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Full Length Research Paper

Identification and quantification of caffeoylquinic acid derivatives in *Cynara scolymus* L. tablets and capsules

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***Cynara scolymus* L. leaves, popularly known as globe artichoke, have cholagogue and choleretic properties which are attributed to caffeoylquinic acid derivatives. Although, there are already pharmacopoeial methods for quality control of artichoke dry leaf extract, it only shows the assessment of chlorogenic acid determination. This study aimed to adapt and validate a method for simultaneous determination of neochlorogenic, chlorogenic, cryptochlorogenic, cynarin and isochlorogenic acids A and C in tablets or capsules of globe artichoke leaf extract herbal medicines. Also, it evaluated the content of these caffeoylquinic acid derivatives in three commercial products and leaf extract. The validation considered the parameters of selectivity, precision, linearity, quantification and detection limits and accuracy. The method proved to be selective, precise and accurate. Commercial products and leaf extract had significantly different concentrations of standard compounds which may bring an impact on therapeutic outcomes. The method proposed was suitable for the identification and quantification of caffeoylquinic acid derivatives in tablets and capsules and could be used in quality control, ensuring the safety and efficacy of commercially prepared herbal medicines.**

Key words: Herbal medicines, quality control, *Cynara scolymus.*

INTRODUCTION

Over the last years, the search for natural treatments, including herbal medicines, has been growing due to the potential value for health and well-being. Commonly, these treatments are mistakenly considered to be risk-free, only because they are “natural”. However, adverse events from herbal medicines have been reported and therefore deserve attention. The World Health Organization (WHO) emphasizes the importance of providing traditional medicine with quality, safety, and efficacy to ensure access to health for the entire population (Robinson and Zhang, 2011; WHO, 2013).

Several factors such as climatic variations, growing conditions, and extractive processes can cause a wide variability of the chemical substances contained in the leaves, which can contribute to alterations in quality, safety and efficacy of the herbal medicines. High-performance liquid chromatography (HPLC) allows the separation, identification and quantification of these

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Table 1. Mobile phase gradient.

Time (min)	Pump A (%)	Pump B (%)
0 – 1	92	8
1 – 20	92 → 75	8 →25
20 – 33	75	25
33 – 35	75 →0	25 → 100
35 – 40	0 → 92	100 → 8

substances, and thus it is a valuable tool for the quality control of these products (Tistaert et al., 2011; Govindaraghavan and Sucher, 2015; Applequist and Miller, 2013).

The plant species, *Cynara scolymus* L. is popularly known as globe artichoke and belongs to the Asteraceae family (Trópicos, 2016). This species is widely used in the traditional medicine for the treatment of digestive disorders (Boughrara and Belgacen, 2015; Nogueira et al., 2016; Lima et al., 2016; Moradi et al., 2016). The scientific literature mainly reports its effects on liver diseases and often associates its effects to the presence of phenolic compounds such as caffeoylquinic acid derivatives (Pereira et al., 2016; Mohamed et al., 2016; Colak et al., 2016; Mocelin et al., 2016).

Although, there are already pharmacopoeial methods for quality control of artichoke dry leaf extract, it lacks further information regarding the determination of compounds other than chlorogenic acid. This study aimed to adapt and validate a method for simultaneous determination of neochlorogenic, chlorogenic and cryptochlorogenic acids, isochlorogenic acid A and C, and cynarin in tablets or capsules of artichoke leaf extract herbal medicines. The used method was based on European Pharmacopoeia - 7th edition (2011) for identification and quantification of chlorogenic acid in the globe artichoke dry leaf extract. Also, this method was used to evaluate the content of caffeoylquinic acid derivatives in three commercial herbal products and a dry leaf extract of *Cynara scolymus* L.

MATERIALS AND METHODS

Reagents and standards

All solvents were HPLC grade purchased from Tedia® and Sigma-Aldrich®. The water was purified using a Milli-Q system (Millipore). The standards 3-O-caffeoylquinic acid (chlorogenic acid), 4-O-caffeoylquinic acid (cryptochlorogenic acid), 5-O-caffeoylquinic acid (neochlorogenic acid) and 1,5-di-O-caffeoylquinic acid (cynarin) were obtained from Sigma-Aldrich®; 3,5-dicaffeoylquinic acid (isochlorogenic acid A) and 4,5-dicaffeoylquinic acid (isochlorogenic acid C) were purchased from ChromaDex®.

Plant material

C. scolymus L. dried leaves (batch number: 1004154) were obtained from *Panizza Fitoterápicos* Laboratory.

Herbal medicines

Three commercial products (named A, B and C) were obtained from the local market in Brasilia, Brazil. The products presented 200, 335 and 300 mg of *C. scolymus* dry leaf extract per tablet or capsule, respectively. Products A and B were purchased in the tablet dosage form, while the product C was purchased in the capsule dosage form. The manufacturer did not provide information about the harvest and extraction processes.

Preparation of *Cynara scolymus* L. extract

The dried leaves of *C. scolymus* L. were powdered in a knife mill (Marcone, MA580) and submitted to infusion. The infusion was performed in the proportion of 1 g of the powdered dried leaves to 10 mL of distilled water at 70°C. The extract was filtered, cooled down and then lyophilized (SP Scientific, Advantage Plus XL-70).

HPLC analysis

HPLC analysis was carried out using the chromatograph LaChrom Elite (Hitachi, Tokyo, Japan) equipped with L2455 DAD detector, L2200 injector, L2130 pump and L2300 column oven. Data were obtained with EZChrom Elite software, version 3.3.2 SP1. The method was adapted from the quality control monography of artichoke dry leaf extract presented by European Pharmacopoeia - 7th edition (EDQM, 2011). It was equipped with a C18 column (250 x 4.6 mm, five µm) with the temperature maintained at 40°C, flow rate of 1.2 mL/min, injection volume of 25 µL and detection at 330 nm. The mobile phase was composed of a gradient of phosphoric acid 0.5% (pump A) and phosphoric acid 0.5% in acetonitrile (pump B) (Table 1).

Sample preparation

Commercial herbal medicines were prepared at a concentration of 4 mg/mL. Using not less than 20 tablets or capsules, an accurately weighed portion of the powder, equivalent to 100 mg of the labeled amount of artichoke dry leaf extract, was solubilized in 25 mL of a mixture of methanol and water (3:7). The solution was filtered through a 0.45 µm membrane filter.

C. scolymus L. leaf extract (LE) was prepared at a concentration of 5 mg/mL by solubilizing the extract in a mixture of methanol and water (3:7). Also, the solution was filtered through a 0.45 µm membrane filter.

Standard solution

All standard solutions were freshly prepared at the concentration of 25 µg/mL in methanol.

Method validation

The method was validated considering the parameters of selectivity, precision, linearity, quantification and detection limits and accuracy. ICH guidelines and Brazil's regulations were used as reference (ICH, 1996; Brazil, 2003).

Selectivity

Selectivity evaluation was performed in triplicate by acidic and basic hydrolysis induction. Samples were solubilized in hydrochloric acid (HCl) 1 M or sodium hydroxide (NaOH) 1 M and incubated at 60°C

Table 2. Peak areas variations of samples with acidic and basic hydrolysis compared to samples without hydrolysis

Peaks	Acidic hydrolysis	Basic hydrolysis
Neochlorogenic acid	- 20.21%	CD
Chlorogenic acid	- 90.31%	CD
Cryptochlorogenic acid	- 45.99%	CD
Cynarin	+ 18.10%	- 66.25%
Isochlorogenic acid A	CD	CD
Isochlorogenic acid C	CD	CD

*CD: Complete degradation.

for 1 h in a water bath. After cooling to room temperature, samples were neutralized and diluted in methanol to a concentration of 4 mg/mL. The method selectivity was given by the comparison of retention time and peak areas related to samples with and without hydrolysis.

Linearity

The linear relation between peak areas and concentration of markers were performed by three analytic curves with six concentrations for each standard compound. Samples were prepared at concentrations of 1, 2.5, 5, 10, 25 and 50 µg/mL. The linearity equations were calculated by linear regression, using the software GraphPad Prism® Version 6.0.

Limit of detection (LOD) and limit of quantification (LOQ)

Both LOD and LOQ were determined from slope (*s*) and standard deviation of Y-intercept (SD) of the linearity curve, according to the following equations:

$$LOD = \frac{SDx3}{S} \qquad LOQ = \frac{SDx10}{S}$$

Precision

Precision was determined by repeatability (intraday precision). Six replicates of the same sample (4 mg/mL) were analyzed on the same day. Precision was expressed as relative standard deviation (RSD), not accepting results higher than 5% (Brasil, 2003).

Accuracy

The accuracy was performed by standard addition method with three concentrations (80, 100 and 120%) in triplicate. These concentrations were achieved with 500 µL of artichoke herbal medicine sample added to 500 µL of a mix containing all markers at the concentrations required to obtain the final theoretical concentration of 80, 100 and 120%. Accuracy was expressed as the ratio between the determined concentration mean and the corresponding theoretical concentration.

Evaluation of commercial products and leaf extract

Three commercial products and the leaf extract were evaluated for the presence of the caffeoylquinic acid derivatives, using the validated method. Identification of the markers was carried out by comparing the retention times and UV spectra of the peaks presented in the sample with the peaks of the standards. The amount of each marker in the sample was calculated two different ways: expressed as chlorogenic acid, calculated by applying the peak areas in the formula obtained by the linearity curve of chlorogenic acid; and calculated by applying the peak areas of each marker in the formula obtained by the linearity curve of the corresponding standard.

Statistical analysis

All the statistical analyses were performed using the software GraphPad Prism® 6.0. Data were expressed as mean ± standard deviation (SD). The one-way ANOVA followed by Tukey post-test was carried out to compare the content of markers between the commercial products. Results with *p*-value less than 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Selectivity

The induction of acidic and basic hydrolysis led to partial or complete peak degradation (Table 2) and formation of degradation products, but there was no co-elution of these products with the peaks of interest. Although, the cynarin peak presented an area increase after acid hydrolysis, this growth showed no statistical significance ($p > 0.005$ at t-Student test). These results demonstrate the ability of the method to accurately measure a compound in the presence of others substances or degradation products.

Linearity and limit of detection (LOD) and limit of quantification (LOQ)

The peak area versus concentration of each marker showed a linear response in a range of 1 to 50 µg/mL. The correlation coefficients (*r*) were higher than 0.99, as required by the guidelines. The limits of detection and quantitation represent the lowest amount of the markers which can be detected and quantified, respectively (Table 3).

Table 3. Linearity, limit of detection (LOD) and limit of quantification (LOQ).

Markers	*r*	Equation	LOD (µg/mL)	LOQ (µg/mL)
Neochlorogenic acid	0.997	Y= 191859x - 103767	1.05	3.49
Chlorogenic acid	0.999	Y= 203957x - 17409	0.20	0.68
Cryptochlorogenic acid	0.995	Y= 268182x + 13926	0.74	2.47
Cynarin	0.996	Y= 355651x + 105198	0.27	0.89
Isochlorogenic acid A	0.998	Y= 516598x - 17972	0.73	2.44
Isochlorogenic acid C	0.998	Y= 343767x + 48808	0.80	2.66

Table 4. Method precision.

Markers	RSD (%)
Neochlorogenic acid	1.65
Chlorogenic acid	2.22
Cryptochlorogenic acid	0.90
Cynarin	1.96
Isochlorogenic acid A	2.94
Isochlorogenic acid C	3.31

RSD: Relative standard deviation.

Precision

All values of intra-day precision, performed on the same day, with the same analyst and same instrumentation, were within acceptable limits with relative standard deviation not greater than 5%, demonstrating the method repeatability (Brasil, 2003). Results are presented in Table 4.

Accuracy

The accuracy, which represents the agreement between the obtained results and the accepted true value, was performed by the standard addition method. Regarding the standards chlorogenic acid, cryptochlorogenic acid, cynarin, isochlorogenic acid A and isochlorogenic acid C, the accuracy values were within the acceptable limits with relative standard errors not greater than 15%. However, relative standard error values of neochlorogenic acid were between 17 and 19%, which are higher than the acceptable limits. Therefore, the method has no proper accuracy for this standard. The results are shown in Table 5.

Evaluation of commercial products and dry leaf extract

After method validation, it was possible to obtain the chromatographic profiles, identify and quantify the caffeoylquinic acid derivatives in commercial globe artichoke herbal medicines and globe artichoke leaf extract (LE). Caffeoylquinic acid derivatives were identified by retention times and UV spectra (Figure 1). The chromatographic profile of LE showed a major peak corresponding to chlorogenic acid, minor peaks corresponding to neochlorogenic acid, cryptochlorogenic acid, isochlorogenic acid A and C and other minor peaks of unidentified substances (Figure 2). All commercial products presented neochlorogenic, chlorogenic, cryptochlorogenic, isochlorogenic acids A and C. However, commercial product B had more peaks in relation to the other products and was the only one that presented cynarin (Figure 3).

The literature often associates the therapeutic effects of the leaves of *C. scolymus* with cynarin, emphasizing it as the main compound of the species. However, these studies were carried out mainly in European Countries (Pagano et al., 2016; Petrovic et al., 2008; Falco et al., 2015; Kaymaz et al., 2017). In this study, the absence of cynarin was noted in the leaf extract and two of the three commercial herbal medicines. Also, previous studies performed with globe artichoke leaves grown in Brazil reported only traces of cynarin (Noldin et al., 2003; Costa et al., 2005). Moreover, the high chemical instability of cynarin makes it difficult to detect it in the final product (Guimarães et al., 2007; Carvalho et al., 2004). Further studies are needed to elucidate the presence of cynarin in globe artichoke leaves grown in Brazil, and the therapeutic implications of its absence.

Regarding the concentration of chemical markers, a significant difference was observed among the herbal medicines and mainly between the herbal medicines and the leaf extract. The leaf extract showed a significantly higher amount of chlorogenic acid and, therefore, a higher total amount of caffeoylquinic acid derivatives. The commercial herbal medicine B showed a proportion of chemical constituents closer to the leaf extract in relation to the others products (Table 6). These differences found in the chemical composition can be attributed to several factors including harvesting, climatic variations and extractive process (Tistaert et al., 2011; Govindaraghavan and Sucher, 2015; Applequist and Miller, 2013). Therefore, it is important to establish the standardization of extracts with the proper foundation of safety and efficacy.

Although, information regarding the extractive process is necessary for the registration of the herbal medicine by

Table 5. Accuracy and relative standard error (E%).

Theoretical concentration (µg/mL)	Determined concentration (µg/mL) ± SD	Accuracy (%)	E (%)
Neochlorogenic acid			
5.38 (80%)	6.34 ± 0.09	117.83	17.83
6.72 (100%)	8.05 ± 0.03	119.00	19.00
8.06 (120%)	9.47 ± 0.04	117.53	17.53
Chlorogenic acid			
33.12 (80%)	35.86 ± 0.13	108.26	8.26
41.40 (100%)	46.33 ± 0.04	111.90	11.90
49.69 (120%)	54.71 ± 0.18	110.11	10.11
Cryptochlorogenic acid			
2.64 (80%)	2.62 ± 0.07	99.08	0.92
3.30 (100%)	3.37 ± 0.003	102.12	2.12
3.96 (120%)	4.02 ± 0.002	101.53	1.53
Cynarin			
1.10 (80%)	1.23 ± 0.005	111.82	11.82
1.37 (100%)	1.53 ± 0.005	111.40	11.40
1.64 (120%)	1.77 ± 0.004	108.08	8.08
Isochlorogenic acid A			
17.77 (80%)	14.78 ± 0.05	83.16	16.84
22.21 (100%)	19.10 ± 0.02	86.00	14.00
26.66 (120%)	23.16 ± 0.09	86.88	13.12
Isochlorogenic acid C			
8.22 (80%)	7.98 ± 0.01	97.11	2.89
10.28 (100%)	10.09 ± 0.01	98.20	1.80
12.33 (120%)	11.90 ± 0.01	96.55	3.45

Determined concentration represented by mean ± standard deviation (SD). Accuracy was expressed as the ratio between the determined concentration mean and the corresponding theoretical concentration.

Table 6. Concentration of markers per 100 mg of extract.

Markers	Concentration (mg/100 mg of extract)			
	A	B	C	LE
Neochlorogenic acid	0.195 ± 0.011	0.122^{b} ± 0.010	0.208 ± 0.005	0.079^{a} ± 0.007
Chlorogenic acid	0.373 ± 0.019	1.608 ± 0.147	0.412 ± 0.012	6.000^{a} ± 0.485
Cryptochlorogenic acid	0.116 ± 0.005	0.134 ± 0.012	0.148 ± 0.004	0.113 ± 0.013
Cynarin	-	0.066 ± 0.004	-	-
Isochlorogenic acid A	0.080 ± 0.002	0.582^{c} ± 0.046	0.078 ± 0.003	0.365^{a} ± 0.034
Isochlorogenic acid C	0.101 ± 0.004	0.168 ± 0.016	0.105 ± 0.003	0.051^{a} ±0.005
Total	0.865	2.68	0.95	6.61

Concentration of markers per 100 mg extract. Data represented by mean ± standard deviation (SD). LE: Leaf extract. Comparisons carried out by one-way ANOVA followed by Tukey post-test. a: different from A, B and C (p<0.05); b: different from C (p<0.001); c: different from A and C (p<0.005).

the regulatory agency in Brazil (Anvisa), they are not usually provided transparently by the industry. Therefore, this information should be made available to health professionals in the commercial product professional leaflets, which could strengthen the confidence of professionals in prescribing herbal medicines (Evans and Avila, 2016).

The results were also expressed as chlorogenic acid (Table 7), to allow the comparisons with the requirements established by Anvisa. According to Anvisa's regulation,

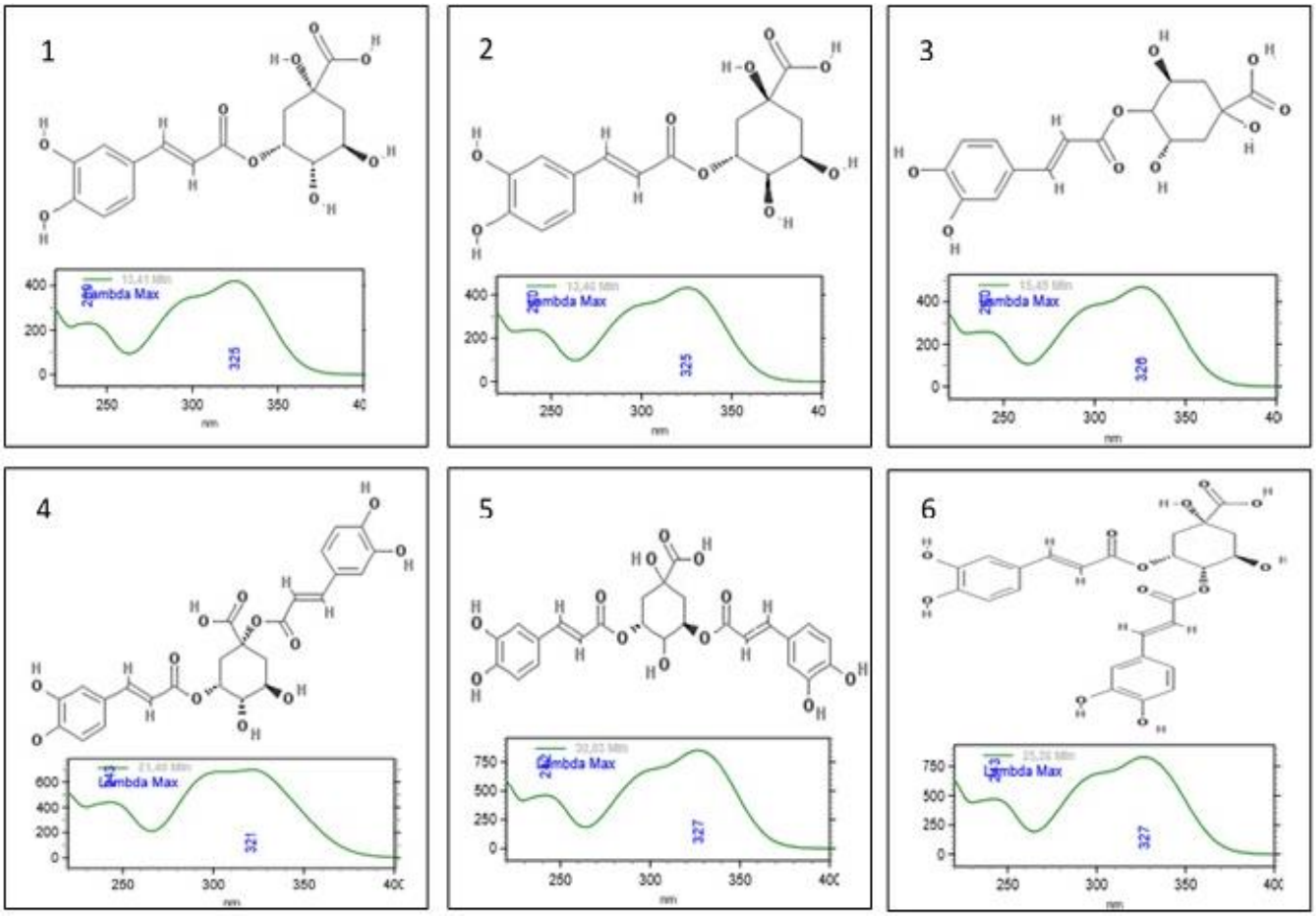

Figure 1. UV Spectra at 330 nm and chemical formulas. 1: Neochlorogenic acid; 2: chlorogenic acid; 3: cryptochlorogenic acid; 4: cynarin; 5 isochlorogenic acid A; 6: isochlorogenic acid C.

Table 7. Concentration of markers per 100 mg of extract expressed as chlorogenic acid.

Markers	Concentration (mg/100 mg of extract)			
	A	B	C	LE
Neochlorogenic acid	0.173 ± 0.010	0.104[b] ± 0.009	0.185 ± 0.005	0.066[a] ± 0.007
Chlorogenic acid	0.373 ± 0.019	1.608[c] ± 0.147	0.412 ± 0.012	6.000[a] ± 0.485
Cryptochlorogenic acid	0.156 ± 0.007	0.180 ± 0.015	0.198 ± 0.005	0.151 ± 0.018
Cynarin	-	0.130 ± 0.006	-	-
Isochlorogenic acid A	0.203 ± 0.006	1.473[c] ± 0.117	0.198 ± 0.008	0.925[a] ± 0.086
Isochlorogenic acid C	0.179 ± 0.006	0.292[c] ± 0.026	0.184 ± 0.004	0.093[a] ±0.008
TOTAL	1.08	3.79	1.18	7,23

Concentration of markers per 100 mg of extract expressed as the equivalent of chlorogenic acid. Data represented by mean ± standard deviation (SD). LE: Leaf extract. Comparisons carried out by one-way ANOVA followed by Tukey post-test. a: different from A, B and C (p<0.005); b: different from C (p<0.005); c: different from A and C (p<0.005).

the active markers can vary by ±15% in relation to the labeled amount (Brazil, 2014). According to the products leaflets, each capsule or tablet in commercial products A and B contained, respectively, 1.4 and 7 mg of caffeoylquinic acid derivatives, expressed as chlorogenic acid equivalent. The commercial herbal medicines A and B showed a variation of 55 and 81.14%, respectively, in comparison with the labeled amount, which is higher than

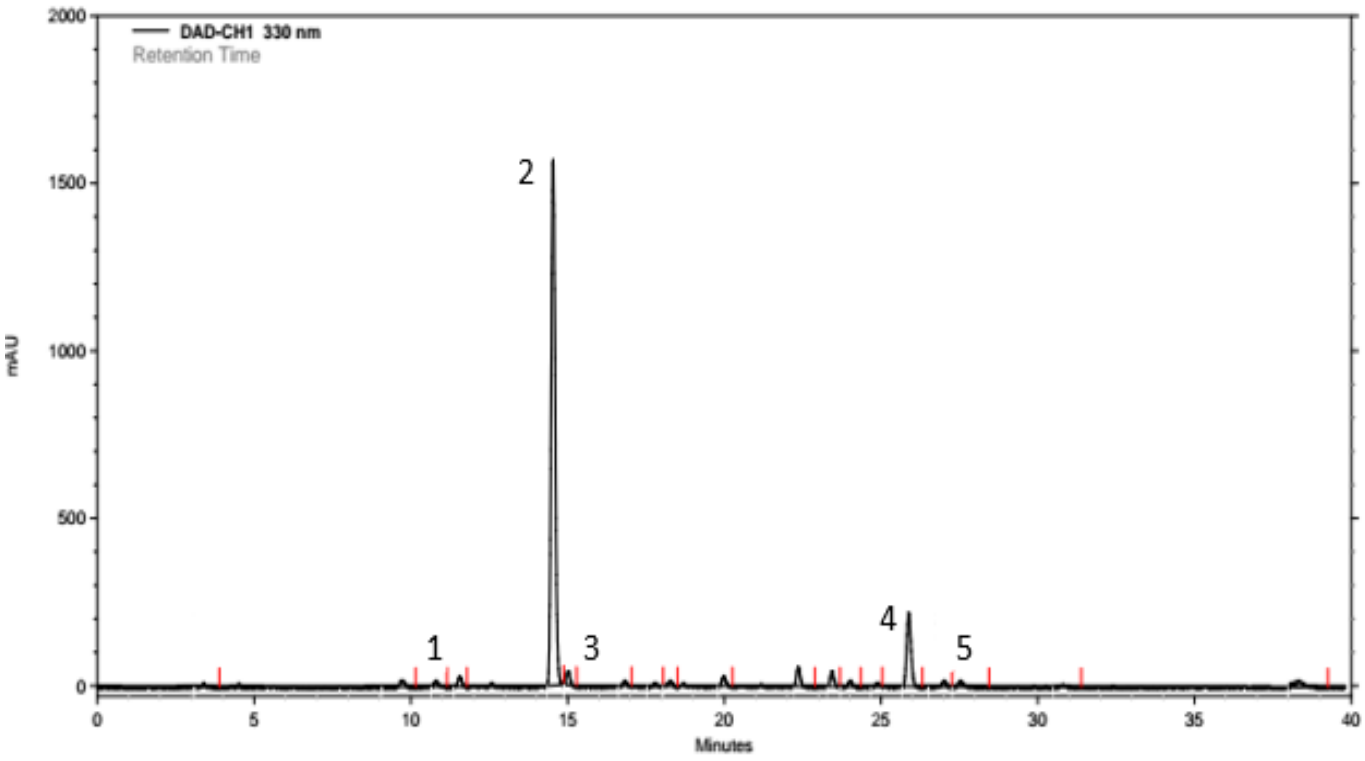

Figure 2. Chromatographic profile of *C. scolymus* L. leaf dry extract, obtained by HPLC-DAD with detection at 330 nm. 1: Neochlorogenic acid; 2: chlorogenic acid; 3: cryptochlorogenic acid; 4: isochlorogenic acid A; 5: isochlorogenic acid C.

the allowable variation. Herbal medicine C should have 1.5 mg of cynarin per capsule. However, as previously discussed, the analysis showed no detectable amounts of cynarin in the sample.

The Brazilian regulatory agency recommends a daily intake of 24 to 48 mg of caffeoylquinic acid derivatives expressed as chlorogenic acid (Brasil, 2014). According to the products leaflets, six capsules or tablets should be administered per day. The commercial products A and C showed lower amounts of caffeoylquinic acid derivatives in the finished product as compared to the recommended daily dose (13.02 and 21.18 mg, respectively), while product B showed a higher amount (76.08 mg). Therefore, all herbal medicines were in disagreement with the dose established by Anvisa. Besides demonstrating a clear noncompliance with the Brazilian sanitary regulation, these results can represent a negative impact on therapeutic outcomes and the occurrence of adverse events, which include, mainly, digestive disorders (EDQM, 2011).

Conclusion

Considering the synergistic action of plant constituents, an analysis focused on multiple markers, may be more representative in terms of quality (Xie and Leung, 2009; Guo et al., 2016; Long et al., 2015). The proposed method for the simultaneous identification and quantification of chlorogenic, cryptochlorogenic, neochlorogenic, isochlorogenic acids A and C and cynarin can be a suitable tool for quality assessment of globe artichoke herbal medicines. A considerable variability in the proportion of chemical constituents was observed in the three samples of herbal medicines containing globe artichoke dry leaf extract. This variation can potentially represent public health risks, and this study emphasizes the importance of monitoring the quality of herbal medicines to ensure safety and efficacy of these products.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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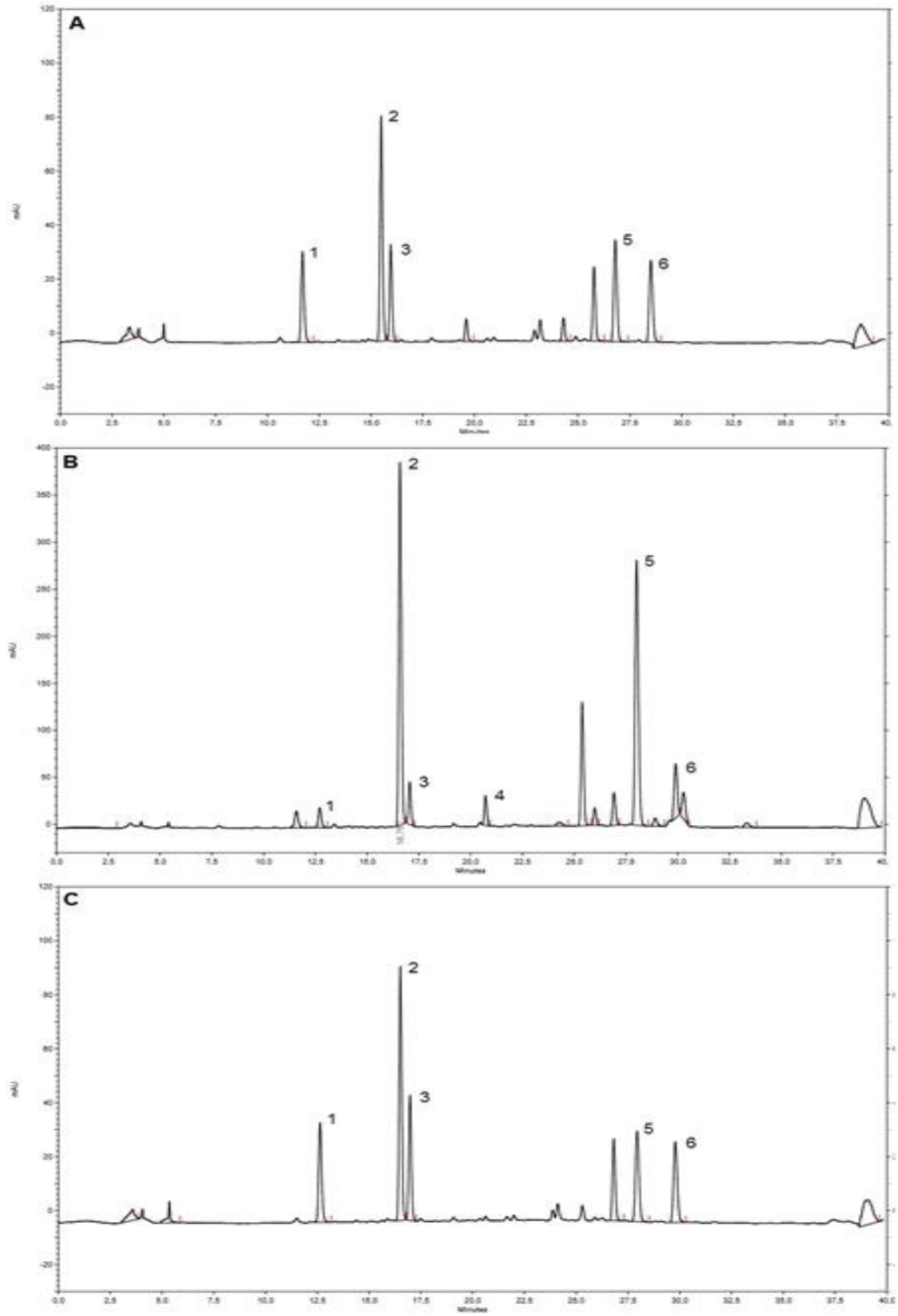

Figure 3. Chromatographic profiles of commercial products A, B and C, obtained by HPLC-DAD with detection at 330 nm. 1: Neochlorogenic acid; 2: chlorogenic acid; 3: cryptochlorogenic acid; 4: cynarin; 5: isochlorogenic acid A; 6: isochlorogenic acid C.

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