Radical scavenging, antioxidant, and cytotoxic activities of the methanolic extracts from different organs of *Ternstroemia pringlei*

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Abstract: *Ternstroemia pringlei* (Rose) Standl. (Theaceae) is widely used in Mexican traditional medicine to treat a diverse array of illnesses including rheumatoid pains, and is listed as one of the most consumed medicinal plants in the country. We selected *T. pringlei* given the strong relationship between oxidative stress and arthritis pathology, and investigated antioxidant potential of leaf, petal, fruit and seed methanolic extracts. Our method included assessing the *in vitro* free radical scavenger activity using the 2,2´-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) test, as well as the *in vivo* antioxidant action in the H_2O_2 protection model with *Saccharomyces cerevisiae*. Leaves and fruits afforded the most active extract in the ABTS assay, with antiradical activity of IC_{50}=33.91 and 38.09µg/mL, respectively; while fruit extracts at 250µg/mL proved the most protective action against H_2O_2 oxidative stress. All extracts were non-cytotoxic against HF-6 (colon), PC-3 (prostate), MCF-7 (breast), SiHa (cervical) cancer cell lines and also toward the HFS-30 fibroblast normal skin cell line (IC_{50}>20µg/mL). Leaf methanolic extracts afforded ternstroside B, a known phenylethanoid glycoside with a strong free radical scavenging action. The presence of this kind of metabolites opens new research perspectives for the plant.

Keywords: *Ternstroemia pringlei*; antioxidant; antiradical; phenylethanoid glycoside; ternstroside B.

INTRODUCTION

Ternstroemia pringlei, commonly known as "tila flower", is one of the 12 species of the genus *Ternstroemia* found in Mexico. Since powerful antiradical phenylethanoid glycosides as ternstrosides A-F have been isolated from species of *Ternstroemia* (Jo *et al*., 2006), interest to search for these kind of compounds in members of the genus has been aroused. Infusions prepared from flowers and fruits of this species have been used since very ancient times to treat different conditions such as nerve disorders, insomnia, cough and rheumatic pain (Huerta 1997), *e.g*. in the state of Michoacán *T. pringlei* has been documented to alleviate rheumatic conditions (Atlas de las Plantas de la Medicina Tradicional Mexicana 2015). It is also well documented that the pathophysiology of arthritis involves the production of Reactive Oxygen Species (ROS) (Comar *et al*., 2013).

In the context of the present study, the strong antioxidant action reported to the phenylethanoid glycosides (Marino *et al*., 2012; Kondeva-Burdina *et al*., 2013), especially concerning the action described for the ternstrosides found in *T. japonica* (Jo *et al*., 2006), encouraged us to perform a pharmacological investigation of the antioxidant, and radical scavenger of the methanolic extracts from four organs of *T. pringlei*. We also performed a phytochemical study to investigate the presence of phenylethanoid glycosides in the plant extracts. Since this plant is highly consumed in Mexico, the cytotoxic activities of the methanolic extracts were also investigated.

In *T. pringlei,* we have previously documented the sedative action of jacaranone, a quinone compound, isolated from the fruits methanolic extract by means of a bioassay-guided study (Lozada-Lechuga *et al.*, 2010).

MATERIALS AND METHODS

Plant material

Leaves, petals, fruits without seeds, and seeds of *T. pringlei* were collected in 2013, at the locality of Huitzilac, Morelos State, Mexico, at coordinates 19°1´ 42´´ N, and 99° 13´ 88´´ W. A voucher specimen (No. 26349) was authenticated by Juan Carlos Juarez and deposited at the Autonomous University of Morelos State Herbarium (HUMO).

Extracts preparation for the assays

The extracts of leaf, petal, fruit with calyx and seed were prepared separately. Petals were separated from the blossom flowers, and seeds were removed from ripened

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fruits for analysis. Plant material was dried at room temperature and grounded to a fine powder by using an electric mill. Subsequently, 10g of each material was subjected to sonication in 50mL methanol for 20min and then filtered in a Whatman N°2 filter paper. Each sample was processed to complete a series of three consecutive extractions. All three extracts were combined and evaporated in a rotavapor at 40°C until dryness, in order to obtain a crude methanolic extract for pharmacological assays.

In vitro antiradical ABTS assay

The ABTS assay was adapted from Re *et al*., 1999. The assay was performed in 96-well microplates where 20µL of the sample (extract) was added to 230µL of the ethanolic ABTS•+ solution previously adjusted to an absorbance of 0.70 (±0.1) at 754nm. The absorbance value was read at 30°C for a period of 1, 4 and 6min after mixing. For the negative and positive controls 20µL of methanol (vehicle); And 20µL of trolox and *Camellia sinensis* solutions (positive controls) were added to 230 µL of ABTS•+ solution.

Autographic assay on TLC using ABTS•+ solution

All methanolic crude extracts of *T. pringlei* were applied at the same amount of 0.6mg extract on the thin layer chromatography (TLC). For the control, quercetin 2µL of the stock solution of 4mg/1500µL methanol was used. The TLC was developed using a mobile phase composed with chloroform: methanol (4:1) and the plates were observed under short ultraviolet light and revealed using vainillin-H_2SO_4 (1%, v/v) and heating at 100°C. For the autographic assay the ABTS•+ reagent was sprayed over the TLC after the chromatographic development.

In vivo antioxidant assay

This test was performed using the yeast model reported by Golla and Bhimathati (2014). The wild-type yeast strain of *Saccharomyces cerevisiae* was donated by Dr. Omar Ayala Homero Pantoja from the Institute of Biotechnology at the National Autonomous University of Mexico (UNAM), Mexico. The yeast was grown in liquid yeast peptone dextrose (YPD) medium at 28°C in agitation at 200rpm. Cells were collected at the exponential growth phase (OD_{600} of 1.0-1.3/mL), corresponding to 1 x10^8 cells/mL, which were placed in a 24-wells plates with the extracts at 50,100, 250, 500 and 1000µg/mL and incubated for 1h, at 28°C and 170 rpm. Ascorbic acid and quercetin (100uM) were assayed as positive controls for the antioxidant activity. Subsequently, the treated cells with the extracts and controls were stressed by addition of 4mM hydrogen peroxide prepared in phosphate buffered saline (PBS) solution, at pH 7.4 and incubated again for 2h at the same previous conditions. Cell viability was determined by counting colony forming units (CFU). For that purpose cell dilution of 1:10,000 were made using YPD liquid medium, seeded in YPD solid medium plates and incubated for 48h at 28°C. All experiments were conducted in triplicate and reported as mean of cell percentage of survival, which represents the protective antioxidant activity. The viability of the cells without any treatment (negative control) corresponded to 100% viability. The protective antioxidant percentage (% PA) of the samples was calculated considering the cell viability after treatment with the extract (% CVE) during 1h and then exposed to H_2O_2 (% CVP) for 2h, as expressed in the following formula: (100 / % CVE) x % CVP.

Cytotoxicity assay

In vitro cytotoxic activity was determined by the sulforhodamine B protein staining assay against the HF-6 (colon), PC-3 (prostate), MCF-7 (breast), SiHa (cervical) cancer cell lines, as well as toward HFS-30 fibroblast normal skin cells. Extracts with inhibitory concentration $\leq$20µg/mL were considered active according to the National Cancer Institute (NCI) guidelines (Skehan *et al*., 1990). Podophyllotoxin and taxol were used as positive controls.

Isolation and identification of ternstroside B (1)

For isolation purposes the dried and powdered leaves (400 g) of *T. pringlei* were macerated with MeOH (2L ×72h × 3 times) to afford the crude extract. It was subjected to an open column chromatography over silica gel (1:10, w/w), and eluted with a $CHCl_3$-MeOH gradient from 90% to 50% $CHCl_3$. A total of 40 fractions (150mL each) were collected, analyzed by TLC employing as mobile phase $CHCl_3$-MeOH (8:2, v/v), developed with vanillin-H_2SO_4 (1%, v/v) and heated. Eluates were pooled according to chromatographic similarities. Fractions 15-26 were combined to yield 1.4g of a sample enriched with compound 1. This fraction was again subjected on column chromatography, but in this case using a ternary mobile phase $CHCl_3$-EtOH-H_2O (80:40:5, v/v, down layer phase was used) to afford 127mg of semi-purified fraction of 1. The final purification of 1 was achieved by preparative-scale high-resolution liquid chromatography (HPLC) on C-18 column (7 µm, 19 x 300mm) with an isocratic phase of CH_3CN-H_2O (2:8, v/v), and flow rate of 8mL/min.

The ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra, as well as the 2D experiments HHCOSY, HSQC, and HMBC were recorded on a Bruker Avance DMX500 spectrometer, using TMS as internal standard. Mass data acquisitions were performed using Xcalibur 2.3 software and a LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific). The ionization was performed with heated electro spray ionization in the positive and negative mode.

The crude methanolic extracts at 4mg/650µL CD_3OD were registered in a Varian Mercury 400 MHz apparatus in order to obtain their ^1H NMR spectra, which were

compared to each other to verify the presence of phenolic signals.

STATISTICAL ANALYSIS

All assays determinations were conducted in triplicate. Mean ± SD values were calculated and the data were subjected under ANOVA followed by Dunnett's test to compare controls with treatments. Also the Tukey´s test for multiple comparisons of the means was carried out to find differences at $P<0.05$ between treatments. The statistical analysis of the results was performed using an SAS 9.5 program.

RESULTS

Crude extracts yields

Different organs of the plant *T. pringlei* afforded methanolic crude extracts with varied yields expressed in dry weight (g/Kg): leaves (197.0), petals (169.3), fruits (161.8), and seeds (86.2). Leaves showed the highest yield from crude extracts, followed in order by petals, fruits and seeds.

Table 1: ABTS IC_{50} antiradical activity of different organs methanolic extracts

Extract	ABTS (IC_{50} µg/mL)*
Leaves	33.91^{B}±1.00
Petals	123.79^{C}±9.71
Fruits	38.09^{B}±2.09
Seeds	105.86^{C}±1.28
Trolox	0.72^{A}±0.04
Camellia sinensis	3.91^{A}±0.62

*Means followed by the same letter are not significantly different at p<0.05 level in the multiple comparisons by Tukey´s range test.

ABTS antiradical activity

The four extracts showed strong antiradical activity with IC_{50} values from 33.91 to 123.79µg/mL (table 1). The highest ABTS cation radical scavenging activity was observed for the leaves followed by fruit methanolic crude extracts, with IC_{50} values of 33.91 and 38.09 µg/mL, respectively, which were not statistically different as shown by Tukey analysis.

Antioxidant activity in the yeast model

Before testing the protective action of the extracts against the oxidative stressing agent H_2O_2, the extract-treated yeast cells viability was evaluated to check the cell toxicity caused by the extract alone (fig. 1A). Leaf extracts slightly affected the yeast cell survival, with 72.5% cell viability when treated with the extract at 50 µg/mL, which was not statistically different to the negative control. However, the leaf extracts at concentrations of 100-1000µg/mL turned out to be more toxic to the yeast cells, decreasing the survival percentage to 60.6-15.5%, which was now statistically different to the negative control. The same tendency was observed with the seed extracts, where the lower concentrations of 50, 100 and 250µg/mL displayed cell viability percentage of 76.3, 89.9 and 76.7%, respectively, showing no statistical differences with untreated cells. In contrast, the concentrations at 500 and 1000µg/mL were the most toxic, with 20.1 and 13.4% cell viability. In the case of the extracts from fruits, all the tested concentrations resulted in viability between 43.1-63.3%, which was statistically different to the negative control. For the petal extracts, the concentrations between 50-500µg/mL displayed high levels of cell viability of 85.9-99.5%, with the same significance compared to the untreated cells and the controls quercetin and ascorbic acid, which did not affect the cell viability. For a better understanding of the extract toxicities upon viability, the half inhibitory concentration (IC_{50}) was calculated. The IC_{50} for leaf and seed extracts were 202±29.13µg/mL and 257.6±83.53 µg/mL, respectively, revealing high toxicities in regard to the fruit (IC_{50} 882.95±154.48µg/mL) and petal (IC_{50}> 1000µg/mL) extracts.

When *S. cerevisiae* cells were treated with H_2O_2 at 4mM, the control (untreated cells) viability decreased drastically to 3.9%. Nevertheless, the presence of *T. pringlei* extracts protected the cells against the oxidative stress (fig. 1B). The most efficient protective antioxidant percentage (% PA) was observed with the fruit extracts at 250µg/mL, displaying a cell viability of 54.0%. This value was 1.96 and 4.28 times higher than the positive controls quercetin (27.5%) and ascorbic acid (12.6%), both tested at 100 µM, respectively. Although the petal extracts were not toxic for the yeast cells, except at the concentrations of 1000µg/mL, those extracts exhibited the lowest % PA, with values between 9.4-12.0 %.

TLC profiling of the extracts revealed with vainillin-H_2SO_4 and the ABTS•+ solution

Camellia sinensis methanolic extract (0.6mg) and quercetin (5.3µg) were used as positive controls (fig. 2). The high antiradical property of the *C. sinensis* extract was evidenced by the intense decoloring of its complex spot. Quercetin resulted in a dimly spot with a retention factor (R_f) of 0.64, which was positive to the ABTS•+ scavenging reaction. Likewise to the *C. sinensis* profiling, leaf methanolic extracts also displayed an intense spot around R_f 0.20-0.40, but revealed pink by the vainillin-H_2SO_4 chromopher, and were highly positive to the ABTS reaction. The fruit methanolic extracts showed red spots at R_{fs} 0.10 and 0.41 and a pink spot R_f at 0.16, which were all positive as ABTS•+ scavengers. Petal methanolic extracts displayed three defined spots at R_f of 0.59, 0.30, and 0.18, revealing pink using the vainillin-H_2SO_4 reagent and decoloring the ABTS•+ reagent. Seed methanolic extracts did not showed defined spots on TLC revealing pink or red neither were positive to the ABTS test (fig. 2A, 2B and 2C).

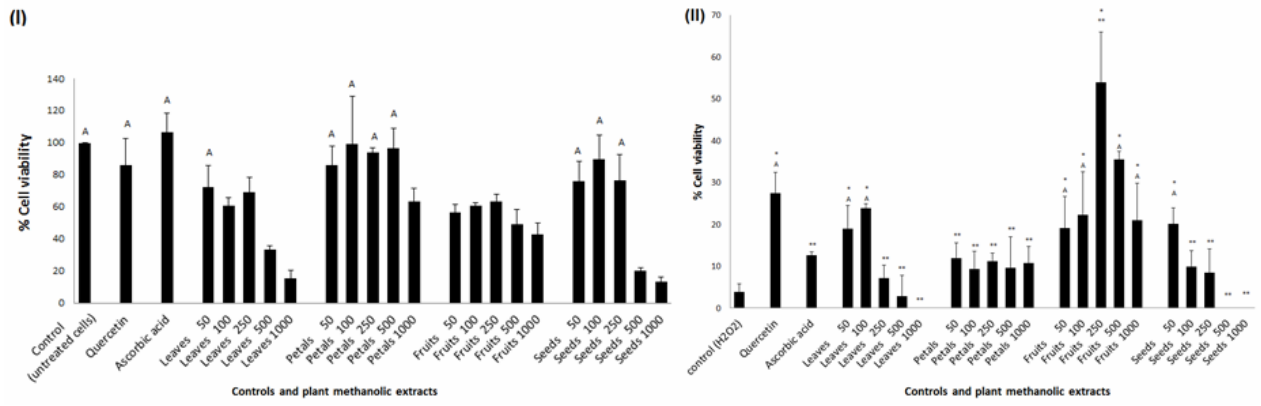

Fig. 1: Percentage of yeast cell viability (I) and cell protection (% survival) towards the H_2O_2 (4mM) oxidative stress (II), for different concentrations of the leaf, seed, fruit and petal methanolic extracts from *T. pringlei* (µg/mL). Quercetin and ascorbic acid at 100µM were used as positive controls. Letter "A" indicates the same grouping in the multiple comparisons by Tukey´s range test. (*) Significance differences of the treatment in comparison to the control group only treated with the H_2O_2 and (**) differences regarding to quercetin at Dunnett´s test level.

Cytotoxic activity

All the four methanolic extracts were non cytotoxic against the tested human cell carcinomas HF-6, PC-3, MCF-7, SiHa and normal fibroblast HFS-30, showing IC_{50} □ 20µg/mL.

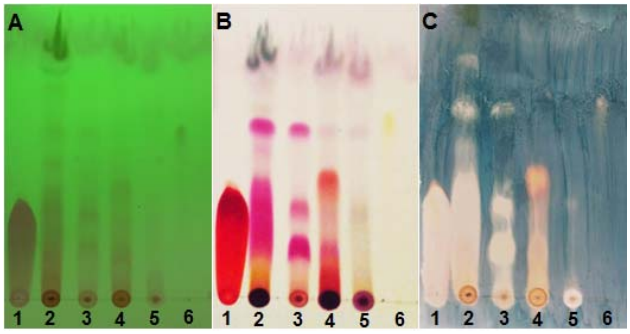

Fig. 2: TLC plates visualized with short UV (A), revealed with vainillin/H_2SO_4/heat (B), and with ABTS•+ (C). (1) *Camellia sinensis*, (2) leaf, (3) petal, (4) fruit, (5) seed MeOH crude extracts, and (6) quercetin. $CHCl_3$: CH_3OH, 4:2, was employed as mobile phase; silica gel with indicator was used as stationary phase.

Isolation of compound 1

After diverse fractionation of the leaf crude methanolic extract of *T. pringlei* by open chromatographic column procedures, a rich fraction containing compound 1 was obtained. The final HPLC purification of 1 using a preparative-scale C-18 column resulted in a peak with a retention time of 16 min, which was collected to afford 5 mg of pure compound 1.

Identification of ternstroside B (1)

Compound 1 was identified as the known ternstroside B by means of 1D and 2D NMR and mass experiments. Its ^1H and ^{13}C NMR spectra as well as the peak at m/z 473 $[M + Na]^+$ for ($C_{22}H_{26}O_{10}Na$) were fully consistent with the spectral data of ternstroside B reported by Jo *et al*., 2006.

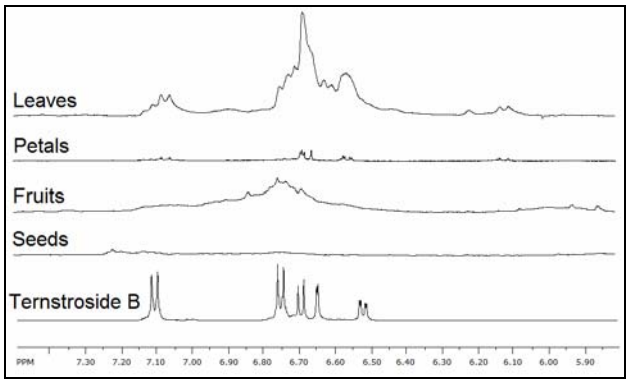

Fig. 3: ^1H NMR detail of the aromatic region from the crude methanolic extracts of leaves, petals, fruits, and seeds. Spectra were run with samples at the same concentration of 4mg/650µL CD_3OD at 400 MHz (Varian). ^1H NMR spectra of ternstroside B (compound 1) was recorded in CD_3OD at 500 MHz (Bruker).

DISCUSSION

Local market in Mexico for *T. pringlei* "tila flower" is mainly focused on the tea prepared from the dried fruit. This commercialized fruit is always composed of a calyx, with or without pericarp. In a previous study we identified and isolated the sedative compound jacaranone from the fruit extracts of *T. pringlei* using a bioguided study (Lozada-Lechuga *et al.*, 2010). Up to now, very little attention had been devoted to its leaves, reason for which we decided to investigate and afforded compound 1. After purification of 1 by HPLC, the 1D and 2D NMR spectroscopy and mass spectrometric analysis identified

its structure as ternstroside B, a phenylethanoid glycoside previously isolated from *T. japonica*. Ternstroside B exhibited potent antioxidative activity in three different tests: hydroxyl radical (•OH) inhibitory activity (IC_{50} 4.66 μM), total reactive oxygen species inhibition activity (IC_{50} 62.52μM) and peroxynitrite ($ONOO^-$) scavenging action (IC_{50} 2.36μM) performed by Jo *et al.* (2006).

In the present work the TLC profiling of *T. pringlei* anatomical part extracts proved to be very different (fig. 2). Leaves and fruits share no similarities in terms of metabolites, at least for those visualized at the employed chromatographic conditions. While the TLC of leaf extracts evidenced a complex mixture of compounds between R_f 0.20-0.40 (large pink spot), from where ternstroside B (R_f 0.31) was isolated, fruits' extract did not present this compound. Instead, fruits produced a slightly more polar compound with an R_f 0.41, which was revealed as a red spot. Leaf and fruit extracts displayed the highest scavenging action over the ABTS•+, with IC_{50} values of 33.91 and 38.09μg/mL, respectively. These values are not so distant from the IC_{50} of 12.99μg/mL reported to *Camellia sinensis* extract (green and black tea), both very well known for their antioxidative properties (Pereira *et al.*, 2014). Antiradical action of *T. pringlei* extracts might also be compared with plants from the genus *Stachys*, which are also well known for their antioxidative properties, as well as for a marked presence of phenylethanoid glycosides. The methanolic crude extract of the leaves of *Stachys lavandulifolia* showed an IC_{50} value of 22.8μg/mL in the antiradical ABTS test (Tundis *et al*., 2015). Also, in the same test model, the pomegranate seeds ethanolic extracts (*Punica granatum*), a plant largely reported as beneficial for human health due to its potent antioxidant action, displayed an IC_{50} of 12.87 μg/mL (Lucci *et al*., 2015).

The ABTS•+ autographic assay on TLC showed that the leaf methanolic extracts containing tersntroside B were the most active extract, which is consistent with the *in vitro* ABTS assay. It was evidenced by the ABTS•+ scavenging test on TLC that both *C. sinensis* and *T. pringlei* leaf methanolic extracts have strong and comparable activities.

Despite that at every tested concentration fruit extracts affected cell viability, each concentration exhibited protective action against the H_2O_2 damage. However, extract at 250μg/mL displayed the highest activity within all tested samples, which was even higher than positive controls.

Petal extracts showed a less complex TLC profiling (fig. 2), and displayed the highest yeast cell viability, but none of their assayed concentrations were able to protect *S. cerevisiae* cells from oxidative stress.

A plant extract is a complex entity composed by thousands of metabolites. In this sense all plants possess antiradical as well as antioxidant action at a certain level. However, the accumulation of particular groups of metabolites contributes to the real benefit of a plant for the human health. It is well known that phenolics are considered a major group of molecules with antioxidant activity in plants (Vieira *et al.*, 2010). To investigate this assertion, *T. pringlei* extracts were studied through ^1H NMR analysis in relation to the presence of phenolics, because their aromatic signals can be easily identified at the low field region around 6-8 ppm. The presence of phenolic signals in the ^1H NMR is mostly in the leaf and fruit methanolic crude extracts, corroborating the correlation of high antiradical and antioxidant properties of these extracts to the phenolic content. The occurrence of aromatic signals in those extracts, which features are also attributed to the presence of phenylethanoid glycosides among other possible phenolic metabolites, might explain the high activities found in leaf and fruit extracts and the poor results obtained with petal and seed methanolic crude extracts.

Considering that both, leaf and fruit methanolic extracts displayed the most potent antiradical and antioxidant activities, for the first time we evidenced that Tila tea using the fruits of *T. pringlei* has the potential to induce a positive balance in human oxidative stress. The present study opens new questions about the antioxidant potential of *T. pringlei* extracts, and additional investigations based on specific targets must be considered in future studies, *e.g*. the regulation of antioxidant enzymes including glutathione peroxidase, catalase, superoxide dismutase. Taking into account that *Ternstroemia* species produce a diversity of metabolites like triterpenes, saponins and flavonoids, those compounds may also play antioxidant and other pharmacological actions (Balderas-López *et al*., 2013; Jo *et al*., 2006; Yo *et al*., 2005). The isolation of ternstroside B for the first time from *T. pringlei* increased the interest in this plant as a source of phenylethanoid glycosides, as well as its antioxidant benefit in human health.

CONCLUSION

Leaf and fruit methanolic extracts of *T. pringlei* possess high antiradical and antioxidant actions, which can afford advantages to Tila´s products in terms of food quality improvement.

The identification of ternstroside B in the Mexican species *T. pringlei* corroborates the close chemo-taxonomic relationship with the Asian species *T. japonica*, in which this compound was first described. Furthermore the well-known relationship between anti-ROS compounds and anti-rheumatic action may explain the ethnomedical use of this species in Mexico.

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