Distillable ionic liquid extraction of tannins from plant materials†

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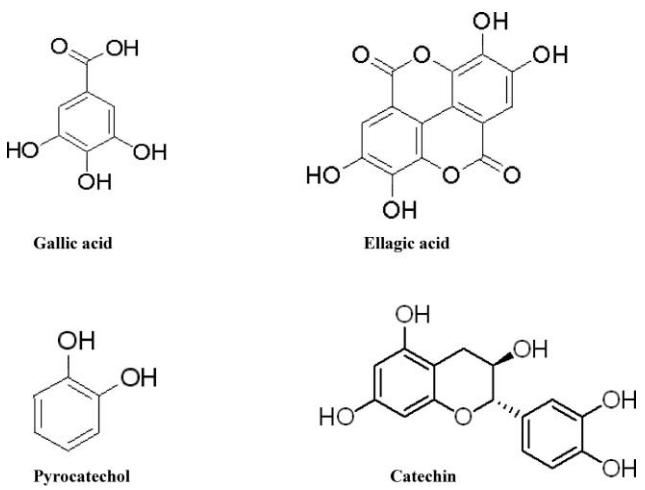
A distillable ionic liquid, *N*,*N*-dimethylammonium *N′N′*-dimethylcarbamate (DIMCARB), has been employed at room temperature to extract hydrolysable tannin materials from plant sources such as catechu (*Acacia Catechu*) and myrobolan (*Terminalia Chebula*). The extraction efficiencies (85%) were found be significantly higher compared to traditional extraction methods which utilise large quantities of water. Moreover, in the ionic liquid extraction method selectively higher concentrations of ellagic acid have been extracted from these raw materials. The results of hyde powder tests have revealed that the extracted materials have higher content of tannins compared to traditional extraction methods and this will be useful for tanning leather. Microbiological tests have shown that the ionic liquid extracted materials possess good shelf life and resist fungal growth.

Introduction

The term "tannin" has been used since the end of the eighteenth century to describe a family of water soluble organic substances present in plant extracts that effect the transformation of animal hide into leather. Vegetable tannins (Scheme 1) are water soluble phenolic rich compounds,[1] having molecular weights between 500 and 3000 Da, capable of binding water soluble proteins.[2] They can crosslink collagen through a number of hydrogen bonding sites.[3] Vegetable tannins can be classified into hydrolysable and condensed tannins.[4] Hydrolysable tannins contain either gallotannins or ellagitannins and on hydrolysis (by acids, bases or certain enzymes) produce glucose and gallic acid. In ellagitannins, one or more hydroxydiphenoyl residues are linked to glucose as a diester. This residue on hydrolysis yields ellagic acid. Condensed tannins are the polymers of a mixture of either flavan-3-ols or flavan-3,4-diols. They are often referred to as 'flavolans' and have become popularly known in recent times as proanthocyanidins. Catechin, gallocatechin and epi-gallocatechin are all precursors of condensed tannins. Tannins have been extensively used in the fields of pharmaceuticals, food processing and tanning processes.[5-7] The pharmacological properties of tannins have been investigated based on recent advances in the structural study of tannins in medicinal plants and various properties of tannins including antitumor, anticarcinogenic, antimicrobial and antiviral effects have been revealed.[8-11] It was also shown that tannin extracts have been found to be useful against controlling HIV activity.[12] The work of Chung *et al.* has shown that tannins and their precursors can be beneficial as well as detrimental to human health depending upon the concentration of the exposure.[13] These effects are also attributed to the interactions of tannins with certain biomolecules in the organism.



Scheme 1 Chemical structures of useful tannin materials.

In the leather industry, the hydrolysable vegetable tannin materials have also been used as a substitute for "chrome tanning". Nonetheless chromium (as Cr(III)) tanning remains the most popular tanning method in many parts of the world in terms of the properties of the leather produced. However the toxicity of chromium (both the trivalent and hexavalent forms) is a serious issue. The conventional chrome tanning method results in very poor utilisation of the chromium, thereby creating a highly toxic effluent.[14] Although the leather industry uses the trivalent chromium (basic chromium sulfate), considerable quantities of hexavalent chromium have been found both in the leather and the effluent.[15] The post tanning processes, often carried out at high pH, typically involve conditions capable of oxidising Cr(III) to Cr(VI).[16] Cr(III) is also converted to the hexavalent state by light or heat in the presence of the oxidised fats present in leather.[17] The +6 state is highly toxic, mutagenic

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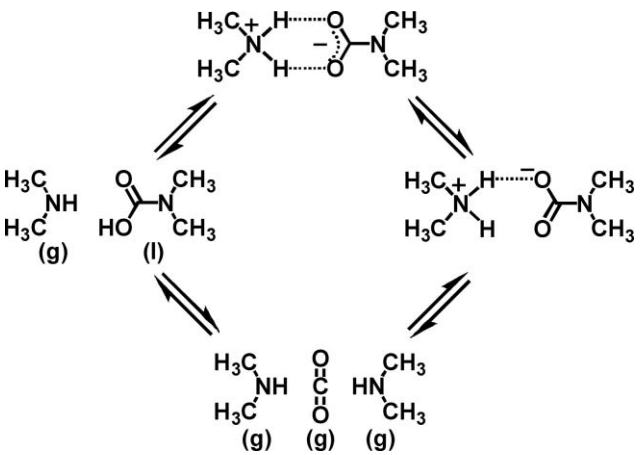
and carcinogenic, causing damage to skin, mucous membrane and respiratory tract.[18] The presence of residual concentrations of chromium in leather have resulted in the banning of chrome tanned leather in the European Union, however it is still in widespread use throughout other parts of the world. The United Nations Industrial Development Organization (UNIDO) has recommended that chrome tanning be avoided and the use of vegetable tannins as an alternative.[19] Hence, there is a growing and urgent need to provide inexpensive vegetable tanning agents from renewable resources.

Conventional state of the art of technologies for the extraction of hydrolysable vegetable tannin materials require[20] relatively harsh conditions, such as high temperature (70 °C), use of counter current techniques and high solvent/solid weight ratio, but often suffer from poor extraction efficiencies. Hence there is a need for an alternative technology that is more efficient both in terms of yield and energy cost in order to allow the production of tannins at a lower cost. In this study, distillable ionic liquids have been employed at room temperature to improve extraction efficiencies and obtain selective extraction of gallotannins. The IL used belongs to the protic class of ionic liquids[21-22] that are 'distillable'. This particular ionic liquid is formed by combining CO_2 and dimethylamine in an approximately 1 : 2 ratio. A two-step proton transfer takes place to form the dimethyl ammonium ion and the dimethylcarbamate ion (DIMCARB), as shown in Scheme 2. The attractive feature of DIMCAB is that it can be distilled at around 45 °C, or lower at reduced pressure, during which process it reforms CO_2 and dimethylamine. DIMCARB has been employed in organic synthesis and electrochemical applications.[23-24]



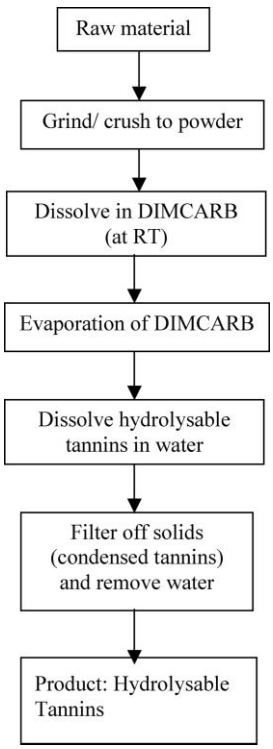
Scheme 2 Dynamic equilibria in the DIMCARB system.

The main objective of the present study was to use this *N*,*N*-dimethylammonium *N*′*N*′-dimethylcarbamate (DIMCARB) ionic liquid as an alternate solvent for extracting vegetable tannin materials from species such as catechu (*Acacia Catechu*) and myrobolan (*Terminalia Chebula*) at high efficiency and with lower water utilisation. The advantage of using this ionic liquid is its easy removal from the system by a low temperature distillation to allow reuse with fresh material. These raw materials were chosen because of their potential to produce a range of tannins having applications in medicines, tanning, dyeing and adhesives. In fact these materials are used as edible nuts and consumed for different reasons including as traditional medicines. Another objective was to investigate the selectivity of the extraction process. Microbiological assays of the extracted materials (both as a raw powder and applied onto a collagen substrate) have also been carried out to assess the stability of the materials.

Results and discussion

Extraction of tannins

The extraction method is summarised in Scheme 3. The DIMCARB extracts the soluble tannins, leaving a small residue of insolubles. It was found to be difficult to filter this mixture efficiently and therefore in this work it was found preferable to remove the DIMCARB and then dissolve the soluble tannins in water for recovery. A larger scale process may be able to directly filter the DIMCARB solution.



Scheme 3 Flowchart for extracting vegetable tannin materials.

In order to investigate the extraction at different time intervals the reaction was terminated at known time intervals and the results are shown in Fig 1. The results show higher extraction for both catecheu and myrobolan materials using DIMCARB ionic liquid in shorter intervals (for instance, a maximum extraction of 80% in 6 h was observed for catecheu material and then levels off to 85% in 24 h) *whereas* only 64% was obtained from the conventional extraction of catecheu material after 24 h of reaction time.

A 16 hour series of extractions was then carried out in triplicate to calculate the average extraction efficiencies of both the DIMCARB and water extracted products (Table 1). The results show that the products of DIMCARB extraction show higher extraction efficiencies compared to the water extraction.

Table 1 Extraction efficiency of DIMCARB ionic liquids after 16 hours

Product	Extraction efficiency (%)			
	Run 1	Run 2	Run 3	Average (± s.e.m.[a])
DIMCARB extracted product from catecheu	84	86	86	85 ± 1
DIMCARB extracted product from myrobolan nut	73	74	78	75 ± 2
Water extracted product from catecheu	61	65	66	64 ± 2
Water extracted product from myrobalan nut	51	51	54	52 ± 1

[a] s.e.m. = standard error in the mean.

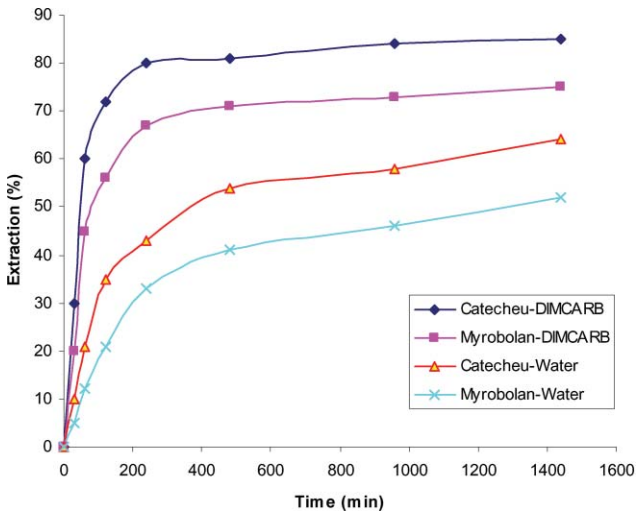


Fig. 1 Comparison of extraction of catecheu and myrobolan plant materials using DIMCARB and water.

The present DIMCARB process of tannin extraction can be made more energy efficient by dissolving the raw materials in DIMCARB at room temperature, filtering to remove undissolved constituents and evaporating DIMCARB at low temperature. The extract is then preserved at room temperature and at a desired time the extract is dissolved in water to obtain the hydrolysable tannins in aqueous solution. This aqueous tannin solution can be directly used for the leather tanning process.

The higher extraction in ionic liquid could be due to the high solvency towards the phenolic components of the extractants and/or the ability of the IL to deprotonate them as part of the dissolution reaction. The DIMCARB liquid can be thought of as a pure acid/base buffer in the sense that it has high capacity to both absorb and release protons. The solubility of the extracted products was determined at room temperature and these are summarised in Table 2. The pH of the aqueous solutions was also measured (ESI S1†); the data shows that the DIMCARB extracted products are more basic compared to the water extracted products and that therefore are likely to have extracted into this medium in their conjugate base form and have been recovered as the dimethylammonium salt.

Table 2 Solubility of the extracted products

Solvent	Catecheu (DIMCARB)	Catecheu (water)
Water	Mostly soluble	Completely soluble
Methanol	Partly soluble	Partly soluble
Ethanol	Slightly soluble (after 24 h)	Slightly soluble

HPLC analysis

The extracted products were analysed by HPLC for identification. Known mixtures of standards (gallic acid, ellagic acid, (+) catechin hydrate, pyrocatechol) were injected into the HPLC column and their retention times were used for the identification (Table 3) of products in the extracted samples; the chromatograms showing the elution of standard samples are shown in ESI S2†. Note that the elution solvent contains trifluoroacetic acid to ensure that the extracts are converted to their acid forms. A typical chromatogram of myrobolan samples in ionic liquid and water are shown in ESI S1 and S4†. A quantification was also made by taking the known concentration of the standards and the extracted samples to calculate the exact amount of each compound present in the extracted samples. The results are given in Table 4. The results show higher extraction of ellagic acid from both of the raw materials for ionic liquid extracted samples compared to water extracted. In addition, in the case of the catecheu raw material, the extraction of catechin hydrate was substantial when ionic liquid was used as a solvent. This clearly shows the efficiency of the ionic liquid over water in selectively extracting the components from the vegetable tannin material.

LC-MS analysis

The ionic liquid and water extracted samples were subjected to LCMS analysis to confirm the identity of the separated samples. The results are shown in ESI S5† confirming the presence of ellagic acid, pyrocatechol and catechin hydrate in the DIMCARB extracts of catecheu and myrobolan. In the case of water extracted samples, gallic acid was not observed for catecheu samples; in other cases, although the parent ions of ellagic acid, catechin hydrate and pyrocatechol were observed, they were present in relatively lower concentrations as evidenced by the HPLC analysis.

Microbial assay

The results of microbial assay for the raw materials, and water and ionic liquid extracted samples, are summarised in ESI S6†. It shows that both the catecheu raw material samples had bacterial and fungal contaminants; the myrobolan nut samples had low levels of such contaminants. In the case of the catecheu (DIMCARB extract) samples there was no colony growth in nutrient agar and hose blood agar medium. However, there was a low level of bacterial growth in malt extract agar. The DIMCARB extracted product, in spite of the water washings, retains the dimethylammonium cation in the recovered salt and

Table 3 Retention time and % area of mixed pure standards and extracted vegetable tannin samples

Sample	Retention time (min)	% Area
Gallic acid (pure)	5.7	66
Ellagic acid (pure)	13.9	3
Catechin hydrate (pure)	9.9	17
Pyrocatechol (pure)	12.1	11
Catecheu (DIMCARB extract)	5.4 (G), 13.9 (E), 9.9 (C), 12.4 (P)	4 (G), 17 (E), 31 (C), 16 (P)
Catecheu (water extract)	13.9 (E), 9.9 (C), 12.4 (P)	7 (E), 44 (C), 6 (P)
Myrobolan nut (DIMCARB extract)	5.7 (G), 14.0 (E)	7 (G), 27 (E)
Myrobalan nut (water extract)	5.7 (G), 13.8 (E)	5 (G), 1 (E)

G = Gallic acid E = ellagic acid C = catechin hydrate P = pyrocatechol

Table 4 Quantitative HPLC analysis for extracted tannin materials

Sample	Gallic acid (mg g^{-1})	Ellagic acid (mg g^{-1})	(+) Catechin hydrate (mg g^{-1})	Pyrocatechol (mg g^{-1})
Catecheu (DIMCARB extract)	1.8	59.7	27.0	13.0
Catecheu (water extract)	ND	28.6	19.3	8.3
Myrobolan nut (DIMCARB extract)	1.5	296.4	ND	ND
Myrobolan nut (water extract)	0.5	8.1	ND	ND

ND = not detected

Table 5 Tannin contents of extracted samples

Sample	Tannin content (%)
Catecheu (DIMCARB extract)	56
Catecheu (water extract)	27
Myrobolan nut (DIMCARB extract)	32
Myrobolan nut (water extract)	21

the presence of the tannins as salts is probably responsible for the antifungal action reflected in the microbial assay of the product. In the case of water extracted product there were colonies of growth observed in the nutrient agar medium. This could be due to the presence of large gram positive cells, which were possibly eukaryotic rather than bacterial cells. The myrobolan samples extracted into DIMCARB showed no contamination.

Hyde powder test

In order to assess the tannin contents of the extracted materials, the hyde powder test has been used as a standard method, as described by Garro *et al*.[25] In this test, an aqueous solution of the extracted materials is allowed to filter through the hyde powder and since collagen is the main content of the powder, it can absorb only the tannins and the non-tannins can be separated and evaporated. The tannin absorbed by the hyde powder can be determined gravimetrically and the results are given in Table 5. The results show that the ionic liquid extracted raw materials have higher tannin contents than the water extracted samples.

The dried samples were allowed to stand for the observance of fungal growth. The photographs of Cutecheu samples extracted from ionic liquid and water were shown in ESI S7 and S8†. The DIMCARB extracted product shows that there was no fungal growth whereas in the water extracted sample (ESI S8†) there were moulds of fungal growth indicating that the presence of ionic liquid in these samples can prevent the bacterial growth and provide good life to the matrix. This can serve as a potential advantage in the use of these tannins in leather tanning applications.

Conclusions

Thus distillable ionic liquids, such as DIMCARB, can be successfully used to extract valuable hydrolysable tannin materials such as ellagic acid in substantial quantities, in contrast to conventional extraction methods. The DIMCARB extracted products have a higher tannin content compared to traditional solvent extracts. The DIMCARB extract product is also more stable against bacterial moulds as evidenced by microbial analysis and has good shelf life. The quantities of water used in these processes are also a significant difference. Typically the water extraction standard method requires a water to product ratio of 15 to 20 : 1 by mass. In the DIMCARB process described here, the unoptimised water to product ratio is ~7 : 1. In an optimised process this may be lowered further and if DIMCARB filtration could be achieved then water may be avoidable altogether.

Materials and methods

The raw materials namely catecheu (*Acacia Catechu*) and myrobolan (*Terminalia Chebula*) were purchased from Nadan Herbal Pharmacy, W.A, Australia and used without further purification. The synthesis of DIMCARB ionic liquid was carried out as per the literature procedure.[26] The DIMCARB extraction was carried out as shown in Scheme 3. Typically, 5.0 g of either myrobalan nut or catecheu was treated with 25.0 g of DIMCARB ionic liquid, the mixture stirred at room temperature for the desired period of time and then the ionic liquid was distilled from the reaction mixture. Then water (25 ml) was added to the flask to dissolve the hydrolysable components and then the mixture was filtered. The filtrate contains the hydrolysable tannins and they were recovered as a powder by evaporating the water. A control experiment was carried out

using these raw materials by the standard method[20] which involves a hot water extraction utilising 5 g of raw materials and 50 ml water.

HPLC analysis

The HPLC analyses were carried out on an Agilent 1200 Series (Agilent Technologies, Switzerland), equipped with a quaternary pump delivery system, an auto sampler and an Agilent diode-array UV-Vis detector (DAD) 1200 Series. Integration and data analyses were performed using EZ Chrom Elite (version 3.2.1) by Agilent. A Deltapak 15 μm C18, 30 cm × 3.9 mm i.d. (Waters, Japan) column was used. All solvents were filtered and degassed by stripping with helium. A binary gradient elution was employed using the following solvent systems: mobile phase A, 0.1% TFA in water and mobile phase B, 0.1% TFA in acetonitrile (v/v). The flow rate was maintained at 1 mL min^{-1} and 20 μL of each sample was injected after filtering through a 0.45 μm (Teflon) filter disk. The solvent gradient elution system was as follows: 0–10 min, 95% A, 5% B; 10–20 min, 95–75% A, 5–25% B; 20–30 min, 75–65% A, 25–35% B; 30–31 min, 65–55% A, 35–45% B; 31–35 min, 55–0% A, 45–100% B; 35–46 min, 0–95% A, 100–5% B. The quantification of the tannins was performed at 254 nm.

LC-MS analysis

The identification of compounds was performed using a Micromass ZMD (Micromass, UK), orthogonal source with Electrospray Ionisation Mass Spectrometry (ESI) probe quadrupole mass spectrometer equipped with a Gilson 306 pump (Middleton, USA), a Gilson 215 auto sampler with Gilson 819 injector module and an Agilent diode-array UV-Vis detector (DAD) 1100 Series (Agilent Technologies, Germany). Integration and data analyses were performed using MassLynx software (version 3.5). The chromatographic conditions included a reversed-phase column, Alltech Alltima 5 μm C18, 15 cm × 4.6 mm i.d. (Alltech Associates, Australia). The mobile phases were A; 100% Milli Q water and B; 100% MeOH, with gradient elution at 0.6 mL min^{-1} followed as: 0–15 min, 90% A, 10% B; 16–17 min, 10% A, 90% B; 17–40 min, 90% A, 10% B. The quantification of the tannins was performed at 254 and 270 nm. The mass range was measured at 100–1500μ (m/z). The ESI conditions were 3 kV capillary voltage, desolvation nitrogen gas flow rate was 15 mL min^{-1}, source block and desolvation temperatures were 80 °C and 150 °C, respectively.

Microbial assay

Tannins are polyphenolic compounds which occur in a variety of plants and are the fourth most abundant plant materials after cellulose, hemicellulose and lignin. Fungal tannases are very diverse and efficiently degrade different types of hydrolysable tannins.[27] Tannases produced by bacteria can degrade tannic acid and also natural tannins like chestnut, tara, oak and myrobalan tannins.[28,29] In the literature, however, there are no systematic studies on the detection of bacteria on the vegetable tannin raw materials and the extracts. Hence an attempt was made in this study to analyze the raw materials and the extracts using microbial assay by placing a small amount of the sample into 400 μL of sterile saline and then vortexing to re-suspend as much material as possible. Aliquots of 100 μL from each re-suspended sample were then inoculated onto the surface of two nutrient agar plates, namely, one horse blood agar plate and one malt extract agar plate. One of the nutrient agar plates was incubated overnight at 37 °C, and the other nutrient agar plate was incubated overnight at room temperature. The malt extract agar plate was incubated at 30 °C overnight. The presence or absence of growth following incubation was noted by the observation of colonies or fungal growth on the plates. Cells from the resulting colonies were Gram stained and the Gram reaction noted.

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