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Research Article

## Macro-microscopic and LCMS markers for identification and authentication of herbal formulations of *Taraxacum officinale* and *Launaea procumbens*

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### ABSTRACT

Quality and efficacy of botanical formulations are largely dependent on authenticity of raw materials used. *Taraxacum officinale* Weber (TO) and *Launaea procumbens* (Roxb.) Ramayya & Rajagopal (LP) are well known Indian traditional herbs used in a variety of herbal preparation for the treatment of a number of diseases. The morphological resemblance in leaves and flowers of both the species led to the problems of adulteration and ultimately affects the quality and efficacy of their products. The current investigation was aimed to provide the macro-microscopic and LCMS markers for identification and authentication of genuine plant materials of TO and LP. Morphologically, TO can be differentiated with bright yellow colored inflorescence arranged as solitary terminal capitulum on the un-branched peduncle, pappus hairs pale white and unequal, rhizomatous root with milky latex. While in LP, capitulum was arranged in lax corymbs on branched peduncle. Powder microscopy of TO revealed the presence of distinct schizogenous oil glands and laticifers which were absent in LP. Moreover, liquid chromatography-mass spectrometry analysis showed the presence of Quinic acid, Protocatechuic acid, Chlorogenic acid and Umbelliferone in both the plants with their quantitative variability, whereas Scopoletin showed distinguishing LCMS marker as it was present only in TO samples.

**Keywords:** *Launaea procumbens*; LCMS markers; Pharmacognosy; *Taraxacum officinale*.

### INTRODUCTION

The traditional system of medicine has been used by mankind since time immemorial and has great value, not only in traditional healthcare system but also in identification of newer chemical entities for modern therapeutics. *Taraxacum officinale* (TO), family Asteraceae locally known as *Dudhee*, is a well-known ancient medicinal herb. The plant species is used to treat a variety of diseases such as jaundice, skin, liver, kidney, hepatitis, migraine, spleen and pulmonary disorders etc. (Benigni et al., 1964; Frawley and Lad, 2001; Foster and Johnson, 2008; Sweeney et al., 2005). In Chinese, Arabian and Native American traditional medicine, it is also known to treat a number of diseases. In modern therapeutics, the plant is also known to use for the treatment of microbial infections, body swelling, breast cancer, pneumonia, hepatitis, hypo-lepidemia, oxidative stress, and gallbladder problems (Bae et al., 2005; Schutz et al., 2005; Sigstedt

et al., 2008; Clare et al., 2009; Choi et al., 2010; Jeon et al., 2008; Takasaki et al., 1999a; 1999b; Choi et al., 2002). A recent report also reveals some new insights of TO as a remedy for the treatment of osteoporosis (Gargouri et al., 2016). However, the triterpenoids of this plant have been suggested to exhibit cytotoxicity with low toxicity against mammary tumours (Bishayee et al., 2011; Takasaki et al., 1999a; 1999b). Number of its products for different therapeutic applications were already commercialized in national and international market (Charters et al., 2003; Guo, 2000; Stevenot, 2000).

*Launaea procumbens* (LP) is traditionally well known to treat rheumatism (Parekh and Chandra, 2006), kidney disorders (Ahmad, 2006; Khan et al., 2010), male hormonal imbalance, cardiovascular and reproductive disorders (Singh et al., 2007). As a nutritional supplement, LP contains salicylic acid, vanillic acid, synergic acid, 2-methyl-resercol and gallic acid (Shaukat et al., 2003) and showed antioxidant, anticancer and anti-inflammatory properties. Leaves of this plant contain xyloside,  $\beta$ -sitosterol and its acetate, taraxasterol. The plant is also known as a rich source of bioactive secondary metabolites such as flavonoid, phenolic acids, tannins, cardiac glycosides and coumarins, etc. (Khan et al., 2010). The high content of bioactive compounds in the extract of this plant could

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be responsible for its anti-inflammatory and antioxidant effects against various health-related disorders (Etuk et al., 2009; Vardavas et al., 2006; Khan et al., 2010). These evidences reveal the immense medicinal importance of both the species in traditional as well as in modern therapeutics. The quality and effectiveness of these products mainly depend upon the proper identification, authentication and their sustainable availability. Determination of secondary metabolites for pharmaceutical applications in traces that co-elute with a parent drug is one of the most challenging tasks (Kumari et al., 2015). A large number of methods for qualitative and quantitative determination of plant secondary metabolites have been developed. Moreover, the recent developments in Liquid chromatography hyphenated Mass spectrometry techniques for phytochemical analysis have led to the high frequency of its application (Singh et al., 2015a; Kumar et al., 2015; Kumari et al., 2015). Furthermore, Ultra high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) proved to be much more sensitive and rapid technique for analysis of multiple compounds in a complex plant sample. Moreover, the global resurgence of interest in the herbal market led an unmet need for standard markers for authentication, prevention of adulterations and to determine the efficacy, safety and reproducibility in the quality of herbal products. This study presents a macro and microscopic description of LP and TO along with a qualitative and quantitative determination of five bioactive marker compounds viz. Quinic acid, Protocatechuic acid, Chlorogenic acid, Umbelliferone, Scopoletin through UHPLC-MS/MS to lay down their standard quality control markers.

## MATERIALS AND METHODS

### Plant Material

Fresh plant material of TO and LP were collected from Doonagiri forest range of district Almora, Kumaon, Uttarakhand, India and identified by one of the senior author (KRA) according to Flora of District Garhwal (Gaur, 1999). Voucher specimens (KRA-24497 and KRA-24484) were housed in departmental herbarium CSIR-Central Drug Research Institute, Lucknow, India. The gross morphological description of both the plant species was based on the shape, size, color and morphological appearance of vegetative and reproductive parts.

### Microscopic Measurement

Plants samples were fixed in the solution containing formalin, glacial acetic acid and 70% ethanol (5:5:9 v/v) for two days. Free hand transverse sections (TS) were prepared and stained according to Brian & Turner (1975). For powder microscopy, materials were thoroughly washed, dried in a hot air oven (35±2°C), grinded and stored at 25±2 °C in air tight container and used for further investigations. All precautions were

undertaken to avoid any types of microbial contamination. A small quantity of grinded powder was sieved through 85 meshes and kept in saturated chloral hydrate solution for 48 hrs, stained and mounted in glycerine for microscopic observation.

## Phytochemical Studies

### Chemicals

All the standards viz. Quinic Acid (QA), Protocatechuic Acid (PA), Chlorogenic Acid (CA), Umbelliferone (UM), and Scopoletin (SC) were procured from Sigma-Aldrich, St. Louis, MO (USA).

### Sample Preparation

The powdered samples were kept in 95 % ethanol for overnight and filtered through Whatman no.1 filter paper. The filtrate was dried under vacuum at 40 °C using a rotary evaporator. The dried residue thus obtained was considered as ethanolic extract and was stored at 4 °C before further analysis.

### Optimization of UHPLC-MS/MS method for quantitative analysis

The selected marker compounds (Table 1) were quantified through UHPLC-MS/MS analysis using a 4000 QTRAP™ MS/MS system. ACQUITY UPLC C18 column was used with BEH (Ethylene-bridged hybrid) particles which enable a high-performance separation of targeted compounds owing to its wide and stable pH range. Quantitative determination was done using multiple-reaction monitoring (MRM) mode, which is highly sensitive and targeted technique to monitor and quantify even a single targeted compound within a complex mixture of sample. 2µL volume of each sample was injected. The mobile phase composed of aqueous solution containing 0.1% formic acid and acetonitrile was performed under optimized UHPLC conditions (flow rate of 0.35 mL/ min, gradient system: 7-10% (B) initial to 2.0 min, 10-35% (B) 2.0 to 3.0 min, 35-95% (B) 3.0 to 4.3 min) and provided the highest signal intensity for marker compounds. According to our previously standardised methods (Singh et al., 2015b; Singh et al., 2016; Bajpai et al., 2015) LCMS compound dependent factors i.e. declustering potential (DP), entrance potential (EP), collision energy (CE) and cell exit potential (CXP) were optimized for high performance separation of each compound (Table 1).

### Validation

The accuracy of developed MRM data (Table 2) such as linearity, lower limits of detection (LOD), limits of quantification (LOQ), precisions, stability and recovery methods was validated as per the standardized method described by Bajpai et al., (2015); Branch (2005). Calibration curves were established from a series of 5 experiments for each standard and determined through squared linear correlation coefficient (r).

## RESULTS

### Macroscopic description *Taraxacum officinale*

Perennial herbs consist of a rhizomatous tap root with milky latex. Taproot is thick, unbranched and lateral roots are thin. Leaves radical (arising directly from roots), flat, spreading on the ground, oblanceolate-oblong or linear, 5-10 x 1-3.5 cm, young leaves were entire but mature ones toothed, lobes acute more or less dentate. The flowering stalks are long (6-12 cm) and upstanding and carry a solitary terminal inflorescence. Flowering head discoid, 1.5 to 3.5 cm across, ray florets are yellow and numerous in number. Involucre bracts green, outer one ovate-lanceolate. Inner ones are lanceolate. Flowers bright yellow, pappus hairs pale-white and unequal.

### *Launaea procumbens*

Perennial herbs, 10-50 cm high, with creeping stolons and upstanding branching pattern. Leaves radical in rosettes, oblong-oblanceolate or spatulate, 5-20 X 1-4 cm, leaf margins are closely denticulate with cartilaginous teeth. The midrib is prominent. Ligules yellow, Achenes pale yellow, 2-4 mm long, heads arranged in corymb on dichotomously branched and much longer peduncle, pappus white, soft, longer than achenes. Ray florets are faded yellow and few when compared to TO.

### Microscopic Studies

#### TS of TO Leaf

The transverse section passing through midrib region shows convex at both the side but broad hump at the abaxial side (Fig. 1A). The transverse section passing through lamina shows a dorsiventral structure with its mesophyll differentiated into palisade and spongy photosynthetic tissue. The palisade layer made up of 3-4 rows of columnar cells which occupy a little less than half of the width of the mesophyll. Trichomes are visible on the lower side of the lamina (Fig. 1B). The upper and lower epidermal cells are single layered, thick walled and covered with a cuticle. The hypodermis is collenchymatous, 5-8 layered, cells smaller in size with dense angular thickening as compared to ground tissue. The ground tissue is collenchymatous embedded with five collateral vascular bundles arranged in an arc at the middle of the midrib. The centrally located vascular bundle is much larger in size as compared to lateral ones. Each vascular bundle capped with broad parenchymatous bundle sheath (Fig. 1C). The outer margin of bundle sheath comprises of fibers and latex canals as beaded appearance. Xylem and phloem are well developed. Xylem vessels are arranged in radial rows. 4-5 peculiar schizogenous oil glands are also arranged in an arc towards the adaxial side in the midrib region.

#### TS of LP Leaf

The transverse section passing through midrib region shows slight depression at the adaxial side and broad hump at the abaxial side (Fig. 2A). The transverse section passing through lamina shows a dorsiventral structure with its mesophyll differentiated into palisade and spongy tissue. The upper and lower epidermal cells are single layered, thick walled and covered with a cuticle. The hypodermis is 1-2 layered; the size of the hypodermal cells is almost similar to epidermal cells and much smaller in size as compared to the ground tissue. The ground tissue is collenchymatous embedded with 4-5 collateral vascular bundles arranged in an arc at the middle of the midrib (Fig. 2B) Lateral vascular bundles towards the lamina region are much smaller in size as compared to middle ones. Each vascular bundle capped with broad parenchymatous bundle sheath. The outer margin of bundle sheath comprises of fibers as beaded appearance. Xylem and phloem are well developed. Xylem vessels are arranged in radial rows.

#### TS of TO root

TS of the root is more or less circular in outline (Fig. 3A). Outermost 3-4 layered cork which is not uniform, Cork is followed by 10-12 layered parenchymatous cortex, cells polygonal in shape. Endodermis and pericycle are not distinct. Most of the region shows well developed secondary vascular tissue i.e. phloem and xylem (Fig. 3B). Secondary phloem with alternating rings of parenchyma & laticifers occupied a major part of the cross-section (Upton, 2016). It consists of phloem parenchyma, sieve tubes, companion cells, fibers, radiating medullary rays and latex canals. The Central region is occupied by secondary xylem which consists of xylem vessels, fibers, parenchyma, medullary rays and latex canals. Secondary Xylem shows the presence of vessels with mostly narrow lumen and scattered.

#### TO Powder

The powder is muddy green color, odorless and bitter in taste. Under the microscope (Fig. 4A-E) it shows several multicellular glandular and non-glandular trichomes, latex canals, schizogenous oil glands, fibers and xylem vessels with annular to scalariform secondary wall thickenings. Stomata were anomocytic in nature where subsidiary cells are indistinct from epidermal cells.

#### TS of LP root

TS of the root is circular in outline (Fig. 5A-B). Cork is 5-7 layered; cells are laterally compressed and arranged in radial rows. Cork is followed by 12-15 layered parenchymatous cortex embedded with some latex canals. Cortical cells are somewhat polygonal in shape. Endodermis and pericycle are not distinct. Most of the region shows secondary vascular tissue i.e. phloem and xylem. Xylem occupied half of the cross section. Phloem is well-developed consists of phloem

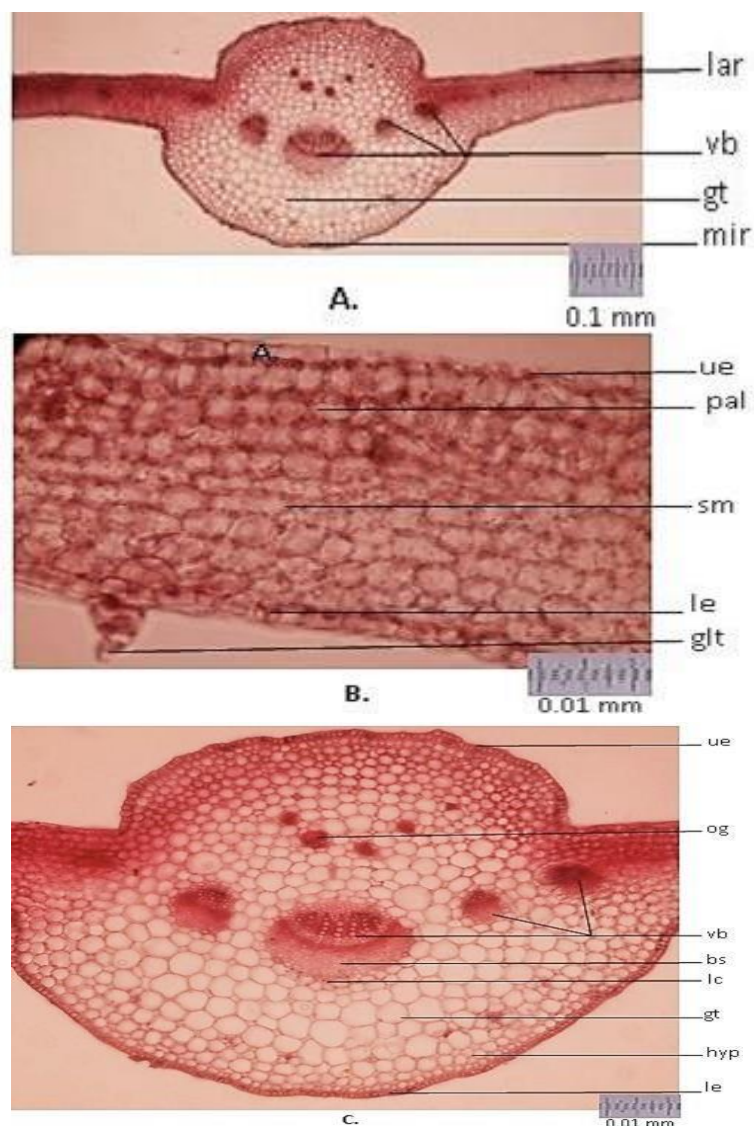


Figure 1: A-C Transverse section of TO leaf

Table 1: Compound dependent parameters for UHPLC-MS/MS method

Peak No.	RT <sup>a</sup> (min)	Compound	Precursor ion Q1 (Da)	DP <sup>b</sup> (V)	Product ion Q3 (Da)	CE <sup>c</sup> (eV)	CXP <sup>d</sup> (V)
1	0.71	Quinic Acid	191.0 [M-H] <sup>-</sup>	-67	85.0	-33	-6
2	1.44	Protocatechuic Acid	153.0 [M-H] <sup>-</sup>	-64	109.0	-22	-9
3	1.48	Chlorogenic Acid	353.0 [M-H] <sup>-</sup>	-60	191	-30	-10
4	1.91	Umbelliferone	161.1 [M-H] <sup>-</sup>	-135	132.8	-28	-12
5	2.30	Scopoletin	191.0 [M-H] <sup>-</sup>	-103	175.7	-20	-10

<sup>a</sup>retention time; <sup>b</sup>declustering potential; <sup>c</sup>collision energy; <sup>d</sup>cell exit potential

parenchyma, sieve tubes, companion cells, fibers, medullary rays and latex canals. The Central region is occupied by well-developed secondary xylem which consists of xylem vessels, fibers, parenchyma, medullary rays and latex canals. Xylem is diffuse porous along with broad and narrow lumen vessels more or less scattered. Medullary rays are very broad and radiating.

#### LP Powder

The powder is olive green color, odorless and bitter in taste. Under the microscope (Fig. 6A-D) it shows latex canals, fibers and xylem vessels with scalariform to

reticulate secondary wall thickenings. Unicellular simple trichomes are present but less numerous. Stomata were anomocytic in nature.

#### UHPLC-MS/MS determination

Various compound dependent parameters of LCMS determination viz. RT, precursor ion (MH), regression equation, DP, CP etc. for accurate identification and validation of identified compounds were optimized and validated (Table 1 and 2). Peak values of UHPLC-MS/MS determinate compounds are labelled as 1-5 in figure 8. Quantitative determination of these identified compounds showed the presence of 4 in LO except

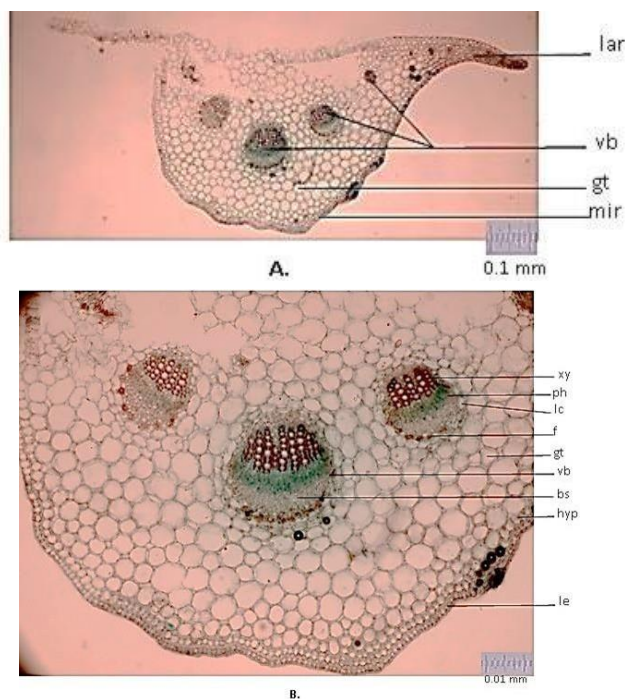


Figure 2: A & B Transverse section of LP leaf

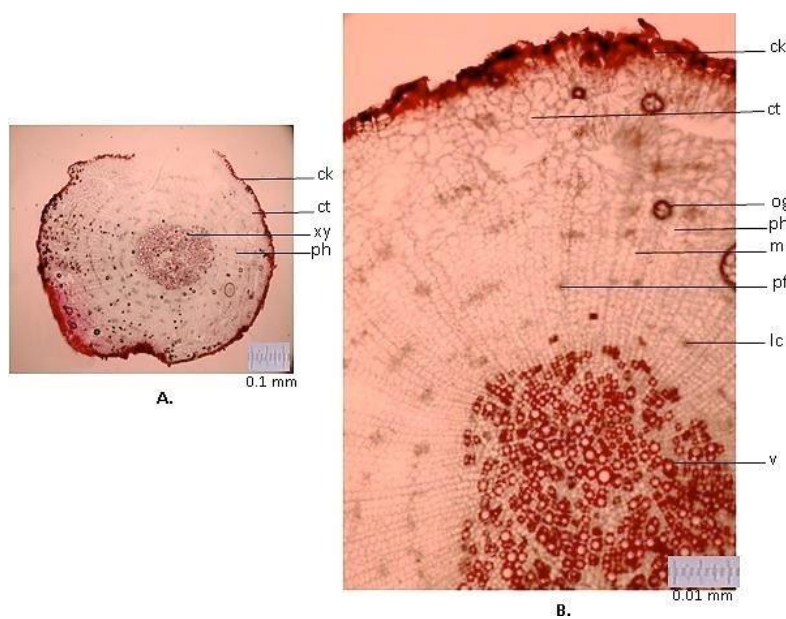


Figure 3: A-B Transverse section of TO root

Table 2: Calibration curves, LOD and LOQ data of investigated compounds by UHPLC–MS/MS

Analytes	Regression equation	R <sup>2</sup>	Linear Range (ng/ml)	LOD <sup>a</sup> (ng/ml)	LOQ <sup>b</sup> (ng/ml)	Precision RSD <sup>c</sup> (%)		Stability RSD (%) (n=5)	Recovery	
						Intraday (n=5)	Interday (n=9)		Mean (n=5)	RSD (%)
QA	y=608 x +115	0.9987	5-75	0.38	1.15	0.18	1.74	0.99	98.68	0.93
PA	y = 8260 x + 1430	0.9998	0.25-250	0.04	0.12	1.13	1.52	1.17	102.05	1.17
CA	y = 120 x - 58.6	0.9999	5-250	0.11	0.33	0.98	1.61	0.23	98.71	1.52
UM	y = 231 x + 149	0.9994	1-500	0.08	0.25	0.20	0.93	1.19	102.35	1.76
SC	y = 69.7*x - 10.4	0.9982	1-500	0.24	0.73	0.42	0.78	0.09	97.62	1.16

<sup>a</sup>Limit of detection; <sup>b</sup>Limit of quantification; <sup>c</sup>relative standard deviation

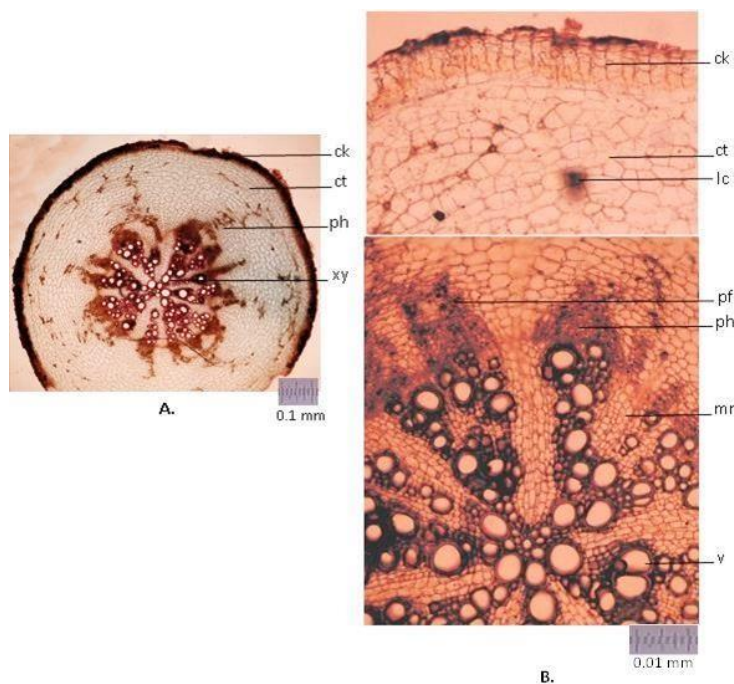


Figure 4: A-B Transverse section of LP root

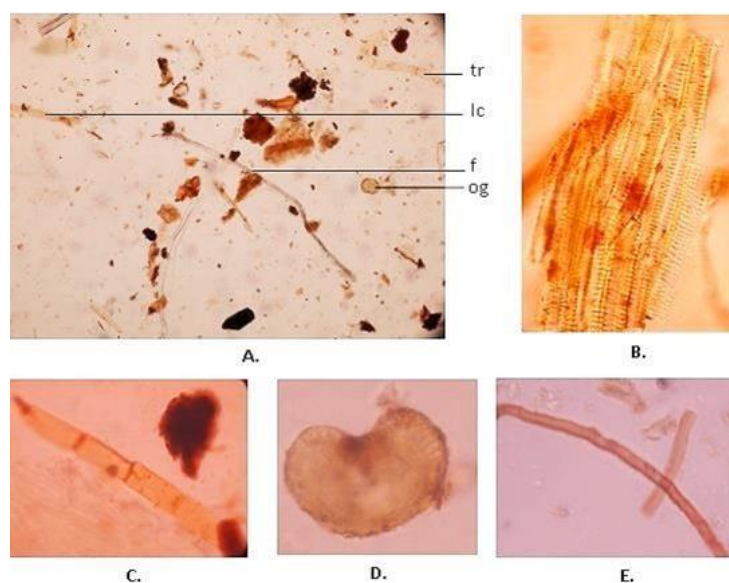


Figure 5: A-E powder microscopy of TO

Table 3: Quantitative and comparative LCMS determination of marker compounds in TO and LP samples<sup>a</sup> (µg/g)

Sample Code <sup>b</sup>	Quinic Acid	Protocatechuic Acid	Chlorogenic Acid	Umbelliferone	Scopoletin
LP	262.17	19.52	1836.67	698.83	nd
TO	162.83	32.33	3326.67	717.00	52.75

<sup>a</sup>presented values were mean of three replications (n = 3); <sup>b</sup>LP and TO refers to wild plants of *L. procumbens* and *T. officinale* respectively; nd: not detected

scopoletin (Table 3). Quantitative determination showed the higher content of Quinic acid (262.17 µg/g) in LP as compared to (162.83 µg/g) TO. However, protocatechuic acid, chlorogenic acid and umbelliferone were found much higher in TO as compared to LP (Table 3). Extracted ion chromatograms (XIC) of selected standard compounds is represented

by a single peak (Fig. 7). Also the overlay spectra confirming the presence of marker compounds in plant samples is shown in Fig. 8. The comparative variability of marker compounds in TO and LP are presented in Fig. 9.

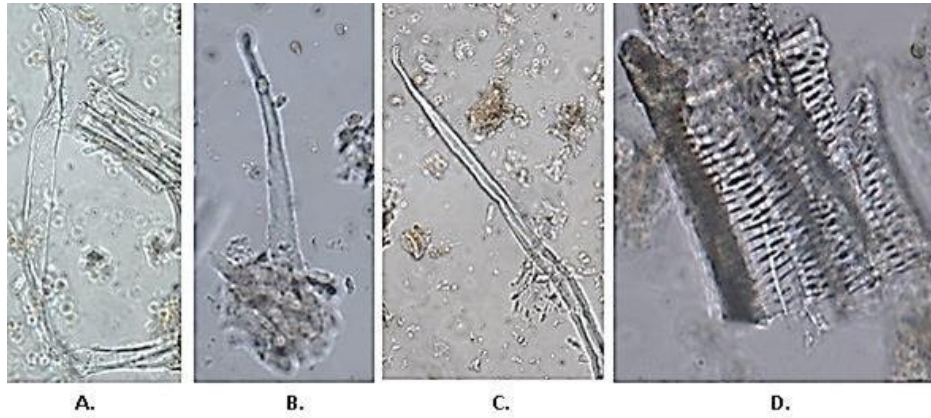


Figure 6: A-D Powder microscopy of LP

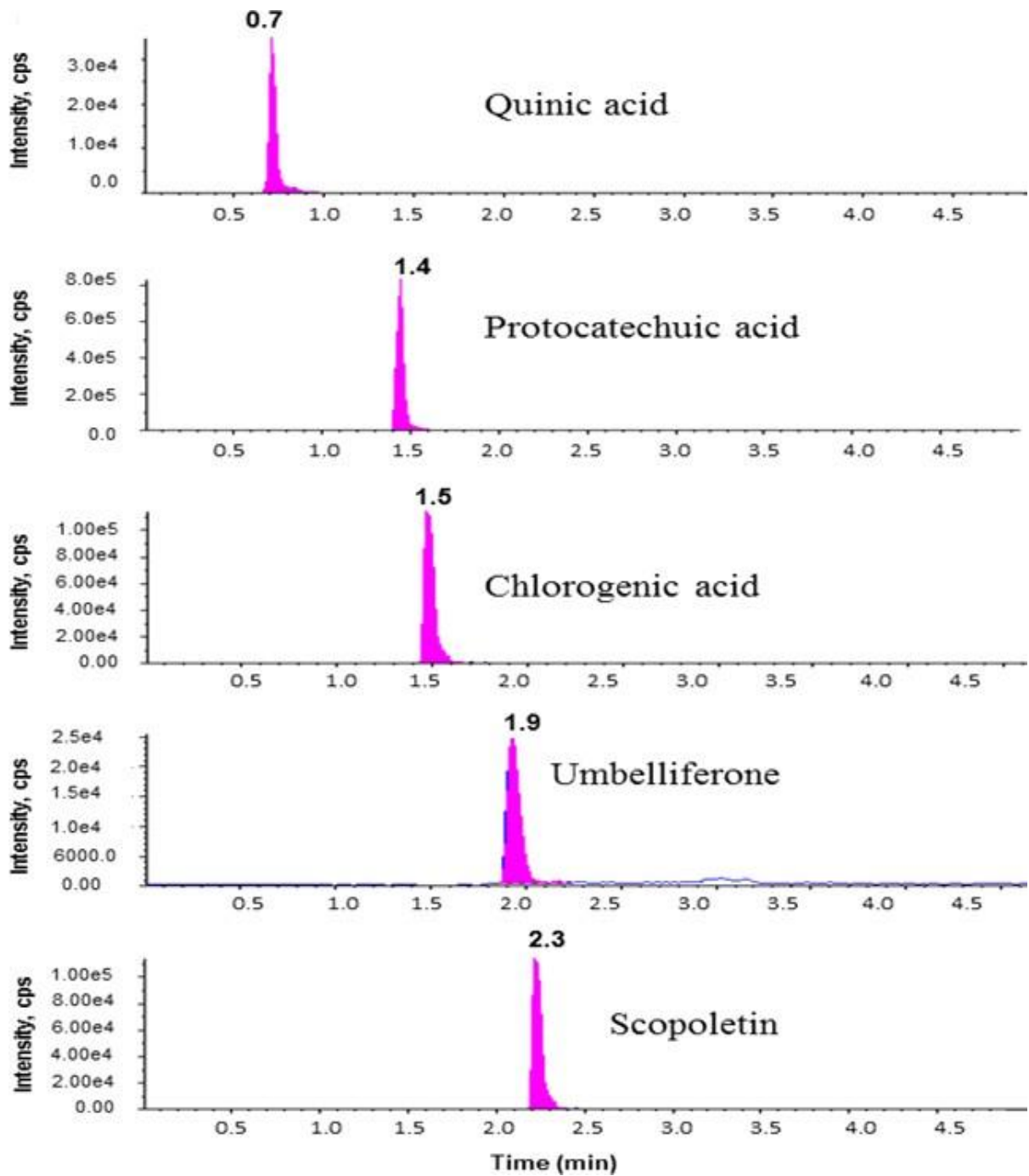


Figure 7: Extracted ion chromatograms (XIC) of selected marker compounds

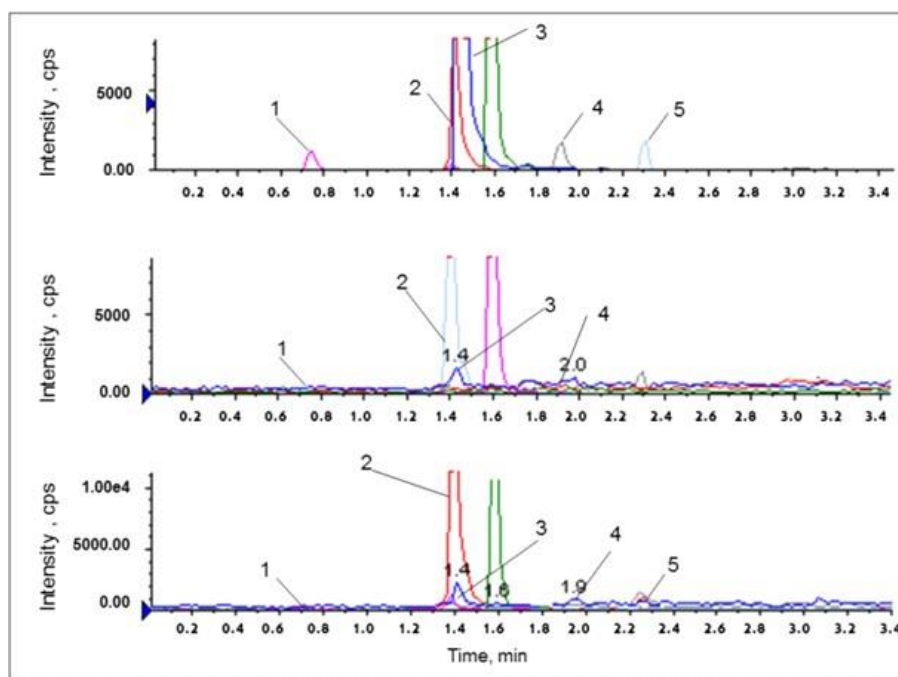


Figure 8: XIC of A) standard mixture; B) LP; C) TO

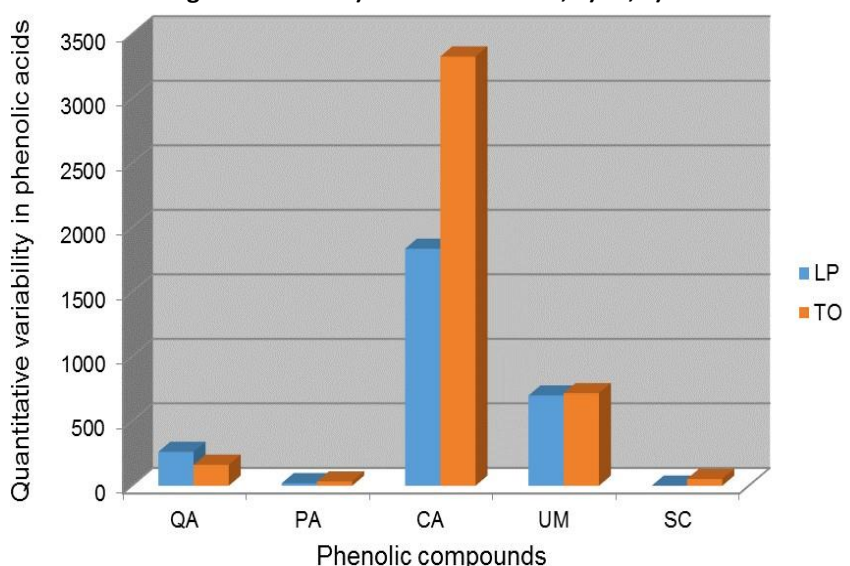


Figure 9: Quantitative variabilities in content of marker compounds within LP as compared to TO samples

## DISCUSSION

The close morphological resemblance between both the plant species can be distinguished by a marked difference in its solitary terminal inflorescences, numerous ray floret and shorter and unbranched peduncle in TO. Furthermore, Microscopic studies of leaves and powder of TO reveal the presence of several multicellular trichomes with pointed ends and considered as an important microscopical feature (Hallahan et al., 2000) and could play a key taxonomical role to distinguish these closely related species. In contrast, the presence of similar nature of chemical constituents except scopoletin supports the view of chemotaxonomical identification for which they were kept within the same family i.e. Asteraceae. Remarkably, the quantitative determination showed

that the protocatechuic acid, chlorogenic acid and umbelliferone was observed in higher quantity in TO as compared to LP (Table 3) and therefore may be considered for quality and efficacy of its herbal products. Traditionally, TO is a well-known herbal drug formulation used for the treatment of a number of liver problems. Moreover, chlorogenic and protocatechuic acid are the important constituents of TO and known as hepatoprotective agents (Kapil et al., 1995; Kakkar and Bais, 2014). Determination of chlorogenic and protocatechuic acid in TO validates its traditional claims for hepatoprotective activities and presence in higher quantity may also support the efficacy of the products.



## CONCLUSION

Our previous field experiences on ethnobotanical, ethnopharmacological as well as herbal market surveillances of these plants reveal a strong possibility of adulteration in their products due to their close morphological resemblance. Identification and authentication of raw materials especially the medicinal herb readily used in clinical practices is one of the important parameters for quality, safety and efficacy of any herbal products. Both the species reported in this paper are important and known for their various traditional as well as pharmaceutical applications. Thus the identified macro-microscopic and LCMS markers of both the species may serve as standard pharmacognostical markers for authentication of their products and limit the chances of adulteration during product development. These parameters may also assure to maintain the quality and efficacy of batch to batch products related with these plant species.

## DISCLOSURE STATEMENT

The authors reported no potential conflict of interest.

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