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SHORT COMMUNICATION

Green ultrasound-assisted extraction of lichen substances from *Hypotrachyna cirrhata*. Ethyl lactate, a better extracting agent than methanol toxic organic solvent?

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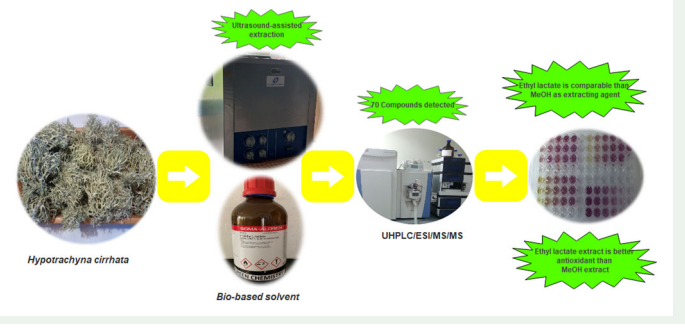
ABSTRACT

For the first time, we report a green extraction of lichen substances assisted by high power ultrasounds from *Hypotrachyna cirrhata* using ethyl lactate. This sustainable alternative was comparable, both in isolation and detection of lichen substances, to methanol. In the metabolomic analysis, a total of 77 lichen substances were detected comprising depsides, depsidones, dibenzofurans, organic acids, and lipids. Although the UHPLC/ESI/MS profiles were similar, the antioxidant activity was higher for the ethyl lactate extract. Ethyl lactate can replace toxic organic solvents, such as methanol, in order to provide more sustainable green chemistry methods.



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1. Introduction

Solvents commonly used, both in academic and chemical industries, are volatile organic compounds (VOCs) in most cases, which inevitably lead to environmental pollution. In that sense, the scientific community and industry need to replace the intensive use of VOCs by alternative green solvents, which is a challenging task in some

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cases. Green chemistry is the use of a set of 12 principles, which reduce or eliminate the use and generation of hazardous substances, during the manufacture and application of chemical products (Soquetta et al. 2018; Chatel and Varma 2019).

Over the past years, a number of alternative solvents such as water, supercritical fluids, Bio-based solvents, ionic liquids (ILs), and Deep Eutectic solvents (DES) have been reported. Ethyl lactate (EL) has been approved by the FDA (USA) for use in foods, and is considered as bio-based solvent (Pereira 2014; Vovers 2017). Therefore, these features have been considered by natural product chemists to extract and isolate secondary metabolites from terrestrial organisms, such as flavonoids, alkaloids, phenolics, terpenoids, and phenylpropanoids (Kua et al. 2016; Villanueva-Bermejo et al. 2017).

This study focuses on lichens, and their extraction, using ethyl lactate due to the scarcity of studies involving lichens and green solvents, specially ethyl lactate. Lichens produce compounds known as lichen substances, which include depsides, depsidones, depsones, lactones, anthraquinones, dibenzofurans, and pulvinic acid derivatives. These interesting organisms have displayed wide biological activity (Shukla et al. 2010; Lakatos 2011; Calcott et al. 2018).

The aim of our work is the comparison of the ultrasound-assisted extraction and maceration of lichen substances from *H. cirrhata* by UHPLC/HESI/orbitrap/MS/MS. In this context, ethyl lactate and methanol were used as extraction solvents

2. Results and discussion

From *H. cirrhata*, protolichesterinic acid (**1**), ergosterol peroxide (**2**) and 2,4-dihydroxy-3-hydroxymethyl-6-methylbenzaldehyde (**3**) were isolated and identified on the basis of their spectroscopic analysis (^{1}H-NMR). Both extracts showed the presence of compounds **1**-**3** with isolated yields almost similar (see Figure S1 in SM). This fact implies that ethyl lactate could replace the methanol extraction solvent in spite of the green solvent's higher cost, both in energy use and money (Pereira et al. 2011). Despite the huge differences of the solvating properties (Hildebrand and Hansen parameters) of ethyl lactate over methanol it was not possible to find a correlation from the point of view of isolation and solubility of lichen substances in those solvents. However, hexane and methanol have a negative impact on the environment and living beings. As an alternative strategy to overcome the use of toxic organic solvents for extraction, we agreed to use green solvents as ethyl lactate. This solvent is biodegradable, ozone friendly, non-corrosive, non-toxic and considered as a GRAS solvent (Pereira et al. 2011). Since no difference was observed in the isolation process, we decided to check the obtained extracts by maceration and ultrasound by UHPLC/ESI/MS/MS. The main advantage of this ultrasonic technique is the shorter extraction time of only 30 min, in contrast to the 24-72 h required by the maceration method. In the case of maceration with methanol, untargeted metabolomics tentatively identified 69 compounds from the methanolic extract, and 70 metabolites from ethyl lactate extract (for detail, see Table S1 in SM). Both solvents showed a comparable number of extracted compounds. Methanol was more efficient when extracting polyhydroxylated fatty acids, while ethyl lactate extracted more unknown compounds probably of phenolic nature more efficiently. This difference in the extraction index could be due to their distinct polarity

based on the dielectric constants (methanol: 32.6; ethyl lactate: 13.1) (Pereira et al. 2011; Kua et al. 2016). The qualitative analysis by UHPLC/ESI/MS/MS was performed based on their full MS and MS/MS fragmentation patters (Calla-Quispe et al. 2020). Our results indicate that the green solvent was more efficient for the extraction of unknown compounds than MeOH was. In this case, ethyl lactate showed more tendency to extract, due to its lower polarity, than methanol (Pereira et al 2011). No match was found for the unknown compounds based upon LC/MS/MS data and literature search.

Regarding biological activity, ethyl lactate extract showed better in vitro antioxidant activity than methanol extract. These activities were evaluated through in vitro analysis, such as DPPH, FRAP, ABTS (TEAC), and TPC (see Table S3 in SM) (Guerrero-Castillo et al. 2019), and compared to other lichen extracts (Fernandez-Moriano et al. 2016; Sieteiglesias et al. 2019).

Ethyl lactate extract (6.4%) showed a higher yield than methanol extract (4.6%; Table S3), of a similar range to the previously reported methanol extracts from *Hypotrachyna* species (Fernandez-Moriano et al. 2016; Sieteiglesias et al. 2019). Our results showed a high extraction yield for the green extract by using ultrasound correlated with their high phenolic content, unlike with the maceration process.

The antioxidant activities of the two extracts are shown in Table S3 (SM). Through the DPPH assay, ethyl lactate extract displayed higher DPPH radical scavenging activity (IC_{50}= 105.7 μg/mL) than methanol extract (IC_{50}=348.8 μg/mL). These differences on IC_{50} of each extract could be explained due to a greater presence of phenolic compounds in the green extract with the capacity to scavenger free radicals. FRAP assay (Guerrero-Castillo et al. 2019) for ethyl lactate extract (196.4 ± 0 μM TE/g) was higher than those of methanol extract (30.1 μM TE/g) to reduce Fe^{+3} to Fe^{+2}. Finally, both ethyl lactate and methanol extracts were subjected to an ABTS assay. Green extract exhibited a higher value (69.28 μg/mL) than methanol extract (81.92 μg/mL).

Previous reports have demonstrated the antioxidant activity of lichens and other biological activities. Some closer works were reported by Gomez-Serranillo group (Fernandez-Moriano et al. 2016; Sieteiglesias et al. 2019). They demonstrated that two methanolic extracts of H. formosa and Parmotrema perlatum from India exhibited neuroprotective activity and high antioxidant activity evidenced by ORAC, DPPH and FRAP assay. So far, no research group had used green solvents for the extraction of lichens substances. Several methods using green solvents had been used only on plants research, such as ethyl lactate, for the extraction of secondary metabolites (Kua et al. 2016; Villanueva-Bermejo et al. 2017; D'Archivio et al. 2018; Silva et al. 2019; Torres-Valenzuela et al. 2020). These results could support the use of ethyl lactate instead of traditional organic solvents in the extraction of lichen substances based on alternative solvents in Green Chemistry.

3. Conclusions

The use of green solvents in the extraction of natural products is vital to reduce a negative impact on the environment. We have shown that ultrasound-assisted extraction by using ethyl lactate as green solvent is comparable to the maceration-assisted

extraction using methanol regarding its phytochemical profile. In regards to the *in-vitro* antioxidant assays (DPPH, FRAP, ABTS and TPC) ethyl lactate extract showed higher activity than methanol extract did. This non-conventional technique, of using ethyl lactate as an extraction solvent, was simple, fast, effective, safe and comparable to conventional techniques, with the exception of biological activity. Finally, we highly consider green solvent's 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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