Simultaneous Extraction of Bioactive Limonoid Aglycones and Glucoside from *Citrus aurantium* L. Using Hydrotropy

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Citrus limonoids were demonstrated to possess potential biological activities in reducing the risk of certain diseases. Limonoids are present in citrus fruits in the form of aglycones and glucosides. At present, limonoid aglycones and limonoid glucosides are extracted in multiple steps using different solvents. In order to understand their potential bioactivity, it may be beneficial to isolate and purify these compounds using environment friendly methods. A new method of extraction and purification of limonoids was established using a hydrotrope polystyrene adsorbent resin. Extraction of aglycones and glucosides was achieved in a single step, using an aqueous solution of sodium cumene sulphonate (Na–CuS). Sour orange (*Citrus aurantium* L.) seed powder was extracted with 2 M Na–CuS solution at 45 °C for 6 h. The filtered extract was diluted with water and loaded on an SP 700 adsorbent column. The column was washed with distilled water to remove the hydrotrope and then eluted using water and methanol in different compositions to obtain three compounds. The structures of the isolated compounds were confirmed by NMR spectroscopy as deacetyl nomilinic acid glucoside (DNAG), deacetyl nomilin (DAN) and limonin (LIM).

Key words: Citrus, Deacetyl Nomilinic Acid Glucoside, Deacetyl Nomilin, Sodium Cumene Sulphonate

Introduction

Recently, the evidences of health-maintaining properties of citrus bioactive compounds have been increased tremendously (Vanamala *et al.*, 2006; Poulose *et al.*, 2007; Jayaprakasha *et al.*, 2007a). Obviously, the demand for the isolation of these bioactive compounds using the well-defined as well as economical extraction and purification methods becomes very important. Several methods of extraction and purification of limonoids using different solvents are reported (Sawabe *et al.*, 1999; Schoch *et al.*, 2002; Jayaprakasha *et al.*, 2007b; Mandadi *et al.*, 2007). These methods include extraction of citrus raw material using solvents such as hexane, ethyl acetate, acetone, ethanol and methanol. Conventionally, purification of aglycones from crude extracts is accomplished by chromatography using alumina or silica while purification of individual glucosides is reported using ion exchange resins and adsorbent columns (Bennett and Hasegawa, 1982; Bennett *et al.*, 1988; Sawabe *et al.*, 1999; Schoch *et al.*, 2002; Mandadi *et al.*, 2007). Since the abundance of certain limonoids in citrus fruits is at very low levels, extraction and purification of limonoids are lengthy and tedious. Recently, an improved method was developed in our lab for the purification of limonoid glucosides on a multi gramme scale (Jayaprakasha *et al.*, 2006, 2007b). However, the above-mentioned method involves solvents for the extraction and purification.

Recent attempts in our lab and elsewhere were made to extract limonoid aglycones and glucosides using supercritical fluid extraction (SFE) (Miyake *et al.*, 2000; Patil *et al.*, 2004; Yu *et al.*, 2006, 2007). The SFE methods are considered to be green methods providing an environment friendly process. However, scaling up of these methods is tedious and not cost-effective. In certain cases, solvents used to modify the supercritical CO_2 are at hazardous levels making these methods less environment friendly. In search of environment friendly extraction methods, researchers explored the hydrotropy phenomenon and investigated it for potential process applications. Hydrotropes are highly water-soluble organic salts, and hydrotropy is the phenomenon of increasing the solubility of

water-insoluble or sparingly water-soluble organic compounds in aqueous solutions in the presence of hydrotropes (Balasubramanian *et al.*, 1989; Srinivas *et al.*, 1997). Recently, water-insoluble bioactive compounds, such as curcuminoids, diosgenin and piperin, were successfully extracted using water-based hydrotropic solutions (Raman and Gaikar, 2002; Dandekar and Gaikar, 2003; Mishra and Gaikar, 2004). In the present study, water-insoluble and water-soluble limonoids were extracted simultaneously using hydrotropy. The extract was purified to obtain three limonoids, the structures of which were identified by NMR spectroscopy.

Material and Methods

Raw materials and chemicals

Sour orange (*Citrus aurantium* L.) seeds were obtained from Texas A&M University, Kingsville, Citrus Center, Weslaco, TX, USA. All solvents used were of HPLC-, ACS-grade and obtained from Fisher Scientific (Atlanta, GA, USA). 2 M aqueous sodium cumene sulphonate (Na–CuS) was obtained from Stepan Company (Romeoville, IL, USA). Polystyrenic Sepabead SP700 synthetic adsorbent (SP700) was obtained from Supelco (Bellefonte, PA, USA).

Instruments

HPLC analysis was carried out using a Perkin Elmer HPLC instrument (Salem, MA, USA) equipped with a Perkin Elmer Series 2000 pump coupled with a Perkin Elmer Series 2000 autosampler and a Perkin Elmer 235 C diode array detector. Purified compounds were identified using NMR spectroscopy (Varian Inova 300, Palo Alto, CA, USA).

Extraction

Shells of the dried seeds were collected by removing kernels and powdered. The schematic diagram of hydrotropic extraction and purification is illustrated in Fig. 1. The extraction was conducted in a fully baffled cylindrical vessel (20 cm × 20 cm) equipped with a four-blade turbine impeller of 7.5 cm diameter. Raw material (250 g) was suspended in 2.5 L of Na–CuS solution (2 M) and maintained at 45 °C, and extraction was conducted for 6 h. The extract was then filtered and diluted 20 times using water and loaded on an adsorbent column.

Purification of limonoids

SP700 (3.5 kg) was packed in a glass column (100 cm × 12 cm) with PTFE end fittings. Resin

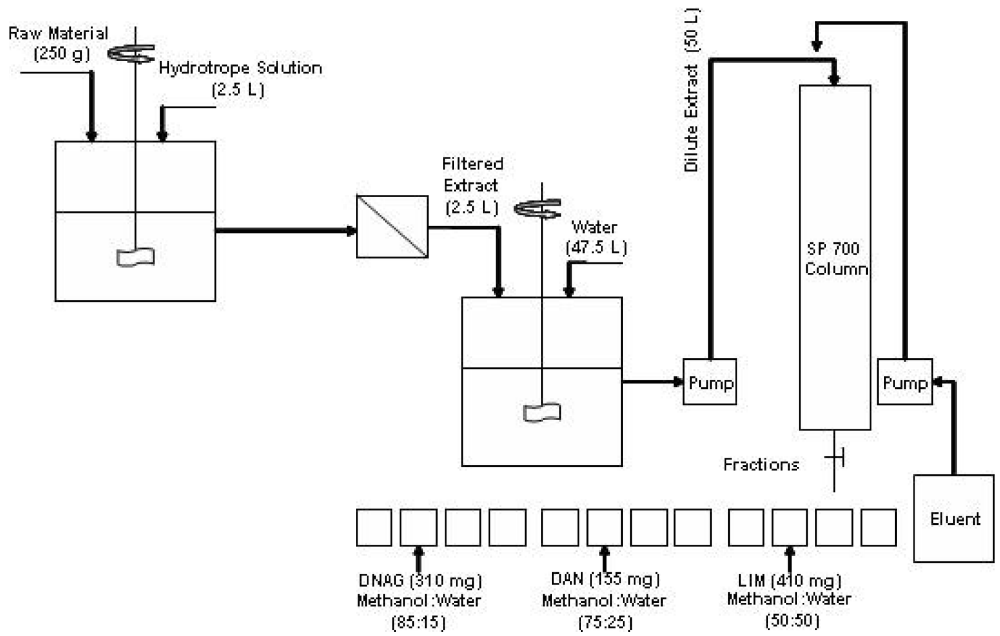

Fig. 1. Schematic diagram of hydrotropic extraction and purification of limonoids.

was washed with 5 L of methanol using a peristaltic pump followed by rinsing overnight with water. Dilute extract (50 L) was loaded onto the SP700 column and washed with 50 L of water to remove the hydrotrope. Further, the column was eluted with a mixture of water/methanol and methanol. Fractions of 1 L each were collected and concentrated using a rotary evaporator (Büchi, New Castle, DE, USA) till 10 mL and stored in a refrigerator for crystallization.

HPLC analysis

Column fractions and purified compounds were analyzed by HPLC using a C_{18} Gemini column (250×4.6 mm, 5 μm particle size) (Torrence, CA, USA) and detected at 210 nm (Vikram *et al.*, 2007). The HPLC column was eluted at a flow rate of 1.0 mL/min using a binary solvent system of 3 mM phosphoric acid (solvent A) and acetonitrile (solvent B). The gradient elution was conducted, starting at 85% of solvent A, reduction to 77% in 5 min, 74% after 25 min, further reduction to 60% at 30 min and completing the gradient at 54% at the end of 45 min. The column was equilibrated for 5 min with 85% solvent A and 15% solvent B before next run.

Results and Discussion

Sour orange seeds are considered as a good source of limonoid aglycones as well as glucosides (Bennett *et al.*, 1991). The conventional extraction process of both limonoid derivatives involves the use of different solvents and series of steps where the solvent polarity increases in successive steps (Mandadi *et al.*, 2007). In order to reduce the use of organic solvents and to achieve extraction of both aglycones and glucosides in a single step, hydrotropic extraction was employed. Dilute hydrotropic extract was loaded on an SP-700 column followed by water washing. HPLC analysis of water washing showed the presence of the hydrotrope, however no limonoids were detected. Column was washed repeatedly till the hydrotrope concentration went below the detection level in the sample. Elution of the column was completed using water/methanol with a linear increase of methanol in the mobile phase. All the fractions (1 L) were concentrated and stored at 4 °C to obtain crystallized compounds.

Compound **1** was obtained from water/methanol 85:15. It was separated by filtration. HPLC analysis of compound **1** showed one peak and the yield was 310 mg. Water/methanol 75:25 (v/v) gave compound **2** with the yield of 155 mg. Compound **3** (410 mg) was obtained from water/methanol 50:50 and 40:60. HPLC analysis of the compounds **1**–**3** showed single peaks. All three compounds were identified by NMR spectroscopy as deacetyl nomilinic acid glucoside(DNAG, **1**), deacetyl nomilin (DAN, **2**) and limonin (LIM, **3**) (Table I; Fig. 2). The chemical shifts of our values were matched with reported values (Manners *et al.*, 2000; Mandadi *et al.*, 2007).

Table I. ^{13}C NMR chemical shifts of isolated limonoids.

Carbon	^{13}C NMR chemical shift		
	LIM	DAN	DNAG
1	78.6	68.2	73.5
2	35.3	38.6	37.5
3	170.0	171.0	174.2
4	79.8	83.4	73.5
5	58.4	49.8	47.3
6	36.1	38.8	39.5
7	207.7	208.0	213.3
8	50.5	52.2	51.2
9	46.7	43.9	39.8
10	45.3	44.1	44.4
11	17.8	16.5	16.8
12	29.3	31.5	27.1
13	37.5	37.2	43.6
14	66.6	66.0	70.4
15	53.8	53.2	58.1
16	167.2	166.7	169.4
17	77.6	77.9	78.0
18	17.0	16.1	24.4
19	64.8	16.0	16.2
20	120.2	120.1	125.8
21	143.5	143.5	141.5
22	110.3	109.9	112.5
23	141.5	141.0	140.4
24	19.8	20.1	19.4
25a	29.9	32.6	32.3
26b	21.5	23.3	27.5
Glu C-1			104.2
Glu C-2			74.3
Glu C-3			76.8
Glu C-4			70.9
Glu C-5			76.3
Glu C-6			61.6

The main challenge during the extraction step is to provide access to limonoids and then dissolve water-soluble and -insoluble compounds in the hydrotrope in a single step. When considering the cellular nature of the plant, the extraction process includes two main steps. During step one, the solvent destabilizes the cell wall and increases the accessibility of limonoids, followed by step two in which limonoids are dissolved and brought in to

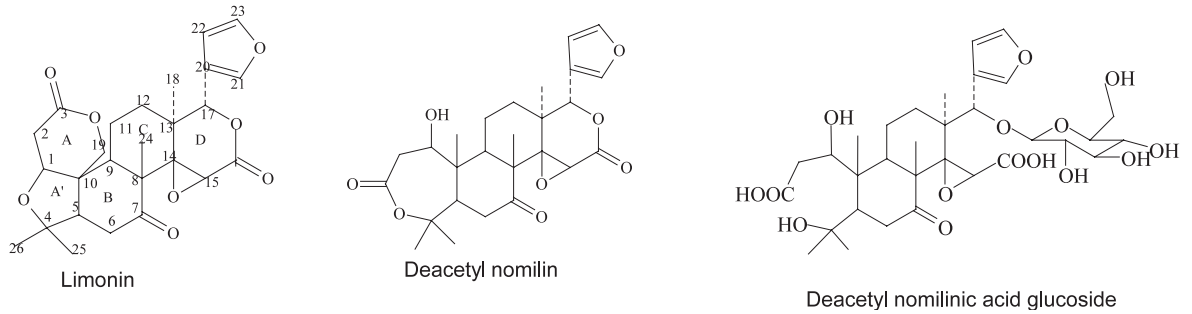

Fig. 2. Structures of limonoids **1**–**3** isolated in the present study.

the bulk solution. The extraction efficiency is thus the function of the hydrotrope capacity of penetrating cell structure and dissolving limonoids. Previous studies showed that hydrotrope solutions penetrate the cell wall by destabilizing and make intra-cellular compounds accessible for the extraction in aqueous hydrotrope solvents (Raman and Gaikar, 2002; Dandekar and Gaikar, 2003; Mishra and Gaikar, 2004).

Simultaneous dissolution of accessible aglycones and glucosides in same aqueous solution is also important to achieve extraction in a single step. Hydrotropes, with hydrophilic as well as hydrophobic moieties in the same molecule, are expected to form large stack-like but somewhat open structures (Srinivas *et al.*, 1997). This characteristic property of hydrotropic solvents shows a polar water phase as well as a less polar aggregate phase in the same solution which is an ideal medium for dissolving both aglycones and glucosides. The medium polar aglycone molecules would enter the hydrophobic layers of these assemblies, intercalating themselves between the layering molecules, and in turn may stabilize the layered structures producing a cooperative solubilizing isotherm (Balasubramanian *et al.*, 1989). The bulk water phase in the same system dissolves the polar glucoside molecules.

An extraction step was followed by a purification step in which, to separate limonoid aglycones from the extract, breaking of hydrotropic assemblies was necessary. Hydrotropic extract thus was diluted with water (20 times) to bring the Na–CuS concentration (0.1 M) in the solution well below its minimum hydrotrope concentration (MHC). At a lower concentration of the hydrotrope (below MHC), it was expected that limonoid aglycones, being water-insoluble, will precipitate and thus can be recovered. However, precipitation of aglycones was not observed on dilution. This demonstrates greater affinity of Na–CuS towards the aglycones. In our previous study, we reported greater affinity of alkyl benzene sulphonates towards curcuminoids and recovery of curcuminoids by dilution using acidic water (Dandekar and Gaikar, 2003). Similarly, while Na–CuS and aglycone association was weak in the dilute extract, it was not enough to separate the aglycones.

Dilute extract was loaded on an SP700 column followed by water washing. Polystyrenic adsorbents are known to adsorb and separate limonoids from various citrus and byproduct extracts (Schoch *et al.*, 2002; Jayaprakasha *et al.*, 2007b). It is possible that the column adsorbed limonoids while the highly polar hydrotrope was rinsed out with water. Column elution was achieved using water/methanol with a linear gradient increase of methanol in the mobile phase what facilitated the elution of compounds based on their polarity. Concentration of the fractions facilitated the saturation of the limonoids leading to crystallization.

In conclusion, extraction of both the limonoid aglycones and glucosides was successfully achieved in a single step without using organic solvents. The main challenge during the extraction step was to extract water-soluble and -insoluble compounds in the same media in a single step which was completed using hydrotropic solvents. Later, the extract was purified by column chromatography and eluted with water and methanol to obtain three bioactive compounds.

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