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Employment of hyphenated approach for metabolomics fingerprinting of phenolics from *Torilis leptophylla* roots

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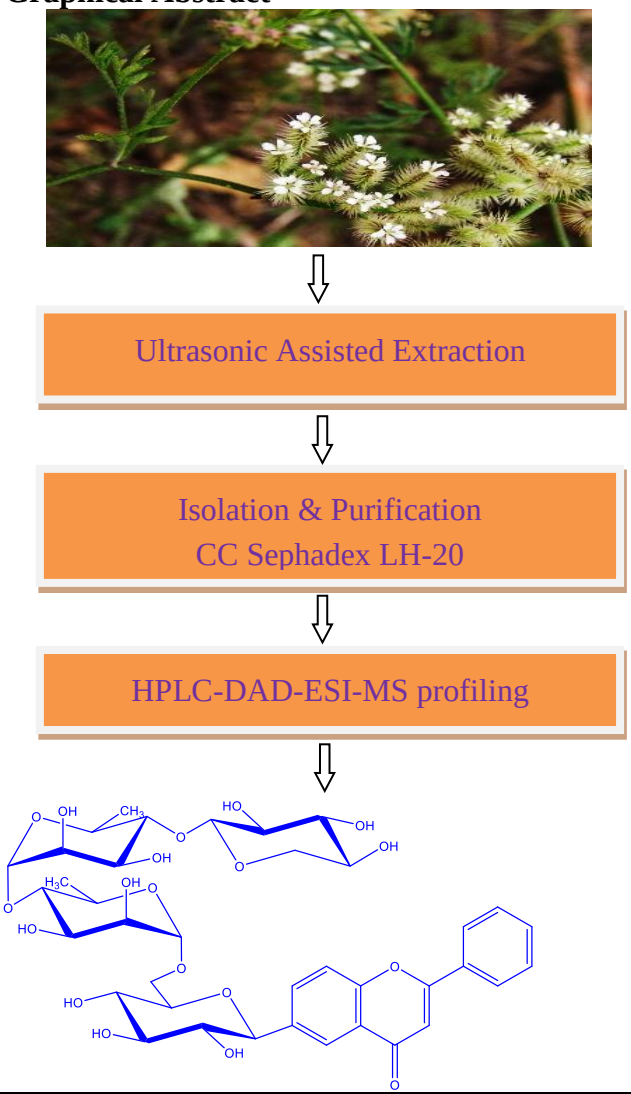
Graphical Abstract



Abstract

Torilis leptophylla synonymously called bristle fruit hedge parsley is widely appraised for its folkloric use to combat liver and gastrointestinal disorders. In order to have a complete picture of its phytoconstituents, an *in extenso* HPLC-MS analysis was carried out that led to the identification of 11 phenolic compounds in the roots of *Torilis leptophylla* including a *C*-linked glycoside reported here for the first time as Flavone-6-*C*-*β*-D-xylopyranosyl-(1→4)-*α*-L-rhamnopyranosyl-(1→4)-*α*-L-rhamnopyranosyl-(1→4)-*β*-D-glucopyranoside. The present study paves a way for establishing the identity of metabolites, metabolomic fingerprinting and provides authentic basis for the better use of *Torilis leptophylla* in *in vivo* applications.

Introduction

Torilis species (Apiaceae), popular worldwide as hedge parsleys, comprises 11-13 species of aroma containing plants possessing diverse phytoconstituents and pronounced biological activities. Only three species of genus Torilis has been reported from various domains of Pakistan [1]. *Torilis leptophylla* is an annual herb that is traditionally a well known medication to relieve hepatic, gastric and intestinal disorders. Due of its potential to act as a disinfectant, the plant is highly efficacious against some pathogens [2]. Furthermore *Torilis leptophylla* leaves are taken as vegetable while stem and branches serve as fodder [3].

The comprehensive profile of *Torilis leptophylla* roots has not been documented earlier. In order to discern the full medicinal prospects of *Torilis leptophylla* roots, it is required to have a complete picture of its phytoconstituents therefore, the present study aimed at characterization, isolation and metabolomic fingerprinting of phenolics using HPLC-DAD-ESI-MS analysis.

Materials and Methods

Extraction

Torilis leptophylla roots extracts were prepared by infusion technique. Plant material (100 grams) was added to 300 mL of methanol: water (70: 30). Contents were irradiated at 35 KHz frequency and 220 volts by using Elma Ultrasonic LC-30H instrument for 15 minutes and stored at room temperature. After 24 hours infusions were filtered and the whole process was repeated thrice. Combined supernatants were evaporated to dryness *in vacuo* using Heidolph 4000-efficient rotary evaporator. As a consequence, crude extract having syrupy consistency was obtained.

Acid Hydrolysis

5 gm *Torilis leptophylla* roots were added to 2M HCl and methanol in (1:1) and contents were refluxed on a boiling water bath for 3 hours. Extract was cooled, filtered and filtrate was extracted thrice, each time with 25 mL of ethyl acetate to separate the pool of aglycones and sugars. Aqueous and ethyl acetate layers were separated and evaporated to dryness on a rotary evaporator. Aglycones (Agly) remained in ethyl acetate layer whereas sugars (Hyd) moved to the aqueous layer.

Spots of standard sugars and Hyd were applied on the TLC plate, developed, sprayed and heated in an oven at 105 ^{0}C for 5-10 min. until the brownish spots appeared. The sugars present in the sample were identified by comparison of their R_f values with those of the standard sugars [4]. The sugars were identified by using precoated silica gel $60F_{254}$ (Merck) as a stationary phase.

Column chromatography

Column chromatography was carried out by using Sephadex LH-20, a dextran gel that swells in water resulting in shrinkage of pore volume. Exploring the size exclusion chromatographic technique, slurry of sephadex made in MeOH : H_2O (70:30) was introduced into column having dimensions 17 × 1.5 cm, plugged with glass wool. 1.5 mL of herbal extract was introduced on the top of sephadex.

Elution was carried out by the same solvent and monitored by UV. Separation took place on the basis of molecular sizes. Larger molecules being eluted first followed by smaller sized molecules. Different fractions were collected which were later subjected to HPLC-DAD-ESI-MS analysis.

HPLC-DAD-ESI-MS analysis

Parameters

The flavonoids composition of *Torilisleptophylla* was determined by injecting 5 μL of extract in Agilent Technology 1200 LC instrument's stainless steel column (4.6 X 150mm) packed with 5 μmthickness of Agilent eclipse extra dense bonding (XDB) reverse phase C18 silica. Elution was carried out by using binary solvent system mobile phase (deionized water labeled as solvent A and HPLC grade acetonitrile labeled as solvent B). 0.1% formic acid was added in both the solvents. Sixty minutes gradient elution program was followed as 10% of acetonitrile at 0 minutes, 10% of acetonitrile at 15 minutes, 40% of acetonitrile at 40 minutes, 80% of acetonitrile at 50 minutes and 10% of acetonitrile at 60 minutes. Flow of mobile phase was maintained at a rate of 0.5 mL/min. Diode array detector was adjusted to detect three different wavelengths (254 nm, 320 nm and 370 nm). On the other hand mass spectrometer was equipped with electrospray ionization mode (ESI), Ion trap analyzer and photomultiplier tube as detector. Spectra were recorded in negative ionization mode between mass range of 50-2200 *a.m.u.*

Samples preparation

Clear concentrated hydroalcoholic extracts were subjected to HPLC-DAD-ESI-MS analysis to explore the phenolic compounds.

Results and Discussion

HPLC-DAD-ESI-MS Analysis

HPLC Chromatogram of the isolated fraction of hydro-alcoholic extract of TL is shown in fig.1. This pure fraction was obtained from column chromatography using sephadex as a stationary phase and MeOH : H_2O (70:30) as mobile phase.

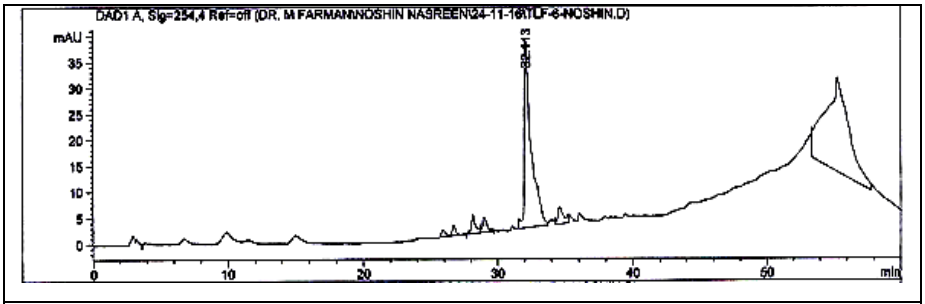
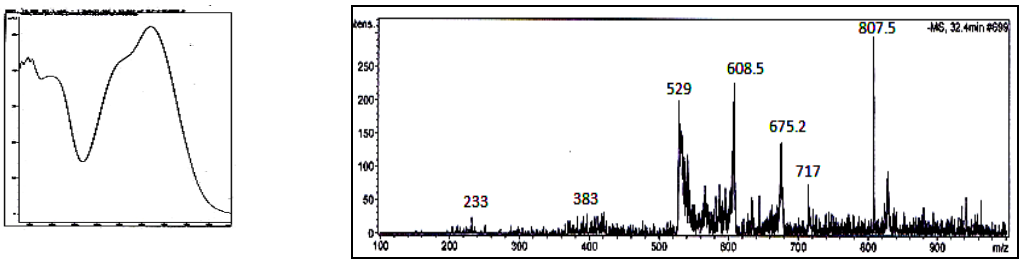


Fig.1 HPLC chromatogram of isolated fraction TL-F6

Compound eluted at retention time of 32.4 min when the composition of mobile phase was 28% acetonitrile in deionized water. More proportion of water revealed more hydrophilic nature of the eluted compound. *i.e.*, tetraglycosides. DAD response showed characteristic band pattern of flavones where band II corresponding to ring A appeared at 240 nm. The intensity of band I was higher as compared to band II. Hypsochromic shift of 10 nm from normal value indicated glycosylation on ring

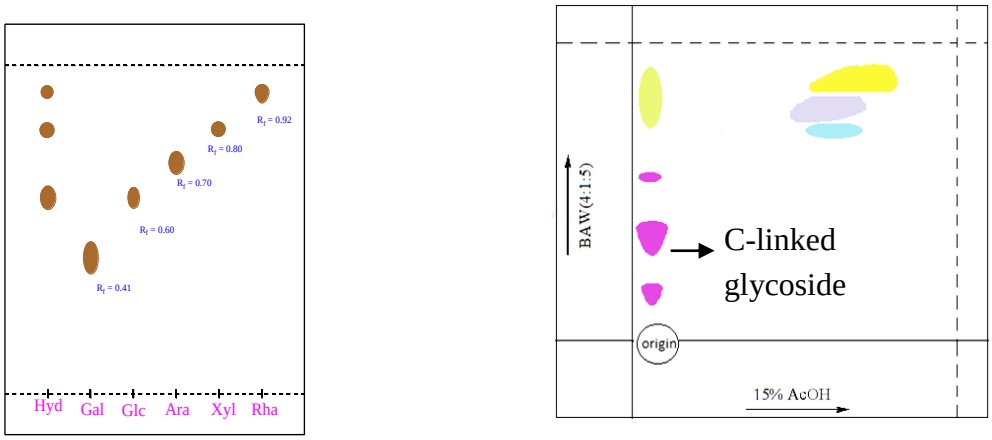
A. Typical value of absorption of band I at 330 nm indicated no substitution on B ring.The shape, intensities and λ_{max} values of the two bands were closely related to flavone-6-*C*-tetraglycoside [5]. Spectral data for compound TL-F6 is shown in fig. 2.



(a) (b)

Fig. 2 Spectral data for compound TL-F6 a)DAD response b) ESI-MS spectrum of compound TL-F6

The ESI-MS spectrum of the compound TL-F6 recorded in the –ve ionization mode showed deprotonated molecular ion at m/z 807. Sequential losses of 132, 146, 146 and 149 mass units probably justified the consecutive attachment of Sugars. The pictorial representation of TLC and PC analyses of sugars and aglycones obtained from acid hydrolysis is shown in fig. 3.



Stationary phase= Silica gel 60F_{254}(Merck) impregnated with Sodium dihydrogen phosphate

Mobile phase = Acetone :Water (9:1)

Spraying reagent =Aniline hydrogen phthalate

Stationary phase = Cellulose

Mobile phase

1D = BAW (4:1:5)

2D = 15% AcOH

Fig. 3 TLC and PC analyses of Hyd and AGLY obtained from acid hydrolysis

The TLC and PC analyses of the hydrolysate provided extra information in probing the structure of glycoside. Nature of sugars was determined from mass losses however the identity was established from co-chromatography of hydrolysate. Loss of pentose, hexose and deoxyhexose sugars were assigned to xylose, glucose and rhamnose respectively.

The PC analysis provided supplementary information regarding the *C*-linked nature of glycosides. Loss of 149 mass units and additional 13 mass units with aglycone fragment disclosed the *C*-linked nature of aglycone.

Linkages between the sugars were determined on the basis of literature cited. In nature [1→4] linkage of xylose to any other sugar, [1→4] linkage of rhamnose to glucose and [1→4] linkage between two rhamnose moieties is known. The structure was determined keeping in view the DAD response, the differences in m/z, sequential losses of sugars and deductions from TLC and PC analyses.

Deprotonated molecular ion appeared at m/z 807 $[M-H]^-$and it determined m/z 808 as the molecular mass of the compound. The fragment at m/z 717 indicated $^{0,2}X$ cleavage of xylose *i.e.,* $[M-H-^{0,2}X_{xyl}]^-$[6].The peak corresponding to m/z 675 indicated the cleavage of xylose moiety *i.e.,* $[M-H-xyl]^-$. Signal at m/z 529 resulted due to sequential cleavage of xylose and rhamnose from the deprotonated molecular ion *i.e.*,$[M-H-xyl-rham]^-$. Fragment corresponding to m/z 383 indicated losses of xylose and two rhamnose moieties from the compound *i.e.,* $[M-H-xyl-rham-rham]^-$. Appearance of peak at m/z 283 suggested the sequential losses of one xylose, two rhamnose and a *C*-linked glucose moiety from the compound *i.e.,* [M-H-xyl-rham-rham-glc(*C*-linked)]$^-$. The justification of major fragments formed in ESI-MS further ensured that the compound was Flavone-6-*C*-*β*-D-xylopyranosyl-(1→4)-*α*-L-rhamnopyranosyl-(1→4)-*α*-L-rhamnopyranosyl-(1→4)-*β*-D-glucopyranoside. Spectral data for the characterization of compounds in *Torilis leptophylla* roots by HPLC-DAD-ESI-MS analysis is shown in table 1.

Table 1: Spectral data for the characterization of compounds in *Torilis leptophylla* roots by HPLC-DAD-ESI-MS analysis

Compounds Codes	**DAD λ_{max}value**	**Pseudo molecular Ion**	**Fragment ions**	**Identified compounds**
TLF-6	240,330	$[M-H]^-$	807,717,675,608, 529,383,233	Flavone-6-*C*-*β*-D-xylopyranosyl-(1→4)-*α*-L-rhamnopyranosyl-(1→4)-*α*-L-rhamnopyranosyl-(1→4)-*β*-D-glucopyranoside
TLF-7	230,325	$[M-H]^-$	693,531,427,369	Flavone-7-*O*-*β*-D-glucopyranosyl-(1→6)- *β*-D-glucopyranosyl-(1→4)- *β*-D-xylopyranoside
TLF-8	220, 240sh, 326	$[M-2H+ACN]^-$	887,643,319	Ampelopsin-7-*O*-*α*-L-3˝-propanoylrhamnopyranosyl-(1→6)-*β*-D-3˝-caffeylglucopyranoside

TLF-9	230,320	$[M-H]^-$	563,503,329,269, 239	Apigenin-7-*O*-*β*-D-xylopyranosyl-(1→4)-*β*-D-glucopyranoside
TLF-10	268, 342	$[M-H+H_2O]^-$	889,737,685,563, 269	Apigenin-7-*O*-*β*-D-glucopyranosyl-(1→4)-*β*-D-rhamnopyranosyl-(1→6)-*β*-D-glucopyranosyl-(1→4)-*β*-D-xylopyranoside
TLF-11	240, 340	$[M-H]^-$	563,473,269	Apigenin-4′-*O*-*β*-D-glucopyranosyl-(1→4)-*β*-D-xylopyranoside
TLF-12	248, 350	$[M-H+H_2O]^-$	595,477,449,269	Apigenin-7-*O*-rutinoside
TLF-13	270, 334	$[M-H+CH_3OH+CH_2O]^-$	329,267	3-Methyl-7-deoxy galangin
TLF-14	265,350	$[M-H+Acetyl]^-$.	327,285	7-acetyl luteolin
TLF-15	248, 350	$[M-H+2H_2O+CH_3OH]^-$	337,269	Apigenin
TLF-16	252, 335	$[M-H+ACN]^-$.	262,221	Flavone

Conclusions

The detailed study on metabolomic finger printing of *Torilis leptophylla* roots was successfully conducted and an innovative and upto date phytoconstituents profile of the genus was developed. The research proved that *Torilis leptophylla* is a vast fountain of secondary metabolites that can be utilized in a better way for future drug development.

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