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Enhanced extraction and biological activity of 7-hydroxymatairesinol obtained from Norway spruce knots using aqueous solutions of ionic liquids†

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In the past few years, an increasing demand for therapeutic drugs obtained from natural products has been faced. Amongst the bioactive natural compounds with pharmacological properties, 7-hydroxymatairesinol (HMR) has been commercially available since 2006 as a nutritional supplement due to its anticarcinogenic and antioxidative properties. The extraction of HMR from biomass is usually performed with sequential soxhlet extractions using volatile organic solvents (*e.g.* acetone, and water–acetone and water–ethanol mixtures). To avoid the use of volatile organic solvents, aqueous solutions of analogues of glycine-betaine ionic liquids (AGB-ILs) were here studied as alternative solvents for the extraction of HMR from knots of Norway spruce trees. A response surface methodology (RSM) was used to optimize the extraction operational parameters, in which extraction yields up to 9.46 wt% were obtained with aqueous solutions of AGB-ILs at 1.5 M and 25 °C. Furthermore, the cytotoxicity of the IL aqueous solutions containing the HMR extract was assessed in a macrophage cell line, as well as their anti-inflammatory potential *via* reduction of lipopolysaccharide-induced cellular oxidative stress. It was found that the antioxidant and anti-inflammatory potentials of IL aqueous solutions enriched in HMR are more promising than the recovered HMR-rich solid extracts and that these solutions can be safely used in nutraceutical and cosmetic applications. These results bring new perspectives into the design of new integrated approaches for the extraction and direct application of naturally derived high-value compounds using adequate ILs, without requiring an additional step involving the target product recovery and solvent recycling.

Introduction

Lignans are a specific family of phenolic compounds (comprising a 2,3-dibenzylbutane skeleton) that can be found in different morphological parts of a wide variety of plant species.[1] Lignans and their derivatives possess a large number of relevant biological properties and thus have been used in nutraceutical and pharmacological applications.[2,3] Norway spruce (*Picea abies*) knots, *i.e.* the part of branches that is embedded in the stem, are an exceptional source of lignans (5–15 wt%), such as hydroxymatairesinol (HMR), matairesinol, α-conidendrin, conidendrinic acid, isolariciresinol, secoisolariciresinol, liovile, picearesinol, lariciresinol, and pinoresinol.[2,4] HMR represents 65–80% of the total lignan content,[5] and although it is found in several biomass sources, such as wheat, triticale, oat, barley, millet, corn bran, and amaranth whole grain,[2] Norway spruce knots are a particularly attractive source of HMR. Moreover, these knots can be separated from wood chips at pulp mills by sedimentation in water.[6] HMR (Fig. 1i) occurs naturally in Spruce knots as a mixture of two stereoisomers differing in the stereochemistry of C-7, namely (7*R*,8*R*,8′*R*)-(−)-allo-hydroxymatairesinol (HMR1) and (7*S*,8*R*,8′*R*)-(−)-7-hydroxymatairesinol (HMR2). The most abundant isomer is HMR2, usually present in a 3 : 1 HMR2 : HMR1 ratio.[7]

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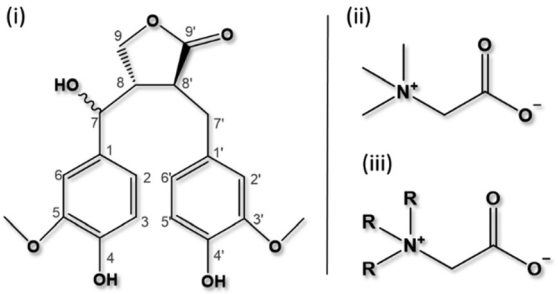

Fig. 1 (i) Chemical structures of hydroxymatairesinol (HMR), (ii) betaine and (iii) betaine analogues.

HMR has attracted a lot of attention since it is a direct metabolic precursor of the mammalian lignan, enterolactone (ENL), presenting therefore promising biological properties.[5] In fact, the daily intake of HMR increases the blood levels of ENL with proved benefits in the prevention of breast and prostate cancers, in menopause, and in heart diseases; additionally, HMR displays a strong antioxidant activity.[5] Due to its valuable properties, HMR has been commercially available as a nutritional supplement since 2006.[6]

Currently, HMR is extracted from knots using volatile organic solvents, such as acetone, and mixtures of ethanol or acetone with water.[2] Others methods/solvents for the extraction of lignans have been reported, including supercritical fluid extraction, pressurized liquid extraction, microwave-assisted extraction, and ultrasonic-based extraction.[2] These methods require, however, the use of sophisticated equipment and are highly energy-consuming.[8]

To overcome some of the concerns associated with the use of volatile organic compounds, either regarding their environmental footprint or when used for the extraction of target compounds envisioned for human consumption, ionic liquids (ILs) and their aqueous solutions have emerged as promising solvents.[9] For instance, Bioniqs Ltd[10] (UK) has proposed the use of ILs as alternative solvents for the extraction of artemisinin from *Artemisia annua*. Chowdhury *et al.*[11] suggested the application of pure ILs for the extraction of ellagic and gallic acids, pyrocatechol and catechin, from *Acacia catechu* and *Terminalia chebula*. In addition to pure ILs, which usually display high viscosity and melting points above room temperature, more recently, aqueous solutions of ILs have been investigated.[9] In addition to the use of a greener and lower cost solvent, *i.e.* water, aqueous solutions of ILs display a lower viscosity, enhancing thus the mass transfer and reducing the energy consumption.[9] Furthermore, IL aqueous solutions have been described as more efficient and selective solvents.[12] The high efficiency achieved using IL aqueous solutions has been related with the ability of ILs to disrupt the biomass structure;[9] yet, a more sound explanation, and particularly when dealing with aqueous solutions, depends on the enhanced solubility of biomolecules in aqueous solutions of ILs when compared with their solubility in the respective pure solvents. The solubility of biomolecules in aqueous solutions of ILs goes through a maximum along the IL concentration – a phenomenon that has been attributed to the ILs' ability to act as hydrotropes by the formation of IL–biomolecule aggregates.[13] Additionally, surface-active ILs can be used to increase the solubility of more hydrophobic target compounds, and have been described as promising solvents for the extraction of tanshinones from the Chinese herbal medicine Salvia miltiorrhiza bunge[14] and of piperine from black pepper.[15]

Although ILs present favourable environmental characteristics compared to conventional organic solvents, mainly due to their negligible vapour pressure under ambient conditions, their potential toxicity and low biodegradability should also be taken into consideration.[16–20] In fact, most studies reported in the literature regarding the use of ILs for the extraction of natural compounds are based on imidazolium ILs,[9] which are not the best choice when considering their cost, toxicity and biodegradability. Aiming at moving towards more sustainable solvents, the synthesis of ILs from non-toxic starting materials and their applications are nowadays major topics of research.[21] In addition to the well-studied cholinium-based ILs, those derived from glycine-betaine (GB-ILs) (Fig. 1ii) or its analogues (AGB-ILs)[22] (Fig. 1iii), a naturally occurring and low cost amino acid, can be seen as promising alternatives as extraction solvents, yet are still underexplored. In fact, there is only a single study reporting the use of GB-ILs (2-(dodecyloxy)-*N*,*N*,*N*-trimethyl-2 oxoethanaminium chloride) in the extraction of value-added compounds from biomass.[15] Glycine-betaine can be found in sugar beet molasses (up to 27 wt%), after the extraction of saccharose, and is still an underexploited by-product of the sugar industry.[22] Furthermore, glycine-betaine and their derivatives are currently used as food supplements,[23] as well as in cosmetic lotions and formulations.[24]

Taking into account the properties of HMR and advantages of the AGB-ILs described before, we propose here the use of aqueous solutions of AGB-ILs as alternative solvents for the extraction of HMR from Norway spruce knots by solid–liquid extraction at low temperatures (25 °C). An initial screening of several ILs was conducted, in which some well-studied and commercially available imidazolium-based ILs were also considered for comparison purposes. A response surface methodology (RSM) was then used to optimize the extraction yield by means of operational conditions, namely IL concentration (C), solid–liquid ratio (S/L, biomass weight/solvent weight), and extraction time (t). All extractions were carried out at 25 °C to minimize the energy consumption. Enhanced extraction yields of HMR (up to 9.45 wt%) were achieved using aqueous solutions of AGB-ILs at 1.5 M, a solid–liquid ratio of 0.01, for 280 min at 25 °C. Furthermore, the biological properties of the aqueous IL solutions containing HMR, such as their antioxidant and anti-inflammatory activity and cytotoxicity, were assessed, showing that there is no need to recover the HMR from the extraction media and that these can be directly used in nutraceutical and cosmetic applications – a major advantage of the used systems given the complexity of recovering non-volatile aprotic ILs.

Results and discussion

Evaluation of different ILs under constant operational conditions

Several aqueous solutions of ILs were investigated to infer their ability for extraction of HMR from Norway spruce knots. A list of the studied ILs, including a definition of their acronyms, is provided as an endnote.‡ The chemical structure of the ILs considered is shown in the ESI.† For this initial evaluation, aqueous solutions of imidazolium-based and AGB-based ILs at 0.5 M were used, as well as water and acetone. Imidazolium-based ILs were considered since they are the most commonly used type of IL in the extraction of value-added compounds from biomass,[9] and thus for comparison purposes. The same operational conditions were maintained in all experiments, namely a biomass–solvent ratio of 0.10 and 0.02 and an extraction time of 180 min at 25 °C. The reported HMR extraction yields correspond to the percentage ratio between the weight of extracted HMR (1 and 2) and of dry biomass. The results obtained are depicted in Fig. 2 (detailed data are provided in the ESI†).

The obtained extraction yields of HMR with IL aqueous solutions range between 5.1 and 7.4 wt%, corresponding to higher yields than those achieved with water (4.4 wt%) or acetone (5.0 wt%), and showing the relevance of ILs in increasing the extraction yield of hydrophobic compounds, such as HMR (octanol–water partition coefficients (K_{ow}), $\log(K_{ow}) \approx 2.1$).[25] Furthermore, the extraction yield decreases with the increase of the solid–liquid ratio (less solvent for a given weight of biomass). The extraction yield of HMR also depends on the IL chemical structure, with the aqueous solutions of 1-butyl-3-methylimidazolium bromide and 1-butyl-3-methylimidazolium tosylate appearing as the best solvents, meaning that ILs composed of aromatic ions lead to higher extractions yields. Electron-rich aromatic π-systems can establish π···π and strong hydrogen-bonding interactions with target solutes such as HMR. These favourable interactions seem to be responsible for the slightly increased extraction performance of imidazolium-based ILs over AGB-ILs.

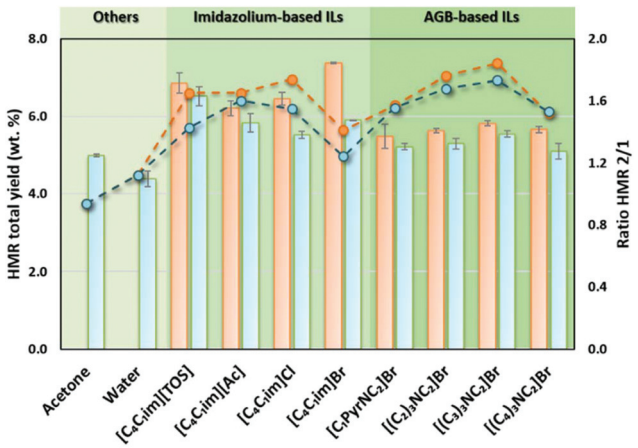

Fig. 2 Yield of HMR extracted from Norway spruce knots with different aqueous solutions of ILs (at 0.5 M), acetone and water (T = 25 °C, t = 180 min) for a S/L ratio = 0.10 (■) and for a S/L ratio = 0.02 (■). Ratio of HMR2/HMR1 for a S/L ratio = 0.10 (●) or for a S/L ratio = 0.02 (●).

‡Acronyms of ionic liquids: 1-butyl-3-methylimidazolium chloride, [C_4C_1im]Cl; 1-butyl-3-methylimidazolium tosylate, [C_4C_1im][TOS]; 1-butyl-3-methylimidazolium acetate, [C_4C_1im][AC], 1-butyl-3-methylimidazolium bromide, [C_4C_1im]Br; *N*-(1-methylpyrrolidyl-2-ethoxy-2-oxoethyl)ammonium bromide, [C_1PyrNC_2]Br; triethyl[2-ethoxy-2-oxoethyl]ammonium bromide, [$(C_2)_3NC_2$]Br; tri(*n*-propyl)[2-ethoxy-2-oxoethyl]ammonium bromide, [$(C_3)_3NC_2$]Br and tri(n-butyl)[2-ethoxy-2-oxoethyl]ammonium bromide, [$(C_4)_3NC_2$]Br.

As previously mentioned, HMR exists in Norway spruce knots in a mixture of two stereoisomers, usually present in a 3 : 1 HMR2 : HMR1 ratio.[7] The ratio of the two stereoisomers does not significantly change with the IL chemical structure (Fig. 2). However, considering the ratio of HMR2 : HMR1 of about 3 : 1, it seems that aqueous solutions of ILs are more effective for the extraction of HMR1 than of HMR2.

Based on the results discussed above and considering, on the one hand, that the imidazolium-based IL only slightly increased the extraction yield and, on the other hand, the lower cost and lower toxicity of [$(C_2)_3NC_2$]Br, this last IL was used to optimize the extraction operational conditions by a response surface methodology (RSM). Furthermore, the selection of short chain length derivatives is further supported by the well known fact that the toxicity of ILs increases with the increase of the alkyl side chain length.[20,26]

Optimization of the operational conditions by a response surface methodology

A RSM was used to optimize the operational conditions to improve the HMR extraction yield. This methodology allows the exploitation of the relationship between the response (extraction yield of HMR) and the independent variables/conditions which influence the extraction yield.[27] To this end, a 2^3 (3 factors and 2 levels) factorial planning was executed. The parameters studied were the extraction time (t, min), solid–liquid ratio (S/L ratio, weight of dried biomass per weight of solvent) and IL concentration (C, M). The influence of the three variables on the extraction yields of HMR is illustrated in Fig. 3. Variance analysis (ANOVA) was used to estimate the statistical significance of the variables and their interactions. The experimental points used in the factorial planning, the model equation, the extraction yield of HMR obtained experimentally and the respective calculated values, and the correlation coefficients obtained, as well as all the statistical analyses, are shown in the ESI.† The R^2 value obtained is 0.954 and the average relative deviation between the experimental and the predicted values is 0.33%. Thus, no significant differences were observed between the experimental and calculated responses, supporting the good description of the experimental results by the statistical models developed.

The statistical analysis shown in the ESI† and the data depicted in Fig. 3 show that the IL concentration is the most significant parameter, with a region of maximum extraction yield at moderate IL concentrations (1.5 M). In fact, this result is in good agreement with the ILs' ability to act as hydro-

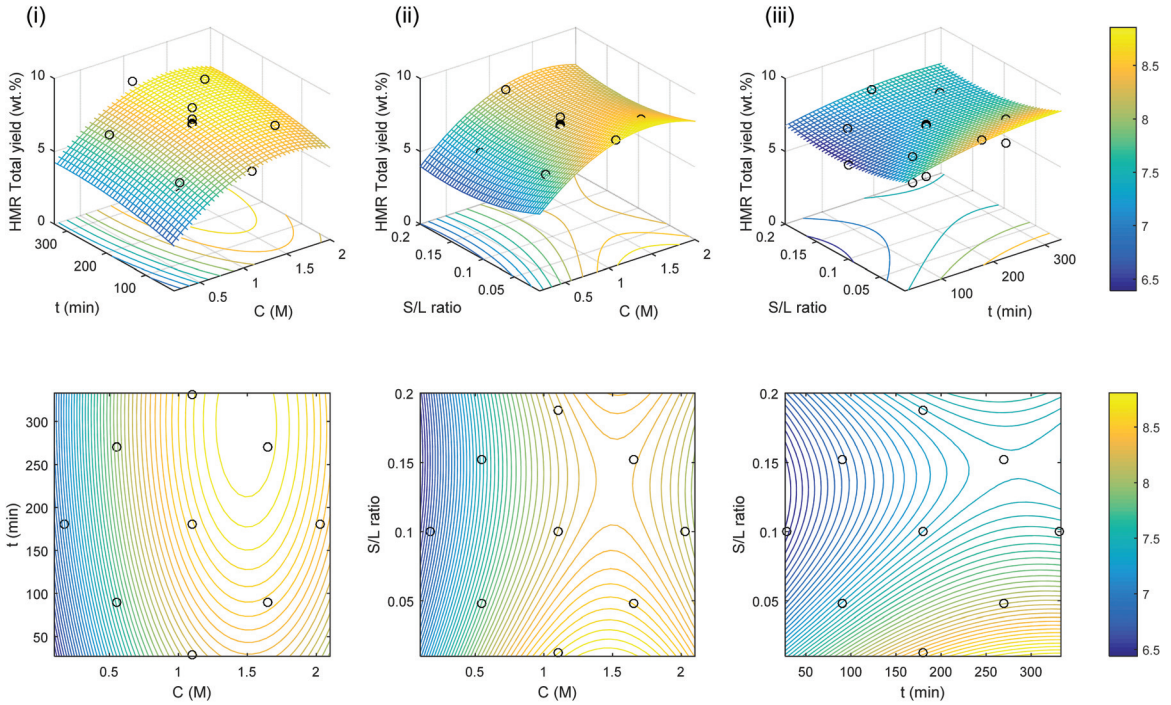

Fig. 3 Response surface (top) and contour plots (bottom) of the yield of total HMR extracted using aqueous solutions of $[(C_2)_3NC_2]Br$ at 25 °C with the combined effects of: (i) extraction time (*t*) and IL concentration (*C*); (ii) solid–liquid ratio (S/L ratio) and concentration (*C*); and (iii) solid–liquid ratio (S/L ratio) and extraction time (*t*).

tropes,[13] where a maximum in the solubility of biomolecules with the IL concentration is observed. The extraction time also influences the HMR extraction yield. In general, the amount of extracted HMR increases with time, reaching a maximum at 280 min. The solid–liquid ratio is also relevant, although with a behaviour that depends on other variables. Albeit all parameters have a significant effect on the extraction yields of HMR, as can be seen in the pareto chart shown in the ESI,† their significance decreases in the order: concentration of IL > extraction time > solid–liquid ratio. The optimized conditions found for the extraction of HMR at 25 °C are an IL concentration of 1.5 M, an extraction time of 280 min, and a solid–liquid ratio of 0.01, giving an HMR yield of 9.46 wt%. Regarding the evaluation of higher extraction times, as can be seen in the ESI,† under the other optimized conditions the change of the extraction time does not bring significant differences in the HMR extraction yield, falling within the associated uncertainties. These results highlight the potential of IL aqueous solutions to extract HMR from biomass under mild conditions. It should be also noted that this value is higher or in the same range of those obtained by soxhlet extraction (8.42 wt%), using longer extraction times (at least 360 minutes) and higher temperatures (60–80 °C).[2]

Solvent saturation and optimization of the extraction process from a critical perspective

First, the reuse of the biomass was investigated (details of the experimental procedure and results are described in the ESI†) to ascertain whether all the HMR present was extracted in a single extraction step. Although we could use a soxhlet extraction to infer the total amount of HMR present in the studied biomass, this approach is not feasible here since we obtained higher HMR extraction yields using the optimized operational conditions with IL aqueous solutions (extraction yield of 9.46 wt% *versus* 8.42 wt% obtained by soxhlet extraction with volatile organic solvents). Therefore, the only way to address the total amount of HMR present in the biomass was to use several "fresh" and consecutive aqueous solutions of ILs, aiming at avoiding limitations in the extractions that could arise from the IL aqueous solution saturation. This type of experiment allowed us to conclude that the studied biomass contains *ca.* 10.5 wt% of HMR, as discussed below. As expected, when a lower solid–liquid ratio is used, the complete extraction of HMR from knots is almost achieved. However, to maximise the cost-efficiency and sustainability of the developed method, it is necessary to saturate the IL solution on the target compound, and this variable was not considered in the multifactorial optimization. Thus, from a critical perspective, the use of a higher solid–liquid ratio is a better approach since the IL aqueous solution saturation is faster achieved, although in this approach a small amount of HMR (2–3 wt%) still remains in the biomass and is lost. Even so, a lower quantity of solvent is employed, resulting thus in a lower consumption of solvents and in more sustainable extraction technologies. Envisaging the industrial application of the process under study, it should be economical and fast; a solid–liquid ratio of

0.10 is the most advisable and was chosen in the subsequent studies.

Aiming at developing a more cost-effective and more environmentally friendly extraction technique, the reusability of the extraction solvent without any pre-extraction step was also investigated. To this end, the 1.5 M $[(C_2)_3NC_2]Br$ aqueous solution was used in six successive extractions under the following operational conditions: a solid–liquid ratio of 0.1 for 280 min at 25 °C. After each extraction, the solid–liquid mixture was filtered and the aqueous solution was reused with a new batch of Norway spruce knots. Remarkably, in the first five samples, *ca.* 7 wt% of HMR was extracted in each step (see the ESI†), with the overall solution reaching a HMR total concentration around 5 times higher (36.3 g L^{-1}). This means that the IL aqueous solution was far from saturation and can be reused at least five times without any loss in extraction performance. We also determined the HMR (standard) solubility at 25 °C in a solution of $[(C_2)_3NC_2]Br$ at 1.5 M, where a saturation value of 37.5 g L^{-1} was found, supporting the experimentally demonstrated use of the IL aqueous solutions for five consecutive extraction steps. Detailed results and details of the experimental procedure are described in the ESI.†

In spite of the high efficiency of IL aqueous solutions for the extraction of bioactive compounds, the non-volatile nature of aprotic ILs represents a major drawback when envisaging the target product recovery since a simple evaporation step cannot be applied.[9,28] There are some proposed alternatives for the recovery of the target products or IL removal, such as back-extraction using organic solvents,[12,29] distillation of the compounds,[30] induced precipitation with anti-solvents,[31,32] and adsorption on macroporous materials[33] or anion-exchange resins;[34] yet, some of these are laborious and costly.[9] Accordingly, we started by studying the possibility of precipitating HMR from the IL-aqueous solutions using potassium acetate (data shown in the ESI†), often used to form an adduct with HMR.[35,36] By applying this process we were able to recover 75% of the extracted HMR with a purity of 65%. However, considering that in this work, we used more biocompatible ILs derived from glycine-betaine, which are currently used as food supplements,[23] and in cosmetic formulations,[24] and although a recovery step by the induced precipitation of HMR could be feasible as well as the IL recycling, we evaluated the possibility of using directly the aqueous IL HMR-rich extracts without the need for an additional isolation/recovery step. To appraise this possibility, the biological properties (antioxidant an anti-inflammatory activity and cytotoxicity) of the IL-HMR-extract aqueous solutions were further assessed.

Biological activities of the IL aqueous solutions enriched in HMR and respective extracts

The antioxidant activity of the aqueous solutions of ILs containing HMR, as well as of HMR extracts recovered from the IL aqueous solutions or organic solvents, was determined using the 2,2-diphenyl-2-picrylhydrazyl hydrate (DPPH) radical scavenging assay, with ascorbic acid as the reference. The antioxidant activities of the HMR extracts obtained with acetone,

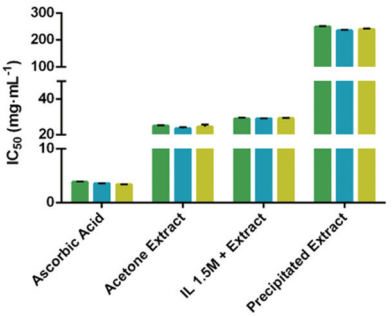

Fig. 4 IC_{50} values (mg mL^{-1}) after 0.5 (■), 1.5 (■) and 2 h (■) of exposure to DPPH.

of the precipitated HMR-rich extract from the aqueous IL solutions, and of the $[(C_2)_3NC_2]Br$ 1.5 M aqueous solution containing HMR are shown in Fig. 4 and in the ESI.† The antioxidant activity was also evaluated for the aqueous IL solution as a control. The extracts obtained by acetone extraction and aqueous IL solutions show IC_{50} values in the same range (23.5 ± 0.8 and 24.3 ± 1.3 μg mL^{-1} at 1.5 h, respectively), and are not influenced by the presence of the IL (as confirmed by null IC_{50} values of the aqueous solution of IL used as the control). On the other hand, the precipitated extract from the IL aqueous solution presents the lowest antioxidant activity, further supporting the usefulness of directly using IL aqueous solutions containing HMR instead of recovering it, which also implies an additional step in the process. HMR is the major phenolic compound extracted from the selected biomass; yet, other phenolic compounds commonly found in Norway spruce knots[37] can also be extracted contributing to the higher antioxidant activity observed. Due to their lower amounts, they may not precipitate by the addition of potassium acetate. Moreover, according to Willför *et al.*[38] some synergistic effects may occur in less pure extracts, thus leading to a higher antioxidant activity.[38]

All HMR-rich extracts display a lower antioxidant capacity than ascorbic acid (Fig. 4); however, when compared to other phenolic extracts, such those obtained from *Terminalia chebula* (IC_{50} of 14 μg mL^{-1}),[39] *Cynara cardunculus* L. var. *altilis* (IC_{50} of 34.3 ± 2.3 μg mL^{-1}),[40] and *Malpighia emarginata* DC (405 to 1744 mg AAE per 100 g of fruit),[41] we obtained a high antioxidant activity (21.8 ± 7.0 μg AAE per mg of extract).[2,3,6]

In order to address the potential applications of the IL aqueous solutions containing HMR, their cytotoxicity and cellular antioxidant/anti-inflammatory activity were determined. A macrophage cell line was used since these cells are central players in inflammatory responses. When the cells are stimulated with a strong inflammation inducer, bacterial lipopolysaccharide (LPS), this leads to the production of reactive oxygen species (ROS) causing oxidative stress. This can be used as a tool to assess the cellular antioxidant activity of the produced HMR-rich extracts. First, the cell viability was studied at different IL and extract concentrations (Fig. 5i). For the HMR extract in aqueous IL solutions there is a decrease in the cell viability for concentrations of HMR above

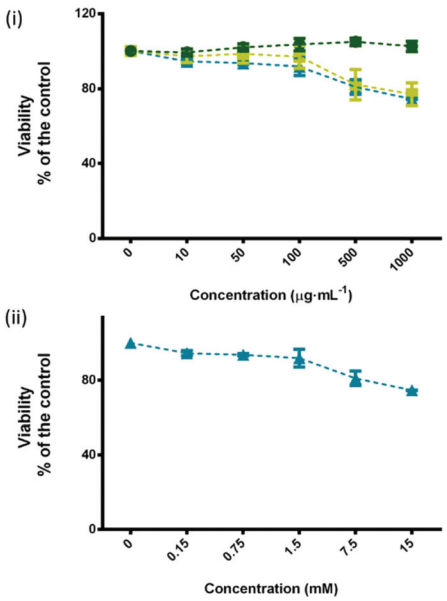

Fig. 5 (i) Cell viability when exposed to increasing concentrations of pure IL (▲), HMR in the IL–water solution (■) and the precipitated HMR-rich extract (●). (ii) Cell viability when exposed to increasing concentrations of the IL (▲) used for the extractions of HMR.

100 μg mL^{-1}. This toxicity results from the presence of the IL since the precipitated HMR-rich extract does not affect the cell viability at higher concentrations. Cell viability assays were also carried out for aqueous solutions of $[(C_2)_3NC_2]Br$ at different concentrations (Fig. 5ii), showing that concentrations of IL above 1.5 mM start to affect the cells. Nevertheless, and according to the US Food and Drug Administration (FDA), in human consumption the HMR amount cannot exceed 500 μg mL^{-1}, and at concentrations of 1000 μg mL^{-1}, HMR causes an increase in adenylate kinase (AK) activity which is responsible for the loss of cell membrane integrity.[42] Therefore, a daily maximum of 1 mg kg^{-1} of body weight consumption is recommended.[42]

The effect of the precipitated HMR-rich extract, $[(C_2)_3NC_2]Br$, and HMR extract in aqueous $[(C_2)_3NC_2]Br$ (all at 100 μg mL^{-1}) on cellular oxidative stress is shown in Fig. 6. The images were obtained through fluorescence microscopy. Cell nuclei stained with Hoechst 33342 appear in blue, while cellular ROS would appear in green through the H2DCFDA probe. In general, none of the samples shows oxidative damage in the cells at the tested concentrations, as shown by the lack of green colouration in the second and third lines of Fig. 6.

In a second approach we addressed the capacity of the precipitated HMR and the HMR in IL aqueous solutions to reduce LPS-induced oxidative stress (Fig. 7). Overall, HMR has indeed considerable cellular antioxidant activity, reducing the LPS-induced production of ROS, as shown by the decrease of intensity in the cells' green colour, when compared with the cells stimulated only with LPS. The aqueous solution of $[(C_2)_3NC_2]Br$, on the other hand, does not display significant antioxidant activity. These results are in agreement with the results

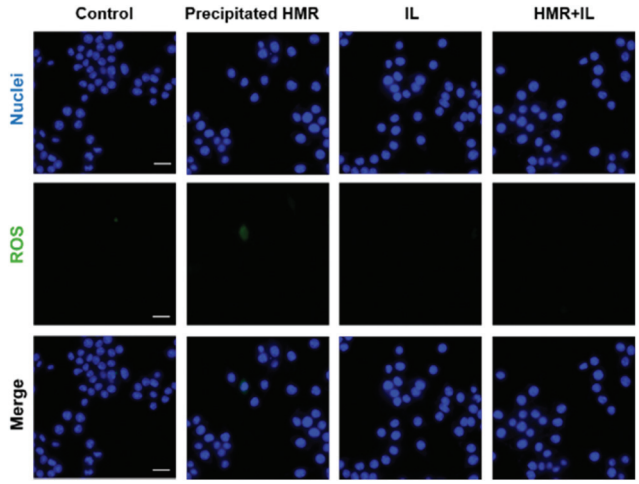

Fig. 6 Macrophage cellular oxidative stress in the presence of different samples of HMR and pure IL. Scale bar: 20 μm.

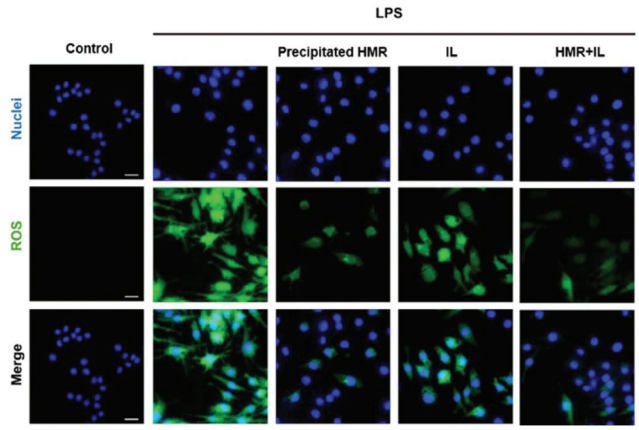

Fig. 7 Cellular oxidative stress in LPS-stimulated macrophages treated with the precipitated HMR, the HMR in IL solution and pure IL. Scale bar: 20 μm.

obtained in the DPPH assays discussed above. Moreover, commercially available HMR has been tested in the ROS induced oxidative stress[5] in THP-1 acute monocytic leukaemia cells, with similar results, showing that with 100–300 μM (0.37–1.12 mg mL^{-1}) of HMR there is a significant reduction of ROS levels. Thus, the HMR extracts in aqueous solutions of $[(C_2)_3NC_2]Br$ show a similar behaviour to a commercially available extract[5] with a reported purity of 91%, confirming their potential for direct application in nutraceutical formulations.

Our studies on the cytotoxicity of the $[(C_2)_3NC_2]Br$ aqueous solutions support their biocompatible nature and high antioxidant activity of the HMR rich extracts, in the range of concentrations used to perform efficient extractions. Therefore, the HMR-rich extracts obtained can be used directly in cosmetic, nutraceutical and pharmaceutical applications without requiring an additional step of HMR recovery and IL recycling.

Finally, the equation suggested by Passos *et al.*[9] was used to evaluate the scale-up viability of the proposed extraction

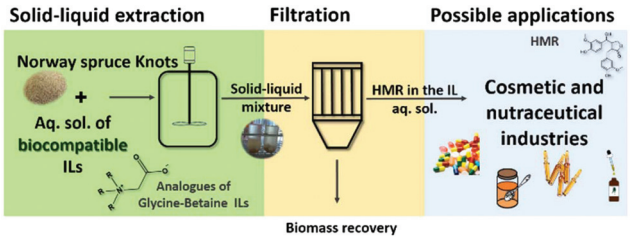

Fig. 8 Schematic overview of the proposed process for the extraction of HMR from Norway spruce knots using aqueous solutions of AGB-based ILs.

process. The proposed equation[9] is a simplified model that relates the return associated with the extraction of a particular value-added compound when ILs are used as extraction solvents. In our case, the cost of the IL is the main factor in the final product cost, mainly because we do not propose the recycling of the IL and instead it is present in the final HMR-rich formulations. Therefore, for this process to be economically viable it is necessary to employ ILs with a cost lower than 11 € kg^{-1}, which is perfectly achievable if the process is scaled up and industrial reagents are acquired[43] (*cf.* the ESI† for detailed information). In summary, the cost-efficient extraction method presented here, summarized in Fig. 8, using aqueous solutions of AGB-ILs instead of volatile organic solvents, opens new perspectives for the recovery of HMR from biomass sources, while envisaging their widespread use (at a lower cost) in the most diverse food, nutraceutical and pharmaceutical applications. It should be, however, highlighted that after the extraction of HMR with IL aqueous solutions, the remaining biomass can be further used in other applications within an integrated biorefinery approach.

Conclusions

HMR and its derivatives have been extensively investigated for nutraceutical, cosmetic and pharmaceutical applications. Aiming at developing a more sustainable approach for HMR extraction from biomass, we here demonstrate the outstanding performance of IL aqueous solutions as alternative solvents. A RSM was applied to assess the significance of the most important factors, namely the IL concentration, extraction time and solid–liquid ratio (weight of biomass per volume of solvent). The best results were obtained with HMR extraction yields up to 9.45 wt%, obtained at 25 °C with an aqueous solution of 1.5 M of $[(C_2)_3NC_2]Br$, a solid–liquid ratio of 0.01, and for 280 min. This yield is higher than that obtained with volatile organic solvents, using higher temperatures and with longer extraction times. Finally, the HMR extracts obtained, especially the HMR extract in $[(C_2)_3NC_2]Br$ aqueous solution, present a high antioxidant activity, and thus ROS damage inhibition *in vitro*, without being harmful to the cells. These results prove that aqueous solutions of ILs containing HMR have the potential to be safely used in cosmetic and nutraceutical applications. Furthermore, some estimated costs to implement the process on a large scale were provided, demonstrating its feasibility. In summary, this study brings new perspectives on the use of aqueous solutions of appropriate ILs to replace the commonly used volatile organic solvents for the extraction of lignans and similar components from biomass, without requiring an additional step of product recovery/isolation – a major drawback when dealing with non-volatile ILs.

Experimental

Materials

Norway spruce knots (90–95% actual knot material) were separated from over-thick industrial wood chips by sedimentation in water after drying and grinding.[6] Before extraction, knots were immersed in liquid nitrogen and milled to pass through a 40–60 mesh sieve. A reference sample of HMR (HMR1/HMR2, >96% purity) was prepared by precipitation with potassium acetate and flash silica chromatography of a spruce knot extract.

The solvents used for the extraction included distilled water, acetone (purity ≥99.99 wt%) from VWR chemicals and aqueous solutions of ILs. The ILs $[C_4C_1im]Cl$, $[C_4C_1im][Tos]$, $[C_2C_1im][AC]$ and $[C_4C_1im]Br$ were purchased from Iolitec. The ILs $[C_1PyrNC_2]Br$, $[(C_2)_3NC_2]Br$, $[(C_3)_3NC_2]Br$ and $[(C_4)_3NC_2]Br$ have been synthesized and characterized by us according to the literature.[44] All IL samples were dried for at least 24 h under vacuum and at a moderate temperature (≈50 °C) before use. Their purities were further confirmed by ^1H and ^{13}C NMR spectra and shown to be ≥98–99 wt%. The water content of all ILs, after the drying procedure, was <1000 ppm as determined by Karl-Fischer titration.

The mobile phase used in the HPLC analysis was composed of methanol (purity ≥99.99 wt%) from Fisher Chemical, and ultra-pure water (purity ≥99.99 wt%) from Merck, both of HPLC grade. Syringe filters (0.45 μm) acquired from GE Healthcare, Whatman, were used.

2,2-Diphenyl-2-picrylhydrazyl hydrate (DPPH) was acquired from Sigma-Aldrich and methanol (HPLC grade) from VWR. LPS from *Escherichia coli* (serotype 026:B6), penicillin and streptomycin were obtained from Sigma Chemical Co. (St Louis, MO, USA). Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) and Hoechst 33342 were bought from Fisher Scientific (Leicestershire, UK).

Methods

AGB-based IL synthesis

The synthesis of AGB-ILs was carried out according to a previously reported procedure.[44] Briefly, a solution of trialkylamine (0.55 mol) in ethyl acetate (150 mL), cooled to 4 °C, was added dropwise to 2-bromoacetic acid ethyl ester (0.22 mol). The mixture was then stirred at room temperature for 1 day.

The precipitate produced during the reaction was filtered and washed with ethyl acetate. Recrystallization of the residue by ethanol/ethyl acetate (10 : 90, v : v) resulted in a white powder that was filtered, washed with ethyl acetate and dried under vacuum. The chemical structures of the synthesised ILs, reaction yields, and some of their properties can be found in the literature.[44]

HMR extraction

Solid–liquid extractions of HMR from Norway spruce knots were carried out using a commercial Carousel from Radleys Tech that was able to both stir and maintain the temperature within ±0.5 °C, and was protected from light. In all experiments the stirring was kept constant at 1000 rpm. All aqueous solutions containing known amounts of ILs and biomass were prepared gravimetrically within $\pm 10^{-4}$ g. Several concentrations of IL, and different solid–liquid ratios, and times of extraction were ascertained. For comparison purposes, water and acetone were used as solvents for the extractions. After the extraction step, water or acetone or IL aqueous solutions were separated from the biomass by centrifugation (at 4000 rpm for 30 minutes using an Eppendorf centrifuge 5804).

HPLC-DAD

The quantification of HMR in each solution was carried out by HPLC-DAD (Shimadzu, model PROMINENCE). HPLC analyses were performed with an analytical C18 reversed-phase column (250 × 4.60 mm), Kinetex 5 μm C18 100 Å, from Phenomenex. The mobile phase consisted of 40% of methanol and 60% of ultra-pure water. The separation was conducted in isocratic mode, at a flow rate of 0.8 mL min^{-1} and using an injection volume of 10 μL. DAD was set at 280 nm. Each sample was analysed at least in duplicate. The column oven and the autosampler were operated at a controlled temperature of 30 °C. Calibration curves were prepared using the pure HMR sample dissolved in methanol. HMR1 and HMR2 display a retention time of 8.4 and 7.8 minutes, respectively, and the sum of their areas was used for calibration purposes. The HMR1/HMR2 ratio was calculated from the relative proportion of the two chromatographic peaks.

Response surface methodology (RSM)

A RSM was applied to simultaneously analyse various factors (operational conditions) and to identify the most significant parameters which enhance the HMR extraction yield. In a 2^k surface response methodology there are k factors that contribute to a different response, and the data are treated according to a second order polynomial equation:

$$y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} {X_i}^2 + \sum_{i<j} \beta_{ij} X_i X_j \quad (1)$$

where y is the response variable and β_0, β_i, β_{ii} and β_{ij} are the adjusted coefficients for the intercept, linear, quadratic and interaction terms, respectively, and X_i and X_j are independent variables. This model allows the drawing of surface response curves and through their analysis the optimal conditions can be determined.[27] $[(C_2)_3NC_2]Br$ was selected to perform a 2^3 factorial planning with the aim of optimizing the extraction yield of HMR. The 2^3 factorial planning used is provided in the ESI.† The obtained results were statistically analysed with a confidence level of 95%. Student's t-test was used to check the statistical significance of the adjusted data. The adequacy of the model was determined by evaluating the lack of fit, the regression coefficient and the F-value obtained from the analysis of variance (ANOVA). The StatSoft Statistica 10.0© software was used for all statistical analyses, and Matlab 2015b, from MathWorks, was used for generating the response surfaces and contour plots.

Antioxidant activity assays

The antioxidant activities of the different HMR extracts were determined using the 2,2-diphenyl-1-picrylhydrazyl radical ($DPPH^{\bullet}$) scavenging assay. The principle of the assay is based on the colour change of the $DPPH^{\bullet}$ solution from purple to yellow, as the radical is quenched by the antioxidant.[45] This change in colour was monitored by visible spectroscopy at 517 nm. The antioxidant activity was expressed in IC_{50} values, defined as the inhibitory concentration of the extract necessary to decrease the initial DPPH radical concentration by 50%, as well as in g of ascorbic acid equivalents per kg of dry weight (μg AAE per mg of extract).[46] Taking into account the IC_{50} definition, a lower IC_{50} value reflects a better DPPH radical scavenging activity. Further details are given in the ESI.†

Cell culture

Raw 264.7, a mouse leukaemic monocyte macrophage cell line from the American Type Culture Collection (ATCC number TIB-71), was cultured in DMEM supplemented with 10% non-inactivated FBS, 100 U mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin at 37 °C under a humidified atmosphere of 95% air and 5% CO_2. Cells were routinely inspected for morphological changes by microscopy observation and subcultured every two days until passage 25.

Cell viability assays

In order to investigate the biocompatibility of the HMR extracts, their effect on macrophage viability/metabolic activity was assessed by a resazurin assay.[47] Briefly, 0.05×10^6 cells per well in a 96 well plate were exposed to indicated concentrations of the various HMR extracts for 24 h. Two hours before the end of exposure, a resazurin solution was added to each well to a final concentration of 50 μM. Absorbance was then read at 570 and 600 nm using a Synergy microplate reader (Biotek, Winooski, VT, USA).

In vitro cell viability and antioxidant activity assays

The potential antioxidant activity of $[(C_2)_3NC_2]Br$ and HMR extracts was addressed by their capacity to prevent LPS-induced oxidative stress in macrophages. Raw cells were plated at 0.05×10^6 per well of a μ-Chamber slide (IBIDI GmbH, Germany), allowed to stabilize overnight and then stimulated

with 1 μg mL^{-1} LPS for 16 h. The compounds to be tested were added 1 h prior to LPS stimulation. At the end of the incubation period, cells were washed three times with HBSS (in mM: 1.3 $CaCl_2$, 0.5 $MgCl_2$, 5.3 KCl, 138 NaCl, 0.44 KH_2PO_4, 4.2 $NaHCO_3$, and 0.34 Na_2HPO_4, pH 7.4) and then loaded with 5 μM H_2DCFDA and 0.5 μg mL^{-1} Hoechst in HBSS for 30 min at 37 °C in the dark. Cells were washed three times with HBSS, and analysed with an Axio Observer Z1 fluorescent microscope (Zeiss Group, Oberkochen, Germany) at 63× magnification.

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