Antihyperglycemic Activity and Standardization of the Bioactive Extract of *Cleome droserifolia* Growing in Egypt

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ABSTRACT

Background: *Cleome droserifolia* herb is well known in the Egyptian folk medicine for the treatment of diabetes. However, a standardized active extract of the herb was never prepared for incorporation into a pharmaceutical dosage form. **Materials and Methods:** Comparative high performance liquid chromatography (HPLC) chromatographic profiles were established in order to study the ethnopharmacological use of the antihyperglycemic herb using a validated reversed phase-HPLC method which was developed for standardization of the active aqueous extract. A biologically guided fractionation of the antihyperglycemic aqueous extract was carried out *in vivo* using alloxan induced diabetic rats. **Results:** The aqueous extract contained the highest percent of the total active flavonol glycosides (78.20%) compared to the 70% and 50% ethanolic extracts (51.17 and 42.66%, respectively). The aqueous extract and its ethyl acetate fraction possessed the highest antihyperglycemic activities. A standard calibration curve, established for the major bioactive methoxylated flavonol glycoside (kaempferol-4′-methoxy-3,7-dirhamnoside) at a concentration range of 44-174 µg/ml, showed good linearity with a correlation coefficient (R2) of 0.998. The recovery of the method was 100.5%. A high degree of precision (relative standard deviation values <5%) was achieved. The limits of detection and quantification were 0.01 and 0.02 µg/ml, respectively, indicating the sensitivity of the method.**Conclusion:** The aqueous extract contained the highest percent of the total active flavonol glycosides. The extract, standardized to contain not <1.5 ± 0.06% of kaempferol-4′-methoxy-3,7-dirhamnoside, was tested at three different dose levels showing a 63.3% activity of that of metformin at100 mg/kg body weight. Furthermore, it raised the blood insulin level by 146.26% at this dose level.

Keywords: Antihyperglycemic, *Cleome droserifolia,* high performance liquid chromatography standardization, kaempferol-4′-methoxy-3,7-dirhamnoside, validation

INTRODUCTION

The dried herb of *Cleome droserifolia* (Forssk.) Del, a plant of the Cleomaceae family,[1] locally known as Samwah is well known in the Egyptian folk medicine as its decoction has been used by the Bedouins of the southern Sinai for the treatment of diabetes.[2] The herb is also sold by herbalists in the Egyptian market as an antihyperglycemic agent. Several articles discussed the efficacy and safety of *C. droserifolia*.[2-7]

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Nevertheless, a standardized extract of the herb was never prepared nor incorporated into a pharmaceutical preparation for the treatment of diabetes.

In a previous work of our group, cell-based bioassays were applied to determine active compounds, which could be used as bioactive markers to standardize the herbal extract of *C. droserifolia* and hence assure its quality and efficacy.[8,9] The aqueous extract of *C. droserifolia* and its ethyl acetate fraction showed significant insulino-mimetic effects in peripheral tissues. We isolated three flavonol glycosides; isorhamnetin-3-O-β-D-glucoside (F1), quercetin-3'-methoxy-3-O-(4'-acetylrhamnoside)-7-O-α-rhamnoside (F2) and kaempferol-4'-methoxy-3,7-dirhamnoside (F3) from the ethyl acetate fraction. These compounds proved to possess a high antihyperglycemic activity, which was twice as active as insulin in stimulating glucose uptake in differentiated C2C12 skeletal muscle cells.[8,9]

The aim of the present study was to support our previous *in vitro* results by *in vivo* experiments, compare two hydroalcoholic extracts of *C. droserifolia* to the aqueous extract regarding the active flavonol glycoside content, and prepare a standardized active extract through a validated reversed phase-high performance liquid chromatography (RP-HPLC) method using the major bioactive methoxylated flavonoid glycoside. Kaempferol-4'-methoxy-3,7-dirhamnoside (F3) was selected as a marker for HPLC standardization as it was the major bioactive compound we previously isolated from the ethyl acetate fraction.

MATERIALS AND METHODS

Plant material

The aerial parts of *C. droserifolia* were obtained from the Medicinal Plants Society, Saint Catherine, Sinai in 2008 and 2009. The plant was authenticated by Assistant Prof. Dr. M. Gebali (Plant Taxonomy and Egyptian Flora Department, National Research Center, Giza, Egypt). A voucher specimen (no. 313) was deposited at the herbarium of the Pharmacognosy Department, Faculty of Pharmacy, Cairo University, Cairo, Egypt.

Preparation of extracts and fractions

For comparative HPLC analysis of the extracts, 50 g of the air-dried aerial parts of *C. droserifolia* were extracted with water, 50% and 70% ethanol in water (2 × 500 ml) on cold, each time for 3 days and the volume was adjusted to 1 L in a volumetric flask.

The aqueous extract of the air-dried aerial parts of *C. droserifolia* as well as its fractions; the n-hexane, chloroform, ethyl acetate and *n*-butanol fractions were prepared as mentioned in Ezzat and Abdel Motaal, 2012.[8]

Chemicals

Alloxan was purchased from Sigma-Aldrich (St. Louis, MO, USA). Carboxymethyl cellulose sodium (CMC-Na) was purchased from Acros Organics (NJ, USA), while heparin sodium was purchased from Merck (Dramstadt, Germany). Insulin kit (Coat-A-Count Insulin) was purchased from Siemens, Medical Solutions Diagnostics (LA, USA). All reagents for extraction were of analytical grade (ADWIC, Egypt). Metformin, CID Co., Egypt. Chromatographic grade-double distilled water; analytical grade *O*-phosphoric acid and HPLC grade methanol were purchased from Merck, Germany. The acidic aqueous solution used for HPLC analysis was filtered through Agilent Ecno 0.45 µm polytetrafluoroethylene membrane filter and degassed in an ultrasonic bath before use. The marker flavonoid, kaempferol-4'-methoxy-3,7-dirhamnoside (F3), was previously isolated and identified by the authors.[8]

Animals

Adult male rats of Sprague-Dawley strain (130-150 g body weight) were obtained from the animal house of the National Research Center, Giza, Egypt. They were kept under the same hygienic conditions and were fed by the basal diet recommended by the American Institute of Nutrition.[10] Animals were housed for 2 days under standard conditions (well ventilated, temperature 22 ± 2°C, relative humidity 50-60% and 12 h day and night cycle). Food consisted of normal rat chow and water was provided *ad libitum*. Care was taken to avoid stressful conditions. All experimental procedures were performed between 8:00 and 10:00 a.m. All the animal experimental work was carried out after the approval of the Institutional Animal Ethical Committee.

RP-HPLC analysis of the extracts and fractions

An HPLC method was developed for the comparative study of the different extracts of *C. droserifolia* namely the aqueous, 50% and 70% extracts in order to reach the best solvent for extraction of the active flavonol glycosides. An Agilent technologies 1100 series HPLC was used, equipped with an Agilent 1200 series G1322A quaternary pump and degasser, a G1314A variable wavelength detector and an Agilent ChemStation software, Santa clara, California, United States software. Samples (1 mg/ml methanol) were injected into a lichrosphere 100 RP-18, 5 µm, 250 mm × 4 mm column (Merck, Germany) maintained at a temperature of 25°C, guarded by a 5 µm, 10 mm × 4 mm guard column. The mobile phase used was acetonitrile (solvent A) and 0.3% *O*-phosphoric acid in water (solvent B). A continuous gradient elution (10-75% A in B) for 25 min was carried out at a flow rate of 1.0 ml/min. The injection volume was 20 µl and detection was made at 325 nm. The same RP-HPLC method was used for the analysis of the chloroform, ethyl acetate and *n*-butanol fractions (1 mg/ml methanol, each).

Screening for *in vivo* antihyperglycemic activity

The 70% ethanolic and aqueous extracts of the aerial parts of *C. droserifolia* were assessed for their antihyperglycemic activity in rats over 28 days. In addition, the *n*-hexane, chloroform, ethyl acetate and *n*-butanol fractions of the aqueous extract were tested. All samples were tested at the same dose level of the biguanide metformin (150 mg/kg body weight). Diabetes was induced intraperitoneally in

the rats with a single dose of alloxan (150 mg/kg body weight).[11] The antihyperglycemic activity of the most active aqueous extract of the aerial parts of *C. droserifolia* was assessed in a further experiment over 28 days using three different dose levels; 50, 75 and 100 mg/kg body weight.

The control diabetic group in each experiment received a single daily dose of 1% CMC-Na. The vehicle, metformin and plant extracts were given orally by gavage as single daily treatments for 4 weeks. Blood samples in each experiment were collected from tail vein and fasting blood glucose levels of the overnight fasted animals at 0 time, and at days 14 and 28 from the treatment.

Estimation of plasma insulin level

At the end of the 28th day in the second experiment, 3 h after the last dose of the vehicle or the aqueous extract, blood samples were withdrawn from the orbital sinus of rats under light ether anesthesia into heparinized tubes. Samples were centrifuged at 3500 g for 15 min for separation of plasma. Plasma samples were separated and kept at −20°C for analysis when required. Insulin concentrations were determined by radio immunoassay procedure using insulin kit.

Standardization of the aqueous extract using RP-HPLC

Sample preparation

The aqueous extract (20 mg) was dissolved in 1 ml of 40% methanol in H_2O, using an ultrasonic, and loaded on an EX trelut® prepacked column (Merck). Elution was carried out using 7 × 1 ml, 40% methanol in H_2O, and the volume completed to 10 ml in a volumetric flask.

Construction of the standard calibration curve

Serial dilutions of F3 (50, 100, 120, 160, 200 μg/ml) were prepared from a stock solution having a final concentration of 200 μg/ml, formed by weighing accurately 5 mg in a 25 ml volumetric flask and dissolving in methanol. The purity of the standard F3 used was 87%, where the area-percent method was used to correct for the purity. A standard calibration curve was established using the corrected concentrations (44, 87, 105, 139 and 174 μg/ml). Each sample was injected in triplicates.

Validation of the RP-HPLC method

Linearity was determined by injecting five different concentrations of F3 standard solution (44-174 μg/ml). The accuracy was calculated as the percent recovery of spiked aqueous extract samples with F3 sample at a concentration of 139 μg/ml. To determine the intra- and inter-day precision of the method, F3 was assayed at two different concentrations (44 and 87 μg/ml) on 1 day in six replicates and on 3 separate days in triplicates. Robustness of the method was determined by employing two different sample weights of the aqueous extract (20 mg and 23 mg). Two different analysts carried out the analysis of the aqueous extract in three consecutive days to assess the ruggedness of the method. The sample solution was kept at 4°C and its stability was tested at 0, 7 and 44 h. Limit of quantification (LQ) and limit of detection (LD) were determined based on the standard deviation of the response (σ) and the slope of the calibration curve (S) following the International Conference on Harmonization Guidelines;[12] LQ = 10 (σ/S), LD = 3.3 (σ/S).

Statistical analysis

Data were expressed as mean ± standard error of the mean. Statistical analyses were performed using one-way analysis of variance, unless otherwise indicated. If the overall *F* value was found statistically significant ($P < 0.05$), further comparisons among groups were made according to *post-hoc* Tuckey's test. All statistical analyses were performed using Statistical Package for the Social Sciences GraphPad In Stat 3 software (La Jolla, CA, USA).

RESULTS

HPLC standardization of the active extracts and fractions

Comparing the RP-HPLC chromatograms of *C. droserifolia*, it was evident that the aqueous extract contained the highest percent of the total active flavonol glycosides (78.20%, major peaks at R_t 7-10 min), followed by its ethyl acetate fraction (65.4%) (Figures 1 and 2). While, the 70% and 50% ethanolic extracts contained 51.17 and 42.66% of total flavonol glycosides, respectively (Figure 2).

The aqueous extract was standardized to contain not less than 1.5 ± 0.06% of F3 (kaempferol-4'-methoxy-3,7-dirhamnoside), while the ethyl acetate fraction was standardized to 2.7 ± 0.06% of F3.

In vivo antihyperglycemic activity

Both the aqueous and 70% ethanolic extracts of the aerial parts of *C. droserifolia* were chosen for the *in vivo* activity as they contained relatively high content of total active flavonol glycosides. They showed a significant antihyperglycemic response as they reduced the blood

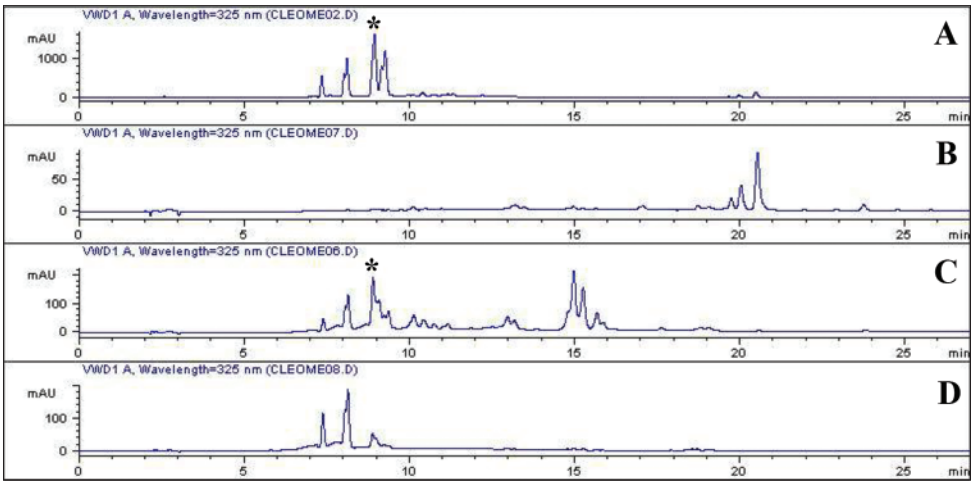

Figure 1: Reversed phase-high performance liquid chromatography chromatograms of the aqueous extract of *Cleome droserifolia* and its successive fractions. (a) Aqueous extract 50 g/L; (b) chloroform fraction 1 mg/ml; (c) ethyl acetate fraction 1 mg/ml; (d) butanol fraction 1 mg/ml; *compound F3 at R_t at 9.09.

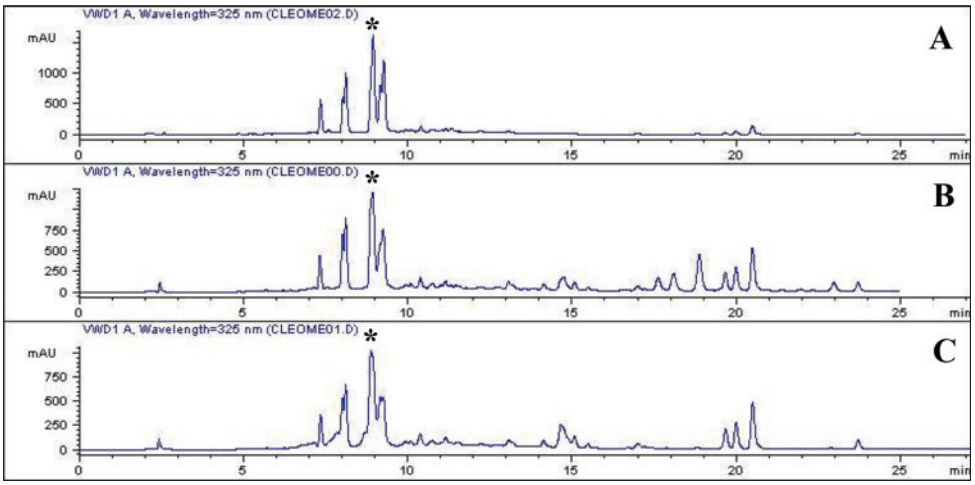

Figure 2: Reversed phase-high performance liquid chromatography chromatograms of the different extracts (50 g/L) of *Cleome droserifolia*. (a) Aqueous extract; (b) 50% ethanol extract; (c) 70% ethanol extract; *compound F3 at Rt at 9.09.

glucose levels by 47.6 and 38.5%, respectively, after 4 weeks of administration (Table 1). The ethyl acetate among other fractions of the parent aqueous extract showed the highest activity, as it caused 44.7% reduction in blood glucose level after 4 weeks of administration (Table 1).

The most active standardized aqueous extract was then tested at different dose levels (Table 2), the results showed that after 4 weeks the blood glucose levels were reduced by 31.25, 36.70 and 46.47% at a dose of 50, 75 and 100 mg/kg body weight, respectively.

Estimation of plasma insulin level

The aqueous extract moreover raised the levels of the blood insulin by 30.23, 92.78 and 146.24% at a dose of 50, 75 and 100 mg/kg body weight, respectively (Table 3).

Validation of the RP-HPLC method

Linear regression analysis of compound F3 was performed by plotting the mean peak area versus concentration (Figure 3). The correlation coefficient (R^2) of the standard calibration curve was 0.998 indicating linearity of the peak area in the range of 44-174 µg/ml. The LD and LQ for F3 were 0.01 and 0.02 µg/ml, respectively. Precision of the method during the intra- and inter-day run are given in Table 4. The relative standard deviation (RSD) was taken as a measure of precision. The data were within the acceptance criteria (< 5%). Sample of F3 proved to be stable in methanol solution within 44 h at 4°C (RSD 2.20%, Table 4).

Recovery tests were carried out to further investigate the accuracy of the method. The percent recovery of spiked

Table 1: Antihyperglycemic effect of the different extracts of *C. droserifolia*

Group	Time				
	0	2 weeks		4 weeks	
	M±SE (mg/dl)	M±SE	% of change	M±SE	% of change
Diabetic rats (Db) non treated	243.7±8.2	256.8±9.6	-	256.8±9.6	-
Db+metformin	257.3±11.4	129.8±4.3*	49.5	81.9±3.2*	68.2
Db+70% ethanolic extract	249.2±8.2	216.2±7.6*	13.2	153.2±4.6*	38.5
Db+aqueous extract	256.8±10.1	173.2±6.2*	32.5	141.9±5.5*	47.6
Db+*n*-hexane fraction	246.9±7.8	214.3±8.6*	13.2	198.6±7.1*	19.5
Db+chloroform fraction	251.9±8.6	186.8±7.4*	25.8	138.9±5.8*	41.8
Db+ethyl acetate fraction	258.4±7.1	187.4±6.3*	27.5	135.3±4.1*	44.7
Db+*n*-butanol fraction	258.3±10.2	224.9±8.4	13.3	203.7±6.5*	21.1

Extracts, fractions, and the standard metformin were given at a dose of 150 mg/kg body weight. *Statistically significant difference from 0 time at $P<0.01$. M: Mean, SE: Standard error ($n=6$)

Table 2: Antihyperglycemic effect of the different doses of the aqueous extract of *C. droserifolia*

	Blood glucose level mg/dl (M±SE)				
	Diabetic non-treated (Db)	Db+ 50 mg/kg	Db+ 75 mg/kg	Db+ 100 mg/kg	Metformin (150 mg/kg)
0	254.9±6.4	248.2±9.6	261.3±7.1	264.9±7.6	259.7±8.1
2 weeks	262.3±7.9	224.9±5.8	196.4±6.5*	192.3±6.2*	146.3±5.2*
4 weeks	258.9±7.7	178.4±4.9*	163.9±5.7*	138.6±4.3*	87.8±2.4*

*Statistically significant difference from 0 time at $P<0.01$. M: Mean, SE: Standard error ($n=6$)

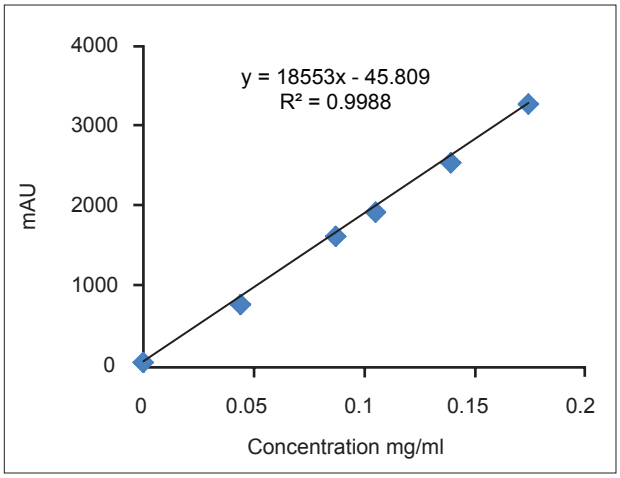

Figure 3: Standard calibration curve of the flavonoid glycoside F3 using reversed phase-high performance liquid chromatography.

aqueous extract samples with F3 sample at a concentration of 139 μg/ml was 100.5% with RSD of 2.25 (Table 5).

Influence of small changes in the chromatographic method, such as change in the sample weight and analyst, was also assessed to determine the robustness and ruggedness of the method, respectively. Results (RSD < 3%, Table 6) were also in favor of the developed RP-HPLC method.

DISCUSSION

In our previous work, the aqueous extract of the aerial parts of *C. droserifolia* was shown to have a similar effect to insulin in increasing the basal glucose up-take in cultured C2C12 skeletal muscle cells, while the ethanolic extract did not show any effect *in vitro*.[9] However, several studies proved the *in vivo* antihyperglycemic properties of the ethanolic extract at dose levels of 310 and 900 mg/kg

Table 3: Effects of oral administration of the different doses of the aqueous extract on plasma insulin levels in diabetic rats

	Blood insulin level uUL/ml (M±SE)			
	Diabetic non-treated (Db)	Db+ 50 mg/kg	Db+ 75 mg/kg	Db+ 100 mg/kg
0	8.9±0.1	8.6±0.1	9.7±0.4	9.3±0.2
2 weeks	8.1±0.1	9.4±0.2	14.6±0.5*	16.2±0.6*
4 weeks	8.5±0.1	11.2±0.3*	18.7±0.4*	22.9±0.7*

*Statistically significant difference from 0 time at $P<0.01$. M: Mean, SE: Standard error ($n=6$)

Table 4: Precision and stability of compound F3 ($n=3$)

Compound F3 (μg/ml)	Intra-day RSD (%)	Inter-day RSD (%)	Stability
44	4.25	3.82	
87	2.04	2.22	2.20

RSD: Relative standard deviation

Table 5: Recovery of compound F3 in the active aqueous extract ($n=3$)

Compound F3 original (μg/ml)	Spiked (μg/ml)	Found (μg/ml)	Recovery (%)	RSD (%)
139	199	199.99	100.50	2.25

RSD: Relative standard deviation

Table 6: Robustness and ruggedness of the RP-HPLC method (*n*=3)

		Mean (mg/ml)±SD*	RSD (%)
Ruggedness	Analysts (1 and 2)	0.064±0.0004	0.65
Robustness	20 mg ethyl acetate fraction	0.0628±0.0007	1.12
	23 mg ethyl acetate fraction	0.0631±0.0010	2.63

*Mean concentration of F3 in the aqueous sample as calculated from the standard calibration curve (*n*=3), RP-HPLC: Reversed phase-high performance liquid chromatography, SD: Standard deviation

body weight in alloxan-induced diabetic mice and rats, respectively.[2,3] By comparing the different extracting solvents using the RP-HPLC chromatographic profiles, it was evident that the aqueous extract was rich in the active flavonol glycosides followed by the 70% ethanolic extract, where the 50% ethanolic extract contained the least amount. Hence, both the aqueous and 70% ethanolic extracts of *C. droserifolia* were tested *in vivo* in alloxan-induced diabetic rats at 150 mg/kg body weight. The aqueous extract showed a higher activity. Consequently its four fractions were assessed for their activities. The ethyl acetate fraction, again containing relatively high content of the total flavonol glycosides, showed the highest activity, which was comparable to the mother aqueous extract. This strongly supports and confirms our previous *in vitro* assays, which showed that the ethyl acetate fraction and its isolated flavonol glycosides possessed significant insulin–like properties in peripheral tissues.[9]

The activity of the aqueous extract was thus tested at different dose levels, showing significant antihyperglycemic activities, especially at 100 mg/kg body weight, where its activity was 63.3% of that of metformin at 150 mg/kg body weight. Furthermore, oral administration of the aqueous extract for 4 weeks increased significantly the insulin blood levels, compared with the diabetic rats, which may be through stimulation of the activity of the remnant pancreatic β-cells.

This provided an explanation for the promising activity of the aqueous extract known traditionally strongly correlating it to the total flavonol glycosides content, and also suggested the potentiality of preparing a standardized aqueous extract of *C. droserifolia* for use against hyperglycemia.

Compound F3 was chosen as the bioactive marker for standardization of the aqueous extract being the major active compound which we previously isolated,[8,9] besides giving a sharp peak, which was not overlapping with other peaks using the developed fingerprint chromatogram.

To obtain a satisfactory chromatogram for the aqueous extract, several sample preparation methods were tried until reaching the optimum method, comparing the numbers, areas and resolution of the chromatographic peaks. The EXtrelut® prepacked column was conditioned with either 3 × 1 ml H_2O followed by 3 × 1 ml methanol or with methanol followed by water, when the aqueous extract (10 mg) was dissolved in 1 ml methanol or in H_2O, respectively. Elution was carried out with different mixtures of methanol: H_2O (25, 30, 40, 50, 75 and 100%).

In order to obtain valid chromatographic conditions, different RP-HPLC parameters were compared and optimized, including mobile phases (methanol/water and acetonitrile/water with different modifiers as acetic acid and *O*-phosphoric acid), various gradient and isocratic elutions, as well as, different flow rates. Best conditions were determined and the established aqueous extract chromatogram was used for standardization of the extract.

The active aqueous extract was standardized to contain not <1.5 ± 0.06% of F3 (kaempferol-4'-methoxy-3,7-dirhamnoside).

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