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Chemical Profile, Antioxidant Activity, Flavonoids Content and Leaf Anatomy of *Odontocarya vitis* (Menispermaceae)

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Abstract: *Odontocarya vitis* (Menispermaceae) is endemic to the Atlantic Forest from Brazil, and its family is known in folk medicine to treat inflammation and kidney problems. To the best of our knowledge, there is no description in the literature of chemical, biological activity, and leaf anatomy aspects of this species. The aim of this study was to contribute with data from *O. vitis* evaluating the chemical composition, the antioxidant activity and flavonoids content of the stems and leaves, and the leaf anatomy. The chemical composition was determined by gas-phase chromatography coupled with mass spectroscopy, and long-chain hydrocarbons, steroids, and terpenoids were identified. The antioxidant activity of the methanolic crude extracts was determined by scavenger of the free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) and showed better results for the stems extracts (7.50±0.81 g extract/g DPPH). The flavonoids content was determined by the colorimetric method and evidenced the presence of these metabolites, with a strong negative correlation between the EC_{50} values and flavonoids content. Leaf anatomy was evaluated by optical microscopy and showed hypostomatic epidermis with normocytic stomata, dorsiventral mesophyll, and collateral vascularization. These studies can contribute to the knowledge of this species and evidence its potential as a source of active substances.

Keywords: DPPH; flavones and flavonols; GC/MS; morphology.

1. Introduction

Menispermaceae (Ranunculales) is a pantropical family comprising a current estimate of 72 genera and 526 species of mostly climbing plants [1,2]. Traditionally known as the "moonseed family", its ethnobotanical uses range from arrow poisons (curare) to medicinal plants [3]. Their pharmacological activities include antioxidant, bronchodilator, anti-inflammatory, hyperglycemia, cardiopathy, and many of them are associated with the presence of alkaloids, but other constituents such as pectins, flavonoids alkamides, and essential oils were also reported in the family [4-9].

Although *Odontocarya* (Burasaieae) is among the largest genera, with about 48 species [2,10], phytochemical studies are limited to *Odontocarya tamoides* (DC.) Miers, with the diterpene jateorin isolated from the ethanol extract of stems [11], and *Odontocarya paupera* (Griseb.) Diels, with the identification of β-amyrin acetate, friedelin, β-sitosterol, pachulen, and a naphthoquinone in the petroleum ether extract of leaves and stems [12]. Performing further studies on the chemical diversity of other species from the genus is of paramount importance to the rational search of bioactive compounds and as contributions to herbal drugs authentication[13], in addition to anatomical descriptions, which are restricted to stems and secondary growth patterns of *Odontocarya vitis* (Vell.) J.M.A. Braga [14], an endemic species from Brazil [15].

Given the incipient chemical and anatomical data in the literature concerning the genus *Odontocarya* and the importance of plants as health resources [16], this outstanding study aimed at characterizing the chemical composition, antioxidant activity, and flavonoids content of crude extracts from stems and leaves and the leaf anatomy of *O. vitis*.

2. Materials and Methods

2.1. Plant material.

The samples were collected in Atlantic Forest fragments in southeastern Brazil: Prainha, Rio de Janeiro, RJ (Braga 10-002) and Morro do Itaoca, Campos dos Goytacazes, RJ (Braga 11-003), and deposited in the herbarium collection of the Instituto de Pesquisas Jardim Botânico Rio de Janeiro, with registration numbers RB811237 and RB811238, respectively. For the accomplishment of the chemical and biological studies, CGEN authorization was obtained (CGEN nº A511EB8).

2.2. Preparation of the extracts.

Stems and leaves were dried in an oven with forced air circulation (Solab SL) at 40ºC and later reduced to small fragments using a blender (Tron). The processed material was submitted to extraction by exhaustive static maceration with hexane and methanol (VETEC). The solvent was reduced in a rotary evaporator (Buchi R 114).

2.3. Gas chromatography coupled to mass spectrometry (GC/MS).

Stems and leaves hexanic crude extracts were analyzed by GC/MS to evaluate their constituents. For the analysis, it was used an Agilent Technologies chromatograph (model 6890N) coupled to a mass detector (model 5973N) with an automatic injector (model 5683), HP-5MS capillary column (30mx0.25mmx0.25μm), and Wiley Library database. Helium was used as carrier gas at a flow rate of 2 mL/min and the detector temperature beginning at 150°C,

increasing 10°C per minute to 300°C, remaining at 300°C for 15 minutes. The injected concentration was 1 mg/mL. The mass spectra of the compounds were also compared to data available in NIST Mass Spectral Library.

2.4. Antioxidant activity.

Total antioxidant activity (TAA) of the stems and leaves methanolic crude extracts were evaluated by a spectrophotometric method based on the scavenging of the free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) (Sigma-Aldrich) [17]. The absorbance readings were taken in a UV-VIS spectrophotometer (Biospectro SP 220). The experiment was carried out in three independent assays in triplicate. Rutin and butylhydroxytoluene (BHT) from previews work were used as positive controls [18].

2.5. Flavonoids content.

The flavonoids content, expressed as flavones and flavonols, was determined for the stems and leaves of methanolic crude extracts. A colorimetric assay that involved the reaction with aluminum chloride PA (Proquímios) was used [17]. The absorbance readings were taken in a UV-VIS spectrophotometer (Biospectro SP 220). The experiment was performed in three independent trials, each one in triplicate. Rutin was used as a positive control.

2.6. Statistical analysis.

The results were expressed as mean ± standard deviation from the evaluation of three independent assays. Comparisons among the triplicate means of the three independent trials for the same sample were performed using the one-way analysis of variance (ANOVA). The Tukey-Kramer test was applied in cases of rejection of the null hypothesis by ANOVA. A comparison among samples in the antioxidant activity was performed using ANOVA and confirmed by Tukey-Kramer; for flavonoids content, was used unpaired t-test. Pearson's correlation test was performed between the results obtained for EC_{50} values and flavonoids content.

2.7. Leaf anatomy.

For the structural characterization, fully expanded leaves were selected from the 3rd to 4th nodes, fixed in 50% FAA, and transferred to 50% ethanol [19]. Fragments of the petiole, leaf blade, edge, and midrib were embedded in Historesin (Leica®). Cross-sections (3-5 μm) were obtained using a semi-automated rotary microtome Leica RM2245 and stained with 0.05% toluidine blue in a 0.1 M phosphate buffer (Sigma-Aldrich). The epidermis was dissociated by the Jeffrey method [19]. The samples were photomicrographed with an Olympus DP73 digital camera fitted to an Olympus BX50 microscope.

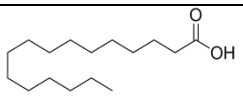
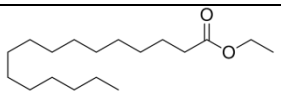
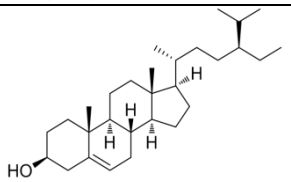
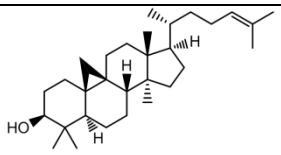
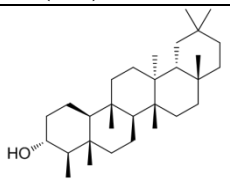
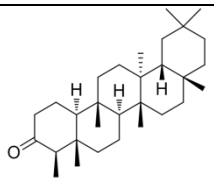
3. Results

3.1. Gas chromatography coupled to mass spectrometry (GC/MS).

The data analysis evidenced higher metabolic diversity in the hexanic crude extracts of stems than the leaves hexanic crude extract (Figure 1). The mass spectra of the substances present in the stems and leave extracts indicated the presence of long-chain hydrocarbons (Figure 1 and Table 1).

In the stems, palmitic acid (8.691 min) was identified in greater abundance, and in the leaves, the hydrocarbon hentriacontane (17.786 min) represented the major component (Figure 1 and Table 1). The remaining four most abundant compounds of each extract are listed in Table 1.

Table 1. Retention time, molecular weight, suggested compound, and the corresponding structure present in the hexanic crude extracts of stems and leaves of *Odontocarya vitis.*

Stems hexanic crude extract			
Retention time (min)	**Molecular weight (m/z)**	**Compound**	**Structure**
8.691	256	Palmitic acid	
9.012	284	Ethyl palmitate	
17.749	408	Nonacosane	$CH_3(CH_2)_{27}CH_3$
19.522	489	β-sitosterol derivative	
20.140	426	Cycloartenol	
Leaves hexanic crude extract			
16.376	408	Nonacosane	$CH_3(CH_2)_{27}CH_3$
17.786	436	Hentriacontane	$CH_3(CH_2)_{29}CH_3$
19.547	466	Dotriacontanol	$CH_3(CH_2)_{30}CH_2OH$
22.301	428	Friedelanol	
22.565	426	Friedelin	

The substances were compared with the Wiley Database and NIST Spectral Library.

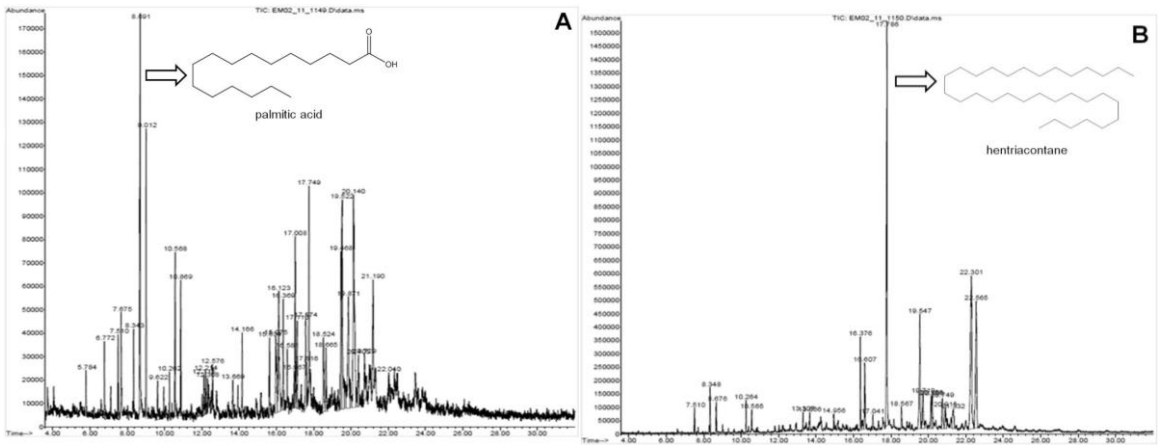


Figure 1. The chromatogram obtained for hexanic crude extracts of *Odontocarya vitis* with major substances (**A**) stems extract; (**B**) leaves extract.

3.2. Antioxidant activity.

The results for the antioxidant activity assay with methanolic crude extracts of stems and leaves of *O. vitis* are presented in Table 2, and the results for positive controls Rutin and BHT, were obtained in previous work [18].

Table 2. Antioxidant activity, expressed as EC_{50} values, of *Odontocarya vitis* methanolic crude extracts and positive controls Rutin [18] and Butylhydroxytoluene (BHT) [18].

Samples	**EC_{50} (g extract/g DPPH)**
Stems methanolic crude extract	7.50±0.81
Leaves methanolic crude extract	15.60±2.00
Rutin	0.579±0.035
BHT	0.748±0.125

DPPH = 1,1-diphenyl-2-picrylhydrazyl.

EC_{50} = concentration of sample able to reduce in 50% the initial concentration of DPPH.

The curves obtained by linear regression showed a good coefficient of determination ($r^2 > 0.90$). The statistical treatment by ANOVA did not show significant differences among the three independent trials for each sample ($p \geq 0.05$) and significant differences between the extracts and extracts and controls ($p \leq 0.05$), confirmed by the Tukey-Kramer test.

The reaction kinetics showed different patterns for the analyzed extracts according to their concentrations. For concentrations of 5, 10, and 25 µg/mL, a fast reaction kinetic pattern was observed since the maximum percentages of the remaining DPPH were reached before 5 minutes reaction. The other concentrations presented a slower profile, with the maximum remaining DPPH concentrations being reached between 15 and 30 minutes of reaction (Figure 2). The maximum antioxidant activity for both extracts was reached at 250 µg/mL. The methanolic crude extract of stems demonstrated the best result since the remaining DPPH was 27.80% (Figure 2A). The other concentrations of both extracts presented remaining DPPH greater than 50% (Figure 2). As observed in previous work, Rutin showed fast kinetics and BHT, intermediate kinetics, with a remaining DPPH of 5% [18].

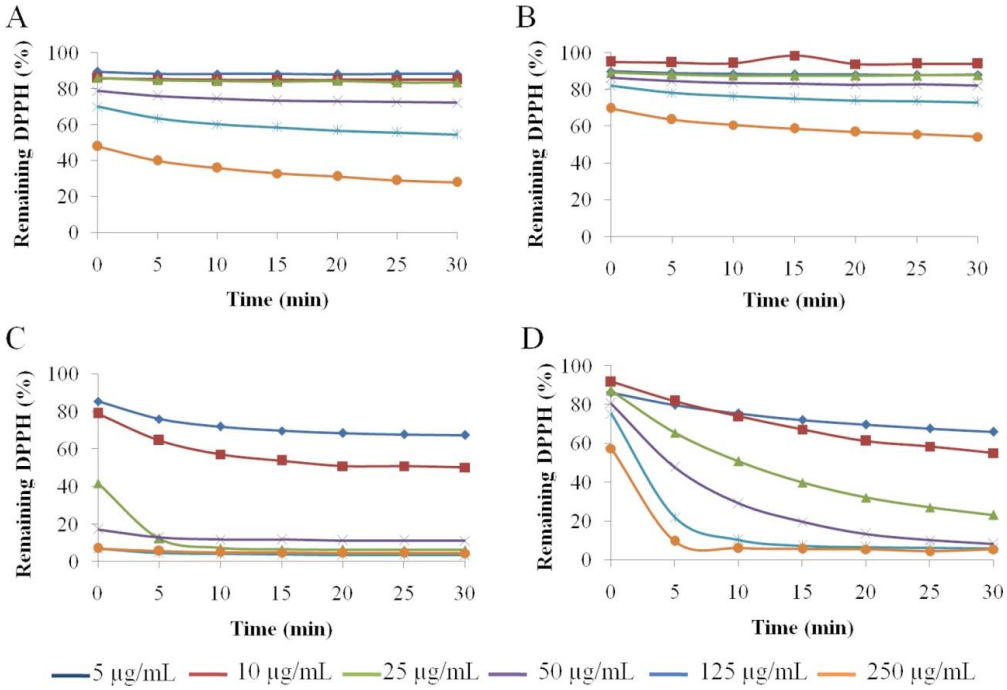


Figure 2. Reaction kinetics with 1,1-diphenyl-2-picrylhydrazyl (DPPH): (**A**) methanolic crude extract of stems of *Odontocarya vitis*; (**B**) methanolic crude extract of leaves of *Odontocarya vitis*; (**C**) Rutin [18];(**D**) Butylhydroxytoluene (BHT) [18].

3.3. Flavonoids content.

The flavonoids content in methanolic crude extracts of stems and leaves of *O. vitis* are listed in Table 3.

Table 3. Flavonoids content, expressed as rutin equivalent, in the stems and leaves methanolic crude extracts of *Odontocarya vitis*.

Samples	Flavonoid content (%)
Stems methanolic crude extract	53.50±6.60
Leaves methanolic crude extract	32.61±2.95

The curves obtained by linear regression showed a good coefficient of determination ($r^2 > 0.90$), and the statistical treatment by ANOVA showed significant differences among the three independent trials for each extract ($p \leq 0.05$). The Tukey-Kramer test does not confirm these differences. A significant difference between the samples ($p = 0.0075$) was confirmed by the t-test. The Pearson's correlation test showed a strong negative correlation ($r = -1.0$) between the EC_{50} values and flavonoids content.

3.4. Leaf anatomy.

The petiole of *O. vitis* was circular in cross-section, the epidermis uniseriate, glabrous, and covered by a thin cuticle. The subjacent layers composed by collenchyma cells followed by cortical and medullary parenchyma delimited by a ring of 11-12 radially elongated collateral bundles (Figure 3A). The midrib was plano-convex in cross-section, the epidermis uniseriate, glabrous and covered by a thin cuticle, the subjacent layers composed by 5-8 collenchyma strata followed by parenchyma, and the vascular system consisted of a major central bundle and two accessory bundles facing the adaxial side (Figure 3B).

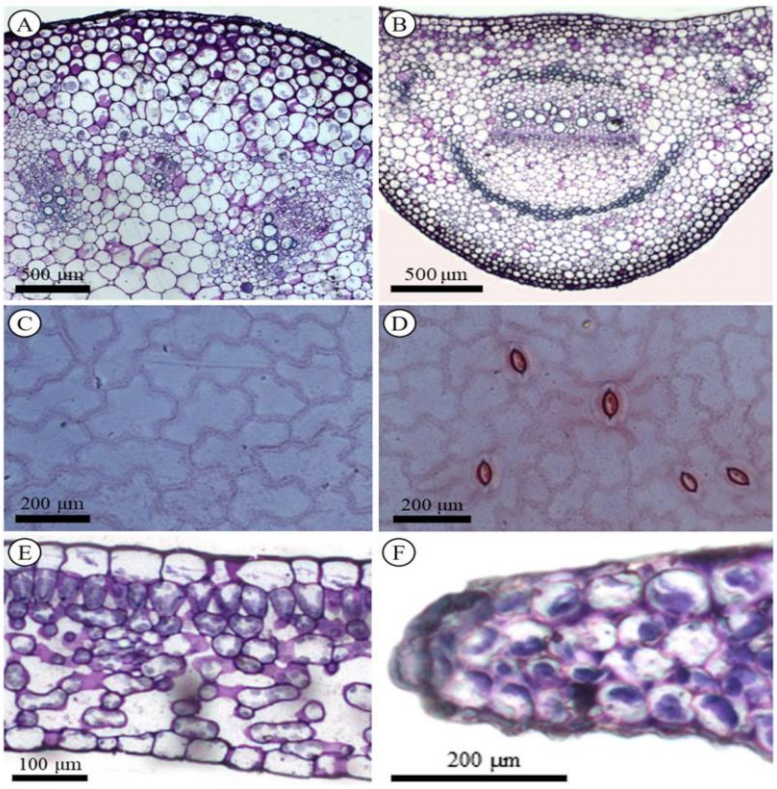


Figure 3. Leaf of *Odontocarya vitis* in cross-section (**A**, **B**, **E**, and **F**) and front view (**C** and **D**): (**A**) petiole; (**B**) midrib; (**C**-**E**) intercostal region, (**C**) adaxial and (**D**) abaxial epidermal surfaces, (**E**) dorsiventral mesophyll; (**F**) edge.

The major bundle was circular and collateral, with a straight interface between phloem and xylem conduits and a discontinuous fiber sheath consisting of two arcs on adaxial and abaxial sides. Accessory bundles were circular, collateral, and surrounded by a discontinuous fiber sheath (Figure 3B). In the front view, the epidermis is hypostomatic in the intercostal region, with anomocytic stomata on the abaxial surface and ordinary cells with sinuous walls on both surfaces (Figure 3C-D). In cross-section, the epidermis was uniseriate, glabrous, covered by a thin cuticle, and the mesophyll was dorsiventral, with collateral bundles surrounded by a discontinuous fiber sheath (Figure 3E). The leaf edge was straight and narrow in cross-section, the epidermis was uniseriate, glabrous, covered by a thin cuticle, followed by parenchyma cells (Figure 3F).

4. Discussion

Production of terpenes by species of the *Odontocarya* genus has already been described with the isolation and identification of the diterpene jateorine from the stems ethanolic extract of *Odontocarya tamoides* [11]; and β-amyrin acetate, friedelin, β-sitosterol and pachulen from the leaves and stems petroleum ether extracts of *Odontocarya paupera* [12]. The presence of steroids and triterpenoids was identified in *Legnephora moorei* (F. Muell.) Miers with the isolation of friedelin and a mixture of steroids, among themsitosterol [20]. In fractions from roots and twigs, extracts of *Penianthus zenkeri* Diels were identified as steroids and terpenoids [21]. Stem and root extract phytochemical screening of *Cocculus pendulus* Diels. also showed the possible presence of steroids and triterpenoids [22]. Acids and esters were already isolated from the aerial part of *Tiliacora triandra* (Colebr.) Diels [23].

The chemical profile of hexane extracts of *Odontocarya vitis* revealed the presence of some bioactive compounds, such as β-sitosterol in stems and friedelin in leaves, both showing pharmacological properties reported in the literature as anticancer, antidiabetic, anti-inflammatory, antimicrobial, antioxidant, and antipyretic [24-29]. In the Burasaieae tribe, in addition to the *Odontocarya* species, friedelin was also described in *Penianthus camerounensis* A. Dekker [30]; and β-sitosterol in species from the genus *Tinospora*, such as *T. cordifolia* (Willd.) Miers ex Hook. f. & Thomson, *T. crispa* (L.) Hook. f. & Thomson, *T. sinensis* (Lour.) Merr., and *T. oblongifolia* (Engl.) Troupin [31].

Natural antioxidants, such as flavonoids, can control the imbalance of the free radicals, compounds responsible for oxidative damages. Due to flavonoids' antioxidant activity, other biological properties can be underlined, such as anticancer activity and protection of the cardiovascular and neurological systems [32]. The extracts of *O. vitis* showed considerable antioxidant activity, being the best result for the crude methanolic extract of the stems, with EC_{50} of 7.50±0.81 g extract/g DPPH. However, they were less active than the positive controls, Rutin and BHT [18]. From the kinetic point of view, the extracts presented a fast kinetic profile in the lower concentrations, with remaining DPPH greater than 50%, except for the stems methanolic crude extract, of 27.80%. A different pattern was observed for Rutin and BHT. The difference between the extracts and controls could be justified by the fact that the crude extracts are composed of several substances in smaller quantities, whereas the controls are pure substances [17].

The flavonoids content, expressed by flavones and flavonols, in the polar extracts of *O. vitis* suggested the presence of these secondary metabolites in the stems and leaves, but with a higher occurrence in the stems methanolic crude extract (53.50±6.60%). The correlation between EC_{50} values and flavonoids content showed a strong tendency to decrease EC_{50}, while

increasing the flavones and flavonols content. Thus, suggesting that the presence of these metabolites could be related to antioxidant activity.

To the best of our knowledge, there were no reports in the literature for antioxidant activities and the presence of flavonoids in *Odontocarya* species. However, the ethyl acetate extract of fruits of *Haematocarpus validus* Bakh. f., the methanolic flowers extract of *Coscinium fenestratum* Colebr. and the ethanolic stem extract of *Tinospora crispa* treated with DPPH evidenced antioxidant activity [33-35]. In another study involving *Coscinium fenestratum* it was observed moderate antioxidant activity in pulp fruit crude extracts, besides the possible presence of flavonoids in phytochemical screening and flavonoids content assays [36]. In the phytochemical screening of *Cissampelos sympodialis* Eichlerthe presence of flavonoids in the leaves ethanolic extracts was suggested [37].

From the aerial parts of *Cissampelos pareira* L., the flavone cissampeloflavone was isolated [38]. In *Stephania tetrandra* S. Moore, glycosylated flavones and the biflavonoids stephaflavone A and stephaflavone B were isolated from aerial parts extracts [39]. In *Tinospora crispa*, a species from the same tribe of *Odontocarya*, flavones, such as apigenin, diosmetin and genkwanin, were isolated from stems extracts [40].

Anatomical studies on the genus *Odontocarya* are restricted to stem descriptions of *O. vitis* [14], being this the first work to describe leaf anatomy in the taxa. In the Burasaieae tribe, anatomical studies of leaves focus on *Tinospora* species, aiming at providing parameters for authentication of raw material related to medicinal plants and dietary supplements based on *T. sinensis*, *T.crispa*, and *T. cordifolia* [41, 42]. Phylogenetically, the genus *Odontocarya* and the mentioned *Tinospora* species are considered close relatives [43]. While in *O. vitis* the contour observed in cross-sections of the petiole and midrib were plano-convex, it was biconvex in *T. sinensis*, *T. crispa*, and *T. cordifolia* [41].

The cell composition and arrangement of epidermal, fundamental, and vascular tissues observed in cross-sections of the petiole, midrib, and intercostal region of *O. vitis* were similar within the three species, except for the presence of trichomes in the petiole, midrib, and leaf blade of *T. sinensis*, the presence of a sclerenchymatous cap surrounding the vascular system in the petiole of *T. crispa*, and the absence of accessory bundles in the midrib of *T. cordifolia* [41]. Another common feature between *O. vitis* and the described *Tinospora* species was the hypostomatic leaf blade with anomocytic stomata on the abaxial surface [41]. This analysis could provide correct identification and standardization of the plant material; in addition, anatomical characters, such as the presence and position of trichomes, could be used to diagnose dry and processed plants for quality control [44-46]. Concerning the discrimination between *T. cordifolia* and its adulterant *Pergularia daemia* (Forssk.) Chiov., plant anatomy was considered an important tool for authenticity analyses of fresh and dried material [42].

5. Conclusions

Although *Odontocarya vitis* belongs to a family known for its use in folk medicine, there are few reports in the literature regarding the species. This study suggested the presence of secondary metabolites, such as terpenoids, steroids, and flavonoids, the latter probably responsible for the observed antioxidant activity of polar extracts.The anatomy of the leaf of *O. vitis* is similar to the other related species of the Menispermaceae family, such as *Tinospora*. However, features such as the petiole contour, the presence or absence of trichomes, and aspects of the vascular system may represent important diagnostic characteristics. In this way,

this article can contribute to delimit the circumscription of species and to the knowledge of their potential as a source of active substances.

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Conflicts of Interest

The authors declare no conflict of interest.

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