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SHORT COMMUNICATION

A sustainable application for the extraction of lichen metabolites from *Usnea cornuta*: nontargeted metabolomics and antioxidant activity

Grover Castañeta[a], Beatriz Sepulveda[b], Reinaldo Vargas[c], Olimpo Garcia-Beltran[d], Mario Simirgiotis[e] and Carlos Areche[a]

[a]Departmento de Química, Facultad de Ciencias, Universidad de Chile, Santiago, Chile; [b]Departamento de Ciencias Químicas, Universidad Andrés Bello, Quillota, Chile; [c]Departamento de Biología, Universidad Metropolitana de Ciencias de la Educación, Santiago, Chile; [d]Facultad de Ciencias Naturales y Matemáticas, Universidad de Ibagué, Ibagué, Colombia; [e]Instituto de Farmacia, Facultad de Ciencias, Universidad Austral de Chile, Valdivia, Chile

ABSTRACT

In this study, isolation and purification of lichen substances from *Usnea cornuta* were performed using conventional solvents, green solvents and green technologies. In addition, several lichen compounds were tentatively identified by UHPLC/ESI/MS/MS and usnic acid, diffractaic and galbinic acids were quantified as well. Limonene, ethyl lactate and methanol, were compared regarding their extraction properties and antioxidant capacities, determined by DPPH, ORAC, and FRAP assays. In the ethyl lactate, methanol and limonene extracts, 28 compounds in all, were detected for the first time by high resolution UHPLC-MS/MS fingerprinting. Untargeted metabolomics tentatively identified 14 compounds from the methanolic extract, 4 from limonene extract, and 20 metabolites from ethyl lactate extract. The green extract of ethyl lactate showed a similar antioxidant capacity to toxic methanol extract, except at ORAC assay where it was higher. Therefore, ethyl lactate can replace methanol, to provide more sustainable green chemistry methods.

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CONTACT Carlos Areche areche@uchile.cl; Beatriz Sepulveda bsepulveda@uc.cl

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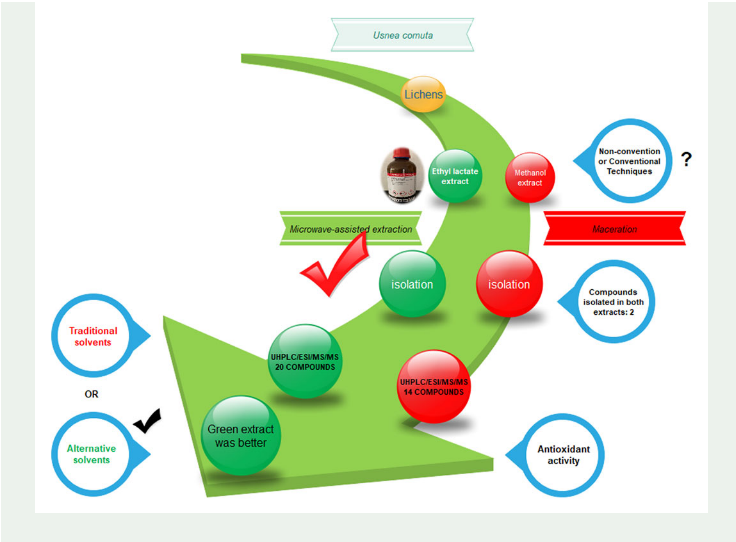
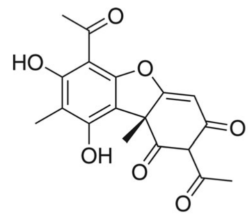


1. Introduction

Isolation of natural products from natural sources is usually performed using conventional techniques based on the use of *n*-hexane, dichloromethane, chloroform, and methanol, all known as volatile organic solvents. They are used in extraction and chromatography methods in most research groups in universities. In this context, environmental regulations for decreasing the emissions of organic solvents, both industrial and academic, have become of public knowledge for the last two decades (Cañadas et al. 2020). Green solvents have emerged as promising candidates, and a good alternative, to petrochemical solvents since they are environmentally friendly, being derived from agricultural crops. Limonene is considered as a cyclic monoterpene, and is the major by-product of the citrus fruits industry. The D-limonene is more common in nature and is considered as a GRAS (Generally Recognized As Safe) solvent by the Food and Drug Administration. Its properties have indicated the high suitability of limonene as green solvent in order to replace toxic solvents such as n-hexane and toluene (Chemat et al. 2019). Ethyl lactate (EL) is considered a green solvent derived from the processing of corn. It is biodegradable, noncorrosive, noncarcinogenic and nonozone depleting (Doble et al. 2007). Therefore, the obtention and separation of extracts using green and sustainable methods from natural resources is an important topic on Natural Products for the design of cleaner processes with lower energy requirements, according to the principles of the Green Chemistry (Chemat et al. 2019). Today, the extraction of secondary metabolites can be achieved using conventional (Soxhlet, maceration, and hydrodistillation) or non-conventional techniques (microwave, ultrasound, pressurized liquid, supercritical fluids, pulsed electric field, high-voltage discharges, and high hydrostatic pressure) (Soquetta et al. 2018).

The goals of this work were the isolation and purification of lichen substances from *Usnea cornuta* using a combination of green technology and green solvents for the first time. Then, the extracts were analyzed through UHPLC/HESI/MS/MS (Ultra-High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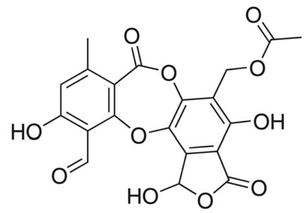
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Figure 1. Compounds isolated from *Usnea cornuta* extracts.

Performance Liquid Chromatography/Heated ElectroSpray Ionization/Mass/Mass) for full untargeted metabolomic analyses. Finally, the antioxidant activities and phenolic contents of all extracts were tested and compared.

2. Results and discussion

Extraction with methanol (conventional technique), EL [Microwave Assisted Extraction (MAE)] and EL [Ultrasound Assisted Extraction (UAE)] yielded 13.5%, 24.8% and 15.3% respectively. This fact implies that EL could replace the methanol extraction solvent despite of the green solvent's higher cost, both in energy use and money (Doble et al. 2007). Therefore, these findings indicated that the non-conventional techniques based on MAE were better than maceration.

From methanol, and EL (MAE) extracts, usnic acid **1** and galbinic acid **2** (see Figure 1) with yield by 3.75 g and 4.0 g for methanol, and 5.5 g and 4.9 g for EL were isolated, respectively. This finding shows that isolation of metabolites using green solvents is better than toxic organic solvents based on the yield obtained. As an alternative strategy to overcome the use of toxic organic solvents for extraction, we agreed to use green solvents, as EL. This solvent is biodegradable, ozone friendly, non-corrosive, non-toxic and considered as a GRAS solvent (Pereira et al. 2011).

In the case of maceration with methanol, untargeted metabolomics using UHPLC-ESI-MS/MS tentatively identified 14 compounds from the methanolic extract, 4 from limonene extract, and 20 metabolites from EL extract [for detail, see Table S1 in SM (Supplementary Material)]. The potential of each extract was grouped as follows: depsidones, depsides, organic acids, xanthones, dibenzofurans, cycloaliphatic acids, lipids, polyols and unidentified compounds (Calla-Quispe et al. 2020; Salgado et al. 2020; Sepulveda et al. 2021). Methanol was more efficient when extracting cycloaliphatic acids (peaks 33 and 36) and organic acids (peaks 3-4), EL extracted more depsidones, and limonene extracted unknown compounds probably of apolar nature more efficiently. This finding showed that the identification of metabolites using EL is better than limonene and methanol based on UHPLC/ESI/MS/MS.

In relation to quantitative analysis, usnic acid (**1**) was the main compound in the EL extract using MAE at 100 °C. (138.55 ± 0.20, mg/g extract, see Table S2 in SM). Increasing temperature does not have incidence in the extraction of this main compound (data not shown). However, the most concentrated sample regarding usnic acid (**1**) was the methanol extract (181.96 ± 0.38 mg/g extract), but galbinic acid (**2**) concentration was also the highest (12.62 ± 0.08 mg/g extract). Usnic acid (**1**) has been reported as the most important bioactive compound in extracts of the genus *Usnea* (Chae et al. 2021) because of its abundance, easy isolation, multiple biological activities, and promising contribution as a component of phytopharmaceuticals (Prateeksha et al. 2016). Popovici et al. 2021 found in *U. barbata* variable concentrations of usnic acid (**1**) in four extracts obtained by different solvents: ethyl acetate (376.73 mg/g), acetone (282.78 mg/g), methanol (137.60 mg/g) and ethanol (127.21 mg/g). In *U. barbata* the usnic acid (**1**) content present in methanolic extracts, ethyl acetate extracts, and pure conditions, shows high proportions of cytotoxicity on cancer cells, highlighting its potential anti-tumor activity (Tang et al. 2020; Popovici et al. 2021). Pathak et al. 2016 found efficient antidermatophytic activity in *U. orientalis* extract, mediated by the high concentration of usnic acid (**1**). Moreover, usnic acid in *U. longissima* is found in high concentration and is evidenced with a significant effect on different cancer cell lines (Reddy et al. 2019). As for other chemical compounds, galbinic acid (**2**) in *U. undulata* (Elix and Engkaninan 1975) and diffractaic acid in *U. diffracta* (Okuyama et al. 1995), show medium-high concentrations with recognized biological activities.

Regarding to antioxidant activity, all extracts were evaluated in vitro tests (see Table S3 in SM). The EL green extract showed a similar antioxidant capacity to toxic methanol extract, except at ORAC (Oxygen Radical Absorbance Capacity) assay where it was higher. Some Usnea lichens extracts were proven to show antioxidant capacities; for example, ethyl acetate and methanol extracts from *U. pictoides* and supercritical CO_2 extracts of *U. barbata* showed good DPPH (1,1-Diphenyl-2-PicrylHydrazyl) radical trapping activity (Behera et al. 2005; Zugic et al. 2016) and *U. ghattensis* methanol extract prevented lipid peroxidation by 87% followed by 65% in Trolox at 20 µg/ml (Behera et al. 2005; Verma et al. 2008). This lichen species also displayed superoxide, DPPH, nitric oxide, and hydroxyl radical-scavenging activity, 89%, 89.6%, 94.8%, and 89.6%, respectively (Verma et al. 2008). In our study, all extracts obtained by the limonene, methanol and EL solvents were subject to DPPH, ORAC, FRAP (Ferric Reducing Antioxidant Power) assays and the analyses of Total Phenolic Compounds (TPC) contents. EL extract showed similar antioxidant DPPH bleaching capacity to the methanol extract (46.25 µg/mL and 45.58 µg/mL, respectively, Table S3); however, with ORAC capacity, EL extract showed 562.61 µM/g of dry lichen and methanol extract only 288.15 ± 0.05 µM/g of dry lichen. FRAP measures the presence of reductants in extracts, which causes the reduction of ferric complex to ferrous form. FRAP measurements in our extracts displayed similar trends, 245.36 ± 1.52 µmol equivalents of Trolox/g of the dry plant, for the EL extract, versus 315.23 ± 2.37 µmol equivalents of Trolox/g of the dry plant, for the methanol extract. The antioxidant activity of extracts from *U. cornuta* assessed by the different systems could be attributed to their high total polyphenolic contents. However, TPC values were 28.64 ± 0.01 and 21.71 ± 0.01 µmol GAE/g extract, respectively, for the EL and methanol extracts, which is much higher to the one reported for the methanol extract of *U. pictoides* (60 µg per g lichen) (Pavithra et al. 2013), and close to the one reported from *U. barbata* ethanol extract and water maceration extract (25.7 and 14.4 mg per g of dry lichen, respectively) (Zugic et al. 2016). However, in our study, more phenolic compounds were detected in the green EL extract. These similar activities could be attributed to different quantities and nature of phenolic compounds in the extracts. Regarding the active compounds, norstictic acid from *U. articulata*, psoromic acid from *U. complanata*, stictic acid from *U. articulata* were the main reported phenolic compounds with attributed antioxidant properties (Fernández-Moriano et al. 2016). In this paper, the antioxidant property of the lichen could be attributed to the significant number of depsidones, especially salazinic acid, which possesses two phenolic groups in the molecule and probably plays a significant role in antioxidant activity.

3. Conclusions

The use of green solvents combined with green technologies in the extraction of natural products, is vital to reduce negative impacts on the environment. In this process, non-conventional techniques combined with green solvents, were used to produce extracts and were also compared with a conventional method, as maceration for isolation and purification of natural products from the lichen *U. cornuta*. Our results were evidenced both isolating the two compounds (usnic acid and galbinic acid) and by an untargeted metabolomics study (UHPLC/ESI/MS/MS). The LC/MS/MS plan provided 20 compounds for EL extract, 4 for limonene and 14 metabolites for MeOH extract from the lichen *U cornuta*. In relation to quantitative analysis, usnic acid was the main compound in the all extracts and the highest amount was in the methanolic extract followed by the EL extract. Galbinic acid concentration was also in the same way. In regards to the *in-vitro* antioxidant assays, EL extract showed higher activity than the methanol extract based on ORAC assay. This finding proved that extraction of metabolites, using green solvents combined with MAE, is better than toxic organic solvents, such as methanol, except on preparative scale. Green solvents can be used for the good extraction of organic compounds from lichens, as well as for purification by column chromatography. Finally, we highly consider green solvents' potential to replace toxic organic solvents, such as methanol, in order to supply more sustainable methods.

Disclosure statement

No potential conflict of interest was reported by the authors.

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ORCID

Carlos Areche http://orcid.org/0000-0001-5246-1368

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