000;66:228–30.
[63] Tsuda S, Egawa T, Ma X, Oshima R, Kurogi E, Hayashi T. Coffee polyphenol caffeic acid but not chlorogenic acid increases 5′AMP-activated protein kinase and insulin-independent glucose transport in rat skeletal muscle. J Nutr Biochem 2012;23:1403–9.
[64] Erk T, Williamson G, Renouf M, Marmet C, Steiling H, Dionisi F, et al. Dose-dependent absorption of chlorogenic acids in the small intestine assessed by coffee consumption in ileostomists. Mol Nutr Food Res 2012;56:1488–500.
[65] Monteiro M, Farah A, Perrone D, Trugo LC, Donangelo C. Chlorogenic acid compounds from coffee are differentially absorbed and metabolized in humans. J Nutr 2007;137:2196–201.
[66] Farah A, Monteiro M, Donangelo CM, Lafay S. Chlorogenic acids from green coffee extract are highly bioavailable in humans. J Nutr 2008;138:2309–15.
[67] Stalmach A, Edwards CA, Wightman JD, Crozier A. Identification of (poly)phenolic compounds in concord grape juice and their metabolites in human plasma and urine after juice consumption. J Agric Food Chem 2011;59:9512–22.
[68] Bel-Rhlid R, Pagé-Zoerkler N, Fumeaux R, Ho-Dac T, Chuat JY, Sauvageat JL, et al. Hydrolysis of chicoric and caftaric acids with esterases and *Lactobacillus johnsonii* in vitro and in a gastrointestinal model. J Agric Food Chem 2012;60:9236–341.