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Short Communication

pH-Zone-refining centrifugal partition chromatography for preparative isolation and purification of steroidal glycoalkaloids from *Solanum xanthocarpum*

pH-Zone-refining centrifugal-partition chromatography (CPC) was successfully applied in the separation of complex polar steroidal glycoalkaloids of close R_f values, directly from a crude extract of *Solanum xanthocarpum*. The experiment was performed with a two phase solvent system composed of ethyl acetate/butanol/water (1:4:5 by volume) where triethylamine (5 mM) was added to the upper organic mobile phase as an eluter and TFA (10 mM) to the aqueous stationary phase as a retainer. Separation of 1 g of crude extract over CPC resulted in two distinct pH-zones. The fractions collected in pH-zone i afforded 72 mg of solasonine while the fractions collected in pH-zone ii were slightly impure, hence were purified over medium pressure LC, which afforded 30 mg of solasonine and further 15 mg of solamargine (SM). The steroidal glycoalkaloids, SM and solasonine were isolated in 93.3 and 91.6% purity, respectively. The isolated alkaloids were characterized on the basis of their ^1H , ^{13}C -NMR, and ESI-MS data.

Keywords: pH-Zone-refining centrifugal-partition chromatography / Solamargine / Solanaceae / *Solanum xanthocarpum* / Solasonine

Received: May 8, 2009; revised: June 12, 2009; accepted: June 12, 2009

DOI 10.1002/jssc.200900323



1 Introduction

Solanum xanthocarpum Schrad. & Wendl. (syn. *S. virginianum*/*S. surattense* F. Solanaceae) is a green perennial herb, commonly known as Kantakari in Hindi and Yellow Berried Nightshade in English. It is found throughout India as a weed on roadsides and on dry wastelands [1]. The fruits are globular berries, white and green strips when young but yellow when mature [2]. The plant has been reported to possess antispasmodic, antitumor, cardio-tonic, hypotensive, antianaphylactic, and cytotoxic activities [3–5]. The stem, flowers, and fruits are bitter and carminative and are prescribed for relief in burning sensation in the feet accompanied by vesicular eruptions [6]. Root is an expectorant, used in Ayurvedic medicine for cough, asthma, chest pain, flatulence, sore throat, and toothache. Fruits are used in indigestion and as an anthelmintic. The *kondh* tribes of Dhenkanal district of

Orissa, India use the hot aqueous extract of the matured fruits as a traditional medicine for the treatment of diabetes mellitus and recently significant hypoglycemic activity in the aqueous extract of *S. xanthocarpum* fruits has also been confirmed [7]. The fruits are also known for several medicinal uses like anthelmintic, antipyretic, laxative, anti-inflammatory, antiasthmatic, and aphrodisiac activities [8]. The hot aqueous extract of dried fruits is used for treating cough, fever, and heart diseases [9]. The fruit paste is applied externally to the affected area for treating pimples and swellings [10]. The plant is reported to contain steroidal glycoalkaloids, solasonine [9], and solamargine (SM) [11] as shown in Fig. 1.

Biological investigations of SM showed significant anticancer activity against human hepatoma cells (Hep3B) [12] and superior cytotoxicity against the four human lung cancer cell lines, H441, H520, H661, and H69 for which IC_{50} were 3, 6.7, 7.2, and 5.8 μM , respectively [13]. These studies demonstrate that SM may be a new and superior agent for the treatment of hepatoma and lung cancers. SM [14] and solasonine are also used for treating the skin tumor [15] and recently a novel chemotherapeutic and immunomodulating combination of solasonine and SM [1:1] “Coramsine” has been developed for the treatment of renal cell carcinoma and for malignant melanoma, respectively (<http://www.fda.gov/>

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Abbreviations: FCPC, fast centrifugal partition chromatography; MPLC, medium pressure LC; SM, solamargine

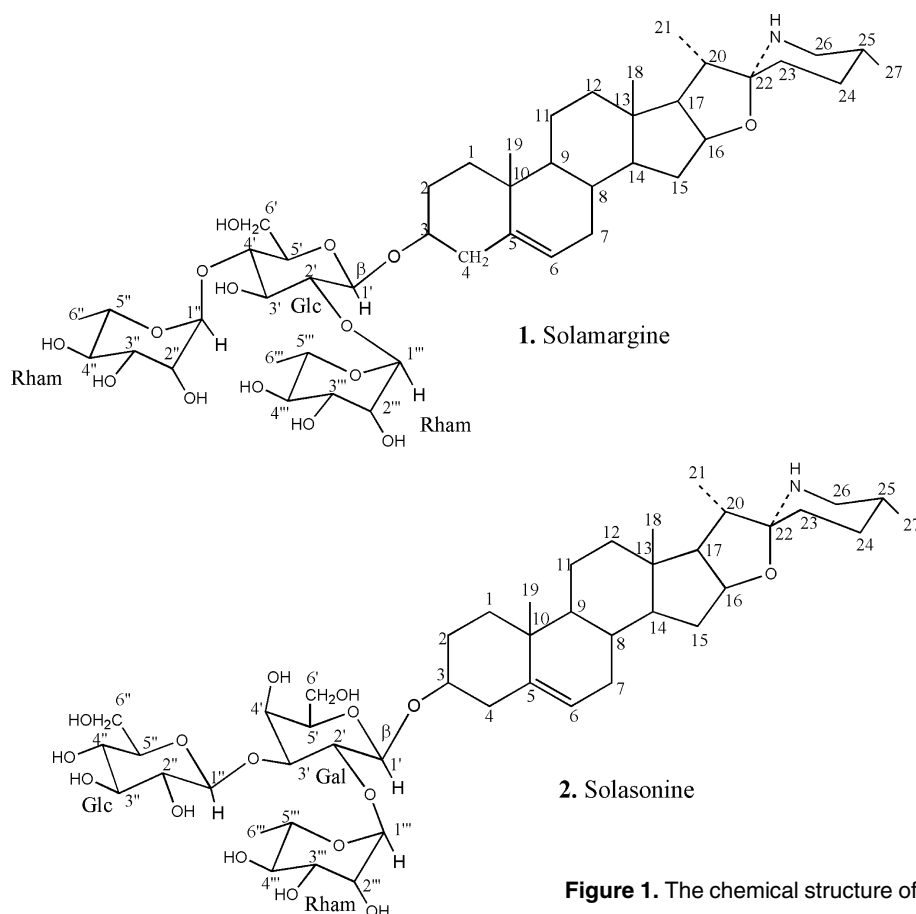


Figure 1. The chemical structure of solamargine (1) and solasonine (2).

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The presence of steroidal glycoalkaloids, SM and solasonine in various *Solanum* species is well documented [16, 17], but due to high polarity and close R_f values, their separation and purification from other constituent of the plant by classical methods are tedious, time taking and require multiple chromatographic steps, which results in low recovery [18–21] of these important steroidal glycoalkaloids.

pH-Zone-refining counter current chromatography (CCC) has been developed by Ito and coworkers [22–26] as a preparative purification method for the separation of compounds whose electric charge depends on pH-value. The method enables separation of organic acids and bases into a succession of highly concentrated rectangular peaks with minimum overlap that elute according to their pK_a values and hydrophobicities. pH-Zone-refining CCC provides many advantages over the conventional CCC such as more than 10-fold increases in the sample loading capacity, high purity, and high concentration of fractions, concentration of minor constituents, and minor impurities *etc.* The method has been successfully used for the separation of a wide variety of natural products [27], alkaloids [22, 25, 28, 29], structural [30] and geo-

metrical [31] isomers, acidic [24, 32] and basic [33] amino acid derivatives, synthetic colors [24, 34], and chiral compounds [35]. Similarly pH-zone-refining CPC method has also been successfully used for the separation of bioactive natural products [28] and recently we applied this method for the large scale separation of clavine alkaloids from the seeds of *Ipomoea muricata* [36].

The purpose of the present study was to develop a more efficient pH-zone-refining CPC method using a two phase solvent system with retention capabilities optimized for the preparative separation and purification of two alkaloids, SM and solasonine, from the complex crude alkaloid extract of *S. xanthocarpum*.

2 Experimental

2.1 Apparatus

The separations were performed using Kromaton Technologies (Angers, France) apparatus, fast centrifugal partition chromatography (FCPC) with a rotor of 20 circular partition disks (1320 partition cells, column capacity: 200 mL). Rotation speed could be adjusted from 200 to 2000 rpm, producing a centrifugal force field in the partition cell of about 120 g at 1000 rpm and 470 g at

2000 rpm. The column was connected to injector or to the detector through high pressure rotary seals. A four port valve installed on the CPC allows its operation both in ascending and descending modes. The CPC was connected to a Waters (USA) 2525 modular gradient pump. The samples were introduced into the CPC column – after equilibration of the two phases – via a Rheodyne injection valve (ALTech association, Deerfield, IL, USA) equipped with 20 mL sample loop. The effluent was monitored with Waters PDA 2996 detector equipped with a preparative flow cell set at wave length 203 nm. Fractions were collected manually and pH was checked with Consort pH Meter model C861 (Japan).

2.2 Reagents and materials

All the organic solvents used for FCPC and Silica gel H for medium pressure LC (MPLC) were of analytical grade and purchased from Merck (Mumbai, India). Ammonium dihydrogen phosphate buffer, orthophosphoric acid, ACN, and water used for HPLC analysis were of HPLC grade and were also purchased from Merck (Mumbai, India). The aerial parts of *S. xanthocarpum* (Kantkari) were identified and collected by Dr. S. C. Singh, Botany and Pharmacognosy Division, Central Institute of Medicinal & Aromatic Plants, Lucknow, India in the month of May, 2008 and a voucher specimen (No. 12470) is deposited in the Herbarium Division of the institute.

2.3 Preparation of the sample

The aerial parts of *S. xanthocarpum* were air-dried under shade and then powdered. This powdered material (1.3 kg) was extracted with methanol (3 × 6 L, 24 h each) at room temperature. The combined methanol extract was subjected for complete solvent removal at 40°C under vacuum. This dried methanolic extract was dissolved in distilled water (2000 mL) and successively extracted with hexane, dichloromethane, ethyl acetate, and water saturated *n*-butanol to yield respective extracts. The butanol extract so obtained was further extracted with 2% aqueous HCL solution (8 × 200 mL) to dissolve the alkaloids as water soluble salts. This acidic extract was then basified (in cold) with 10% NaOH solution up to pH 12 (to liberate free alkaloids in solution) and extracted with *n*-butanol saturated with water (4 × 500 mL). The combined butanol extract was subjected under vacuum distillation at 60°C to yield butanol extract (9.7 g). This was stored in a refrigerator (6°C) for the subsequent FCPC separation.

2.4 Preparation of solvent system and sample solution

The biphasic solvent system used in the present study consisted of EtOAc/BuOH/water (1:4:5 by volume). The sol-

vent mixture was vigorously shaken and then allowed to settle until the phases became limpid. The upper organic phase was made basic with TEA (eluter) to obtain a final concentration of 5 mM and TFA (retainer) was added to the lower aqueous stationary phase to obtain a final concentration of 10 mM. The sample solution was prepared by dissolving 1 g of crude alkaloid extract (BuOH extract pH 12) in 10 mL solution consisting of 6 mL of stationary phase (containing TFA as retainer) and 4 mL of mobile phase (without triethyl amine as eluter).

2.5 Separation procedure for FCPC

Keeping the rotor speed at 400 rpm and flow-rate 6 mL/min, the column was first filled with aqueous stationary phase containing TFA (10 mM as retainer) in ascending mode. Then the rotor speed was increased to 1400 rpm and the basic upper organic layer containing TEA (5 mM as eluter) was pumped into the column as mobile phase in ascending mode with a flow rate of 2 mL/min. This increased the back pressure from 33 to 55 bar, causing displacement of about 25% of stationary phase. When equilibrium was maintained between mobile and stationary phase no more stationary phase was pumped out. Then the sample was injected through the sample port. After loading the sample, fractions were collected at a regular interval of 2 min. The effluent from the outlet of the column was continuously monitored with a Waters PDA 2996 detector at 203 nm and the evolution of the pH was continuously monitored using a pH meter. After elution of the desired compounds, rotor speed was further reduced to 400 rpm and column was washed with a mixture of water/methanol (1:1) in ascending mode at a flow rate of 6 mL/min until complete stationary phase was pumped out. On completion of the experiment (as per advice of the manufacture) the column was filled with a mixture of water/methanol (1:1).

The fractions collected in the pH-zone “i” at retention times 31–37 min resulted in the isolation of SM (1, 72 mg), while the fractions collected in the pH-zone “ii” (174 mg), showed presence of minor impurities on TLC, hence were further purified by MPLC using TLC grade Silica gel H (4 g, average particle size, 10 µm). Fractions of 5 mL each were collected. Fractions 21–31 eluted with chloroform/methanol (92:8) resulted in the isolation of SM (1, 15 mg). Fractions 35–50 eluted with chloroform/methanol (90:10) resulted in the isolation of solasonine (2, 30 mg). The MPLC was completed in 4 h.

2.6 Purity evaluation of FCPC fractions

Purity evaluation of FCPC fractions was first performed by thin-layer-chromatography on normal phase silica gel plates 60 F₂₅₄ Merck (Darmstadt, Germany) with the solvent system chloroform/methanol/water (65:38:10). Visualization was performed with Dragendroff's reagent. Fur-

ther purity evaluation of FCPC fractions was performed on a Dionex Summit P680 HPG preparative binary gradient pump (Sunnyvale, CA, USA) equipped with Dionex PDA-100 detector. The experiments were conducted at room temperature ($25 \pm 1^\circ\text{C}$). The isolated alkaloids SM and solasonine were analyzed by HPLC (DIONEX) with a Chromolith performance RP-18e column (100×4.6 mm ID, Merck) at 203 nm with acetonitrile-100 mM ammonium dihydrogen phosphate buffer ($\text{NH}_4\text{H}_2\text{PO}_4$) acidified to pH 2.5 with 85% orthophosphoric acid, as mobile phase with 1 mL/min flow rate. The optimal isocratic solvent condition was 22% ACN and 78% buffer.

2.7 Analytical control and structure elucidation

^1H , ^{13}C , DEPT-135, and 2D-NMR experiments HSQC and HMBC were performed on a Bruker 300 MHz instrument (^1H 300 MHz; ^{13}C 75.5 MHz). Chemical shifts (δ) are reported in ppm relative to the residual solvent signals ($\text{C}_5\text{D}_5\text{N}$ δ_{H} 7.19, 7.55, 8.71 ppm and δ_{C} 123.5, 135.5, 149.9 ppm). ESI-MS experiments were performed on a LCMS-2010 V (Shimadzu, Kyoto, Japan) simultaneously in positive (detector voltage 1.6 kV) and negative (detector voltage 1.3 kV) ionization under scan mode. Mass spectrometer consists of a Q-array-octapole-quadrupole mass analyzer with an ESI in positive and negative ionization mode and coupled to the monolith column. Sensitivity optimization was performed by injection of a papaverine standard. Mass spectrometric detection conditions were as follows: curved desolvation line (CDL) temperature and heat block temperature: 250°C ; probe voltage + 4.5 kV; detector voltage 1.5 kV; CDL voltage -20 V and Q-array bias was 50 V. Nebulizing gas was nitrogen at a flow rate of 1.5 L/min. The scan speed of the mass analyzer was 2000 m/z per sec within the range of 400–1000 m/z . A positive full scan mode for screening and library-assisted identification was used whereas time schedule selected-ion mode (SIM) in +ve ionization mode of the characteristic abundant adduct ions.

3 Results and discussion

3.1 FCPC separation

The aim of the present study was to assess the applicability of FCPC for the isolation of very polar steroidal glycoalkaloids from the butanol extract of acid-base extraction of *S. xanthocarpum*. In an initial step, the crude butanol extract at pH 12 was investigated by silica gel TLC where characteristic colorization using Dragendorff's reagent indicated the presence of polar alkaloids.

pH-Zone-refining CPC has been successfully applied to the separation of alkaloids [21, 22, 28]. For the successful separation of alkaloids in the present application, a suitable two-phase solvent system was necessary, which

should provide ideal partition coefficient (K) values in both acidic ($K_{\text{acid}} \ll 1$) and basic ($K_{\text{base}} \gg 1$) condition as well as good solubility of the sample in the solvent system [37]. Although we tried several solvent systems, which have been used for the separation of various kinds of compounds [18, 32], none of them gave suitable “ K ” values necessary for FCPC separation. Finally best results for equal partitioning of *S. xanthocarpum* alkaloids between the two nonmiscible phases were obtained with a system composed of EtOAc/BuOH/water (1:4:5 by volume), which give suitable “ K ” values and good solubility of sample. The separation was performed in ascending mode using the lower aqueous solvent layer as stationary phase. The selected solvent system exhibited favorable retention capabilities of aqueous stationary phase (75%) in the FCPC rotor-system during the complete separation.

Figure 2 shows a typical pH-zone refining centrifugal partition chromatogram obtained from the separation of 1 g crude alkaloid extract of *S. xanthocarpum* by normal displacement mode with 5 mM TEA (as eluter) in upper organic mobile phase and 10 mM TFA (as retainer) in lower stationary phase. The alkaloids were eluted as irregular rectangular peaks where absorbance plateaus were observed at retention time 31–37 min and 37–45 min respectively.

The measurement of the collected fractions also revealed two pH-zone “i” & “ii” which respectively correspond to the above absorbance plateaus, revealed the successful separation of SM (1, 72 mg) at retention times 31–37 min in pH-zone “i”, while the compound isolated at retention times 37–45 min in pH-zone “ii” showed some minor impurities on TLC, hence was further purified using a short MPLC column to yield solasonine (2, 30 mg) and SM (1, 15 mg, cf. experimental). From Fig. 2, it is evident that considerable amounts of impurities were eluted in the front and the back of the main peak, forming multiple peaks. In this way a total 87 mg of SM (1) and 30 mg of solasonine (2) were obtained with 93.3% and 91.6% purity respectively on the basis of their HPLC profile as shown in Fig. 3.

The observed elution order for RP₁₈ HPLC is not necessarily identical with that for FCPC operating in the normal mode using an aqueous stationary phase. In this arrangement FCPC theory predicts that less polar components are eluted initially and the increasing polarity of components causes a higher affinity to the stationary phase leading to longer retention times. Interestingly, solasonine (2) with the highest polarity of other separated components eluted towards the end of the FCPC run at 37–45 min.

It is important to note that the separation and purification of SM and solasonine from the crude butanol extract of *S. xanthocarpum* was initially performed by several steps using conventional silica gel column chromatography [16, 17], which yielded a small amount of SM and solaso-

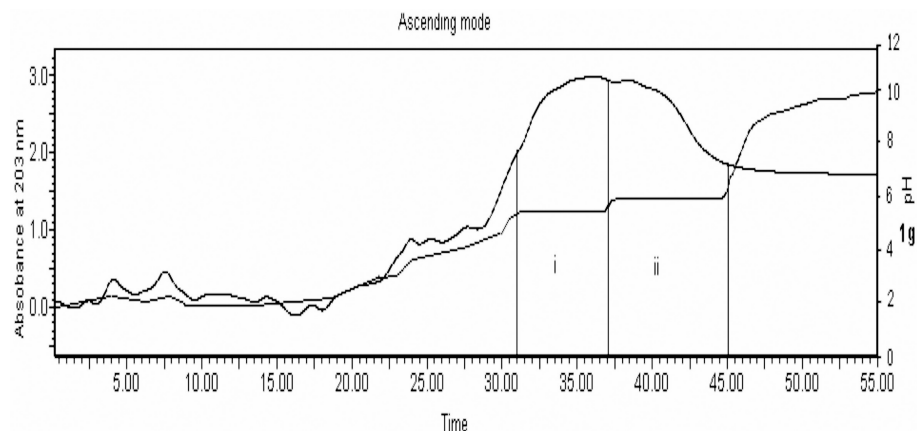


Figure 2. FCPC chromatogram of the crude butanol extract (pH 12) of *Solanum xanthocarpum*, i solamargine, $R_t = 31\text{--}37$ min; ii solasonine, $R_t = 37\text{--}40$ min. Column capacity: 200 mL; sample loop: 20 mL; revolution speed: 1400 rpm, sample size 1 g. pH-Zone-refining CPC two-phase solvent system: ethyl acetate/butanol/water (1:4:5 by volume) where triethylamine (5 mM) was added to the upper organic mobile phase as an eluter and TFA (10 mM) to the aqueous stationary phase as retainer; elution mode: ascending; flow rate: 2 mL/min; detection: 203 nm; retention of stationary phase: 75%.

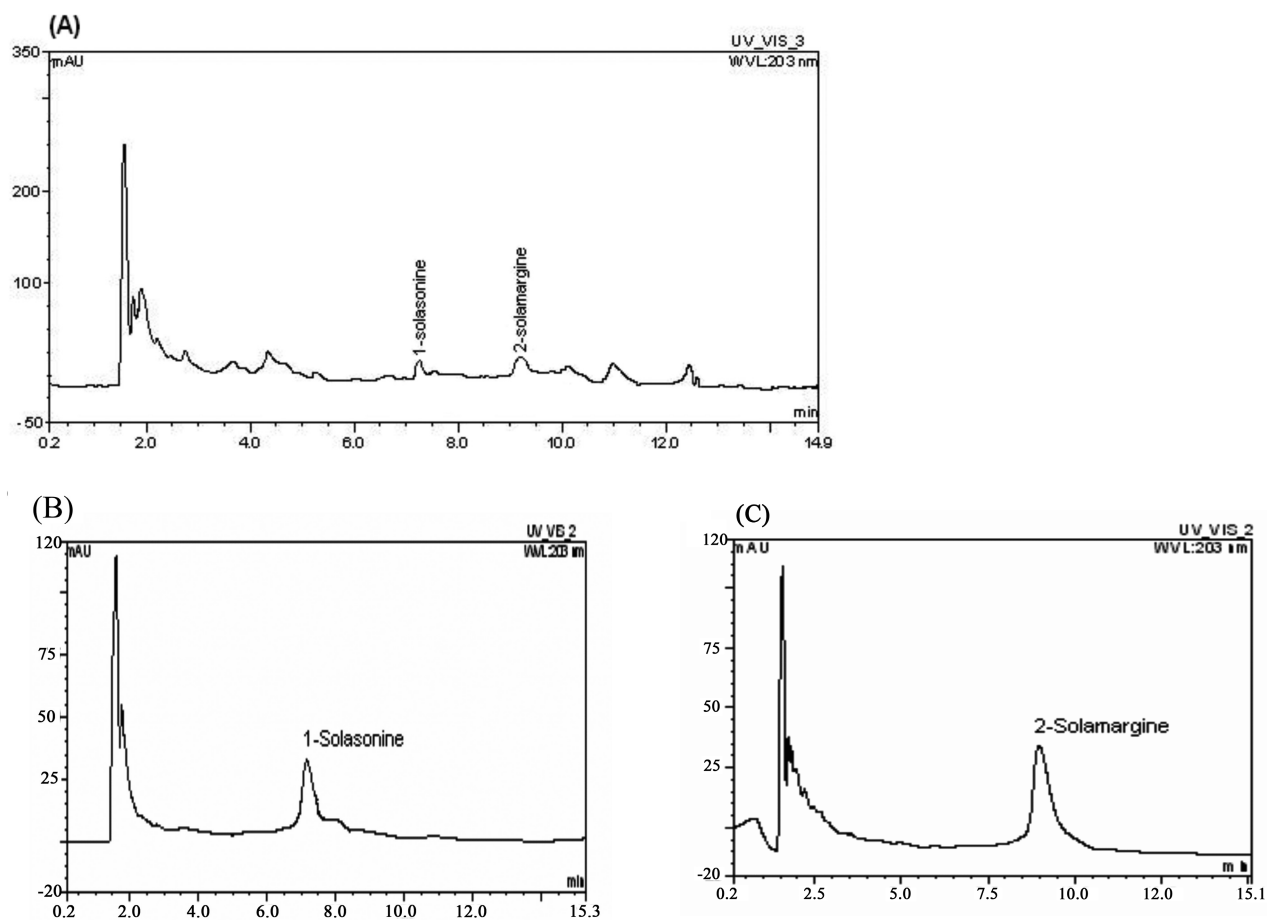


Figure 3. HPLC analysis of original sample and two pH plateau fractions from pH-zone-refining CPC separation of *Solanum xanthocarpum* using 1 g sample size (Fig. 2). HPLC column: chromolith performance RP-18e (100 × 4.6 mm ID); column temperature: (25 ± 1 °C); mobile phase: acetonitrile-100 mM ammonium dihydrogen phosphate buffer ($\text{NH}_4\text{H}_2\text{PO}_4$) acidified to pH 2.5 with 85% orthophosphoric acid (22:78 v/v); flow rate: 1 mL/min; UV detection wavelength: 203 nm; injection volume 10 μL . (A) Crude butanol extract (pH 12), (B) solasonine from pH-zone ii in Fig. 2 (purified by MPLC) and (C) pH-zone i in Fig. 2 (solamargine).

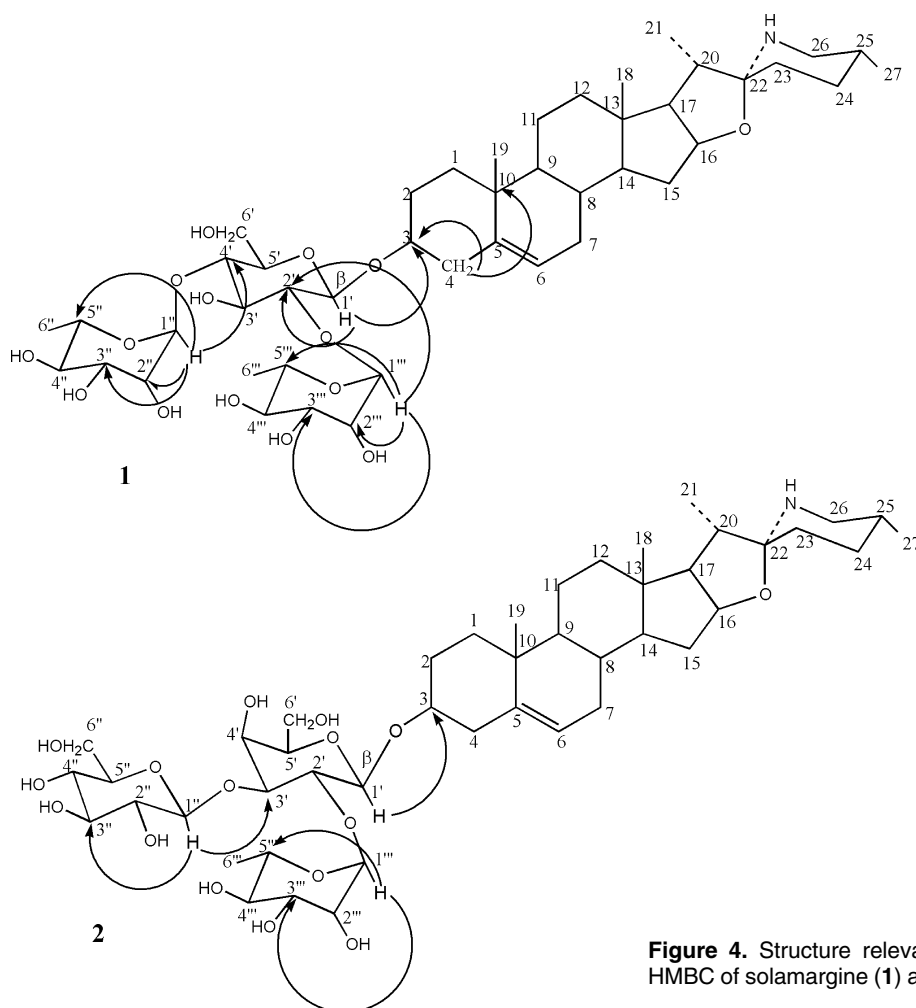


Figure 4. Structure relevant long-range HC-correlation in the HMBC of solamargine (**1**) and solasonine (**2**).

nine. Similarly conventional rotational locular counter current chromatography (RLCCC), roplet counter current chromatography (DCCC) and PTLC were also used for the separation of steroidal glycoalkaloids from the crude extract by Sarg *et al.* [38], but the yield and sample sizes were small.

pH-Zone-refining CPC was successfully implemented for the separation of very polar and close *R_f* steroidal glycoalkaloids at gram level using a rotor of 20 circular partition disks. This was achieved in one step, with a two phase solvent system composed of EtOAc/BuOH/H₂O (1:4:5 by volume). In comparison with open column silica gel or conventional RLCCC and DCCC methods, FCPC represents very low solvent consumption, convenient and easy to handle for a sample of the size used in this experiment.

3.2 Determination of chemical structures

Structure elucidation of compounds **1** and **2** was performed by heteronuclear NMR connectivity and correlation techniques (HSQC and HMBC) and corroborated

the steroidal alkaloid aglycon units as well as the glycosidic linkages. The relevant ^{2,3}J-CH correlations for SM (**1**) and solasonine (**2**) are summarized in Fig. 4. The positive ESI-MS of SM and solasonine showed *m/z* at 868 [M+H]⁺ (cf, Fig. 5A) and *m/z*: 884 [M + H]⁺ (cf, Fig. 5B) respectively. The ¹H and ¹³C NMR chemical shift assignments data (cf, supplementary file) were in complete agreement with reported data for SM and solasonine (16, 17).

4 Concluding remarks

In conclusion, an effective pH-zone-refining centrifugal partition chromatographic (CPC) method was developed for preparative isolation and purification of SM and solasonine from the Indian Ayurvedic medicinal plant *S. xanthocarpum*. These results clearly demonstrate the high effectiveness of the methodology for selective isolation, and indicate its advantage for providing significant amounts of bioactive steroidal glycoalkaloids, which may be available for further biological *in vitro* and *in vivo* studies as well as marker compounds for quality control

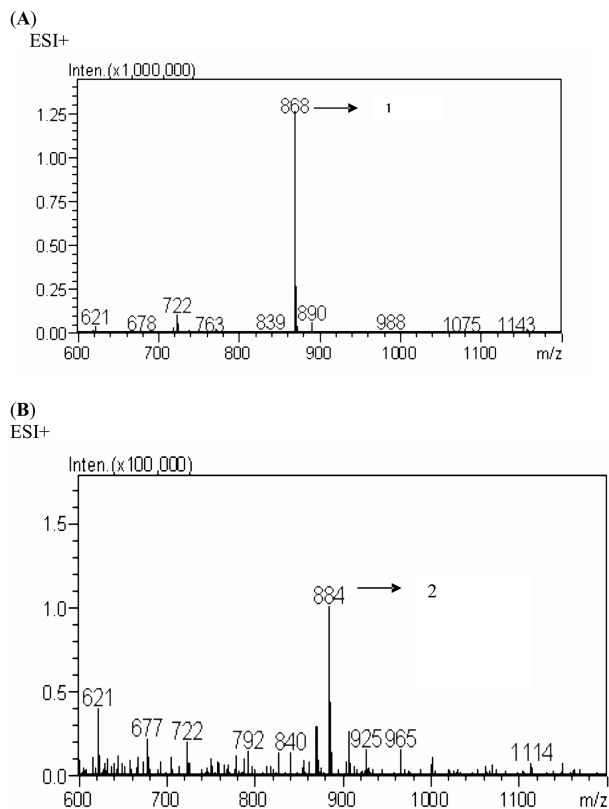


Figure 5. ESI-MS (positive ionization mode) chromatogram, (A) solamargine $[M+H]^+$ m/z 868, (B) solasonine $[M+H]^+$ m/z 884.

of the drugs. The present method may also be successfully applied for the separation of various other alkaloids from natural products.

Financial support for this research by Golden Triangle Partnership (AYUSH-CSIR-ICMR) programme of CSIR is gratefully acknowledged. We also thank all of our colleagues for their excellent assistance.

The authors declared no conflict of interest.

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