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***Copaifera langsdorffii* supercritical fluid extraction: chemical and functional characterization by LC/MS and in vitro assays.**

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ABSTRACT

30 *Copaifera langsdorffii* is a native Brazilian plant containing important amounts of polyphenols and diterpenes with biological activity. To obtain extracts enriched in these two types of compounds, supercritical fluid extraction (SFE) conditions have been optimized by using a Response Surface Methodology (RSM) and considering as a factors: extraction pressure and temperature and percentage of co-solvent (ethanol). The response variables selected were the

35 concentration of the two most representative compounds in *C. langsdorffii*, kaurenoic acid (diterpenic acid) and quercitrin (glycosylated flavonol), total flavonoids content, and antioxidant activity (measured by DPPH and TEAC assays). According to the statistical analysis of the experimental design, extraction temperature and ethanol percentage were the main factors influencing the selectivity towards the extraction of the target compounds. Two optimized

40 extracts were obtained: 1) containing high amount of total flavonoids, high concentration of quercitrin and low EC₅₀ for the DPPH assay, named HQE- High Quercitrin Extract (100 bar, 70°C and 50% of ethanol, in the region of gas-expanded liquids, GXLs) and 2) containing high value of kaurenoic acid extract, named HKAE-High Kaurenoic Acid Extract, (390 bar, 70°C and pure CO₂). Chemical characterization by LC/MS of HQE showed the presence of galloylquinic

45 acids and flavonoids while, on the other hand, HKAE presented only nonpolar compounds such as kaurenoic acid. Moreover, a solid-liquid extraction (SLE) with methanol was carried out as a benchmark protocol for comparative purposes. SFE presented approximately 10-fold higher content of total flavonoids than the extracts obtained by SLE.

50 Keywords: *Copaifera langsdorffii*, supercritical fluid extraction, response surface methodology, flavonoids, kaurenoic acid, quercitrin, antioxidant activity

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1 Introduction

The *Copaifera sp* genus belongs to the botanical family of *Leguminales Juss.*, sub-family *Caesalpinioideae Kunth.*, but it can be also found in the literature by its previous classification:

55 Fabaceae Lindley. *Copaifera* is a native Brazilian plant known popularly as *C. langsdorffii*; only in Brazil more than 20 species from the *Copaifera sp* genus have been described, all of them with similar therapeutic claims [1, 2]. Several scientific studies on *Copaifera sp* reported important pharmacological activities, among them: anti-inflammatory, analgesic, antimicrobial, antitumor, antioxidant, wound healing, gastro protective, anti-helminthic, leishmanicide, 60 trypanomicide and muscle relaxant [1-6].

Among the different species of *Copaifera*, *C. langsdorffii* has been suggested as a promising natural source of products for the pharmaceutical market. The oral use of *C. langsdorffii* leaves was reported to be effective in relieving pain and eliminating renal calculi in nephrolithiasic patients [7]. The *in vitro* disruption of oxalate crystals and the *in vivo* assay using ethylene 65 glycol-induced nephrolithiasis in rats was reported by Oliveira and co-workers employing *C. langsdorffii* leaves' extracts [8]. An *in vivo* study reported a decrease of the number and weight in calcium oxalate calculi previously introduced in the bladder of rats treated with hydroethanolic extract of *C. langsdorffii* leaves [9].

Phytochemical investigations on *C. langsdorffii* leaves reported 6 to 10% of phenolic 70 compounds, 3% of the amino acid N-methyl-trans-4-hydroxy-L-proline and sesquiterpenes as α -cubebene, α -copaene, ciperene, β -copaene, caryophyllene, β -humulene, muurolene, β -selinene, δ -cadinene and γ -cadinene [10-12]. Furthermore, recent works reported the isolation and identification of kaurenoic acid, caryophyllene oxide, kaurenol, hydroxy-labdaenoic acid, quercitrin and afzelin [13]. Quercitrin (quercetin-3-O-rhamnoside) is a glycosylated flavonol 75 with a molecular weight of 448.38 g/mol, practically insoluble in cold water, moderately soluble

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in hot water and freely soluble in ethanol [14]; several activities have been described for

quercitrin, such as antioxidant [15, 16], anti-inflammatory and anti-complement system [17, 18], enzymatic inhibition of HMG-CoA reductase and angiotensine-I converser [19], tyrosinase [20], cyclooxygenase-2 [15], myeloperoxidase [21] and inhibition of substances such as nitric oxide
80 [15]. Moreover, in a recent study [22] galloylquinic acids have been identified as compounds present in *C. langsdorffii* leaves. It is well know that galloylquinic acids are very good antioxidant compounds since they can present from one to four molecules of gallic acid linked to a molecule of quinic acid.

On the other hand, diterpene acids, such as kaurenoic acid (also known as ent-kaur-16-en-18-oic
85 acid), are also an important part of *C. langsdorffii* chemical compounds. Kaurenoic acid (**Figure 1**) presents a molecular weight of 302.45 g/mol and a pKa value of 4.70 ± 0.40 , being soluble in hexane and ethyl acetate [23]; diterpenes from kaurane series present many biological effects such as antimicrobial, antifungal [24], anti-inflammatory [25], vasodilatory [26], wound healing [27] and anti-psoriasis [28]. Besides, some previous studies on the oil, extracts, fractions and
90 isolated substances, such as kaurenoic acid, from the *Copaifera* sp genus reported antitumor activity [29-35].

Extraction protocols for plant antioxidant phenolic compounds are very diverse due to the variation and complexity of phenolic substances and to the variety of vegetable matrices. The extraction yield, phenolic content and antioxidant activity of the extracts are strongly dependent
95 not only on the specific plant material and its bioactive components, but also on the solvent and extraction method employed [36]. There are several extraction processes described in the literature, ranging from more traditional such as maceration, percolation and Soxhlet extraction [37] to modern extraction methods such as those based on the use of compressed fluids (including supercritical fluids (SCF), gas-expanded liquids (GXLs), pressurized liquids and

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subcritical water extraction) [38]. Supercritical fluid extraction (SFE) offers considerable advantages compared to traditional extraction processes because it provides higher selectivity, shorter extraction times, higher efficiency and do not use toxic organic solvents avoiding the environment and sample contamination [39, 40]. SFE has been largely applied to extract different chemical constituents from plants including low [41] and high to medium polarity compounds (such as phenolics) [39, 40, 42]. Furthermore, to our knowledge this extraction process has never been used to evaluate the compounds' extraction profile from *Copaifera* sp leaves. Moreover, since the *Copaifera* genus presents polyphenols and diterpene compounds, both with important biological activities, the goal of the present work has been the study and optimization, via experimental design, of the supercritical fluid extraction of two representative compounds of these groups: kaurenoic acid and quercitrin, from the *C. langsdorffii* leaves.

2 Materials and methods

2.1 Materials

The leaves of the *Copaifera langsdorffii* Desf., Fabaceae, tree were collected at the Ribeirão Preto Campus of the University of São Paulo, SP, Brazil in July, 2012 and a voucher specimen (SPFR 10120) was deposited in the herbarium of the Faculdade de Filosofia, Ciência e Letras de Ribeirão Preto, University of São Paulo. The herbal material was cleaned and dried in ventilated atmosphere of $30\pm5^{\circ}\text{C}$ temperature, milled in a cutting mill to obtain particles between 0.5 to 1.0 mm and stored in a closed plastic bag in fresh and dried environment at 25°C until its use.

The kaurenoic acid standard was kindly given by the Laboratório de Farmacognosia from Faculdade de Ciências Farmacêuticas de Ribeirão Preto and the quercitrin standard was acquired from Sigma Aldrich (Madrid, Spain).

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Acetonitrile (LC-MS quality, LabScan, Gliwice, Poland), water (purified using a Milli-Q system,

Millipore Corporation, Billerica, USA) and acidified water with phosphoric acid (Sigma-Aldrich, Madrid, Spain) were used as mobile phases for HPLC analysis.

125 The solvents used in the supercritical extraction process were CO₂ premier quality provided by Carbueros Metálicos (Air Products Group, Madrid, Spain) and ethanol absolute 99% provided by Panreac Quimica S.A. (Barcelona, Spain). Methanol was purchased from Sigma-Aldrich (Madrid, Spain).

2,2-Diphenil-1-pyrcil hydrazyl hydrate (DPPH, 99%), gallic acid, 6-hydroxy-2,5,7,8-
130 tetramethylchroman-2-carboxylic acid (Trolox, ≥ 97%), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS, ≥ 99%) and AlCl₃ anhydrous were purchased from Sigma-Aldrich (Madrid, Spain).

2.2 Solid-liquid extraction

A solid-liquid extraction (SLE) was performed as a reference extraction in order to compare the
135 results of yield, total flavonoids, kaurenoic acid and quercitrin concentration and antioxidant activities with the extracts obtained by SFE. For that, 1 g of the dried milled leaves of *C. langsdorffii* was weighed in a flask and added of 50 mL of methanol. The extraction was kept for 24 h at room temperature under shaking of 150 rpm. After this time, the extracts were filtered and dried under vacuum.

140 2.3 Supercritical fluid extraction

Supercritical fluid extractions were performed in a homemade supercritical fluid extractor using pumps for CO₂ and ethanol PU2080 from Jasco (Tokyo, Japan) and a manual back pressure regulator LF-540 from Pressure Tech (Hadfield, United Kingdom). Three factors were considered for the optimization of the SFE process: pressure (100-400 bar), temperature (40-
145 70°C) and the amount of ethanol (0-50%) that was used as modifier. Optimization was

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performed using a Box-Behnken experimental design. Experiments were carried out in randomized order. A kinetic study of the extraction process at the central conditions of the experimental design was carried out to select the extraction time that was set at 120 min. The other factors were maintained constant: carbon dioxide flow rate, 1 mL/min (measured at 50 bar) and mass of plant, 1g.

Once obtained the optimized conditions according to the results of the Box-Behnken design and the statistical analysis, the scale up of the extraction conditions to obtain the extract enriched in kaurenoic acid was performed in a Helix Spe-ed SFE equipment (Applied Separations, Inc, Allentown, PA, USA) which was loaded with 50 g of *C. langsdorffii* dried and milled leaves. The other conditions as pressure, temperature and modifier were selected as provided by the experimental design.

2.4 Physico-chemical extract characterization

2.4.1 Yield (θ)

To obtain the soluble solids (SS) extracted in the experiments, a thermogravimetric method was used. For that, the liquid extracts were dried in an evaporator under vacuum and, after full dryness, they were weighted. These value were compared to the total mass of plant used (M_p) to obtain the yield [43].

$$\theta = \frac{SS \times 100}{M_p} \quad (1)$$

2.4.2 Total flavonoid quantification (TF)

The method of complexation with aluminum chloride (55.23 mg of $AlCl_3$ anhydrous in 5 mL followed by a dilution of 10 fold) in methanol was applied to quantify the total flavonoid content using quercetin as standard (4, 6, 8, 10, 12 and 14 $\mu\text{g/mL}$). The samples were diluted in methanol to obtain a concentration of total flavonoid of approximately 8 $\mu\text{g/mL}$ or 0.5 mg/mL of dried

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extract (330 fold dilution). A final concentration of 1.6 mM of aluminum chloride in the reaction

media was obtained. The samples were analyzed at the wavelength of 425 nm in a micro plate reader, model Synergy HT (Biotek, Winooski, USA). The total flavonoids yield was calculated as milligrams per gram of soluble solids. The analyses were performed in triplicate [44, 45].

2.4.3 Chromatographic method development and validation

The kaurenoic acid and quercitrin quantifications were done by high performance liquid chromatography (HPLC). The chromatographic analyses were carried out using an Agilent Liquid Chromatograph equipped with a DAD model G1315B Agilent 1100 series and an ALS injector G1329A Agilent 1200 series. The optimized chromatographic conditions allowing the elution and complete resolution of the compounds of interested consisted of a ACE C18-AR column (150 mm × 4.6 mm, d.p. 3 μm, Advanced Chromatography Technologies Ltd, Aberdeen, UK) using as mobile phases purified acetonitrile (A) and water (B) eluted according to the following gradient: 0 min, 90% B; 5 min, 80% B; 10 min, 65% B; 15 min, 55% B; 20 min, 0% B; 30 min, 0% B; 35 min, 90% B. The optimum flow rate was 0.8 mL/min and the injection volume was 20 μL. The diode array detector recorded the spectra from 200 to 700 nm. The compounds quercitrin (Q) and kaurenoic acid (KA) were quantified at 210 nm. The kaurenoic acid and quercitrin concentrations were expressed as milligrams per 100g of soluble solids. The method was validated regarding the selectivity, linearity, precision, accuracy, limit of detection and quantification.

To quantify kaurenoic acid and quercitrin, the solids of each extract were dissolved in 10 mL and then an aliquot of 50 μL of this solution were diluted in 950 μL of methanol. As the extracts obtained in the runs 5, 6, 9 and 10 of the Box-Benhken design presented low values of solids as well as total flavonoids, they were diluted in 0.5 mL and then an aliquot of 50 μL was diluted in

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950 μL of methanol, as was performed to dilute the standards, to allow the quantification of the compounds in the developed method.

2.4.4 LC/MS analysis

195 The optimized extracts were qualitative analyzed in a LC/MS-ESI Accela composed of a photodiode array detector, an autosampler, a quaternary pump and a TSQ quantum access (Thermo, San Jose, USA). The column used was the ACE C18-AR column (150 mm $\times$ 4.6 mm, d.p. 3 μm) using as mobile phases purified methanol (A) and water acidified with 0.2% v/v of formic acid (B) eluted according to the following gradient: 0 min, 25% A; 25 min, 55% A; 30
200 min, 100% A; 35 min, 100% A; 40 min, 25% A; 45 min, 25% A; 50 min, 100% A. The flow rate was 0.6 mL/min and the injection volume was 20 μL . The electrospray ionization (ESI) conditions were: capillary voltage: 3500V, end plate: 500V, capillary exit: 120V. Dependent scan types were obtained using skimmer offset of 6V as first event and the peaks that presented signal threshold higher than $8 \cdot 10^5$ counts received collision energy of 20V.

205 2.4.5 Antioxidant activity

2.4.5.1 DPPH (EC_{50})

The DPPH scavenging activity was evaluated according to [44, 45]. Five concentrations of each sample were prepared. A volume of 25 μL of the sample was added to 975 μL of the 60 μM DPPH solution. These solutions were maintained in reaction during 30 minutes in the dark. The
210 DPPH scavenging activity was measured at 516 nm in a micro plate reader, model Synergy HT (Biotek, Winooski, USA). The percentage of DPPH scavenging activity was calculated as shown in equation 2. The concentration corresponding to 50% of DPPH inhibition (EC_{50}) was determined by fitting a linear model to the calibration curve (7.5, 15, 30, 45, 60 and 90 mM). The EC_{50} of the samples were compared with EC_{50} of quercetin and BHT. The analyses were
215 performed in triplicate.

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$$\% \text{ inhibition} = \frac{Abs_{516} \text{ control} - Abs_{516} \text{ sample}}{Abs_{516} \text{ control}} \quad (2)$$

2.4.5.2 TEAC

The TEAC assay was performed according to [46]. The ABTS radical cation (ABTS^{•+}) was produced by adding 7 mM ABTS with 2.45 mM potassium persulfate allowing to react in the dark at room temperature for 12–16 h before use. The aqueous ABTS^{•+} solution was diluted with 5 mM phosphate buffer (pH = 7.4) to an absorbance of 0.70 (±0.02) at 734 nm. Ten microliters of sample (different concentrations) was added to 1 mL of diluted ABTS^{•+} radical solution. After 50 min at 30°C, 300 µL of the mixture was transferred into a well of the microplate and the absorbance was measured at 734 nm in a microplate spectrophotometer reader (Synergy HT, BioTek Instruments). Trolox was used as reference standard and results were expressed as TEAC values (mmol Trolox/g extract). These values were obtained from at least four different concentrations of each extract tested in the assay giving a linear response between 20 and 80% of the blank absorbance. All analyses were performed in triplicate.

2.4.6 Statistical analysis

The Box-Behnken design results were analyzed by ANOVA regarding p<0.5 as significant and response surface were plotted to help to evaluate the results. For that, the statistical software Statistica® 11.0 version and MiniTab® 7.0 version were used. One-sample statistical analyses were evaluated by t-test in the GraphPad Prism 5.03 version software.

3 Results and Discussion

3.1 Chromatographic method development

A chromatographic method was developed in order to quantify the kaurenoic acid and quercitrin in the *C. langsdorffii* extracts. After developing the method, it was validated regarding its selectivity, linearity, accuracy, precision, limit of detection and quantification [47].

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In order to evaluate the selectivity of the method towards the separation of quercitrin and

240 kaurenoic acid, the SLE extract, that presented the most complex profile, was injected and compared with the standards and the blank (solvent). Furthermore, the UV-vis spectra were evaluated to corroborate that no co-elution was observed under the optimized separation conditions. Quercitrin eluted at 11.095 min and kaurenoic acid at 23.625 min; quercitrin spectrum showed two characteristic maximum wavelengths at 255 and 349 nm while kaurenoic
245 acid spectrum showed a maximum at 210 nm. No interferences were observed with the rest of compounds eluted in the chromatogram. Figures of merit of the developed HPLC method for quantification of kaurenoic acid and quercitrin are shown in Table 1.

3.2 Solid-liquid extraction (SLE)

The extraction yield, total flavonoids, antioxidant activity and kaurenoic acid and quercitrin
250 concentration of the extracts obtained by SLE are presented in **Table 2**. The yields obtained, around $32.12 \pm 0.93\%$ w/w, are in accordance with values achieved previously in our laboratory by SLE and Soxhlet (SOX) extraction using ethanol as solvent [49]. Also, the total flavonoids of the *C. langsdorffii* extract obtained by an optimized SLE condition presented 10.2 ± 0.25 mg/g and quercitrin of 7.70 ± 0.02 mg/g, both values lower than the SLE values observed in this study
255 [49]. These results may vary because of the plant variability in its secondary compounds' metabolism is influenced by solar irradiation and/or the amount of raining in the season [50].

3.3 Supercritical Fluid Extraction

3.3.1 Kinetics

Initially, the kinetics of the supercritical fluid extraction process was evaluated considering 250
260 bar and 55°C (central point of the experimental design) to find a suitable extraction time. There is an increasing extraction rate until 80 minutes (constant extraction rate, CER), followed by a

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period of falling extraction rate of about 40 min; after that, there is no significant increase in the amount of soluble solids extracted. Thus, 120 minutes was selected as the extraction time.

3.3.2 Box-Behnken optimization of SFE

265 As mentioned, a Box-Behnken experimental design was used to optimize the SFE process. The matrix of the design, together with the results of the different studied responses, are presented in **Table 3**. It is worth to mention that the variability of the process, studied at the central point (experiments 13, 14 and 15), was below 10% for all the responses.

As can be seen, total flavonoids are not always proportional to the yield values. For instance, in
270 run 3, the highest yield was obtained (14.5% w/w) corresponding to a medium value of flavonoids content (50.9 mg/g) while run 2 provided a medium yield (7.0% w/w) and the highest content in flavonoids (76.6 mg/g). This, in fact, demonstrates the selectivity of the process towards the extraction of selected target compounds such as flavonoids.

Comparing the results of the SFE and SLE, it can be observed that SLE presented approximately
275 3-fold higher yield values and around 10-fold less total flavonoids than the extracts obtained by SFE, thus showing the selectivity associated to the extraction of flavonoids under supercritical conditions. As for kaurenoic acid content, it can be seen that SFE is able to extract 10-fold more than SLE, being a better extraction method than SLE to extract non-polar compounds using non-toxic solvents such as CO₂.

280 Comparing SFE and SLE in terms of antioxidant activity, it can be observed that both presented similar EC₅₀ values, thus corroborating the fact that the antioxidant activity is not strictly related to the flavonoids content. For instance, the best DPPH antioxidant activity (run 11, 7.581 µg/mL) was neither related with the highest yield nor with the highest total flavonoid content; thus, the antioxidant activity can be due to the presence of several compounds other than flavonoids,
285 belonging or not to the polyphenolic group [51, 52]. Values obtained for DPPH in the present

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work were similar to those reported for supercritical extracts of other plants such as *Rosmarinus officinalis* [53] or *Solanum paludosum* [54]. Compared to the antioxidant activity of quercetin (standard) and BHT (EC₅₀ values equal to 1.94 and 48.46 µg/mL, respectively), it can be concluded that *C. langsdorffii* extracts have a strong antioxidant activity.

290 As for the antioxidant activity measured by using the TEAC method, a positive correlation was observed between the TEAC values and the total flavonoids content in the central point of the Box-Behnken design. Runs 13, 14 and 15, which presented the best TEAC values (2.261, 2.111 and 2.123 mmol/g, respectively) also presented the highest values of total flavonoids (66.635, 68.293 and 72.921 mg/g, respectively). However, this correlation was different for the SLE
295 extracts where the values of total flavonoids were 3-fold lower than in the central points of the SFE Box-Behnken design and the TEAC values were similar. Once more, it is possible to notice that the antioxidant activity is not strictly dependent of the flavonoids, and probably other antioxidant compounds are also extracted but not quantified by the methods employed.

3.3.3 Statistical analysis

300 As mentioned, 3 factors were selected to optimize the extraction of bioactive compounds of *C. langsdorffii* using SFE: extraction pressure, extraction temperature and ethanol percentage. After performing the ANOVA for each of the responses (data not shown) the model was fitted; **Table 4** shows the regression coefficients for the different factors and the statistics for goodness of fit of the models for all the response variables. As a general trend, it can be observed that ethanol
305 significantly influenced all the responses (except kaurenoic acid) in its linear and quadratic form. This means that the yield, total flavonoids, quercitrin and antioxidant activity (evaluated by both, DPPH and TEAC) are maximum when ethanol is at its highest level (50%) and they decrease if the ethanol concentration increases or decreases (see **Figures 2A, B, D, E and F**). On the other hand, for kaurenoic acid, ethanol was significant only in its linear form and its coefficient was

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negative, meaning that the kaurenoic acid values are the highest when ethanol percentage is the lowest (see Figure 2C). This is in agreement with the reported solubility of kaurenoic acid, which is maximum in non-polar solvents [23]. Moreover, considering extraction yield, temperature was also a significant factor in its linear form ($p=0.034$); so, yields can be the highest when the temperature is equal to or above 70°C (see Figure 2A).

As can be observed in Figures 2C and 2D, the maximum extraction of kaurenoic acid and quercitrin are obtained under completely different percentages of ethanol which, in fact, was the main factor influencing their extraction under supercritical fluids conditions. In this sense, when using high percentages of ethanol as modifier, extracts preferentially enriched in quercitrin were obtained while kaurenoic acid was barely extracted. As mentioned, this is in agreement with the different solubility reported for both types of compounds.

As for the antioxidant activity, measured using the DPPH *in vitro* assay, the ANOVA also presented the interaction between the temperature and ethanol as significant; that means that ethanol percentage must be around 50% (high) and temperature has to be low (<40°C) to provide the lowest values of EC₅₀. For the response related to the antioxidant activity measured using TEAC *in vitro* assay, the ANOVA also presented as significant the quadratic effect of the pressure and temperature, besides the effect of the ethanol. This means that the TEAC values are the highest when the ethanol is around 50%, the pressure 250 bar and the temperature 55°C. In the response surface (Figure 2F) it is possible to observe exactly the stationary point of maximum TEAC value.

It has to be pointed out that, in general, the correlation coefficient (R^2) and the adjusted correlation coefficient (R^2_{adj}) of the models presented very similar values thus indicating a good fit; only for the kaurenoic acid, these coefficients presented values far from each other (0.829 and 0.521, respectively) pointing out that the estimative values of the model, including the

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optimized predicted values, will not be so precise since other factors different from those

335 considered in the optimization may have an influence in the final response.

3.3.4 Supercritical fluid extraction conditions optimization

From the results obtained it was possible to observe that not all the studied responses could be optimized at the same time. Therefore, two different features set to the extracts were selected for optimization: 1) maximize yield, total flavonoids content and quercitrin concentration and
340 minimize EC₅₀ (DPPH assay), this extract will be called High Quercetrin Extract (HQE); 2) maximize kaurenoic acid concentration, this extract will be called High Kaurenoic Acid Extract (HKAE). The TEAC antioxidant activity was neglected because it decreased the optimum extraction conditions, the desirability function as well as the estimative responses of the other responses.

345 **Table 5** shows the goals, experimental conditions and response values (predicted and experimental) for HQE considering the optimum extraction conditions: pressure, 100 bar; temperature, 70°C and a percentage of ethanol as modifier, 50%. As can be seen, there was no statistically difference ($\alpha=0.05$ on t-Student test) between the predicted and real values for the responses. By this way, the proposed model studied in this work can be considered validated and
350 appropriate to estimate these responses.

In parallel, the model provided by the Box-Behnken design was used to reach the conditions in order to obtain the HKAE. The provided values of the desired responses are presented in **Table 6**, together with the real values obtained experimentally. The optimum extraction conditions provided by the model to maximize kaurenoic acid content in the extract were as follows:

355 pressure, 390 bar; temperature, 70°C and pure CO₂. The predicted response for the kaurenoic acid value was 198.68 mg/g and the desirability was 0.870. The extraction was performed at optimum conditions by triplicate; the analysis of the extracts resulted in a kaurenoic acid

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concentration of 262.92 ± 14.28 mg/g. This value was statistically different ($\alpha=0.05$ on t-test with

a Welch’s correction) from the value provided by the software of 198.68 mg/g. This result can be
360 due to the fact the correlation coefficient (0.829) and the adjusted correlation coefficient values
were far from each other (0.520) which means it was expected that the estimative values in the
model could vary from the real values.

3.2. Scale up extraction

Once obtained the optimized conditions, the scale up of the extraction in order to obtain the
365 extract enriched in kaurenoic acid was performed allowing a scale up of 50-fold. The conditions
as pressure, temperature and ethanol percentage were selected as provided by the experimental
design.

The result for the kaurenoic acid values in the scaled-up extracts was of 266.39 ± 14.96 mg/g.
This value is similar to the result of the real kaurenoic acid content in the optimized extract
370 obtained in the bench scale (262.92 ± 14.28 mg/g). The results of yields were also similar in both
scales, $1.00 \pm 0.20\%$ w/w for the bench supercritical fluid equipment and $1.87 \pm 0.54\%$ w/w for the
scaled-up equipment. Observing these two results, it is possible to infer the scale up of the SFE
was successful making possible to amplify the extraction 50-fold.

3.3. LC/MS analysis

375 The *C. langsdorffii* optimized extracts were analyzed by using HPLC coupled to MS-ESI in
negative ionization mode in order to carry out a qualitative characterization of bioactive phenolic
compounds in *C. langsdorffii* leaves. The peaks of the chromatogram that presented signal
threshold higher than 8.10^5 counts received collision energy of 20V in order to fragment the
compound and make possible to evaluate its fragmentation profile. So, the peak identification
380 was performed on basis of retention time, the absorption spectrum in the UV-visible region, the

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mass spectrum obtained by MS-ESI, the fragmentation profile and previous reports in the

literature related to the genus *Copaifera* or to the family Fabaceae [13, 22, 55, 56].

The profile of the extract with high values of quercitrin (HQE) showed several peaks corresponding to acidic diterpene, polyphenols and polar compounds among which 21 were tentatively characterized. The compounds are reported in **Table 6** presenting their retention time, molecular weight (MW), experimental m/z , maximum UV-visible bands ($\lambda_{\max}$), proposed fragment and proposed tentative identification.

Although in the literature several researches concerning the analysis of the oil obtained from the trunk of *Copaifera* trees have been described, few works can be found identifying compounds from the leaves of *Copaifera*, specially the phenolic and polar compounds. Therefore, to identify the major compounds of the extracts obtained from *C. langsdorffii* leaves'extracts by using HPLC coupled to diode array and mass spectrometry detectors can be considered an important contribution.

As mentioned, researches performed of *C. langsdorffii* leaves in the 1980's reported that they present from 6 to 10% of phenolic compounds, 3% of the amino acid N-metyl-*trans*-4-hidroxi-L-proline and sesquiterpenes (α -cubebene, α -copaene, ciperene, β -copaene, caryophyllene, β -humulene, muurolene, β -selinene, δ -cadinene and γ -cadinene, caryophyllene oxide) [10-12]. Furthermore, recent works reported that their leaves present acidic diterpenes (kaurenoic acid, hydroxy-labdanolic acid), alcoholic diterpenes (kaurenol) and flavonoids (quercitrin and afzelin) [13]. In a more recent study [22], 26 galloylquinic acids, some of them methylated, were identified as polar compounds presented in the *C. langsdorffii* leaves.

In the present study, we observed that the extracts obtained under optimum conditions employing 50% of ethanol as modifier, that is, those extracts containing the highest concentration of polar compounds, presented good results of antioxidant activity (using both,

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together with the flavonoids, can be the main responsible for the antioxidant activity of the *C. langsdorffii* leaves' extracts. Meanwhile, the SFE extracts using pure carbon dioxide did not presented any detectable antioxidant activity.

410 Quercitrin standard presented a retention time of 21 min, a molecular weight of 447⁻ m/z [M-H] and a UV-vis spectrum with maximum wavelength of 256 and 348 nm (**Figure 3**). Its fragmentation profile provided a mass of 301⁻ m/z which corresponds to the loss of the glycoside providing the quercetin.

Flavonoids represent a subgroup of phenolic phytochemicals [57] and quercitrin is one
415 representative. It is reported to be an oxidative stress protector, which normalized the intestinal oxidative status in rats after oral administration [58]. Other flavonoid found in the HQE was afzelin that is the glycosylated form of kaempferol, a flavonol. Afzelin was reported to be able to inhibit intestinal absorption of glucose what can be an interesting use as a nutraceutical product [59].

420 As could be observed, kaurenoic acid, a diterpenic acid, was also present in the HQE and HKAE. The kaurenoic acid standard was analyzed and presented a retention time of 35.8 min, a molecular weight of 301⁻ m/z [M-H] and UV-vis spectrum with no maximum wavelength, as expected since it does not present double bonds.

Kaurenoic acid has been reported to possess several biological activities such as: antimicrobial,
425 antifungal [24], anti-inflammatory [25], vasodilator [26], wound healing [27], anti-psoriasis [28], trypanocide, cytotoxic, potent stimulators of uterine contraction [60, 61] and antitumor activity [29-35]. Besides, the kaurenoic acid has been used in semi-synthesis as a precursor to obtain more selective and/or more bioactive derivative as, for example, to enhance tripanomicidal activity [61] and even to obtain compounds with enhanced antiplasmodial activity [60].

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430 Galloylquinic acids were widely found in the HQE; several of them are isomers since they are present in the methylated form and the methyl group can vary its position as can be observed in Figure 3. Various galloyl derivatives of quinic acid were found to be inhibitors of human DNA polymerases. Among them, 3,4,5-tri-O-galloylquinic acid was the most potent inhibitor of DNA polymerase α [62].

435 The LC/MS analysis of the HKAE (optimum conditions: 390 bar; temperature, 70°C and pure CO₂) was also performed. As expected, these conditions favored the extraction of non-polar compounds from the leaves of *C. langsdorffii*.

4. Conclusion

440 In conclusion, temperature and ethanol percentage were the factors that mostly influenced the SFE of the chemical compounds from *C. langsdorffii* leaves. Besides, the use of response surface methodology allowed the modulation of the composition of the extract providing extracts enriched in either quercitrin or kaurenoic acid. A high content of kaurenoic acid (HKAE) could be obtained at 390 bar, 70°C of temperature and pure CO₂, while an extract rich in phenolic
445 compounds can be obtained at 100 bar, 55°C and using 50% ethanol as modifier (HQE). The qualitative LC/MS analysis allowed the tentative identification of galloylquinic acids in the HQE optimized extracts; those compounds were not present in the HKAE extracts, as expected. SFE was an efficient method for the extraction of bioactive compounds from *C. langsdorffii* leaves while avoiding the use of toxic organic solvents that could be later on found in the product or
450 delivered it to the environment.

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References

- 455 [1] V.F. Veiga, A.C. Pinto, The *Copaifera* L. genus., Quim Nova, 25 (2002) 273-286.
[2] V.F. Veiga, A.C. Pinto, M.A.M. Maciel, Medicinal plants: safe cure?, Quim Nova, 28 (2005) 519-528.
[3] V. Cascon, Copaíba - *Copaifera* spp., in: J.C.T. Carvalho (Ed.) Fitoterápicos antiinflamatórios: aspectos químicos, farmacológicos e aplicações terapêuticas, Tecmedd, 460 Ribeirão Preto, 2004, pp. 221-256,.
[4] L.A.F. Paiva, L.A. Gurgel, E.T. De Sousa, E.R. Silveira, R.M. Silva, F.A. Santos, V.S.N. Rao, Protective effect of *Copaifera langsdorffii* oleo-resin against acetic acid-induced colitis in rats, Journal of Ethnopharmacology, 93 (2004) 51-56.
[5] L.A.F. Paiva, L.A. Gurgel, R.M. Silva, A.R. Tome, N.V. Gramosa, E.R. Silveira, F.A. Santos, V.S.N. Rao, Anti-inflammatory effect of kaurenoic acid, a diterpene from *Copaifera langsdorffii* on acetic acid-induced colitis in rats, Vascular Pharmacology, 39 (2002) 303-307.
465 [6] J.P.B. Sousa, N.P.D. Nanayakkara, A.A. Berretta, J.K. Bastos, Leishmanicidal and antimalarial activities of crude extracts from aerial parts of *Copaifera langsdorffii* and isolation of secondary metabolites, Journal of Pharmacy Research, 5 (2012) 4103-4107.
470 [7] J.K. Bastos, A.R.F. Brunharoto, C. Brunharoto-Júnior, L.P.F. Brunharoto, Process of obtation and uses of the extracts, fractions and isolated substances from *Copaifera* species in the treatment of urinary lithiasis in human beings and animals, in: Instituto Nacional de Propriedade Intelectual, Brasil, 2005.
[8] R.B. de Oliveira, E.B. Coelho, M.R. Rodrigues, A.R.M. Costa-Machado, J.P.B. de Sousa, A.A. Berretta, J.K. Bastos, Effect of the *Copaifera langsdorffii* Desf. Leaf Extract on the Ethylene Glycol-Induced Nephrolithiasis in Rats, Evidence-Based Complementary and Alternative Medicine, (2013) doi.org/10.1155/2013/131372.
475 [9] A.P.S. Brancalion, R.B. Oliveira, J.P.B. Sousa, M. Groppo, A.A. Berretta, M.E. Barros, M.A. Boim, J.K. Bastos, Effect of hydroalcoholic extract from *Copaifera langsdorffii* leaves on urolithiasis induced in rats, Urological Research, 40 (2012) 475-481.
480 [10] J.H. Langenheim, C.L. Convis, C.A. Macedo, W.H. Stubblebine, *Hymenaea* and *Copaifera* leaf sesquiterpenes in relation to lepidopteran herbivory in southeastern Brazil, Biochemical Systematics and Ecology, 14 (1986) 41-49.
[11] C.A. Macedo, J.H. Langenheim, A further investigation of leaf sesquiterpene variation in relation to herbivory in 2 Brazilian populations of *Copaifera langsdorffii*, Biochemical Systematics and Ecology, 17 (1989) 207-216.
485 [12] C.A. Macedo, J.H. Langenheim, Microlepidopteran herbivory in relation to leaf sesquiterpenes in *Copaifera langsdorffii* adult trees and their seedling progeny in a Brazilian woodland, Biochemical Systematics and Ecology, 17 (1989) 217-224.
490 [13] J.P.B. Sousa, A.P.S. Brancalion, M.G. Junior, J.K. Bastos, A validated chromatographic method for the determination of flavonoids in *Copaifera langsdorffii* by HPLC, Natural Product Communications, 7 (2012) 25-29.
[14] Quercitrin, National Center for Biotechnology Information, PubChem Compound Database, Accessed in 19 November 2014. Available from:
495 <http://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?sid=87225407>
[15] S. Kim, H. Kim, S. Choi, K. Park, H. Choi, M. Lee, Anti-oxidative and inhibitory activities on nitric oxide (NO) and prostaglandin E2 (COX-2) production of flavonoids from seeds of *Prunus tomentosa* Thunberg, Archives of Pharmacal Research, 31 (2008) 424-428.

Cite as:

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- [16] G.H. Xu, I.J. Ryoo, Y.H. Kim, S.J. Choo, I.D. Yoo, Free radical scavenging and antielastase activities of flavonoids from the fruits of *Thuja orientalis*, Archives of Pharmacal Research, 32 (2009) 275-282.
- [17] W. Li, D.K.J. Lin, Optimal foldover plans for two-level fractional factorial designs, Technometrics, 45 (2003) 142-149.
- [18] B.S. Min, S.Y. Lee, J.H. Kim, J.K. Lee, T.J. Kim, D.H. Kim, Y.H. Kim, H. Joung, H.K. Lee, N. Nakamura, H. Miyashiro, M. Hattori, Anti-complement activity of constituents from the stem-bark of *Juglans mandshurica*, Biological & Pharmaceutical Bulletin, 26 (2003) 1042-1044.
- [19] E.K. Kwon, D.Y. Lee, H. Lee, D.O. Kim, N.I. Baek, Y.E. Kim, H.Y. Kim, Flavonoids from the buds of *Rosa damascena* inhibit the activity of 3-Hydroxy-3-methylglutaryl-coenzyme A reductase and angiotensin I-converting enzyme, Journal of Agricultural and Food Chemistry, 58 (2010) 882-886.
- [20] H.S. Rho, S.M. Ahn, B.C. Lee, M.K. Kim, A.K. Ghimeray, C.W. Jin, D.H. Cho, Changes in flavonoid content and tyrosinase inhibitory activity in kenaf leaf extract after far-infrared treatment, Bioorganic & Medicinal Chemistry Letters, 20 (2010) 7534-7536.
- [21] L.O. Regasini, J.C.R. Velloso, D.H.S. Silva, M. Furlan, O.M.M. Oliveira, N.M. Khalil, I.L. Brunetti, M.C.M. Young, E.J. Barreiro, V.S. Bolzani, Flavonols from *Pterogyne nitens* and their evaluation as myeloperoxidase inhibitors, Phytochemistry, 69 (2008) 1739-1744.
- [22] Motta, Desenvolvimento e validação de método analítico por CLAE-UV-DAD para quantificação e análise sazonal de derivados galoilquínicos nas folhas de *Copaifera langsdorffii*, in: Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, 2014, pp. 160.
- [23] Kaurenoic, National Center for Biotechnology Information, PubChem Compound Database, Accessed in 19 November 2014. Available from: <http://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?sid=14036>
- [24] E.P. Padla, L.T. Solis, C.Y. Ragasa, Antibacterial and antifungal properties of ent-kaurenoic acid from *Smallanthus sonchifolius*, Chinese Journal of Natural Medicines, 10 (2012) 408-414.
- [25] R.J. Choi, E.M. Shin, H.A. Jung, J.S. Choi, Y.S. Kim, Inhibitory effects of kaurenoic acid from *Aralia continentalis* on LPS-induced inflammatory response in RAW264.7 macrophages, Phytomedicine, 18 (2011) 677-682.
- [26] C.R. Tirapelli, S.R. Ambrosio, F.B. da Costa, S.T. Coutinho, D.C.R. de Oliveira, A.M. de Oliveira, Analysis of the mechanisms underlying the vasorelaxant action of kaurenoic acid in the isolated rat aorta, European Journal of Pharmacology, 492 (2004) 233-241.
- [27] N. Balekar, T. Nakpheng, N.G. Katkam, T. Srichana, Wound healing activity of ent-kaura-9(11),16-dien-19-oic acid isolated from *Wedelia trilobata* (L.) leaves, Phytomedicine, 19 (2012) 1178-1184.
- [28] F. Gelmini, G. Beretta, C. Anselmi, M. Centini, P. Magni, M. Ruscica, A. Cavalchini, R.M. Facino, GC-MS profiling of the phytochemical constituents of the oleoresin from *Copaifera langsdorffii* Desf. and a preliminary in vivo evaluation of its antipsoriatic effect, European Journal of Pharmacology, 440 (2013) 170-178.
- [29] B.C. Cavalcanti, D.P. Bezerra, H.I.F. Magalhães, M.O. Moraes, M.A.S. Lima, E.R. Silveira, C.A.G. Câmara, V.S. Rao, C. Pessoa, L.V. Costa-Lotufo, Kauren-19-oic acid induces DNA damage followed by apoptosis in human leukemia cells, Journal of Applied Toxicology, 29 (2009) 560-568.
- [30] L.V. Costa-Lotufo, G.M.A. Cunha, P.A.M. Farias, G.S.B. Viana, K.M.A. Cunha, C. Pessoa, M.O. Moraes, E.R. Silveira, N.V. Gramosa, V.S.N. Rao, The cytotoxic and embryotoxic effects of kaurenoic acid, a diterpene isolated from *Copaifera langsdorffii* oleo-resin, Toxicon, 40 (2002) 1231-1234.

Cite as:

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- [31] N.d.M. Gomes, C.d.M. Rezende, S.P. Fontes, A.M.C. Hovell, R.G. Landgraf, M.E. Matheus, A.d.C. Pinto, P.D. Fernandes, Antineoplastic activity of *Copaifera multijuga* oil and fractions against ascitic and solid Ehrlich tumor, Journal of Ethnopharmacology, 119 (2008) 179-184.
- [32] S.R.M. Lima, V.F. Veiga, H.B. Christo, A.C. Pinto, P.D. Fernandes, *In vivo* and *in vitro* studies on the anticancer activity of *Copaifera multijuga* Hayne and its fractions, Phytotherapy Research, 17 (2003) 1048-1053.
- [33] M.O. Moraes, M.C. Fonteles, M.E.A. Moraes, M.I.L. Machado, F.J.A. Matos, Screening for anticancer activity of plants from the Northeast of Brazil, Fitoterapia, 68 (1997) 235.
- [34] A. Ohsaki, L.T. Yan, S. Ito, H. Edatsugi, D. Iwata, Y. Komoda, The isolation and *in-vivo* potent antitumor-activity of clerodane diterpenoid from the oleoresin of the Brazilian medicinal plant, *Copaifera-langsdorffii* Desfon, Bioorganic & Medicinal Chemistry Letters, 4 (1994) 2889-2892.
- [35] C.D. Souza, J.M. Felfili, Uso de plantas medicinais na região de Alto Paraíso de Goiás, Acta Botanica Brasilica, 20 (2006) 7.
- [36] M. Contini, S. Baccelloni, R. Massantini, G. Anelli, Extraction of natural antioxidants from hazelnut (*Corylus avellana* L.) shell and skin wastes by long maceration at room temperature, Food Chemistry, 110 (2008) 659-669.
- [37] Brazilian Pharmacopoeia, in, ANVISA. Agência Nacional de Vigilância Sanitária. Fundação Oswaldo Cruz, Brasília, DF, 2010.
- [38] M. Herrero, A. Cifuentes, E. Ibáñez, Sub- and supercritical fluid extraction of functional ingredients from different natural sources: Plants, food-by-products, algae and microalgae: A review, Food Chemistry, 98 (2006) 136-148.
- [39] T. Anaëlle, E. Serrano Leon, V. Laurent, I. Elena, J.A. Mendiola, C. Stéphane, K. Nelly, L.B. Stéphane, M. Luc, S.-P. Valérie, Green improved processes to extract bioactive phenolic compounds from brown macroalgae using *Sargassum muticum* as model, Talanta, 104 (2013) 44-52.
- [40] B. Beňová, M. Adam, P. Pavlíková, J. Fischer, Supercritical fluid extraction of piceid, resveratrol and emodin from Japanese knotweed, Journal of Supercritical Fluids, 51 (2010) 325-330.
- [41] J.H.Y. Vilegas, E. de Marchi, F.M. Lancas, Extraction of low-polarity compounds (with emphasis on coumarin and kaurenoic acid) from *Mikania glomerata* (‘guaco’) leaves, Phytochemical Analysis, 8 (1997) 266-270.
- [42] E. Shortle, J. Kerry, A. Furey, D. Gilroy, Optimisation of process variables for antioxidant components from *Crataegus monogyna* by supercritical fluid extraction (CO₂) using Box-Behnken experimental design, Journal of Supercritical Fluids, 81 (2013) 112-118.
- [43] P.H. List, P.C. Schmidt, Phytopharmaceutical technology, Heyden & Son Limited, London, 1989.
- [44] A.R.D. Costa, F.S. Marquiasfavel, M.M.D.L.L. Vaz, B.A. Rocha, P.C.P. Bueno, P.L.M. Amaral, H.D. Barud, A. Ap Berreta-Silva, Quercetin-PVP K25 solid dispersions: Preparation, thermal characterization and antioxidant activity, Journal of Thermal Analysis and Calorimetry, 104 (2011) 273-278.
- [45] L.E. Dowd, Spectrophotometric Determination of Quercetin, Analytical Chemistry, 31 (1959) 1184-1187.
- [46] T.L. Miron, M. Plaza, G. Bahrim, E. Ibáñez, M. Herrero, Chemical composition of bioactive pressurized extracts of Romanian aromatic plants, Journal of Chromatography A, 1218 (2011) 4918-4927.

Cite as:

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- 595 [47] ICH, Validation of analytical procedures: text and methodology Q2(R1), in: International conference on harmonisation of technical requirements for registration of pharmaceuticals for human use, 2005.
- [48] ANVISA. Agência Nacional de Vigilância Sanitária, Ministério da Saúde, RE 899, 29th may, 2003, Brasília, DF, Brasil.
- 600 [49] A.R.M. Costa-Machado, Obtenção de produtos a partir das folhas de *Copaifera langsdorffii* Desf.: otimização da extração e secagem em spray dryer utilizando planejamentos experimentais, in: Departamento de Ciências Farmacêuticas, Universidade de São Paulo, Ribeirão Preto, 2011, pp. 115.
- [50] J.P. de Sousa, M.F. Leite, R.F. Jorge, D.O. Resende, A.A. da Silva Filho, N.A. Furtado, A.E. Soares, A.C. Spadaro, P.M. de Magalhaes, J.K. Bastos, Seasonality role on the phenolics from cultivated *Baccharis dracunculifolia*, Evidence-Based Complementary and Alternative Medicine, (2011) doi: 10.1093/ecam/nep077.
- 605 [51] A. Kazan, H. Koyu, I.C. Turu, O. Yesil-Celiktas, Supercritical fluid extraction of *Prunus persica* leaves and utilization possibilities as a source of phenolic compounds, Journal of Supercritical Fluids, 92 (2014) 55-59.
- 610 [52] W. Kukula-Koch, N. Aligiannis, M. Halabalaki, A.-L. Skaltsounis, K. Glowinski, E. Kalpoutzakis, Influence of extraction procedures on phenolic content and antioxidant activity of Cretan barberry herb, Food Chemistry, 138 (2013) 406-413.
- [53] M. Herrero, M. Plaza, A. Cifuentes, E. Ibáñez, Green processes for the extraction of bioactives from Rosemary: Chemical and functional characterization via ultra-performance liquid chromatography-tandem mass spectrometry and in-vitro assays, Journal of Chromatography A, 1217 (2010) 2512-2520.
- 615 [54] S. Siqueira, V.d.S. Falcão-Silva, M.d.F. Agra, C. Dariva, J.P.d. Siqueira-Júnior, M.J.V. Fonseca, Biological activities of *Solanum paludosum* Moric. extracts obtained by maceration and supercritical fluid extraction, Journal of Supercritical Fluids, 58 (2011) 391-397.
- 620 [55] Taylor & Francis group, Dictionary of Natural Products, 19 November 2014. Available from: www.dnp.chemnetbase.com
- [56] J.A. Rothwell, J. Pérez-Jiménez, V. Neveu, A. Medina-Ramon, N. M'Hiri, P. Garcia Lobato, C. Manach, K. Knox, R. Eisner, D. Wishart, A. Scalbert, Phenol-Explorer 3.0: a major update of the Phenol-Explorer database to incorporate data on the effects of food processing on polyphenol content, Accessed in 19 November, 2014. Available from: <http://phenol-explorer.eu/compounds>.
- 625 [57] C.A. RiceEvans, N.J. Miller, G. Paganga, Structure-antioxidant activity relationships of flavonoids and phenolic acids, Free Radical Biology and Medicine, 20 (1996) 933-956.
- [58] J. Galvez, J.P. Delacruz, A. Zarzuelo, F.S. Demedina, J. Jimenez, F.S. Delacuesta, Oral-administration of quercitrin modifies intestinal oxidative status in rats, General Pharmacology, 25 (1994) 1237-1243.
- 630 [59] P. Rodriguez, F. Gonzalez-Mujica, J. Bermudez, M. Hasegawa, Inhibition of glucose intestinal absorption by kaempferol 3-O-alpha-rhamnoside purified from *Bauhinia megalandra* leaves, Fitoterapia, 81 (2010) 1220-1223.
- [60] R. Batista, P.A. García, M.A. Castro, J.M. Miguel del Corral, N.L. Speziali, F. de P. Varotti, R.C. de Paula, L.F. García-Fernández, A.s. Francesch, A. San Feliciano, A.B. de Oliveira, Synthesis, cytotoxicity and antiplasmodial activity of novel ent-kaurane derivatives, European Journal of Medicinal Chemistry, 62 (2013) 168-176.
- 635 [61] R. Batista, J.L. Humberto, E. Chiari, A.d.B. de Oliveira, Synthesis and trypanocidal activity of ent-kaurane glycosides, Bioorganic & Medicinal Chemistry, 15 (2007) 381-391.

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640 [62] C.O. Rezende Jr, C. Rigotto, W. Caneschi, C.A.M. Rezende, M. Le Hyaric, M.R.C. Couri, C.M.O. Simões, M.V. de Almeida, Anti-HSV-1 and antioxidant activities of dicaffeoyl and digalloyl esters of quinic acid, Biomedicine & Preventive Nutrition, 4 (2014) 35-38.

Cite as:

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Tables

Table 1. Figures of merit of the HPLC developed method for quantification of kaurenoic acid and quercitrin

	linear range μg/mL	Linear regression curve	R ²	Recovery test			Analytical limits (μg/mL)	
				Low (80%)	Medium (100%)	High (120%)	LOD	LOQ
kaurenoic acid	10-110	y=23.362x + 46.646	0.9997	98.0±4.0%	100.0±2.8%	104.5±3.1%	0.443	1.341
quercitrin	1-40	y=98.104x - 65.378	0.9954	100.6±2.2%	100.2±4.5%	100.8±1.0%	0.241	0.730

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Table 2. Responses evaluated for the standard extracts obtained using SLE.

	<i>Yield</i> (% w/w)	<i>Total flavonoids</i> (mg/g)	<i>Kaurenoic acid</i> (mg/g)	<i>Quercitrin</i> (mg/g)	<i>EC₅₀</i> (μg/mL)	<i>TEAC</i> (mmol/g)
A	31.83	20.587	24.136	9.610	9.074	2.737
B	34.18	24.229	25.869	10.369	10.280	2.438
C	36.30	24.529	21.778	10.259	8.148	2.472

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Table 3. Box-Behnken experimental design, presenting the coded factors: X_1 , X_2 and X_3 ; and the uncoded factors: pressure (P), temperature (T) and ethanol (E). Responses evaluated for the Box-Behnken experimental design.

Run	X_1 (P)	X_2 (T)	X_3 (E)	Yield (% w/w)	Total flavonoids (mg/g)	Kaurenoic acid (mg/g)	Quercitrin (mg/g)	EC_{50} (μ g/mL)	TEAC (mmol/g)
1	-1 (100 bar)	-1 (40°C)	0 (25%)	8.33	72.569	103.982	10.493	14.118	0.982
2	+1 (400 bar)	-1 (40°C)	0 (25%)	7.01	76.618	115.233	9.251	12.596	1.016
3	-1 (100 bar)	+1 (70°C)	0 (25%)	14.50	50.918	73.127	13.151	10.162	1.182
4	+1 (400 bar)	+1 (70°C)	0 (25%)	11.18	66.906	82.194	12.114	10.461	1.048
5	-1 (100 bar)	0 (55°C)	-1 (0%)	0.31	0.657	81.063	7.468	131.726	0.244
6	+1 (400 bar)	0 (55°C)	-1 (0%)	0.55	20.324	152.476	4.578	169.987	0.283
7	-1 (100 bar)	0 (55°C)	+1 (50%)	12.28	59.356	69.818	11.859	11.540	1.173
8	+1 (400 bar)	0 (55°C)	+1 (50%)	10.81	56.127	67.462	9.672	7.867	1.232
9	0 (250 bar)	-1 (40°C)	-1 (0%)	0.69	8.322	191.870	6.612	197.334	0.251
10	0 (250 bar)	+1 (70°C)	-1 (0%)	1.06	8.420	218.243	4.942	91.410	0.247
11	0 (250 bar)	-1 (40°C)	+1 (50%)	10.49	56.981	69.388	10.991	7.581	1.268
12	0 (250 bar)	+1 (70°C)	+1 (50%)	11.98	55.142	53.930	12.258	8.168	1.133
13	0 (250 bar)	0 (55°C)	0 (25%)	7.67	66.635	87.239	9.153	10.547	2.261
14	0 (250 bar)	0 (55°C)	0 (25%)	8.21	68.293	78.486	10.511	11.989	2.111
15	0 (250 bar)	0 (55°C)	0 (25%)	9.13	72.921	86.389	9.491	9.705	2.123

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Table 4. Model coefficients and regression adjustment of mathematical models obtained by Response Surface Methodology.

<i>Effect</i>	<i>Yield</i> (% w/w)		<i>Total flavonoids</i> (mg/g)		<i>Quercitrin</i> (mg/g)		<i>Kaurenoic acid (mg/g)</i>		<i>EC₅₀</i> (µg/mL)		<i>TEAC</i> (mmol/g)	
	<i>Coefficients</i>	<i>P</i>	<i>Coefficients</i>	<i>P</i>	<i>Coefficients</i>	<i>P</i>	<i>Coefficients</i>	<i>P</i>	<i>Coefficients</i>	<i>P</i>	<i>Coefficients</i>	<i>P</i>
<i>Intercept</i>	8.339	0.000	69.283	0.000	9.718	0.000	84.0380	0.007	10.747	0.317	2.165	0.000
<i>X₁¹</i>	-0.734	0.229	4.559	0.054	-0.920	0.079	11.1720	0.388	4.171	0.513	-0.000	0.992
<i>X₁²</i>	0.899	0.307	-0.315	0.911	0.614	0.364	-15.5284	0.413	2.622	0.776	-0.550	0.000*
<i>X₂</i>	1.551	0.034*	-4.138	0.072	0.640	0.186	-6.6223	0.599	-13.929	0.066	0.012	0.709
<i>X₂²</i>	1.016	0.254	-2.215	0.446	0.920	0.195	25.1243	0.208	-1.535	0.867	-0.558	0.000*
<i>X₃</i>	5.344	0.000*	23.735	0.000*	2.648	0.001*	-47.8818	0.010*	-69.413	0.000*	0.473	0.000*
<i>X₃²</i>	-3.249	0.009*	-34.852	0.000*	-1.938	0.025*	24.1951	0.223	66.911	0.001*	-0.882	0.000*
<i>X₁*X₂</i>	-0.499	0.539	2.985	0.299	0.051	0.934	-0.5461	0.975	0.455	0.959	-0.042	0.367
<i>X₁*X₃</i>	-0.428	0.597	-5.724	0.077	0.176	0.778	-18.4423	0.320	-10.484	0.266	0.005	0.914
<i>X₂*X₃</i>	0.232	0.772	-0.484	0.858	0.734	0.269	-10.4576	0.559	26.628	0.025*	-0.033	0.475
<i>R²</i>	0.963	-	0.986	-	0.927	-	0.829	-	0.977	-	0.994	-
<i>R²_{adjusted}</i>	0.898	-	0.961	-	0.795	-	0.521	-	0.936	-	0.984	-
<i>Lack of fit</i>	0.138	-	0.230	-	0.003*	-	0.207	-	0.012*	-	0.519	-

* significant values

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Table 5. Goals, experimental conditions and response values (predicted and experimental) for HQE at optimum conditions: pressure, 100 bar; temperature, 70°C and 50 % ethanol as modifier.

<i>Responses</i>	<i>Goal</i>	<i>Lower</i>	<i>Target</i>	<i>Upper</i>	<i>Weight</i>	<i>Desirability</i>	<i>Predicted</i>	<i>Real</i>
Yield (% w/w)	Max	0.30	15.0	15.0	1	1.000	17.07	16.34±3.09
Total flavonoids(mg/g)	Max	0.65	77.0	77.0	1	0.695	53.73	53.28±6.08
EC ₅₀ (µg/mL)	Min	7.50	7.50	197.00	1	0.999	7.53	10.52±2.23
Quercitrin (mg/g)	Max	4.50	13.00	13.00	1	1.000	14.66	15.22±2.06

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Table 6. Goals and values to the provided responses to obtain the HKAE at optimum conditions: pressure, 390 bar; temperature, 70°C and pure CO₂.

<i>Responses</i>	<i>Goal</i>	<i>Lower</i>	<i>Target</i>	<i>Upper</i>	<i>Weight</i>	<i>Desirability</i>	<i>Predicted</i>	<i>Real</i>
Kaurenoic acid (mg/g)	Max	67.50	218.20	218.20	1	0.870	198.68	262.92±14.28

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Peak number	t_R (min)	MW	m/z-	λ_{max} (nm)	Proposed compound	Fragmentation profile	
						m/z-	Proposed Fragment
1	2.82	-	386.99	225/266(s)	NI	341.04	NI
2	4.11	358.27	356.90	225/276	galloylquinic acid with 1 methyl group	191.02	quinic acid
						183.01	methyl gallate
						172.92	gallic acid
3	4.52	358.27	356.90	225/276	galloylquinic acid with 1 methyl group	190.98	quinic acid
						182.94	methyl gallate
						173.07	gallic acid
4	5.25	358.27	356.90	225/277	galloylquinic acid with 1 methyl group	191.09	quinic acid
5	7.52	510.37	509.06	225/277	di-galloylquinic acid with 1 methyl group	356.94	galloylquinic acid with 1 methyl
6	7.62	510.37	509.05	226/277	di-galloylquinic acid with 1 methyl group	356.94	galloylquinic acid with 1 methyl
7	8.14	510.37	509.09	225/276	di-galloylquinic acid with 1 methyl group	356.94	galloylquinic acid with 1 methyl
						342.92	galloylquinic acid
8	9.85	524.37	523.06	226/276	di-galloylquinic acid with 2 methyl groups	356.93	galloylquinic acid with 1 methyl
						338.92	galloylquinic acid with 1 methyldehydrated
9	9.90	524.37	523.06	226/276	di-galloylquinic acid with 2 methyl groups	356.95	galloylquinic acid with 1 methyl
						338.94	galloylquinic acid with 1 methyldehydrated
10	11.12	524.37	523.06	226/276	di-galloylquinic acid with 2methyl groups	356.93	galloylquinic acid with 1 methyl

Table 7. Results of the LC/MS analysis from the HQE in the negative mode.

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						339.02	galloylquinic acid with 1 methyl dehydrated	
11	13.43	524.37	523.06	226/278	di-galloylquinic acid with 2methyl groups	356.93	galloylquinic acid with 1 methyl	
12	15.57	676.48	675.02	227/277	tri-galloylquinic acid with 2methyl groups	523.01	di-galloylquinic acid with 2 methyl groups	
13	19.85	524.37	523.06	226/276	di-galloylquinic acid with 2methyl groups	338.92	galloylquinic acid with 1 methyl dehydrated	
14	19.40	690.48	689.08	227/278	tri-galloylquinic acid with 3methyl groups	523.05	di-galloylquinic acid with 2 methyl groups	
15	21.00	448.38	446.99	256/348	quercitrin*	300.96	quercetin	
16	24.79	432.38	430.99	264/(s)341	Afzelin	284.96	kaempferol	
17	31.91	344.32	342.92	270/335	Eupatorin	327.96	loss of a methyl group	*compared to the standard. NI, non-identified.
						312.95	loss of two methyl groups	
18	32.00	344.27	342.96	269/341	galloyl quinic acid	298.99	galloyl quinic acid decarboxylated	
						283.92	NI	
19	32.19	330.29	328.92	257/367	Dimethylquercetin	313.96	methylquercetin	
20	32.84	-	329.05	243/269	NI	293.07	NI	
						195.19	NI	
21	33.58	332.26	331.08	269/404	gallic acid 4-O-glucoside	287.10	gallic acid 4-O-glucoside decarboxylated	
22	35.99	302.45	301.01	-	kaurenoic acid*	-	-	

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Figures

Figure 1. Chemical structures of (A) quercitrin and (B) kaurenoic acid.

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Figure 2. Response surface for (A) yield, (B) total flavonoids, (C) kaurenoic acid, (D) quercitrin, (E) EC_{50} for DPPH and (F) TEAC.

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Galloylquinic acid

Tri-galloylquinic acid

Di-galloylquinic acids

Cite as:

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Figure 3. Representative chemical structures of galloylquinic acids involved in this study. R₁, R₂ and R₃ can be hydrogen or methyl group.