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Inhibition of α-amylase activity by extracts from *Leptocarpha rivularis* stalks obtained with supercritical CO_2

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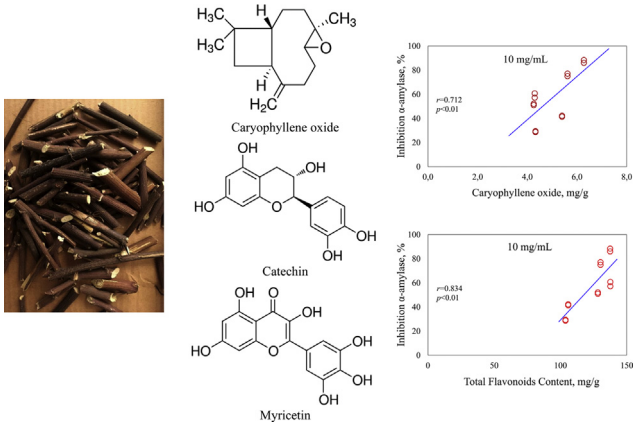
HIGHLIGHTS

- The highest inhibitory capacity against α-amylase was reached at 60 °C and 40 MPa.
- The highest caryophyllene oxide contents was achieved at 60 °C and 40 MPa.
- The highest contents of catechin and myricetin were obtained at 40 °C and 40 MPa.
- Important flavonoids such as catechin, myricetin, resveratrol and kaempferol were quantified.
- The capacity of the extract to inhibit α-amylase enzyme was demonstrated.

GRAPHICAL ABSTRACT



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ABSTRACT

We studied supercritical extraction with ethanol-modified CO_2, combining different levels of temperature (40 and 60 °C) and pressure (20, 30 and 40 MPa), to obtain extracts from *Leptocarpha rivularis* stalks with inhibitory capacity against α-amylase, an enzyme associated with diabetes mellitus. The extracts were measured for their ability to inhibit α-amylase, and for contents of total flavonoids, catechin and myricetin and caryophyllene oxide. The highest inhibitory capacity against α-amylase was obtained with extract at 60 °C and 40 MPa, producing the highest total flavonoids (137.6 mg/g) and caryophyllene oxide (6.28 mg/g) contents. The highest contents of catechin (1.98 mg/g) and myricetin (0.12 mg/g) were obtained at 40 °C and 40 MPa. Part of the hypoglycemic mechanism of the extract would be associated with the inhibitory capacity against α-amylase, due to the presence of flavonoids and caryophyllene oxide.

1. Introduction

Diabetes mellitus is a disease characterized by high levels of blood glucose that the body's cells cannot use, due to defects in the secretion and/or action of the hormone insulin [1]. This disease results in changes in the metabolism of carbohydrates, fats and proteins, causing hyperglycemia, hyperlipidemia and atherosclerosis.

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Diabetes mellitus is an important cause of death in people aged under 60 years. Worldwide, it affected more than 415 million people in 2015 and this number is expected to increase to 642 million by 2040. One of the available therapies is to decrease the absorption of glucose, through inhibition of starch breakdown enzymes such as α-amylase [2].

Pancreatic α-amylase is a key enzyme in the digestive system. It catalyzes the hydrolysis of the α-1,4 glycosidic linkages of complex carbohydrates like starch to a mixture of smaller oligosaccharides consisting of maltose, maltotriose and a series of oligoglycans [3]. It has been shown that the activity of human pancreatic α-amylase in the small intestine correlates with an increase in the levels of blood glucose; controlling these is, therefore, an important aspect of the treatment of diabetes mellitus [4]. Controlling glucose production from complex carbohydrates is considered to be effective in controlling diabetes.

The literature contains reports of the properties of flavonoids (*e.g.* catechin, myricetin) as inhibitors of carbohydrate hydrolyzing enzymes relevant to diabetes mellitus [5,6]. The specialized literature reports studies on the benefits of catechins, including anti-diabetic [7], anti-oxidant [8], anti-carcinogenic, cardioprotective and neuroprotective [9] effects. The beneficial properties of myricetin to preserve health include anti-oxidant [10], anti-diabetic [11], cardioprotective [12] and anti-inflammatory [13] effects. On the other hand, most volatile components in essential oils are terpenoids, which are secondary plant metabolites derived from isoprene. Monoterpenes, sesquiterpenes, and oxygenic derivatives in herbal plant extracts are known to inhibit the activity of carbohydrate hydrolyzing enzymes. Analgesic, anti-inflammatory activity [14], cardioprotective [15] effects have been reported in caryophyllene oxide.

Extraction with supercritical carbon dioxide (SC-CO_2) produces extracts at low temperatures, thus avoiding the degradation of active compounds. Supercritical extraction allows extraction with greater selectivity and efficiency, by controlling temperature and pressure conditions. Manipulation of the operating temperature and pressure regulates the CO_2 density, which determines the solvent power of CO_2. Furthermore, the addition of polar co-solvents (*e.g.* ethanol) in a low concentration allows for interactions with the solute of various types (*e.g.* dipole–dipole, dipole–induced dipole, or induced dipole–induced dipole (dispersion), and specific interactions such as a hydrogen bond) [16], which improves the co-solvent effect on the solubility of polar compounds in the mixed solvent rather than in pure SC-CO_2. Furthermore, CO_2 is widely used because it is non-toxic, chemically stable, environmentally acceptable and easily separated from the extract since it exists as a gas under normal atmospheric conditions.

Medicinal plants are characterized by the presence of secondary metabolites such as flavonoids and terpenoids, and these traditional herbs have been used since ancient times by native peoples for their medicinal properties. *L. rivularis* (known as "palo negro") is an evergreen bush belonging to family the Asteraceae that grows in the south of Chile; it is used as a treatment for different illnesses such as cancer and hyperglycaemia [17].

The α-amylase inhibition capacity of extracts from *L. rivularis* obtained under supercritical extraction conditions have not been investigated. Uquiche et al. [18] studied the effect of the modified concentration and extraction pressure on the bioactive properties of an extract from *L. rivularis* stalks. Stalk extracts obtained under a selected extraction condition demonstrated the ability to inhibit α-amylase (56.8 ± 4.4 %), showing its potential benefits for health. The aim of our work was to evaluate the effect of temperature and pressure conditions on the inhibitory capacity of α-amylase and bioactive compounds of extracts from *L. rivularis* stalks obtained using ethanol-modified CO_2.

2. Materials and methods

Analytical grade ethanol and methanol were procured from J.T. Baker (New Jersey, USA). DPPH (2,2-diphenyl-1-picrylhydrazyl) was purchased from Calbiochem Co. (California, USA). Acetonitrile, methanol and water, all chromatography grade (LiChrosolv Reag. Ph Eur), and DMSO (dimethyl sulfoxide) and formic acid were obtained from Merck KGaA (Darmstadt, Germany). Iron (II) sulfate heptahydrate and iron (III) chloride anhydrous were purchased from ACROS Organics (New Jersey, USA). Potassium sodium tartrate tetrahydrate, sodium hydroxide, α-amylase from *Bacillus licheniformis*, TROLOX (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), DNSA (3,5-Dinitrosalicylic acid), pNPG (p-Nitrophenyl β-D-glucopyranoside), p-Nitrophenol, 1,2,4,5-tetramethylbenzene, caryophyllene oxide, gallic acid, catechin, kaempferol, myricetin, naringenin, quercetin, resveratrol, maltose monohydrate from potato were procured from Sigma-Aldrich (Missouri, USA). Soluble starch was acquired from Scharlau Chemie S.A. (Barcelona, Spain). Phosphate buffer pH 7 was obtained from Winkler Ltda. (Santiago, Chile).

2.1. Pretreatment and characterization of substrate

Samples of *L. rivularis* stalks as substrate were provided by Agrícola Los Esteros (La Unión, Chile) (39°52′S, 73°14′W). The stalks were dried in an oven (Memmert UF110, Schwabach, Germany) at 50 °C for 10 h. The moisture content of the substrate was determined gravimetrically, based on drying the substrate in an oven at a temperature of 105 °C until it reaches a constant weight. The substrate was ground in a Moulinex chopper (model AD5661AR, Moulinex, Ecully, France). The particle size distribution was determined using a series of ASTM sieves with different mesh sizes and the Ro-Tap testing sieve shaker (model RX-29-10, W.S. Tyler, Mentor, OH, USA). Average particle diameter (d_p) was calculated using the standard method [19]. The bulk density of the substrate (ρ_{bulk}, kg/m^3) was determined using the liquid displacement method. The apparent density of substrates (ρ_a, kg/m^3) was determined gravimetrically, weighing the material loaded in a 100 cm^3 graduated cylinder using a standard tapping procedure. The bed porosity ($\varepsilon = 1 - \rho_a/\rho_{bulk}$) of the substrate was determined using the values of bulk density and apparent density. Measurements were made in triplicate. The substrate samples were stored packed in plastic bags in the absence of oxygen under dry, dark conditions until later use.

2.2. Supercritical extraction

Supercritical extraction assays were carried out in a Spe-ed SFE-2 process development unit (model 7072, Applied Separations, Allentown, PA), loading 24 g of substrate in a 100 cm^3 extraction vessel (30 mm internal diameter). The pressure (20–40 MPa) was controlled manually with an air-booster pump; the temperature of the air bath (convection oven) containing the extraction vessel (40 and 60 °C) was controlled automatically. Depending on the extraction conditions, 3.8–5.1 L NTP (Normal Temperature and Pressure)/min of food grade (99.95 % pure) CO_2 (Linde Chile S.A.) was fed to the extraction vessel. The 20-min static extraction period was followed by a dynamic extraction period, which varied between 79 and 105 min in order to obtain a total solvent consumption of 30 kg CO_2/kg d.s. The expansion valve was kept at 100 °C. The air inside the extractor was purged by careful displacement with CO_2. The extractions were carried out in duplicate. Ethanol as co-solvent was pumped at a flow rate between 0.17 and 0.23 mL/min into the CO_2 feed line to achieve a modifier concentration of 2 wt.%, using an HPLC pump (Knauer, model K-501, Berlin, Germany) (flow accuracy<1 %, at 1 mL/min, 12 MPa). The extract was collected in previously weighed vials (60 mL). The collected

extracts were subjected to a stream of nitrogen to evaporate the ethanol.

2.3. Analysis of extract

2.3.1. Inhibition of α-amylase

The inhibition of α-amylase was determined according to Rahali et al. [20] with some modifications [18,21]. The activity of α-amylase was measured by the colorimetric method, based on hydrolysis of starch as a substrate and reaction of the released maltose with 3,5-dinitrosalicylic acid (DNSA). The maltose was quantified by spectrophotometry reading at 540 nm and using a calibration curve. A positive control was carried out with acarbose.

2.3.2. Individual quantification of flavonoids

Individual flavonoids contents were quantified according to Kim [22] with some modifications [19]. Quantification was carried out using using a HPLC–DAD 1260 infinity (Agilent Technologies, CA) fitted with a quaternary pump, equipped with an Agilent Zorbax rapid resolution high-definition (RRHD) SB-C18 column (2.1 mm i.d. × 100 mm, 1.8 μm, catalog number: 858758-902). Quantification was carried out by the internal standard method using naringenin. The HPLC-DAD was controlled using the Agilent ChemStation Software.

2.3.3. Total flavonoids content

Total flavonoids content was quantified according to Zhishen et al. [23] with some modifications [20]. Quantification was performed by spectrophotometry at 510 nm in an UV–vis spectrophotometer Genesys 10S (Thermo Fisher Scientific Inc., Madison, WI). The total flavonoids content was expressed as mg of quercetin equivalent per gram of extract (mg/g), based on the standard calibration curve.

2.3.4. Individual quantification of sesquiterpene

Caryophyllene oxide was quantified according to Gong et al. [24] with some modifications [25], using a GC-FID 6850 (Agilent Technologies, CA) equipped with a flame ionization detector and 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.

2.3.5. Type of inhibition

The type of α-amylase inhibition of the selected extract was determined according to the procedure described by Sabiu et al. [26]. A solution of α-amylase in phosphate buffer at a concentration of 2 U/mL was used. Soluble starch was used as substrate at different concentrations, [*S*]: 0, 2, 8 and 15 mg/mL. Assays were carried out with extract solution in DMSO at different concentrations (0, 0.5, 6 and 10 mg/mL). Extract solutions (1000 μL) were mixed with 2000 μL of starch in a test tube and incubated for 3 min at 37 °C. Next, 1000 μL of enzyme solution were added and allowed to react for different time intervals: 0, 0.5, 1.0, 1.5, 2, 2.5 and 3 min. Then 400 μL of DNSA were added to 800 μL of the mixture. It was boiled for 15 min, allowed to cool, and 3600 μL of deionized water were added. The activity of α-amylase was quantified by measuring the maltose equivalents at different time intervals, calculated with absorbance reading at 540 nm and using a calibration curve for maltose. The reaction velocities (*V*, mmol of maltose released per minute) were calculated with this data. A blank solution was prepared, replacing 1000 μL of enzyme per 1000 μL of phosphate buffer. Finally, a mixture to correct the apparent change of maltose at each time interval was prepared without the addition of substrate and extract, replacing them with deionized water and DMSO respectively. A Lineweaver-Burk plot (1/*V versus* 1/[*S*]) with the data kinetic was constructed and the type of inhibition against α-amylase was determined. Experiments were repeated two times.

Table 1
Experimental design for extraction assays.

Set	*T*, °C	*P*, MPa
1	40	20
2	40	30
3	40	40
4	60	20
5	60	30
6	60	40

Table 2
Characteristics of milled *L. rivularis* stalks.

Characteristics	
Moisture content (%)	5.72 ± 0.2
Average particle size (d_p, mm)	0.74 ± 0.04
Particle density (kg/m^3)	751.8 ± 6.8
Bed density (kg/m^3)	246.1 ± 2.3
Bed porosity (%)	67.3 ± 0.5

2.3.6. Antioxidant activity

The antioxidant capacity of the selected extract was measured using DPPH radical scavenging assay and ferric-reducing antioxidant power (FRAP) assay. The DPPH assay was performed according to Brand-Williams et al. [27] with some modifications [24]. The anti-radical activity (DPPH assay) was measured by spectrophotometry at 510 nm and expressed as the equivalent of millimol TROLOX (TE) per kilogram of extract (mmol of TE/kg) based on a standard calibration curve. The FRAP assay was carried out according to Benzie and Strain [28] with some modifications. The antioxidant power was measured by spectrophotometry at 593 nm and expressed as millimol Fe^{+2} equivalents per kilogram of extract (mmol Fe^{+2}/kg) based on the standard calibration curve constructed using aqueous solutions of $FeSO_4{\cdot}7H_2O$.

2.4. Experimental design and statistical analysis

Experiments to evaluate the effects of temperature (40 and 60 °C) and pressure (20, 30, 40 MPa) on α-amylase inhibition, content of total flavonoids, catechin, myricetin and caryophyllene oxide, were performed according to the 2 × 3 factorial design shown in Table 1. Extraction assays were carried out in duplicate. In the statistical analysis of the results, the differences were established with a confidence level of 95 % using Design-Expert Software, version 6.0.1 (Stat-Ease, Inc., Minneapolis, MN, USA).

3. Results and discussion

3.1. Substrate characteristics

The stalks used as substrate were dried at 50 °C. Studies on drying in vegetable raw material have reported that a temperature equal to or greater than 60 °C caused a reduction in the phenol and flavonoid content in *Stevia rebaudiana* leaves [29] and phenol content in quinoa seeds (*Chenopodium quinoa* Willd.) [30]. There was no effect at 50 °C; however, at 80 °C a notable reduction of these compounds was observed. Table 2 presents the characteristics of the substrate used for the extraction assays. The average particle size was considered suitable for extraction, since with very fine particles (*e.g.* <0.1 mm) compaction of the substrate occurs within the extractor, leading to channeling of solvent flow through the vessel which decreases extraction efficiency. The substrate moisture

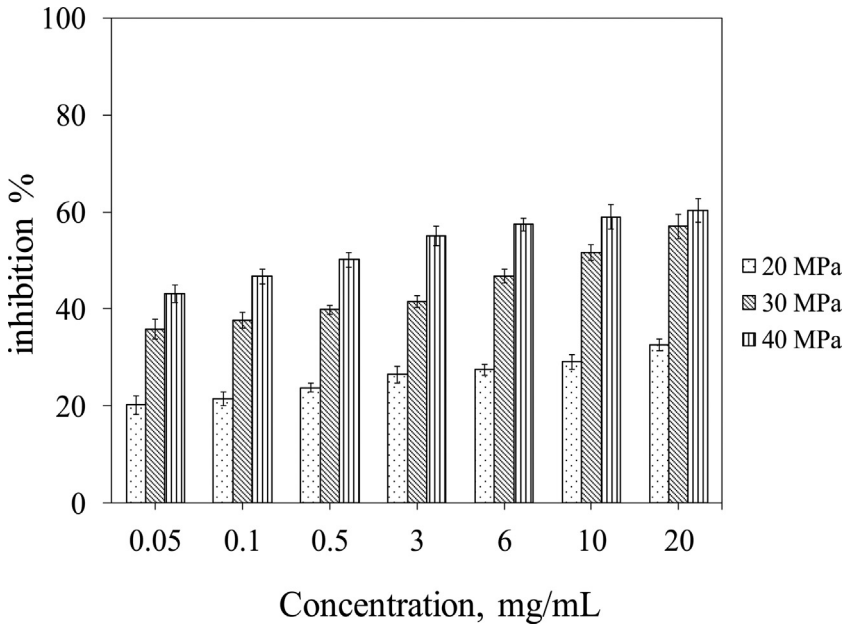

Fig. 1. Inhibition of α-amylase (%) as a function of extract concentration (mg/mL) and pressure (*P*, MPa) at 40 °C.

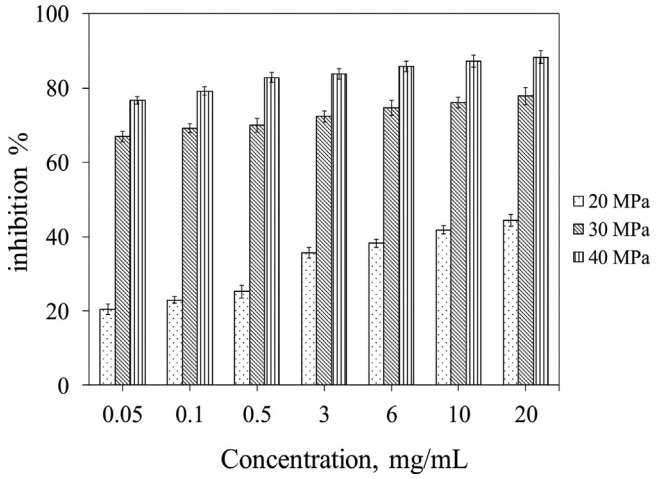

Fig. 2. Inhibition of α-amylase (%) as a function of extract concentration (mg/mL) and pressure (*P*, MPa) at 60 °C.

was between 3 and 12 %, a range in which the effect of moisture is insignificant on the efficiency of supercritical oil extraction [31]. The porosity of the packed bed is an important factor in the mass transfer rate and is directly related with the extraction efficiency [32]. If the porosity were low, it would cause problems of solvent flow channeling through the extractor [33].

3.2. Inhibition of α-amylase

The inhibitory capacity of the extracts obtained at 40 °C (Fig. 1) and 60 °C (Fig. 2) against the enzyme α-amylase increased as the extraction pressure increased from 20 to 40 MPa, for each extract concentration evaluated (0.05–20 mg/mL). For the effect of temperature, it was observed that the enzyme inhibition of extracts obtained at 60 °C was higher than at 40 °C for each pressure. An analysis of variance was performed to assess the effect of temperature (*T*) and pressure (*P*) on inhibitory capacity. Significant effects of *T*, *P* and *T*×*P* interaction were found for each extract concentration (0.05–20 mg/mL). A Tukey analysis showed that the inhibitory capacity at 20 MPa was significantly lower ($p < 0.01$) than those obtained at 30 and 40 MPa. No significant differences were observed between these two pressures. A higher effect of pressure on inhibitory capacity ($p < 0.01$) was observed at 60°C than at 40°C, which demonstrates the importance of the *T*×*P* interaction. An increase in the extraction pressure increases the density of CO_2 and therefore its solvent power, so that higher bioactive compound extraction was obtained at 30 and 40 MPa than at 20 MPa. Density determines the number of interactions between CO_2 and the molecules of organic compounds; if sufficient interactions occur, the cohesion forces between the individual molecules of the organic compound are broken and solubilization will occur, increasing compound extraction. This higher effect of the increase in pressure at 60 °C than at 40 °C was sufficient to counteract the effect of temperature on density; the higher temperature favored greater bioactive compound extraction by a positive effect of the temperature on the solute vapor pressure.

3.3. Individual contents of catechin and myricetin

The contents of individual flavonoids such as catechin and myricetin would partially explain the inhibitory capacity of the extracts, because both concentrations increased with the pressure. Fig. 3 shows that at both temperatures (40 and 60 °C) the catechin content increased as the pressure increased from 20 to 40 MPa. The inhibition of α-amylase by catechins is reported in the literature. Lo Piparo et al. [34] reported that catechin inhibited α-amylase activity by 13 %; while acarbose (a commercial drug used to control glucose levels in diabetic patients) showed 9.9 % inhibition. Koh et al. [35] reported higher α-amylase inhibitory capacity by tea catechins than by acarbose. Likewise, Fig. 4 shows that the myricetin content increased as the pressure increased from 20 to 40 MPa at both 40 °C and 60 °C. Studies have demonstrated the inhibitory capacity of myricetin against α-amylase. Gu et al. [36] reported a greater inhibitory capacity of myricetin against α-amylase than that of other flavonoids.

3.4. Total flavonoids content

Fig. 5 shows the effect of pressure (20–40 MPa) on the total flavonoids content as a function of temperature (40 and 60 °C). The

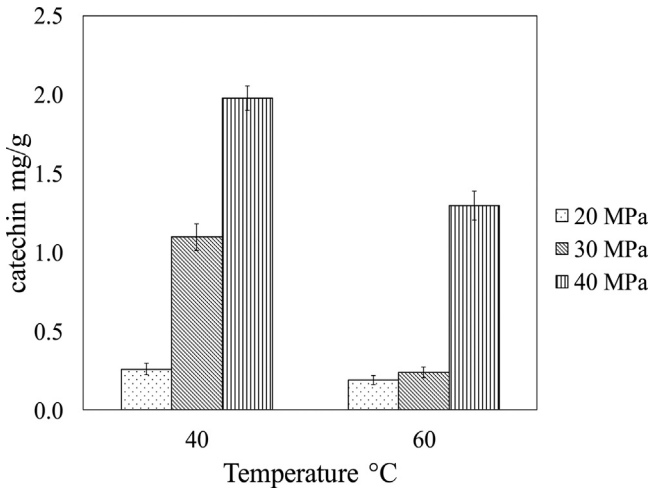

Fig. 3. Catechin content (mg/g) in extract as a function of temperature (T, °C) and extraction pressure (P, MPa).

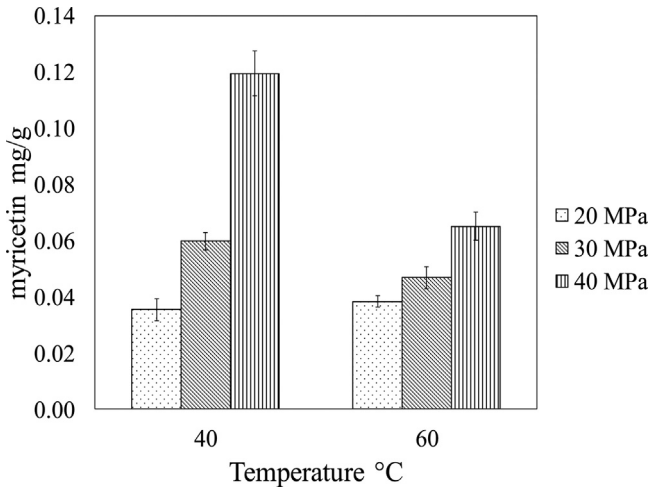

Fig. 4. Myricetin content (mg/g) in extract as a function of temperature (T, °C) and extraction pressure (P, MPa).

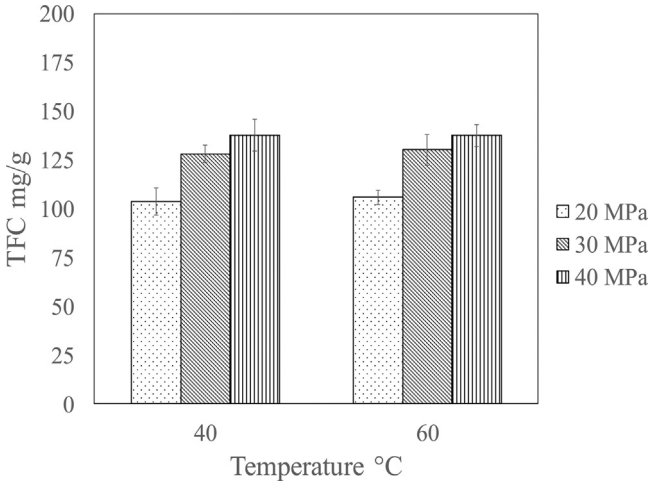

Fig. 5. Total flavonoid content (mg/g) in extract as a function of temperature (T, °C) and extraction pressure (P, MPa).

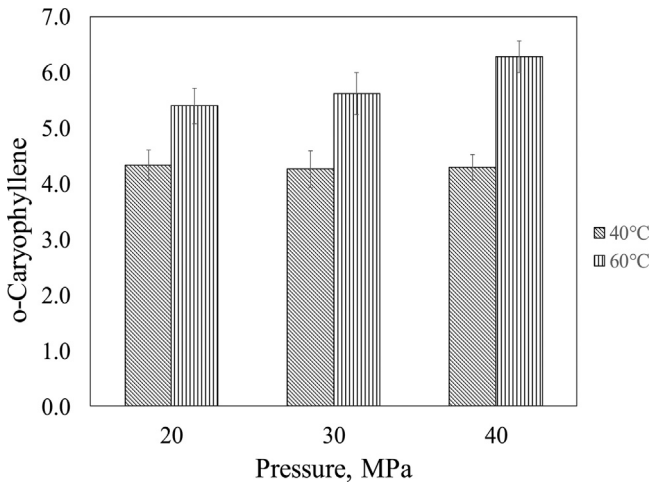

Fig. 6. Caryophyllene oxide content (mg/g) in extract as a function of temperature (T, °C) and extraction pressure (P, MPa).

pressure had a significant effect ($p < 0.05$): the concentrations in the extracts obtained at 30 and 40 MPa were higher than at 20 MPa. The temperature and the $T \times P$ interaction had no effect. The properties of flavonoids as inhibitors of carbohydrate hydrolyzing enzymes relevant to diabetes mellitus have been reported in the literature. Shu et al. [5] reported the capacity to inhibit pancreatic α-amylase enzyme of flavonoids from *Polygonatum odoratum*.

3.5. Individual content of caryophyllene oxide

The caryophyllene oxide content in extracts increased when the extraction temperature increased to 60 °C (Fig. 6). However, at 40 °C there was no pressure effect. This behavior would partially explain the effect of the caryophyllene oxide concentration on α-amylase inhibition, since at 60 °C the increase in pressure was accompanied by an increase in the inhibitory capacity against α-amylase and the caryophyllene oxide concentration. In the literature no studies were found on the inhibition of carbohydrase enzymes by caryophyllene oxide, but there are studies on the inhibitory capacity of essential oils against α-amylase. Compounds such as monoterpenes and sesquiterpenes – part of the essential oils – present inhibitory capacity against α-amylase.

Essential oils from orange and lemon peel [37], clove (*Eugenia aromatica* Kuntze) [38] and sweet basil (*Ocimum basilicum*) leaves [39] have been investigated. The essential oil present in lemon peel exhibited higher inhibitory activity against the α-amylase enzyme than that in orange peel [37]. Clove essential oil inhibited between 35 % and 78 % of α-amylase activity [38].

3.6. Correlation analysis

In Figs. 1 and 2, the inhibitory capacity (%) of extracts was reported as a function of the extraction conditions. Statistical analysis showed that the inhibition capacity of the extracts correlated positively with their total flavonoids content ($p < 0.01$) and with their caryophyllene oxide content ($p < 0.01$) (Fig. 7). To exemplify, the following figures show the positive correlation between inhibitory capacity against α-amylase and total flavonoids content (Fig. 7A) and caryophyllene oxide content (Fig. 7D), for extracts with a concentration of 6 mg/mL. Similar correlations were observed for extract concentrations of 10 mg/mL (Fig. 7B and E) and 20 mg/mL (Fig. 7C and F), and also for the rest of the concentrations (0.05, 0.1, 0.5, 3 mg/mL) (figures not shown). In the case of individual flavonoids, the inhibitory capacity (%) did not correlate ($p > 0.05$) with the concentration of catechin and myricetin. This suggests

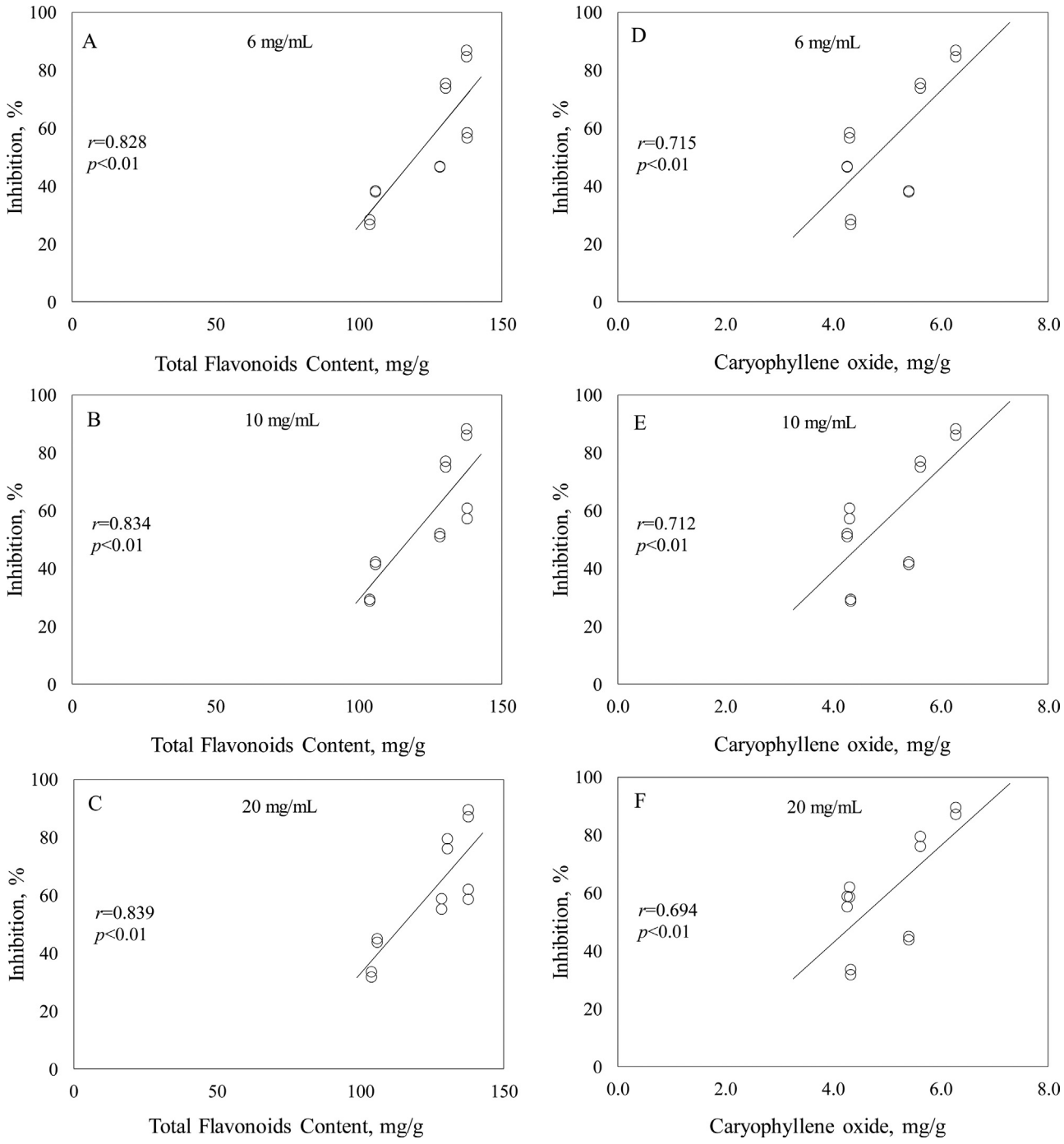

Fig. 7. Positive correlation between the % inhibition of α-amylase with the content of total flavonoids (A–C) and oxide caryophyllene (D–F) for different extract concentrations (6–10 mg/mL).

that other flavonoids present in *L. rivularis* extracts could explain the behavior of the inhibitory capacity against α-amylase with the extraction conditions. A complementary analysis of the selected extract shows that other flavonoids are present, such as kaempferol and resveratrol (Table 3).

3.7. Complementary analysis

Since the extract with the highest inhibitory capacity against α-amylase and the highest total flavonoids content (137.6 mg/g) was obtained at 60 °C and 40 MPa, this condition was selected for complementary analysis of the extract. Table 3 also shows the

Table 3
Characteristics of extract from *L. rivularis* stalks obtained with ethanol-modified CO_2 (2 wt.%) at 60 °C and 40 MPa.

Characteristics	
Antioxidant activity	
DPPH (IC_{50}, mg/mL)	2.57 ± 0.19
FRAP (mmol Fe^{+2}/kg)	68.48 ± 7.62
Total Flavonoids content (mg/100 g)	137.6 ± 5.7
Flavonoids individual content (mg/100 g)	
Catechin	129.6 ± 9.3
Kaempferol	2.1 ± 0.2
Myricetin	6.5 ± 0.5
Resveratrol	2.6 ± 0.2

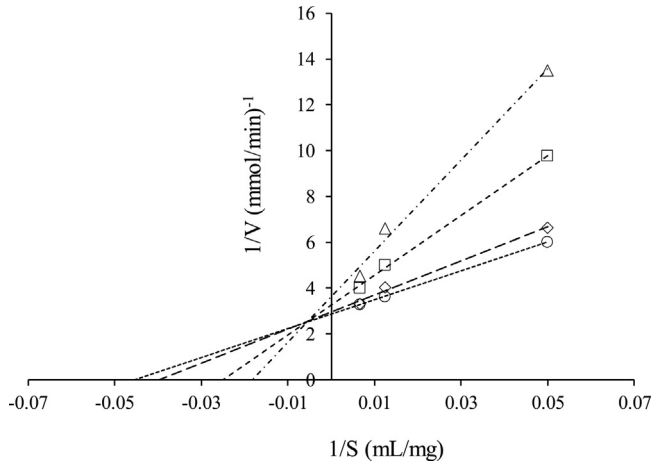

Fig. 8. Lineweaver-Burke plot analysis of the inhibition kinetics of α-amylase inhibitory in the absence and presence of SC-CO_2 extract from *L. rivularis* stalks. △: 10 mg/mL, □: 6 mg/mL, ○: 0.5 mg/mL, ◊: mg/mL. Values are mean of duplicates experiment.

antioxidant activity and content of other individual flavonoids. A total flavonoids content of 176.6 mg/g was reported for extract from *L. rivularis* leaves using ethanol-modified CO_2 [21]. Quantification of individual compounds for the selected extract showed contents (mg/100 g) of 129.6 for catechin, 2.1 for kaempferol, 6.5 for myricetin, 2.6 for resveratrol and 6.29 for caryophyllene oxide (Table 3). A catechin content between 8.1 and 13.5 mg/100 g in supercritical extract of spearmint leaves (*Mentha spicata* L.) has been reported in the literature [40]. Uquiche et al. [18] reported the following compound contents for extract from *L. rivularis* stalks, using ethanol-modified CO_2: catechin 32.5 mg/100 g, quercetin 16.4 mg/100 g, resveratrol 36.6 mg/100 g and caryophyllene oxide 7.3 mg/g. Although differences are observed between the values in Table 3 and those reported in the literature, these can be attributed to differences in substrate characteristics (agronomic conditions), extraction conditions and solvents; however, the values are of the same order of magnitude and demonstrate important properties of the extract from *L. rivularis* stalk.

The literature contains reports of important bioactive properties for kaempferol, resveratrol and caryophyllene oxide, for example: for kaempferol, antioxidant activity [41], anti-cancer properties [42], cardioprotective effect [43] and anti-diabetic [44] effects and for resveratrol, anti-cancer properties [45], anti-inflammatory, anti-diabetic [46] and cardioprotective [47] effects.

Table 3 shows the antioxidant activity of the selected extract measured by DPPH assay (IC_{50} = 2.6 mg/mL) and FRAP assay (68.5 mmol Fe^{+2}/kg). The DPPH assay measures the antioxidant activity of the extracts, attributed to their hydrogen-donating potential and ability to stabilize a free radical, compared to a standard antioxidant [27]. The IC_{50} value indicates the amount of extract that is needed to reduce the formation of free radicals by 50 % by the action of antioxidant compounds in the extract. FRAP represents the reducing capacity of antioxidant compounds in a redox reaction, in which one reactive species is reduced at the expense of the oxidation of another. Uquiche et al. [21] reported 150.4 mmol Fe^{+2}/kg for extract from *L. rivularis* leaves using ethanol-modified CO_2.

3.8. Type of inhibition

Fig. 8 shows measurements of the initial velocity *V* of the reactions catalyzed by α-amylase for various substrate concentrations [*S*] in the presence and absence of *L. rivularis* extract. The double reciprocal plot shows that a straight line was obtained for α-amylase using starch as substrate. Fig. 8 shows that both the slope and the intercept increased as the extract concentration increased. These results indicate that the type of inhibition corresponded to a mixed non-competitive inhibition, exerted by the extract on α-amylase; it is characterized by a decrease in the hydrolysis reaction velocity (0.351–0.276 μmol/min) proportional to the concentration of the extract (0–10 mg/mL), and by an increase in the constant K_m (22.2–55.0 mg/mL) (Table 4). Mixed non-competitive inhibition would indicate that the extract compounds (*e.g.* flavonoids) can bind to enzymes or to the enzyme-substrate complex other than at the active site (allosteric site), decreasing the reaction velocity. This result suggests that the α-amylase inhibitory activity of *L. rivularis* extract would be a mechanism by which it exerts a hypoglycemic effect. It has been reported in the literature that extracts from malted cereal [48] and *Phyllanthus amarus* [49] showed mixed non-competitive inhibition against α-amylase. Shobana et al. [50] and Sabiu et al. [26] reported non-competitive inhibition against α-amylase by extracts from corn silk (*Zea mays*) and millet seed coat, respectively.

Table 4
Kinetic parameters of α-amylase inhibition.

[*I*] (mg/mL)	V_{max} (μmol/min)	K_m (mg/mL)
0	0.351 ± 0.011	22.192 ± 0.833
0.5	0.340 ± 0.004	25.313 ± 1.154
6	0.308 ± 0.027	40.245 ± 2.592
10	0.276 ± 0.026	55.003 ± 1.964

V_{max}: maximum velocity.
K_m: Michaelis-Menten constant.

4. Conclusions

This work studied the extraction of compunds with inhibitory capacity against α-amylase from *L. rivularis* stalks, using ethanol-modified CO_2 under combined conditions of temperature (40–60 °C) and pressure (20–40 MPa). Both temperature and pressure had a positive effect on the α-amylase inhibitory capacity of *L. rivularis* extracts. Extraction pressure was the factor that most influenced the contents of total flavonoids, catechin, myricetin and caryophyllene oxide. The enzyme kinetics assay demonstrated a mixed non-competitive inhibition of α-amylase activity. The inhibitory capacity against α-amylase correlated positively with the contents of total flavonoids and caryophyllene oxide. The highest inhibitory capacity against α-amylase and the highest contents of total flavonoids and caryophyllene oxide were achieved under conditions of 60 °C and 40 MPa. This result suggests that part of the hypoglycemic mechanism of the extract would be associated with its inhibitory capacity against α-amylase. Extracts of *L. rivularis* would serve as an alternative treatment for diabetes due to the presence of flavonoids and caryophyllene oxide. Future work could investigate isolation of the active principles responsible for this inhibitory activity, and also carry out *in-vivo* studies.

Declaration of Competing Interest

The authors whose names are listed immediately below declare don't have no conflict of interest. This work has not been submitted for publication nor has it been published in whole or in part elsewhere. Further that it is not under consideration for publication elsewhere, and that, if accepted, it will not be published elsewhere in the same form or other idiom.

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