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Simultaneous isolation and identification of phytoconstituents from *Terminalia chebula* by preparative chromatography

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ABSTRACT

A reversed-phase preparative HPLC method with UV spectrophotometric detection has been developed for the simultaneous isolation of eight hydrolysable tannins from dried fruits of Terminalia chebula, a traditional herbal medicine. Isolation of phytoconstituents was achieved by preparative HPLC using C18 column and acetonitrile – 0.2% formic acid in water as mobile phase. The purities of the phytoconstituents were determined by HPLC and their structures were elucidated by spectroscopic (UV, ^{1}H-NMR, ESI-MS) techniques. These phytoconstituents can be used as marker compound to develop suitable identification test for raw materials, to determine the assay of active constituents of known therapeutic activity as well as stability of the extracts.

Keywords: Terminalia chebula; Hydrolyzable tannin; Preparative HPLC; ^{1}H -NMR; ESI-MS

INTRODUCTION

India has a rich heritage of traditional herbal medicine since ancient times. These herbal medicines formed the basis of healthcare worldwide since earliest days of mankind. They are still used and have considerable importance in international trade. There is huge biodiversity in India including varied geography, climatic changes and absence of standard practice of cultivation thereby leading to variations in composition and concentration of phytoconstituents. Adulteration and contaminations are regular events in herbal dug manufacturing. To ensure that raw materials used in the manufacturing of the drugs are not only authentic but also of prescribed quality; identification and evaluation of raw materials has become fundamental need of herbal industry. Thus finger printing and marker compound analysis by chemical and validated chromatographic techniques are gaining importance for use in standardizing herbal medicinal formulations [1].

The fruit of *Terminalia chebula Retizus* (Combrataceae) commonly known in India as Harad (Sanskrit: Haritaki) is found throughout India and Southeast Asia in deciduous forest and areas of light rainfall. There are seven varieties of haritaki all of which are more or less used in similar fashion but vary in specific usages and quality [2]. *T. chebula* has been reported to an exhibit variety of biological activity including anticancer, antioxidant, antidiabetic, antibacterial, antiviral, purgative, astringent and blood purifier[3-8]. In *T. chebula*, 33% of the total phytoconstituents are hydrolysable tannins (which may vary from 20-50%) and are responsible for pharmacological activity. These tannins contain phenolic carboxylic acid like gallic acid, ellagic acid, chebulic acid and gallotannins such as 1,6 di-*O*-galloyl-β-D-glucose, 3,4,6 tri-*O*-galloyl-β-D-glucose, 2,3,4,6 tetra-*O*-galloyl-β-D-glucose, 1,2,3,4,6 penta-*O*-galloyl-β-D-glucose. Ellagitannin such as punacalagin, casurarinin, corilagin and terchebulin and others such as chebulanin, neochebulinic acid, chebulagic acid and chebulinic acid reported in literature [9, 10]. Various methods have been reported for extraction of phytoconstituents from *T. chebula* for studying their pharmacological activities.

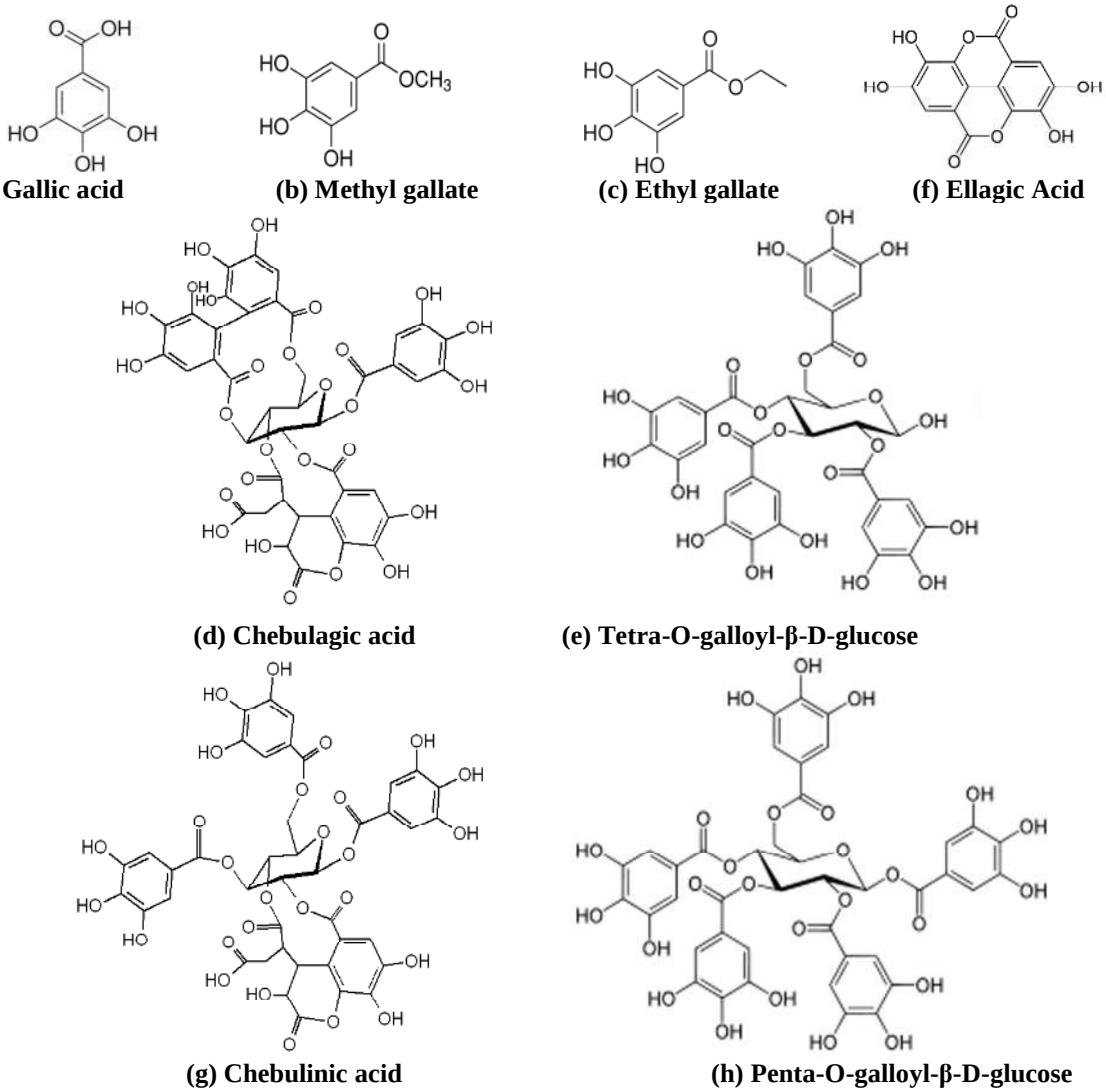

Fig. 1. Compounds from *T. chebula* fruit extract: (a) Gallic acid, (b) Methyl gallate, (c) Ethyl gallate, (d) Chebulagic acid, (e) Tetra-*O*-galloyl-*β*-D-glucose, (f) Ellagic acid, (g) Chebulinic acid, (h) Penta-*O*-galloyl-*β*-D-glucose

These methods comprise of extraction followed by size exclusion or reversed phase chromatography [3, 4]. However these methods consist of multiple steps and are unsuccessful in simultaneous preparative separation of phytoconstituents. Here in we present a new method comprising of simultaneous preparative separation of phytoconstituents of *T. chebula*. These phytoconstituents can be used as marker compounds to develop suitable identification test for raw materials, to determine the assay of active constituents of known therapeutic activity as well as stability of the extracts. This technique will not only be useful in establishing the correct botanical identity but also in regulating the chemical sanctity of a herb as well as raw material used in polyherbal formulation.

EXPERIMENTAL SECTION

General Procedures

For preparative chromatography a Waters Semi- preparative HPLC system equipped with Empower software, Waters Delta 600 Multisolvent delivery system and a UV-vis dual wavelength detector (Waters 2487), a system controller (Waters 600), and a Rheodyne injector 7725i with a 5 ml sample loop was used. HPLC-DAD analyses were carried out on a Waters Alliance system equipped with Empower software and DAD detector (Waters 2695). NMR was recorded on a Bruker Ultrashield spectrometer 400 MHz. The spectra were recorded in CD_3OD, Acetone-d_6 or DMSO-d_6 with TMS as internal standard. MS studies were done on Applied biosystem QTRAP LCMS-3200 with ESI probe. The ESI probe was operated in negative mode.

Chemicals

Used chemicals and solvents were purchased from Merck, India and were of either analytical or HPLC grade. Millipore water for HPLC was used for all experiments.

Plant Material

The dried fruit of *Terminalia chebula Retizus* (combrataceae) were procured locally in Mumbai, India. It was authenticated at Agharkar Research Institute Pune, India, and a voucher specimen (F-138) was deposited in the herbarium. The fruits (200 gm) were hammered into small pieces, and extracted (3X) with 70% aq methanol. (1 L) by stirring for 1 hour. Methanol was evaporated at reduced pressure by rotary evaporator yielding 70 g dry extract. 30 g of dry extract was redissolved in 750ml of HPLC grade water followed by treatment with an equal volume of hexane (3X) to defat the extract followed by chloroform to remove highly nonpolar compounds those may retain on column. The aqueous layer was then finally extracted with ethyl acetate. Ethyl acetate layer dried with anhydrous sodium sulphate and evaporated under reduced pressure on rotary evaporator to yield a residue (Figure 2).

High-Performance Liquid Chromatography (HPLC) Analysis

Separations were performed on a Inertsil ODS-3, (100 × 4.6 mm, 3 μm) column using following method. Mobile phases (A) 0.2% formic acid in water (B) acetonitrile. A gradient solvent system was used; 0-2 min, 10% B; 2-16 min, 35% B; 16-17 min, 10% B; 17-25 min, 10% B; the flow rate was 1.0 ml/min with monitoring at 220 and 272 nm.

Isolation by Preparative HPLC

The residue from ethyl acetate layer was dissolved in minimum amount of acetonitrile to obtain a suspension. The suspension was filtered through whatman 42 filter paper. The residue was kept aside for characterization purpose while the filtrate was taken up for separation by preparative chromatography. Separations were performed on a Luna 10µ C18

(2) 100A, (250 × 30 mm, 10 μm) column. The mobile phases used were (A) 0.2% formic acid in water (B) acetonitrile.

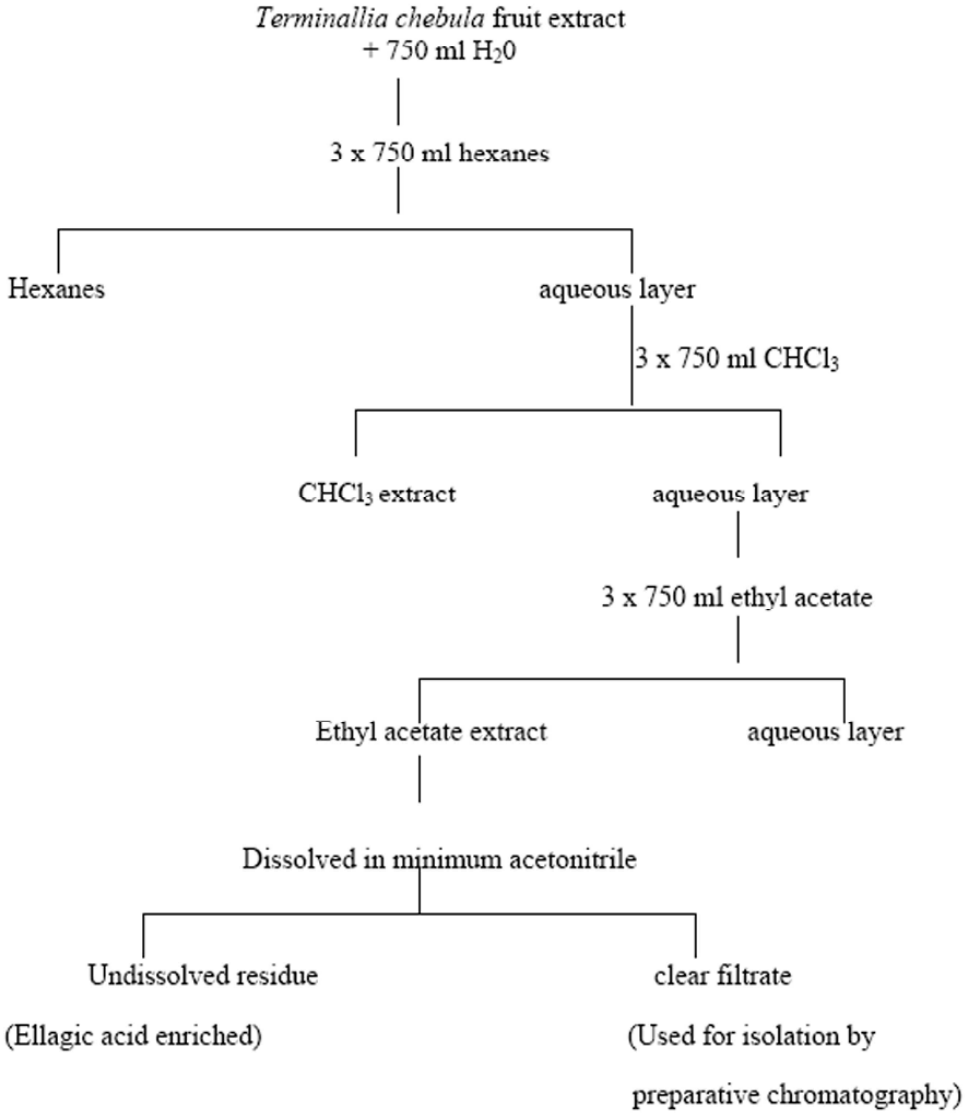

Fig. 2. Scheme showing the isolation of hydrolysable tannins from *T. chebula* fruit extract

A gradient solvent system was used: 0-5 min, 10% B; 5-38 min, 45% B; 38-43 min, 50% B; 43-44 min, 10% B; and 44-50 min, 10% B. The flow rate was 40 mL/min with monitoring at 220 and 272 nm. Fractions 1 (36 ml), 2 (45 ml), 3 (35 ml), 4 (37 ml), 5 (36 ml), 6 (38 ml), 7 (20 ml) and 8 (18 ml) were collected and stored at -20 °C until further use. The separations were repeated, and combined fractions were evaporated under reduced pressure on rotary evaporator. Fractions 1-6 were pure fraction but fraction 7 and 8 were obtained as mixture of two compounds in different proportions. Fraction 7and 8 were reinjected on the Phenomenex Luna 10µ prep Phenyl-hexyl (250 × 30 mm, 10 μm) column. Mobile phase and gradient program used was same as above except that the flow rate was 10ml/min. Identification of phytoconstituents in fraction was done by comparing their retention time, ESI MS and UV λmax with standard or reported literature values [10-13].

Structural identification

The identification of the isolated phytoconstituents was carried out by ESI-MS, UV λmax and ^{1}H NMR

Fraction 1 (Gallic acid): White amorphous powder UV λmax 215, 271 nm (methanol); ESI-MS (negative mode) *m*/*z* 169 [M - H].

Fraction 2 (Methyl gallate): White amorphous powder UV λmax 215, 271 nm (methanol); ESI-MS (negative mode) *m*/*z* 182.9 [M - H]; ^{1}H NMR (CD_3OD); δ 7.10 (2H, s); δ 3.85 (3H, s).

Fraction 3 (Ethyl gallate): White amorphous powder UV λmax 215, 271 nm (methanol); ESI-MS (negative mode) *m*/*z* 196.95 [M - H]; ^{1}H NMR (CD_3OD); δ 7.12 (2H, s); δ 4.28 (2H, qd); δ 1.36 (3H, t).

Fraction 4 (Chebulagic acid): Pale yellow powder UV λmax 222, 276 nm (methanol); ESI-MS (negative mode) *m*/*z* 953.5 [M - H]; ^{1}H NMR (Acetone-*d*6); δ 7.21 (2H, s, H-galloyl); δ 6.67 & 6.53 (each 1H, s, H-HHDP); glucose moiety, δ 5.97 (1H, d,); δ 5.24 (1H, d, *J*=4 Hz); δ 5.13 (1H, d, *J*=1.3 Hz); δ 4.79 (2H, d); δ 4.47 (1H, t, *J*=8 Hz); δ 4.40 (1H, d); chebulloyl, δ 7.54 (1H, s) δ 4.97 (1H, d); δ 3.86 (1H, dd, J=4 Hz); δ 2.22 (2H, d); δ 2.06 (1H, d).

Fraction 5 (**Tetra-*O*-galloyl-*β*-D-glucose**)**:** Pale yellow powder UV λmax 217, 278 nm (methanol); ESI-MS (negative mode) *m*/*z* 787.4 [M - H]; ^{1}H NMR (CD_3OD) δ 7.15, 7.06, 7.04, 6.95 (each 2H, s, H-galloyl); glucose moiety, δ 6.11 (1H, d, *J*=12 Hz); δ 5.57 (1H, d, *J*=12 Hz); δ 5.44 (1H, dt, *J*=8Hz, *J*=12Hz); δ 4.65 (1H, m);δ 4.56 (1H, m); δ 3.97 (2H, d, *J*=8);

Fraction 6 (Ellagic acid): White powder UV λ max 253, 364 nm (methanol); ESI-MS (negative mode) *m*/*z* 301.1 [M - H]; ^{1}H NMR ($CDCl_3$) as tetra acetate δ 7.87 (2H, s, aromatic); δ 2.35 (6H, s, CH_3CO_2); δ 2.30 (6H, s, CH_3CO_2).

Fraction 7 (Chebulinic acid): Pale yellow powder UV λmax 222, 276 nm; ESI-MS (negative mode) *m*/*z* 955.4 [M - H]; ^{1}H NMR (CD_3OD) δ 7.21 (2H, s, H-galloyl); δ 7.10 (2H, s, H-galloyl); δ 6.99 (2H, s, galloyl); glucose moiety, δ 6.52 (1H, d, *J*=1.64 Hz); δ 6.24 (1H, d, *J*=4 Hz); δ 5.15 (1H, d, *J*=3 Hz); δ 4.88 (1H, d *J*=7.6 Hz); δ 4.86 (2H, d, *J*=7.6 Hz); δ 4.47 (1H, t, *J*=8Hz); chebulloyl, δ 7.55 (1H, s); δ 5.01 (1H, d); δ 3.91 (1H, d, J=8 Hz); δ 3.87 (1H, dd, J=8 Hz, J=4 Hz) δ 2.22 (2H, d).

Fraction 8 (Penta-*O*-galloyl-*β*-D-glucose) : Pale yellow powder UV λmax 217, 278.nm (methanol); ESI-MS (negative mode) *m*/*z* 939.5 [M - H]; ^{1}H NMR (Acetone-*d*6) δ 7.19, 7.12, 7.06, 7.03, 6.98 (each 2H, s, H-galloyl); glucose moiety, δ 6.33 (1H, d); δ 6.02 (1H, d); δ 5.66 (1H, d); δ 5.61 (1H, d);δ 4.60 (1H, m); δ 4.32 (2H, m).

RESULTS AND DISCUSSION

In the present work, total eight compounds viz. gallic acid, methyl gallate, ethyl gallate, chebulagic acid, tetra-*O*-galloyl-*β*-D-glucose, ellagic acid, chebulinic acid and penta-*O*-

galloyl-*β*-D-glucose from *T. chebula* were isolated on reverse phase chromatography (Fig. 3.). The isolation of target phytoconstituent from *T. chebula* was carried out by extraction in 70% aqueous methanol. Methanol was distilled out and the aqueous layer was sequentially extracted with hexane, chloroform and finally ethyl acetate (Fig. 2.). These fractions were analyzed by HPLC and compared with crude extract. It was observed that the major peak of interest were present in ethyl acetate fraction. Ethyl acetate layer was evaporated under reduced pressure to obtain residue to which acetonitrile was added to give suspension. The suspension was filtered. It was observed that the undissolved material contained predominantly ellagic acid while the filtrate contained remaining compounds (Fig. 2.).

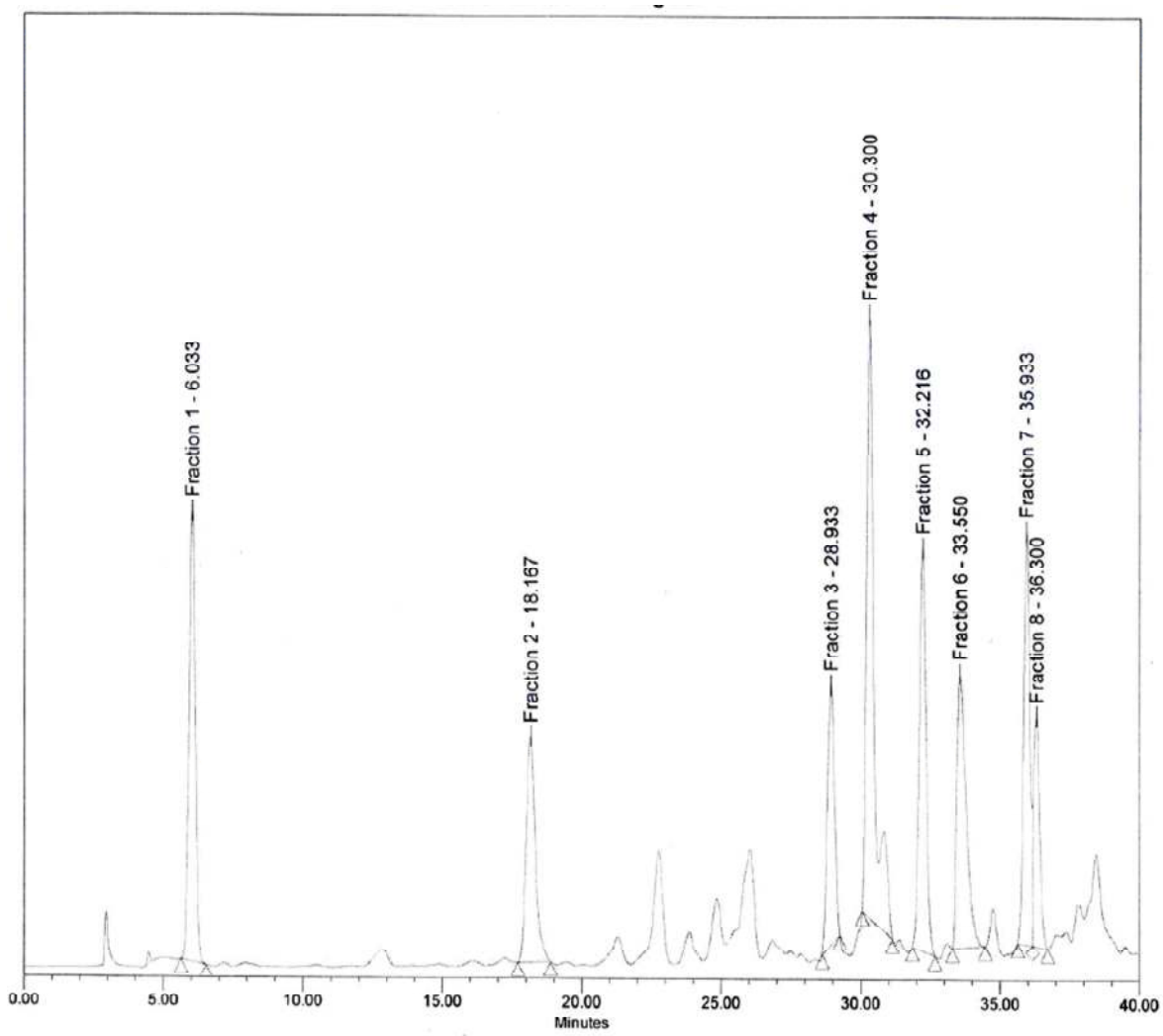

Fig. 3. Preparative HPLC chromatogram (at 272 nm) EA extract of *T. chebula* fruits

T. chebula contains hydrolysable tannins bearing phenolic as well as carboxylic groups the mobile phase selected was acidic containing 0.2% formic acid. Methanol and acetonitrile were tested as organic modifier. Between them, acetonitrile gave satisfactory results i.e. Shorter retention times, sharper peaks, and better resolution. As the ethyl acetate extract was mixture of polar and nonpolar compounds gradient mode was used to avoid broad peaks.

Initially, Phenomenex Luna 10µ C18 (2) 100A, (250 × 30 mm,10 μm.) column was used for separation. Gallic acid being a polar compound, eluted out early (identified by comparing retention time and UV λ_{max} with standard gallic acid) followed by methyl gallate and ethyl gallate. But in case of ellagic acid, it retained on the column and eluted after tetra-*O*-Galloyl-

β-D-glucose. Chebulagic acid and chebulinc acid have similar structures, but in case of chebulagic acid, the biphenyl linkage between two galloyl group increases the polarity thereby causing it to elute earlier than chebulinic acid [9]. The retention time of penta-*O*-Galloyl-*β*-D-glucose and chebulinic acid were very close they eluted out as mixture. However this mixture injected on Phenomenex Luna 10µ prep Phenyl-hexyl (250 × 30 mm, 10 μm) column these peaks was well separated.

CONCLUSION

We conclude that, eight hydrolysable tannins were successfully isolated with high purities (>98%) using preparative HPLC along with UV spectrophotometric detection. Because of high purities of the isolated phytoconstituents they can be used as marker in HPLC validation of single herb as well as polyherbal formulation. The advantages of preparative HPLC are the method is fully automated thereby giving better reproducibility with minimal errors and also it takes lesser time for completion.

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