Revista de Ciências Farmacêuticas Básica e Aplicada
*Journal of Basic and Applied Pharmaceutical Sciences*

**Rev Ciênc Farm Básica Apl**, 2018;39:e639
ISSN 1808-4532

# Chemical variability between different organs of the medicinal plant *Casearia sylvestris*

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## ABSTRACT

**The phytochemical profile of essential oils and extracts from *Casearia sylvestris* leaves, flowers and fruits have been investigated here. Leaf and flower extracts were prepared by sonication and analyzed by thin-layer chromatography and high-performance liquid chromatography. The phenolic content was determined by ultraviolet spectrophotometry. Leaves, flowers, and fruits essential oils were extracted by hydrodistillation. The highest extracts yields were 20.3% (leaves) and 23.4% (flowers) with ethanol 70%. Essential oil extraction yields were 0.3% (leaves) and 0.1% (flowers and fruits). Bicyclogermacrene was the major component in all essential oil. Thin-layer chromatography suggests a chemical profile similar for leaves and flowers. The leaves and flowers phenolic contents were similar (14.0 and 15.0%, respectively). Chromatographic analyses indicated the predominance of casearin clerodane diterpenes in leaves ($\lambda_{max}$ 232-235), whereas in flowers diterpenes with a different standard diene in side-chain C13(16) and C14 ($\lambda_{max}$ 223-229). The different phytochemical profile of *C. sylvestris* flowers as compared to the leaves could be explored by the search for new bioactive components. This is the first report on the fruit and flower *C. sylvestris* essential oil composition. These data could be used as quality control of herbal medicine derived from *C. sylvestris* leaves.**

**Keywords: Bicyclogermacrene. Clerodane Diterpenes. Quality control. Essential Oil.**

## INTRODUCTION

*Casearia sylvestris* Swartz (Salicaceae), popularly known as guaçatonga in Brazil, is a medicinal plant found in Central and South America (Ferreira, et al., 2011). In folk medicine, the plant is employed as wound healing, anti-inflammatory, antiseptic, topical anesthetic, and for the treatment of gastritis and snakebites. The antiulcerogenic, antiophidic, anti-inflammatory and wound healing activities have been proven through pharmacological assays using its extracts, essential oil, and isolated secondary metabolites (Ferreira et al., 2011; Pierri et al., 2017; Santos et al., 2010; Raslan et al., 2002).

The main secondary metabolites in *C. sylvestris* are flavonoids, gallic acid derivatives, sesquiterpenes and clerodane diterpenes. Clerodane diterpenes are considered as taxonomic markers of *Casearia* genus (Xia et al., 2015) and they were detected in different *C. sylvestris* organs (Carvalho et al., 2010). Diterpenes with a diene in the side-chain at C13(16) and C14 predominate in stems, flowers and roots, whereas diterpenes with a diene at C12*Z* and C14 predominate in leaves.

*C. sylvestris* leaves afford an essential oil (EO) in yields ranging from 0.2 to 2.5% (v/w) and EO composition varies according to the harvesting period (morning or afternoon) (Tininis et al., 2006). Sesquiterpenes are the main EO components, with predominance of bicyclogermacrene, *E*-caryophyllene and germacrene D. On the other hand, there are no previous reports on the chemical composition *C. sylvestris* EO from flowers and fruits (Esteves et al., 2005; Spósito et al., 2019; Moreira et al., 2019; Bou et al., 2013; Sousa et al., 2007).

The aim was to analyze the chemical composition of extracts and essential oils from *C. sylvestris* leaves, flowers and fruits by means of comparison of the thin-layer chromatography (TLC), high-performance liquid chromatography with diode-array detection (HPLC-PAD), and gas-chromatography mass spectrometry (GC-MS) chromatographic profiles with emphasis on diterpenes and sesquiterpenes, which are the main bioactive compounds in *C. sylvestris*.

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## MATERIAL AND METHODS

*Plant material*

*C. sylvestris* leaves, flowers, and fruits were collected at Medicinal and Toxic Botanical Garden of School of Pharmaceutical Sciences of São Paulo State University (UNESP), Araraquara-SP in September 2016 (coordinates: "21º81'4.6" S; "48º20'21.5" W). A voucher specimen (AGS 102) was deposited at the Herbarium Maria Eneyda P. K. Fidalgo in the Botanical Institute of São Paulo; Brazil. The access to the genetic heritage was authorized by the National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SisGen) under license number A37C0DA. The dried plant material and the fresh fruits were stored at -20 ºC.

*Solvents*

P.A. grade solvents (Qhemis®) were used in the extract preparation; photometric and TLC analyses. For HPLC and GC-MS analyses, methanol, acetonitrile and hexane chromatographic grade (J.T. Baker®), and ultrapure water (18 MΩ) obtained from Milli Q purifier (Synergy®) were employed.

*Extract preparation*

The leaves and flowers were oven-dried with air circulation at 40 °C for 7 days and grounded with the aid of a knife mill. Dried and powdered leaves (20 g each) were extracted by sonication (UNIQUE®, USC-2800, 40 KHz) with ethyl acetate: hexane: isopropanol 91:08:01 (v/v), (3 x 200 mL) for 20 min each extraction; yielding the ethyl acetate leaf and flower extract (EA-L and EA-F; respectively) The same method was carried out for extraction with 70% ethanol, which was preceded by hexane extraction; then yielding the extracts ethanol leaf and flower (EE-L and EE-F, respectively).

*Determination of total phenolic content*

The total phenolic content in EE-L and EE-F extracts was determined by UV-Vis photometry (Singleton et al., 1999). Extract samples (5.0 mg) were solubilized in 1.0 mL (water: ethanol, 1:1; v/v). Aliquots of 0.1 mL of the samples were added in a 10.0 mL volumetric flask followed by 6.0 mL of deionized water and 0.5 mL of the Folin Ciocalteu's reagent (Sigma-Aldrich®) and the mixture was homogenized. After 5 min, 1.5 mL of 20% sodium carbonate solution was added and the volume was filled to 10 mL with deionized water. After 2 h, the absorbance values of sample solutions were determined in a UV-Vis spectrophotometer (Shimadzu®, UV-1800) at 760 nm. For the analytical curve, solutions of gallic acid standard were prepared at concentrations of 1.25, 2.50, 4.00, 5.00, 7.50, 9.00, 10.00, 10.50 and 20.00 µg/mL (ethanol: water, 1:1, v/v). The assays were performed in triplicate.

*Clerodane diterpenes chromatographic profile*

***Thin Layer Chromatography analysis***

The extracts EA-L; EA-F; EE-L; and EE-F were solubilized in ethyl acetate (5.0 mg/mL) and applied to aluminum plates (Sigma-Aldrich® silica gel, 20 x 20 cm x 0.25 µm). Hexane: ethyl acetate: isopropanol 70:28:02 (v/v) was used as mobile phase. Anisaldehyde sulfuric acid (110 °C, 10 min) was employed as spray reagent. The standards used were casearin B, casearin X, and caseargrewiin F (Santos et al., 2010).

***High performance liquid chromatography with photodiode array ultraviolet detector (HPLC-PDA/UV) analysis***

The sample pretreatment was performed by solid-phase extraction (SPE-C18 E Phenomenex® Strata ™ 1.5 x 1.0 cm, 55 µm). The samples (EA-L 6.0 mg, EA-F 7.5 mg) were solubilized in 1.0 mL (methanol: water, 98:2, v/v), applied into the cartridge, eluted with 4.0 mL of the eluent. The solutions were dried, solubilized in 1.0 mL (methanol) and filtered through membrane (0.22 µm, PVDF Millipore®). Chromatographic analysis was performed using Shimadzu® Proeminence® equipment (LC-20AT pump; SPD-M20A PDA detector, SIL-20 automatic injector, CTO-20 column oven, DGU-20AS degasser, LCSolution® software) and Hypersil Gold® C18 column (250 x 4.6 mm, 5 µm). The mobile phase was methanol: acetonitrile: water in linear gradient mode from 22:44:34 to 47:53:00 (v/v) for 42 min, followed by 47:53:00 (v/v) for 5 min, 0.8 mL/min flow rate, detection: 190-700 nm, injection volume 20 µL (Claudino et al., 2013). Diterpene quantification in the extracts was performed through the analytical curve of caseargrewiin F (Santos et al., 2010) at concentrations of 0.035, 0.070, 0.140, 0.280 and 0.580 mg/mL.

***Essential oil extraction and GC-MS analyses***

The EO of *C. sylvestris* leaves; flowers and fruits (80.0 g of each) were extracted separately by hydrodistillation in a Clevenger-type apparatus. The EOs were collected with ethyl ether and the residual water was discarded using sodium sulfate anhydrous, followed by filtration and drying (Brasil, 2010). GC-MS analysis were performed on a Shimadzu® QP 2010 Plus gas chromatograph under the following conditions: Rtx-5MS column (30 m x 0.25 mm x 0.25 µm film thickness), temperature program from 60 to 240 °C at 3 °C/min, 1:20 split mode, injector temperature of 240 °C. Helium (99.999%) was used as the carrier gas at a linear velocity of 1.33 mL/min. The detector used was a mass spectrometer fitted with an electron ionization (EI) source operating at 70 eV and with registration a scan interval of 0.5 s for masses from 40 to 600 Da. EO components were identified on the basis of their retention indices relative a series of *n*-alkanes ($C_8$-$C_{40}$) and the retention index (Van Den Dool & Kratz, 1963), which was calculated according to Adams (2007), as well as on the comparison of the experimental indices with those from literature. The chemical structures were computer-matched with reference spectra of the NIST 08 and WILEY 7 mass spectral libraries and their fragmentation standards were compared with those reported by Adams (2007).

## RESULTS

*Extract analyses by TLC and HPLC-PDA*

In order to analyze the diterpenes of *C. sylvestris* leaves and flowers, extraction was performed with a mixture of solvents selective to casearins (Claudino et al., 2013). The 70% ethanol extracts EE-L and EE-F were obtained aiming to compare the total phenolic content in leaves and flowers. The extract yields are presented in Table 1 and chromatogram profile (HPLC-PDA) in Figure 1.

In TLC analyses, several bands with $R_f$ values between 0.2 and 1.0 were observed for EA-L and EA-F (Table 1). The diterpenes casearin X ($R_f$: 0.35) and caseargrewiin F ($R_f$: 0.25) were identified in both extracts; whereas casearin B was not identified in the analyzed extracts. Moreover; EA-L displayed ten chromatographic bands with the same $R_f$ values as EA-F.

The total phenolic compounds content expressed as gallic acid (760 nm, equation y = 0.1022x + 0.1049, $R^2$ 0.9967) was similar for EE-L and EE-F - 14.0 and 15.0% (w/w), respectively.

**Table 1.** Data on chemical analysis of *C. sylvestris* leaves and flowers extracts.

| Extracts | Yield (%) | TLC bands[1] | HPLC-PDA[2] (223-229 nm) | HPLC-PDA[3] (232-235 nm) | Total phenolic (%) |
|---|---|---|---|---|---|
| EA-L | 5.9 | 20 | 0 | 20 | |
| EE-L | 20.3 | 0 | 0 | 0 | 14.0 |
| EA-F | 4.2 | 16 | 26 | 12 | |
| EE-F | 23.4 | 9 | 0 | 0 | 15.0 |

[1]Total bands on chromatoplates. [2]Total chromatographic peaks in UV spectrum ($\lambda_{max.}$ 223-229) in chromatograms. [3]Total chromatographic peaks in UV spectrum ($\lambda_{max.}$ 232-235) in chromatograms.

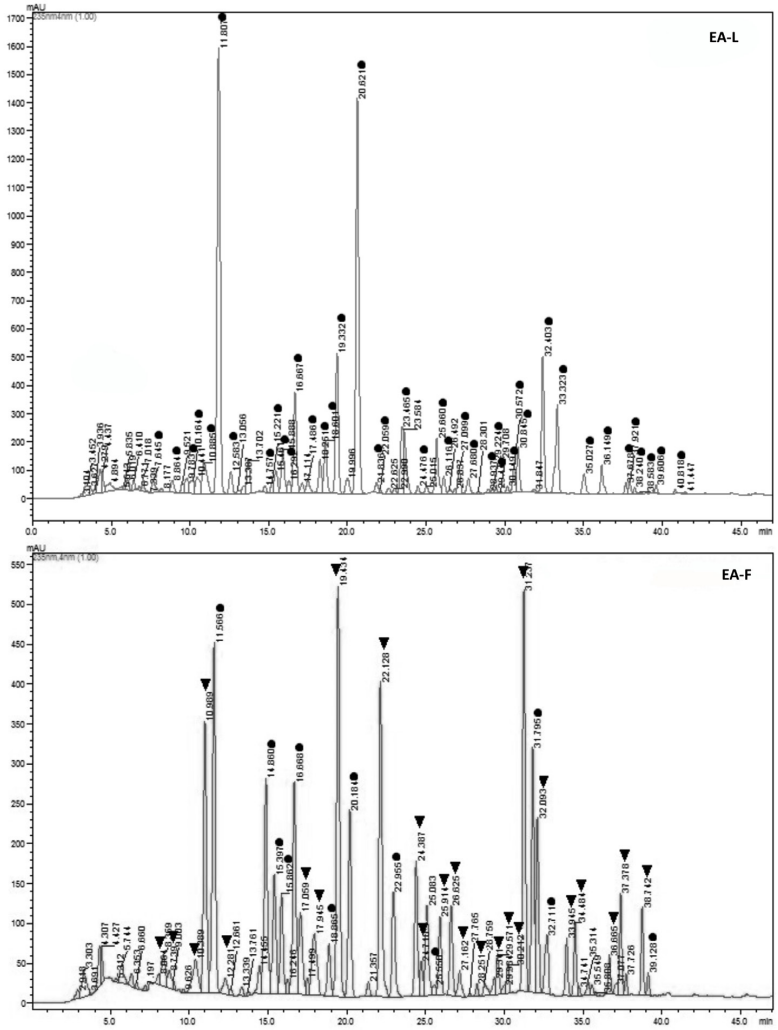


**Figure 1**. EA-L and EA-F HPLC chromatograms. The symbol (●) indicate peaks with UV spectrum in $\lambda_{max}$ 232-235 nm and symbol (▼) indicate peaks with spectrum in $\lambda_{max}$ 223-229 nm. Chromatographic conditions were according to Claudino et al. (2013).

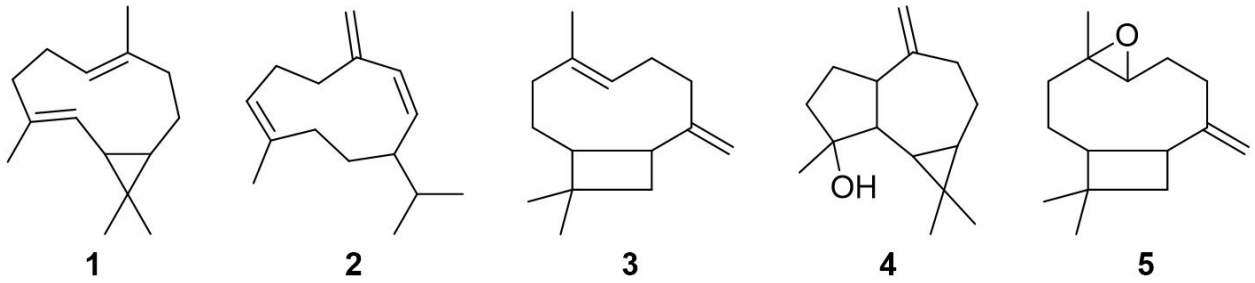


**Figure 2.** Chemical structure of the bicyclogermacrene (**1**), germacrene D (**2**), *E*-caryophyllene (**3**), spathulenol (**4**) and caryophyllene oxide (**5**).

**Table 2.** Chemical composition of EO of *C. sylvestris* leaves, flowers and fruits, identified by GC-MS.

| Components | Leaves (%) | Flowers (%) | Fruits (%) |
|---|---|---|---|
| aromadendrene | 2.6 | 1.0 | ----- |
| bicyclogermacrene | 67.2 | 45.9 | ----- |
| (*E*)-caryophyllene | 0.9 | 9.8 | ----- |
| *α*-copaene | ----- | 0.3 | 1.3 |
| *β*-elemene | 3.1 | 1.3 | ----- |
| germacrene D | 2.3 | 17.5 | ----- |
| *α*-gurjunene | 0.5 | ----- | ----- |
| *α*-humulene | ----- | 1.0 | ----- |
| *δ*-cadinene | ----- | 0.9 | 2.1 |
| **Sesquiterpene hydrocarbons** | **76.6** | **77.7** | **3.4** |
| spathulenol | 1.3 | 2.9 | 27.9 |
| caryophyllene oxide | ----- | ----- | 17.4 |
| viridiflorol | 2.1 | 0.5 | 4.2 |
| globulol | ----- | ----- | 1.6 |
| guaiol | ----- | ----- | 4.1 |
| humulene epoxide II | ----- | ----- | 3.8 |
| *α*-cadinol | ----- | ----- | 6.0 |
| *α*-muurolol | ----- | ----- | 3.6 |
| **Oxygenated sesquiterpenes** | **3.4** | **3.4** | **68.6** |
| **Identified** | **80.0** | **81.1** | **72.0** |

*EO analysis by GC-MS*

EO yields for fresh leaves, flowers and fruits were 0.3, 0.1 and 0.1% (w/v), respectively. Bicyclogermacrene (**1**; Figure 2) was the major component in the essential oil from leaves (67.2%) and flowers (45.9%). On the other hand, the major compound in the fruit essential oil was spathulenol (**4**; Figure 2) with 27.9%.

The complete chemical composition of the leaf, flower, and fruit EOs is shown in Table 2. Sesquiterpenes hydrocarbons were predominant in leaf EO (76.6%) and flower EO (77.7%), respectively, whereas oxygenated sesquiterpenes (68.6%) were predominant in fruits EO. Monoterpenes, phenylpropanoids or other volatiles were not identified in the EO.

## DISCUSSION

The differentiation between clerodane diterpenes of *C. sylvestris* with different diene patterns in the side-chain may be performed through UV spectra analyses, as proposed (Carvalho et al., 2010). In the leaf extracts; diterpenes with conjugated double bond at C12 and C14 ($\lambda_{max}$ 232-235 nm) predominate; whereas in the flowers predominate diterpenes with conjugated double bound at C13(16) and C14 ($\lambda_{max}$ 223-229 nm). As described (Carvalho et al., 2010), the results of this work confirm the predominance of casearin-like diterpenes (diene at C12 and C14) in the leaves through the number of peaks in the chromatograms: twenty peaks with $\lambda_{max}$ 232-235 nm were observed in the EA-L HPLC chromatogram; whereas only twelve peaks with the same $\lambda_{max}$ were detected in the EA-F chromatogram (Figure 1). With respect to peaks with $\lambda_{max}$ 223-229 nm; no peaks were observed in EA-L, on the other hand, twenty-six were observed in the EA-F chromatogram.

The total phenolic content values were consistent with the value of 11.9% determined for the leaf ethanolic extract (Carvalho et al., 2018). The diene model for clerodane diterpenes shows differences in UV absorbance and UV spectra from chromatogram peak obtained by HPLC-PDA analyses may be used to differentiate leaves and flowers. The extract components of *C. sylvestris* flowers may present different pharmacological activities or do not have the same effect as the compounds in the leaf extracts. In the case of an herbal medicine produced with *C. sylvestris* leaves, the flowers can be adulterants and this HPLC-PDA method may be employed for the quality control of the plant drug (raw material).

In literature; the yield reported for the leaf EO ranges from 0.2 to 2.5% (w/w), and the major compounds described were bicyclogermacrene (**1**, Figure 2); germacrene D (**2**, Figure 2), *δ*-cadinene, *α*-zingiberene, *α*-humulene, *E*-caryophyllene (**3**, Figure 2) and spathulenol (**4**, Figure 2) fruits (Esteves et al., 2005; Spósito et al., 2019; Moreira et al., 2019; Bou et al., 2013; Sousa et al., 2007). However, the chemical composition of *C. sylvestris* flowers and fruits EO has not been reported to date. The variation of the major chemical components between the essential oils obtained from different *C. sylvestris* organs may be associated with different biological activities that these components play in the various stages of the plant, such as flowering and fruiting (Silva et al., 2010).

Among the chemical analyses performed on leaves and flowers extracts, only HPLC-PDA analyses showed potential for *C. sylvestris* organs differentiation on the basis of clerodane diterpenes composition. The EO showed variation on chemical composition of the different *C. sylvestris* organs. However, the compounds identified in both flowers and fruits have also been identified in leaves and may not differentiate these organs in other specimens. In this case; chemical variability studies on EO composition of these organs might be further performed to understand these differences.

This study contributes to the knowledge on the *Casearia sylvestris* phytochemical profile, especially for flowers and fruits EO composition. This data may be employed in the quality control of herbal medicines based on *C. sylvestris* leaves to determine the fruits and flowers as adulterants even in powdered material.

*ACKNOWLEDGMENTS*

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoa de Nível Superior - Brasil (CAPES) – Finance Code 001.

## RESUMO

*Variabilidade química entre diferentes órgãos da planta medicinal Casearia sylvestris*

**Nesse trabalho o perfil fitoquímico de óleos essenciais e extratos de folhas, flores e frutos de *Casearia sylvestris* foi investigado. Os extratos de folhas e flores foram preparados por sonicação e analisados por cromatografia em camada fina e cromatografia líquida de alta eficiência. O teor de fenólicos totais foi determinado por fotometria. Os óleos essenciais de folhas, flores e frutos foram extraídos por hidrodestilação. Os maiores rendimentos de extratos foram 20,3% (folhas) e 23,4% (flores) com etanol 70%. Os rendimentos de extração de óleo essencial foram de 0,3% (folhas) e 0,1% (flores e frutos). O biciclogermacreno foi o principal componente do óleo essencial. A cromatografia em camada delgada sugere que as folhas e flores possuem um perfil químico semelhante. O teor fenólico de folhas e flores foi semelhante (14,0 e 15,0%, respectivamente). As análises cromatográficas indicaram a predominância diterpenos clerodânicos do tipo das casearinas nas folhas ($\lambda_{max}$ 232-235), enquanto que nas flores diterpenos com um padrão de dienos diferente nas cadeias C13 (16) e C14 ($\lambda_{max}$ 223-229). O diferente perfil fitoquímico das flores de *C. sylvestris* em relação às folhas pode ser explorado pela busca de novos componentes bioativos. Este é o primeiro relato sobre a composição de óleos essenciais de frutos e flores de *C. sylvestris*; cujos dados podem ser utilizados no controle de qualidade de fitoterápicos derivados de folhas de *C. sylvestris*.**

**Palavras-chave: Biciclogermacreno. Diterpenos Clerodânicos. Controle de Qualidade. Óleo Essencial.**

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Received on November 2nd 2018
Accepted on December 1st 2018