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Journal of Chromatography A, 1083 (2005) 32–41
JOURNAL OF CHROMATOGRAPHY A
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Quantitative determination of the group of flavonoids and saponins from the extracts of the seeds of *Glinus lotoides* and tablet formulation thereof by high-performance liquid chromatography

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Received 18 March 2005; received in revised form 13 May 2005; accepted 24 May 2005

Abstract

The total flavonoids and saponins of the seeds of *Glinus lotoides* in the crude extracts and tablet formulation thereof were quantified by reversed-phase high-performance liquid chromatographic (RP-HPLC) methods with UV detection. The saponins were analyzed after acid hydrolysis in 3 M HCl at 100 °C for 1 h. Vicenin-2 and mollugogenol B were isolated and used as reference substances for the quantification of total flavonoids and saponins, respectively. The identity and purity (>97%) of the standards were confirmed by spectroscopic (UV, MS, and NMR) and chromatographic (HPLC) methods. The flavonoids and saponins of the crude extract of the seeds and tablet formulation were separated by RP-HPLC (Nucleosil RP-18 column, 250 mm × 4.6 mm) using linear gradient elution systems of acetonitrile–water–0.1 M H_3PO_4 for flavonoids and methanol–water for saponins. Satisfactory separation of the compounds was obtained in less than 30 and 25 min, for the flavonoids and saponins, respectively. The methods were validated for linearity, repeatability, limits of detection (LOD) and limits of quantification (LOQ). Repeatability (inter- and intra-day, $n = 6$ and 9, respectively) showed less than 2% relative standard deviation (RSD). The LOD and LOQ were found to be 0.075 and 0.225 mg/mL, respectively, for vicenin-2 and 0.027 and 0.082 mg/100 mL, respectively, for mollugogenol B. The content of flavonoids and saponins of six single tablets was between 95 and 103% for flavonoids and 94–98% for saponins. The validated HPLC methods were employed to standardize a fingerprint of a laboratory produced purified extract, which could be used as a secondary standard for the routine quality control. Accordingly, the purified extract was found to contain 21.3% flavonoids (vicenin-2, 10%) and 25.4% saponins (glinuside G, 14.2%).
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Keywords: *Glinus lotoides*; Vicenin-2; Mollugogenol B; Glinuside G; RP-HPLC; Quantitative determination

1. Introduction

Glinus lotoides Linné (Molluginaceae), locally known as "Mettere" is an annual or short-lived perennial prostrate herb, the seeds of which are traditionally used in Ethiopia for the treatment of tape worm infection [1,2]. A description of the plant, the traditional mode of administration, the in vitro and in vivo taenicidal activity against *Tenia saginata* and *Hymenolepis nana* worms have been reported [3–6]. Preliminary pharmacological studies undertaken on the plant indicate that the extract does not affect the blood pressure, heart rate or the ECG of anesthetized rabbits, bile production in guinea pigs, or contraction of frog isolated heart after oral administration [7,8]. In an attempt to formulate the extracts of the plant into dosage forms, the effects of some extraction processes, such as extracting solvents like various concentrations of methanol in water and methods of extract drying like freeze-drying and vacuum oven-drying

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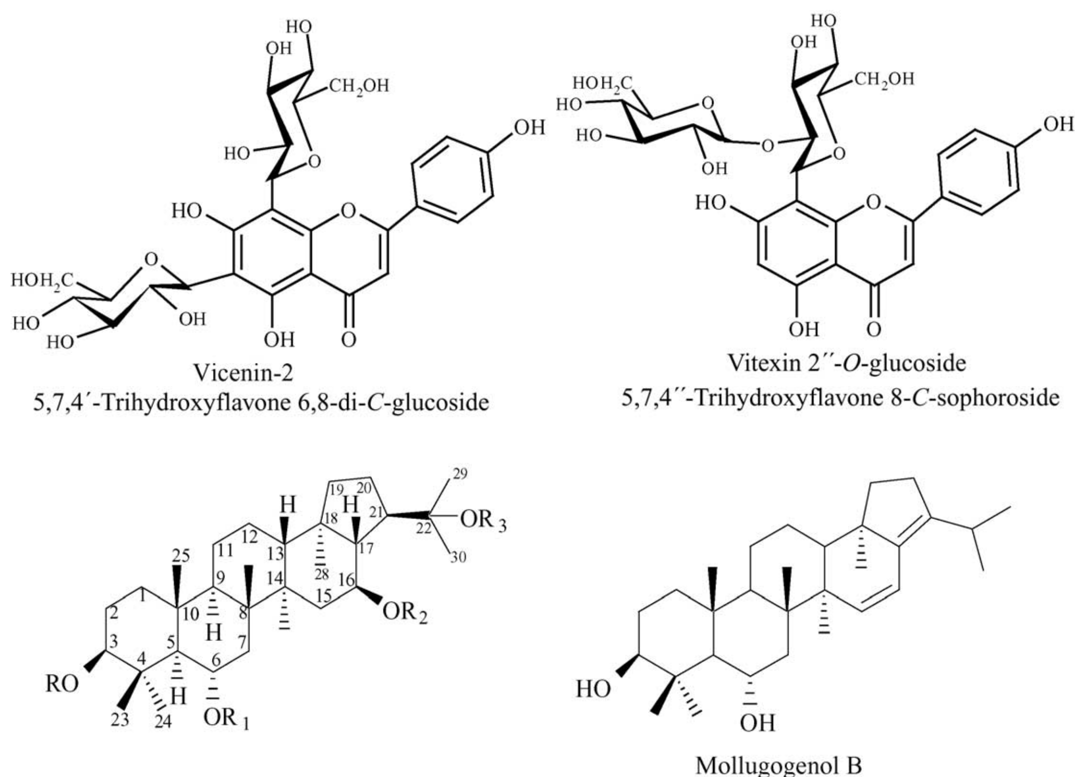
doi:10.1016/j.chroma.2005.05.095



Saponins/Aglycon	R	R1	R2	R3	Formula	Mol. Weight
Mollugogenol A	H	H	H	H	$C_{30}H_{49}O_4$	473
Glinuside F	Xyl	Xyl	Xyl	H	$C_{45}H_{76}O_{16}$	872
Glinuside G	Rha-Xyl	H	H	Rha	$C_{47}H_{80}O_{16}$	900
Glinuside H	Rha-Xyl	Xyl	Xyl	H	$C_{51}H_{86}O_{20}$	1018
Glinuside I	Rha-Xyl	Xyl	H	Rha	$C_{52}H_{88}O_{20}$	1032
Succulentoside B	Xyl	Ara	Xyl	H	$C_{45}H_{76}O_{16}$	872

Abbreviations: Xyl, xylose; Rha, rhamnose and Ara, arabinose.

Fig. 1. Flavonoids and saponins of *Glinus lotoides* and mollugogenol A and B.

on the yield and physicochemical properties of the saponin-containing extracts of *G. lotoides* have been investigated and standardized [9].

The major constituents of the seeds of *G. lotoides* are hopane-type triterpenoidal saponins and *C*-glycoside flavones, which are the main bioactive components of the herb, contributing to the cestocidal and pharmacological activity [4–8]. Recently, five hopane-type saponins (glinusides F, G, H, I, and succulentoside B) and two flavonoids (vicenin-2 and vitexin-2′′-*O*-glucoside) have been isolated from the seeds of the plant and their structures were elucidated (Fig. 1) [10]. Similarly, seven hopane-type saponins (lotoidesides A, B, C, D, E, F, and succulentoside B) have been isolated from *G. lotoides* [11]. In addition, Abegaz and Tecle isolated one triterpenoidal saponin with an oleanane skeleton from the seeds of the plant [12]. Furthermore, five hopane triterpenoidal saponins (glinusides A, B, C, D, and E) have been reported from the aerial parts of *G. lotoides* var. *dictamnoides* growing in Egypt [13,14].

The major saponins of *G. lotoides* consist of the same aglycon, mollugogenol A, which is converted into mollugogenol B during acid hydrolysis (Fig. 1). Unlike the native saponins of *G. lotoides*, mollugogenol B is UV-active due to the formation of a diene chromophore in ring D and E [10].

High-performance liquid chromatographic (HPLC) and thin-layer chromatographic (TLC) methods are generally used for the analysis and identification of pharmaceuticals based on their chromatographic retention data. However, identification problems arise when standards are not available, which is the case for most phytopharmaceutical preparations. In an effort to standardize the crude extract, colorimetric and UV-spectrophotometric methods have been developed for quantitative determination of total saponins in the crude extracts using a purified extract and β-escin, a mixture of

triterpenoidic saponins obtained from *Aesculus hippocastanum*, as standards [15]. However, there has been no attempt so far to isolate and quantify the flavonoids and saponins of the seeds of the plant.

The present work, therefore, reports on the development and validation of reversed-phase (RP)-HPLC methods for the identification and quantitative determination of total flavonoids and saponins of *G. lotoides* in the crude extract and tablet formulation thereof using vicenin-2 and mollugogenol B as standard substances, respectively. This is the first attempt to quantify the flavonoids and saponins of *G. lotoides* using HPLC.

2. Experimental

2.1. Materials

Fruits of *G. lotoides* were purchased from the local market, 'Merkato' in Addis Ababa, Ethiopia, in September 2001. The identity was confirmed by the National Herbarium, Department of Biology, Faculty of Science, Addis Ababa University, Ethiopia, and a voucher specimen (No. 003444) has been deposited. Sodium methoxide, aluminum chloride, hydrochloric acid, sodium acetate, boric acid, acetonitrile, *n*-hexane, methanol, *n*-butanol, diethyl ether were all of pharmaceutical grade (Merck, Darmstadt, Germany). All HPLC solvents were of HPLC grade.

2.2. Preparation of standards

2.2.1. Extraction and isolation of vicenin-2 and mollugogenol B

The seeds of *G. lotoides* (500 g) were separated from the fruits, powdered and defatted with 5 L *n*-hexane using ultraturrax (10000 rpm, 10 min). The defatted powdered seeds were extracted with 5 L 60% methanol in water using ultraturrax. The extract was filtered, concentrated under reduced pressure and dried. The dried extract (90 g) was dissolved in water and partitioned between equal volumes of *n*-butanol and water. On separation, the butanol fraction was dried (16 g), dissolved in a small volume of methanol and poured into a large volume of diethyl ether. The precipitate formed, containing mainly flavonoids and saponins, was separated by centrifugation and dried (12 g). This extract is referred to as purified extract and was used as a starting material for the isolation of the saponins and flavonoids as well as a secondary standard for the routine quality control of the crude extract and the tablet formulation thereof.

The purified extract (302 mg) was eluted on sephadex LH-20 column (28 cm × 1.5 cm i.d.) with methanol at a rate of 4 mL/min providing 20 fractions. These were combined into two major fractions [Fraction 1 (88 mg) and Fraction 2 (150 mg)] after TLC control on analytical RP-18 TLC plates using a mixture of MeOH–H_2O (4:1) as eluent and 5% vanillin–H_2SO_4 reagent for detection. Fraction 1, containing mainly flavonoids, was separated by reversed-phase HPLC (CC250/4 Nucleosil 100-7 C_{18} Macherey-Nagel, GmbH & Co. KG, Düren, Germany) with a guard column (ChroCART 4-4 Lichrospher 100 RP-18, 5 μm, Merck KGaA, Darmstadt, Germany) using 15% CH_3CN in water as a mobile phase at a flow rate of 1.0 mL/min to provide 5,7,4′-trihydroxyflavone-6,8-di-*C*-glucoside (vicenin-2) (6.1 mg) and 5,7,4′-trihydroxy-flavone-8-*C*-sophoroside (vitexin-2″-*O*-glucoside) (3.6 mg). Glinusides F, G, H, I, and succulentoside B were isolated from Fraction 2 [10].

The purified extract (500 mg) was hydrolyzed with 3 M HCl at 100 °C for 1 h and the saponin aglycons were extracted with diethyl ether. The major hydrolysis product (30 mg) was isolated using RP-HPLC, using the same column and guard column described above and the mobile phase acetonitrile and UV detection at 254 nm. The structure was elucidated with NMR as mollugogenol B.

2.2.2. Structural confirmation of vicenin-2 and mollugogenol B

The isolated flavonoids were identified by a combination of UV spectrophotometry in methanol and after the addition of the classical alkali and metal reagents [16]. ESIMS and NMR analyses were performed for structural confirmation of the flavonoids as well as mollugogenol B.

UV spectra of the isolated standards were recorded on a Perkin-Elmer Lambda 16 UV–vis spectrometer (Überlingen, Germany). 1D (^{1}H, ^{13}C, and DEPT-135) and 2D (^{1}H COSY, HMQC, and HMBC) NMR spectra were obtained at 300 K on Bruker AVANCE DMX-600 or ARX-400 NMR spectrometers (Bruker S.A., Wissembourg, France) locked to the major deuterium signal of the solvent, CD_3OD. Chemical shifts are reported in δ ppm and coupling constants in Hz.

The mass spectra of the substances were obtained using a Thermo Finnigan TSQ Quantum triple quadrupole mass spectrometer (Thermo Finnigan, San Jose, CA, USA) equipped with an electrospray interface coupled to an integrated syringe pump system working with a flow rate of 10–20 μl/min. The electrospray interface was locked in a 90° position toward the orifice and operated at 3.8 kV in positive ionization mode and at 2.6 kV in negative ionization mode. Capillary temperature and sheath gas were optimized at 280 °C and 30 (arbitrary) units. Auxiliary gas was not used. The collision induced dissociation spectra (CID-spectra) were recorded with collision energy 20 eV for positive and 35 eV for negative mode. Source CID voltage was set to a constant value of 10 V for both Q1 and Q3 full scan and CID spectra, and the argon collision gas for MS/MS experiments was maintained at a pressure of 1.3×10^{-6} bar.

Vicenin-2: UV (MeOH, log ε): 272 (4.26), 330 (4.29); (+$AlCl_3$): 279, 305, 351, 387; (+$AlCl_3$/HCl): 280, 304, 344, 383sh; (+NaOMe): 282, 334, 399; (+NaOAc): 282, 395; (NaOAc/H_3BO_3): 284, 320, 406 nm. ESIMS (positive mode) *m/z* 595 $[M+H]^+$, 475 $[(M+H)\text{-}120]^+$, 433 $[(M+H)\text{-}162)]^+$, 355 $[(M+H)\text{-}2(120)]^+$, 313$[(M+H)\text{-}(120+162)]^+$, 271 $[(M+H)\text{-}2(162)]^+$ (negative mode) *m/z* 593 $[M-H]^-$, 473

[(M − H)-120]$^-$, 383 [(M − H)-(120 + 90)]$^-$, 353 [(M − H)-2(120)]. ^{1}H NMR: δ 7.95 (H-2′ and H6′, d, J 9 Hz), 6.93 (H-3′ and H-5′, d, J 9 Hz), 6.70 (H-3, s). ^{13}C NMR: δ 181.11 (C-4), 162.72 (C-2), 160.78^a (C-5), 160.77^a (C-7), 159.25^a (C-4′), 155.01 (C-9), 128.35 (C-2′, C-6′), 121.70 (C-1′), 115.65 (C-3′, C-5′), 108.32 (C-6), 104.45 (C-10), 102.03 (C-8), 101.64 (C-3), 81.47 (C-5″), 80.87 (C-5‴), 78.77 (C-3″, C-3‴), 74.27 (C-1‴), 74.00 (C-1″), 71.43 (C-2‴), 71.10 (C-2″), 70.39 (C-4″), 69.86 (C-4‴), 61.08 (C-6″), 60.60 (C-6‴). ^{a}Assignments interchangeable.

Mollugogenol B: UV (MeOH, log ε): 260sh (3.98), 251 (4.16), 243 (4.12). ^{1}H NMR: δ 6.28 (H-16, d, J 10 Hz), 5.68 (H-15, d, J 10 Hz), 4.04 (H-6a, ddd, J 10, 10, 4), 3.15 (H-3, dd, J 10.5, 6), 2.82 (H-22, qq, J 7.7), 2.42 (H-20a, ddd, J 16, 10.5, 6), 2.25 (H-20b, dd, J 16, 9), 1.33 (H-23, s), 1.24 (H-27, s), 1.04 (H-30, d, J 7), 1.03 (H-26), 1.02 (H-29, d, J 7), 0.98 (H-24, s), 0.93 (H-28, s), 0.92 (H-25, s). ^{13}C NMR: δ 141.7 (C-21, s), 140.3 (C-17, s), 136.0 (C-15, d), 120.7 (C-16, d), 79.6 (C-3, d), 69.1 (C-6, d), 61.8 (C-5, d), 51.3 (C-9, d), 49.0 (C-18, s), 49.0 (C-13, d), 47.2 (C-8, s), 46.0 (C-7, t), 43.5 (C-14, s), 41.3 (C-19 or C-1, t), 40.5 (C-4, s), 39.9 (C-10, s), 31.5 (C-23, q), 29.0 (C-20, t), 28.0 (C-22, d), 27.8 (C-2, t), 23.4 (C-12, t) 22.8 (C-11, t), 22.0 (C-29, q), 21.6 (C-30, q), 19.2 (C-28, q), 18.7 (C-26, q); 18.0 (C-27, q), 17.2 (C-25, q), 16.1 (C-24, q).

2.3. Sample preparation for HPLC analysis

2.3.1. Crude extract

For the analysis of the flavonoids, 20, 40, 50, and 76 mg dried extract (see details of extraction procedure in Section 2.2.1) were dissolved in 100 mL water and samples of the solution were filtered through a 0.2 μm cellulose acetate filter (Sartorius AG, Goettingen, Germany) before injection into HPLC. Total flavonoids were calculated as vicenin-2 equivalent.

Saponins were analyzed after the dried extract was hydrolyzed with 3 M HCl at 100 °C for 1 h. The hydrolysis products were cooled and the aglycons were extracted three times with diethyl ether. The ether layers were combined, dried, and dissolved in 100 mL methanol. The solution was filtered and injected into HPLC. Optimal hydrolysis conditions were investigated using various acid concentrations (0.5–5.0 M HCl), hydrolysis temperature (25–100 °C), and hydrolysis duration (0.5–8 h), and a method was selected that provided the maximum amount of mollugogenol B with minimum artifacts. Calculation of the total saponins was based on glinuside G, which possess an average molecular weight of *G. lotoides* saponins (Fig. 1).

2.3.2. Analysis of tablet formulation of the crude extract

Oblong tablets (1.4 g) containing 650 mg extract of the seeds of *G. lotoides* were crushed using mortar and pestle and 120 mg of the tablet powder were suspended in a 100 mL volumetric flask with water. The mixture was sonicated for 30 min and a portion of the supernatant was filtered through a 0.2 μm cellulose acetate filter before injection into HPLC. For the analysis of the saponins, 215.4 mg of the tablet powder, equivalent to 100 mg extract, were hydrolyzed with 100 mL of 3 M HCl at 100 °C for 1 h. The aglycons were extracted three times with 100 mL diethyl ether. The ether layers were combined, dried, and dissolved in 100 mL methanol. The solution was filtered and injected into HPLC. Six tablets were analyzed and the content of the flavonoids and saponins was determined. The tablet formulation contained Aeroperl® 300 Pharma (20%), Avicel® PH 101 (26%), Ac-Di-Sol® (6.5%), and magnesium stearate (1%).

2.4. Preparation of calibration curves

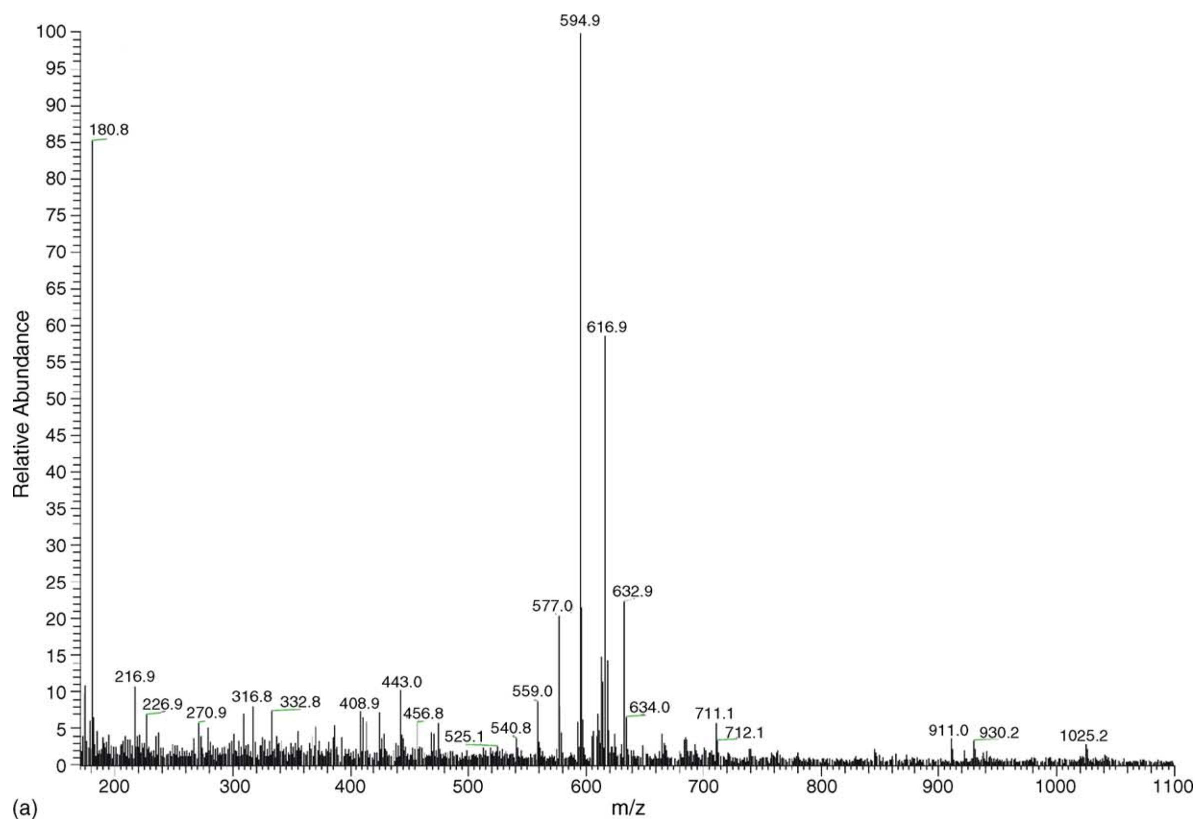
An amount of 5.83 mg vicenin-2 was accurately weighed and dissolved in 100 mL of water (stock solution [5.83 mg/100 mL]). Five additional calibration levels (4.664, 3.489, 2.332, 1.166, and 0.583 mg/100 mL) were prepared by dilution of the stock solution with water. Similarly, 2.37 mg mollugogenol B was accurately weighed and dissolved in 100 mL of methanol (stock solution [2.37 mg/100 mL]). Five additional calibration levels (1.896, 1.422, 0.948, 0.474, and 0.237 mg/100 mL) were prepared by dilution of the stock solution with methanol. The standard solutions, stored at 4 °C, were stable for at least 3 months, as confirmed be re-assaying the solutions. The limits of detection (LOD) and limits of quantification (LOQ) were determined by serial dilution and were calculated according to Gottwald [17].

2.5. Chromatographic conditions

HPLC pump (LC-10AD, Shimadzu Liquid Chromatography, Shimadzu Co., Kyoto, Japan) with a manual Rheodyne injector (7021, Rohnert Park, CA, USA) and a Chromato-Integrator (D-2000, Merck, Darmstadt, Germany) were used for the HPLC analysis. A Millpore® Waters, Model 481 LC spectrophotometer, (Milford, MA, USA) was used at 360 and 254 nm for detection of the flavonoids and hydrolysis products of the saponins, respectively.

Nucleosil RP-18 Columns (CC 250/4.6 Nucleosil 100-5 C_{18}, Macherey-Nagel, GmbH & Co. KG, Düren, Germany) with guard columns (LichroCART® 4-4, Lichrospher® 100-5 RP-18, Merck KGaA, Darmstadt, Germany) were used for the separation of flavonoids and saponins.

A binary linear gradient elution system composed of two mixtures [Mixture A: acetonitrile–water–0.1 M H_3PO_4 (130:825:24) and Mixture B: acetonitrile–water–0.1 M H_3PO_4 (240:374:12)] was used for the analysis of the flavonoids with the following gradient elution: 0–20 min, 100% Mixture A to 100% Mixture B; 20–25 min, 100% Mixture B to 100% Mixture A; 25–30 min, 100% Mixture A. For the separation of the saponin aglycons, the mobile phase composed of 95% (w/w) MeOH in water (A) and 100% MeOH (B) was used with gradient elution as follows: 0–15 min, 100% A to 100% B; 15–20 min, 100%B to 100% A; 20–25 min, 100% A. The solvents were degassed with helium (Type



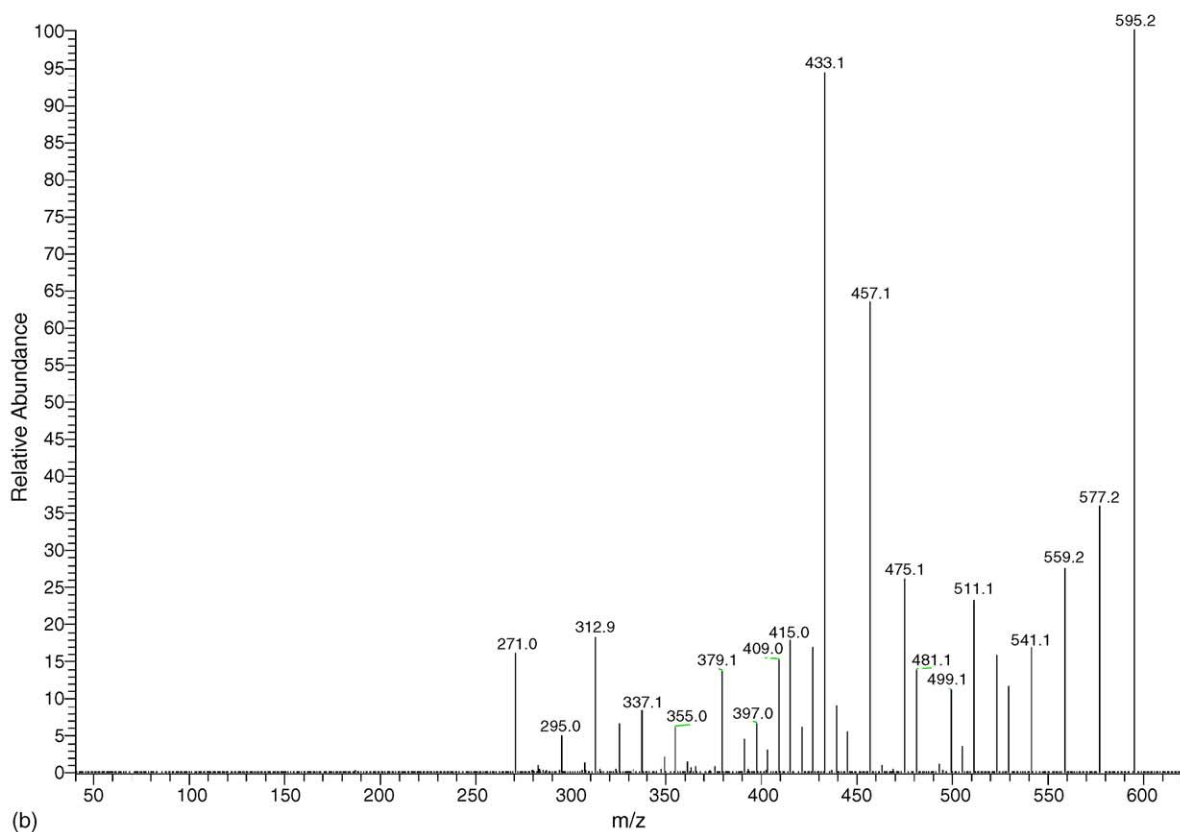
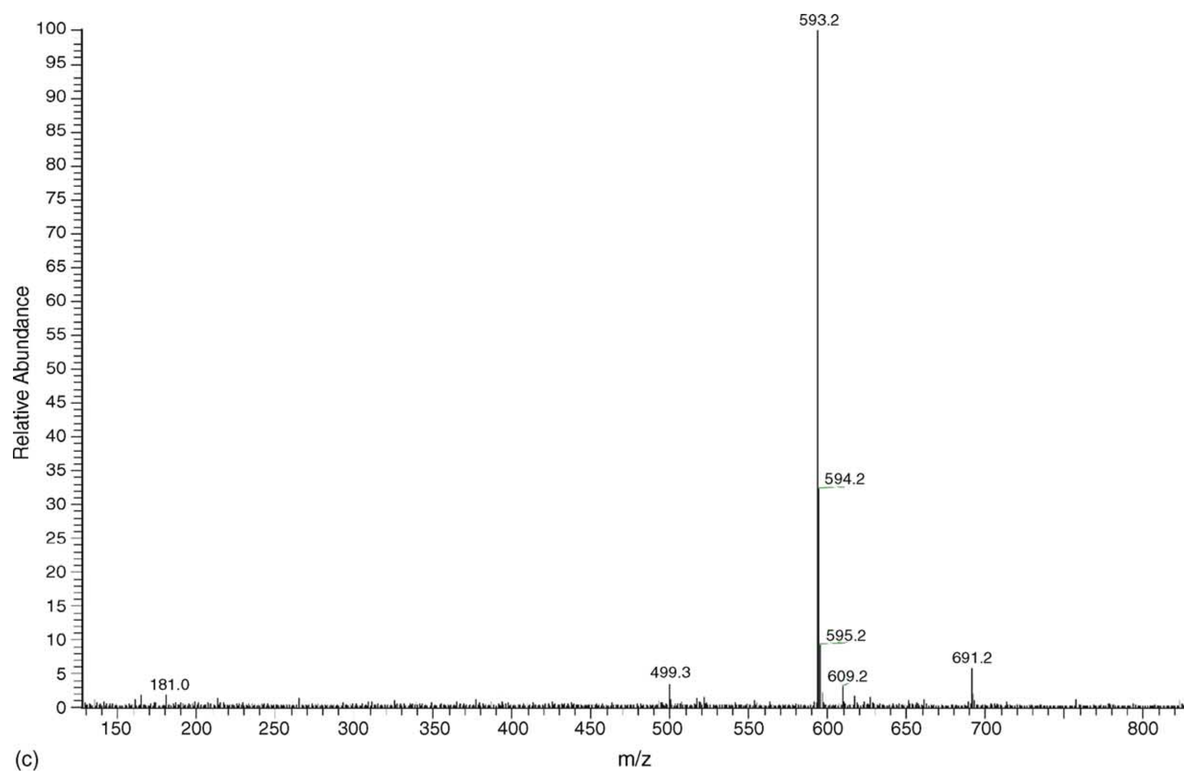


Fig. 2. The mass spectra of the isolated flavonoid: (a) full scan positive mode; (b) CID-fragmentation at *m*/*z* 595; (c) full scan negative mode; and (d) CID-fragmentation at *m*/*z* 593.



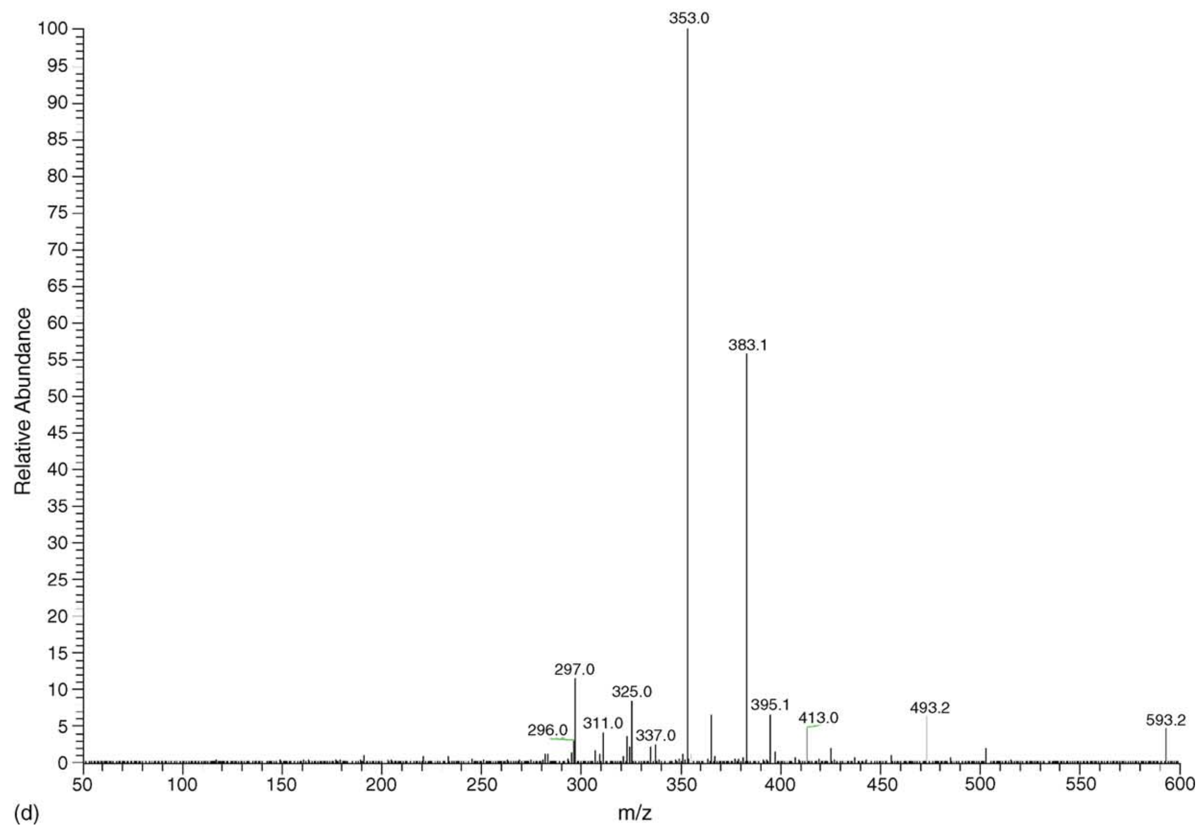


Fig. 2. (*Continued*).

5.0, Messer Griesheim GmbH, Krefeld, Germany). Twenty microlitres of sample were injected and eluted with a flow rate of 1.0 mL/min.

2.6. Method validation

The calibration curves were analyzed using a linear regression model and correlation coefficients. The calibration curves, residuals, and standardized residuals were inspected to assess linearity. The intra- and inter-day repeatability of the method was evaluated using multiple preparations of the sample. The accuracy of the method was verified by spiking 32.8 mg/100 mL solution of the extract with the standard solutions. The recovery of vicenin-2 in 50, 75, 100, 125, and 150% solution was determined by HPLC. The LOD and LOQ were determined from serial dilution and were calculated according Gottwald [17]. The content of flavonoids and saponins in six single tablets were analyzed.

3. Results and discussion

3.1. Preparation of standards

3.1.1. Isolation and characterization of vicenin-2 and mollugogenol B

Due to the absence of commercial standards, vicenin-2 and mollugogenol B were isolated and used as reference substances for the flavonoids and the aglycons of the saponins of *G. lotoides*, respectively. The identities of the isolated standards were confirmed with spectral (UV, MS, and NMR) analyses.

Two flavonoids were isolated form the purified extract of the seeds of *G. lotoides* using the method described earlier [10]. Their UV spectra in methanol using various diagnostic reagents, revealed the presence of flavones with free 4′-, 5-, and 7-hydroxyl groups [15]. ESIMS full-scan spectra of the major flavonoid ($t_0 = 8$) showed molecular ions at *m/z* 594.9 $[M+H]^+$ (Fig. 2a) and 593.2 $[M-H]^-$ (Fig. 2c) with positive and negative modes, respectively. The collision induced dissociation spectra of the flavonoid provided characteristic fragmentation patterns at *m/z* 475$[(M+H)\text{-}120]^+$, 433$[(M+H)\text{-}162)]^+$, 355$[(M+H)\text{-}2(120)]^+$, 313$[(M+H)\text{-}(120+162)]^+$, 271$[(M+H)\text{-}2(162)]^+$ with positive mode (Fig. 2b) and *m/z* 473$[M-H\text{–}120]^-$, 383$[M-H\text{-}120\text{-}90]^-$, 353$[M-H\text{-}2(120)]^-$ negative mode (Fig. 2d) indicating the presence of two hexose sugars, which are *C*-glycosides at C-6 and C-8 [18]. ^{1}H and ^{13}C NMR spectra were identical to the published data for the *C*-flavone glycoside, vicenin-2 [19].

Triterpene saponins isolated from the seeds of *G. lotoides* are derivatives of the same aglycon, mollugogenol A, differing only in their sugar moieties and position of substitution (Fig. 1). In addition to their close structural resemblance, the saponins of *G. lotoides* lack chromophore to be UV-detected, which makes the separation and quantification by HPLC/UV of the individual saponins a challenge. Hence, the saponins of *G. lotoides* were quantified after acid hydrolysis using mollugogenol B as an external standard. The structure of mollugogenol B was confirmed using NMR spectral analyzes and comparison with reported data [20].

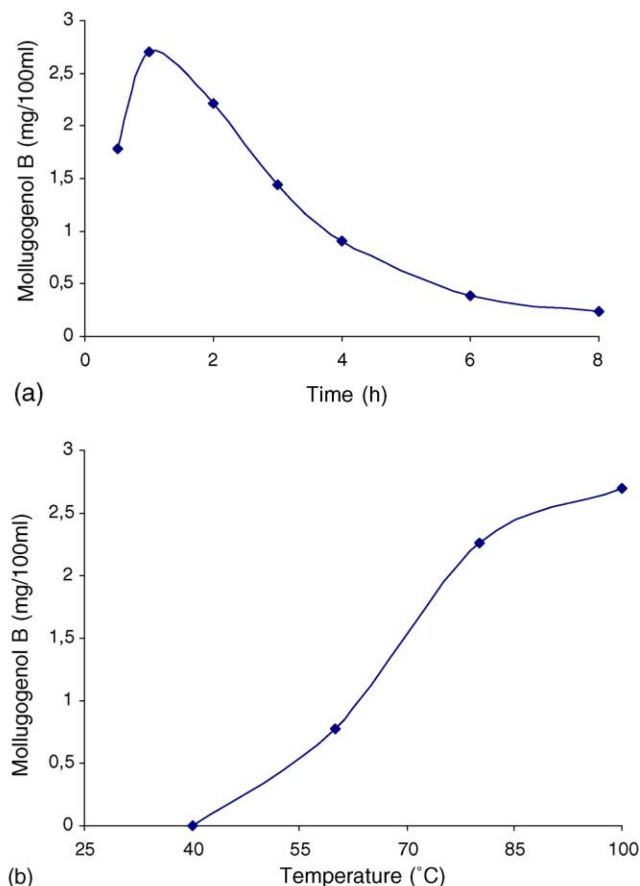


Fig. 3. Effect of time (a) and temperature (b) of acid hydrolysis of the saponins of *G. lotoides* on the mollugogenol B yield. Acid hydrolysis conditions: (a) 3 M HCl at 100 °C and (b) 3 M HCl for 1 h.

During acid hydrolysis, a number of saponins generate not only the genuine aglycon but also artifacts, which can influence the final results. Acid-catalyzed double-bound migration, epimerisation, and dehydration are often observed during hydrolysis [21]. Besides, structurally different saponins show different rates of hydrolysis [22]. Hence, optimization of hydrolysis conditions is essential. As shown in Fig. 3, acid hydrolysis of the saponins using 3 M HCl at 100 °C for 1 h provided the maximum yield of mollugogenol B. The optimum time of hydrolysis was determined by analyzing the hydrolyzes at different time intervals (Fig. 3a). The formation of mollugogenol B reached the maximum after 1 h and subsequent heating under the hydrolysis conditions resulted in a loss of mollugogenol B. As shown in Fig. 3b, the maximum amount of mollugogenol B was obtained using a hydrolysis temperature of 100 °C. Lower acid concentrations (0.5, 1, and 2 M HCl) required longer time to achieve the maximum concentration of mollugogenol B, whereas higher acid

concentrations (4 and 5 M HCl) produced artifacts, which reduced the yield of mollugogenol B.

3.1.2. Determination of purity of the isolated standards

The purity of the standards as determined by HPLC methods at different experimental conditions was found to be more than 97%. For identification and quantification of minor impurities, high concentrations of the isolated vicenin-2 and mollugogenol B, 56 and 47 mg/100 mL, respectively, were injected into HPLC and developed with various solvent systems. Peak areas of the standards were compared to the total peak area of the chromatogram and were found to be 97 and 98% for vicenin-2 and mollugogenol B, respectively.

3.2. Method development and validation

In the development of HPLC method for the determination of total flavonoids in the crude extract as well as in tablet formulation vicenin-2 was used as an external standard. Several solvent systems were evaluated as mobile phase and the gradient water–acetonitrile–0.1 M phosphoric acid system with a flow rate of 1 mL/min was selected. Fig. 4 depicts chromatogram of vicenin-2 (Fig. 4a), crude extract of *G. lotoides* (Fig. 4b) and tablet formulation of the extract (Fig. 4c). The flavonoid at retention time of 10.7 min was identified as vitexin-2″-*O*-glucoside. As shown in Fig. 4c, the HPLC chromatogram of the tablets showed similar flavonoids pattern compared to the crude extract (Fig. 4b) indicating that the HPLC method was not affected by the tablet excipients.

Within the concentration range of 0.583–5.83 mg/100 mL, the relationship between peak area of vicenin-2 (Y) and concentration in mg/100 mL (X) was linear with a regression equation $Y = 260009X - 5250.8$. The linearity of the calibration curve was verified by the correlation coefficient ($r^2 = 0.9997$) as well as inspection of the residuals. The LOD and LOQ were found to be 0.075 and 0.225 mg/100 mL, respectively. Similar LOD and LOQ results were obtained using serial dilution of the standard solution. The amount of total flavonoids in the crude extracts and tablet formulation were calculated as vicenin-2 equivalent from the total peak areas of the chromatogram (Fig. 4b and c).

For the quantitative determination of the saponins after acid hydrolysis, various mobile phase compositions were studied and the gradient methanol–water system was chosen. Fig. 5 depicts typical chromatogram of mollugogenol B (Fig. 5a), the acid hydrolysis of the saponins from the crude

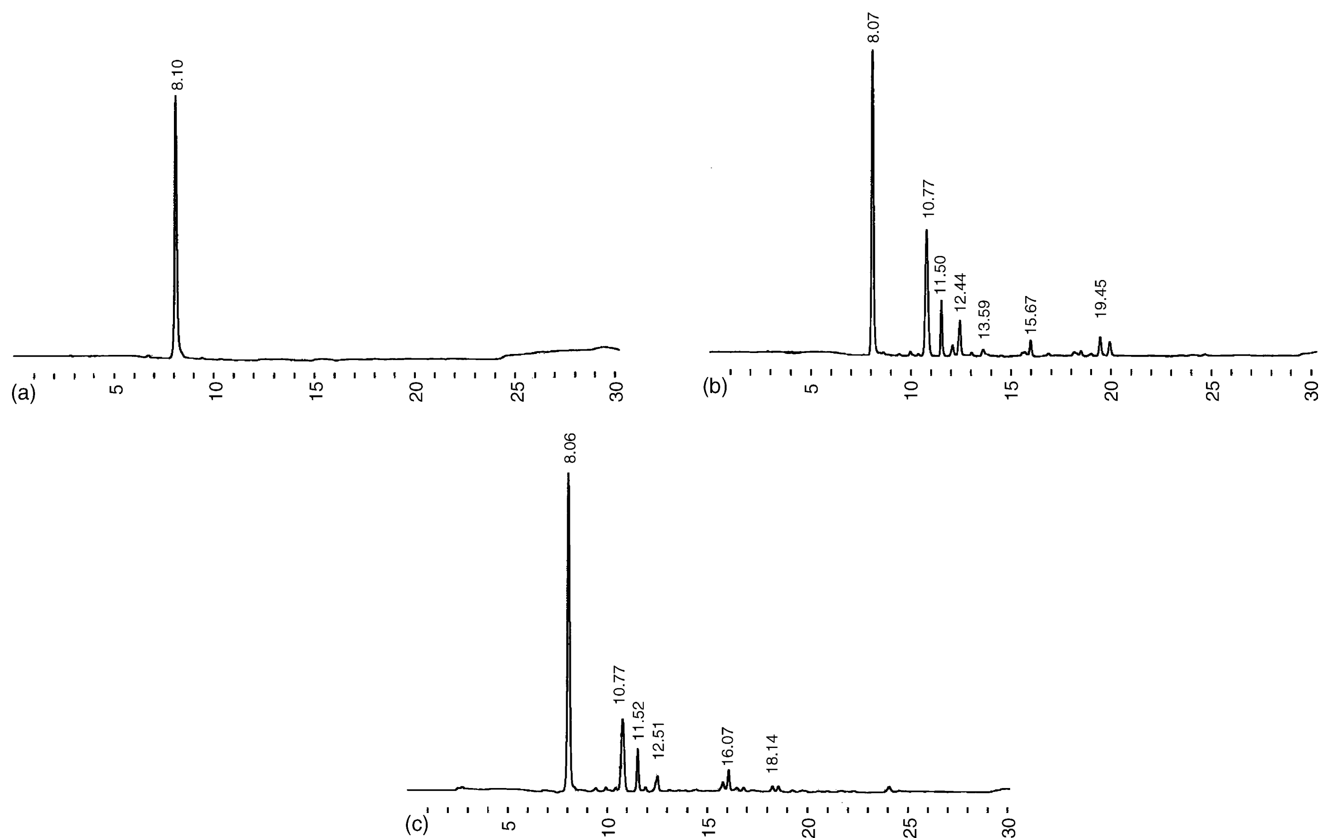


Fig. 4. Chromatograms of: (a) vicenin-2 standard; (b) crude extract of the seeds of *G. lotoides*; and (c) tablet formulation of the extract separated by RP-18 HPLC [column: CC 250/4.6 Nucleosil 100-5 C_{18}, Macherey-Nagel; guard columns: LichroCART® 4-4, Lichrospher® 100-5 RP-18, Merck; mobile phase: (Mixture A: acetonitrile–water–0.1 M H_3PO_4 (130:825:24) and Mixture B: acetonitrile–water–0.1 M H_3PO_4 (240:374:12)) gradient elution: 0–20 min, 100% Mixture A to 100% Mixture B; 20–25 min, 100% Mixture B to 100% Mixture A; 25–30 min, 100% Mixture A.; flow rate 1.0 mL/min; detection: 360 nm; injected sample volume: 20 μL]. Assignment of peaks: vicenin-2 (8 min) and vitexin-2″-*O*-glucoside (10 min).

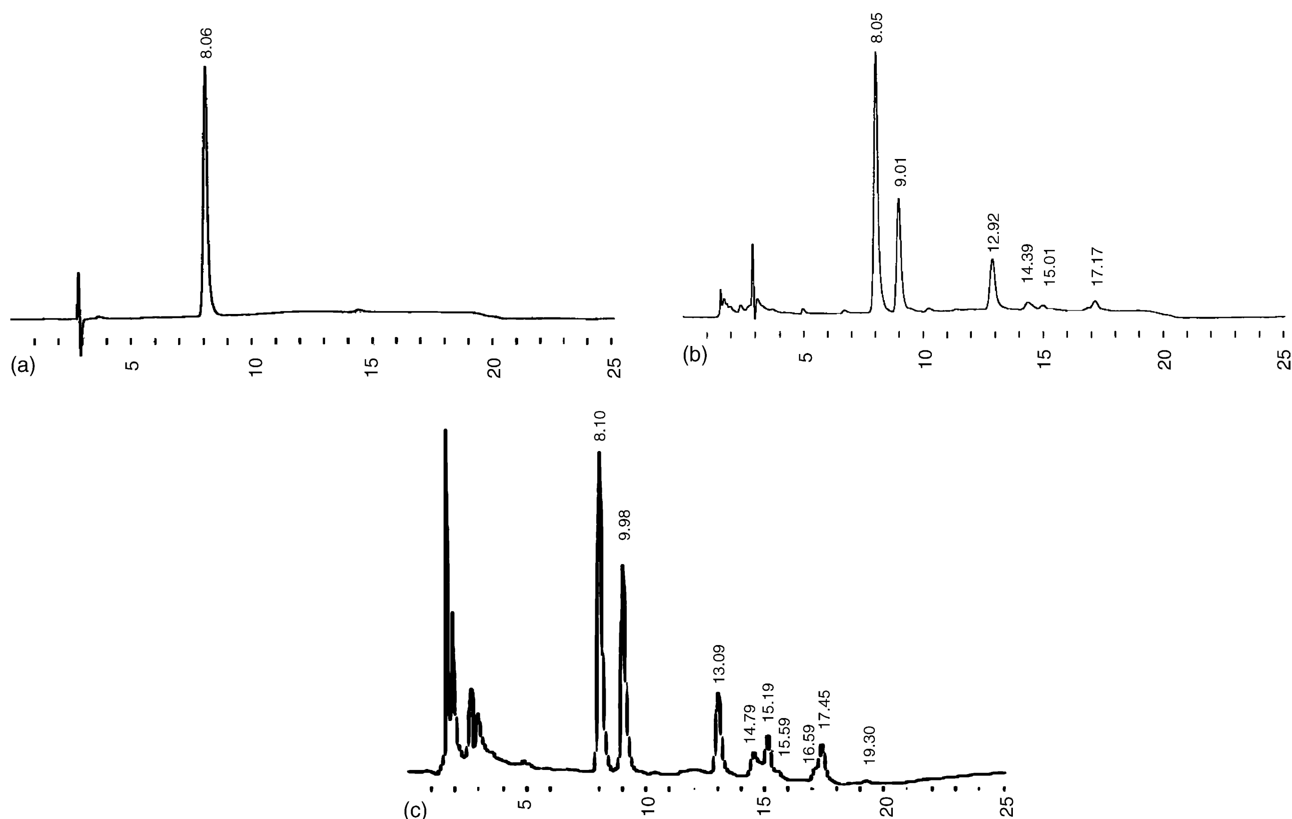


Fig. 5. HPLC Chromatogram of: (a) mollugogenol B as external standard; (b) acid hydrolysis products of the crude extract of the seeds of *G. lotoides*; and (c) tablet formulation of the extract after acid hydrolysis. [column: CC 250/4.6 Nucleosil 100-5 C_{18}, Macherey-Nagel; guard columns: LichroCART® 4-4, Lichrospher® 100-5 RP-18, Merck; mobile phase: 95% (w/w) MeOH in water (A) and 100% MeOH (B) with gradient elution: 0–15 min, 100% A to 100% B; 15–20 min, 100% B to 100% A; 20–25 min, 100% A; flow rate 1.0 mL/min; detection: 254 nm; injected sample volume: 20 μL]. Assignment of peak: mollugogenol B (8 min) other peaks are not identified.

extract of the seeds of *G. lotoides* (Fig. 5b) and the tablet formulation thereof (Fig. 5c). As shown in the figure, separation of the aglycons was completed within 25 min and the major peak at 8 min belongs to mollugogenol B. A linear relationship with the regression equation $Y = 376738X - 703.7$ ($r^2 = 0.9998$) was observed for mollugogenol B in the concentration range of 0.237–2.37 mg/100 mL. The LOD and LOQ were 0.027 and 0.082 mg/100 mL, respectively, which was confirmed with serial dilution. The total amounts of saponins of the crude extract and tablet formulation were determined as glinuside G equivalent. The molecular weight of glinuside G (900) approximates the average molecular weight of the saponins of *G. lotoides* (Fig. 1). Before determination of the total saponins, an equimolecular mollugogenol A was calculated from mollugogenol B.

Accuracy of the analytical method was determined by spiking 32.8 mg of purified extract with standard solutions in the range of 50–150%. The recovery of vicenin-2 was found to be between 98.8 and 101.6%. Reproducibility of the methods was verified by determining six extracts with relative standard deviations of 0.95 and 1.17% for the flavonoids and aglycons, respectively. Intra-day variations ($n = 9$) were found to be 1.86 and 0.50% for the flavonoids and saponins, respectively. The values are in good agreement with USP requirements for a validated method. The content of flavonoids and saponins of six single tablets was between 95 and 103% for flavonoids and 94 to 98% for saponins.

3.3. Preparation of secondary standard

Characterization and utilization of a secondary standard is necessary as extraction and isolation of the pure standards are time consuming and expensive for routine quality control of plant extracts. Hence, a purified extract of the seeds of *G. lotoides* was prepared and characterized, following the extraction and purification of the extract as described above (Section 2.2.1). The amounts of total flavonoids and saponins in the purified extract were determined using the HPLC methods detailed above (Sections 2.3.1 and 2.5).

Accordingly, the secondary standard was found to contain 10% vicenin-2 and 21.3% total flavonoids calculated as vicenin-2 equivalent. The amount of saponins in the secondary standard was 6.3% mollugogenol B and 12.4% total aglycons calculated as mollugogenol B, 14.2% glinuside G and 25.4% total saponins calculated as glinuside G equivalent. The contents of the vicenin-2 and mollugogenol B standards, total flavonoids and saponins in the crude extract,

Table 1
The contents of vicenin-2 and mollugogenol B standards, total flavonoids and saponins of *G. lotoides* in the crude extract, secondary standard and tablet formulation

Compound(s)	Crude extract (%)	Secondary standard (%)	Tablet formulation[a] (%)
Vicenin-2	4.4	10.0	4.3
Total flavonoids[b]	9.3	21.3	9.1
Mollugogenol B	3.1	14.2	3.0
Total saponins[c]	10.8	25.4	10.4

[a] 1.4 g oblong tables containing 650 mg extract of the seeds of *G. lotoides*.
[b] Calculated as vicenin-2 equivalent.
[c] Calculated as glinuside G equivalent.

secondary standard and tablet formulation are summarized in Table 1.

4. Conclusion

HPLC methods for the identification and quantification of the flavonoids and saponins of the seeds of *G. lotoides* have been developed for a routine quantitative analysis of the extracts and the tablet formulation thereof. The recovery of vicenin-2 is good over a wide range of concentrations (50–150%) indicating the accuracy of the analytical method. It has been shown that the purified extract of the seeds could be used as a secondary standard for a routine quality control in tablet formulation of the extract. The methods have been validated for linearity, precision, LOD and LOQ.

Acknowledgment

The financial support of Deutscher Akademischer Austauschdienst (DAAD) to A. Endale is greatly acknowledged.

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