Revista Brasileira de Farmacognosia 27 (2017) 673–678

Brazilian Journal of Pharmacognosy
REVISTA BRASILEIRA DE FARMACOGNOSIA
www.elsevier.com/locate/bjp

Original Article

Leaf anatomy of *Protium ovatum* and its antiproliferative potential in cervical cells

Patrícia F. Rosalem, Thaís B. Picão, Flávia C. Rodrigues-Lisoni, Aline R. Martins*

Departamento de Biologia e Zootecnia, Universidade Estadual Paulista "Júlio de Mesquita Filho", Ilha Solteira, SP, Brazil

ARTICLE INFO

Article history:
Received 29 March 2017
Accepted 12 September 2017
Available online 6 October 2017

Keywords:
Morphology
Plant anatomy
Medicinal plants
Antiproliferative effect
SiHa

ABSTRACT

The aim of this study was to analyze the morphology and anatomy of the leaves of *Protium ovatum* Engl., Burseraceae, and verify the antiproliferative activity in cervical cells. For anatomical analysis, the leaf samples were fixed in formol, acetic acid, alcohol 70, dehydrated, included in hydroxyethyl methacrylate and sectioned at a thickness of 5–10 μm in rotative microtome. The samples were stained with toluidine blue and blades mounted with synthetic resin "Entellan". Histochemical tests and scanning electron microscopy were also performed. To investigate the antiproliferative effect we used the cells strain of human cervix carcinoma and normal keratinocytes. The anatomical analysis demonstrated that the leaf is hypostomatic and the epidermal cells walls were slightly undulate on both faces. The palisade parenchyma occupies most part of leaf mesophyll. The spongy parenchyma is organized into 3–4 layers of cells. Vascular bundles of smaller diameter and secretory cavities are distributed along the leaf mesophyll. The midrib region was formed by a single vascular bundle with xylem in the center surrounded by phloem. Secretory cavities are distributed along the phloem. The histochemical tests revealed the presence of lipids in the secretory cavities and phenolic compounds in almost cell of mesophyll. Scanning electron microscopy analysis showed the smooth leaf cuticle ornamentation with some striated areas. It was observed antiproliferative effect on human cervix carcinoma cell comparing with normal cells.

Introduction

The distribution of family Burseraceae is mostly pantropical and it occurs in tropical America and Africa. This family has seventeen genera and 500 species and its main genera are *Bursera*, *Commiphora*, *Protium* and *Canarium* (Judd et al., 2009). In Brazil, there occur seven genera and about sixty species, mostly native to the Amazon region (Souza and Lorenzi, 2005).

Burseraceae compose an important part of the diversity of wet and dry forests in many parts of the tropics, sometimes responsible for 10–14% of the trees in tropical forest of the lowlands with great variety of species (Daly et al., 2012).

The family is very rich in gums and resins and well known for the incense extracted from *Boswellia* Roxb. and myrrh extracted from *Commiphora* Jacq. (Lima and Pirani, 2005).

For Lorenzi (2008), the wood is suitable for construction, internal works, floors, carpentry, and joinery. The tree provides good shade and features ornamental qualities and can thus be used in urban and rural afforestation.

In addition to the economic importance of the wood, oil from species of the Burseraceae family is used in the cosmetic industry to produce perfumes and in traditional medicine (Rudiger et al., 2009). In the genera *Bursera* and *Protium*, anti-tumor and stimulant features in the central nervous system have been detected. For species *Bursera tokinensis* Guillaumin, a compound responsible for fighting oral cancer, colon cancer and prostate cancer has been identified (Camargo et al., 2010).

Protium Burm. f. is the most widely distributed genus in Brazil, receiving popular names of "almecegueira" or "pau-de-breu", which are names also used for other family members (Souza and Lorenzi, 2005). In São Paulo State, five species are found, namely *P. heptaphyllum* (Aubl.) Marchand., *P. kleinii* Cuatrec., *P. ovatum* Engl., *P. spruceanum* (Benth.) Engl., *P. widgrenii* Engl. (Lima and Pirani, 2005).

The trunk of species *Protium* and *Tetragastris*, for example, exudes a resin oil, known as "breu", used in industry to produce cosmetics and medicines (Souza and Lorenzi, 2005).

Protium ovatum is a shrub of 0.4–4 m high, with glabrous branches and few lenticels. Its leaves are oval, glabrous,

* Corresponding author.
E-mail: aline@bio.feis.unesp.br (A.R. Martins).

https://doi.org/10.1016/j.bjp.2017.09.001

composed, with 3–5 coriaceous leaflets. The species blooms from April to September when fruits can be observed. Reports indicate the presence of a thickened underground system that sprouts after a fire event (Lima and Pirani, 2005).

The species can be confused with *P. heptaphyllum*; however, some features differ such as the presence of lateral oval leaflets with truncated or corded base in *P. ovatum* (Lima and Pirani, 2005).

The purpose of this study was to analyze the morphology and anatomy of leaves of *P. ovatum*. We also evaluated the potential antiproliferative effect of neoplastic cells compounds on morphology and cell proliferation, observing how the phytotherapic features act and how these changes can participate in the tumor process.

Materials and methods

Plant material

The material (*Protium ovatum* Engl., Burseraceae) was collected in Fazenda de Ensino, Pesquisa e Extensão (FEPE – UNESP) located in Selvíria, MS. Leaves were sampled from three individuals of the same species, characterizing the work in triplicate.

Samples of plant material was identified and incorporated to the UNESP – Ilha Solteira Herbarium collection (ISA 10294).

Light microscopy

Foliar tissue samples were fixed in FAA 50 (Johansen, 1940) to avoid material loss due to natural degradation. After 48 h, the material was stored in 70% alcohol. In the completely expanded leaf, the middle region of the leaf was analyzed at the midrib and internervural areas. The samples were dehydrated in ethylic series, included in hydroxy-ethyl-methacrylate (Leica Historesin) and the blocks were cut at 5–10 μm thick.

The material was stained with toluidine blue 0.05% in phosphate buffer and citric acid with pH between 4.5 and 6.0 (Sakai, 1973). The slides were mounted with synthetic resin "Entellan".

Histochemical tests were performed in fresh material, hydrated, fixed, using hand-cut technique and/or included in historesin. For lipid detection, we used Sudan IV (Jensen, 1962); for starch, iodized zinc and chloride (Strasburger, 1913); for phenolics, ferric chloride (Johansen, 1940) and for pectic substances and mucilage, Ruthenium red (Johansen, 1940). To verify the natural aspect of the organ, we assembled the cuts of the material only in water, that is, without treatment and observed them under a light microscope.

Photomicrographs of materials prepared in blades were prepared in trinocular microscope of Photonic Bel brand camera Moticam 1000 of 1.3 M Pixel coupled.

Scanning electron microscopy (SEM)

Samples fixed on leaves of three individuals were dehydrated in ethylic series, dried to a critical point, fixed in aluminum bracket with carbon double-sided tape and metallized with a gold layer of 30–40 nm.

The analyses and digitalization of images were made by a scanning electron microscope Zeiss model LEO 435VP, operated at 20 kV with scales printed directly on the electron micrographs. This step was performed at the Center for Research Support in Electron Microscopy Applied to Agriculture (NAP/MEPA) of ESALQ-USP.

Preparation of aqueous extract and verification of antiproliferative activity

Leaves were collected from three individuals, which were dried in an oven at 60 °C for 48 h. Then, the dried leaves were ground in a knife mill. A tea was prepared and remained at rest for 24 h. Afterward, the tea was filtered and the liquid was frozen at 5 °C for 3 days. The material was freeze dried in a lyophilizer Líotop L 108, for 72 h. Three concentrations of water extract were evaluated (10, 100, and 1000 μg/ml) prepared from leaves of *P. ovatum*.

We used the cervical squamous cell carcinoma (SiHa, ATCC – HTB35) and Human normal immortalized keratinocytes (HaCaT, CLS – Cell Line Service 300493) cell lines.

The SiHa and HaCaT cell lines were cultivated in complete medium (MEM supplemented with 10% bovine serum, 10 mM non-essential amino acids, 100 mM sodium pyruvate and 1× antibiotic/antimycotic) and kept at 37 °C and 5% CO_2 atmosphere for 24 h, until they fixed to the substrate. Afterward, the medium was replaced every day until it became confluent. Cell growth and morphology were assessed daily under an inverted microscope and, when cell density was high, the material was submitted to trypsinization and subdivided into two replicas.

Four experiments were carried out with the cell lines SiHa and HaCaT a negative control experiment (culture medium), one with a concentration of 10 μg/ml, another with 100 μg/ml and another with 1000 μg/ml *P. ovatum*.

To analyze the cellular proliferation, in the SiHa and HaCaT cells, it was performed a growth curve, to counting the cultivated cells, they were seeded at the concentration of 5×10^4 in 500 μl of complete medium for 24 h. After this period, the medium was replaced by a serum free medium, with the purpose of maintaining the cells in the same cellular phase. After further 24 h this medium was replaced again with complete medium, added *P. ovatum* extract. The cells were analyzed in three different times (24, 48 and 72 h) and for each concentration (10, 100, and 1000 μg/ml), which was carried out in triplicate. The cellular morphology was evaluated with inverted microscopy Olympus CKX41.

The cells were harvested, stained with trypan blue and counted using the Countess Automated Cell Counter (Invitrogen) that separated dead and live cells. After this cell proliferation assay, we applied the statistical analysis of variance test where time and concentration were assessed (*T* test) to discover the one with greater statistically significant difference in relation to cellular growth, where $p < 0.5$ was considered significant (Tukey test).

Cytotoxicity and viability assay

The cells were subdivide into the experimental groups and handled according to the manufacture's protocol from the CellTiter 96® AQUeous One Solution Cell Proliferation Assay (Promega), which uses the tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt; MTS], and an electron coupling reagent [phenazine ethosulfate (PES)]. The analyses were made at the time of 24, 48 and 72 h and was evaluated by comparing the cellular viability across different concentrations, as well as the IC_{50} calculus (inhibitory concentration to 50% of the cells). The software used to perform the statistic were Graph Pad version 7.02, then, the statistic test used to do the comparison between the groups was the variance of analysis (ANOVA) and it was considered to be significant the probability value less than 0.05.

Results and discussion

Leaf anatomy

The anatomical analysis (Figs. 1 and 2) showed that the leaf is hypostomatic and that the walls of epidermal cells are slightly wavy on both sides. Sparse tector trichomes with few cells in length were

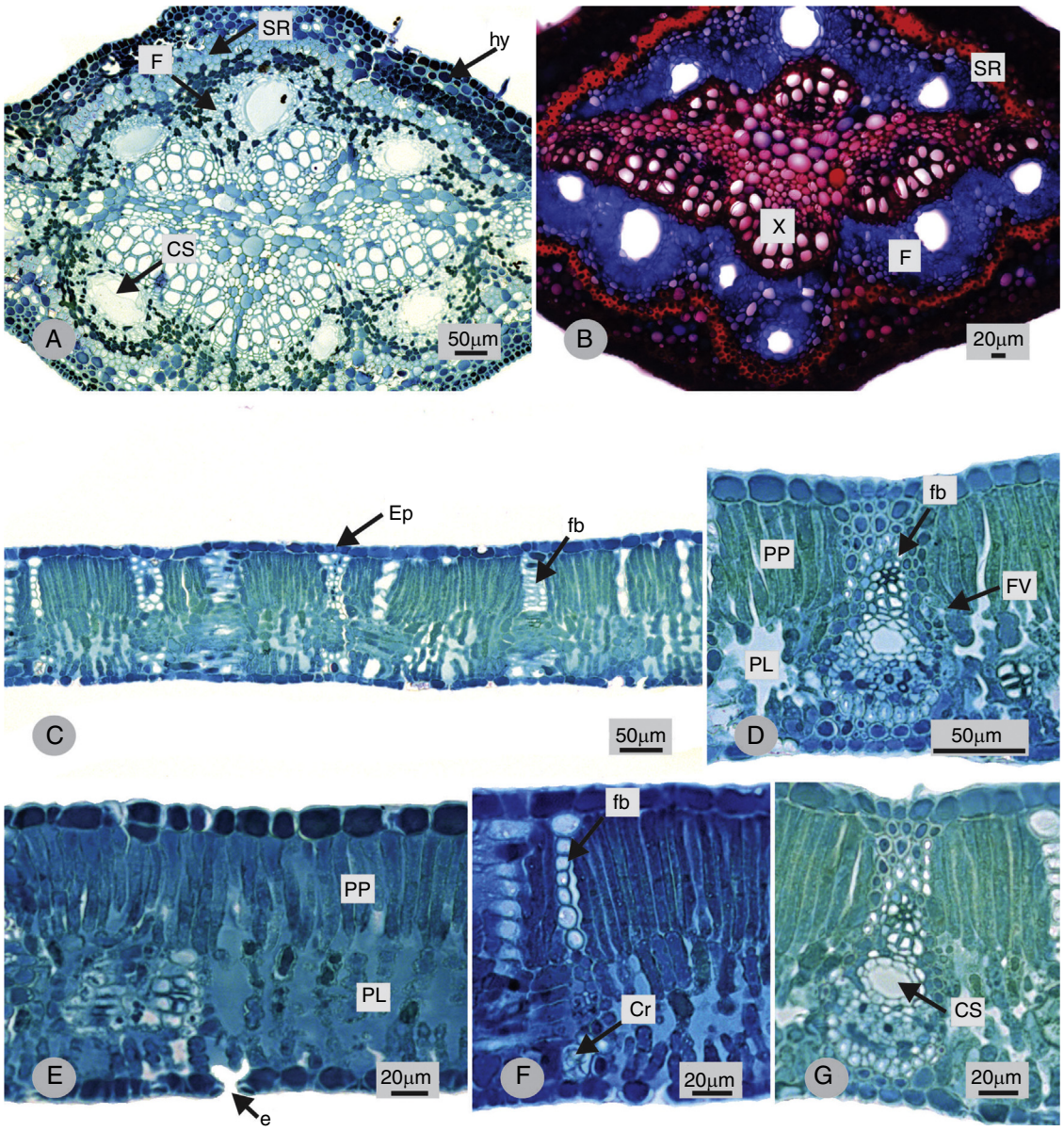

Fig. 1. Cross sections of *Protium ovatum* leaf. (A, B) Midrib region. (C–G) Internervural leaf area. Arrows in (A) and (G) indicate secretory cavities. Detail of (B) showing the xylem and phloem. (C) Detail of uniseriate epidermis. (D, E) Detail of the palisade parenchyma and spongy parenchyma. (F) Idioblast detail containing crystals. CS, secretory cell; Cr, idioblast containing crystal; e, stomata; EP, epidermis; F, phloem; fb, fibrovascular bundles; FV, vascular bundle; hy, hypodermis; PP, palisade parenchyma; PL, spongy parenchyma; Sr, sclerenchyma ring; X, xylem.

found in the adaxial epidermis (Figs. 1A, 2F, 3B). In cross section, the epidermis is unisseriate as well as the palisade parenchyma (Fig. 1C and D). The palisade parenchyma occupies most leaf mesophyll. The spongy parenchyma is organized into 3–4 layers of cells (Fig. 1E). Vascular bundles of smaller diameter and secretory cavities are distributed along the leaf mesophyll.

The midrib present unisseriate epidermis and hypodermis, 4–5 parenchyma layers of cells and a single vascular bundle containing xylem more centered surrounded by phloem (Fig. 1A). Secretory cavities (approximately 8) are distributed along the phloem (Fig. 1A and B). Such vascular tissues are surrounded by a sclerenchyma ring of 2–3 layers, demonstrated by double staining with safranin and astra blue (Fig. 1B). The histochemical analyses also revealed the existence of phenolic compounds in parenchyma and phloem cell of midrib (Fig. 2A).

Compared with leaves of *Protium kleinii* described by Boeger et al. (1997), there are some similarities, such as the palisade parenchyma consisting of a single layer of elongated cells, and reasonable differences, such as the layer of cells in the spongy parenchyma, which in *P. kleinii*, consists of five layers of cells and in *P. ovatum*, 3–4 layers.

Bieras and Sajo (2009) reported that the species *P. heptaphyllum* has, similar to *P. ovatum*, secretory cavities by phloem elements, in addition to the hypostomatic leaf and the presence of phenolic substances in the parenchyma cells of the mesophyll. However, unlike *P. ovatum*, *P. heptaphyllum*, as described by the authors above, *P. heptaphyllum* does not present trichomes and its palisade parenchyma is spongy and loosely organized.

Histochemical tests (Fig. 2) are important because they are greatly help morphoanatomical identification and characterization of plants, revealing classes of chemical compounds of medicinal importance (Martins and Appezzato-Da-Gloria, 2006).

The histochemical analyses performed on the leaves of *P. ovatum* revealed the existence of phenolic compounds and starch grains in

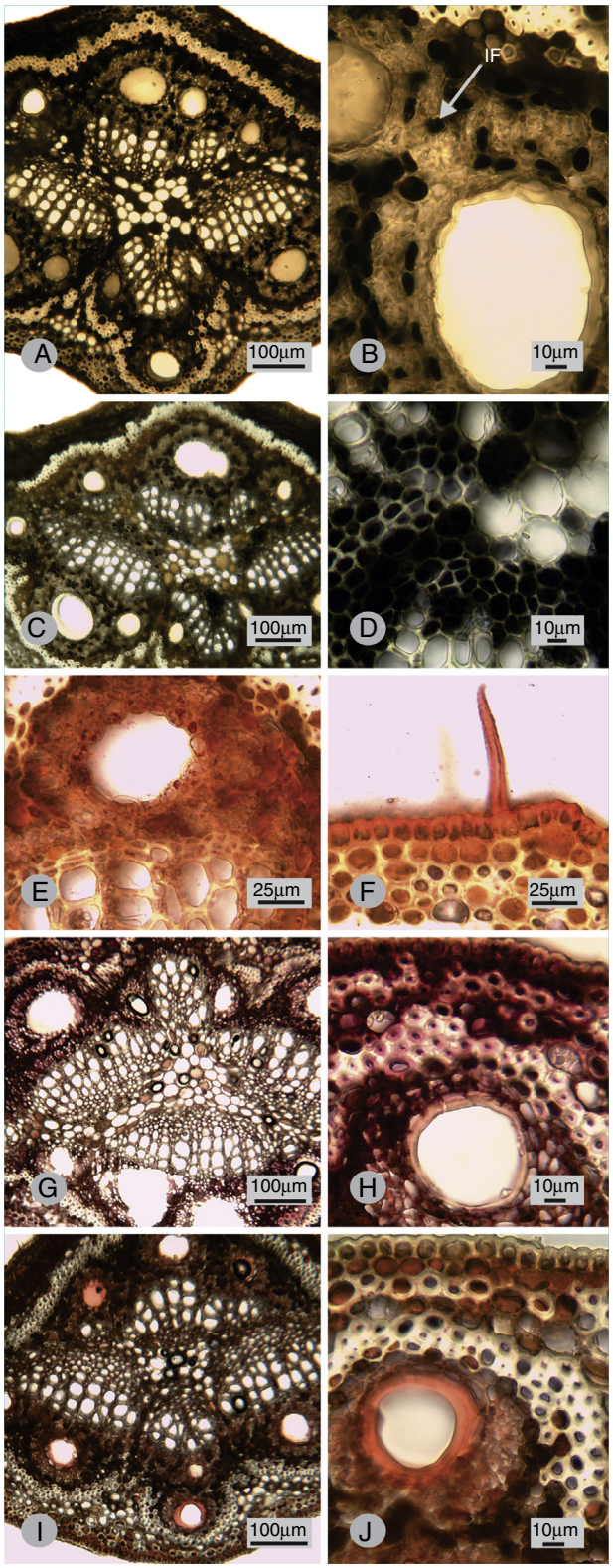

Fig. 2. Cross sections of *Protium ovatum* leaf. Histochemical tests with positive reactions to ferric chloride (A, B), iodinated zinc chloride (C, D), Sudan IV and Sudan black (E, F), ruthenium red (G, H) and xylidine ponceau (I, J). IF, phenolic idioblasts.

leaf mesophyll (both in the palisade and spongy parenchyma), in the epidermis and vascular tissues. Lipids were found mainly in the leaf cuticle and secretory cavities, which also had a positive reaction to protein substances, indicating that the type of secretion from the cavities must be mixed. Pectic substances react with less intensity around the secretory cavities and in the phloem (vascular tissue).

According to Silva et al. (2009), *Protium* species present an expressive number of terpenes in their phytochemestry constituints. This terpenes are divided in monoterpens (α-pinene, β-pinene, *p*-cymene), sesquiterpenes (α-cubebene, β-caryophyllene) and triterpenes (α-amyrenone, α-amyrin and β-amyrin). The *p*-cymene is the most usual monoterpene, found in great concentration in the oleoresin of *Protium grandifolium* (46.5%). Moreover, α-amyrin and β-amyrin (triterpenes) are also found in extracts of the Burseraceae family. These mono and triterpene compounds are widely used by pharmacological industry due their antimicrobial, anti-inflammatory, antiviral and antidiuretic activities (Bandeira et al., 2007).

Scanning electron microscopy (SEM)

Observing images in the scanning electron microscope (SEM) and comparing them to descriptions of Metcalfe and Chalk (1979), we observed that the cuticle of *P. ovatum* in some points is smooth on the adaxial leaf surface (Fig. 3A, B and E).

On the abaxial face, the cuticle is smooth with grooved areas (Fig. 3C, D and F). According to Metcalfe and Chalk (1979), the cuticle of *Picrasma quassioides* Benn., Simaroubeceae, is also smooth in the SEM analysis and it is grooved in species *Viburnum vernicosum* Gibbs. (Adoxaceae). These species are classification models in SEM analyses of leaf cuticles.

Small sparse tector trichomes were also observed on the adaxial surface and stomata at large quantities on the surface abaxial (Fig. 3B). The cuticle on both sides is very thick and it is almost impossible to verify the cell boundary. On both sides, it was also observed various types of hyphae of unknown fungi.

Analysis of cell morphology, proliferation and citotoxicity of SiHa and HaCaT cells

SiHa cells morphology is characterized by a monolayer of nucleated cells in the control group without changes after treatment with *P. ovatum*. The same was observed in HaCaT cells.

Analyzing cell proliferation, it was possible to observe the cell growth inhibitor effect caused by the action of *P. ovatum* for the control of cells. It seems that, when used, the compounds act in tumorigenic cells causing cell death, hindering growth of these cells (Fig. 4). The statistical analysis indicated a significant decrease for SiHa cells. The concentration of 1000 μg/ml at 72 h showed greater efficiency in reducing the proliferation of SiHa cells. However, another drug, doxorubicin HCL, tested at concentration 10 μM, also presented cytotoxic effect on SiHa cell line with less than 50% viability (Escobar et al., 2009).

The cytotoxicity analysis of SiHa cells showed that the action of *P. ovatum* was not cytotoxic.

Aqueous extracts of plants are of great importance, as they can act less aggressively and, at times, lessen the viability of cancer cells, for example, *Erythroxylum suberosum* A. St.-Hil., Erythroxylaceae. The aqueous extract applied in combination to radiation therapy presented 63% of cytotoxicity in cancer cells and oropharynx (Macedo et al., 2016). Knowing the antiproliferative potential of *P. ovatum* water extract, it was necessary to test the phytotherapic condition in normal keratinocytes cells (HaCaT). Thus, it was possible to observe that the compound, when used in normal cells, also causes cell death. When the compound was used for 24 and 72 h at low concentrations of 10 and 100 μg/ml, it caused moderate cell death. When it was used at very high concentrations, such as 1000 μg/ml, it killed a large number of normal cells. Therefore, it is preferable to use it at low concentrations, but for long periods, as shown in Fig. 4A and B.

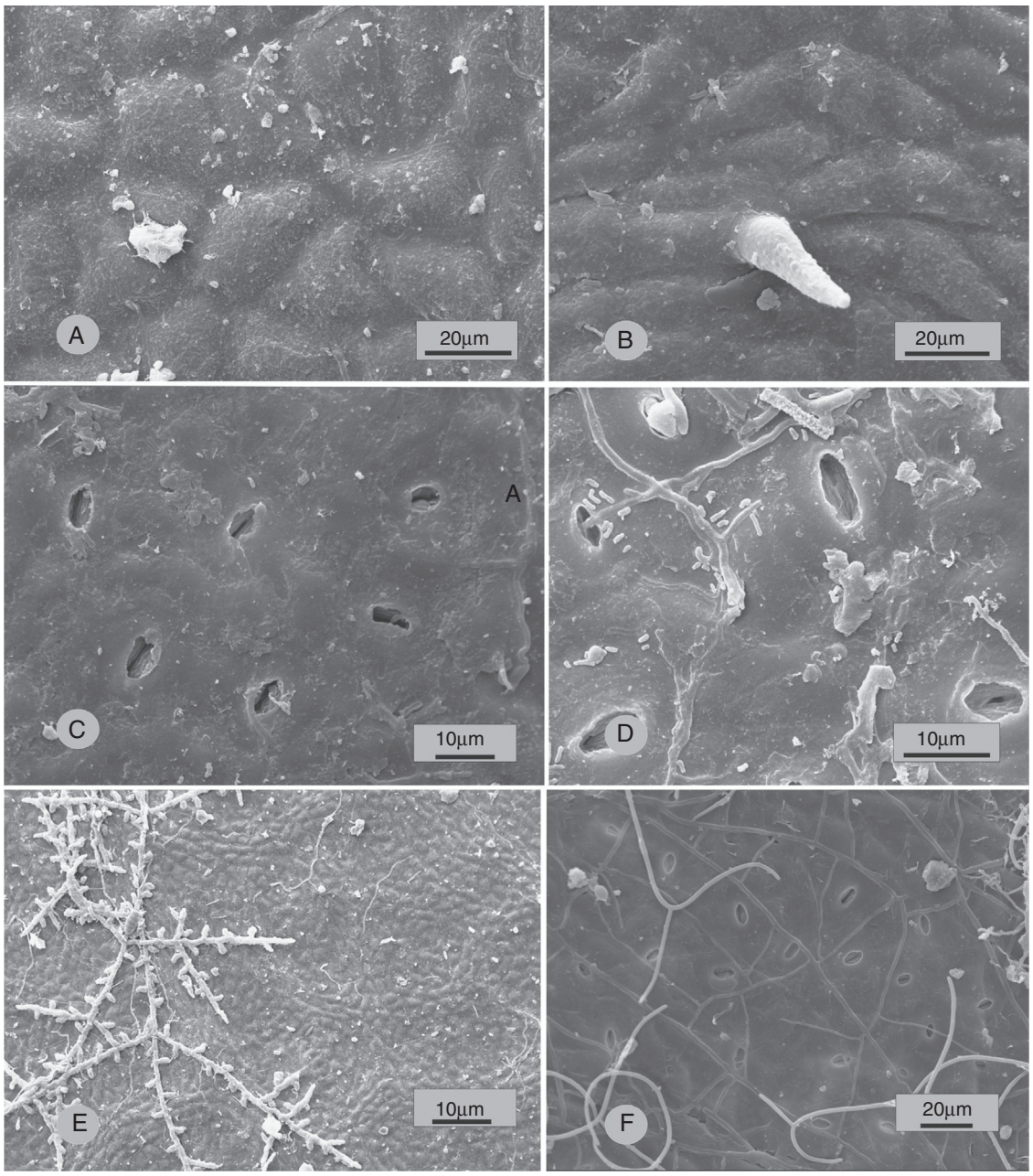

Fig. 3. Electron micrographs of *Protium ovatum* leaf surfaces. (A, B, E) Adaxial leaf surface. (C, D, F) Abaxial leaf surface. (B) Detail of trichomes structure. (D) Stomata structure. (E, F) Detail showing fungi species on both leaf surfaces.

It was concluded that the morphoanatomical analyses were able to provide data on the structure found in leaves of *P. ovatum*, such as hypostomatic leaf and walls of epidermal cells that were slightly wavy on both sides, unisseriate epidermis, as well as the palisade parenchyma. Secretory cavities (eight cavities) are distributed along the leaf mesophyll, predominantly located in the midrib in the phloem region. Histochemical tests revealed the mixed composition (lipids and proteins) in the secretory cavities and significant presence of phenolics in parenchyma cells of the mesophyll. SEM analyses indicated thick cuticle whose ornamentation was classified as smooth in some parts and slightly grooved in other parts.

Compared to the treatment with the *P. ovatum* leaf extract, changes were not observed in the morphology of SiHa cells, but there has been a reduction in cell growth, possibly due to the antiproliferative effect of the extract on cervical carcinoma cell cultures.

Ethical disclosures

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this study.

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Authors' contributions

PFR and ARM contributed in collecting commercial sample, running the laboratory work, analysis the data and writing the section

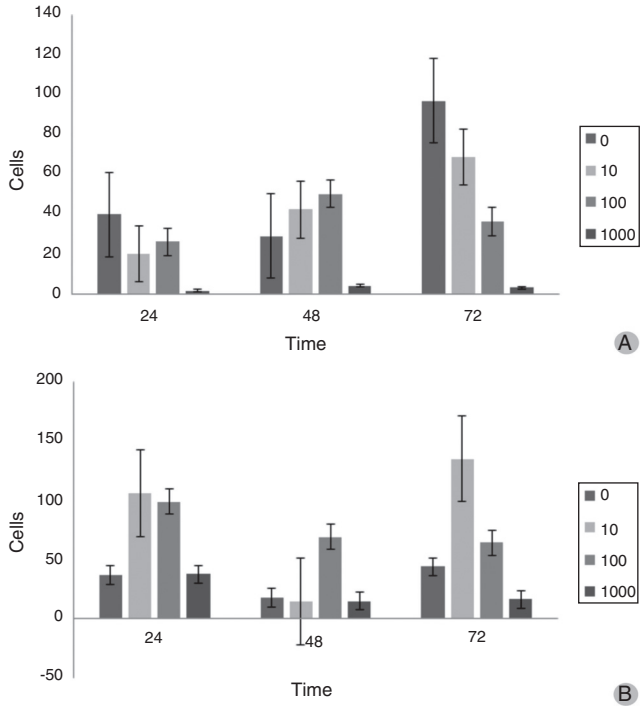

Fig. 4. *P. ovatum* treatment effect in SiHa (A) and HaCaT (B) cell proliferation. The cells were cultured in complete MEM medium and treated with *P. ovatum* [10,100 and 1000 μg/ml] for 24, 48 and 72 h. Graphs with x = time (h) and y = number of cells $\times 10^4$.

related to the information about *Protium* leaf anatomy. PFR TBP and FCRL contributed in citotoxicity of SiHa and HaCaT cells. PFR and ARM designed the study, contributed to critical reading and final editing of the manuscript. All the authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

The authors would like to thank Prof. Dr. Alan R. Panosso and Wellington Y. Hirai for the statistical analysis; Prof. Dr. José Rubens Pirani for the species identification; Prof. Dr. Eloiza Helena Tajara da Silva (Faculty of Medicine of São José do Rio Preto/FAMERP – SP) for cervical tumor cell line (SiHa) and Prof. Dr. Andréia Machado Leopoldino (USP/FMRP, SP) for human normal keratinocytes (HaCaT). Financial support from FAPESP (2017/021000-3).

References

Bandeira, P.N., Lemos, T.L.G., Costa, S.M.O., Santos, H.S., 2007. Obtenção de derivados da mistura triterpenoídica α- e β-amirina. Rev. Bras. Farmacogn. 17, 204–208.
Bieras, A.C., Sajo, M.G., 2009. Leaf structure of the cerrado (Brazilian savanna) woody plants. Trees 23, 451–471.
Boeger, M.R.T., Alves de Brito, C.J.F., Negrelle, R.R.B., 1997. Relação entre características morfo-anatomicas foliares e esclerofilia em oito espécies arbóreas de um trecho de Floresta Pluvial Atlântica. Arq. Biol. Tecnol. 40, 493–503.
Camargo, J.E.R., Afonso, A.N.T., Rojas-Rozo, R.A., Castro, C., Murcia, T.R., 2010. Efeitos citotoxicos in vitro de extratos y frações de *Bursera tomentosa* (Jacq.) Triana and Planch., Burseraceae, frente a linhas celulares tumorais humanos. Rev. Bras. Farmacogn. 20, 588–593.
Daly, D.C.B., Fine, P.V.A., Martínez-Habibe, M.C., 2012. Burseraceae: a model for studying the Amazon Flora. Rodriguésia 63, 21–30.
Escobar, L.M., Alfonso, P.A.R., Aristizábal, F.A.G., 2009. Valoración de dos métodos de tinción en ensayos de citotoxicidad sobre líneas celulares tumorales. Rev. Colombiana Biotecnol. 11, 49–56.
Jensen, W.A., 1962. Botanical Histochemistry: Principle and Practice. W.H. Freeman and Company, San Francisco.
Johansen, D.A., 1940. Plant Microtechnique. McGraw-Hill, New York.
Judd, W.S., Campbell, C.S., Kellogg, E.A., Stevens, P.F., Donoghue, M.J., 2009. Sistemática Vegetal um Enfoque Filogenético. Artmed, Porto Alegre, pp. 437–438.
Lima, L.R., Pirani, J.R., 2005. Burseraceae. In: Wanderley, M.G.L., Shepherd, G.J., Melhem, T.S., Martins, S.E., Kirizawa, M., Giulietti, A.M. (Eds.), Flora Fanerogâmica do Estado de São Paulo. FAPESP/RiMa, São Paulo, pp. 163–168.
Lorenzi, H., 2008. Árvores Brasileiras. Instituto Plantarum de Estudos da Flora LTDA, Nova Odessa, pp. 89.
Macedo, T.B.C., Elias, S.T., Torres, H.M., Yamamoto-Silva, F.P., Silveira, D., Magalhães, P.O., Porto, A.L., Guerra, E.N.S., Silva, M.A.G., 2016. Cytotoxic effect of *Erythroxylum suberosum* combined with radiotherapy in head and neck cancer cell lines. Braz. Dental J. 27, 108–112.
Martins, A.R., Appezzato-Da-Gloria, B., 2006. Morfoanatomia dos órgãos vegetativos de *Smilax polyantha* Griseb. (Smilacaceae). Rev. Bras. Bot. 29, 555–567.
Metcalfe, C.R., Chalk, L., 1979. Anatomy of the Dicotyledons: Volume I: Systematic Anatomy of the Leaf and Stem. Oxford Science Publications, New York.
Rudiger, A.L., Silva, C.C., Veiga Junior, V.F., 2009. EDXRF analysis of Amazonian Burseraceae oleoresins. J. Braz. Chem. Soc. 20, 1077–1081.
Sakai, W.S., 1973. Simple method for differential stainning of paraffin embedded plant material using toluidine blue. Stain Technol. 43, 247–249.
Silva, J.R., de, A., Zoghbi, M., das, G.B., Pinto, A., da, C., Godoy, R.L.O., Amaral, A.C.F., 2009. Analysis of the hexane extracts from seven oleoresins of *Protium* species. J. Essent. Oil Res. 21, 305–308.
Souza, V.C., Lorenzi, H., 2005. Botânica Sistemática: Guia Ilustrado Para Identificação das Famílias de Angiospermas da Flora Brasileira, Baseado em APG II. Instituto Plantarum de Estudos da Flora Ltda, Nova Odessa, pp. 435–436.
Strasburger, E., 1913. Handbook of Practical Botany. George Allen and Company Ltda, London.