Morphoanatomical characterization and chemical study of the internal portion of the stem bark of *Sambucus australis* Cham. & Schltdl

ALERICO, G.C.[1]*; VIGNOLI-SILVA, M.[2]; LANDO, V.R.[2]
*[1]Universidade Federal do Rio Grande do Sul, Av. Ipiranga 2752, 90.610-000, Porto Alegre, RS, Brazil. [2]Universidade Federal de Ciência da Saúde de Porto Alegre, Av. Sarmento Leite 245, 90050-170, Porto Alegre, RS, Brazil. *Autor para correspondência: gabrielaalerico@gmail.com*

ABSTRACT: *Sambucus australis* Cham. & Schtdl. (Adoxaceae) is an arboreal species native to the south of Brazil, known as "sabugueiro". The internal part of the stem bark of this species is used to produce a homemade ointment in some regions of the state of Rio Grande do Sul. The purpose of this study is to characterize the morphoanatomy and identify the compounds present in the internal part of the stem bark of *S. australis* through chemical and histochemical methods. In addition, the best extraction conditions for the sample were determined. It was possible to quantify the rutin and total phenolic compounds, as well as define the Soxhlet method with an 80% hydroethanolic solution as the best method for extracting these compounds from the bark of the species. The portion of the stem bark that is popularly used could also be determined. Based on the results, new studies will be performed in order to identify other characteristics of the species and the possible reasons that sustain its traditional use.

Keywords: *Sambucus australis*, bark, cicatrization, traditional use.

RESUMO: Caracterização morfoanatômica e estudo químico da porção interna da casca do caule de *Sambucus australis* Cham. & Schltdl. *Sambucus australis* Cham. & Schtdl. (Adoxaceae) é uma espécie arbórea nativa do sul do Brazil, conhecida como "sabugueiro". A parte interna da casca do caule dessa espécie é utilizada na produção de uma pomada caseira em algumas regiões do estado do Rio Grande do Sul. O objetivo desse estudo é caracterizar a morfoanatomia e identificar compostos presentes na parte interna da casca do caule de *S. australis* por métodos químicos e histoquímicos. Ademais, realizou-se avaliações para determinar as melhores condições extrativas para a amostra. Foi possível quantificar rutina e compostos fenólicos totais, além de determinar o método Soxhlet com solvente hidroetanólico 80% como o melhor método para extrair esses compostos da casca da espécie. Ainda foi possível determinar a porção da casca do caule utilizada popularmente. Com base nos resultados, novas investigações serão realizadas a fim de determinar mais características da espécie e as possíveis razões que corroboram o uso tradicional.

Palavras-chave: *Sambucus australis*, casca, cicatrização, uso tradicional.

INTRODUCTION

Plants are widely used to medical purposes and have a significant role in the traditional health and in the development of drugs. Besides, more than 50% of all drugs available, derivate from natural products (Newman & Cragg, 2012).

In this context, *Sambucus* L. genus, belonging to Adoxaceae family (Judd et al., 2009), is widely known by its species properties, for example, *S. nigra* L. had its immunomodulatory functions showed by Barsett et al. (2012). Other species include *S. chinensis* Lindl (Zhang et al., 2010) and *S. australis* Cham & Schltdl (Rao et al., 2011), from which were isolated a few flavonoids and ursolic acid respectively, whereas *S. ebulus* L. has anti-inflammatory activity (Ebrahimzadeh et al., 2006) and *S. canadensis* L. was pointed out by its antioxidant action (Ozgen et al., 2010).

Sambucus australis, popularly known in Brazil as "sabugueiro", and "sabugueiro-do-Brasil", has an arborescent habit and is a native species from Brazilian forest formations from Amazônia and Mata Atlântica. Its occurrence was registered in the

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North (Amazonas), Northeast (Rio Grande do Norte, Paraíba, Pernambuco and Bahia), Southeast (Minas Gerais and São Paulo) and South regions (Paraná, Santa Catarina e Rio Grande do Sul) from Brazil (Sobral, 2010). As a medicinal plant, *S. australis* has been used to treat several conditions, and is cited as one of Brazilian native species with current or potential economic value (Coradin et al., 2011).

There are few studies with *S. australis* leaves and flowers; Scopel et al. (2010) and Rao et al. (2011). The last showed, with his study, the preventive action of ursolic acid in the abdominal adipogenesis and its promising use as an anti-obesity multiple-target agent. However, in Northern region from Rio Grande do Sul state, the internal portion of the *S. australis* stem bark is used too, by some people, in the production of a homemade ointment (Personal communication of the first author's grandfather). According to popular reports, the ointment, when applied over skin burns, shows analgesic and wound healing effects, the last described only to *S. ebulus* (Süntar et al., 2010). Also, *S. australis* appears in ethnobotanical surveys conducted in Rio Grande do Sul state, as a species used traditionally for wound-healing purposes (Somavilla & Canto-Dorow, 1996; Barros et al., 2007; Vendruscolo, 2004). Besides, in one of these studies, the part used as remedy is the internal portion of the stem bark (Vendruscolo, 2004). It is important to highlight the need for new wound healing agents, considering the implications of a chronic wound for example. Chronic wounds are a burden on the health care system, whereas regarding the high costs attached or the loss of life quality of the patients (Sen et al. 2009).

Barks of other two Brazilian native species are use in Brazil by the herbal industry, one from *Erythrina verna* Vell.(Fabaceae), popularly known as "mulungu" and the other from *Stryphnodendron adstringens* (Mart.) Coville (Fabaceae), known as "barbatimão" (Farmacopeia Brasileira, 2000; Rambo et al., 2013). Macroscopically, the barks from the two species differ from each other in form, appearance, texture and color. Other exotic species barks utilized in Brazil to medicinal purpose are *Cinnamomum cassia* (L.) J. Presl (Lauraceae), *Quillaja saponaria* Mol. (Quillajaceae), *Salix alba* L. (Salicaceae) (Farmacopeia Brasileira, 2010), *Cinchona pubescens* Vahl (Rubiaceae), *Cinnamomum verum* J.S. Presl (Lauraceae) (Farmacopeia Brasileira, 2000) and *Rhamnus purshiana* DC. (Rhamnaceae) (Farmacopeia Brasileira, 1996). The comparison of the several barks is important for differentiating the material used to produce drugs. The purpose of this paper is to investigate the morphoanatomic and chemical characteristics of the stem bark's internal portion of *S. australis*, material used to produce the homemade ointment, in Northern region from Rio Grande do Sul state, based on the ethnopharmacological relevance of the species.

MATERIALS AND METHODS

Plant material

The internal part of the stem bark of *S. australis* was collected in the municipality of Passo Fundo (28°15'58"S; 52°24'59"W), northern Rio Grande do Sul. The collection occurred in January 2013, the sample was identified and a voucher was deposited in the Herbarium (ICN) of Universidade Federal do Rio Grande do Sul (Voucher number: ICN 184327). The sample was dried at room temperature, hidden from sunlight, and powdered with a blender.

Morphoanatomical and histochemical analysis

For the identification of the bark tissue involved on the study, a horizontal slice of bark was collected and softened in a mixture of water, ethanol and glycerin (2:2:1) for ten days. Transversal and longitudinal histological sections were carried out with razor blades and observed in optical microscope.

Histochemical tests, referred in several studies (Rambo et al 2013; Brazilian Pharmacopoeia 2010. Nunes et al, 2007) and methodologies suggested by some authors (Leite 2009; Macêdo, 1997; Argüeso, 1986) were applied. With fresh material, the histochemical tests were performed for alkaloids with Dittmar reagent (Leite, 2009), for lignins with acidic phloroglucinol (Leite, 2009), starch grains with Lugol reactive (Leite, 2009), cellulose with toluidine blue (Macêdo, 1997), phenolic compounds with ferric chlorate (Leite, 2009), calcium oxalate crystals with sulfuric acid 50% (Macêdo, 1997), lipids with Sudan IV (Leite, 2009), saponins with concentrated sulfuric acid (Macêdo, 1997) and tannins with ferric chlorate 10% and sodium carbonate 2% (Argüeso, 1986).

Chemical analysis

The variables for the extraction conditions study were extraction method, temperature, time, solvent, sample mass and solvent volume. To compare the different extraction parameters, the quantification values of rutin and total phenolic content were used.

The sample was extracted by maceration, ultrasound-assisted, Soxhlet and reflux, each one with two different time extraction, being 30 and 60 minutes for the first two, and 60 and 240 minutes for the following. And, maceration was done under different temperature conditions, room temperature

(25°C) and 60°C. All extractions were performed using methanol, then the most promising one was evaluated with hidroethanolic solution 80% as solvent.

Rutin quantification was done by UV-VIS spectrophotometry, 356nm wavelength, using an analytical curve with rutin standard (Sigma-Aldrich). And total phenolic content was determined by Folin-Ciocalteau method according to Fazio et al. (2013), the results are showed as milligrams of gallic acid equivalent (GAE) per gram of dried sample (GAE/g).

Statistical analysis

Results are presented as mean ± standard deviation**.** Data was submitted to variance analysis (Anova) and Tukey Test, and analyzed with software Minitab 1.6. Results were considered significate different if $p < 0.05$.

RESULTS AND DISCUSSION

Morphoanatomic and organoleptics characteristics

In a transversal section, the stem is macroscopical characterized by the dark colored peridermis, followed by a thin cream or pale yellow colored layer of phloem, with fibrous aspect and characteristic odor. In sequence, the organ presents a layer of xylem, well developed, with a lighter color than the other tissues, followed by a fistulous medulla. Stem bark is known as all the tissues located externally to the vascular cambium of this organ (Metcalfe & Chalk, 1979). However, the target of this study is only the internal portion of the stem bark, correspond to the phloem. Some stem barks, used as vegetal drugs as *Cinnamomum cassia* (L.) J. Presl e *Stryphnodendron adstringens*, are constituted by peridermis and phloem (Farmacopéia Brasileira, 2010), while others, as *Cinnamomum verum* e *Quillaja saponaria* Mol. are only constituted by peridermis. The internal portion of the stem bark of *S. australis* presents some similarities with *Q. saponaria*, especially regarding the flat shape, smooth texture and pale yellow color, according to the macroscopical description of the drug, presented on the Brazilian Pharmacopoeia (2010) for the last species. However, the phloem fibers characteristics can help in the differentiation of both species. In *S. australis* the fibers are angle, with thick walls and small lumen, and do not present the tortuous aspect, as described for *Q. saponaria fibers* (Farmacopeia Brasileira, 2010).

Microscopical characteristics

In transversal section, the phloem presents continuous distribution. The tissue is composed by axial parenchyma cells, sieve tube elements, companion cells and sclerenchyma fibers. Still composing the phloem, there are radial parenchyma cells, which form cell rays, sometimes more elongated than the axial (Figure 1).

The fibers are angle, with thick walls and small lumen; they are arranged in tangential groups, alternately distributed with little and few evident groups of conductive cells (sieve tube elements and companion cells) and well developed layers of axial parenchyma cells. Intercalated to these layers, there are the well-defined parenchyma rays, formed by well-organized parenchyma cells (Figure 1).

The fibers exhibit simple pits. Conductor cells are fewer than fibers and parenchyma cells, which have several shapes and sizes. In the parenchyma cells interior are alkaloids, calcium oxalate prismatic crystals, lipid drops and a large amount of starch grains. These cellular constituents react positively to histochemical tests with Dittmar reagent, sulfuric acid, Sudan IV and Lugol reactive, respectively (Figures 2 and 3). Nunes et al. (2007) report the absence of idioblasts with calcium oxalate sandy crystals in the *S. australis* flowers' tissues, neither have they mentioned the presence of other calcium oxalate sandy crystal forms. Large amounts of lipid drops (volatile and non volatile oil globules) and starch grains were found in the *S. australis* flowers analysis (Nunes et al., 2007).

The result for alkaloids was evident when the histochemical test was applied in fresh material. Besides the evidence of alkaloids in the interior of parenchyma cells, the fibers walls were also colored, indicating a positive result with Dittmar reagent. However, alkaloids are not molecules commonly

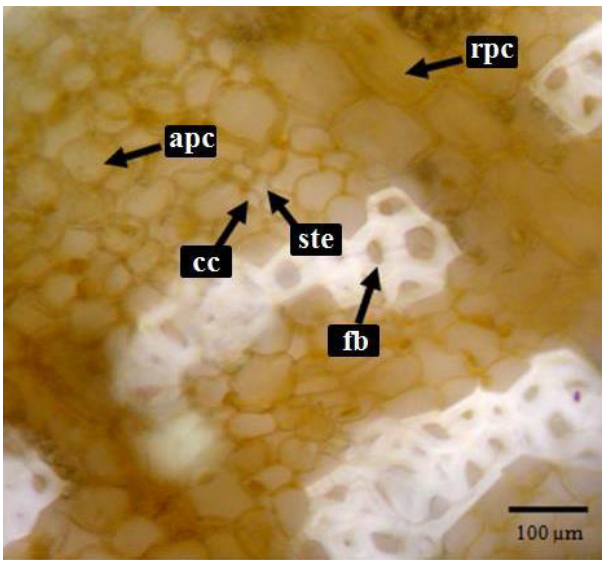

FIGURE 1. *Sambucus australis* Cham. & Schltdl. Phloem in transversal section. cc: companion cells; apc: axial parenchyma cell; rpc: radial parenchyma cell; ste: sieve tube element; fb: fiber. (Fresh material, no dye).

found in cell wall constitution. Also, it was observed positivity for the cellulose and lignin tests. The presence of these metabolites makes the species interesting from the chemical view and can stimulate further studies.

Regarding the histochemical tests applied to detect specifically phenolic compounds, saponins and tannins, the results were negative, in agreement with the results found by Alice et al. (1991). The author also described the absence of alkaloids on the flowers, but found positivity for flavonoids and terpenoids/steroids (Alice et al., 1991). However, total phenolic compounds were detected in the extract analysis (as will be presented later), which agrees to the results found by Alice et al. (1991), regarding the flavonoids. Nunes et al. (2007) did not applied histochemical tests for phenolic compounds, saponins and tannins, so there is no report f the presence of these compounds on *S. australis* flowers.

In tangencial longitudinal view, axial parenchyma cells show a more elongated aspect when compared to the radial parenchyma cells, being the characteristics cited above for the conductor cells and fibers, also evidenced here (Figure 4).

Chemical study

The success of bioactive compounds extraction depends on many factors such as plant material, solvent, temperature, pressure and time (Azmir et al., 2013), in this study, some of this factors were evaluated in order to optimize the extraction process of the stem bark of *S. australis*. In Table 1 are presented the quantification results of rutin and total phenolic compounds.

The extraction method that presented the best results was Soxhlet, followed by reflux, ultrasound-assisted and maceration. This information

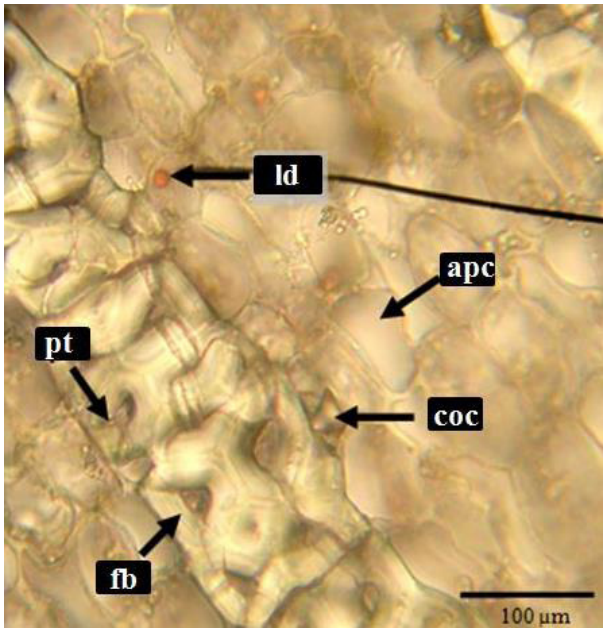

FIGURE 2. *Sambucus australis* Cham. & Schltdl. Phloem in transversal section. coc: calcium oxalate crystal; apc: axial parenchyma cell; fb: fiber; ld: lipid droplet; pt: pits. (Stained with Sudan IV).

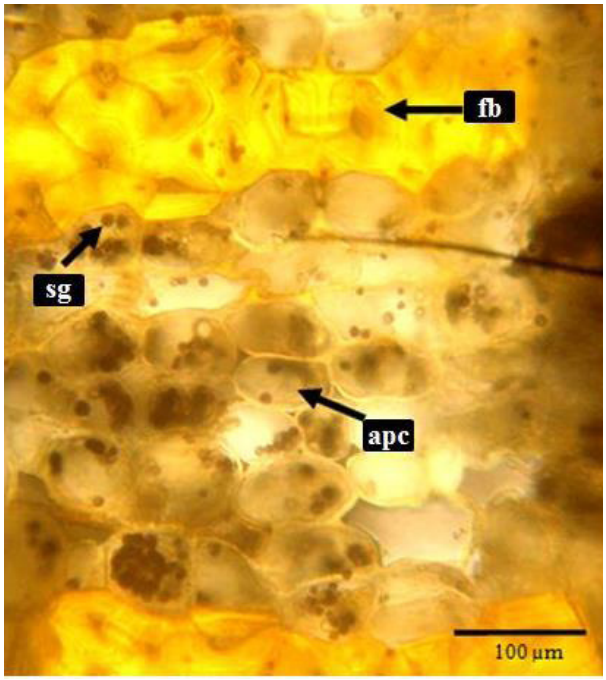

FIGURE 3. *Sambucus australis* Cham. & Schltdl. Phloem in transversal section. apc: axial parenchyma cell; fb: fiber; sg: strach grain. (Stained with lugol).

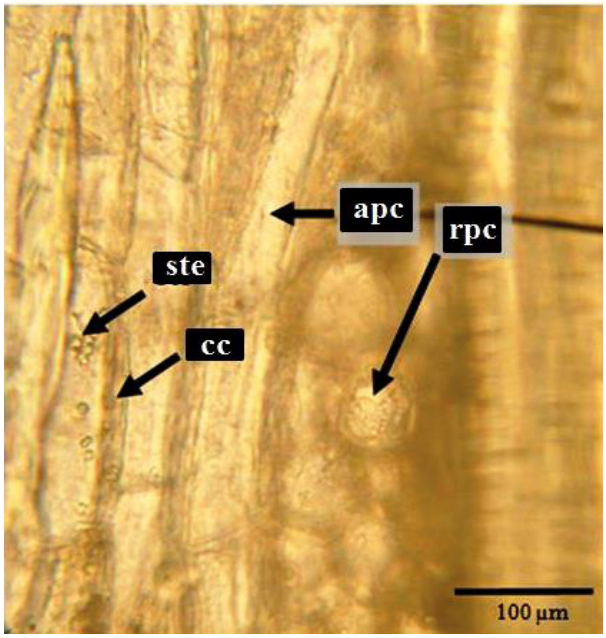

FIGURE 4. *Sambucus australis* Cham. & Schltdl. Phloem in transversal section. cc: companion cells; apc: axial parenchyma cell; rpc: radial parenchyma cell; ste: sieve tube element. (Fresh material, no dye).

TABLE 1. Results for different extraction methods.

Extraction Method	Time (minutes)	Rutin (mg/g)	Total Phenolic Compounds (mg GAE/g)
Hot Maceration	30	0.72±0.10^{b}	45.02±5.25^{bcd}
	60	0.63±0.05^{b}	49.81±6.01^{bcd}
Maceration	30	0.26±0.04^{c}	25.49±10.07^{d}
	60	0.27±0.01^{c}	26.16±12.47^{d}
Ultrasound-assisted	30	0.81±0.04^{b}	64.57±1.50^{ab}
	60	0.66±0.09^{b}	52.32±9.39^{bc}
Soxhlet	60	0.29±0.12^{c}	27.08±6.16^{cd}
	240	1.05±0.01^{a}	81.54±12.59^{a}
Reflux	60	0.70±0.11^{b}	44.82±10.56^{bcd}
	240	0.81±0.06^{b}	66.38±7.23^{ab}

Values that don't share a letter are statistically different ($p<0.05$).

is consistent with the consulted literature, in which the Soxhlet method presented better results than maceration (Scalia et al., 1999) and ultrasound-assisted extraction (Péres et al., 2006). These findings may be due the fact that in Soxhlet extraction, the sample remains continuously in contact with the extractor solvent, and also due the ability of the system to retain high temperatures. Furthermore, is a simple technique and has no need of filtration after the process. However, there are some disadvantages, as the large amount of solvent needed and the fact that the system provides no agitation (Carvalho et al., 2009; Luque de Castro & Priego-Capote, 2010).

Except for the Soxhlet method, the time of extraction was not a key factor in this study. On the contrary, the literature points the variable time as important for the quality of extractions, Lapornik et al. (2005) compared time extractions of 1, 12 and 24 hours, and demonstrated that longer time extractions can provide 6-7 times higher values of polyphenols. This supports the Soxhlet results, once this technique demands longer extractions, differently from maceration and ultrasound-assisted extraction. Also, Hamissa et al. (2012) found that the extraction time is important in the extraction of total phenolic compounds and total flavonoids.

Extraction temperature was found statistically relevant for the extraction of rutin and polyphenols. The comparison between maceration at 25°C and at 60°C provided similar results to the literature, which demonstrate that high temperatures improve the extraction of polyphenols (Leite, 2009; Hamissa et al., 2012).

It was possible to determine that the extraction solvent plays an important role in the process, once the solvent must extract the wanted compounds from the plant material. Methanol, ethanol and their combinations are commonly used for the extraction of phenolics from plant materials (Dai & Mumper, 2010). Table 2 present the quantification results of rutin and polyphenols for methanol and ethanol 80%.

In this study, the most polar solvent, ethanolic solution 80%, showed the best results for the analyzed compounds. This result is supported by Shi et al. (2005) and Chua (2013), who considered alcoholic mixtures the best choice for extraction of polyphenols. Also, ethanol is preferred because is a non-toxic solvent.

The presence of high quantities of phenolic compounds in the sample evaluated may support the traditional of this plant, once this class of chemical compounds is known to stimulate the proliferation of skin cells, helping wound-healing (Ratz-Łyko et al., 2015).

CONCLUSION

The results allowed demonstrating the importance of optimized extraction conditions and an appropriate solvent to the interest molecules. Since a four hours Soxhlet extraction, using ethanol 80% as solvent was considered the most efficient for rutin and polyphenols from the inner stem bark of *S. australis.* Also, the botanical analysis allowed determining the portion used as remedy as the phloem, as well as detected metabolites *in situ*.

TABLE 2. Results of rutin and phenolic compounds for different solvents.

Extraction Solvent	Rutin (mg/g)	Phenolic Compounds (mg GAE/g)
Methanol	1.05±0.01^{b}	81.55±12.60^{b}
Ethanol 80%	1.61±0.10^{a}	137.79±26.31^{a}

Values that don't share a letter are statistically different ($p<0.05$).

The macroscopical aspect of the internal part of the stem bark presents itself in a flat shape, with smooth texture and a pale yellow color. The phloem is composed by axial parenchyma cells and radial parenchyma cells, sometimes more elongated than the axial cells, sieve tube elements, companion cells and angle fibers. Through histochemical testing, the presence of alkaloids, starch grains, calcium oxalate crystals and lipid drops was detected. The species should be include in further phytochemical and pharmacological studies, since is widely used in Brazilian traditional medicine.

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