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Inhibitory capacity of extracts and main constituents of hop flowers

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Abstract

Background Hops (Humulus lupulus Linn.) produce flowers that are used on an industrial scale to impart sensory properties to beer and have some pharmacologic properties already published in the literature. Gout, type 2 diabetes and Alzheimer's are diseases the appearance/aggravation of which is related to the action of enzymes such as xanthine oxidase, α-glucosidase and acetylcholinesterase, respectively, which are associated with oxidative stress. Our hypothesis is that the extracts and chemical constituents isolated from the flowers of hops act as enzyme inhibitors and scavenge free radicals. Hexane and methanol extracts were obtained from flowers of different cultivars produced in Brazil and from commercial pellets, with the contents of bitter acids and xanthohumol determined by high-performance liquid chromatography (HPLC), after QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) extraction and were evaluated for their pharmacologic properties.

Results The extracts and analytical standards showed a significant capacity against oxidative stress in all evaluated methods ($DPPH^{\cdot}$, $ABTS^{\cdot+}$, $HO^{\cdot}$). Xanthohumol and extracts with a high concentration of this compound demonstrated a strong capacity to inhibit the xanthine oxidase and significantly reduce the formation of uric acid. The results with the *α*-glucosidase revealed promising pharmacologic action for all samples, even more effective than the commercial drug (acarbose). The acetylcholinesterase inhibition assays were effective for hexane extracts and ICE-4, with values obtained close to the eserine.

Conclusion It can be concluded with the results obtained that extracts and patterns of hop flowers showed promising potential to combat oxidative stress and complementary treatment of gout, type 2 diabetes and Alzheimer's disease.

Keywords Pharmacologic properties, Hop, Bitter acids, Xanthohumol, Antioxidant capacity, Enzyme inhibition

Background

Plant species and their chemical constituents enable the development of different pharmaceutical products to minimize oxidative stress and act as enzymatic inhibitors against various diseases [1, 2]. An example of a currently studied plant species is hops (*Humulus lupulus* Linn.), which belongs to the order Rosales and the family Cannabaceae [3, 4]. Hop flowers contain a diversity of secondary metabolites, such as: bitter acids (*α*-humulones and *β*-lupulones), xanthohumol and essential oils, which have significant medicinal action,

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such as anti-inflammatory, anticancer, antioxidant, glycemic reduction, antiviral and antifungal properties already reported in the literature [3, 5–8].

Despite the diversity of the metabolites present, the main application of hops is in the brewery market, because its flowers (which have a high commercial value) contain concentrated glands of a yellowish resin, called lupulin, which gives the beer sensorial properties (bitterness, aroma and flavor), as well as antimicrobial and antioxidant actions [3, 5–7, 9]. The α-humulones undergo isomerization reactions during the boiling of the brewing wort, producing substances with greater solubility in the drink [10].

The analysis of the antioxidant capacity against the hydroxyl radical ($HO^{\cdot}$) has become a method of significant scientific interest, considering that it is the most harmful free radical produced by the body due to its short half-life rendering it difficult to scavenge in vivo. The search for new bioactives that can be incorporated into the human diet to minimize oxidative stress caused by it remains a field of interest yet to be explored [11, 12].

The formation of uric acid in the human body is carried out by the enzyme xanthine oxidase and its accumulation causes the deposition of monosodium urate crystals in tissues and joints. At concentrations higher than 7 mg/dL, it can cause hyperuricemia and gout, with xanthine oxidase inhibitors [13, 14] members of a battery of drugs used for its treatment. Elevation of the uric acid content in the bloodstream may be associated with other diseases such as hypertension, atherosclerosis, kidney stones and diabetes [14, 15]. Different works also report a close relation between high levels of uric acid and the incidence of myocardial infarction [16]. The polyphenolic species mangiferin demonstrated a high capacity to inhibit the formation of uric acids via the xanthine oxidase system [17]; however, there are no reports in the literature demonstrating this action for hop extracts or their main constituents.

The α-glucosidase enzyme is responsible for breaking down complex carbohydrates from food, catalyzing the hydrolysis reactions of these macromolecules into mono and disaccharides, enabling their digestion, promoting an increase in glucose levels in the bloodstream (hyperglycemia), under which conditions elevated levels can trigger insulin resistance or complete failure in its production, hypertriglyceridemia, cardiovascular diseases and arterial hypertension [18–22]. The drug acarbose® acts as an enzymatic regulator of α-glucosidase in medicines for the treatment of type 2 diabetes, while fractions of humulones and humulinones isolated in the laboratory have already demonstrated potential inhibition in in vitro studies [20].

The neurodegenerative pathology of Alzheimer's mainly affects the elderly population and currently affects, at some stage, about 4.02% of individuals aged over 60 years [23, 24]. This disease is the most common form of dementia and affects about 4.6 million patients per year [25, 26]. Currently, a promising means for treating this disease is to increase acetylcholine levels between neuronal synapses, using substances that act as acetylcholinesterase enzyme inhibitors [27].

Therefore, in this work we analyzed the antioxidant properties and enzyme inhibition capacities related to gout, type 2 diabetes and Alzheimer's disease, of extracts obtained from hop flowers produced in Brazil and their analytical standards. Additionally, the amounts of bitter acids and xanthohumol of the samples studied were measured using HPLC to identify possible correlations between quantitative data and the results of pharmacologic actions.

Methods

Chemicals and materials

Milli-Q® deionized water (Darmstadt, Germany); hexane, methanol, dimethylsulfoxide and acetonitrile were purchased from Bio-Grade® (Durham, USA); hydrochloric acid and sodium bicarbonate from Synth® (Diadema, São Paulo); magnesium sulfate from Êxodo Científica® (Sumaré, São Paulo); acetic acid and magnesium chloride from Dinâmica® (Indaiatuba, São Paulo); Folin–Ciocalteu reagent from Merck® (Darmstadt, Germany); sodium chloride from Neon® (Suzano, São Paulo); monobasic and dibasic potassium phosphate, monobasic and dibasic sodium phosphate, hypoxanthine, ferric chloride hexahydrate, salicylic acid, ethylenediaminetetraacetic acid (EDTA), $DPPH^{\cdot+}$ (1,1-diphenyl-2-picrylhydrazyl), $ABTS^{\cdot+}$ [2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)], *p*-nitrophenyl-α-D-glucopyranoside, acetylthiocholine iodide (ATCI), acetylcholinesterase (AChE), 5,5′-dithiobis[2-nitrobenzoic acid] (DTNB), bovine serum albumin fraction V, Trizma hydrochloride solution, xanthohumol, mangiferin, bovine milk xanthine oxidase and *α*-glucosidase (*Saccharomyces cerevisiae*) all obtained from Sigma-Aldrich® (Saint Louis, USA); acarbose (Glucobay®) from Bayer® (Leverkusen, Germany); international calibration extract of hop ICE-4 (standard mixture of bitter acids, purity 69.2%) was obtained from Labor Veritas® (Zurich, Switzerland).

Plant material

Commercial pellets of two varieties of hop flowers (Saaz–Hallertau® and Herkules–Barth-Haas Group®) were purchased at a brewery supply store in the city of Fortaleza (Brazil), with more than 2 years of storage until the period of analysis.

Hop cones of the Chinook cultivar (Chinook-1: mature hop/Chinook-2: first bloom) and Cascade (first bloom) were provided by two members of the Brazilian Association of Hop Producers (Aprolúpulo®), from their plantations in the cities Brasília (Federal District) and Monte Alegre (Rio Grande do Norte). The samples were received in vacuum-sealed metallized packages, with humidity between 8 and 12% (w/w). These cones were collected from replicated plants from seedlings imported with certification and registration at the Brazilian Ministry of Agriculture, Livestock and Supply (MAPA), acquired from the Lúpulo Ninkasi® nursery (Teresópolis, Brazil). All samples were kept at 5 °C under cold storage, until the analysis period.

Obtaining hexane and methanolic extracts by the Soxhlet system

The extracts of the different cultivars were obtained using the ground plant materials (in a bladed processor; Moulinex-DP700, France) using a Soxhlet system. Initially, the extraction of bitter acids was performed with hexane (150 mL—3×3 h), followed by extraction of the most polar species, including xanthohumol, with methanol (150 mL—3×3 h). The proportion of solvents used was 10 g of plant material to 450 mL of each solvent (hexane and methanol). The solutions were concentrated using a rotary evaporator and dried with a constant nitrogen flow at 25° C [12]. The extracts produced were stored at 5 °C, in a solid state, under cold storage until the experimental procedures were carried out.

Total phenolic content: Folin–Ciocalteu method

The total phenolic content was performed according to the procedure described by Cicco and collaborators with adaptations [28]. 100 µL of extract solution dissolved in methanol (1 mg/mL) was inserted into Eppendorf tubes, followed by the addition of 500 µL of 10% Folin–Ciocalteu reagent in H_2O (v/v) and vortexing (Kasvi-K40/1020, South Korea) for 1 min. The reaction was started by adding 400 µL of 7.5% (w/v) aqueous sodium carbonate solution and incubating at 40 °C for 20 min. without shaking.

The same procedure was carried out with the standard gallic acid at different concentrations (2500–19.53125 µg/mL) to obtain the standard calibration curve and determine the total phenolic content in mg of gallic acid equivalents per gram of extract (mg EAG/g). After incubation, 100 µL of each system was transferred to microplates and the absorbances were measured in an ELISA spectrophotometer (Enzyme-Linked Immunosorbent Assay—Biotek, Winooski, USA) at 740 nm. All samples from the different experiments in this research were obtained in triplicate ($n=3$).

Chemical quantitation of bitter acids and xanthohumol using QuEChERS extraction and high-performance liquid chromatography (HPLC-DAD)

To verify the amount of the compounds of interest in the evaluated cultivars (bitter acids and xanthohumol), the methodology proposed by Marques and collaborators was used for quantitative analyzes in hop flowers [29].

The compounds of interest were obtained through salt-assisted extraction (QuEChERS), using 1 g of powdered plant material. The supernatant solutions were collected, diluted in acetonitrile (1:10–1:20) and centrifuged (13,000 rpm, 25 °C, 5 min) for chromatographic analyses. The extractions were performed in triplicate and evaluated individually by liquid chromatography (HPLC-DAD).

Chromatographic evaluations were performed using a high-performance liquid chromatography with DAD detection (Agilent 1260 Infinity, Germany), equipped with a C-18 reverse phase column (Agilent eclipse plus – 3.5 µm, 4.6×100 mm). The mobile phases were methanol (A) and 2% aqueous acetic acid solution (B), with a chromatographic run time of 35 min, with a flow rate of 1.0 mL/min. The injection volumes used were 10 and 20 µL, with the following gradient flow: 0–5 min (75% A), 5–15 min (100% A) and 15–35 min (75% A).

Bitter acids showed better resolution at a wavelength of 326.4 nm and xanthohumol at 340.4 nm; these being the wavelengths used for quantitation of these compounds [29]. Standard calibration curves, statistical treatment and calculations of compound concentrations (in g/100 g) were performed using Microsoft Excel 365 software.

DPPH$^{\cdot}$ radical scavenging antioxidant assay

Assays were performed in 96-well microplates, adding 20 µL of methanol solutions of extracts and standards (BHT, xanthohumol) at seven different concentrations (15.625–1000 µg/mL), for the standards and extracts of the Herkules and Chinook-1 varieties, and (62.5–4000 µg/mL) for the extracts of the Chinook-2, Saaz and Cascade cultivars, in triplicate. The reaction was started by adding 180 µL of a methanol solution of the DPPH$^{\bullet}$ radical (200 µmol/L) in the absence of light for 30 min [12]. Absorbances were detected in an ELISA spectrophotometer at the maximum absorption wavelength of the DPPH$^{\bullet}$ radical (515 nm) and IC_{50} calculations using Origin 9.5® software.

ABTS$^{\cdot+}$ antioxidant assay

To determine the antioxidant capacity against the ABTS$^{\cdot+}$ radical cation, an adaptation of the method proposed by Torres and collaborators was performed [30].

The radical cation was obtained by the reaction between 5 mL of ABTS aqueous solution (7 mmol/L) with 88 µL of potassium persulfate solution (140 mmol/L) for 16 h in the absence of light. In microplates, 20 µL of methanol solutions of extracts and standards was added in seven different concentrations (0.016–4 mg/mL), followed by the addition of 180 µL of freshly prepared $ABTS^{\cdot+}$ radical solution and subsequent analysis by spectrophotometry (ELISA) at 734 nm. BHT antioxidant was used as a positive control and IC_{50} calculations using Origin 9.5® software.

Antioxidant assay hydroxyl radicals and inhibition of the enzyme xanthine oxidase

The simultaneous assay of xanthine oxidase inhibition and hydroxyl radical scavenging was carried out according to the methodology described by literature [12, 31]. Different volumes (500, 250, 100, 50, 25 and 10 µL) of methanol solutions of extracts/ICE-4 (10,000 µg/mL) and xanthohumol (1000 µg/mL) were added to Eppendorf tubes prior to solvent evaporation. One mL of phosphate buffer (pH = 6.6) was added and vortexed for 1 min and placed in an ultrasonic bath for 10 min. Subsequently, 10 µL of xanthine oxidase enzyme (20 U) was added, and the system was incubated in a thermomixer for 3 h (450 rpm at 37 °C), in the absence of light. The reaction was terminated by adding 10 µL of concentrated hydrochloric acid, centrifuged (13,000 rpm, 25 °C, 5 min) for analyzed by HPLC. The operational conditions of the liquid chromatograph (HPLC–DAD) were the same as described above; however, the injection volume was 10 µL and a chromatographic run of 50 min of with the following gradient flow: 0–11 min (0% of A), 11–35 min (20% A), 35–40 min (40% A), 40–45 min (0% A) and 45–50 min (75% A).

The detection and calculations of uric acid levels (evaluation of the ability to inhibit the enzyme xanthine oxidase) were performed at 278.4 nm, while the dihydroxybenzoic acids (2,3 and 2,5-DHBA), related to the antioxidant capacity, were and quantitated at 325.4 nm [12, 31]. The xanthone mangiferin (10,000 µg/mL) was used as a positive control and IC_{50} calculations using Origin 9.5® software.

α-glucosidase enzyme inhibition assay

For this assay, solutions were prepared at different concentrations of the extracts/ICE-4 (15.625–1000 µg/mL), xanthohumol (3.90625–250 µg/mL) and acarbose (78.125–10,000 µg/mL) in sodium phosphate buffer solution (50 mmol/L, pH = 6.8).

In Eppendorfs, 20 µL of samples and 20 µL of α-glucosidase (1 U/mL) were added. After 5 min, 40 µL of *p*-NPG reagent (1 mmol/L) was added and the system incubated for 30 min at (37 °C). Following incubation, 100 µL of 10% sodium bicarbonate aqueous solution was added and 100 µL of the systems was collected for quantitation of absorbances in an ELISA spectrophotometer (405 nm). Assays were carried out without the enzyme (blank), for each concentration value of the extracts, to evaluate the possible interference of the *p*-NPG reagent on the absorbance values obtained [22]. The drug acarbose was used as a positive control at the same concentrations as the extracts.

Acetylcholinesterase enzyme inhibition assay

The determination of the acetylcholinesterase enzyme inhibition activity was based on the methodology described by literature, modified to TLC (thin layer chromatography) [32, 33]. After 10 min of enzyme application, a yellow coloration is observed in the region of samples that do not present inhibitory action and the formation of a white halo in the "spots" of those that present anticholinesterase activity, are calculated from the diameter of the halos (millimeters) [12]. A methanol solution of eserine (1000 µg/mL) was used as a positive standard.

Results

Qualitative and quantitative analysis of chemical constituents

Figure 1 presents the chromatograms obtained for the solutions (1a and 1b) used in the construction of the standard calibration curves (ICE-4 and xanthohumol), in addition to the extracts obtained by QuEChERS extraction from the samples of pellets and hop cones analyzed in this work (1c-1 g).

The bitter acids: cohumulone—α_1, humulone coeluted with adhumulone—α_2, colupulone—β_1 and lupulone coeluted with adlupulone—β_2, appear with peaks of high intensity in the chromatograms of the ICE-4 standard and in all analyzed hop samples, with retention times between 13 and 16 min. The same profile is observed for xanthohumol, but with a shorter retention time (≈ 6 min) than for bitter acids, due to its greater polarity. The chromatograms showed a very characteristic and similar chemical profile in all analyzed samples, regardless of their format (pellets or cones), demonstrating that bitter acids and xanthohumol are the major constituents in this part of the plant, especially when they reach maturation, corroborating data reported by Marques and collaborators [29]. Peaks are also observed at different retention times of species that were not analyzed.

The yields of hexane and methanol extracts from the different cultivars evaluated varied between 5–32 and 15–21 (g/100 g), respectively, and are available in Table 1. The statistical/analytical data obtained for the standard curves and the quantitative results (g/100 g) for bitter acids and xanthohumol in the different samples analyzed

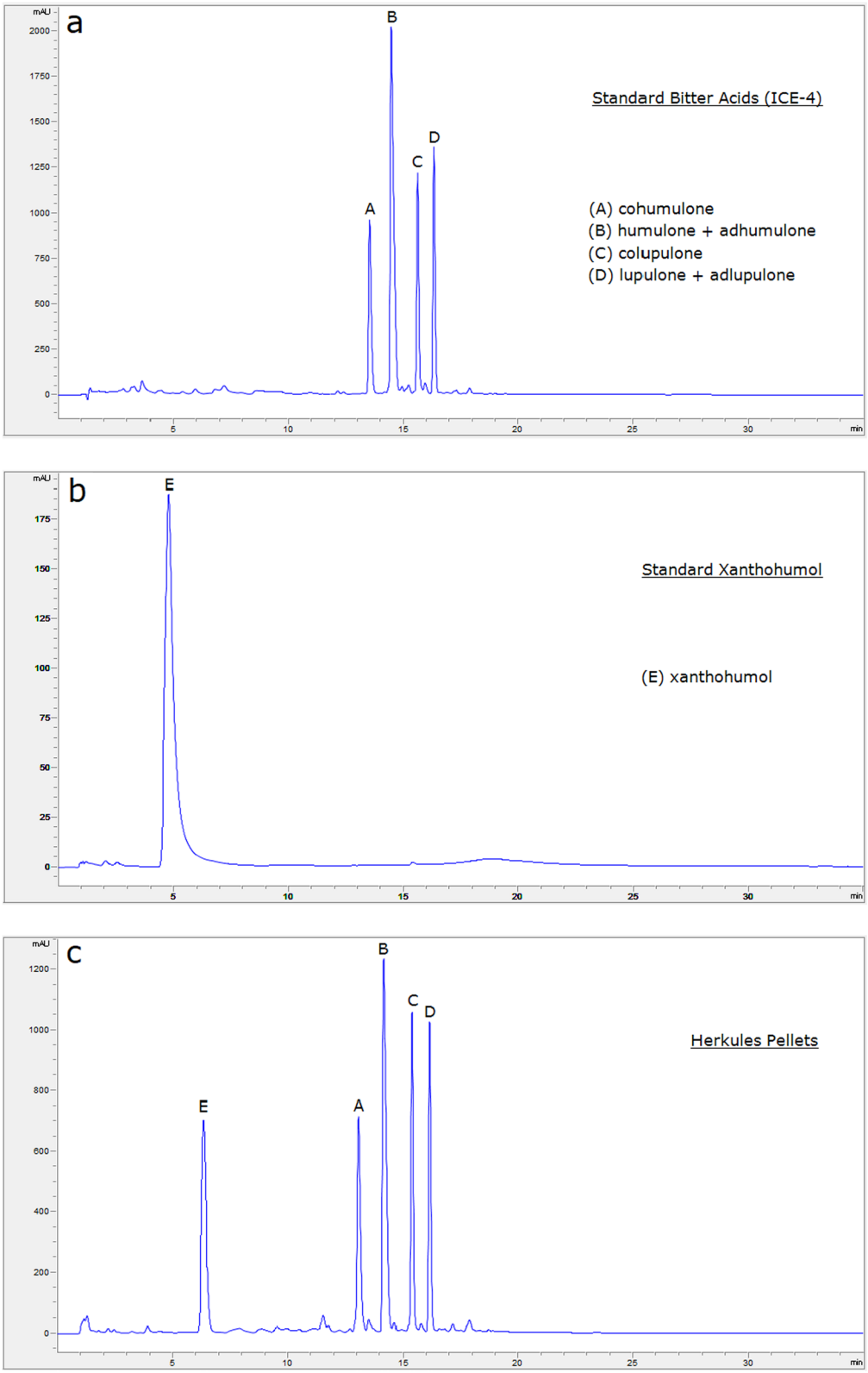

Fig. 1 Chromatograms of hop standards and extracts. **a** Standand Bitter acids, **b** Standard Xanthohumol, **c** Herkules pellets, **d** Saaz pellets, **e** Chinook-1 flowers, **f** Chinook-2 flowers, **g** Cascade flowers

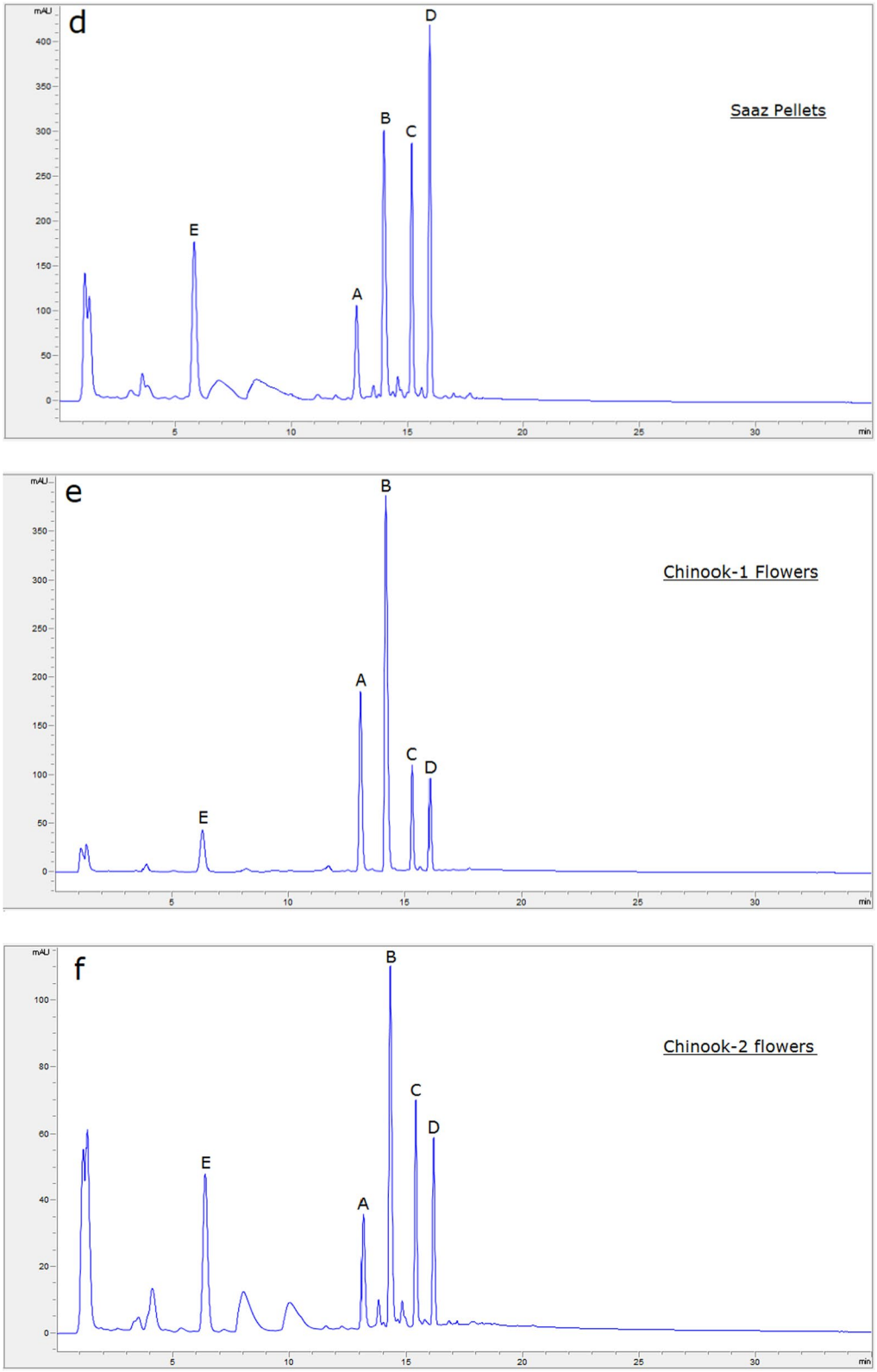

Fig. 1 continued

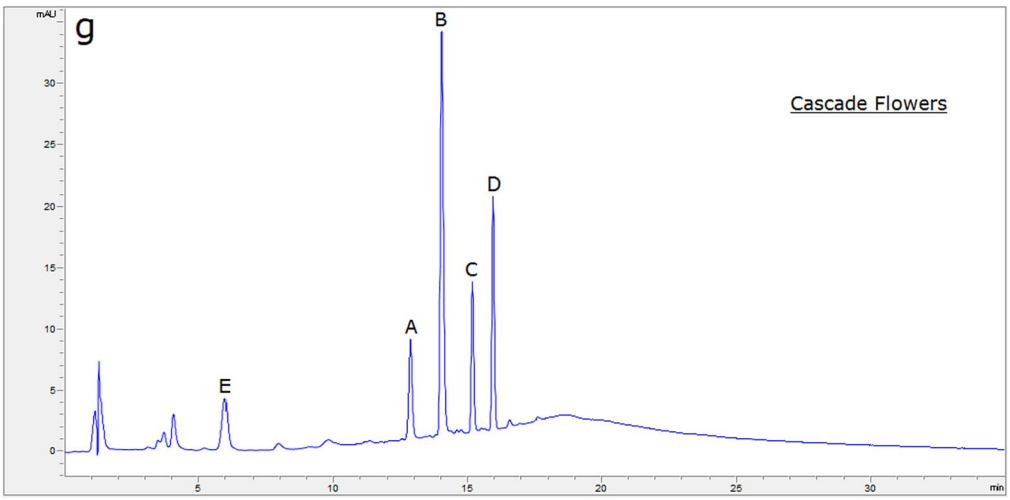

Fig. 1 continued

Table 1 Yield of extracts of the hop varieties analyzed (Soxhlet)

Extracts yield (g/100 g)					
Samples	**Pellets**		**Cones (Brazil)**		
	Saaz	**Herkules**	**Chinook-1**	**Chinook-2**	**Cascade**
Hexane	14.63	31.97	18.99	8.28	5.25
Methanol	21.56	15.58	17.15	21.37	16.26

This table should be added after the third paragraph of the section "Qualitative and quantitative analysis of chemical constituents"

by liquid chromatography are presented in Table 2. Data are expressed as arithmetic means of three extractions performed for each sample ($n=3$) with their respective relative standard deviations (RSD%).

The Herkules cultivar is known to contain high levels of α-acids, which are widely used in brewing recipes with high IBU (International Bitterness Units). The commercial pellets analyzed of this variety showed α_{total} ($\alpha_1+\alpha_2$) and β_{total} ($\beta_1+\beta_2$) equal to 8.855 and 2.695 (g/100 g), respectively, corresponding to about 70% of the reference values indicated on the label. Among all the varieties studied, this was the one with the highest value of total bitter acids ($\alpha_{total}+\beta_{total}=11{,}550$ g/100 g). The proportion of cohumulone (α_1/α_{total}) observed (31.73%) corroborated the information provided by the manufacturer, with a concentration of xanthohumol (2.165 g/100 g) consistent with a phenolic compound content of 5%.

In the pellets of the Saaz variety, the amounts of α_{total} and β_{total} (1.847 and 2.245 g/100 g) corresponded to practically 80% of the manufacturer's reference values. The percentage of cohumulone (α_1/α_{total}) was 36.54%, higher than the reference range for this variety (23–28%) and the xanthohumol content was the lowest among all the samples (0.010 g/100 g) evaluated. The pellets (Herkules and Saaz) had already been stored for more than 2 years until the analysis period (2021 harvest), indicating good stability of the compounds of interest under storage conditions, considering that the degradation of these metabolites is dependent on storage time and is already well demonstrated in the literature [10].

In the extracts of the cones of the Chinook-1 variety (Brasília–Brazil), obtained from plants with a higher state of maturity, the α_{total} concentration (6.414 g/100 g) verified, corresponded to more than half of that expected for a mature plant of this cultivar, after more than 1 year of storage, indicating good adaptation to the planting region. The low concentration of β-acids is notorious ($\beta_{total}=0.236$ g/100 g) in relation to the α_{total} contents for this sample, with the percentage of cohumulone identified (21.20%), slightly lower than expected for this cultivar. This sample contained the highest amounts of xanthohumol (2.402 g/100 g) among all the evaluated samples, demonstrating that this variety is a good source of this highly commercial bioactive.

The extract of Chinook-2 cones, obtained from first bloom plants in another region of Brazil (Monte Alegre—RN), showed the compounds of interest but the values of α_{total} (1.290 g/100 g) and β_{total} (0.730 g/100 g) were much lower than previously reported for this cultivar. The xanthohumol values were more significant (0.432 g/100 g), indicating that the plants were still in the process of adapting to the planting region. Cones of the Cascade variety had the lowest α_{total} value (0.713 g/100 g) in relation to all samples, and low levels of β_{total} (0.311 g/100 g) and xanthohumol (0.083 g/100 g). Therefore, the results demonstrate that the variety Herkules and Chinook-1

Table 2 Analytical/statistical data from standard curves and quantification of bitter acids and xanthohumol present in hops

Compound	λ (*n*m)	Rt ≈ (min)	Standard curve range (mg/mL) *n* = 7	Standard curve equations	R^2	Commercial pellets g/100 g		Cones (Brazil)		
								Matured hop g/100 g	First Bloom g/100 g	
						Saaz *n* = 3 (RSD%)	Herkules *n* = 3 (RSD%)	Chinook-1 *n* = 3 (RSD%)	Chinook-2 *n* = 3 (RSD%)	Cascade *n* = 3 (RSD%)
Xanthohumol (XN)	340	5.7	0.006–0.18 (*n* = 6)	$y = 44.97 \times + 67.39$	0.999	0.010 (12.86)	2.165 (4.90)	2.402 (5.51)	0.432 (16.98)	0.083 (13.57)
Cohumulone (α_1)	326	12.8	0.027–0.432	$y = 10{,}533 \times - 4.4493$	0.991	0.675 (3.14)	2.810 (4.03)	1.360 (11.57)	0.345 (10.25)	0.327 (15.23)
Humulone + Adhumulone (α_2)	326	14.1	0.079–1.264	$y = 10{,}669 \times - 79.51$	0.997	1.172 (6.04)	6.045 (1.29)	5.054 (17.31)	0.945 (20.20)	0.386 (18.76)
Colupulone (β_1)	326	15.2	0.032–0.576	$y = 7{,}785 \times + 88.62$	0.996	1.260 (7.86)	1.405 (21.47)	0.144 (9.43)	0.355 (17.92)	0.145 (13.54)
Lupulone + Adlupulone (β_2)	326	16.0	0.034–0.538	$y = 10{,}159 \times - 40.358$	0.993	0.985 (7.90)	1.290 (16.55)	0.092 (8.99)	0.375 (14.51)	0.166 (14.99)
Total						4.102	13.715	9.052	2.452	1.107

This table should be added after the fourth paragraph of the section "Qualitative and quantitative analysis of chemical constituents"

λ—analysis wavelength (nm), Rt—retention time (min), RSD—relative standard deviation, R^2—determination coefficient

presented the most expressive results in relation to the contents of bitter acids and xanthohumol and this work is the first report containing quantitative data for the cultivation of these hop varieties in the evaluated planting regions.

The DPPH• radical inhibition capacity was more effective for methanol extracts (ME) than for hexane extracts (HE). The methanol extracts of the Chinook-2 (IC_{50} = 361.429 µg/mL), Saaz (IC_{50} = 446.140 µg/mL), Chinook-1 (IC_{50} = 531.961 µg/mL) and Herkules (IC_{50} = 578.206 µg/mL) showed antioxidant capacity, consistent with the amounts of total phenolic compounds shown in Table 3.

Among the hexane extracts, Herkules and Chinook-1 varieties (IC_{50} = 317.263 and 523.310 µg/mL) showed better antioxidant capacity. Chinook-2 and Saaz cultivars were less effective, but also showed antioxidant capacity (IC_{50} = 1,048.631 and 2,225.621 µg/mL, respectively). The data observed for ICE-4 confirm the high capacity of bitter acids to inhibit DPPH• radicals, with an IC_{50} (171.464 µg/mL) showed values close to the standard BHT, which is a commercial antioxidant widely used in food industry. Xanthohumol was not efficient in this method. All samples showed values higher to the BHT standard (IC_{50} = 114.797 µg/mL).

ICE-4 (IC_{50} = 144.565 µg/mL) and xanthohumol (IC_{50} = 51.338 µg/mL) showed better ability to inhibit the $ABTS^{\bullet+}$ radical cation when compared with hop extracts. With the exception of xanthohumol, all samples showed IC_{50} values higher than BHT (IC_{50} = 65.216 µg/mL), indicating lower efficacy.

Total phenolic content and antioxidant assays ($DPPH^{\bullet}$ and $ABTS^{\bullet+}$)

The results of the total phenolic content in the extracts and the $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ antioxidant assays are shown in Table 3, all methanol extracts contained significant amounts of total phenolic compounds, with higher amounts for the Saaz (53.569 mg GAE/g), Chinook-2 (51.360 mg GAE/g), Chinook-1 (47.591 mg GAE/g) and Herkules (45.505 mg GAE/g) varieties. These data agree with the concentrations of xanthohumol in the extracts as shown in Table 2, in addition to the presence of chromatographic peaks with retention times lower than the bitter acids which were not identified.

Analysis of the inhibition of the hydroxyl radical and the enzymes: xanthine oxidase, a-glucosidase and acetylcholinesterase

The antioxidant assay against the hydroxyl radical and enzyme inhibition assays were carried out for the most active samples against the DPPH• and $ABTS^{\bullet+}$ methods (Saaz, Herkules and Chinook-1), to evaluate the possible medicinal action of the extracts and standards (ICE-4 and xanthohumol) for the treatment of gout, type 2 diabetes and early Alzheimer's disease. The results obtained are shown in Table 4.

Table 3 Analytical/statistical data of total phenolic contents and antioxidant analysis of extracts and standards

Samples	Total phenolics (mg GAE/g) (RSD%)	$DPPH^{\bullet}$—IC_{50} (µg/mL) (RSD%)		$ABTS^{\bullet+}$—IC_{50} (µg/mL) (RSD%)	
	ME	ME	HE	ME	HE
(I) Commercial pellets					
Saaz	53.569 (1.353)	446.140 (11.749)	2,225.621 (10.547)	343.186 (6.294)	1,056.956 (11.522)
Herkules	45.505 (5.261)	578.206 (5.175)	317.263 (8.448)	272.502 (16.94)	187.087 (19.848)
(II) Brazil cones					
Chinook-1	47.591 (12.530)	531.961 (14.447)	523.31 (7.291)	289.239 (7.740)	215.298 (6.703)
Chinook-2	51.360 (10.603)	361.429 (0.777)	1,048.631 (3.314)	307.488 (15.699)	637.697 (1.175)
Cascade	22.703 (8.210)	1,977.854 (7.721)	*	923.808 (2.741)	*
(III) standards					
ICE-4	–	171.464 (9.556)		144.565 (5.865)	
Xanthohumol	–	*		51.338 (13.254)	
BHT	–	114.797 (17.542)		65.216 (10.096)	

GAE gallic acid equivalent, *RSD* relative standard deviation, IC_{50} inhibitory concentration 50%, *ME* methanol extract, *HE* hexane extract

*Activity less than 50% in the analyzed concentrations

Table 4 Analytical data obtained from enzyme inhibition analyses

Samples	Inhibition assays							
	Hydroxyl radical $n=2$ IC_{50}—µg/mL (RSD%)		Uric acid $n=2$ IC_{50}—µg/mL (RSD%)		α-glucosidase $n=3$ IC_{50}—µg/mL (RSD%)		Acetylcholinesterase (mm)	
	ME	HE	ME	HE	ME	HE	ME	HE
(I) Commercial pellets and Brazil cones								
Herkules	1,494.345 (5.694)	2,276.029 (21.696)	2,446.075 (2.347)	*	239.344 (6.100)	177.182 (2.729)	**	9
Saaz	*	*	*	*	90.990 (12.430)	313.816 (12.840)	**	7
Chinook-1	2,889.708 (2.718)	2,457.315 (2.115)	2,437.772 (4.222)	*	35.339 (3.873)	127.091 (3.662)	**	8
(II) standards								
ICE-4	2,957.723 (18.782)		5,213.415 (4.006)		183.081 (2.934)		6	
Xanthohumol	225.241 (7.802)		*		13.184 (10.047)		**	
Mangiferin	711.433 (15.154)		521.804 (14.416)		–		–	
Acarbose	–		–		370.540 (2.871)		–	
Eserine	–		–		–		9	

RSD relative standard deviation, *IC_{50}* inhibitory concentration 50%, *mm* millimeter, *ME* methanol extract, *HE* hexane extract

*Activity less than 50% in the analyzed concentrations

**White halo formation was not observed in the acetylcholinesterase inhibition assay

Discussion

The antioxidant DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ assays showed that the Cascade cultivar extracts were the least active in the analyses, which can be explained due to the lower proportion of bitter acids, xanthohumol and total content of phenolic compounds (Tables 2 and 3). Therefore, all evaluated extracts showed antioxidant capacity, regardless of the assay, which corroborates different publications that demonstrated the antioxidant capacity against DPPH$^{\cdot}$ and ABTS$^{\cdot+}$ radicals for aqueous, methanol extracts, isolated fractions and essential oils for other hop cultivars from Poland and Brazil [34, 35].

Analyses of the scavenging capacity of HO$^{\bullet}$ radicals again displayed the antioxidant action of the extracts, in which the samples with the highest amounts of bitter acids and xanthohumol (Herkules and Chinook-1), determined by HPLC, proved to be the most efficient, especially in the methanol extracts (ME). ICE-4 showed results somewhat similar to the more active extracts ($IC_{50}=2{,}957.723$ µg/mL), again demonstrating the antioxidant capacity of isolated bitter acids. However, all with IC_{50} values higher than the positive standard mangiferin ($IC_{50}=711.433$ µg/mL). The result for xanthohumol ($IC_{50}=225.241$ µg/mL) was approximately three times lower than that of mangiferin, which demonstrates the high capacity of this substance to scavenge hydroxyl radicals and consequently reduce oxidative stress. Thus, the relationship between the high levels of xanthohumol observed for the Herkules and Chinook-1 cultivars (Table 2) and the more effective action of the methanol extracts in relation to the hexane extracts (HE) is proven. Data presented by Kontek and collaborators using hop fractions rich in α-acids and β-acids (fraction A) and β-acids and xanthohumol (fraction B) demonstrated the ability to protect macromolecules against lipid peroxidation and oxidation of protein carbonyl groups caused by the action of hydroxyl radicals [34]. Therefore, the data presented efficiently demonstrated that bitter acids and xanthohumol have a high ability to scavenge hydroxyl radicals separately as well by synergism.

The inhibition activity of the xanthine oxidase enzyme, reducing the formation of uric acid, was significantly only in the methanol extracts of the Chinook-1 ($IC_{50}=2{,}437.772$ µg/mL) and Herkules ($IC_{50}=2{,}446.075$ µg/mL) cultivars at IC_{50} values higher than the standard mangiferin ($IC_{50}=521{,}804$ µg/mL). ICE-4 showed discrete inhibitory activity. Xanthohumol showed maximum inhibition of uric acid formation equal to $43.933 \pm 5.109\%$, starting from a solution 10 times more diluted than the other samples, demonstrating a significant capacity to possibly prevent gout. This result reinforces the effective action observed for the methanol extracts of the Herkules and Chinook-1 varieties that showed high levels of xanthohumol. Bibliographical research demonstrates that this is the first report in the literature about the ability to inhibit the enzyme xanthine oxidase and the formation of uric acid for hop extracts and constituents.

The α-glucosidase enzyme inhibition assays demonstrated the antidiabetic potential of the chemical constituents of hops, since all the extracts (ME and HE) of the different cultivars evaluated, presented higher enzymatic inhibition than the drug acarbose, used as a

positive standard (IC_{50} = 370.540 µg/mL). Among the hexane extracts, the samples with the highest contents of bitter acids (Chinook-1 and Herkules) proved to be the most effective (IC_{50} = 127.091 and 177.182 µg/mL, respectively). The ICE-4 bitter acid pattern presented an IC_{50} value approximately two times more effective than the reference drug, which proves once again the effective action of the synergism between humulones and lupulones in inhibiting the α-glucosidase enzyme, indicating promising action of hop extracts for the treatment of type 2 diabetes and corroborates data from the literature that showed this capacity for fractions of humulones and humulinones isolated in the laboratory and other hop constituents [20].

Xanthohumol showed the best *α*-glucosidase enzyme inhibition action among all the samples (IC_{50} = 13.184 µg/mL), with performance practically 28 times higher to that of acarbose, demonstrating excellent viability of this chemical species to act as an active principle of drugs against type 2 diabetes. Because it is the major polyphenolic constituent of the analyzed samples, it contributed to the high antidiabetic activity observed in the methanol extracts (IC_{50} = 35.339–239.344 µg/mL).

The acetylcholinesterase enzyme inhibition tests showed that only hop hexane extracts and ICE-4 acted as good inhibitors, showing halo sizes ranging from 6 to 9 mm in diameter. The Herkules hexane extract showed halo formation with a size similar to the eserine standard (9 mm), indicating a significant anticholinesterase action and demonstrating that bitter acids are promising substances for increasing acetylcholine levels in the brain. The enzyme inhibition reported by Keskin and collaborators for methanol extracts from a cultivar produced in Turkey was not observed in the methanol extracts evaluated in this work [36].

Conclusion

Hop extracts and their chemical constituents showed significant pharmacologic potential, considering the evaluated methodologies. The results concerning the antioxidant properties showed a high capacity to combat oxidative stress and the samples with the highest levels of bitter acids, determined by HPLC, were the most effective in scavenging the $DPPH^{\cdot}$ radical and $ABTS^{\cdot+}$ radical cation. The extracts with the highest levels of xanthohumol were the most active against the hydroxyl radical, corroborating the IC_{50} values obtained with the pure compound. In the xanthine oxidase enzyme inhibition test, xanthohumol and methanol extracts of hops demonstrated an anti-gout action, abruptly reducing the formation of uric acid in the systems. All samples showed inhibitory capacity of the α-glucosidase enzyme, most of them being more effective than the drug acarbose, showing excellent antihyperglycemic capacity. The inhibition of the acetylcholinesterase enzyme was only verified in extracts with high values of bitter acids (hexane), giving results similar to eserine.

Abbreviations

$ABTS^{\cdot+}$	2,2′-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid) radical
AChE	Acetylcholinesterase
ATCI	Acetylthiocholine iodide
BHT	Butylated hydroxytoluene
$DPPH^{\cdot}$	1,1-Diphenyl-2-picrylhydrazyl radical
DTNB	5,5′-Dithiobis[2-nitrobenzoic acid]
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
GAE	Gallic acid equivalent
HE	Hexane extracts
HPLC-DAD	High-performance liquid chromatography coupled to a diode array detector
$HO^{\cdot}$	Hydroxyl radical
IBU	International bitterness units
IC_{50}	Mean inhibitory concentration
ICE-4	International calibration extract of hop
MAPA	The Brazilian Ministry of Agriculture, Livestock and Supply
ME	Methanol extracts
min	Minute
p-NPG	*para*-Nitrophenyl-*α*-D-glucopyranoside
QuEChERS	Quick, easy, cheap, effective, rugged and safe
rpm	Rotations per minute
RSD	Relative standard deviations
U	Unit
TLC	Thin layer chromatography

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Author contributions

FMGN was involved in methodology, validation, investigation, investigation, data curation, formal analysis, writing—original draft, writing—review, data curation, visualization. SPDM helped in conceptualization, methodology, software, validation, formal analysis, investigation, data curation, writing—original draft, visualization, funding acquisition. MTST contributed to conceptualization, methodology, validation, investigation, resources, writing—review and editing, supervision, project administration, funding acquisition. RWO was involved in conceptualization, methodology, validation, investigation, writing—review and editing, supervision. LRP helped in methodology, validation, formal analysis, investigation. TCL contributed to methodology, validation, formal analysis, software. AFS was involved in methodology, formal analysis, software. CEGM helped in methodology, formal analysis. All authors have read and approved the manuscript.

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Availability of data and materials

The data that support the findings of this study are available from the corresponding author, upon reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication
On behalf of all authors, the corresponding author states that there is no conflict of interest.

Competing interests
All authors declare to have no competing interests.

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