Original Article

Simultaneous determination of epicatechin and procyanidin A2 markers in *Litchi chinensis* leaves by high-performance liquid chromatography

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ABSTRACT

The fruits of *Litchi chinensis* Sonn., Sapindaceae, are renowned for their biological activities. However, their leaves are poorly explored, although they represent an important source of vegetable raw material with biological properties as antioxidant, anti-inflammatory and antinociceptive. An HPLC method was developed and validated for the simultaneous quantification of epicatechin and procyanidin A2 in the leaf hydroethanolic extract of *L. chinensis*. The markers and other unidentified components were separated on a Luna Phenomenex C_{18} column (250 mm × 4.6 mm, 5 μm) with mobile phase composed of acetonitrile: water pH 3.0 (with sulfuric acid), in a gradient run; at 1.0 ml min^{-1}, 30 °C and 278 nm for detection. The method was linear over an epicatechin and procyanidin A2 concentration range of 10–100 μg ml^{-1}. The Limit of Quantification for epicatechin and procyanidin A2 were 1.7 and 2 μg ml^{-1}, respectively. The Relative Standard Deviation (%) values for markers (intra- and inter-day precision studies) were <4.0% and the accuracy was 100 ± 5%. The method was applied to ten samples collected in the state of Santa Catarina (Brazil), which showed 14.8–44.5 and 44.8–69.6 mg g^{-1} of epicatechin and procyanidin A2, respectively. The proposed method could be a valuable tool for quality assessment of *L. chinenis* leaves as well as their herbal derivatives.

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Introduction

Litchi chinensis Sonn., Sapindaceae, is an evergreen tree that is indigenous to the subtropics of southern China and has been grown there for over 3500 years (Holcroft and Mitcham, 1996). This specie is widely distributed in Asia, Africa and Americas, and the main economic interest has been assigned to its fruits, which are used both as a source of food as in cosmetics (Menzel, 2002; Martins, 2005). In Brazil, 'Bengal' and 'Brewster' species have been cultivated since 1810 (Martins, 2005).

There are many reports about the phytochemical composition of *L. chinensis* fruits, especially epicatechin and proanthocyanidins (Ruenroengklin et al., 2008; Zhou et al., 2011; Li et al., 2012), and

pharmacological activities, such as immunomodulatory and anti-tumoral (Wang et al., 2006; Zhao et al., 2007), antioxidant (Yang et al., 2006; Duan et al., 2007) and hepatoprotective (Bhoopat et al., 2011). On the other hand, leaves have been used as poultices for skin disease treatment (Pandey and Sharma, 1989) and also as a source of Traditional Chinese Medicine for heatstroke, flatulence and detoxification treatment (Wen et al., 2014). Analgesic and anti-inflammatory activity was firstly reported for the petroleum ether leaf extracts in 1996 (Bersa et al., 1996), as well as hepatoprotective activity in rats, especially for the leaf methanolic extract (Basu et al., 2012). Recently, the isolation and the antioxidant activity (DPPH, ABTS$^+$ and lipidic lipoperoxidation) of (−)-epicatechin (**1**), procyanidin A2 (**2**), procyanidin B2 (**3**), from methanol and ethanol crude leaf extracts of *L. chinensis*, were reported by our research group (Castellain et al., 2014), and in addition, the methanol extract showed central and peripheral antinociceptive effect in four pre-clinical models. These findings

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suggest the potential of *L. chinensis* leaves as source of material for pharmaceutic and cosmetic herbal based products. Despite the pharmacological interest in this herbal drug, there is no report in the literature of HPLC analytical methodology to support its quality control, concerning the quantification of potential markers, as epicatechin (**1**) and procyanidin A2 (**2**), which had already been reported in the litchi leaves (Castellain et al., 2014). Polyphenols as epicatechin and procyanidin are known to have beneficial effects on the human health and have been quantified in several medicinal plants by LC-UV methods (Lopes et al., 2010; Negri et al., 2010; Sousa et al., 2011), however, since herbal medicines and extracts are a complex matrix, each specie represents an analytical challenge.

Based on these biological activities related to *L. chinensis* leaves, hydroethanolic and glycolic extracts have been studied by our group as source of active pharmaceutical ingredient for medicines and cosmetic herbal based products. For these purposes, it is important to establish markers and analytical methodologies, which could be able to monitor the chemical quality of the herbal raw material and its derivatives, batch to batch, and also as a valuable tool for optimization of extraction and stability indicating studies. In this sense, the present work aims the development and validation of a LC-UV methodology to quantify epicatechin and procyanidin A2 in hydroethanolic extract of *L. chinensis* leaves, applying the methodology to different geographical samples, harvested in the South of Brazil.

Material and methods

Chemicals and reagents

Epicatechin (**1**) and procyanidin A2 (**2**) were isolated from *L. chinensis* leaves. The purity of the isolated compounds was determined by HPLC using the method described as follow (> 99% for EPI and >85% for PA2). The chemical structures were confirmed by NMR ^{1}H and ^{13}C as previously reported (Castellain et al., 2014).

Methanol and acetonitrile (HPLC grade) were obtained from J.T.Baker (Phillipsburg, New Jersey, USA). Water was purified using Easy Pure® equipment (Waltham, Massachusetts, USA). The ethanol was analytical grade, purchased from Dinâmica (Diadema, São Paulo Brazil). All other solvents were analytical grade.

Plant material

Batches of *Litchi chinensis* Sonn., Sapindaceae, leaves were sampled from different cities of Santa Catarina, a state in the South of Brazil, in the summer over the years of 2010–2014: Itajaí (year of 2010, 2014), Blumenau (year of 2010), Florianópolis (year of 2014), Palhoça (year of 2014), Pomerode (year of 2014), and Zimbros (year of 2014). All the samples were authenticated, taking into account the literature (Menzel, 2002) and compared with the voucher specimen, number 52829, which was deposited at the Barbosa Rodrigues Herbarium (BRH, Itajaí-SC, Brazil). The leaves were manually separated, cleaned and dried in an air oven at 35 °C until moisture stabilization. After drying, the plant material was ground in a knife mill (outlet sieve = 2 mm) and the average size of the particles was determined by sieving (Farmacopeia Brasileira, 2010).

### Preparation of extractive solution and soft extract

The extractive solution was obtained by dynamic maceration of milled leaves with ethanol:water 70:30 (v/v) at a plant:solvent ratio of 5:100 (w/v), stirred for 4 h at room temperature, filtered through Sontara® paper, kept in amber glass bottles and stored at room temperature. The dried residue was determined by drying 5 ml (weighed) of extractive solution at 100 °C until constant weight in triplicate.

The soft extract was obtained by evaporation of extractive solution at 50 °C, with the aid of vacuum and water bath, in order to achieve 92% of dried residue. The loss on drying of soft extract was determined by drying 0.5 g at 100 °C until constant weight in triplicate (Farmacopeia Brasileira, 2010).

Sample solutions preparation

Both extractive solution and soft extract were analyzed by HPLC in order to quantify the markers. The extractive solution sample for HPLC analysis was prepared by dissolving 1 ml with 5 ml with solvent solution (1:9 (v/v) acetonitrile:acidified water at pH 3.0 using sulfuric acid). The dried residue was taken into account to express the marker assay in this sample.

The soft extract solution sample for HPLC analysis was prepared by dissolving 300 mg in 5 ml of methanol, in a 10 ml volumetric flask, which was sonicated for 15 min, and the volume was completed with the same solvent (stock sample solution), which was

1   2   3
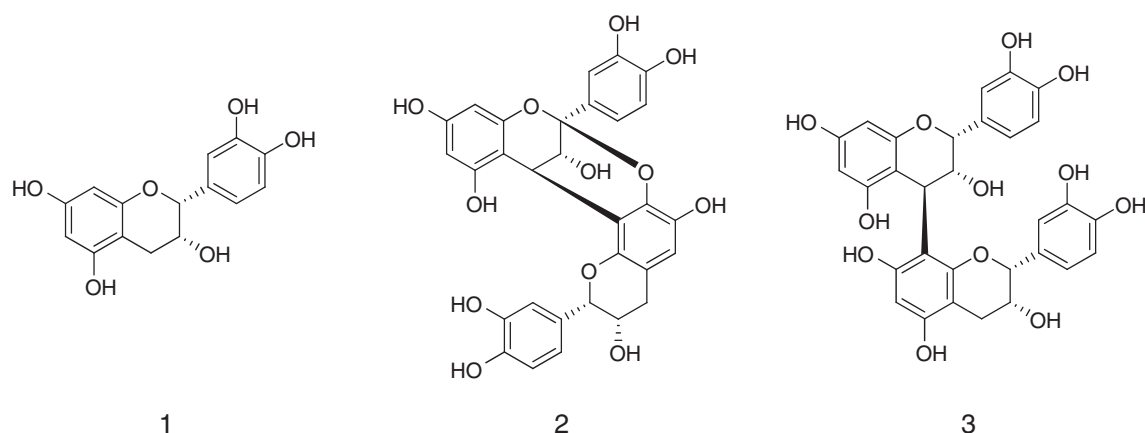
stored at −20 °C. At the moment of analysis, the stock sample solution was thawed at room temperature and diluted (1:10) with solvent solution (1:9 (v/v) acetonitrile:acidified water at pH 3.0 using sulfuric acid) to obtain a concentration of 3 mg ml^{-1}. The loss on drying was taken into account to express the marker assay in this sample.

Standard solution

EPI and PA2 (10 mg) were dissolved with methanol to produce a stock standard solution (1000 μg ml^{-1}). This standard solution was kept in a freezer (−20 °C) and used for the analyses and validation. At the moment of analysis, the stock standard solution was thawed at room temperature and diluted (1:10) with solvent solution to obtain a concentration of 50 μg ml^{-1} (standard solution).

HPLC analysis

A Shimadzu LC-10AD LC system (Shimadzu, Tokyo, Japan) consisting of a binary pump (LC-10ADvp), column oven (CTO-10Avp), an automatic injector (SIL-10AF) and a Shimadzu SPD-M10A photo diode array detector was used. The injections (20 μl) were carried out on a Phenomenex® (Torrance, California, USA) Luna C18 5 μm (250 mm × 4.6 mm) at 30 °C, with detection at 278 nm. For the method development, different solvent systems were assayed in isocratic and gradient conditions using methanol, acetonitrile and acidified water (pH 3.0 with sulfuric acid), at 1 ml min^{-1}. The best gradient was chosen: gradient acetonitrile (A): acidified water pH 3.0 with sulfuric acid (B) of 10:90 (A:B) (0–5 min); 15:85 (5–7 min); 20:80 (7–13 min); 25:75 (13–20 min); 30:70 (20–30 min); 10:90 (30 min) maintaining this composition for 40 min then returning to the initial conditions.

The EPI and PA2 assay in the samples was determined by external standardization, by the simultaneous analysis of a standard solution as described above. Daily, the suitability parameters (resolution – $R > 1.0$, tailing factor – $T < 1.5$, and repeatability of area – RSD% < 2.0) were determined.

Method validation

The method was validated according to the national (Anvisa, 2003) and international guidelines (ICH, 2005), using the extractive solution prepared from the sample (Itajaí – summer, 2010).

The selectivity of the HPLC method was evaluated by comparing the chromatogram of a blank (diluent solution), the mobile phase, and the sample solution, to detect any co-elution interference. The resolution between peaks was analyzed, and also the purity index of markers peaks through PDA detector.

The linearity was evaluated for analytical curves using three different procedures. In the first procedure, the EPI and PA2 standard solution at 1000 μg ml^{-1} was diluted in triplicate, in the range of 10–100 μg ml^{-1}, in seven levels, and injected in duplicate (curve A). Curve A was spiked with a fixed volume of stock sample solution (1 ml in all volumetric flasks of the standard analytical curve) (curve B). Finally, another curve of sample solution was carried out by the dilution of a stock sample solution at 5 mg ml^{-1} in seven different levels (1–5 mg ml^{-1}), in triplicate. All the analytical curves were plotted and statistically evaluated.

The Limit of Detection (LOD) and Limit of Quantification (LOQ) were calculated based on standard deviation of the y-intercepts of equation curves obtained by linear regression (ICH, 2005).

The accuracy of the method was estimated through the analyte recovery test (Anvisa, 2003; ICH, 2005), spiking a 20 mg ml^{-1} stock sample solution with three different level (250, 500 and 750 μl) of stock standard solution (at 1000 μg ml^{-1}) in triplicate. The three levels of standard addition represented 50, 100 and 150% of the markers target concentration, which was previously determined in the sample. The recovery of both markers was calculated after discounting the marker area from the sample solution without standard addition.

Repeatability (intra-day) and intermediate precision (inter-day) were determined through analysis (six replicates) of the sample solution, prepared as described above, with injection in duplicate. The RSD% of the marker assay was determined. This procedure was repeated after two days in order to assess the intermediate precision.

The robustness of the chromatographic method was evaluated by changing the mobile phase flow (0.9, 1.0 and 1.1 ml min^{-1}), the oven temperature of the column (29, 30 and 31 °C), the pH of the mobile phase, and the store conditions of the analytical solutions (0, 24 and 48 h, at room temperature, fridge and freezer). For each experiment, the sample and the standard solutions (prepared as described before) were injected in triplicate. The data (retention time, resolution and assay of markers) were analyzed and the RSD% was calculated.

Statistical analysis

The results were expressed as the mean (RSD%). Statistical significance among groups was calculated by analysis of variance (ANOVA) in which the results were considered significant when the probability was less than 5% ($p < 0.05$), followed by the Tukey test. Statistical analysis was carried out using the software program GraphPad Prism 5.0.

Results and discussion

According to Brazilian and European guidelines, markers are chemically defined as constituents or groups of constituents which are useful to the obtainment of an herbal preparation and an herbal medicinal product, being considered active markers when contribute to the therapeutic activity (EMA, 2011; Anvisa, 2014). Therefore, PA2 and EPI may be considered active markers, based on previous studies of *L. chinensis* leaves (Castellain et al., 2014). The determination of these markers levels in a variety of herbal drug samples contributes to the establishment of specifications for the future development of pharmaceutical or cosmetic herbal based products.

Method development

Regarding the separation of the phenolic compounds of the *L. chinensis* leaf extractive solution (Fig. 1), initially many gradient systems were tested, using acetonitrile, methanol and acidified water (pH 2.5–3.0, with sulfuric or trifluoroacetic acid), at 30–40 °C, 1.0–1.3 ml min^{-1}. Also, different columns (XSelect CSH® hexyl phenyl 3.5 μm, 4.6 mm × 100 mm, Waters; Luna C$_{18}$ 3 μm, 4.6 mm × 150 mm and 5 μm, 4.6 mm × 250 mm, Phenomenex) were tested. The Luna C$_{18}$ 5 μm, 4.6 mm × 250 mm Phenomenex provided a suitable chromatographic profile, with the best separation of both supposed markers, as well as the other compounds. The eluent was monitored by PDA, and the detection wavelength was set at 278 nm which represents the wavelength of maximum absorbance (Fig. 1).

In a run of 40 min, the EPI eluted at 17 min and PA2 at 24 min (Fig. 2a and b). Another minority compound, identified as being PB2 eluted at 16 min. The UV profile of the two major markers is shown in the inserts of Fig. 1, being similar to the other major unknown peaks (peak 1 and 2), with typical UV absorption profile of phenolic compounds. The method meets the previous criteria of suitability.
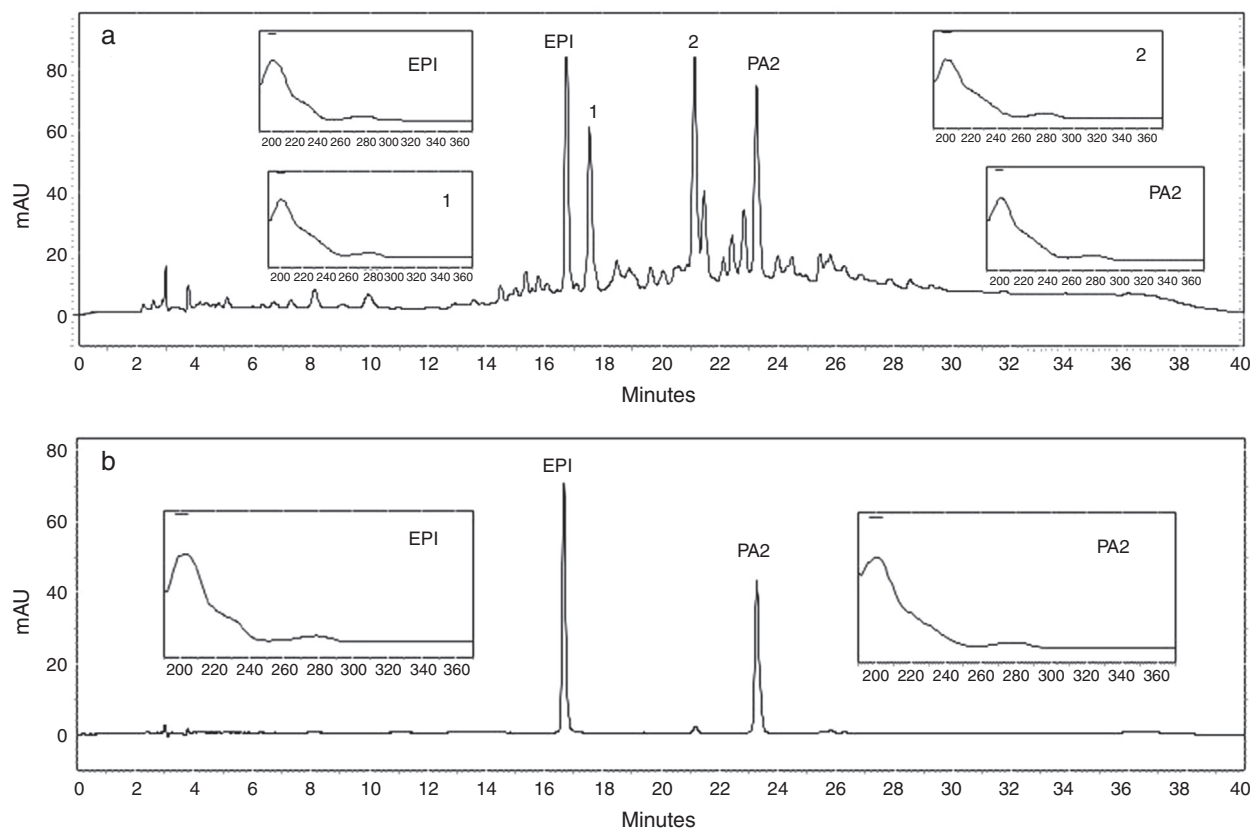
Fig. 1. Chromatographic profile of *Litchi chinensis* sample solution at 3 mg ml^{-1} (a); EPI and PA2 at 100 μg ml^{-1} (b).

Method validation

The selectivity of the method was shown, without co-elution of the peaks (Fig. 1), purity index >0.999, and absence of solvent influence.

The EPI and PA2 calibration curves proved to be linear over the range of 10–100 μg ml^{-1}, as shown by the linear equations and regression coefficients (r^2): $y = 14031x − 8345.3$ ($r^2 > 0.999$), $y = 11630x + 20,426$ ($r^2 > 0.999$), for the EPI and PA2, respectively, demonstrating an acceptable data fit to the regression curve. The standard addition in soft extract (curve B) proved to be parallel to isolated standards (curve A), with similar slopes, without interference of the matrix on the linearity of the method ($y = 13715x + 60,459$, ($r^2 > 0.996$, $y = 11405x + 58,866$, ($r^2 > 0.996$) for the EPI and PA2, respectively), and random distribution of residuals (data not shown). In addition, the analytical curve of soft extract (curve C) in the range corresponding to 1–5 mg ml^{-1} of EPI and PA2 also showed suitable linearity ($r^2 > 0.99$). The method showed high sensitivity, as demonstrated by the low estimated values of LOD and LOQ for both markers, of 0.50 and 1.67 for EPI, respectively, and 0.61 and 2.04 μg ml^{-1}, for PA2, respectively.

The mean recovery of both chemical markers was within the range of 90–107% considered acceptable (AOAC, 2012) in view of the low level of chemical markers in the analyzed samples. The intra- and inter-day precision (Table 1) showed RSD% <5%, which is considered appropriate (AOAC, 2012).

The robustness was estimated after making small variations in the parameters of the methodology, and the results were evaluated based on the mean and RSD% (intra and inter) for area, retention time, resolution and marker assay in the soft extract (Table 2). Although there is influence of deliberate variations in the methodology imposed in relation to the chromatographic parameters (area, retention time and resolution), the method proved to be robust

Table 1
Accuracy and precision for the assay of EPI and PA2 in leaf extract of *Litchi chinensis*.

Accuracy					
Theoretical concentration (μg ml^{-1})		Recovery (%)		RSD (%)	
EPI	PA2	EPI	PA2	EPI	PA2
26.13	22.96	102.5	102.2	0.67	2.69
52.43	45.94	102.8	102.2	2.03	0.72
80.05	69.96	104.6	103.8	0.38	2.09
Precision					
		Amount of EPI (mg g^{-1}) (RSD %)		Amount of PA2 (mg g^{-1}) (RSD %)	
Repeatability (Day 1)		19.58 (2.96)		23.58 (3.71)	
Repeatability (Day 2)		19.97 (1.95)		24.31 (2.54)	
Intermediate precision		19.78 (2.64)		23.95 (3.47)	

considering the assay of both markers in the samples with RSD% inter <5% for most determinations. Among the chromatographic parameters most affected, the resolution showed the greatest variability in the highest levels of temperature, flow and pH of the mobile phase. Regarding the variations imposed on the method, the flow of the mobile phase showed the greatest impact on the chromatographic parameters, including the determination of PA2, highlighting that this parameter should be well controlled during analysis. The sample and standard solutions stored at room temperature, freezers and refrigerator showed to be stable for 48 h, maintaining the peak area of both markers (RSD% ≤ 1.0%).

The leaf extractive solution of *L. chinensis* collected from different cities of the State of Santa Catarina (South of Brazil) were analyzed and showed a similar chromatographic profile (data not

Table 2
Robustness of the assay of EPI and PA2 in leaf extract of *Litchi chinensis*.

|	Mean (%RSD$_{intra}$)							
	Area		R_t (min)		R		Assay (mg/g)	
	EPI	PA2	EPI	PA2	EPI	PA2	EPI	PA2
Temperature (°C)								
29	709,706 (3.60)	711,762 (0.92)	17.34 (0.82)	23.59 (0.11)	2.54 (1.67)	1.14 (4.40)	19.34 (2.94)	16.65 (0.92)
30	710,487 (0.34)	707,988 (1.85)	17.19 (0.21)	23.86 (0.15)	1.22 (0.80)	1.75 (2.94)	19.30 (0.34)	22.15 (1.85)
31	696,520 (0.45)	684,428 (1.24)	17.00 (0.46)	24.02 (0.22)	1.29 (30.92)	1.60 (34.66)	19.03 (0.37)	22.12 (1.24)
%RSD$_{inter}$	(1.87)	(2.16)	(0.78)	(0.73)	(27.06)	(23.68)	(1.76)	(2.20)
Flow (ml min^{-1})								
0.9	794,709 (1.07)	814,976 (2.20)	18.57 (1.18)	25.40 (0.67)	2.00 (29.02)	2.18 (4.49)	19.44 (0.87)	22.87 (2.20)
1.0	710,487 (0.34)	707,988 (1.85)	17.19 (0.21)	23.86 (0.15)	1.22 (0.80)	1.75 (2.94)	19.30 (0.34)	22.15 (1.85)
1.1	662,000 (1.19)	655,750 (1.27)	15.88 (0.09)	22.30 (0.33)	1.05 (8.26)	1.67 (9.33)	19.79 (0.97)	22.08 (1.27)
%RSD$_{inter}$	(7.29)	(5.74)	(6.08)	(4.86)	(28.71)	(34.27)	(1.47)	(5.74)
pH mobile phase								
2.9	727,819 (0.08)	722,094 (0.27)	16.79 (0.23)	23.35 (0.17)	1.33 (1.98)	2.27 (0.25)	19.63 (0.08)	21.75 (0.61)
3.0	710,487 (0.34)	707,988 (1.85)	16.74 (0.18)	23.86 (0.15)	1.21 (4.26)	1.75 (2.94)	19.30 (0.34)	22.15 (1.85)
3.1	704,938 (2.60)	689,595 (1.64)	17.12 (0.57)	23.64 (1.75)	1.78 (3.89)	2.04 (17.93)	19.33 (2.12)	22.38 (1.64)
%RSD$_{inter}$	(1.76)	(8.91)	(1.10)	(5.05)	(13.88)	(21.33)	(1.50)	(2.20)
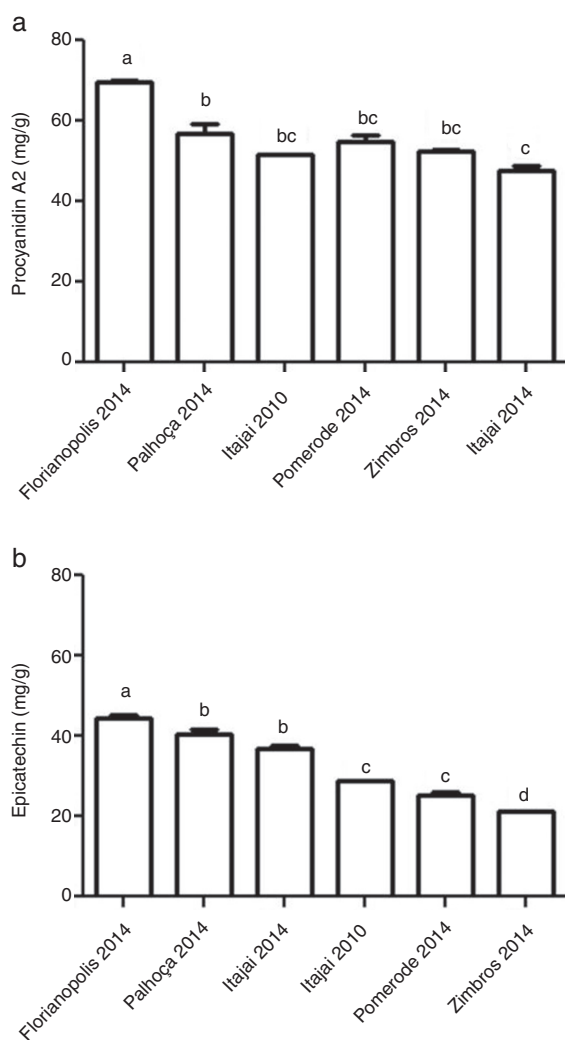
Fig. 2. Procyanidin A2 (a) and Epicatechin (b) concentration and variation among samples of *Litchi chinensis* leaves which were harvested in the summer of different cities of Santa Catarina State (Brazil). Means followed by the same letter over the bars do not differ according Tukey test ($\alpha = 0.05$).

shown) in comparison with the sample from Itajaí. EPI and PA2 were present in all samples (Fig. 2).

The amount of EPI and PA2 may vary depending on the city (five different location) for the same season (Fig. 2), with higher content of PA2 in all samples. On the other hand, the variation of EPI was higher than that of PA2, being the first a more discriminative marker of environmental conditions. Apart from the need to establish the markers ranges, to support the quality specifications of raw plant material it is known that tannins possess an ability as non-enzymatic antioxidants and in high concentrations they may also play a role in UV-protection of plants (Hagerman et al., 1998). Also, Lavola et al. (2003) reported that ambient range of UV-B radiation can influence the chemical composition (flavonoids and tannins) of Scots pine seedlings (*Pinus sylvestris*) and enhancement in nutrients may increase their responsiveness.

Therefore, these active compounds can be established as quality markers for *L. chiniensis* leaves derivatives employed in the development of pharmaceuticals and cosmetics. Its determination in different samples is a crucial factor for the future establishment of quality specifications for vegetable raw material to be used in the pharmaceutical and cosmetics industries. A further analysis of the biological activity of the different samples will establish the minimum limits for markers, contributing to the production of a monograph for the herbal drug.

Conclusion

The LC-UV method for the EPI and PA2 assay was validated and proved to be sensitive, accurate, linear, precise, reproducible, repeatable and robust. The results showed that this method could be readily applied as a suitable quality-control method for the quantification of epicatechin and procyanidin A2 in *L. chinensis* leaves derivatives, and may be used in the future development of herbal medicinal products, as potential markers. Further study is need beyond seasonality, location as parameters for establishment of quality control specifications of this specie as herbal pharmaceutical raw material.

Authors contributions

LCT, LCB and SLZ developed the research, RAF assisted the in micro- and macroscopic identification of the species, CMSB and VCF isolated and characterized the markers, AGC and TMBB advised the

research and prepared the manuscript. All the authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

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References

Anvisa, 2003. Resolução n 899 de 29 de maio de 2003: Guia para Validação de Método Analítico e Bioanalítico Agência Nacional de Vigilância Sanitária. Diário Oficial da União, Brasília (accessed 02.06.03).

Anvisa, 2014. Resolução n° 26 de 13 de maio de 2014. Agência Nacional de Vigilância Sanitária. Diário Oficial da União, Brasília (accessed 14.05.14).

AOAC, 2012. Guidelines for Single Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals., pp. 1–38, Available from: http://www.aoac.org/imis15_prod/AOAC_Docs/StandardsDevelopment/SLV_Guidelines_Dietary_Supplements.pdf (accessed May 2015).

Basu, S., Haldar, N., Bhattacharya, S., Biswas, S., Biswas, M., 2012. Hepatoproctive activity of *Litchi chinensis* leaves against paracetamol-induced liver damage in rats. J. Sci. Res. 7, 77–81.

Bersa, S.E., Sharma, R.M., Gomes, A., 1996. Antiinflammatory effect of petroleum ether extract of leaves of *Litchi chinensis* Gaertn. (Sapindaceae). J. Ethnopharmacol. 54, 1–6.

Bhoopat, L., Srichairatanakool, S., Kanjanapothi, D., Taesotikul, T., Thsnanchai, H., Bhoopat, T., 2011. Hepatoprotective effects of lychee (*Litchi chinensis* Sonn.):bA combination of antioxidant and anti-apoptotic activities. J. Ethnopharmacol. 136, 55–66.

Castellain, R.C.L., Gesser, M., Tonini, F., Schulte, R.V., Demessiano, K.Z., Wolff, F.R., Delle-Monache, F., Netz, D.J.A., Cechinel-Filho, V., de Freitas, R.A., de Souza, M.M., Meyre-Silva, C., 2014. Chemical composition, antioxidant and antinociceptive properties of *Litchi chinensis* leaves. J. Pharm. Pharmacol. 66, 1796–1807.

Duan, X., Jiang, Y., Su, X., Zhang, Z., Shi, J., 2007. Antioxidant properties of anthocyanins extracted from litchi (*Litchi chinensis* Soon.) fruit pericarp tissues in relation to their role in the pericarp browning. Food Chem. 101, 1365–1371.

EMA/CPMP/QWP/2819/00 Rev. 2: Guideline on quality of herbal medicinal products/traditional herbal medicinal products, 2011. http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2011/09/WC500113209.pdf (accessed March 2015).

Farmacopeia Brasileira, 2010. Agência Nacional de Vigilância Sanitária, Brasília, DF, v. 1 e 2, 5th ed.

Holcroft, D.M., Mitcham, E.J., 1996. Postharvest physiology and handling of litchi (*Litchi chinensis* Sonn.). Postharvest Biol. Tech. 9, 265–281.

Hagerman, A.E., Riedl, K.M., Jones, G.A., Sovik, K.N., Ritchard, N.T., Hartzfeld, P.W., Riechel, T.L., 1998. High molecular weight plant polyphenolics (tannins) as biological antioxidants. J. Agric. Food Chem. 46, 1887–1892.

ICH, 2005. Validation of Analytical Procedures: Text and Methodology. International Conference on Harmonization, Geneva.

Lavola, A., Aphalo, P.J., Lahti, M., Julkunen-Tiitto, R., 2003. Nutrient availability and the effect of increasing UV-B radiation on secondary plant compounds in Scots pine. Environ. Exp. Bot. 49, 49–60.

Li, S., Xao, J., Chen, L., Hu, C., Chen, P., Xie, B., Sun, Z., 2012. Identification of A-series oligomeric procyanidins from pericarp of *Litchi chinensis* by FT-ICR-MS and LC–MS. Food Chem. 135, 31–38.

Lopes, G.C., Blainski, A., dos Santos, P.V.P., Diciaula, M.C., Palazzo de Mello, J.C., 2010. Development and validation of an HPLC method for the determination of epicatechin in *Maytenus ilicifolia* (Schrad.) Planch., Celastraceae. Rev. Bras. Farmacogn. 20, 781–788.

Martins, A.B.G., 2005. Lichia. Rev. Bras. Frutic. 27, 349–520.

Menzel, C. (Ed.), 2002. The Lychee Crop in Asia and the Pacific. Regional Office for Asia and the Pacific, Thailand, pp. 1–33.

Negri, G., di Santi, D., Tabach, R., 2010. Bitter acids from hydroethanolic extracts of *Humulus lupulus* L., Cannabaceae, used as anxiolytic. Rev. Bras. Farmacogn. 20, 850–859.

Pandey, R.M., Sharma, H.C., 1989. The litchi ICAR. New Delhi, India, pp. 1–79.

Ruenroengklin, N., Zhong, J., Duan, X., Yang, B., Li, J., Jiang, Y., 2008. Effects of various temperatures and pH values on the extraction yield of phenolics from Litchi fruit pericarp tissue and antioxidant activity of the extracted anthocyanins. Int. J. Mol. Sci. 9, 1333–1341.

Sousa, S., Alves, S.F., de Paula, J.A.M., Fiuza, T.S., Paula, J.R., Bara, M.T.F., 2011. Determinação de taninos e metilxantinas no guaraná em pó (*Paullinia cupana* Kunth, Sapindaceae) por cromatografia líquida de alta eficiência. Rev. Bras. Farmacogn. 20, 866–870.

Wang, X., Yuan, S., Wang, J., Lin, P., Liu, G., Lu, Y., Zhang, J., Wang, W., Wei, Y., 2006. Anticancer activity of litchi fruit pericarp extract against human breast cancer in vitro and in vivo. Toxicol. Appl. Pharmacol. 215, 168–178.

Wen, L., He, J., Wu, D., Jiang, Y., Prasad, K.N., Zhao, M., Lin, S., Jiang, G., Luo, W., Yang, B., 2014. Identification of sesquilignans in litchi (*Litchi chinensis* Sonn.) leaf and their anticancer activities. J. Funct. Foods 8C, 26–34.

Yang, B., Wang, J., Zhao, M., Liu, Y., Wang, W., Jiang, Y., 2006. Corrigendum to: Identification of polysaccharides from pericarp tissue of *Litchi chinensis* Soon. fruit in relation to their antioxidant activities. Carbohydr. Res. 341, 634.

Zhao, M., Yang, B., Wang, J., Liu, Y., Yu, L., Jiang, Y., 2007. Immunomodulatory and anticancer activities of flavonoids extracted from litchi (*Litchi chinensis* Sonn.) pericarp. Int. Immunopharmacol. 7, 62–66.

Zhou, H., Lin, Y., Li, Y., Li, M., Wei, S., Chai, W., Tam, N., 2011. Antioxidant properties of polymeric proanthocyanidins from fruit Stones and pericarps of *Litchi chinensis* Sonn. Food Res. Int. 44, 613–620.