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Supercritical extraction with carbon dioxide and co-solvent from *Leptocarpha rivularis*

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ABSTRACT

The aim of this work was to investigate the effect of temperature (40–60 °C), pressure (10–20 MPa) and co-solvent concentration (0–2 wt.%) on extraction yield from *L. rivularis* leaves using ethanol-modified supercritical carbon dioxide. Extraction assays were carried out in a Process Development Unit using a Box-Behnken design. Extraction yield ranged from 14.7 to 53.1 g/kg dry substrate, with the highest value being achieved at 60 °C, 20 MPa and 2 wt.%. The pressure and the co-solvent concentration had a significant effect on extract recovery. To complement the analysis, recognized bioactive compounds, such as the flavonoids quercetin, kaempferol and resveratrol and the terpenes α-thujone, β-caryophyllene and caryophyllene oxide were quantified in the selected extract. Antioxidant activity and the inhibition of key enzymes linked to diabetes mellitus (α-amylase and α-glucosidase) were exhibited by the extract. These results demonstrate the potential benefit of *L. rivularis* extract for human health.

1. Introduction

Leptocarpha rivularis is a Chilean medicinal plant. Its extract has properties useful for the treatment of various illnesses, including cancer and hypoglycemia (Martínez et al., 1995); these properties are associated with the presence of bioactive compounds, including flavonoids and terpenes. Niemeyer (2009) reported that α-thujone, β-caryophyllene and caryophyllene oxide are the three major terpenes found in *L. rivularis*. Jiménez-González et al. (2018) identified flavonoids in *L. rivularis*, including kaempferol and quercetin.

The extraction of bioactive compounds to be used as nutraceuticals or functional food ingredients requires technologies that allow faster, more efficient extraction while protecting the properties of bioactive compounds. Among existing extraction technologies, supercritical fluid extraction can be made more selective, rapid and efficient by controlling the temperature and pressure, which may be considered the principal factors affecting the behavior of supercritical fluid extraction. Carbon dioxide (CO_2) is the most frequently used solvent because it provides extraction at low temperatures, thus avoiding the degradation of thermolabile compounds.

Since supercritical CO_2 is a nonpolar solvent, it is suitable for the extraction of lipophilic substances; however, its efficiency at solubilizing polar compounds is low. Solubility can be improved by increasing the pressure or adding a polar co-solvent, such as ethanol. Langenfeld et al. (1994) have formulated hypotheses about how co-solvents affect the efficiency of supercritical CO_2 extraction by: (a) increasing the polarity and solvent power of CO_2; (b) covering active sites and preventing readsorption or partitioning of the solutes in the solid matrix; (c) causing the solid matrix to swell, thus allowing the fluid solvent to penetrate the substrate and promoting the transfer of solutes to the solvent; (d) interacting with the solute/solid matrix complex and lowering the activation energy for desorption.

Ethanol is the polar solvent most frequently applied as a co-solvent in supercritical fluid extraction. It has been reported that increasing the ethanol concentration results in a higher extraction yield of bioactive compounds from leaves of rosemary (*Rosmarinus officinalis*) (Bensebia et al., 2009) and spearmint (*Mentha spicata* L.) (Bimakr et al., 2012). Daukšas et al. (2001) studied supercritical CO_2 extraction from sage (*Salvia officinalis* L.) using ethanol as the co-solvent. The extraction yield increased (3.6-fold) with the use 1 wt.% (weight-weight percentage) of ethanol compared to extraction without a co-solvent. However, the extraction yield decreased when the ethanol content was increased to 2 wt.%. Michielin et al. (2009) observed a decrease in the extraction yield when an excess of ethanol was used in supercritical CO_2 extraction from *Cordia verbenacea*. They pointed out that when an excess of ethanol is employed, the co-solvent-extract interaction reduces the CO_2-extract interaction, resulting in a decrease in the extraction yield. Castro-Vargas et al. (2013) pointed out that the increase in ethanol

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concentration can induce the formation of two phases due to CO_2 saturation, reducing the solvent power of the CO_2-ethanol mixture. On the other hand, the use of an excess of ethanol as a co-solvent can reduce the contribution of other factors such as temperature and pressure (Reyes et al., 2014). Thus, increasing the ethanol concentration does not necessarily mean more efficient extraction. The effect of the concentration of the co-solvent on the extraction yield should therefore be investigated.

Response surface methodology (RSM) is a statistical optimization technique used in the extraction process with supercritical CO_2. The experiment design used with RSM is called response surface design; it has been applied to optimize supercritical fluid extraction from leaves of spearmint (Bimakr et al., 2012) and myrtle (*Myrtus communis* L.) (Ghasemi et al., 2011) using ethanol-modified CO_2. The aim of the present work was to study the effect of ethanol as co-solvent, temperature and pressure on the extraction yield from *L. rivularis* leaves using supercritical CO_2 extraction with a response surface design. In addition, the bioactive characteristics of the selected extract were measured, such as: antioxidant activity (DPPH radical scavenging assay, ferric-reducing antioxidant power assay, β-carotene bleaching assay); inhibition of key enzymes of diabetes (α-amylase and α-glucosidase); quantification of flavonoids (quercetin, kaempferol and resveratrol) and terpenoids (α-thujone, β-caryophyllene and caryophyllene oxide).

2. Materials and method

Iron (II) sulfate heptahydrate ≥99.0% ($FeSO_4{\cdot}7H_2O$) (CAS Number: 7782-63-0) and iron (III) chloride anhydrous ≥98% ($FeCl_3$) (CAS Number: 7705-08-0) were procured from ACROS Organics (Morris, NJ). Chloroform ≥99.8% ($CHCl_3$) (CAS Number: 67-66-3), ethanol 99.9% (CH_3CH_2OH) (CAS Number: 64-17-5) and methanol (CH_3OH) ≥99.8% (CAS Number: 67-56-1), all of analysis grade, were procured from J.T. Baker (J.T. Baker, Phillipsburg, NJ). DPPH ≥ 90% (2,2-diphenyl-1-picrylhydrazyl) ($C_{18}H_{12}N_5O_6$) (CAS Number: 1898-66-4) was acquired from Calbiochem Co. (San Diego, CA). Acetonitrile ≥ 99.9% (CH_3CN) (CAS Number: 75-05-08), methanol ≥ 99.9% (CAS Number: 67-56-1) and water (CAS Number 7732-18-5), all of chromatography grade (LiChrosolv Reag. Ph Eur), DMSO ≥ 99.5% (dimethyl sulfoxide) [$(CH_3)_2SO$)] (CAS Number: 67-68-5) and formic acid 98–100% (HCOOH) (CAS Number: 64-18-6) were obtained from Merck KGaA (Darmstadt, Germany). Aluminum chloride 99.99% ($AlCl_3$) (CAS Number 7446-70-0), potassium sodium tartrate tetrahydrate 99% [$KOCOCH(OH)CH(OH)COONa{\cdot}4H_2O$] (CAS Number: 6381-59-5), sodium hydroxide ≥ 97% (NaOH) (CAS Number 1310-73-2), sodium nitrite ≥ 97% ($NaNO_2$) (CAS Number: 7632-00-0), sodium carbonate ≥ 99.5% (Na_2CO_3) (CAS Number: 497-19-8), Lipoxidase from *Glycine max* (soybean) Type I-B, lyophilized powder, ≥50,000 units/mg solid (CAS Number: 9029-60-1), α-amilase from *Bacillus licheniformis* Type XII-A, saline solution, ≥500 units/mg protein (CAS Number: 9000-85-5), α-glucosidase from *Saccharomyces cerevisiae* Type I, lyophilized powder, ≥10 units/mg protein (CAS Number: 9001-42-7), TROLOX 97% (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) ($C_{14}H_{18}O_4$) (CAS Number: 53188-07-1), DNSA 98% (3,5-Dinitrosalicylic acid) [$(O_2N)_2C_6H_2$-2-$(OH)CO_2H$] (CAS Number: 1431-39-6), *p*NPG ≥98% (*p*-Nitrophenyl β-D-glucopyranoside) ($C_{12}H_{15}NO_8$) (CAS Number: 2492-87-7), *p*-Nitrophenol ≥99% ($O_2NC_6H_4OH$) (CAS Number: 100-02-7), β-carotene ≥95% ($C_{40}H_{56}$) (CAS Number: 7235-40-7), 1,2,4,5-tetramethylbenzene 98% [$C_6H_2(CH_3)_4$] (CAS Number: 95-93-2), caryophyllene oxide 99% ($C_{15}H_{24}O$) (CAS Number: 1139-30-6), β-caryophyllene ≥98% ($C_{15}H_{24}$) (CAS Number: 87-44-5), α-thujone ≥96% ($C_{10}H_{16}O$) (CAS Number: 546-80-5), gallic acid ≥98% [$(HO)_3C_6H_2CO_2H$] (CAS Number: 149-91-7), kaempferol ≥97% ($C_{15}H_{10}O_6$) (CAS Number: 520-18-3), naringenin ≥95% ($C_{15}H_{12}O_5$) (CAS Number: 67604-48-2), quercetin ≥95% ($C_{15}H_{10}O_7$) (CAS Number: 6151-25-3), resveratrol ≥99% ($C_{14}H_{12}O_3$) (CAS Number: 501-36-0), maltose monohydrate from potato ≥99% ($C_{12}H_{22}O_{11}{\cdot}H_2O$) (CAS Number: 6363-53-7) and acarbose ≥95% ($C_{25}H_{43}NO_{18}$) (CAS Number: 56180-94-0) were procured from Sigma-Aldrich (St. Louis, MO). Starch, soluble synthesis grade, from potato [$(C_6H_{10}O_5)_n$] (CAS Number: 9005-84-9) was acquired from Scharlau Chemie S.A. (Barcelona, Spain). Phosphate buffer pH 7 (CAS Number: 9005-84-9) was obtained from Winkler Ltda. (Santiago, Chile).

2.1. Substrate

The *L. rivularis* leaves used as substrate were provided by Los Esteros Company (La Union, Chile) (39°52′S, 73°14′W). The substrate had a moisture content of 7.9 ± 0.2 g/100 g d.s. (dry substrate). The substrate was ground and sieved through US sieves using a Ro-Tap testing sieve shaker (model RX-29-10, W.S. Tyler, Mentor, OH). The average particle diameter (d_p) was 0.72 ± 0.03 mm. Samples were stored until use in dry, dark conditions, packed in the absence of oxygen and under refrigeration (5 °C).

2.2. Extraction

Extraction was carried out in a Supercritical Process Development Unit Spe-ed SFE (Applied Separations, Allentown, PA) loading 8 g of substrate in a 50 cm^3 extraction vessel. Depending on the temperature (40–60 °C) and pressure (10–20 MPa), the solvent flow used was between 1.5 and 4.3 L NPT per minute of CO_2 (Linde Chile S.A.). The static extraction period (15 min) was followed by a dynamic extraction period, which was varied between 32 and 93 min in order to obtain a total solvent consumption of 30 kg CO_2/kg d.s. In supercritical fluid extractions involving the use of the co-solvent, ethanol was pumped into the CO_2 feed line at a flow rate between 0.03 and 0.18 mL/min so as to achieve two levels of co-solvent concentration (1 and 2 wt.%), using an HPLC pump (Knauer, model K-501, Germany). The extracts were collected during extraction in pre-weighed glass vials (60 cm^3 capacity). The extracts were subjected to a gentle stream of nitrogen (Linde Chile S.A.) to evaporate the ethanol. The mass of extracts was assessed gravimetrically by the weight difference from the clean, dry vials. Extraction yield (Y) was expressed as grams of extract per kilogram of dry substrate (g/kg d.s.).

2.3. Extract analysis

2.3.1. Total flavonoids content

Total flavonoids content was quantified according to Zhishen et al. (1999) with some modifications. 1250 μL of deionized water and 75 μL of $NaNO_2$ solution (5% w/v) were added to 250 μL of the extract solution (10 mg/mL) in a test tube, and the mixture was incubated at room temperature for 6 min. 150 μL of $AlCl_3$ solution (10% w/v) were added and allowed to stand for 5 min. Then, 500 μL of 1 M solution of NaOH and 275 μL of deionized water were added and absorbance was measured at 510 nm by UV–vis spectrophotometer Genesys 10S (Thermo Fisher Scientific Inc., Madison, WI). A blank was prepared in similar way, replacing the extract solution with ethanol. The total flavonoids content was expressed as mg of quercetin equivalent per gram of extract (mg QUE/g) based on the standard calibration curve.

2.3.2. Individual quantification of target flavonoids

The quercetin, kaempferol and resveratrol contents were quantified according to the method reported by Kim (2016) with some modifications, using a HPLC–DAD 1260 infinity (Agilent Technologies, CA) equipped with a quaternary pump. Stock solutions of samples were prepared at the concentration of 10 mg/mL in methanol. Chromatographic separations were performed in an Agilent Zorbax rapid resolution high-definition (RRHD) SB-C18 column (SB: Stable bond) (2.1 mm i.d. × 100 mm, 1.8 μm, catalog number: 858758-902). The column temperature was 30 °C, the flow rate was 0.3 mL/min and the injection volume was 20 μL. Mobile phases A (water with 0.1% formic

acid) and B (acetonitrile with 0.1% formic acid) were used for gradient elution. The gradient elution program used was as follows: 0% B (0 min), 5% (0–3.5 min), 15% (3.5–7.1 min), 40% (7.1–25 min), 40% (25–26 min), 100% (26–27 min), 100% (27–29 min) and 0% (29–35 min). Individual flavonoids were identified by comparing their retention times with those of pure standards. Solutions of standards were prepared at the concentration of 0.1 mg/mL in methanol. Quantification was carried out by the internal standard method using naringenin. The HPLC-DAD was controlled using the Agilent ChemStation Software.

2.3.3. Individual quantification of target terpenes

The α-thujone, β-caryophyllene and caryophyllene oxide contents were quantified according to Uquiche and Martínez (2016) using an Agilent 6850 series gas chromatograph (Agilent Technologies, CA) with a flame ionization detector equipped with a HP-5 capillary column (0.25 mm i.d. × 30 m, 0.25 μm, catalog number: 19091S-433). The internal standard method was used for quantification, using 1,2,4,5-tetramethylbenzene as the internal standard. The GC-FID was controlled using the Agilent ChemStation Software.

2.3.4. Antioxidant activity

The antioxidant activity of the selected extract was measured at 520 nm by spectrophotometer. DPPH radical scavenging assay (DPPH assay) was carried out according to the method reported by Brand-Williams et al. (1995) with minor modifications (Uquiche and Martínez, 2016). DPPH assay was expressed as mmol TROLOX equivalent (TE) per kilogram of extract (mmol TE/kg) based on the standard calibration curve. The ferric-reducing antioxidant power assay (FRAP assay) was carried out according to the method reported by Benzie and Strain (1996) with minor modifications described in Millao and Uquiche (2016). The FRAP value was calculated and expressed as mmol Fe^{+2} equivalent per kilogram of extract (mmol Fe^{+2}/kg) based on the standard calibration curve constructed using aqueous solutions of $FeSO_4{\cdot}7H_2O$. The β-carotene bleaching assay (BCB assay) was carried out according to the method reported by Koleva et al. (2002) with minor modifications (Uquiche and Martínez, 2016). β-carotene bleaching was expressed as the extract concentration (mg/mL) that provides 50% inhibition (IC_{50}) against oxidation of linoleic acid.

2.3.5. Inhibition of α-amylase

The inhibition of α-amylase was carried out according to the method reported by Rahali et al. (2017) with some modifications, based on colorimetric quantification of the maltose released by the action of the enzyme on the starch. Extract solution was prepared in DMSO at different concentrations (2–20 mg/mL). For the preparation of the color reagent, 20 mL of 96 mM solution of DNSA were mixed with a solution of sodium potassium tartrate (12 g of potassium sodium tartrate dissolved in 8 mL of 2 M sodium hydroxide), and made up to a final volume of 40 mL with deionized water. For the inhibition assay, 200 μL of extract solution were mixed with 400 μL of starch solution (1% w/v in phosphate buffer) and incubated for 3 min at 37 °C. Then 200 μL of enzyme solution (2 U/mL in phosphate buffer) were added and incubated for another 3 min at 37 °C. 400 μL of DNSA solution were added and heated at 95 °C for 15 min; the solution was cooled to ˜20 °C, then 3600 μL of deionized water was added. The absorbance at 540 nm was measured in the spectrophotometer. Maltose was quantified using a calibration curve. A blank was prepared by replacing 200 μL of enzyme solution with 200 μL of phosphate buffer. A control representing 100% of the enzymatic reaction was prepared by replacing 200 μL of extract solution with 200 mL of DMSO solution. The inhibition (%) was calculated using the Eq. 1 below. A positive control was carried out with acarbose.

$$\%\,\text{Inhibition} = \left[1 - \frac{\text{mg maltose}_{\text{sample}}}{\text{mg maltose}_{\text{control}}}\right] \times 100 \quad (1)$$

2.3.6. Inhibition of α-glucosidase

The inhibition of α-glucosidase was determined according to the method reported by Indrianingsih et al. (2015) with some modifications. Solutions of extract in DMSO were prepared at different concentrations (1–3 mg/mL). A 3 mM solution of *p*NPG was prepared as substrate. The enzyme solution was prepared at a concentration of 1 U/mL in phosphate buffer. For the inhibition test 50 μL of extract solution were mixed in a test tube with 125 μL of *p*NPG and 1250 μL of phosphate buffer, and incubated for 10 min at 37 °C. To start the reaction, 50 μL of the enzyme solution were added and incubated for 20 min at 37 °C. Then the reaction was stopped by adding 4000 μL of 0.1 M solution of Na_2CO_3. A blank solution was prepared by replacing 50 μL of the enzyme solution with 50 μL phosphate buffer. A control assay representing 100% of the enzymatic reaction was performed by replacing the 50 μL of the extract solution with 50 μL DMSO. The activity of α-glucosidase was determined by measuring the *p*-nitrophenol released from the hydrolysis of *p*NPG at 400 nm in the spectrophotometer. The inhibition (%) was calculated with the following equation:

$$\text{Inhibition}\,(\%) = \left[1 - \left(\frac{A_{\text{sample}} - A_{\text{blank}}}{A_{\text{control}}}\right)\right] \times 100 \quad (2)$$

2.4. Experimental design

A Box-Behnken design was used to evaluate the effects of the coded temperature (X_1, Eq. 3, where T is temperature in °C), coded pressure (X_2, Eq. 4, where P is pressure in MPa) and coded co-solvent concentration (X_3, Eq. 5, where C is ethanol concentration in wt.%), all expressed in dimensionless units, on the extraction yield (Y, g/kg d.s.).

$$X_1 = \frac{T - 50}{10} \quad (3)$$

$$X_2 = \frac{P - 15}{5} \quad (4)$$

$$X_3 = \frac{C - 1}{1} \quad (5)$$

The independent variables and their levels are shown in Table 1. Experiments were conducted in randomized order to minimize the effects of the uncontrolled factors. The design points are shown in Table 2; six replications of the centre points were used to determine experimental error.

A second-order model (Eq. 6) was used to describe response variable Yas a function of the independent variables (X_1, X_2 and X_3), where A_0 is the constant; A_1 and A_2 are linear coefficients; A_{12} is the cross-product coefficient; and A_{11} and A_{22} are quadratic coefficients. The response surface plot was generated by varying the two variables within the experimental region. The goodness of fit of the model was evaluated by analysis of variance (ANOVA). The coefficients of the second-order model were estimated using Design-Expert Software, version 6.0.1

Table 1
Independent variables and their levels for Box Behnken design.

Independent variables	Variable levels		
	−1	0	+1
Coded temperature, X_1 (°C)	40	50	60
Coded pressure, X_2 (MPa)	10	15	20
Coded co-solvent concentration, X_3 (wt.%)	0	1	2

$X_1 = \frac{T-50}{10}$; $X_2 = \frac{P-15}{5}$; $X_3 = \frac{C-1}{1}$

Table 2
Box Behnken design and experimental data of extraction yield (Y) of *L. rivularis* leaves as a function of temperature (T), pressure (P) and ethanol concentration (C) of supercritical CO_2 extraction.

Set	Independent variables						Response (Y)	
	T °C	P MPa	C wt.%	X_1 (−)	X_2 (−)	X_3 (−)	Experimental g/kg d.s.	Predicted g/kg d.s.
1	40	10	1	−1	−1	0	24.9 ± 1.8	22.6
2	60	10	1	1	−1	0	17.2 ± 2.1	15.6
3	40	20	1	−1	1	0	38.3 ± 2.3	40.0
4	60	20	1	1	1	0	49.7 ± 0.4	52.1
5	40	15	0	−1	0	−1	32.8 ± 2.7	33.0
6	60	15	0	1	0	−1	38.3 ± 0.4	35.5
7	40	15	2	−1	0	1	42.9 ± 1.2	40.7
8	60	15	2	1	0	1	43.9 ± 3.5	43.2
9	50	10	0	0	−1	−1	14.7 ± 2.7	15.2
10	50	20	0	0	1	−1	42.9 ± 1.8	42.2
11	50	10	2	0	−1	1	19.6 ± 2.3	22.9
12	50	20	2	0	1	1	53.1 ± 0.7	49.9
13	50	15	1	0	0	0	38.4 ± 2.7	38.1
14	50	15	1	0	0	0	37.0 ± 3.5	38.1
15	50	15	1	0	0	0	34.8 ± 1.5	38.1
16	50	15	1	0	0	0	36.6 ± 1.7	38.1
17	50	15	1	0	0	0	38.1 ± 2.3	38.1

(Stat-Ease, Inc., Minneapolis, MN). All extraction and analysis assays were carried out in duplicate. The statistical significance was determined with a confidence level of 95%. To characterize the response surface, a canonical analysis was carried out by transforming the model to the canonical form, determining the eigenvalues (λ_i) according to the procedure described by Myers and Montgomery (1995).The canonical form of a quadratic equation determines the relative sensitivity of the response variables to each independent variable. The eigenvalues and their signs determine the type of stationary point according to the following rule: if eigenvalues are positive, the surface has a minimum; if eigenvalues are negative, the surface has a maximum; if eigenvalues are different signs, the surface is a saddle-type (Gacula and Singh, 1984).

$$Y = A_0 + A_1X_1 + A_2X_2 + A_{12}X_1X_2 + A_{11}X_1^2 + A_{22}X_2^2 \quad (6)$$

3. Results and discussion

Table 2 shows experimental results of extraction yield (Y) as a function of temperature, pressure and co-solvent concentration. Extraction yield ranged from 14.7 to 53.1 g/kg d.s., a difference of 3.6-fold. Table 3 summarizes the statistical indicators obtained from the ANOVA applied to the model with significant coefficients only ($p \leq 0.01$). The model was significant (F-value = 59.888, $p \leq 0.0001$). The lack of fit relative to the pure error was non-significant ($p = 0.0961$); there was high signal-to-noise ratio (> 4) and high coefficient of determination ($R^2 = 0.96$ and *adjusted*-$R^2 = 0.95$). The information provided by the statistical indicators was complemented by a good correlation between predicted and experimental responses within the experimental range investigated (Fig. 1).

3.1. Extraction yield

ANOVA was used to evaluate the significance of the regression coefficients of the model (Table 3). A large regression coefficient and a small p-value would indicate a more significant effect on the response variable. Thus, the variable with the largest effect on the extraction yield was the linear coefficient of pressure ($A_2 = +13.46$), followed by a quadratic coefficient of pressure ($A_{22} = +5.561$), the temperature-pressure interaction ($A_{12} = +4.779$) and the linear coefficient of co-solvent concentration ($A_3 = +3.852$). There was no significant effect of

Table 3
Analysis of variance of regression coefficients and statistical appropriateness indicators of the response surface model selected (Eq. 6).

	Values	Sum of squares	DF	F-value	p-value
Model		1803.61	5	59.888	< 0.0001
A_1	+1.284	13.20	1	2.19	0.1669
A_2	+13.46	1449.38	1	240.63	< 0.0001
A_3	+3.852	118.70	1	19.71	0.0010
A_{22}	+5.561	130.98	1	21.75	0.0007
A_{12}	+4.779	91.35	1	15.17	0.0025
Lack of fit		58.12	7	4.08	0.0961
R^2	0.96				
Adjusted-R^2	0.95				
Signal/Noise ratio	25.26				
λ_1	+1.016				
λ_2	–6.497				
λ_3	+2.321				

A_1: linear coefficient of temperature.
A2: linear coefficient of pressure.
A3: linear coefficient of co-solvent concentration.
A22: quadratic coefficient of pressure.
A12: temperature-pressure interaction coefficient.
R2: coefficient of determination.
λi: Eigenvalues of canonical analysis.

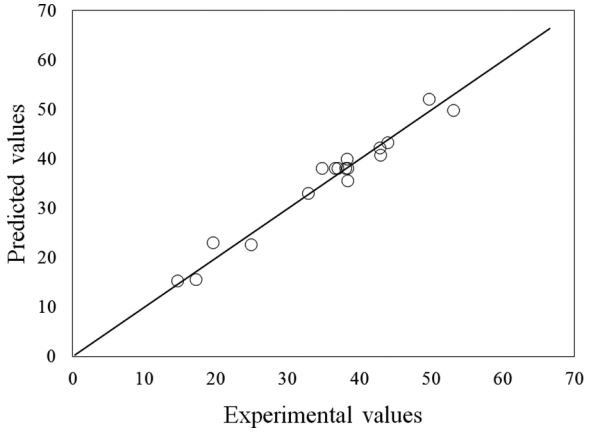

Fig. 1. Good correlation ($r = 0.98$) between predicted and experimental responses shows the goodness of fit of the response surface model for the extraction yield (Y, g/kg dry substrate).

the linear coefficient of temperature ($p = 0.1669$) but the coefficient was not removed to maintain the model's hierarchy. Therefore, the model establishes a statistically significant relationship between the response variable and the independent variables. The response surface graph of Eq. 7 was produced to better visualize the effect of the temperature, pressure and co-solvent concentration on the extraction yield within the experimental region (Fig. 2).

$$Y = 38.1061 + 1.284X_1 + 13.460X_2 + 3.852X_3 - 5.561X_2^2 + 4.779X_1X_2 \quad (7)$$

The temperature had no significant effect on the extraction yield, which shows that the pressure effect on the increased CO_2 density prevails over the vapor pressure increase with the temperature. The linear effect of pressure was important between 10 and 18 MPa, whatever the temperature. Over 18 MPa the quadratic effect of pressure becomes important. Thus, the surface tends to show a plateau at 40 °C, where Y was maintained at ˜36 g/kg d.s. However, because the $T \times P$ interaction was significant ($p = 0.0025$), the pressure effect cannot be discussed in isolation. The positive linear effect of pressure was higher at high temperature than at low temperature. In fact, the extraction yield increased 3.1-fold (15.6 – 48.3 g/kg d.s.) at 60 °C and 1.8-fold

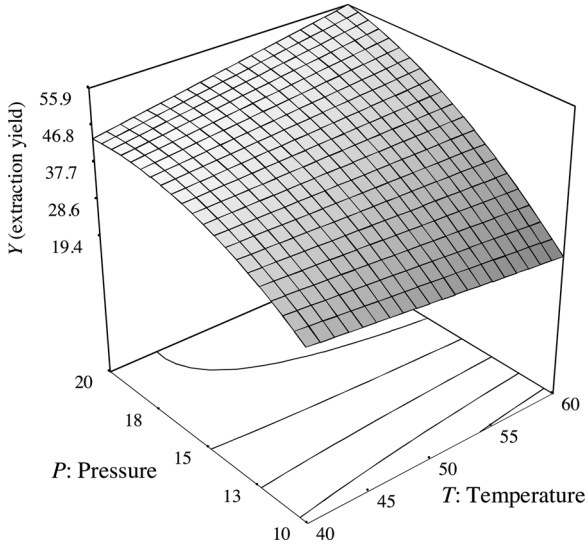

Fig. 2. Response surface graph for extraction yield (Y, g/kg dry substrate) as a function of temperature (T, °C) and pressure (P, MPa) at co-solvent concentration of 2 wt.%.

(22.6–40.0 g/kg d.s.) at 40 °C, when the pressure was increased from 10 to 18 MPa (Fig. 2). The positive effect of pressure on the increase of extraction yield can be explained by the improvement of solvent power of CO_2 due to the increase of its density with pressure. Danh et al. (2010) reported that the extraction yield from vetiver (*Vetiveria zizanioides*) roots using ethanol-modified supercritical CO_2 increased with the pressure (10–19 MPa), and this increase was greater at high temperature (50 °C) than low temperature (40 °C). This behavior was consistent with our results.

The positive effect of co-solvent concentration on extraction yield showed an increase from 34.3 to 42.0 g/kg d.s. when the co-solvent concentration was increased from 0 to 2 wt.% at 50 °C and 15 MPa. The quadratic effect (A_{33}) of co-solvent concentration or its interactions (A_{13} or A_{23}) were not significant (Fig. 2). Bensebia et al. (2009) reported that the extraction yield of rosemary leaves increased with pressure (10–18 MPa) and with the addition of ethanol (0–3 wt.%). The increase in extraction yield with the addition of ethanol has been related to the extraction of more polar compounds from vegetable substrate, which is consistent with the increasing polarity and solvent power of supercritical CO_2 (Langenfeld et al., 1994). However, this behavior disagrees with the results reported by Daukšas et al. (2001), where the increase in ethanol from 1 to 2 wt.% reduced the extraction yield from sage. This demonstrates that an increase in the polar co-solvent does not always mean an increase in extraction yield. Ghasemi et al. (2011) and Khajeh (2011) studied the effect of pressure, temperature and co-solvent on the extraction yield from leaves of *Myrtus communis* and *Satureja hortensis*, respectively. These studies reported that both pressure and co-solvent had a positive effect on the extraction yield. The results of these studies are consistent with our current study.

3.2. Canonical analysis

Partial differentiation of Eq. 7 with respect to independent variables and set to zero was applied to find optimum values of temperature, pressure and co-solvent concentration, which are called stationary points. The stationary points for Eq. 7 were outside the experimental region, so these values were not considered because the fitted model would not be reliable outside that region. We therefore carried out a canonical analysis. According to canonical analysis, the eigenvalues obtained were $\lambda_1 = +1.016$, $\lambda_2 = -6.497$ and $\lambda_3 = +2.321$ (Table 3); as these were of different signs, the surface corresponded to a saddle-type response surface, which is neither a point of maximum nor minimum response. According to this analysis, the selected extraction condition producing the highest extraction yield was 60 °C, 20 MPa and 2 wt.% of ethanol. In terms of absolute value, it was observed that $|\lambda_2| > |\lambda_3| > |\lambda_1|$. Since the absolute value of λ_2 is highest, this confirms that the response surface increases more rapidly in the direction of the pressure variable, followed by the co-solvent concentration variable. Therefore, a region of high pressure and co-solvent concentration was appropriate for a high extraction yield.

Table 4
Bioactive properties of selected supercritical extract of *L. rivularis* leaves using CO_2 modified with ethanol (60 °C, 20 MPa and 2 wt%).

Characteristic	
DPPH (mmol TE/kg)	4.24 ± 0.03
β − carotene bleaching, IC_{50} (mg/mL)	3.87
FRAP (mmol Fe (II)/kg)	159.40 ± 1.33
Total flavonoids content (mg QUE/g)	176.60 ± 2.95
Target terpenoids content (mg/g)	
α-thujone	3.71 ± 0.09
β-caryophyllene	2.88 ± 0.09
Caryophyllene oxide	± 0.24
Target flavonoids content (mg/g)	
Quercetin	0.098 ± 0.003
Kaempferol	0.166 ± 0.008
Resveratrol	1.414 ± 0.265
Inhibition of α-amylase (IC_{50}, mg/mL)	16.9 ± 1.6
Inhibition of α-glucosidase (IC_{50}, mg/mL)	2.7 ± 0.1

3.3. Extract analysis

According to the previous analysis, the condition 60 °C, 20 MPa and 2 wt.% was selected to obtain and characterize the *L. rivularis* extract, resulting in an extraction yield of 56.6 ± 2.2 g/kg d.s., which validates the model with a predicted value of 55.9 g /kg d.s.

3.3.1. Total flavonoids content

The total flavonoids concentration in supercritical extract was 176.6 mg QUE/g (Table 4). These values are within the flavonoids concentration range from herb leaves as substrate, using ethanol-modified supercritical CO_2. Ouédraogo et al. (2018) reported between 99.33–247.78 mg/g for *Odontonema strictum* leaves (55–65 °C, 20–25 MPa); Bimakr et al. (2012) reported 60.57 mg/g (60 °C, 20 MPa) for spearmint (*Mentha spicata* L.) leaves.

3.3.2. Individual quantification of flavonoids

Quantification of individual flavonoids for the selected extract showed contents (mg/g) of 0.098 for quercetin, 1.414 for resveratrol and 0.166 for kaempferol (Table 4). Uquiche et al. (2019) reported the following contents of flavonoids for extract from *L. rivularis* stalks, extracted using ethanol-modified supercritical carbon dioxide: catechin = 0.325 mg/g; quercetin = 0.164 mg/g; resveratrol = 0.366 mg/g. Bimakr et al. (2012) reported between 0.081 and 0.135 mg/g of catechin in supercritical extract from spearmint (*Mentha spicata* L.) leaves. Sulastri et al. (2018) reported a quercetin content of 0.064 mg/g in ethanolic extract from *Moringa oleifera* leaves. Thus, the values reported in Table 4 are comparable with flavonoid content values reported in the literature.

These flavonoids have important bioactive properties. Quercetin exhibits antioxidant properties (Boots et al., 2008; Jan et al., 2010), anti-inflammatory properties (Jan et al., 2010), cardiovascular protection (Boots et al., 2008), and anti-cancer (Boots et al., 2008), anti-bacterial, anti-viral (Jan et al., 2010) and anti-diabetic effects (Shetty et al., 2004). Kaempferol possesses antioxidant, anti-inflammatory, anti-cancer (Calderón-Montaño et al., 2011; Chen and Chen, 2013) and anti-microbial properties, cardiovascular protection (Calderón-Montaño et al., 2011) and anti-diabetic effects (de Sousa et al., 2004). Resveratrol shows antioxidant, anti-inflammatory and anti-cancer

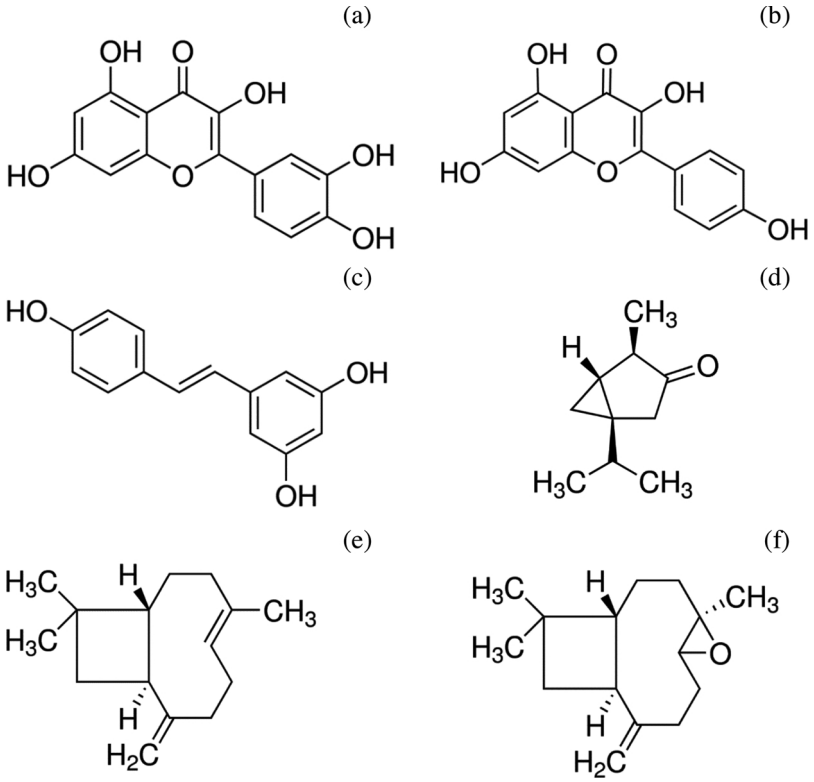

Fig. 3. Molecular structures of flavonoids: (a) quercetin, (b) kaempferol, (c) resveratrol, and terpenoids: (d) α-thujone, (e) β-caryophyllene, (f) caryophyllene oxide.

properties, cardiovascular protection and anti-diabetic effects (Yu et al., 2012).

3.3.3. Individual quantification of terpenes

Quantification of individual terpenes for the selected extract showed contents (mg/g) of 3.71 for α-thujone, 2.88 for β-caryophyllene and 6.29 for caryophyllene oxide (Table 4). Uquiche and Martínez (2016) reported terpenoid contents in extract of *L. rivularis* leaves obtained with supercritical CO_2 (40 °C and 10 MPa): α-thujone = 2.38 mg/g; β-caryophyllene = 4.35 mg/g; caryophyllene oxide = 7.49 mg/g. Quispe-Condori et al. (2008) reported a concentration of between 11.9 and 24.8 mg/g β-caryophyllene in extract from *Cordia verbenacea* using supercritical CO_2. The values are of the same order of magnitude with those presented in Table 4. The terpenes have shown important bioactive capacity. α-thujone exhibits anti-diabetic effects (Alkhateeb and Bonen, 2010) and hypocholesterolemic properties (Baddar et al., 2011). β-caryophyllene possesses anti-cancer (Dahham et al., 2015), anti-inflammatory (Sain et al., 2014) and antioxidant properties (Dahham et al., 2015). Caryophyllene oxide possesses several biological effects such as anti-inflammatory activity (Sain et al., 2014).

3.3.4. Antioxidant activity

The antioxidant activity of the selected extract measured by DPPH assay (4.24 mmol TE/kg) (Table 4) represents the capacity of the extract to stabilize a free radical compared to TROLOX, a standard antioxidant. DPPH is a free radical which becomes yellowish when reduced by an antioxidant hydrogen donor (Brand-Williams et al., 1995). The reaction is accompanied by a decrease in absorbance, which can correlate with the antioxidant content in the extract. Uquiche and Martínez (2016) reported antioxidant activity of 1.85 mmol TE/kg for leaf extract obtained with supercritical CO_2 (40 °C and 10 MPa); and Uquiche et al. (2019) reported 3.02 mmol TE/kg for stalk extract with ethanol-modified CO_2 (60 °C, 40 MPa, 2 wt.%). The antioxidant activity measured by FRAP assay was 159.40 mmol Fe^{+2}/kg (Table 4), which represents its capacity to reduce ferric ion (F^{+3}) to ferrous ion (F^{+2}) in a redox reaction, in which one reactive species is reduced at the expense of the oxidation of another (Benzie and Strain, 1996). The reduction is monitored by measuring the absorbance at 593 nm, and the amount of iron reduced can be correlated with the antioxidant content. Antioxidant activity measured by BCB assay showed the antioxidant capacity of linoleic acid (IC_{50} = 3.87 mg/mL). The IC_{50} value indicates the extract concentration (mg/mL) that provides 50% inhibition, through the donation of hydrogen atoms to neutralize free radicals. Uquiche and Martínez (2016) informed an IC_{50} = 70 mg/mL for supercritical extract of *L. rivularis* leaves.

Antioxidants are bioactive compounds that retard the oxidation of organic matter promoted by free radicals; the latter are involved in the damage of cell components and the development of diseases. In diabetes, the production of free radicals increases due to the increase in oxidative stress, while the production of antioxidants decreases. Thus increased oxidative stress is considered to be one of the most important complications of diabetes. Flavonoids have the capacity to stabilize free radicals because of their ability to donate hydrogen and an electron, resulting in a relatively stable radical (Agati et al., 2012). Moalin et al. (2011) pointed out that the antioxidant activity of flavonoids is dependent of the presence of hydroxyl groups in ring B, and also on the total number of hydroxyl groups (Fig. 3a–c). Terpenes could have an antioxidant role in plants. Terpenes correspond to compounds derived from the isoprene unit. It has been suggested that, on the basis of its chemical structure, the isoprene molecule has the capacity to quench free radicals, which may be associated with the presence of conjugated double bonds (Fig. 3d–f) (Velikova, 2008). For this reason terpenes and their isoprene units are important in protection against oxidative stress.

3.3.5. Enzyme inhibitory activity

Reducing glucose absorption is an important element in the control of diabetes mellitus; it can be achieved by inhibiting α-amylase and α-glucosidase, digestive enzymes that hydrolyze carbohydrate. Uquiche et al. (2019) reported the inhibitory capacity (IC_{50} value) against

Table 5
Percent inhibition of α-amylase and α-glucosidase by selected supercritical extract of *L. rivularis* leaves using CO_2 modified with ethanol (60 °C, 20 MPa and 2 wt%) at varying concentrations (IC_{50}: medium inhibitory concentration).

Enzyme α-amylase			Enzyme α-glucosidase		
Concentration (mg/mL)	Inhibition (%)	IC_{50} mg/mL	Concentration (mg/ml)	Inhibition (%)	IC_{50} mg/mL
2	44.2 ± 2.1	16.9 ± 1.6	1.0	26.5 ± 2.7	2.7 ± 0.1
6	45.5 ± 0.6		1.5	32.1 ± 2.9	
10	47.8 ± 0.3		2.0	43.0 ± 1.6	
20	51.2 ± 0.9		3.0	52.8 ± 0.6	

carbohydrase enzymes of supercritical extract of *L. rivularis* stalks using CO_2 modified with ethanol (60 °C, 40 MPa, 1 wt.%) of 15.1 mg/mL for α-amylase and 2.7 mg/mL for α-glucosidase. The enzyme inhibitory activity values of the extract (Table 5) are within the range reported for the inhibition of α-amylase and α-glucosidase with leaf extract from *Orthosiphon stamineus* (IC_{50}: 36.7 and 4.63 mg/mL respectively) (Mohamed et al., 2012) and *Orthosiphon stamineus* (IC_{50}: 3.21 mg/mL and 3.06 mg/mL respectively) (Ademiluyi et al., 2016). Flavonoids from *Polygonatum odoratum* (Shu et al., 2009) and *Psidium Guajava* (Wang et al., 2010) have shown effective inhibition of α-amylase and α-glucosidase. Constituent terpenes of essential oils from *Ocimum basilicum* (Ademiluyi et al., 2016) have shown inhibitory properties in α-amylase and α-glucosidase. Thus the flavonoids and terpenes contained in the extract have demonstrated beneficial properties against diabetes.

4. Conclusion

The Box-Behnken design was suitable for evaluating extraction, and for determining the conditions to achieve high extraction yield. The pressure and ethanol concentration affected the extraction yield significantly. The effect of the co-solvent was independent of the other factors. The highest extraction yield was reached at 60 °C 20 MPa and 2 wt.% of ethanol. Bioactive compounds were quantified in the selected extract, such as flavonoids: quercetin, kaempferol and resveratrol; and terpenes: α-thujone, β-caryophyllene and caryophyllene oxide. The extract presented antioxidant activity and the capacity to inhibit key enzymes of diabetes (α-amylase and α-glucosidase), showing its potential benefits for health.

Conflict of interest

None.

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