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Antioxidant capacity and cholinesterase inhibitory activity of *Vulpicida pinastri* lichen and its chemical composition

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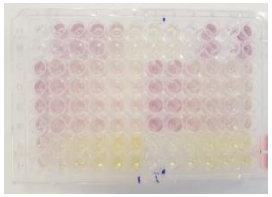
Graphical Abstract

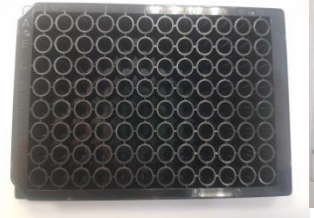
*Vulpicida pinastri, (*Scop.)
J.E. Mattsson & M.J. Lai

Antioxidant *in vitro* test. Phenolic content

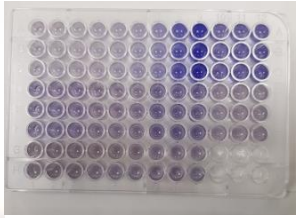
DPPH



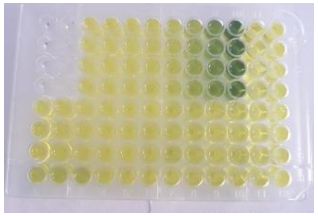
ORAC



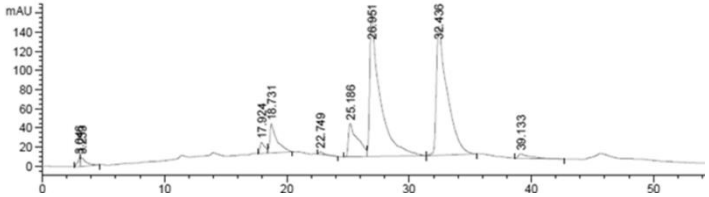
FRAP



FOLIN

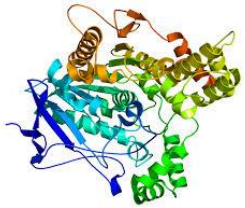


Phytochemical analysis



HPLC-UV

Cholinesterase inhibitory activity



Abstract

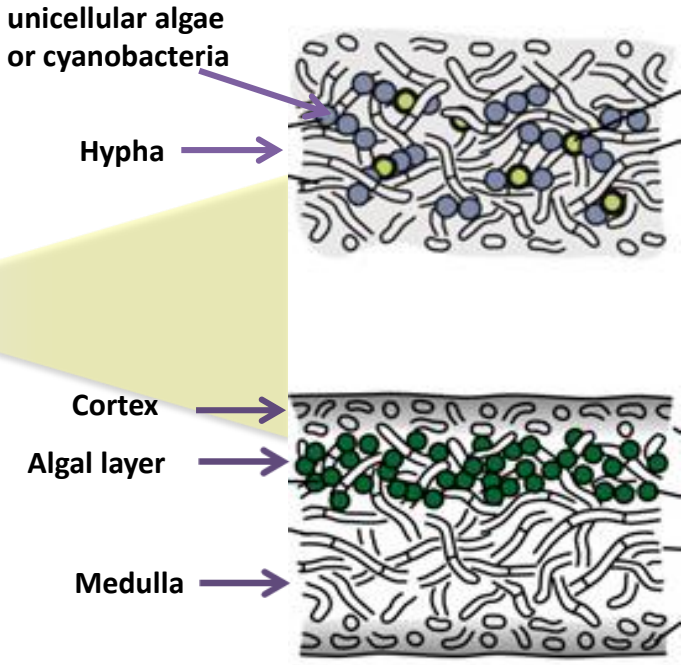
This study reports for first time the antioxidant capacity and cholinesterase inhibitory activity of the methanol extract of the lichen *Vulpicida pinastri* (Scop.) J.E. Mattsson & M.J. Lai and its chemical composition. This lichen specie with a greenish yellow foliose thallus was collected in Puerto Alto del Peñon, Zamora (Spain). Antioxidant capacity was assessed **by *in vitro* tests (DPPH, ORAC and FRAP**), total **phenolic content** by Folin-Ciocalteu method, **cholinesterase inhibitory activity** by Ellman's colorimetric method and chemical composition by **HPLC-UV method**. The results showed that the values for antioxidant capacity were IC50 283.7 ± 31.7 μg/ml for DPPH, 1.5 ± 0.1 μmol TE/mg dry extract for ORAC and 25.4 ± 2.3 μmol of Fe2+ eq/g sample for FRAP. Moreover, total phenolic content had a value of 48, 9 ±4.8 μg GA/mg. Furthermore, IC50 values were 0.19 ± 0.003 mg/mL for acetylcholinesterase inhibitory activity and 0.89 ± 0.018 mg/mL for butyrylcholinesterase inhibitory activity. Finally, the analysis of chemical composition revealed that the major **secondary metabolites** were **vulpinic acid** (9.3 ± 0.8**), pinastric acid** (41.9 ± 1.07) and **usnic acid** (36.5 ± 3.62). In conclusion, *Vulpicida pinastri* is a promising agent to further study for the prevention and treatment of Alzheimer's disease based on its antioxidant and cholinesterase inhibitory activities.

Keywords: lichen; antioxidant; cholinesterease inhibition activity; secondary metabolites

Introduction

Lichens are symbiotic organisms composed by a mycobiont (fungus) and a photobiont (unicellular algae or cyanobacteria). The mycobiont provides algae with water and simulates a humid environment. The algae produces necessary nutrients for the fungus through photosynthesis.

Figure 1. Vulpicida pinastri (Scop.) J.E. Mattsson & M.J. Lai (©Granlav)



Homomer
(homogeneous throughout its thallus)

Heteromer
(stratification in various layers)

Figure 2.©M.Piepenbring, CC BY-SA

Morphology-types:

- **Crustose** — Tightly attached to the substrate
- **Foliose** — Leaf-like
- **Fruticulose** — Similar to branches

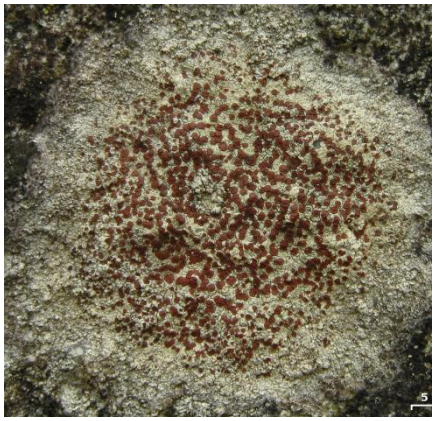


Figure 3. **Crustose lichen morphology**

Figure 4. Foliose lichen morphology

Figure 5. Fruticulose lichen morphology

Introduction: Phylogeny

Parmeliaceae is the largest family of lichenized fungi (80 genera; 2,800 species), and within this, **cetrarioid clade** stands out phylogenetically by number

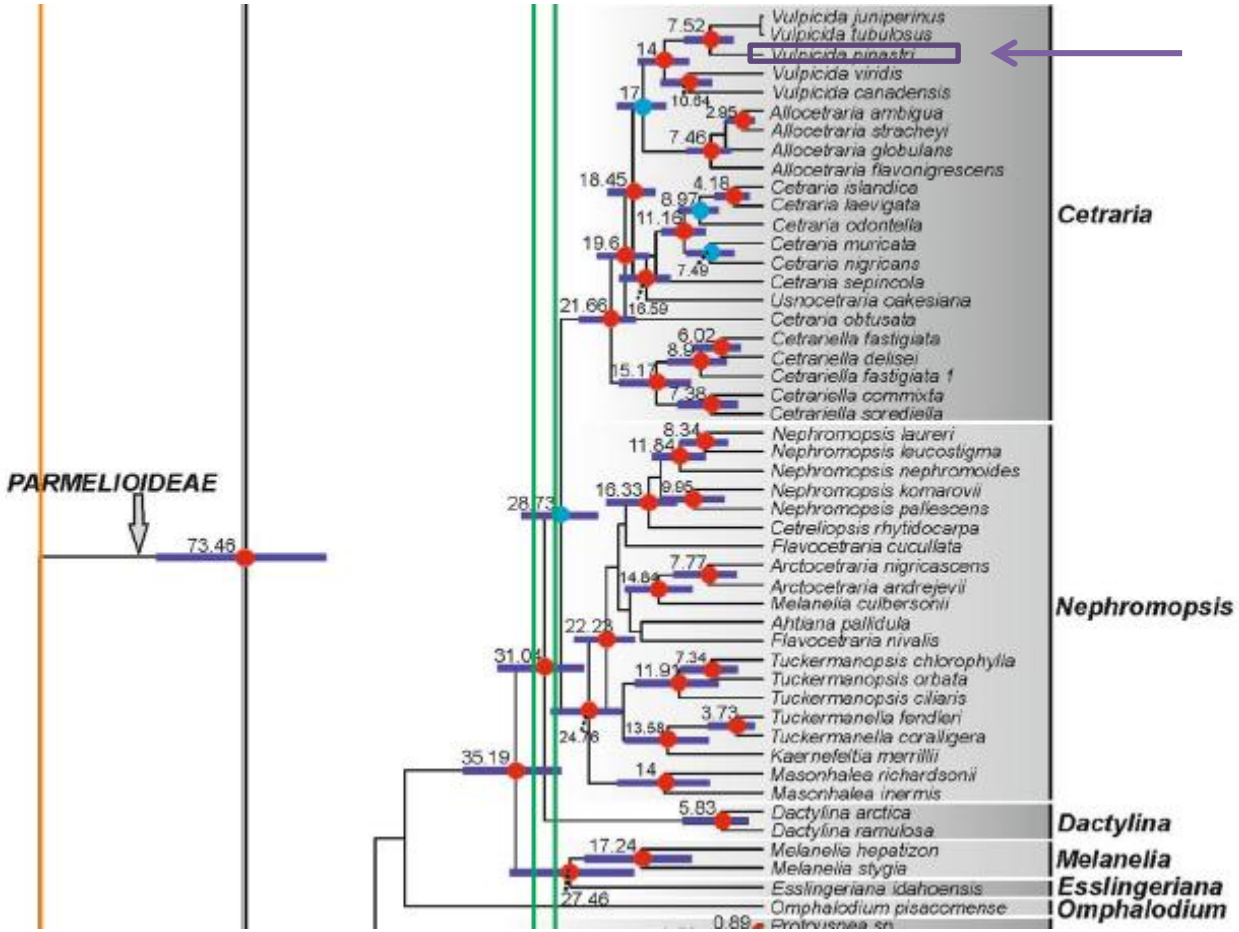
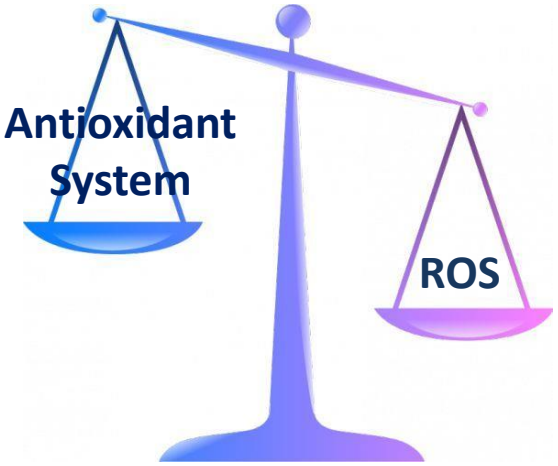


Figure 6. Phylogenetic tree fragment **(Divakar et al., 2017)**

Introduction: Oxidative stress

Imbalance between reactive oxygen species production (H_2O_2, $O_2^{\cdot -}$, $\cdot OH$) and body's antioxidant system (enzymatic and non-enzymatic)



Redox imbalance

Damage

Biological structures: proteins, DNA, lipids

Cell Death: apoptosis

Related to: CV diseases, neurodegenerative diseases, diabetes, cancer, obesity and ageing

ANTIOXIDANT: molecule that inhibit ROS action or slow down biomolecules oxidation

Introduction: Description

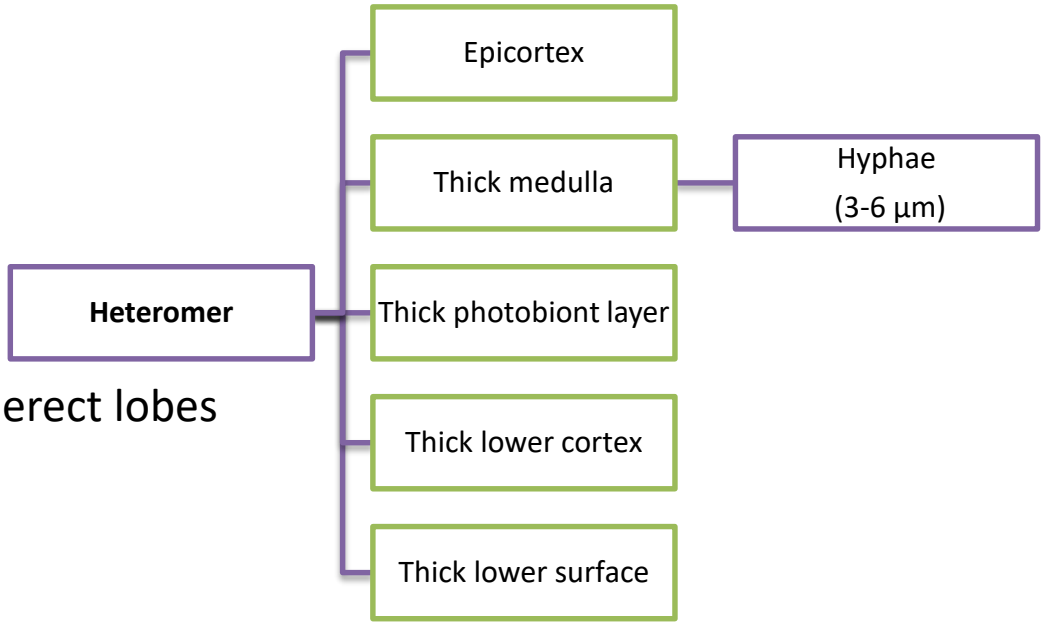
***Vulpicida pinastri* (Scop.) J.E. Mattsson & M.J. Lai > Greenish yellow foliose thallus**

- On **rocks**: rosettes with short lobes

Figure 7. *Vulpicida pinastri* on rocks

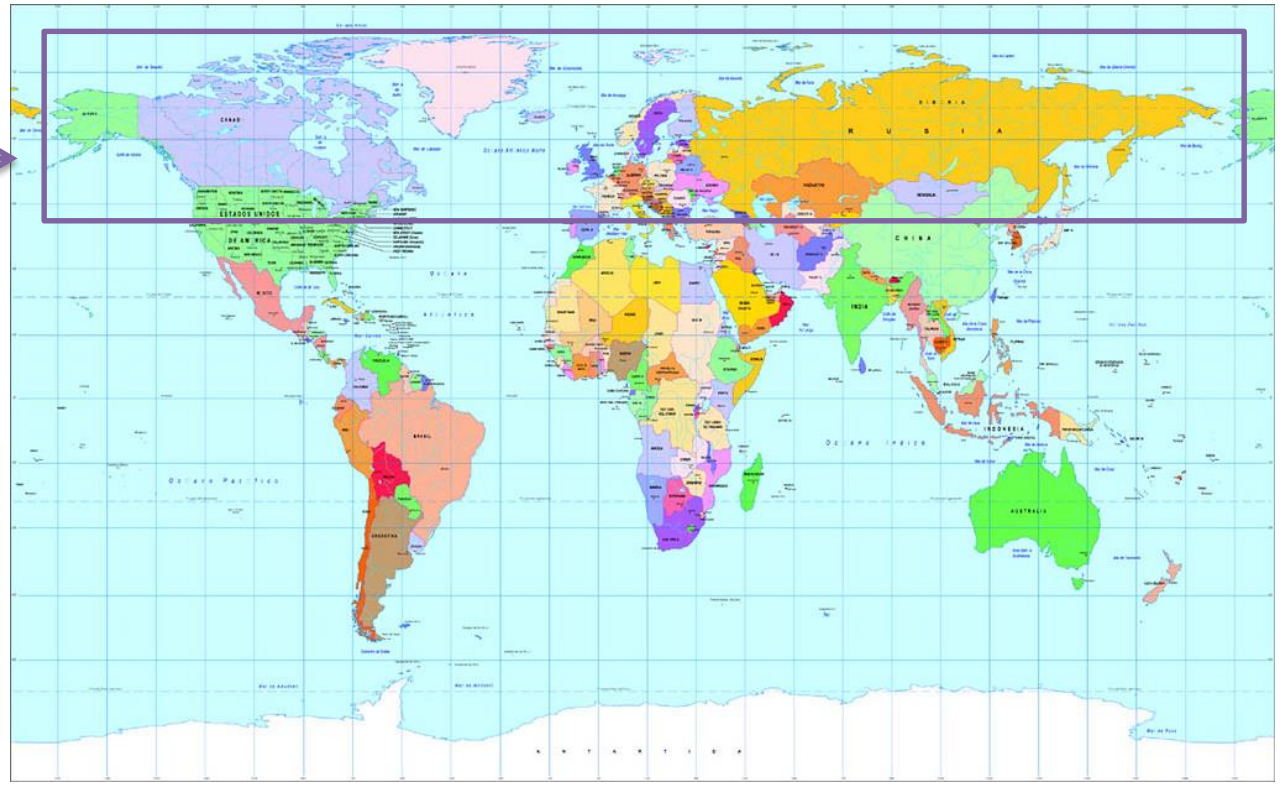
- On **branches**: elongated and erect lobes

Figure 8. *Vulpicida pinastri* on branches



Introduction: Distribution

World distribution: circumboreal, low arctic and lower alpine regions: North America, Europe and Asia



Distribution for lichen sample of this study:
Las Batuecas, Cáceres (Spain)

Introduction: Methods

1. **ANTIOXIDANT *IN-VITRO* TEST**
   - DPPH
   - FRAP
   - ORAC

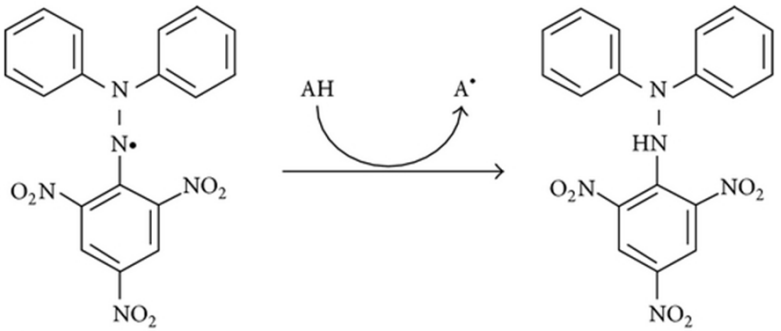
2. **TOTAL PHENOLIC CONTENT**
   - FOLIN- CIOCALTEAU

3. **ANTICHOLINESTEREASE-BUTYRYLCHOLINESTEREASE**
   - ELLMAN´S METHOD

4. **PHYTOCHEMICAL ANALYSIS**
   - HPLC-UV

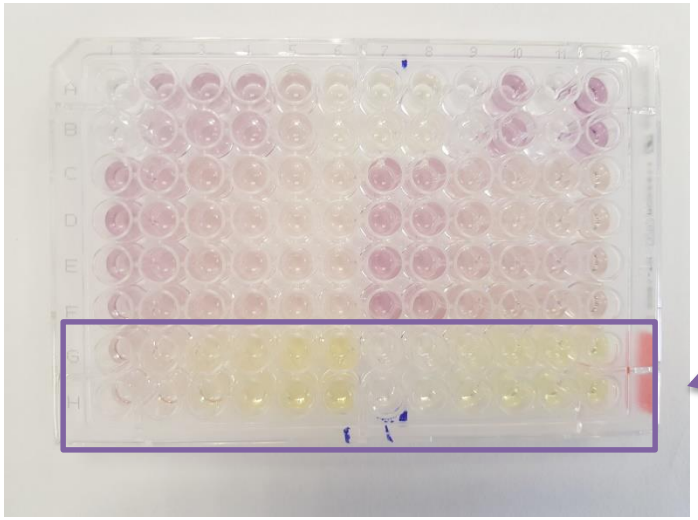
Results and discussion: Antioxidant *In vitro* test -> DPPH

Based on the ability of antioxidants to reduce DPPH (2,2-Diphenyl-1-picrylhydrazyl) in radical form to the DPPH compound.



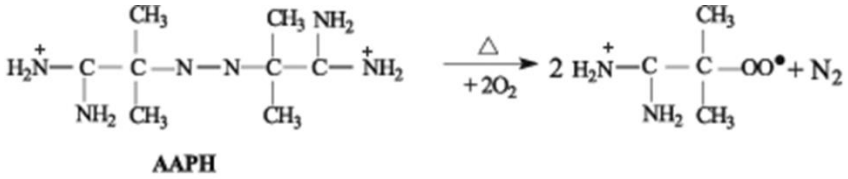
DPPH (ox)
Purple

DPPH (red)
yellow



Lichen species	DPPH EC50 (μg/mL)
Vulpicida pinastri	IC_{50} 283.7 ± 31.7

Results and discussion: Antioxidant *In vitro* test -> ORAC



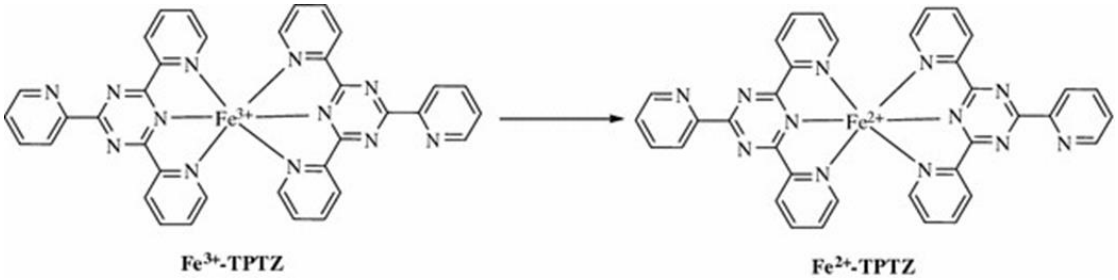
AAPH

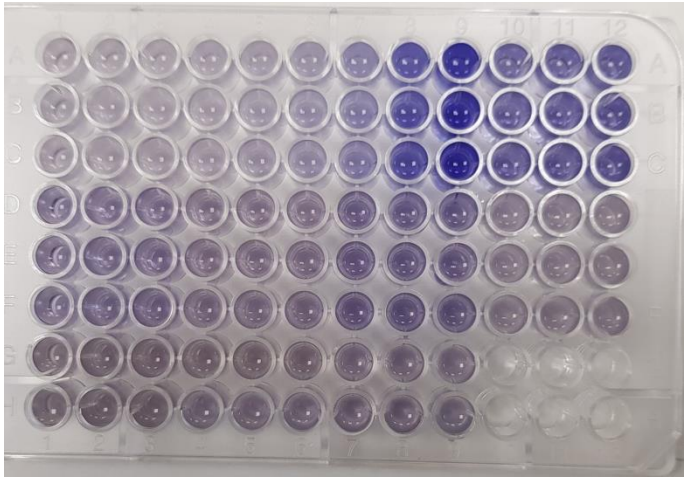
It is based on fluorescence´s loss of fluorescein which decreases in presence of peroxyl radicals.
Peroxyl radicals were generated by thermal decomposition of AAPH.

Lichen species	ORAC (μmol TE/mg dry extract)
Vulpicida pinastri	1.5 ± 0.1

Results and discussion: Antioxidant *In vitro* test -> FRAP

- Redox-linked colorimetric reaction
- Reduction of Fe^{3+} to Fe^{2+} ion is due to antioxidants

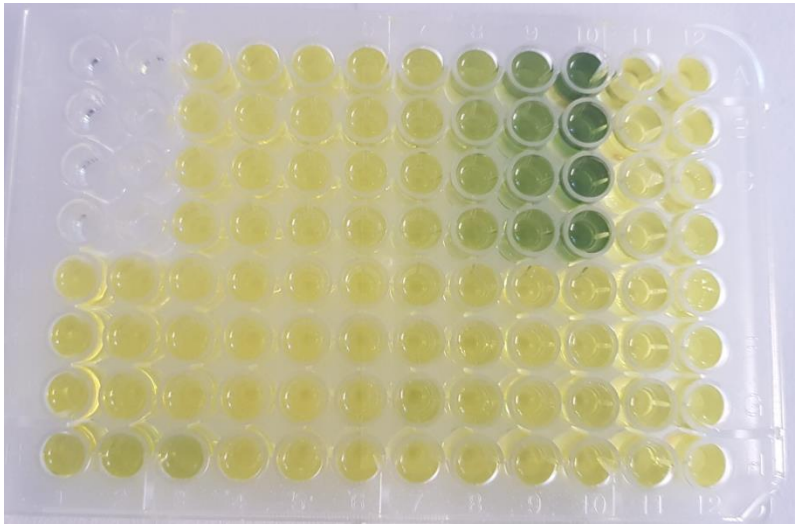




Lichen species	FRAP (μmol of Fe^{2+} eq/g sample)
Vulpicida pinastri	25.4 ± 2.3

Results and discussion: Total phenolic content -> Folin Ciocalteu

- Determination of polyphenols
- Polyphenols react with Folin-Ciocalteu reagent
- This reaction forms a blue chromophore complex that can be quantified by visible-light spectrophotometry



Lichen species	FOLIN (μg GA/mg)
Vulpicida pinastri	48.9 ±4.8

Results and discussion: Cholinesterase Inhibition Activity

- The enzyme inhibitory activities AChE and BChE were determined using Ellman method.
- Cholinesterase activity is measured indirectly by quantifying the concentration of 5-thio-2-nitrobenzoic acid (TNB) ion formed in the reaction between the thiol reagent 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) and thiocholine, a product of substrate (i.e. acetylthiocholine [ATCh]) hydrolysis by the cholinesterase.

Figure 9. Acetylcholinesterase

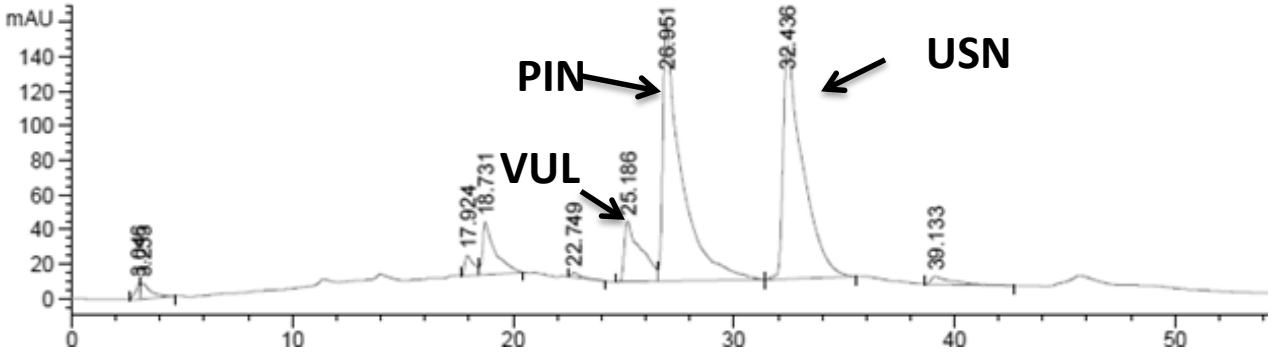
Lichen species	AchEi IC_{50} (mg/mL ± SD)	BchEi IC_{50} (mg/mL ± SD)
Vulpicida pinastri	0.19 ± 0.003	0.89 ± 0.018

Results and discussion: Phytochemical analysis-> HPLC-UV

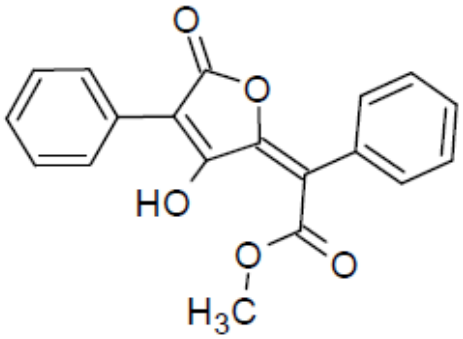
- **Sample**: 250 µg/ml *V. pinastri* methanol extract
- **HPLC**: Agilent 1260 instrument (Agilent Technologies, CA, USA)
- **Software:** Agilent Chemstation
- **Column:** reversed-phase Mediterranea Sea18 column (150 mm × 4.6 mm, 3 µm particle size; Teknokroma, Barcelona, Spain)
- **Phases**:
  - ✓ (A)->1% orthophosphoric acid in milli-Q water
  - ✓ (B)->HPLC-methanol
- **Volume**: 20µL
- **Flow rate:** 0.6 ml/min
- **Column temperature:** 40 °C
- **UV spectrum :**190 and 400 nm
- **Reference chromatograms:** 254 nm

Results and discussion- Phytochemical analysis

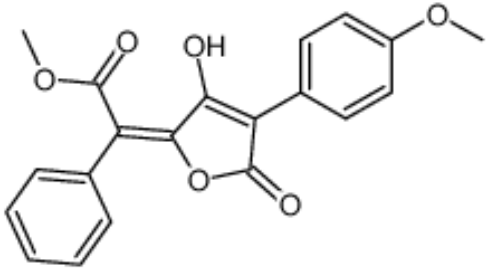
Representative HPLC chromatogram for *V. pinastr*i (250 mg/ml) methanol extract at 254 nm



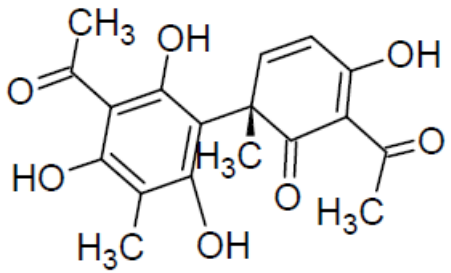
Vulpinic acid



Pinastric acid



Usnic acid



Conclusions

- Among all antioxidant *in vitro* assays, DPPH method shows a moderate antioxidant capacity (IC_{50} 283.7 ± 31.7μg/mL)

- *Vulpicida pinastri* shows that its anti-acetylcholinesterase inhibitory activity (0.19 ± 0.003 mg/mL) was better than its anti-butyrilcholinesterase inhibitory activity (0.89 ± 0.018 mg/mL)

- The major compounds identified by HPLC were vulpinic acid, pinastric acid and usnic acid

- *Vulpicida pinastri* is a promising agent to further study for the prevention and treatment of Alzheimer's disease based on its antioxidant and cholinesterase inhibitory activities

Acknowledgments

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