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Optimization and validation of extraction and quantification methods of antimalarial triterpenic esters in *Keetia leucantha* plant and plasma

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ABSTRACT

The aim of this study is to develop validated methods for the extraction and quantification of antimalarial triterpene esters from *Keetia leucantha* and from plasma samples. These compounds, showing *in vitro* and *in vivo* antiplasmodial activities, were optimally extracted from *Keetia leucantha* twigs using ultrasounds with dichloromethane and from plasma using protein precipitation with acetonitrile. We then developed and validated HPLC-UV quantification methods, which proved to be selective, accurate, linear, true and precise, both in plant and plasma samples for the eight triterpenic esters in mixture. Based on the total error concept as decision criteria, the validated dosage ranges of the triterpene esters mixture were set between 14.68 and 73.37 µg/mL in plants and 15.90 and 106.01 µg/mL in plasma injected solutions, corresponding to 7.95 and 53.01 µg/mL in plasma. These reliable methods were used to determine effectively triterpene esters content in collected samples, that seems highly variable in plant extracts, and will be helpful to further investigate pharmacokinetics parameters of these interesting bioactive compounds.

1. Introduction

Malaria remains the most important parasitic disease worldwide, particularly for children under five years old and pregnant women, with 216 million cases worldwide and about 445,000 deaths in 2016 [1]. Despite efforts to develop vaccines, a protection of at most 36%, mainly for children and infants, could be obtained due to the antigenic variability of these parasites [2]. Furthermore, given the development of resistance even to the current most effective molecules in the market, artemisinin and their derivatives, the treatment of malaria remains a major public health concern [3]. So, the need to discover new drugs prototypes is essential. In this search, investigation of natural resources could be helpful by providing new molecules and skeletons.

Keetia leucantha is a West African tree traditionally used in Benin to treat malaria as a whole plant decoction soup (so containing water soluble but also insoluble compounds) taken three times a day, alone or in association with *Carpolobia lutea* (Polygalaceae) and *Triclisia patens* (Menispermaceae). The twigs dichloromethane extract of this plant has shown a selective antiplasmodial activity *in vitro* against *Plasmodium falciparum* chloroquine-sensitive and –resistant strains as well as an *in vivo* antimalarial activity on *Plasmodium berghei* infected mice [4–6,22]. A bioguided fractionation of this extract allowed the identification of thirteen compounds among which eight triterpenic esters (**8TTE**) resulting from a trans/cis ferulic/coumaric acid esterification of two isomeric triterpenic acids (Fig. 1). These were the most *in vitro* effective and selective identified compounds, being more than ten times more active than corresponding acids. Furthermore, the mixture was shown to be as active as some purified cis/trans forms of individual compounds (the two geometric forms easily interconvert in solution giving an equilibrium of both diastereoisomers) [2]. This activity enhancement had already been associated to coumarate moiety esterification while no significant activity difference due to geometric isomerism was found for other similar compounds [3–5]. The **8TTE** also displayed *in vivo* antimalarial efficacy that can explain at least a part of *Keetia leucantha* activity on *Plasmodium* and no acute toxicity was observed at a total cumulative dose of 150 mg/kg mice body weight given ip [6].

Given the **8TTE** potential as novel antiplasmodial lead, it is important to quantify them accurately in samples from *Keetia* species. This quantification will allow to select samples with the highest antiplasmodial potential and to standardize extracts for traditional use. Moreover, in further investigation, information on pharmacokinetic profile will be needed for dose/effect relation understanding. So, we developed and validated an extraction process from both twigs of *Keetia leucantha* and biological samples, followed by the same HPLC-UV

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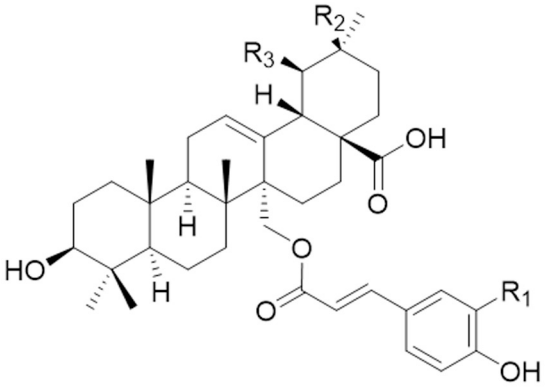
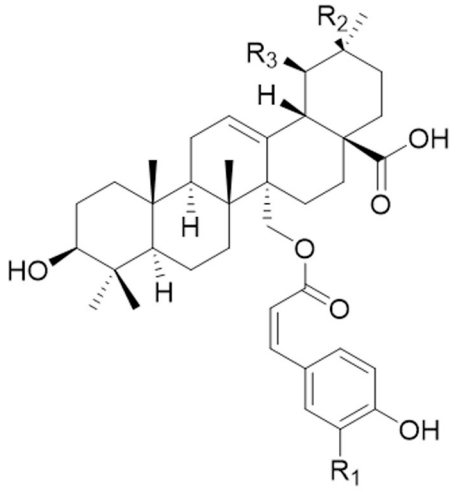


1. R_1 = H, R_2 = CH_3, R_3 = H **(27-O-p-(E)-coumaroyloxyoleanolic acid)**
2. R_1 = H, R_2 = H, R_3 = CH_3 **(27-O-p-(E)-coumaroyloxyursolic acid)**
3. R_1 = OCH_3, R_2 = CH_3, R_3 = H **(27-O-p-(E)-feruloyloxyoleanolic acid)**
4. R_1 = OCH_3, R_2 = H, R_3 = CH_3 **(27-O-p-(E)-feruloyloxyursolic acid)**



5. R_1 = H, R_2 = CH_3, R_3 = H **(27-O-p-(Z)-coumaroyloxyoleanolic acid)**
6. R_1 = H, R_2 = H, R_3 = CH_3 **(27-O-p-(Z)-coumaroyloxyursolic acid)**
7. R_1 = OCH_3, R_2 = CH_3, R_3 = H **(27-O-p-(*Z*)-feruloyloxyoleanolic acid)**
8. R_1 = OCH_3, R_2 = H, R_3 = CH_3 **(27-O-p-(Z)-feruloyloxyursolic acid)**

Fig. 1. Structural formula of antimalarial **8TTE** isolated from *Keetia leucantha*.

quantification of these 8 bioactive compounds. As they possess similar antiplasmodial activity, have very close structures and absorption maxima and form four interconverting diastereoisomers, we decided to develop a method for their global quantification using a Single Standard to Determine Multicomponents (SSDMC) [7–9]. So, the **8TTE** were considered as one single analyte for the quantification.

2. Material and methods

2.1. Chemicals and material

All used organic solvent (VWR, Belgium) were HPLC grade, except dichloromethane used for sample extraction which was of analytical grade and water which had MilliQ quality. The oestradiol valerate was acquired from Aca Pharma NV (Certa, Belgium) and used as internal standard.

The mixture of **8TTE** was isolated from the dichloromethane extract of *Keetia leucantha* twigs as described previously [5]. The purity of this purified mixture was measured as 96.7% using an Accela HPLC system (Thermo Fisher Scientific) (see Section 2.2).

Twigs (**KLT-1**) and leaves (**KLL-1**) of *Keetia leucantha* (K. Krause) Bridson (syn. *Plectronia leucantha* Krause, Rubiaceae) were collected in Benin (Adjarra, Ouémé) in July 2011 and August 2012 and identified at the National Botanic Garden of Belgium in Meise (in comparison to voucher number BR0000005087129). Twigs of *Keetia venosa* (Oliv.) Bridson (syn. *Plectronia venosa* Oliv.) were collected in Benin (**KVT-1**, Djougou, Donga) in December 2009 (voucher number BR0000005088713, the Herbarium of the National Botanic Garden of Belgium, Meise). Several other samples of *K. leucantha* twigs (described in Table 1s) were also collected from different places. Twigs and leaves were dried at room temperature, powdered and sifted with a sieve of 355 μm.

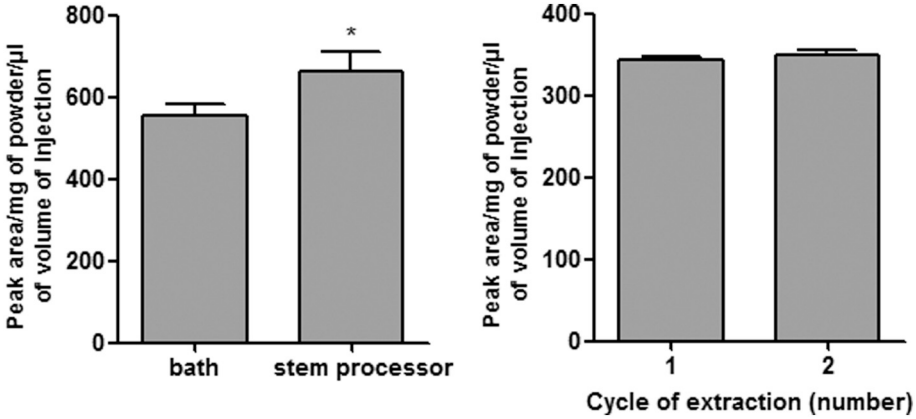


Fig. 2. 8TTE responses with ultrasonication in a bath or with an ultrasound stem processor and after 1 or 2 cycles of extraction (Mann Whitney test, n = 4, $p < 0.05$).

Table 1
Recovery percentages from plant samples.

Concentration levels (µg/mL)	Recovery % (n = 3, m = 4, k = 3)		
	Mean (%)	Sd (%)	CV (%)
9.57	97.88	0.88	0.90
14.68	101.59	3.66	3.60
31.34	99.67	2.33	2.34
73.37	100.26	3.70	3.69
Total	99.85	2.83	2.83

Table 2
Recovery percentages from plasma samples.

Concentration levels[a] (µg/mL)	Recovery % (n = 3, m = 4, k = 3)		
	Mean (%)	Sd (%)	CV (%)
15.90	73.35	7.94	10.82
26.50	75.99	4.37	5.76
53.01	76.84	6.49	8.45
106.01	77.33	9.75	12.61
Total	75.88	6.52	8.59
IS 100 µg/mL	70.75	2.68	3.79

[a] Spiked concentrations

2.2. UV/MS HPLC analyzes

Analyzes were performed on an Accela UHPLC system from Fisher Scientific (Thermo Fisher Scientific, Bremen, Germany) consisting in a photodiode array (PDA) detector, an autosampler equipped with a conventional sample tray compartment with its cooler (set to 4 °C) and an injection system with a sample loop of 50 µL, and a quaternary pump, all piloted by ChromQuest software. The column used was a Phenomenex Lichrospher C18 (2), 250 × 4.6 mm^2 packed with 5 µm particles. 25 µL of samples were injected in the full loop injection mode or no waste one for plant or plasma samples respectively by the autosampler. The column was eluted at constant flow rate of 1 mL/min using a binary solvent system: solvent A, MilliQ water 0.1% formic acid and solvent B, methanol HPLC grade (0–2 min: 60% A, 7–22 min: 15% A, 23–35 min: 60% A). Quantification analyzes were carried out at a wavelength of 310 nm corresponding to the maximum absorption of the triterpene esters and at 281 nm for internal standard (IS) maximum one.

The selectivity of the method and the purity of **8TTE** were evaluated on an LC–MS/MS consisting of the same system as above hyphenated with a Thermo Fisher Scientific LTQ orbitrap XL mass spectrometer from the UCLouvain Massmet platform. HR-MS were measured with ESI source in the positive mode using full-scan MS with a mass range of 100–2000 *m/z*. The orbitrap operated at 30,000 resolution (FWHM definition). All experimental data were acquired using daily external calibration prior to data acquisition. Appropriate tuning of the electrospray ion source was done and the following electrospray inlet conditions were applied: flow rate, 1000 µL/min with a split of 50/50 before reaching mass detector; spray voltage, 5 kV; sheath gas (N_2) flow rate, 20 a.u.; auxiliary gas (N_2) flow rate, 20 a.u.; capillary temperature, 275 °C; capillary voltage, 18 V; tube lens, 45 V. Data acquisition and processing were performed with Xcalibur software.

2.3. Standard solutions

Stock solutions of **8TTE** were prepared in methanol at 500 µg/mL and stored at −20 °C. At the experiment day, they were diluted in methanol to obtain adapted working solutions.

For plant studies, calibration standards were analyzed three times (n = 3) with three series of experiments (k = 3) at four concentration levels (m = 4) (100, 50, 15 and 10 µg/mL). Validation standards were prepared in a mixture of three samples of dichloromethane extracts of *K. venosa* that only contains very low amounts of **8TTE** (< LOQ) to represent a typical mean matrix, stored at 4 °C and spiked with the **8TTE** stock solution (concentration of about 500 µg/mL, considered as true value). They were analyzed three times (n = 3) for three series of experiment (k = 3) at four concentration levels (m = 4) (75, 32.5, 15 and 10 µg/mL).

For plasma studies, six working solutions were prepared at 120, 100, 75, 50, 15 and 10 µg/mL in methanol to be spiked in the blank biological matrix as recommended by EMA guidelines [10]. A suitable internal standard (IS), oestradiol valerate, was added during working solutions preparation from a stock solution of 1 mg/mL in MeOH to obtain a constant 100 µg/mL concentration. 100 µL of these working solutions at the six concentration levels (m = 6) were spiked in 200 µL human plasma, treated and analyzed in triplicate (n = 3) with 3 series of experiments (k = 3) giving the calibration standards. Validation standards were prepared in the same way with four working solutions concentration levels (100, 50, 25 and 15 µg/mL). These are a bit higher than those chosen for plant validation standards to take into account a possible loss during extraction from plasma. Three independent samples (n = 3) at each concentration (m = 4) were analyzed in duplicate for 3 series of experiment (k = 3). All these samples underwent sample preparation procedure described below, giving analyzed calibration solutions at 120, 100, 75, 50, 15 and 10 µg/mL and analyzed validation solutions at 100, 50, 25 and 15 µg/mL.

2.4. Extraction procedure

2.4.1. Ultrasonication with ultrasound stem processor

100 mg dried powdered material was extracted in 5 mL

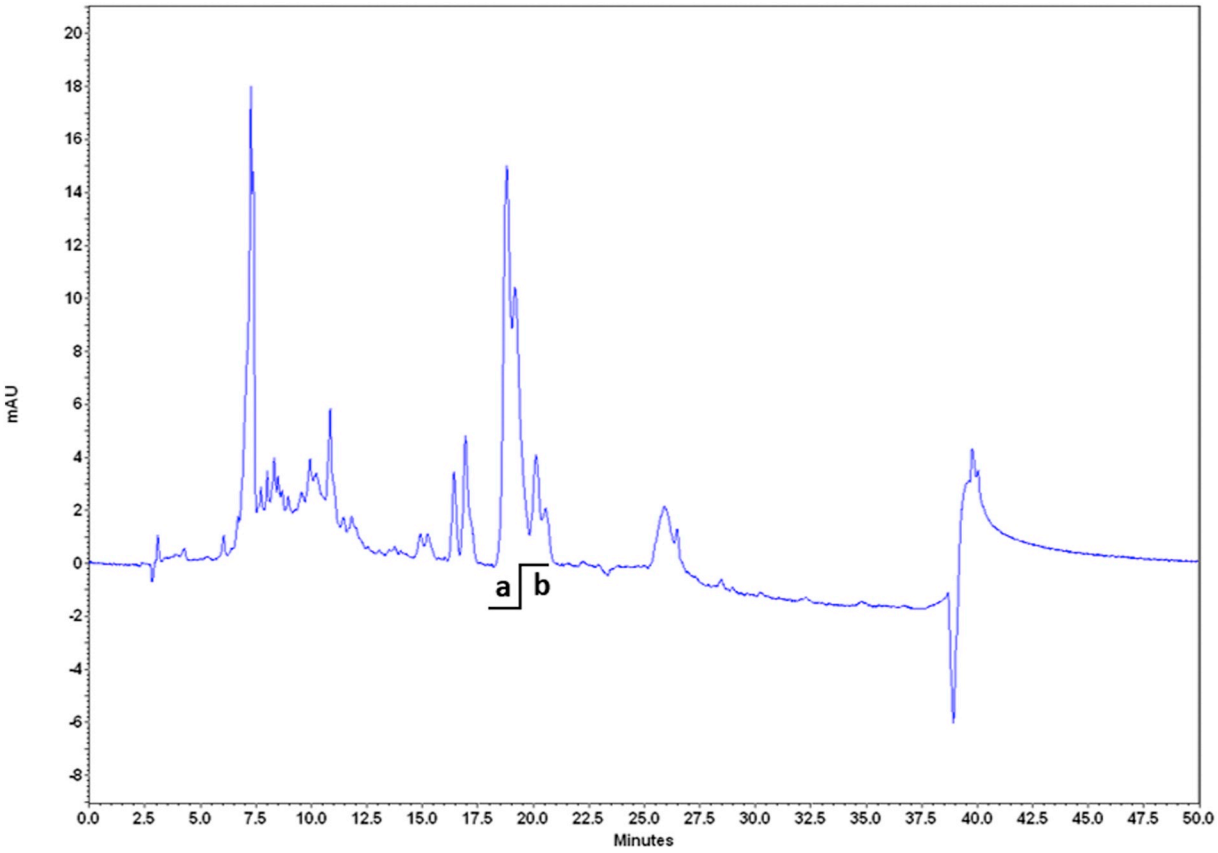


Fig. 3. Chromatogram of a *Keetia leucantha* extract with UV detection at 310 nm (a) triterpene esters **1–6** (b) triterpene esters **7–8**.

dichloromethane and one/two 5 min cycle(s) of sonication in ice with an ultrasound stem processor (Hielscher UP200S (200 W, 24 kHz, 85% of power, pulse each 0.5 s)). At the end of the extraction, the solution was centrifuged at 4000*g* during 10 min and the supernatant was recovered. The pellet was resuspended in 5 mL of dichloromethane and centrifuged again. Supernatants were pooled and evaporated to dryness under reduced pressure with a RapidVap (30 min at 40 °C, 100 mbar and 50% vortex speed).

2.4.2. Ultrasonication in a bath

10 mg dried powdered material was extracted four times with 1 mL of dichloromethane during 20 min in an ultrasound water bath (Omnilab 540S). Between each cycle of extraction, the suspension was centrifuged at 4000 *g* during 10 min, the supernatant was discarded and the pellet was resuspended in 1 mL of dichloromethane. Supernatants were pooled and evaporated to dryness under reduced pressure with a RapidVap (30 min at 40 °C, 100 mbar and 50% vortex speed).

The dried crude extracts obtained were resolubilized in 500 µL of methanol to compare the extraction procedures and in 1000 µL of methanol to compare the number of sonication cycles, filtered through 0.45 µm filter and injected into HPLC.

2.4.3. Protein precipitation

For plasma samples, 100 µL working solution or methanol, for validation or routine analyzes respectively, were spiked in 200 µL plasma. 1 mL organic solvent was added to samples that were submitted to 9184*g* centrifugation during 10 min at 4 °C (Hettich Mikro 200/200R, Sigma-Aldrich). The supernatants were then evaporated 24 h at room temperature and to dryness under gentle N_2 stream. The residues were dissolved in 100 µL methanol, vortex-mixed, sonicated 1 min on ice, vortex-mixed and filtered prior to UV-HPLC analysis. Analyzed solutions were so two times more concentrated than plasma samples.

2.5. Evaluation of the extraction procedure

To determine the extraction procedure for plant extracts, peak area of **8TTE** was determined after 1 or 2 cycles of ultrasound stem extraction and compared with the ultrasound bath one. Results are expressed in area of peak/mg of powder/µL of injection volume. Four independent experiments were realized. The recovery percentages were also calculated from calibration (in MeOH) and validation (in a plant matrix) standards (n = 3, m = 4, k = 3) to verify the completeness of the optimized extraction.

A preliminary extraction test was performed on plasma samples with four different organic solvents mixtures (acetonitrile, methanol, acetonitrile-methanol (2:1), ethyl acetate-hexane (4:1)) [11–14] and the recovery was determined by area comparison between blank plasma, spiked with a *Keetia leucantha* esters-enriched fraction and IS, and working solutions in methanol at the same concentration. Acetonitrile (100%) and acetonitrile-methanol (2:1) extractions were further repeated (n = 2, k = 3, mean ± Sd). The recovery percentages were then calculated with the chosen solvent for pure compounds in matrix compared to solutions in methanol (n = 3, m = 4, k = 3).

2.6. Validation of the method

We validated the method with three independent series of experiments. The same mobile phase was used all along one series. We analyzed the following criteria: response function, linearity, selectivity, precision, trueness, accuracy, limit of detection and limit of quantification, quantification range, stability and matrix effect.

The method selectivity was verified by HPLC-UV/MS (see Section 2.2) for quantification in crude extracts by checking mass spectra at the beginning, the middle and the end of peaks. For quantification in plasma, HPLC-UV chromatograms obtained after sample preparation

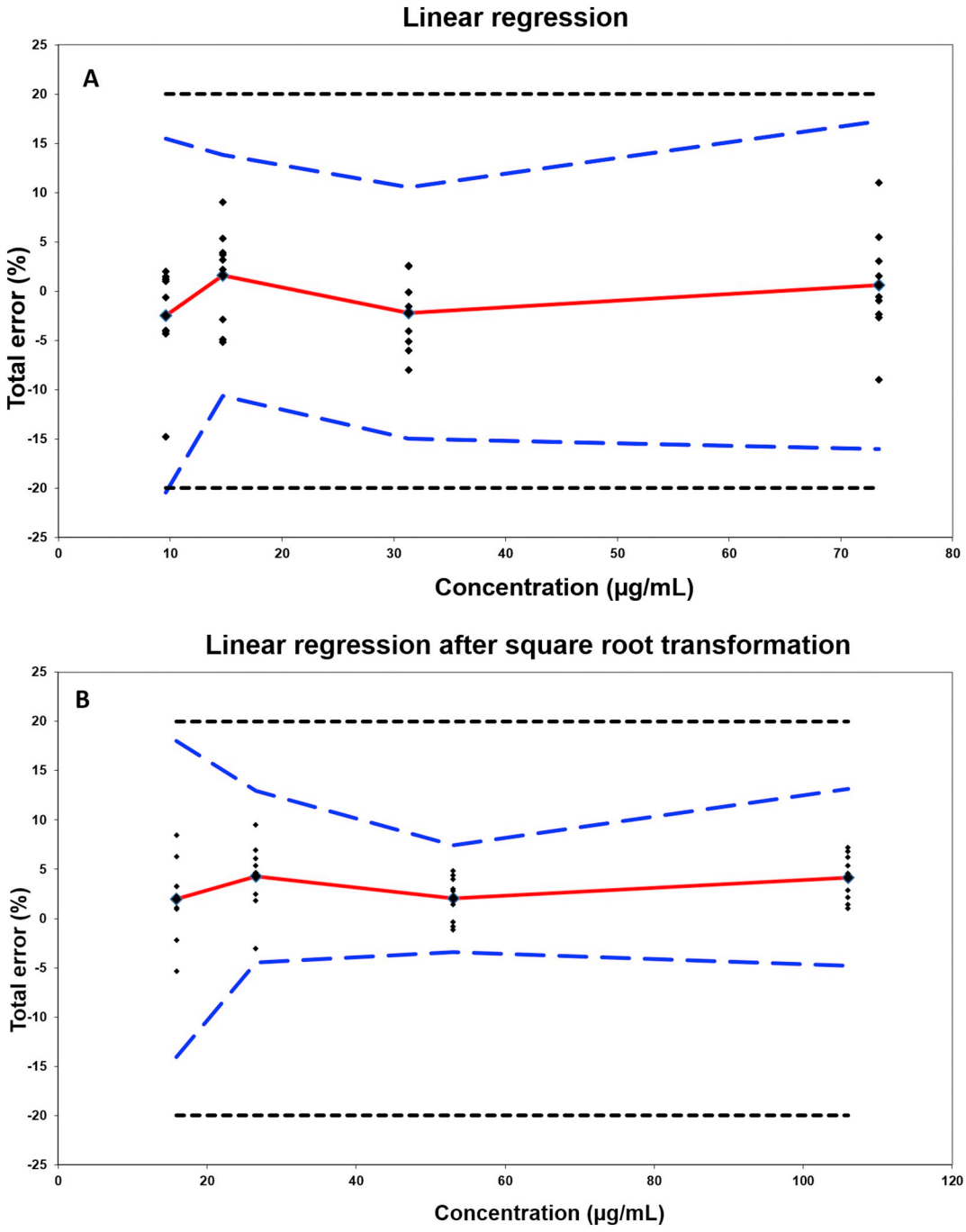


Fig. 4. Accuracy profile of the mixture of **8TTE** obtained with linear regression. The plain line is the relative bias, dashed lines are the β-expectation tolerance limits (β = 95%) and dotted lines represent the acceptance limits (± 20%). The dots represent the relative back-calculated concentrations of the validation standards and are plotted according to their target concentration. A: plant samples and B: plasma samples.

from blank plasma and spiked plasma were compared at the maximum absorption wavelength of **8TTE** (310 nm) and IS (281 nm) to confirm the lack of interference peak in treated blank plasma at interest compounds retention times. The **8TTE** stability was evaluated for the four validation standard concentrations (100, 50, 25 and 15 µg/mL) in three different storage conditions: in methanol (24 h at 4 °C, 24 h and 1 week at −20 °C), in methanol after plasma preparation procedure (24 h at 4 °C) and in plasma (2 weeks at −80 °C). The back-calculated concentrations using corresponding calibration curve and introduced concentrations were compared to assess samples stability (relative standard deviation and relative bias lower than 15%). The dilution effect was evaluated with the highest tested **8TTE** concentration (120 µg/mL) in two different dilution methods: spiked plasma samples at 120 µg/mL were diluted 5-fold and re-analyzed or at the last step of the preparation procedure, the pellet was directly dissolved in 500 µL instead of 100 µL. Once again, the precision and trueness were verified based on calculated relative standard deviation and relative bias. For plant samples, matrix effect was determined with another *Keetia* species extract containing very low esters amount. All plasma samples underwent preparation procedure and same matrix samples were compared to each other.

The validation of our bioanalytical method is in agreement with EMA guidelines defined by trueness and precision values lower than 15% and expressed with the relative bias (RB) and the relative standard

Table 3
Validation results obtained for the quantification method of **8TTE** mixture in *Keetia leucantha* twigs extracts and spiked plasma.

Validation criteria		Concentration levels (μg/mL) for plant samples				Concentration levels[a] (μg/mL) for plasma samples			
		9.57	14.68	31.34	73.37	15.90	26.50	53.01	106.01
Response function		Linear regression Calibration range (4 points) 10–75 μg/mL				Linear regression after square root transformation Calibration range (4 points) 15–100 μg/mL			
Trueness	Relative bias (%)	−2.46	1.59	−2.22	0.60	2.00	4.25	2.01	4.17
Precision	Repeatability (RSD%)	3.97	4.61	2.89	4.56	2.40	3.25	2.32	1.38
	Intermediate precision (RSD%)	5.83	4.87	4.15	5.90	4.47	3.46	2.26	2.51
Accuracy (95% relative limits in %)	β-expectation lower and upper tolerance	−20.43	−10.67	−14.99	−16.03	−14.04	−4.48	−3.45	−4.83
		15.50	13.86	10.54	17.24	18.04	12.97	7.46	13.16
Linearity	Slope	1.008				1.043			
	Intercept	−0.3216				−0.4543			
	R^2	0.9931				0.9983			

[a] Spiked concentrations.

Table 4
Uncertainty estimations of the **8TTE** mixture at each concentration level investigated during the method validation using the selected regression model. The expanded uncertainty was calculated with a coverage factor of 2.

Concentration level (μg/mL)	Uncertainty (μg/mL)	Expanded uncertainty (μg/mL)	Relative expanded uncertainty (%)
Plant sample method			
9.57	0.604	1.208	12.620
14.68	0.773	1.546	10.540
31.34	1.400	2.800	8.990
73.37	4.780	9.560	13.020
Plasma sample method			
15.90[a]	0.817	1.635	10.281
26.50[a]	1.019	2.039	7.693
53.01[a]	1.278	2.557	4.824
106.01[a]	3.121	6.242	5.888

[a] Spiked concentrations

deviation (RSD) respectively [10]. In addition, total error (sum of systematic and random error) was used as decision criteria for the validation process [15–21]. Statistical analyzes were performed using JMP v12 software. The acceptance limits (λ) were set at ± 20%, as usually accepted for complex samples [20,21]. The probability to obtain future results within these limits (β) was set at 95%. Regression parameters were calculated from calibration data using Microsoft excel and Graphpad Prism 5 software.

2.7. Applications

A sample of twigs (**KLT-1**) and leaves (**KLL-1**) of *K. leucantha* from the same collection place and date as well as one sample of twigs of *K. venosa* (**KVT-1**) were analyzed with the validated method for their **8TTE** amount. 26 other *Keetia leucantha* twigs samples from different regions, plant feet and collection date (see Section 2.1 and Table 1s) were also analyzed.

3. Results and discussions

3.1. Optimization of the extraction

We first tested two different extraction methods from plant samples: ultrasonication in a bath (4 × 20 min) or with a stem processor (1 × 5 min). The extraction yield was significantly superior with the ultrasound stem processor than in a bath and in addition more quick and easy to perform (Fig. 2) (Mann Whitney test, n = 4 $p < 0.05$). We also tested two extraction times: one or two 5 min cycle(s) to verify the completeness of extraction. No significant difference was observed in function of the number of cycles (Fig. 2) (Mann Whitney test, n = 4 $p > 0.05$). Consequently, one cycle of extraction with ultrasound stem processor was considered as sufficient for a complete esters extraction and used for all experiments. The recovery percentages, around 100%, also demonstrated the completeness of extraction (Table 1), allowing to work without internal standard, difficult to identify given the complexity of the crude plant extract.

The preliminary extraction test, performed on plasma samples spiked with a *Keetia leucantha* esters-enriched fraction and IS, showed higher recovery percentages (≥70%) with acetonitrile (100%) or acetonitrile-methanol (2:1) than with methanol (100%) or ethyl acetate-hexane (4:1)). So, the use of both solvent mixtures was further repeated and the recovery percentages of **8TTE** and IS were quite similar, with 73.8 ± 5.2 and 69.4 ± 7.7% for acetonitrile and 72.6 ± 11.5 and 62.4 ± 13.6% for acetonitrile-methanol (2:1). Acetonitrile was preferred for its lower variability. The recovery percentages calculated from pure compounds with or without matrix are presented in Table 2 with standard deviation (Sd) and coefficient of variation (CV). They are under 15% but we decided to use an internal standard for plasma analyzes to take into account and further minimize extraction variations and allow more reliable results.

3.2. Validation of the method

3.2.1. Selectivity

Selectivity and peak purity were analyzed by the comparison of retention times and mass spectra of peaks corresponding to the esters with those of the purified mixture of **8TTE** used as reference. Mass spectra were analyzed at six retention times corresponding to the beginning, the middle and the end of both groups of peaks (**a** and **b**) of **8TTE** with positive mode ESI-MS (mass spectra are available in Figs. 1s and 2s in supporting information). Molecular ion peaks at *m/z* 641.38 or 671.39 corresponding to $[M + Na]^+$ of coumaric or ferulic derivatives respectively and fragment spectra were similar to those of references. This indicates the selectivity of the method. As mentioned in the introduction, the method was developed to give a rapid and good separation of the mixture of **8TTE** from other compounds of the extract and did not aim at resolving triterpene esters from each other (Fig. 3). To confirm the method selectivity in plasma, treated blank plasma was also analyzed by HPLC-UV showing no interfering peak at the **8TTE** retention times (at 310 nm) neither at the IS one (at 281 nm). All chromatograms for selectivity determination are available as supporting information (Fig. 3s).

3.2.2. Response function

Method validation aimed to demonstrate quantification reliability in biological matrices. Calibration in methanol are routinely used, even

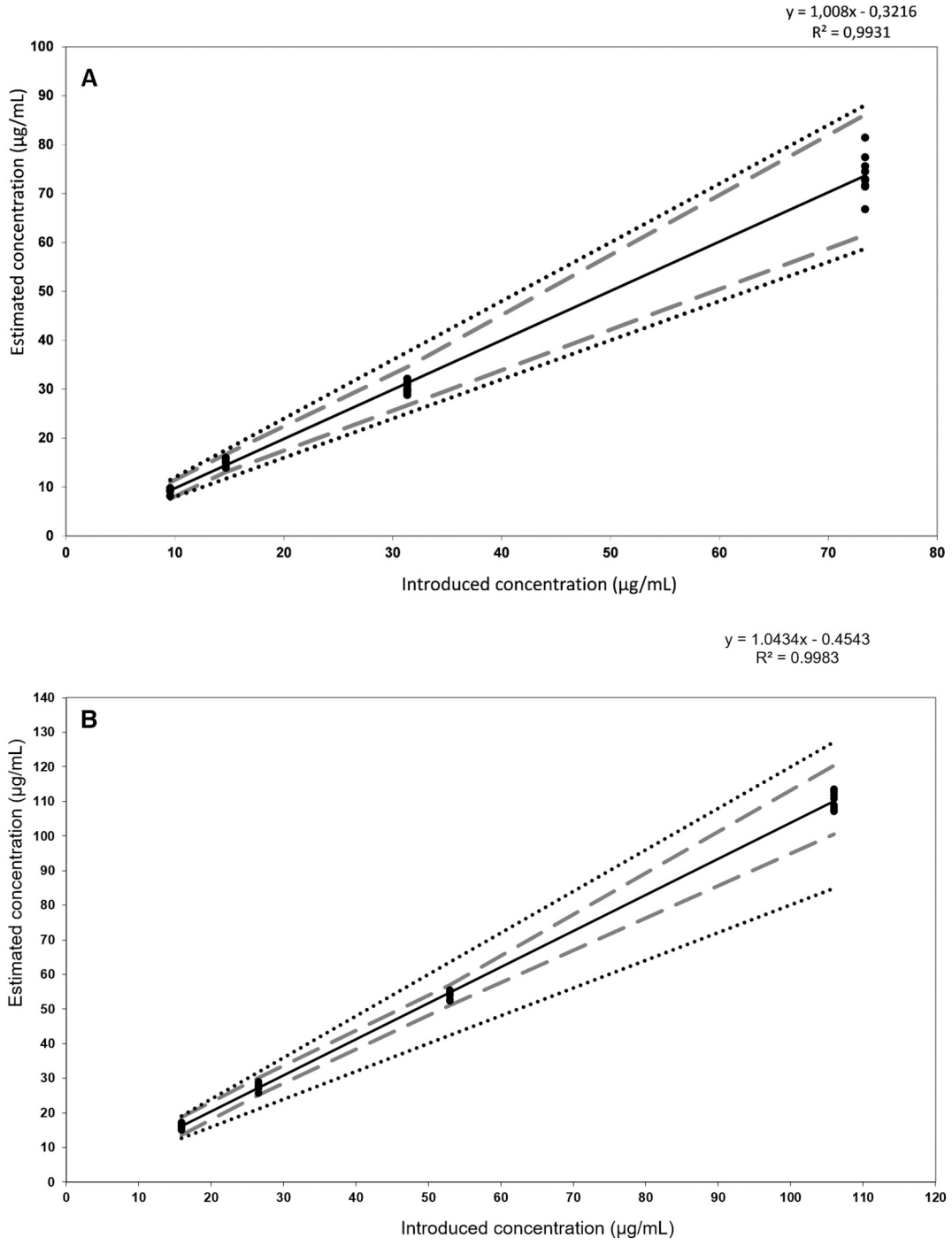


Fig. 5. Linear profiles of the **8TTE** mixture for plant (A) and plasma (B) samples respectively. The plain line is identity line (y = x), the dashed line are the β-expectation tolerance limits (β = 95%) and dotted lines represent the acceptance limits (± 20%).

for plant matrices [20,21] but it is recommended to use matrix calibration for biological matrices as plasma [10,23]. We tested different regression models from calibration standards and determined the respective accuracy profiles to choose the most suitable one [24]. For plant samples, linear regression was selected as the most adequate one with 95% expectation tolerance intervals included inside the ± 20% acceptance limits for each concentration level of the validation standards except the lowest one. For plasma samples, a square root transformation was performed to obtain the best calibration curve model (Fig. 4). This total error concept allows to simplify decision making and reduce risks associated to procedure use [16].

3.2.3. Trueness, precision and accuracy

Trueness [15,25] was calculated at each concentration level of the validation standards and expressed in relative bias (RB). Relative bias was < 3 and 5% for plant and plasma samples respectively showing the excellent trueness of the method (Table 3).

Precision was evaluated intra-day (repeatability) and inter-day (intermediate precision) and expressed as relative standard deviations (RSD) [17,26]. The repeatability was < 4.61 and 3.25% and the intermediate precision 5.90 and 4.47% for plant and plasma samples respectively (Table 3). All the trueness and precision results are in accordance with EMA guidelines criteria (≤15%) [10].

Accuracy profiles, evaluating the sum of systematic and random

Table 5
8TTE content in *Keetia* species samples analyzed by the validated method (n = 3, mean ± Sd) in µg/100 mg of plant.

Sample	**8TTE** (µg/100 mg)
KLT-1	36.87 ± 1.72
KLL-1	LOD < 5.62 ± 1.52 < LOQ
KVT-1	LOD < 1.43 ± 0.35 < LOQ

errors of the test values (total error) [15,17,24–26], are shown in Fig. 4, indicating that the relative upper and lower 95% β-expectation tolerance limits are inside the acceptance limits, set at ± 20% except for the lowest concentration in plant samples. The method can thus be considered as accurate between 14.68 and 73.37 µg/mL for esters quantification in *Keetia* samples and between 15.90 and 106.01 µg/mL for plasma samples analyzed solution corresponding to 7.95 and 53.01 µg/mL in plasma. The accuracy results are presented in Table 3.

3.2.4. Detection and quantification limits

The limit of detection (LOD) is estimated as 1 and 3.2 µg/mL (plant and plasma matrices respectively) by the signal/noise method from the European Pharmacopeia [27]. The limit of quantification was determined as the smallest tested concentration of the 95% β-expectation tolerance limits remaining inside the ± 20% acceptance limits. It was then set at 14.68 and 15.90 µg/mL for plant extract and plasma analyzes respectively. Considering the two-fold concentration of plasma sample during treatment procedure, the method allows a precise quantification from 7.95 µg/mL and detection from 1.6 µg/mL in plasma.

3.2.5. Uncertainty measurement

To characterize the dispersion of the values that could reasonably be attributed to the measurer during routine analysis [21,28], we evaluated the uncertainty of measurement. The expanded uncertainty is calculated by applying a coverage factor of k = 2 (corresponding to a 95% confidence interval around the results where the measure may lie). The maximum relative expanded uncertainty of the mixture of **8TTE** is 13%, inside the ± 20% acceptance limits and all estimated values for each validation standard concentration level are summarized in Table 4.

3.2.6. Linearity

To determine the linearity, which described the relationship between introduced and calculated concentrations, we evaluated the fitness of a regression line between back-calculated concentrations of the validation standards and exact concentrations [18,19]. The estimated concentrations were plotted as a function of the introduced concentrations and the built regression line was compared to the identity line y = x. For both samples kinds, we obtained a slope value close to 1, demonstrating the linearity of the model. Furthermore, the absolute 95% β-expectation tolerance limits are within the absolute acceptance limits, still set at ± 20%, confirming the good linearity of the method (Fig. 5).

3.2.7. Stability, dilution and matrix effects

The **8TTE** stability was verified for methanol stock solution. The trueness and precision for the four tested validation standards concentrations were verified for three different storage conditions and were ≤15%, the criterion value accepted by EMA with a maximum of 20% for the lowest concentration [10].Their stability after plasma sample treatment and in plasma storage condition was also confirmed by the same way as well as the absence of a dilution effect. The results of trueness and precision for the four tested validation standards concentrations are available as supporting information (Tables 2–4s).

Calibration standards with **8TTE** were prepared without matrix (m = 4, n = 3) for plant analytical procedure. To analyze the plant matrix effect, we used a dichloromethane extract of another related

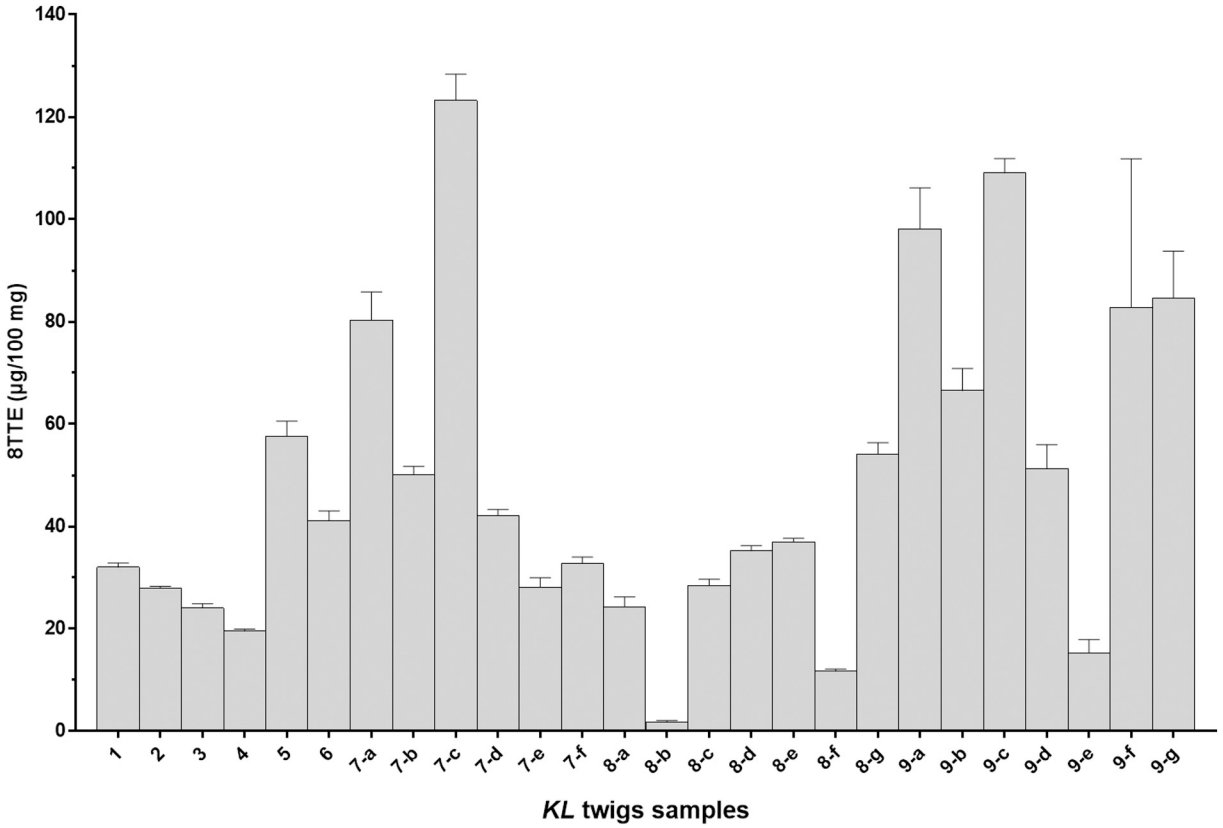


Fig. 6. 8TTE content in several *Keetia leucantha* twigs samples (in µg/100 mg plant).

Keetia species, *Keetia venosa* which contains low quantities of triterpene esters, and added known quantities of triterpene esters. The signal from triterpene esters initially present in this extract was subtracted to triterpene esters peaks obtained with these final solutions. The influence of the matrix was evaluated by comparing the slope and the intercept of the linear regression obtained with and without matrix. No significant difference was observed for the intercept ($p = 0.5127$) nor for the slopes ($p = 0.8273$) (Wilcoxon test, $p > 0.05$). For plasma quantification, the treatment procedure implemented to all samples allowed to overcome the systematic determination of recovery percentage and matrix effect.

3.3. Application to samples of Keetia species

Extracts from *K. leucantha* twigs (**KLT-1**), leaves (**KLL-1**) and *K. venosa* twigs (**KVT-1**) were analyzed with the developed validated method and compared in Table 5.

Esters are mostly present in twigs of *K. leucantha* species with > 5 and 25 fold lower quantities in leaves of *K. leucantha* and twigs of *K. venosa* species respectively for the tested samples. To evaluate their amount variability, several *Keetia leucantha* twigs samples collected in different places and at different date (Table 1s) were analyzed.

Quantification results (Fig. 6) showed a high variability in esters content from different collection place samples but also from different batch collected at the same time in the same area. Our results also showed that older samples (*i.e.* 1–5) may contain higher esters amount than more recent ones (*i.e.* 8b, 8f, 9e), indicating that degradation during conservation time of dried samples, if any, has a lower effect on **8TTE** content than the tree age, shape and sunshine cover. This should be taken into account to investigate the high variation in twigs esters amount.

4. Conclusions

The extraction and quantification methods developed in this work were found to be selective, linear, accurate, true and precise from 14.68 to 73.37 µg/ml and 15.90 to 106.01 µg/mL for plant and plasma samples injected solutions respectively corresponding to 7.95 and 53.1 µg/mL in plasma. The developed methods allowed the detection and quantification of the 8 bioactive triterpenic esters from *Keetia leucantha* twigs in mixture and brought up useful tools for plasma samples analyzes. In one hand, it opens the way to screen and discover samples with higher quantity of these potential antimalarial compounds. In another hand, it will allow their pharmacokinetics investigation.

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Conflict of interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jchromb.2018.11.003.

References

[1] WHO, World Malaria Report, http://www.who.int/malaria/publications/world-malaria-report-2017/report/en/, (2017) , Accessed date: 30 January 2018.
[2] A. Olotu, G. Fegan, J. Wambua, G. Nyangweso, A. Leach, M. Lievens, D.C. Kaslow, P. Njuguna, K. Marsh, P. Bejon, Seven-year efficacy of RTS, S/AS01 malaria vaccine among young African children, N. Engl. J. Med. 374 (2016) 2519–2529, https://doi.org/10.1056/NEJMoa1515257.
[3] E.A. Ashley, M. Dhorda, R.M. Fairhurst, C. Amaratunga, P. Lim, S. Suon, S. Sreng, J.M. Anderson, S. Mao, B. Sam, C. Sopha, C.M. Chuor, C. Nguon, S. Sovannaroth, S. Pukrittayakamee, P. Jittamala, K. Chotivanich, K. Chutasmit, C. Suchatsoonthorn, R. Runcharoen, T.T. Hien, N.T. Thuy-Nhien, N.V. Thanh, N.H. Phu, Y. Htut, K.-T. Han, K.H. Aye, O.A. Mokuolu, R.R. Olaosebikan, O.O. Folaranmi, M. Mayxay, M. Khanthavong, B. Hongvanthong, P.N. Newton, M.A. Onyamboko, C.I. Fanello, A.K. Tshefu, N. Mishra, N. Valecha, A.P. Phyo, F. Nosten, P. Yi, R. Tripura, S. Borrmann, M. Bashraheil, J. Peshu, M.A. Faiz, A. Ghose, M.A. Hossain, R. Samad, M.R. Rahman, M.M. Hasan, A. Islam, O. Miotto, R. Amato, B. MacInnis, J. Stalker, D.P. Kwiatkowski, Z. Bozdech, A. Jeeyapant, P.Y. Cheah, T. Sakulthaew, J. Chalk, B. Intharabut, K. Silamut, S.J. Lee, B. Vihokhern, C. Kunasol, M. Imwong, J. Tarning, W.J. Taylor, S. Yeung, C.J. Woodrow, J.A. Flegg, D. Das, J. Smith, M. Venkatesan, C.V. Plowe, K. Stepniewska, P.J. Guerin, A.M. Dondorp, N.P. Day, N.J. White, Tracking Resistance to Artemisinin Collaboration (TRAC), Spread of artemisinin resistance in Plasmodium falciparum malaria, N. Engl. J. Med. 371 (2014) 411–423, https://doi.org/10.1056/NEJMoa1314981.
[4] C.-Y. Ma, S.F. Musoke, G.T. Tan, K. Sydara, S. Bouamanivong, B. Southavong, D.D. Soejarto, H.H.S. Fong, H.-J. Zhang, Study of antimalarial activity of chemical constituents from *Diospyros quaesita*, Chem. Biodivers. 5 (2008) 2442–2448, https://doi.org/10.1002/cbdv.200890209.
[5] S. Suksamrarn, P. Panseeta, S. Kunchanawatta, T. Distaporn, S. Ruktasing, A. Suksamrarn, Ceanothane- and lupane-type triterpenes with antiplasmodial and antimycobacterial activities from *Ziziphus cambodiana*, Chem. Pharm. Bull. (Tokyo). 54 (2006) 535–537.
[6] C. Beaufay, M.-F. Hérent, J. Quetin-Leclercq, J. Bero, In vivo anti-malarial activity and toxicity studies of triterpenic esters isolated form *Keetia leucantha* and crude extracts, Malar. J. 16 (2017) 406, , https://doi.org/10.1186/s12936-017-2054-y.
[7] W. Lu, Y. Niu, H. Yang, Y. Sheng, H. Shi, L.L. Yu, Simultaneous HPLC quantification of five major triterpene alcohol and sterol ferulates in rice bran oil using a single reference standard, Food Chem. 148 (2014) 329–334, https://doi.org/10.1016/j.foodchem.2013.10.027.
[8] J.-J. Hou, W.-Y. Wu, J. Da, S. Yao, H.-L. Long, Z. Yang, L.-Y. Cai, M. Yang, X. Liu, B.-H. Jiang, D.-A. Guo, Ruggedness and robustness of conversion factors in method of simultaneous determination of multi-components with single reference standard, J. Chromatogr. A 1218 (2011) 5618–5627, https://doi.org/10.1016/j.chroma.2011.06.058.
[9] X.-Y. Gao, Y. Jiang, J. Lu, P.-F. Tu, One single standard substance for the determination of multiple anthraquinone derivatives in rhubarb using high-performance liquid chromatography-diode array detection, J. Chromatogr. A 1216 (2009) 2118–2123, https://doi.org/10.1016/j.chroma.2008.11.104.
[10] European Medicines Agency, Guideline on Bioanalytical Method Validation, EMEACHMPEWP1922172009 Rev 1 Corr 2, http://www.ema.europa.eu/ema/index.jsp?curl=pages/includes/document/document_detail.jsp?webContentId=WC500109686%26mid=WC0b01ac058009a3dc, (2011) , Accessed date: 1 May 2018.
[11] E.-H. Joh, D.-H. Kim, A sensitive liquid chromatography-electrospray tandem mass spectrometric method for lancemaside A and its metabolites in plasma and a pharmacokinetic study in mice, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 878 (2010) 1875–1880, https://doi.org/10.1016/j.jchromb.2010.05.003.
[12] Z.-H. Li, H. Zhu, X.-P. Cai, D.-D. He, J.-L. Hua, J.-M. Ju, H. Lv, L. Ma, W.-L. Li, Simultaneous determination of five triterpene acids in rat plasma by liquid chromatography-mass spectrometry and its application in pharmacokinetic study after oral administration of Folium Eriobotryae effective fraction, Biomed. Chromatogr. BMC. 29 (2015) 1791–1797, https://doi.org/10.1002/bmc.3497.
[13] L. Zhao, W. Li, Y. Li, H. Xu, L. Lv, X. Wang, Y. Chai, G. Zhang, Simultaneous determination of oleanolic and ursolic acids in rat plasma by HPLC-MS: application to a pharmacokinetic study after oral administration of different combinations of QingGanSanJie decoction extracts, J. Chromatogr. Sci. 53 (2015) 1185–1192, https://doi.org/10.1093/chromsci/bmu217.
[14] Z. Yu, W. Sun, W. Peng, R. Yu, G. Li, T. Jiang, Pharmacokinetics in vitro and in vivo of two novel prodrugs of oleanolic acid in rats and its hepatoprotective effects against liver injury induced by CCl4, Mol. Pharm. 13 (2016) 1699–1710, https://doi.org/10.1021/acs.molpharmaceut.6b00129.
[15] P. Hubert, J.-J. Nguyen-Huu, B. Boulanger, E. Chapuzet, P. Chiap, N. Cohen, P.-

A. Compagnon, W. Dewé, M. Feinberg, M. Lallier, M. Laurentie, N. Mercier, G. Muzard, C. Nivet, L. Valat, Harmonization of strategies for the validation of quantitative analytical procedures. A SFSTP proposal–part I, J. Pharm. Biomed. Anal. 36 (2004) 579–586, https://doi.org/10.1016/j.jpba.2004.07.027.

[16] P. Hubert, J.-J. Nguyen-Huu, B. Boulanger, E. Chapuzet, P. Chiap, N. Cohen, P.-A. Compagnon, W. Dewé, M. Feinberg, M. Lallier, M. Laurentie, N. Mercier, G. Muzard, C. Nivet, L. Valat, E. Rozet, Harmonization of strategies for the validation of quantitative analytical procedures. A SFSTP proposal–part II, J. Pharm. Biomed. Anal. 45 (2007) 70–81, https://doi.org/10.1016/j.jpba.2007.06.013.

[17] P. Hubert, J.-J. Nguyen-Huu, B. Boulanger, E. Chapuzet, N. Cohen, P.-A. Compagnon, W. Dewé, M. Feinberg, M. Laurentie, N. Mercier, G. Muzard, L. Valat, E. Rozet, Harmonization of strategies for the validation of quantitative analytical procedures. A SFSTP proposal–part III, J. Pharm. Biomed. Anal. 45 (2007) 82–96, https://doi.org/10.1016/j.jpba.2007.06.032.

[18] P. Hubert, J.-J. Nguyen-Huu, B. Boulanger, E. Chapuzet, N. Cohen, P.-A. Compagnon, W. Dewé, M. Feinberg, M. Laurentie, N. Mercier, G. Muzard, L. Valat, E. Rozet, Harmonization of strategies for the validation of quantitative analytical procedures: a SFSTP proposal part IV. Examples of application, J. Pharm. Biomed. Anal. 48 (2008) 760–771, https://doi.org/10.1016/j.jpba.2008.07.018.

[19] E. Rozet, R.D. Marini, E. Ziemons, B. Boulanger, P. Hubert, Advances in validation, risk and uncertainty assessment of bioanalytical methods, J. Pharm. Biomed. Anal. 55 (2011) 848–858, https://doi.org/10.1016/j.jpba.2010.12.018.

[20] M.H. Rafamantanana, E. Rozet, G.E. Raoelison, K. Cheuk, S.U. Ratsimamanga, P. Hubert, J. Quetin-Leclercq, An improved HPLC-UV method for the simultaneous quantification of triterpenic glycosides and aglycones in leaves of *Centella asiatica* (L.) Urb (Apiaceae), J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 877 (2009) 2396–2402, https://doi.org/10.1016/j.jchromb.2009.03.018.

[21] E. Lautié, E. Rozet, P. Hubert, J. Quetin Leclercq, Quantification of rotenone in seeds of different species of yam bean (*Pachyrhizus* sp.) by a SPE HPLC–UV method, Food Chem. 131 (2012) 1531–1538, https://doi.org/10.1016/j.foodchem.2011.09.125.

[22] J. Bero, H. Ganfon, M.-C. Jonville, M. Frédérich, F. Gbaguidi, P. Demol, M. Moudachirou, J. Quetin-Leclercq, In vitro antiplasmodial activity of plants used in Benin in traditional medicine to treat malaria, J. Ethnopharmacol. 122 (2009) 439–444, https://doi.org/10.1016/j.jep.2009.02.004.

[23] J.B. Lee, A. Zgair, T.H. Kim, M.G. Kim, S.D. Yoo, P.M. Fischer, P. Gershkovich, Simple and sensitive HPLC-UV method for determination of bexarotene in rat plasma, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 1040 (2017) 73–80, https://doi.org/10.1016/j.jchromb.2016.11.024.

[24] E. Rozet, C. Hubert, A. Ceccato, W. Dewé, E. Ziemons, F. Moonen, K. Michail, R. Wintersteiger, B. Streel, B. Boulanger, P. Hubert, Using tolerance intervals in pre-study validation of analytical methods to predict in-study results. The fit-for-future-purpose concept, J. Chromatogr. A 1158 (2007) 126–137, https://doi.org/10.1016/j.chroma.2007.03.102.

[25] E. Rozet, A. Ceccato, C. Hubert, E. Ziemons, R. Oprean, S. Rudaz, B. Boulanger, P. Hubert, Analysis of recent pharmaceutical regulatory documents on analytical method validation, J. Chromatogr. A 1158 (2007) 111–125, https://doi.org/10.1016/j.chroma.2007.03.111.

[26] I.H.T. Guideline, Validation of analytical procedures: text and methodology Q2 (R1), Int. Conf. Harmon. Geneva Switz, 2005, pp. 11–12.

[27] European Directorate for the Quality of Medicines and Health Care, Council of Europe, European Pharmacopoeia, 6th edition, Strasbourg, 2011.

[28] M. Feinberg, B. Boulanger, W. Dewé, P. Hubert, New advances in method validation and measurement uncertainty aimed at improving the quality of chemical data, Anal. Bioanal. Chem. 380 (2004) 502–514, https://doi.org/10.1007/s00216-004-2791-y.