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Calophyllum brasiliense Cambess: An alternative and promising source of shikimic acid

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ABSTRACT

Shikimic acid is the starting material for the synthesis of the antiviral oseltamivir phosphate, a front-line combatant against the human influenza A and B viruses. Nowadays, the demand for shikimic acid is met by seeds of Chinese star anise (*Illicium verum*), which grows exclusively in southern China. In the current work, the *Calophyllum brasiliense* plant is suggested as an alternative source of natural shikimic acid, which was extracted, purified and quantified by high performance liquid chromatography. The presence of shikimic acid in the extract of *C. brasiliense* was confirmed by nuclear magnetic resonance and mass spectrometry analysis. By using a simple and rapid extraction procedure, we showed that the content of shikimic acid in *C. brasiliense* leaves is 3.79%. Because *C. brasiliense* has some additional advantages over Chinese star anise, such as geographical distribution, ecological plasticity, natural regeneration, and versatility regarding large-scale cultivation, it may be a viable and promising source of shikimic acid, with potential industrial uses.

1. Introduction

Shikimic acid (3R,4S,5R-(−)-3,4,5-trihydroxy-1-cyclohexene-1-carboxylic acid; Fig. 1A), a key intermediate of the biosynthesis of aromatic amino acids in microorganisms and plants, is a versatile and promising chemical compound that can be obtained by chemical synthesis and microbial fermentation or directly isolated from plants (Hao et al., 2015). There are many industrial and biotechnological applications for shikimic acid, as it possesses analgesic, antioxidant, anticoagulant, anti-inflammatory and antithrombotic activity (Wang et al., 2011; Estévez and Estévez, 2012). It is also used in dermo-cosmetic preparations and as an exfoliating agent for the stratum corneum (Rawat et al., 2013). Shikimic acid also has neuroprotective effects (Rabelo et al., 2015), and is a precursor in the synthesis of antibacterials, anticancer agents, hormones, herbicides (Cuellar et al., 2015), the production of biorenewable aromatic compounds, and a stabilizer of metal nanoparticles (Candeias et al., 2018). Moreover, in recent years, shikimic acid has gained considerable interest because it is the starting material for the synthesis of the well-known antiviral drug oseltamivir phosphate (Fig. 1B), the active ingredient in Tamiflu®, marketed by Hoffman-La Roche.

Oseltamivir phosphate is commonly chosen for the treatment and prophylaxis of both human influenza A (H1N1and H3N2) and B viruses (WHO, 1980); it is also active against influenza A (H5N1) avian flu (Smith, 2010; WHO, 2007). After being administered orally, the prodrug oseltamivir phosphate is rapidly absorbed from the gastrointestinal tract and converted into the active metabolite oseltamivir carboxylate (Fig. 1C), by the action of hepatic esterases. This metabolite inhibits the active site of the neuramidases of influenza viruses and, as a consequence, reduces its replication (Davies, 2010).

As stated earlier, the commercial production of oseltamivir phosphate requires the precursor shikimic acid (Federspiel et al., 1999; Karpf and Trussardi, 2001; Abrecht et al., 2007). Despite recent advances in the processes of chemical and microbial synthesis as viable alternatives (Ghosh et al., 2012; Kim and Park, 2012; Rawat et al., 2013; Borah, 2015; Kongkathip et al., 2015; Martínez et al., 2015; Frost and Frost, 2016; Bilal et al., 2018; Candeias et al., 2018), non-toxic shikimic acid from plants remains the raw material of choice for the large-scale production of this antiviral. Shikimic acid can be found in the tissues of many plant species, in quantities varying from 0.001% to 24.5% by dry

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mass (Bochkov et al., 2012), including the dry fruits of different *Illicium* species (Avula et al., 2009). Plant species containing shikimic acid are distributed in different regions of the world (Raghavendra et al., 2009; Frost and Frost, 2016). However, only some plant species are good for the isolation of shikimic acid as others contain toxic compounds in their tissues. Thus, like chemical and microbial synthesis processes, the availability of shikimic acid from plants is also a bottleneck.

Currently, the worldwide demand for shikimic acid is met by the seeds of Chinese star anise (*Illicium verum* Hook. f., Illiciaceae), a medium-sized evergreen tree that grows exclusively in southern China (Guizhou, Sichuan, Guangxi, and Yunnan provinces) and Vietnam (Lang Son province) (Avula et al., 2009). The major content of shikimic acid is present in the fruits, which are harvested, and sun dried from September to October (Wang et al., 2011). Up to 80%–90% of the world's star anise is produced in China, and about 66% of the harvest is used for the industrial production of oseltamivir phosphate (ETC group, 2012). As noted, these aspects affect the availability of shikimic acid from Chinese star anise, including the confined geographical distribution of the plants, the major occurrence of the compound only in mature fruits, and seasonal harvests.

Calophyllum brasiliense Cambess (Clusiaseae) is a perennial tree species native to tropical America. In Brazil, it occurs in the Amazon, Cerrado, and Atlantic Forest. Due to the quality of its wood, *C. brasiliense* has been cultivated in several Latin American countries for commercial purposes (Flores, 2002; Piotto et al., 2003a,b; Cole et al., 2011). Moreover, the ability of *C. brasiliense* to grow in poor, rocky, wet and flooded soils makes it an interesting option for reforestation and soil recovery (Filho et al., 2007; Araldi et al., 2015). Over the last few years, one Brazilian company has marketed more than 3 million seeds, contributing to about 360 ha of cultivated plants. Furthermore, *C. brasiliense* has interesting medicinal properties (Ito et al., 2002; Reyes-Chilpa et al., 2004; Souza et al., 2009; Brenzan et al., 2012). For instance, xanthones from the stem bark have cancer chemoprotective activity (Ito et al., 2002). Coumarins from the leaves have antileishmanial activity on *Leishmania amazonensis* (Brenzan et al., 2012). Hydroethanolic extract and dichlromethanic fraction from the stem bark have *in vitro* and *in vivo* anti-*Helicobacter pylori* activities (Souza et al., 2009). Also, Reyes-Chilpa et al. (2004) showed that *C. brasiliense* contains some triterpenes and coumarins with inhibitory effects on HIV-1 reverse transcriptase and human tumor cell lines. These authors obtained shikimic acid crystals after methanolic extraction, indicating that it is present in significant amounts in this plant species. Hence, the aim of the current work was to investigate an alternative source of shikimic acid from a plant with attributes different from those of Chinese star anise, *i.e.*, *C. brasiliense* Cambess. By using a simple and rapid procedure, shikimic acid was extracted and quantified in different tissues of this plant species, and parameters such as tissue amount, accuracy, temperature, and duration of extraction were optimized.

2. Materials and methods

2.1. Chemicals

Acetonitrile (HPLC grade) and formic acid (98%) were purchased from PanReac AppliChemical (Darmstadt, Germany). Shikimic acid (≥99%) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Ultrapure water was obtained from a Milli-Q system (Millipore, Molsheim, France).

2.2. Plant material and general procedures

Seeds and plants of *C. brasiliense* were obtained from Brazilian Institute of Forests (Londrina - latitude 23°11′55′′; Brazil). For plant growth, seeds were planted in 50 mL plastic pots containing a substrate of pine bark, carbonized rice husk, and vermiculite. The pots were kept in a growth chamber at 35 °C with low irradiance for 15 days, and watered daily using a microsprinkler at 100 L h^{-1} for 10 min. After three months, plants were transferred to 250 mL plastic pots containing the substrate and cultivated for four more months. After this procedure, roots, stems and leaves were excised from the plants and dried in an oven at 70 °C until a constant weight was achieved. A similar procedure was conducted for seeds and seed coats, which were manually detached. Dry matter (0.3 g) of each tissue type was ground in a ball mill (Labor, São Paulo, Brazil) for 1 min to provide homogeneous powders for the analysis.

2.3. Extraction of shikimic acid from Calophyllum brasiliense tissues

The shikimic acid extraction was carried out according Ohira et al. (2009), with some modifications. First, 10 mL of water (at 70 °C) was added to a centrifuge tube containing 0.5 g of each dry tissue (seed, seed coat, root, stem, and leaf). The tubes were incubated in a water bath (at 70 °C) for 13 min. During the first 3 min, the sample was homogenized with a glass rod. After extraction, samples were transferred to an ice bath and then centrifuged (Fanem Excelsa 4 centrifuge, São Paulo, Brazil) at 2200×*g* at 4 °C for 5 min. The supernatant was separated, and the pellet was submitted to two more extractions, as described above. The samples obtained were then submitted to chromatographic analysis. Additional experiments were carried out with different amounts of each tissue, temperatures, and extraction times.

2.4. Thin layer chromatography, nuclear magnetic resonance analysis and purification of shikimic acid

Shikimic acid purification and subsequent nuclear magnetic resonance (NMR) analysis were performed to identify the compound in tissues of *C. brasiliense*. For this, chromatography separation was performed on Sephadex LH-20® (Sigma-Aldrich) chromatography column. Thin layer chromatography (TLC) was performed on normal phase pre-coated silica gel 60G (Merck) plates. Visualization of shikimic acid on TLC was accomplished by UV irradiation at 254 nm and 366 nm, and

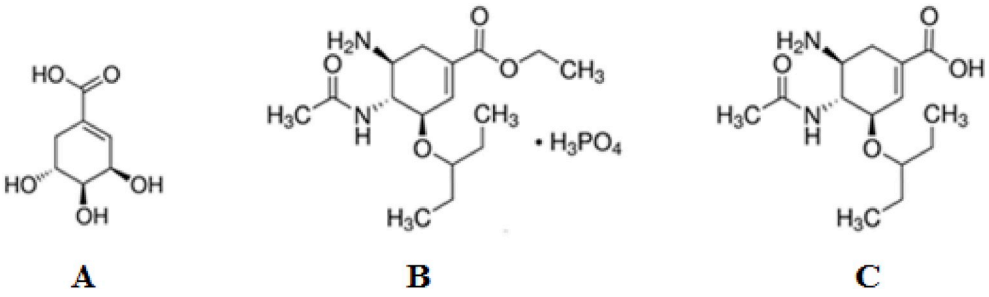

Fig. 1. Chemical structures of shikimic acid (**A**), oseltamivir phosphate (**B**, prodrug) and oseltamivir carboxylate (**C**, active metabolite).

by spraying with a H_2SO_4/anisaldehyde/acetic acid (1: 0.5: 50 mL) solution followed by heating at 100 °C. The NMR spectra were recorded on a Bruker Avance III HD spectrometer (Bruker®, Billerica, USA) operating at 300 MHz and 75.5 MHz, using D_2O (Sigma-Aldrich) as solvent. The optical rotation was measured on a PerkinElmer polarimeter 343 (PerkinElmer®, Waltham, USA) at 20 °C and $\lambda = 589$ nm (sodium D-line), using a 1-cm microcell (*c* 0.20, H_2O). An aliquot of aqueous extract (300 mg) was resuspended in methanol, and the supernatant (155 mg) was submitted to Sephadex LH-20® CC using methanol as eluent to obtain subfractions AC-1 through AC-7. Subfraction AC-3 contained shikimic acid (10.8 mg).

2.5. High performance liquid chromatography analysis and quantification

For shikimic acid quantification, samples of each supernatant were appropriately diluted and filtered through a 0.45-μm disposable syringe filter (Hamilton® Co., Nevada, USA) prior to chromatographic analyses (Bonini et al., 2009). Samples (20 μL) were analyzed on a reverse-phase high performance liquid chromatography (HPLC) system (Shimadzu®, Prominence 20, Tokyo, Japan) equipped with a LC-20AT quaternary pump, a SIL-20A auto-sampler, a CTO-20A column oven, a SPD-M20A diode-array detector, a CBM-20A communications bus module, and a LcSolution workstation system. A reversed-phase Shimpack® CLC-ODS (M) column (5 μm, 250 × 4.6 mm i.d.), protected with a pre-column (10 × 4.6 mm), was used at 30 °C. The mobile phase consisted of a mixture of water:acetonitrile (70:30) with a flow rate of 0.5 mL min^{-1} for an isocratic run of 30 min. Absorption was measured at 220 nm, a wavelength that was previously determined by the spectrophotometric scanning of a shikimic acid authentic standard dissolved in the mobile phase. Parallel controls with shikimate added as an internal standard in the reaction mixture were performed. An eight-point standard curve with shikimic acid concentrations ranging from 10 to 1000 μg mL^{-1} was used to externally quantify shikimate levels in the tissue extracts. The total content of shikimic acid was represented by the sum of data obtained for the three supernatants. Results were expressed as mg shikimic acid g^{-1} dry weight, representing the means of three independent samples ± S.E.

2.6. Direct-infusion mass spectrometry analysis

The presence of shikimic acid in aqueous extract of *C. brasiliense* also was confirmed by mass spectrometry analysis. Samples fractionated from HPLC analyses were collected, lyophilized, appropriately diluted in acetonitrile, and filtered through a 0.45-μm disposable syringe filter (Hamilton® Co., Nevada, USA). The direct-infusion mass spectrometry (MS and MS/MS) analysis was performed using Waters Quattro micro API™ mass spectrometer (Beverly, USA).

Analyses were carried out by the injection of the sample (100 μL in acetonitrile/water 1:1 v/v; 0.05% formic acid) into the mass spectrometer using a syringe pump (0.01 mL min^{-1}). Spectra were obtained in the negative ionization mode setting the capillary voltage at 2 KV; cone voltage, 25 V; source temperature, 150 °C; desolvation temperature, 450 °C; cone gas flow, 25 L h^{-1}; and desolvation gas flow, 900 L h^{-1}. Individual spectrum was produced by accumulation of data over 1 min and the full scan mass spectrum was acquired in the m/z 50–1000 range. For the MS/MS spectra, product ion scan mode was applied. The process of fragmentation of the molecule was carried out by Collision-induced dissociation (CID).

2.7. Statistical analysis

Experiments were independently repeated thrice, and results are given as means ± S.E. Data analysis was performed by using one-way ANOVA followed by Scott-Knott test (Sisvar® package, version 5.6, UFLA, Brazil). A value of $p \leq 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Shikimic acid in Calophyllum brasiliense

The most striking fact revealed in the current research is that *C. brasiliense* is an alternative source of natural shikimic acid. Firstly, shikimic acid was purified from the aqueous extract of *C. brasiliense*; its structure was assigned on the basis of ^1H and ^{13}C NMR and compared with data reported in the literature (Sciubba et al., 2014). $[\alpha]_D^{20} = -105.0$ (*c* 0.20, H_2O); ^1H NMR (300 MHz, D_2O): δ 6.43 (m, 1H), 4.32 (m, 1H), 3.91 (m, 1H), 3.64 (dd, $J = 4.4$ Hz; 8.9 Hz, 1H), 2.68 (dd, $J = 5.4$ Hz; 18.0 Hz, 1H), 2.12 (dd, $J = 7.5$ Hz; 18.0 Hz, 1H); ^{13}C NMR (75.5 MHz, D_2O): 174.5, 135.0, 131.7, 71.9, 66.8, 66.2, 32.3 (see Supplementary material, Fig. S1).

By using a simple, rapid, and reliable extraction procedure, followed by efficient, sensitive, and reproducible analytical methods (HPLC and direct-infusion-MS), shikimic acid was quantified in different tissues of *C. brasiliense*, especially in the leaves. For this, a calibration curve was constructed, by means of HPLC, with shikimic acid concentrations ranging from 10 to 1000 μg mL^{-1} (Fig. 2A). The calibration data were linear in the chosen range of concentrations, indicating a good correlation between the peak area and the shikimic acid concentration. The results revealed a fitted regression with $y = 7323x + 0.084$ ($r^2 = 0.998$). Several injections of shikimic acid showed reproducibility and low standard error. Chromatograms obtained from samples of *C. brasiliense* leaves were clear and with a single peak (retention time = 4.8 min) consistent with the shikimic acid standard (Fig. 2B). The absorption spectra of the sample and standard were also similar (not shown).

The presence of the shikimic acid in samples fractionated from HPLC analyses was confirmed by direct-infusion MS analysis. Fig. 3 shows representative MS spectrum of the shikimic acid standard (Fig. 3A) and of shikimic acid extracted from *C. brasiliense* leaves (Fig. 3C). The MS/MS spectrum were obtained using a product ion scan mode from the precursor ion at $[M\text{-}H]^-$ at $[M\text{-}H]^-$ 173.1 (**C**) and $[M\text{-}H]^-$ *m/z* 172.8 (**D**) of extracted shikimic acid matched with those of the standard compound (**A,** $[M\text{-}H]^-$173.1; **B**, $[M\text{-}H]^-$ 173.0). All these findings confirmed that the peak detected by HPLC (Fig. 2B) was, in fact, shikimic acid. The applied procedure is sensitive and capable of estimating the compound with precision and accuracy. Similar results were found in the seeds, seed coats, roots, and stems of *C. brasiliense* (not shown). Moreover, the analytical methods (HPLC, direct-infusion-MS and NMR) applied herein are satisfactory compared with the extraction and quantification procedures of shikimic acid from plants of the genus *Illicium* and other plant species (Avula et al., 2009; Bochkov et al., 2012).

The extraction efficiency of shikimic acid from different tissues of *C. brasiliense* was determined from samples spiked with standard shikimic acid; accuracy is expressed as (shikimic acid recovered/standard added) × 100%. Regardless of the tissue analyzed, more than 95% of shikimic acid was recovered after its extraction (Table 1), so there was no considerable loss of the compound during the extraction procedure.

3.2. Optimization of the extraction procedure and yield of shikimic acid

Parameters such as the amount of tissue, temperature, and time of extraction were optimized to evaluate the yield of shikimic acid obtained from the seeds, seed coats, roots, stems, and leaves of *C. brasiliense*.

Shikimic acid was extracted thrice from 0.1–1.0 g of each dry tissue (Fig. 4). Independent of the amount of sample used during the extraction procedure, the content of shikimic acid was similar for each tissue. From 0.1 to 1.0 g, the content of shikimic acid had mean values of 0.25 mg g^{-1} (seed), 5.1 mg g^{-1} (seed coat), 16.7 mg g^{-1} (root), 19.2 mg g^{-1} (stem) and 37.9 mg g^{-1} (leaf). The mean extraction percentage of shikimic acid from 0.1 to 1.0 g of seeds (Fig. 5) varied from 84.9% (first extraction), to 14.4% (second extraction) and 0.6% (third extraction). For the seed

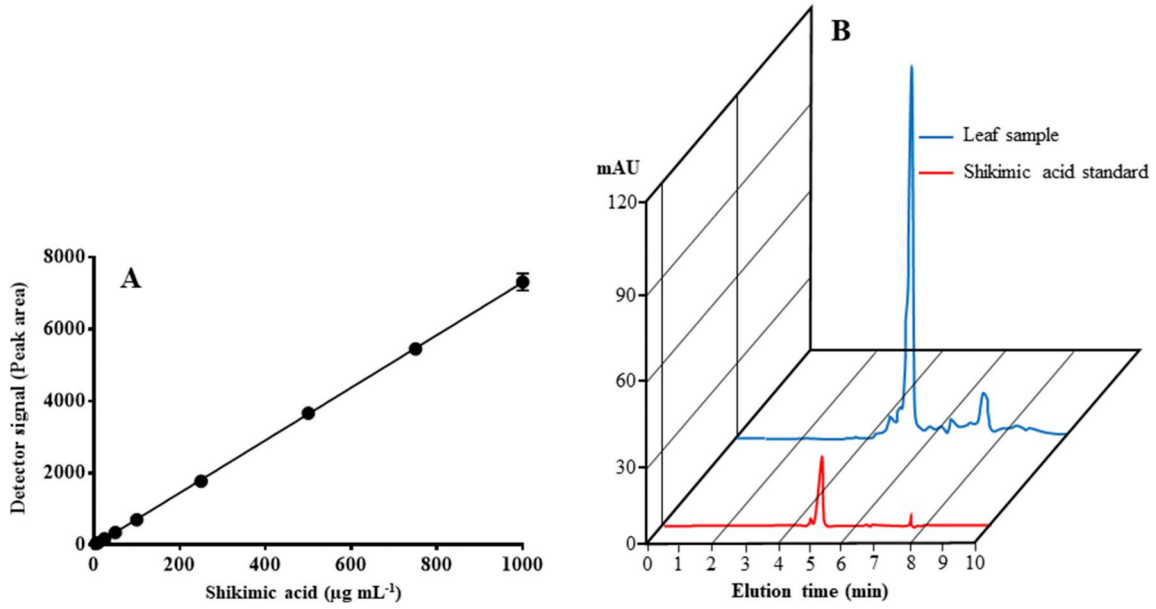

Fig. 2. Standard curve of shikimic acid (**A**), and representative HPLC chromatograms of shikimic acid (as a standard) and in leaves of *Calophyllum brasiliense* (**B**). In **A**, the solid line is the fitted regression line with $y = 7323x + 0.084$ ($r^2 = 0.998$).

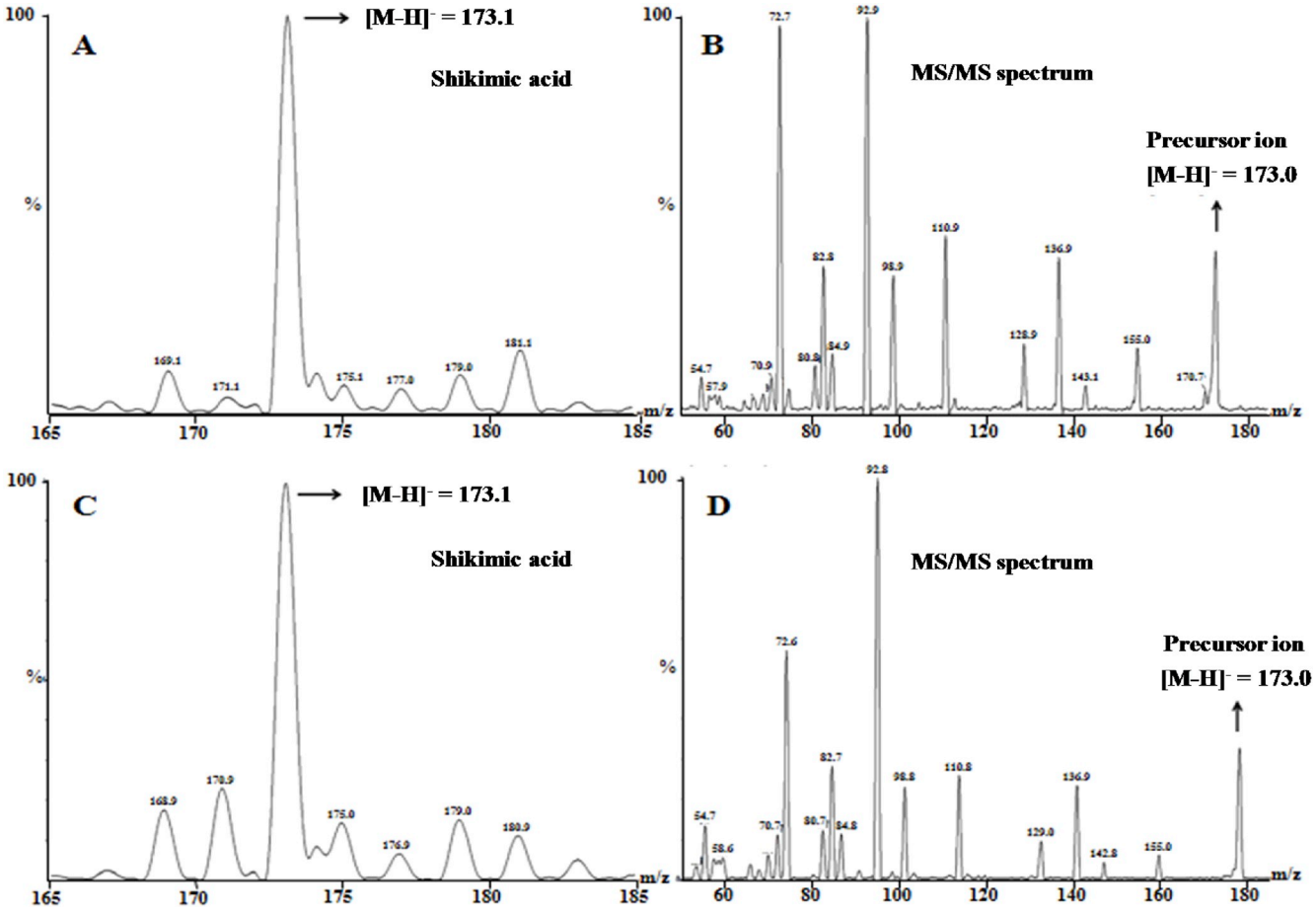

Fig. 3. Representative direct-infusion spectrum MS and MS/MS of a sample of *Calophyllum brasiliense* leaf and of shikimic acid as a standard. **A** and **B**, shikimic acid standard; **C** and **D**, shikimic acid extracted from leaves.

coat, these values were 76.2%, 18.3%, and 5.5%, respectively; for the root, they were 76.3%, 18.2%, and 5.4%, respectively; for the stem, they were 83.2%, 13.6%, and 3.2%, respectively; and for leaves, they were 83.8%, 13.5%, and 2.7%, respectively. The same figures revealed that the extraction percentages decreased from 0.1 to 1.0 g for any tissue on the first extraction, increased during the second extraction, and were

Table 1
Accuracy of the extraction procedure of shikimic acid from different tissues of *Calophyllum brasiliense*. Tissue samples (n = 3 ± S.E.) were spiked with shikimic acid as an internal standard before extraction.

Tissue	Shikimic acid added (mg mL^{-1})	Shikimic acid recovery (mg mL^{-1})	Accuracy (%)
Seed	1.74	1.76 ± 0.02	101.1
Seed coat	1.74	1.66 ± 0.04	95.4
Root	1.74	1.71 ± 0.02	98.3
Stem	1.74	1.66 ± 0.04	95.4
Leaf	1.74	1.69 ± 0.06	97.1

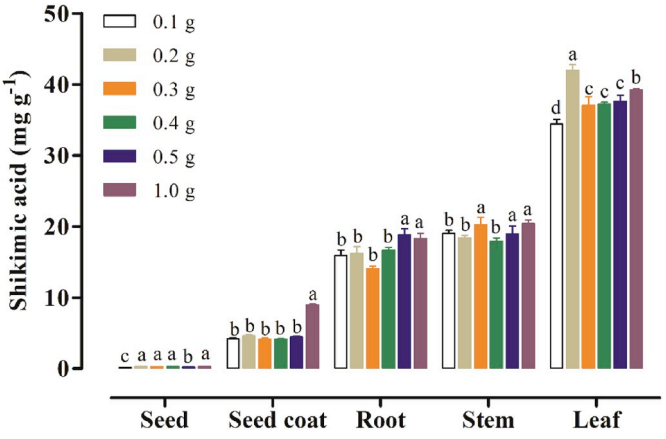

Fig. 4. Shikimic acid contents from different amounts of tissues of *Calophyllum brasiliense*. Shikimic acid was extracted from 0.1 to 1.0 g of each dry tissue (seed, seed coat, root, stem and leaf). Mean values (n = 3 ± S.E.) followed by different letters indicate significance ($p \leq 0.05$) as determined by the Scott-Knott test.

very low or, in some cases undetectable, during the third extraction. The combination of these findings (Figs. 4 and 5) revealed that leaves of *C. brasiliense* contained 3.79% (37.9 mg g^{-1}) shikimic acid.

Currently, Chinese star anise is the main source of commercial production of shikimic acid, but other plant species contain this compound, which accumulates differently in their tissues (Enrich et al., 2008; Avula et al., 2009; Bochkov et al., 2012; ETC group, 2012). Comparative results between plant species indicate that *Illicium religiosum* (fruits), *Illicium lanceolatum* (fruits), *Alangium salvifollium* (roots), *Actaea pachypoda* (whole plant), *Symphytum officinalis* (leaves), *Ribes aureum* (whole plant), *Pistacia lentiscus* (whole plant), and *Terminalia arjuna* (fruits) contain 11.8–24.5% of shikimic acid on a dry mass basis (Avula et al., 2009; Bochkov et al., 2012). However, few of these plants are good for shikimic acid isolation because they are confined to restricted geographic regions of the world (Bochkov et al., 2012; Díaz Quiroz et al., 2014). More recently, varied contents of shikimic acid were found in 58 samples of different plant species from localities of Western Ghats, India (Kshirsagr et al., 2018). A hierarchical cluster analysis revealed that the families Clusiaceae, Ranunculaceae, Anacardaceae, and Simaroubaceae accumulated higher levels of shikimic acid. In fact, quantities of shikimic acid over 8.3 mg g^{-1} in leaves were found in six plant species, and the most promising were *Mammea suriga* (20.07–29.34 mg g^{-1}), *Calophyllum inophyllum* (18.2 mg g^{-1}) and *Garcinia morella* (8.52 mg g^{-1}). Like *C. brasiliense,* these plant species are Clusiaceae; a relevant indication that the members of this family can be potential candidates for further commercial exploration of shikimic acid.

Mostly, shikimic acid is extracted from plant material by using polar protic organic solvents such as ethanol, methanol, and isopropanol (Candeias et al., 2018). However, shikimic acid is highly stable and soluble in aqueous solution, *i.e.*, 204 g/1000 g water at 25 °C (Ohira et al., 2009); this is an important characteristic for its extraction from plants (Hao et al., 2015), and a necessary requirement for steps that require elevated temperatures. To ascertain whether temperature influenced the yield, shikimic acid was extracted from 0.5 g of leaves at a range of temperatures (Fig. 6A). From 25 to 40 °C, the contents of shikimic acid extracted were about 34 mg g^{-1}, while levels significantly decreased to 30 mg g^{-1} at 50–70 °C.

Like temperature, another important factor in the large-scale production of shikimic acid from plants is the extraction time. To determine whether extraction time altered the yield of shikimic acid, subsequent experiments were performed with 5–60 min of extraction (Fig. 6B). No appreciable change was noted from 5 to 10 min of extraction, with 31 mg g^{-1} extracted. From 20 to 60 min of extraction, the content of extracted shikimic acid decreased to 29 mg g^{-1}. Among several procedures (Payne and Edmonds, 2005; Pham et al., 2011; Usuki et al., 2011; Rawat et al., 2013; Cai et al., 2014; Just et al., 2015; Candeias et al., 2018), rapid and efficient extraction of shikimic acid from Chinese star anise has been obtained with hot water (Ohira et al., 2009), but it requires a temperature of 120 °C, or more. Herein, the findings indicate that shikimic acid can be efficiently extracted from the tissue, especially the leaves, of *C. brasiliense* by using water as the extractor, at room temperature for 5 min, using three consecutive extraction steps.

3.3. *Calophyllum brasiliense as an alternative source of shikimic acid*

Shikimic acid is found in many plant species, but Chinese star anise is traditionally its primary source. Although shikimic acid can be found in this plant species, the major problems are its restricted growth in southern China and northeast Vietnam (Avula et al., 2009; EOL, 2019a) and the seasonal harvest of fruits (Wang et al., 2011). Therefore, due to the dire need for alternatives sources of shikimic acid, its isolation from other plant species is an important challenge. Beyond Chinese star anise, another potential source of shikimic acid is the American sweetgum tree (*Liquidambar styraciflua*), which also grows in limited geographical regions of eastern North America and tropical montane regions of Mexico and Central America (EOL, 2019b), and their seeds contain 2.4–3.7% shikimic acid (Lingbeck et al., 2015). Similarly, *Mammea suriga* could be used as a possible source of shikimic acid (Kshirsagr et al., 2018), but it is restricted to the Western Ghats, India, and has not yet been exploited for commercial purposes.

Based on the results obtained herein, it is plausible suggest that *C. brasiliense* is an alternative source of natural shikimic acid; importantly, this plant species has different attributes to those of Chinese star anise. First, *C. brasiliense* has a broad geographical distribution, since it grows abundantly in tropical rain forests from southern Brazil to Central America (EOL, 2019c). It is a versatile plant and well suited to all types of climates and Brazilian soils, and it is characterized by ecological plasticity and excellent natural regeneration (Souza, 2006). Second, *C. brasiliense*, which is called “guanandi” or “Brazilian mahogany”, among other popular names, is widely cultivated in areas of reforestation and soil recovery and has been planted at a large scale. For example, over the last few years, one Brazilian company has marketed more than 3 million seeds, contributing to about 360 ha of cultivated plants. As described herein, shikimic acid has been isolated from *C. brasiliense* plants at about 6–7 months age. Therefore, the extraction steps are not limited to a season in which the plant produces fruits. Finally, an additional advantage is that shikimic acid can be isolated from *C. brasiliense* tissues, especially from leaves, using only water as the extractor, at room temperature, for a short period of time, and without sophisticated equipment. Because leaves produce enhanced biomass, the extraction of shikimic acid from *C. brasiliense* can be economically feasible over fruits of Chinese star anise. In short, all these findings indicate that *C. brasiliense* can be considered a viable and promising source of natural shikimic acid.

It is necessary to highlight that the use of *C. brasiliense* for industrial production of shikimic acid must be preceded by a careful analysis of the presence of possible toxic substances. Organic extracts of the bark and

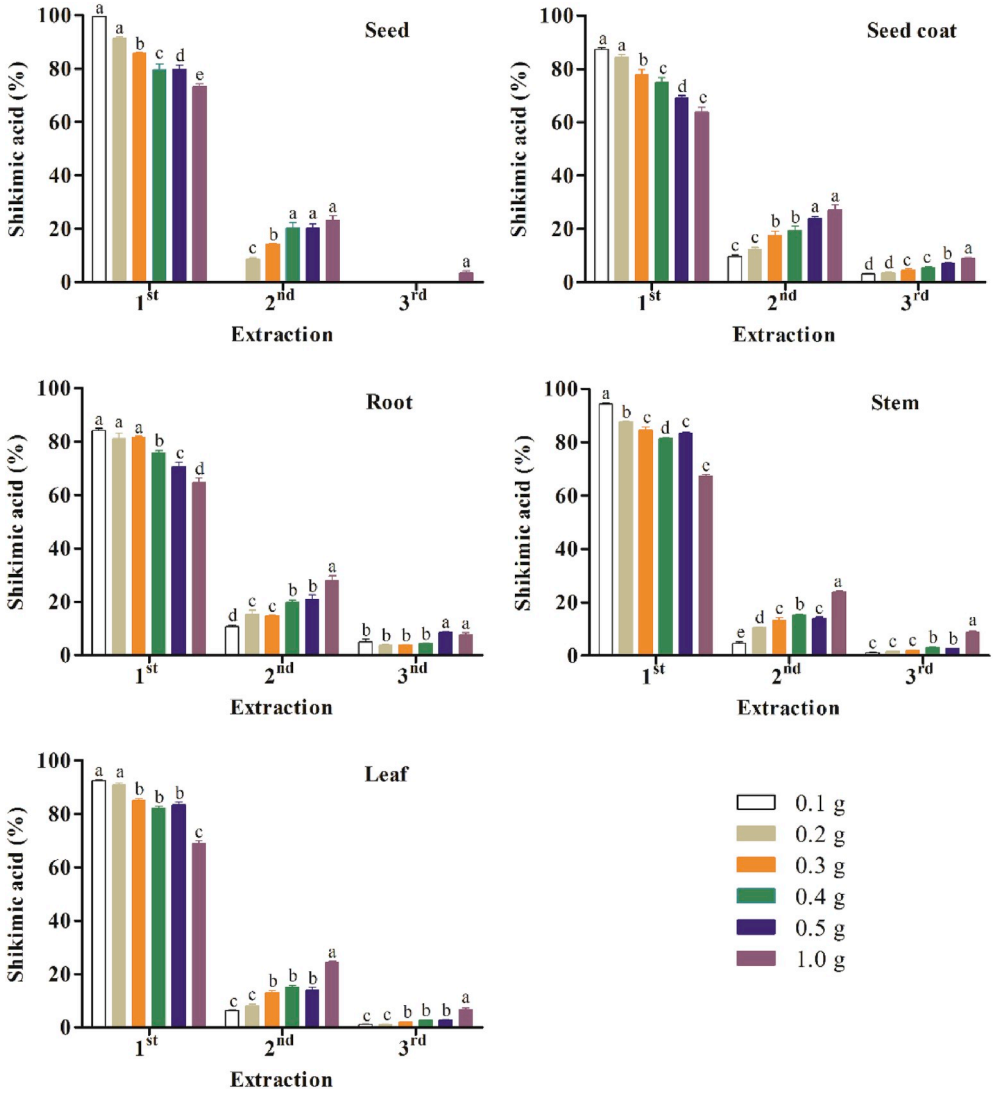

Fig. 5. Percentage of shikimic acid in different tissues of *Calophyllum brasiliense*, following three extraction steps. Shikimic acid was extracted from 0.1 to 1.0 g of each dry tissue (seed, seed coat, root, stem and leaf). Mean values (n = 3 ± S.E.) followed by different letters indicate significance ($p \leq 0.05$) as determined by the Scott-Knott test.

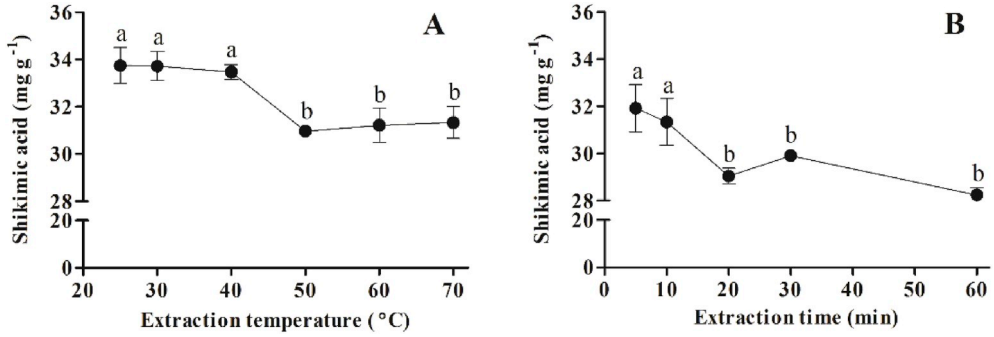

Fig. 6. Shikimic acid contents from *Calophyllum brasiliense* leaves with different extraction temperatures (**A**) and times (**B**). The extraction procedure was performed with 0.5 g of dry tissue. Mean values (n = 3 ± S.E.) followed by different letters indicate significance ($p \leq 0.05$) as determined by the Scott-Knott test.

leaves of this plant species are rich in coumarins and triterpenes (Reyes-Chilpa et al., 2004), xanthones (Ito et al., 2002) and biflavonoids (da Silva et al., 2001; Reyes-Chilpa et al., 2004), which could eventually cause harm to human health. However, these secondary metabolites are poorly soluble in water and must be present in negligible amounts in the aqueous extract of *C. brasiliense*. In addition, the frequent use of *C. brasiliense* in folk medicine for the treatment of rheumatism, varicose veins, hemorrhoids, and chronic ulcers (Brenzan et al., 2012) point out a low risk to humans.

4. Conclusions

The main conclusions that can be drawn from the current work are that a) *C. brasiliense* is an alternative and promising source of shikimic acid with potential industrial uses, and b) shikimic acid can be easily extracted from this plant species, especially their leaves, by using a simple and rapid procedure.

Author contributions

R. Marchiosi and O. Ferrarese-Filho designed the study, analyzed the data and wrote the main manuscript text. W.D. dos Santos helped design the study and analyzed the data. R. Marchiosi, A. P. Ferro and Rodrigo P. Constantin performed all experiments. A. P. Ferro and Renato P. Constantin performed the experiments with HPLC. Anderson V.G. Ramos and Debora C. Baldoqui performed the experiments with NMR. All authors revised and approved the final manuscript.

Declaration of competing interest

We declare that there is no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scp.2019.100188.

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