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Original Article

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

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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,

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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

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