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Anatomical and chemical characteristics of leaves and branches of *Juniperus deppeana* var. *deppeana* (Cupressaceae): A potential source of raw materials for the perfume and sweet candies industries

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ABSTRACT

In North America, the genus *Juniperus* has a high commercial value because of the natural products derived from its wood, bark and leaves. In Mexico, the species of this genus are commonly known as junípero, sabino, enebro and táscate and they could represent potential sources of valuable products. To the best of our knowledge, this is the first report dealing with the anatomy and the phytochemistry of the endemic species *Juniperus deppeana* var. *deppeana*. From the first viewpoint, the resin canals are very large and they occupy one third of leaf volume. In the leaves, they are round, large and turgent, whereas in the branches, they merge and form large, elliptical cavities. The origin of resin ducts in branches is either the cortex or the phloem. They never form in the xylem. The phytochemical analysis of the crude extracts and essential oil of the leaves and branches showed that *J. deppeana* is a rich source of important polyphenolics and essences and might have the potential to be used in formulations containing this plant with applications in different areas, such as cosmetics, food and health care industries.

1. Introduction

The *Juniperus* genus is known in Mexico with the common names of junípero, sabino, enebro and táscate; the latter is also used in the northern states of the country for the genus *Cupressus* (Martínez, 1979). The species constitute the main arboreal component of arid and semi-arid zones in the northern hemisphere (Mao et al., 2010). *Juniperus deppeana* is listed in the Red book of the IUCN as “least threatened”, meaning that "*…there appears to be no serious concern about a threat of extinction for this taxon, as it is said to increase in grasslands especially in the US*." (Farjon, 2013). However, in Mexico some populations are growing in strongly disturbed areas, subjected to cattle grazing and fires. *Juniperus deppeana* var. *deppeana* (sabino) is a tree or shrub that grows from 3 m to 20 m tall, native to North America and distributed from Arizona and New Mexico in South West USA to Veracruz, Mexico (Zanoni, 1982). It belongs to the *Sabina* section, which includes 56 species distributed in South West North America, Asia, the Mediterranean region, Africa and the Canary Islands (Mao et al., 2010).

The genus *Juniperus* has a high commercial value because of the natural products derived from its wood, bark and leaves. In North America, the wood of *J. virginiana* is used to make pencils and the berries of *J. communis* are distilled to produce gin. Also, it is used for firewood, fences, charcoal, rural constructions, furniture, poles, crossties and as forage. In medicinal uses, it is employed in gynecological illnesses, as component of antibiotics; it is recommended for colds, rheumatism, neuralgia, dandruff, blood pressure control, in an illness known colloquially as ‘bad air’ and for ‘soul cleansing’ purposes (Biblioteca Digital de la Medicina Tradicional Mexicana, http://www.medicinatradicionalmexicana.unam.mx/index.php and Fonseca, 2006). So far, there is no technical information available about its

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phytochemistry that the industry can use. Preliminary studies performed by Clark et al. (1990) showed that the methanolic leaves and stem bark extracts and the hexanic heartwood extract of *J. deppeana* exert an antibiotic activity on the *Bacillus subtilis* and *Staphylacoccus aureus* bacteria. Also, the hexanic extract of leaves exerts an action on *Pseudomona aeruginosa*, *Escherichia coli* and *Mycobacterium smegmatis*.

In this context, it is well known that such medicinal properties in some plants including *Juniperus* species are due to the presence of several bioactive phytochemicals also known as secondary metabolites. One group of compounds of great interest for the scientific and biotechnological communities are the polyphenolics, which are one of the most common and widespread groups of substances in nature. In plants, phenolic compounds are involved in several processes such as pigmentation, growth, reproduction, resistance to pathogens and many other functions. Polyphenols have resulted of great interest because they have been described as potent antioxidants as well as antimutagenic, antitumoral, antiatherogenic and cardioprotective agents, *inter alia* (Balasundram et al., 2006; Tai et al., 2011). Thus the traditional uses of *J. deppeana* previously described, could be related in part due to the presence of bioactive secondary metabolites including phenolics among others. So, the main goal of this study was to describe the anatomical and chemical characteristic of leaves and branches of *Juniperus deppeana* var. deppeana (Cupressaceae): as a potential source of raw material for several industries.

2. Materials and methods

2.1. Plant material

Leaves and branches of *Juniperus deppeana* (Cupressaceae) were collected on June 11, 2016 in Tezontepec, 1 Km from the Cantona highway toll, Tepeyahualco municipality, Puebla at Latitude 19°30′54.57" N; Longitude −97°30′2.86" W., elevation 2 420 m.a.s.l.

Voucher samples (C. Madero) were deposited in the Xalapa, Veracruz, Mexico Herbarium (XAL) of the Instituto de Ecología, A. C. The material was dried in an oven (Binder ED115, Germany) at 50 °C during 48 h. Dried plant materials were pulverized using a blades-mill (Pulverisette 15, Fristch, Germany). Then, ground materials were stored in air-free bags at −20 °C until use.

2.2. Leaf anatomy

2.2.1. Sampling – Branches and leaves of three individuals of *J. deppeana* were fixed in a series of formaldehyde, glacial acetic acid, 95% ethanol and water (FAA) solutions, for at least 24 h (Ruzin, 1999). After washing them in tap water, the samples were dehydrated in a graded series of ethanol (15%, 30%, 50%), and passed through a graded series of 95% ethanol, absolute ethanol, tertiary butylic alcohol (TBA) and water, until pure TBA was reached (see Table 1). For paraffin embedding and sectioning, we followed the classical procedure described by Ruzin (1999).

2.2.2. Staining – After removing all the paraffin from the tissues in several changes of xylenes, tissues were re-hydrated in a series of decreasing concentrations of ethanol, up to pure water. Then, sections were stained in a combination of 1% safranin and 0.1% Astra Blue, added with a few drops of glacial acetic acid (Ruzin, 1999). After washing the excess of dye, tissues were dehydrated in a graded series of ethanol (from 15% to 95%). Then, tissues were passed through three changes of absolute ethanol. Ethanol was then gradually replaced with xylenes as described by Ruzin (1999). Finally, tissues were mounted in resin and covered with a glass coverslip. Observations were made under an Eclipse 600 Nikon microscope, in bright field, using a neutral filter and plan achromatic objectives of 10 and 20 X. Pictures were taken with a Cannon EOS Rebel T3i digital camera.

Table 1
TBA Series (Taken from: http://virtualplant.ru.ac.za/Main/FACTFILE/embedding_procedures.htm).

TBA	95% ethyl alcohol	Absolute ethyl alcohol	TBA	Water	Paraffin oil
1	50	0	10	40	0
2	50	0	25	30	0
3	50	0	35	15	0
4	50	0	50	0	0
5	–	25	75	0	0
6	–	–	50	0	50
7	–	–	0	0	100

(volumes per 100 mL).

2.3. Crude extracts and essential oil preparation

The crude extracts of leaves and branches were prepared separately by triplicate using an accelerated solvent extraction system (ASE 350, Thermo Scientific, USA) according to a previous protocol for targeting the extraction of medium polar and polar compounds such as phenolics and discarding fats and oils which were not targeted in the further phytochemical analysis (Luthria and Mukhopadhyay, 2006; Mukhopadhyay et al., 2006). Briefly, 3.0 g of dry material was dispersed in 1.0 g of diatomaceous earth and placed in a 34 mL cell. The cell was filled up with MeOH (HPLC grade) up to a pressure of 1500 psi and heated at 60 °C during 5 min. Then, cell was washed off with 30% of cell volume. Aliquots of 1.0 mL of each sample were placed in 1.5 mL centrifuged tubes and formic acid (MS grade, Sigma-Aldrich, St. Louis, USA) was added to a final concentration of 0.1%. The samples were filtered and placed in 1.5 mL UPLC vials for further LC–MS analysis.

The essential oils from leaves and branches were prepared separately using a steam distillation apparatus (González et al., 2011). The dried material (20 g) was taken into a 1.0 L round bottomed flask and 500 mL of distilled water was added to it and distilled for about 3 h at 100 °C. After cooling, the distillation product was transferred into a separatory 110 funnel and partitioned with 500 mL of dichloromethane (HPLC grade) three times. The organic layers were combined, filtered over anhydrous sodium sulphate and solvent was evaporated by rotary evaporation (Büchi RII, Switzerland). The obtained crude oil was stored at 4 °C until GC–MS analysis.

2.4. Phytochemical analysis

2.4.1. UPLC-ESI–MS–MS analysis

The identification and quantitation of individual phenolic compounds were established by Ultra High Performance Liquid Chromatography (Agilent 1290 series) and dynamic multiple reaction monitoring (dMRM) following the validated protocol conditions (Durand-Hulak et al., 2015). The analytical settings and chromatographic conditions can be consulted in detail in Juárez-Trujillo et al. (2017). dMRM data were obtained on an Agilent 6460 Triple quadrupole (QqQ) mass spectrometer. Compounds identity was confirmed by co-elution with authentic standards under the same analytical conditions above described and using voltage ionization conditions for each compound described in Table S1 (Supporting information). For quantitation of each phenolic compound, a calibration curve in a concentration range of 0.3–30 M was prepared (r^2 values ≥ 0.99 were considered for the linearity range) and quantities were established by using Mass Hunter Workstation Software Version B.06.00 (Agilent Technologies). The results were expressed as the mean of μg/g of sample (dry weight) ± standard deviation (n = 3).

2.4.2. GC–MS analysis

Essential oils were analyzed using a Gas Chromatograph Mass Spectrometer (GCMS-QP2010 Ultra) equipped with a ZB-5MSi column (30 m × 0.25 mm ID × 0.25 μm) (Adams, 2007). Electron impact (70 eV) spectra were obtained. Helium was the carrier gas (0.8 cm/sec,