

A Study on HPTLC Quantification for Quality Control of Myricetin a Nutraceutical from Different Plant Parts of *Myrica esculenta* Linn

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The present study was performed to report the quantification of myricetin present in different extracts (i.e. ethanol, methanol, petroleum ether, chloroform and aqueous extract) of root, leaf, bark, stem and seed of *Myrica esculenta* using TLC densitometric method. Densitometric scanning was performed at 366 nm for the determination and quantitation of standard compounds. The compact spots were achieved at Rf 0.67 corresponding to myricetin. The content of myricetin present in different solvent extracts of *M. esculenta* was $0.2159 \pm 0.002\%$ w/w, $0.3139 \pm 0.003\%$ w/w, $0.0184 \pm 0.004\%$ w/w, $0.0139 \pm 0.003\%$ w/w and $0.2120 \pm 0.005\%$ w/w in ethanolic, methanolic, petroleum ether, chloroform and aqueous extract of *M. esculenta* stem, stem, bark, leaf and leaf plant parts, respectively. The highest myricetin content was found in the order of methanolic stem extract > ethanolic stem extract > aqueous leaf extract > petroleum ether bark extract > chloroform leaf extract of *M. esculenta*. The developed HPTLC method was a precise, simple, convenient and accurate for screening of large number of active compounds present in various solvent extracts. Thus, the study concluded that the developed HPTLC method for quantification of myricetin can be used for a routine quality check and analysis of any formulation and composition containing *M. esculenta*.

Keywords: Densitometric Method; Formulation; HPTLC; *Myrica esculenta*; Myricetin; Quantification; Quality.

Myrica esculenta Linn is a member of the Myricaceae family. It is a Himalayan plant, the local people called it 'Kaiphala'.¹ *M. esculenta* is a well-recognized plant mentioned in the Ayurvedic Pharmacopoeia. This plant has numerous pharmacological activities due to active compounds present in them.² It has been used for its medicinal properties as folk medicine in the

treatment of asthma, cough, chronic bronchitis, inflammation, fever, ulcers, anaemia, diarrhoea and throat disorders.^{3,4} However, the upsurge demand for this plant has led to its illicit harvesting and loss of habitat.⁵ It brings plant species to the stage of extinction. The reported pharmacognostical study of plant showed presence of glycosides, flavonoids, alkaloids, tannins and phenolic compounds.⁶

Myricetin is a polyhydroxyflavonol compound (3, 5, 7, 32, 42, 52-hexahydroxyflavonol), commonly found in the plants of the Myricaceae family.⁷ It is a well-recognized nutraceutical as a key ingredient of various foods and beverages. Myricetin exhibits a range of activities including anticancer, antioxidant, antidiabetic and anti-inflammatory activities.⁸

DNA fingerprinting of any plant extract play an immense role in the quality control of complex herbal drugs due to advancements in chromatographic techniques. In this concern, High-Performance Thin Layer Chromatography (HPTLC) has been gaining much importance. It has become a routine analytical technique due on its reliable results at micro and nanogram levels.^{9,10} HPTLC is a useful technique because of its high sample throughput, cost-effectiveness and minimum sample clean-up requirement. It is also time-efficient and cost-effective.¹¹

Thin Layer Chromatography (TLC) is the fast detection tool of complex compounds. It has an advantage over High-Performance Liquid Chromatography (HPLC) in that TLC detects a large number of compounds, however, the resolution of TLC is poor.¹² Thus, HPTLC can be used for the detection of compounds that cannot be eluted through HPLC. HPTLC chromatograms are found to be a promising tool for fingerprinting of phytoconstituents.¹³ The color pictures of the HPTLC chromatogram looks attractive and more intuitive unlike Gas Chromatography (GC) and HPLC.¹⁴ HPTLC method has an advantage over the limitation of plate efficiency and developing distance. Therefore, in this study HPTLC method was developed for quality standard and quantitation of myricetin in different solvent extracts of *M. esculenta* plant parts. This study would be a milestone for the analysis of the crude drugs regarding quality control and compound analysis.

METHODOLOGY

Reagents and standards

The stock solutions (mg/ml) were prepared fresh with the respective solvents before use. The solution was applied on TLC aluminium plates using a Linomat applicator, which was pre-coated with silica gel (Merck, India). The solvents and chemicals used for experimental purposes

throughout the experiment were of analytical grade.

Collection and authentically of plant material

The Himalayan region of India present in district of Garhwal where Pinder Velly situated in Chamoli was visited and collected the plants of *Myrica esculenta* Linn. It was authenticated by Dr Anurag Chandra, Senior Scientist and Incharge, Forest Research Institute (F.R.I.) Dehradun. The plant specimen was deposited in the herbarium of the institute via voucher no.247/Dis/2018/Syst. Bot./Rev.Gen./4-5.

Preparation of Extracts

The plant parts (root, leaf, bark, stem and seed) were washed with distilled water. It was further dried under shade. The dried parts were grounded to form coarse powder. The plant parts were then passed to sieve no. 14. The extraction was performed by placing individual plant parts (20 g) in a Soxhlet apparatus. The extraction was performed using different solvents (300 ml each) i.e. ethanol, methanol, petroleum ether, chloroform and aqueous extract at 60-65^o for 5 h. The extracts were then filtered and dried in a rotary vacuum evaporator.³

Quantification of *Myrica esculenta* extracts through High-performance thin-layer chromatography (HPTLC)

Preparation of standard solution

Myricetin (1 mg/ml) of concentration was prepared and dissolved in ethanol and then sonicated. The final volume was made up to 10 ml to get the stock solution of 10 µl/ml.

Preparation of plant extracts

Each extract was weighed, to achieve the concentration of 10 mg/10 ml (1000 µg/ml) and dissolved in a respective solvent, make up the volume to 10 ml in a volumetric flask, sonicated and filtered. It was then applied in the concentrations of 1-10 µl on the stationary phase HPTLC Plates.

Chromatography conditions

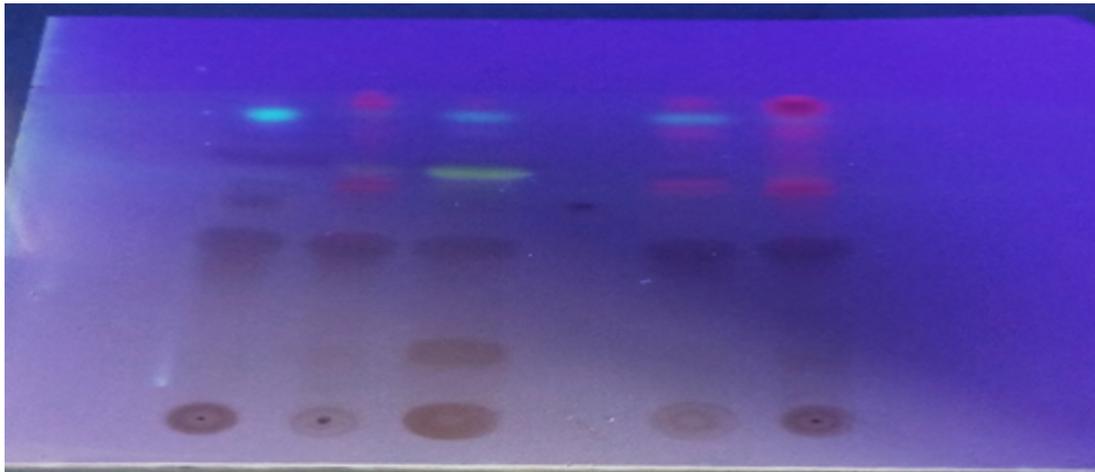
HPTLC chromatography was comprised with a pre-activated HPTLC plate coated with Silica gel 60 F254. HPTLC system was equipped with CAMAG Linomat-V; a sample applicator device and CAMAG TLC scanner-4. The automated TLC applicator Linomat-V was used to apply aliquots of each sample separately to the plate, which was 6 mm wide band and 8 mm from the bottom. CAMAG TLC scanner-4 was used to

perform a densitometric scan at 254 nm and 366 nm with slit dimensions of 6.00×0.30 mm. The micro scanning speed of CAMAG TLC scanner-4 was 20 mm/s. The plates were activated before chromatography at 60°C , which was pre-washed with methanol. The mobile phase was composed of Toluene: Ethyl Acetate: Formic acid: Methanol (3:3:0.6:0.4). The peak height and area as well as spectra were recorded.¹⁵

Detection and quantitation

Thin Layer chromatography was

developed using ascending mode. The plates were dried using a hairdryer. CAMAG glass reagents spray was used for derivatization of chromatograms. It was sprayed with sulphuric acid (5%) in methanol then heated for 5 min at 105°C . The plates were observed at 366 nm after 30 min. Quantitative analysis was performed using CAMAG TLC scanner-4 for scanning the plates, which was equipped with win-CATS-V 1.2.3 software (CAMAG).¹⁶ Figure 1 shows the identification of myricetin. It was confirmed by



Track 1 = stem ethanol, Track 2 = stem methanol, Track 3 = bark petroleum ether, Track 4 = Standard myricetin, Track 5 = leaf chloroform, Track 6 = leaf aqueous extract

Fig. 1. HPTLC chromatoplate of myricetin and extracts of *Myrica esculenta* after derivatization at 366 nm

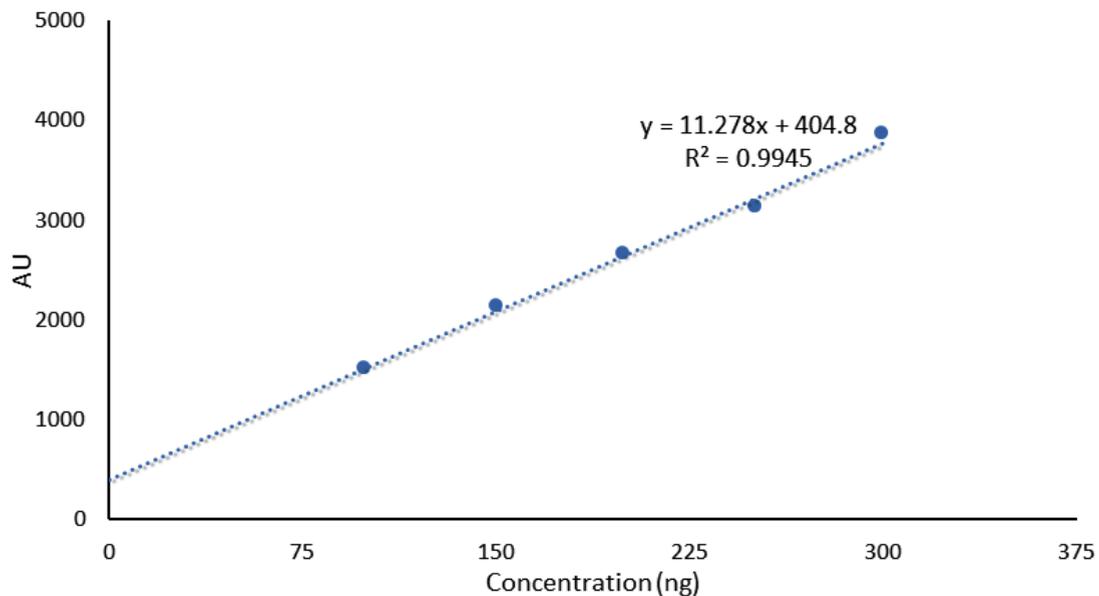


Fig. 2. Calibration curve of standard myricetin (n=6)

superimposing the UV spectra of the samples. The standards within the same were Rf 0.67. In 366 nm scan, myricetin showed in track 4, a sharp peak at Rf value 0.67, which was closely matching with the sample track 1: stem ethanol with Rf value 0.68; sample track 2 stem methanol with Rf value 0.68, sample track 3 bark petroleum ether with Rf value 0.70, sample track 5 leaf chloroform with Rf value 0.69 and sample tract 6 leaf aqueous extract with Rf value 0.69 indicated the presence of myricetin. The characteristic fingerprint profiles were developed through densitometry HPTLC analysis that may be used as a marker for standardization and analysis of the drugs.

Calibration curve of myricetin

The content of myricetin compound in plant extracts was determined by establishing the standard curve with a concentration range from 50-300 ng/spot. The standard sample of myricetin (10 µg/ml) as a stock solution was prepared in

methanol. The HPTLC plates were spotted with the stock solution in the volumes of 5, 10, 15, 20, 25 and 30 µl with concentrations of 50, 100, 150, 200, 250 and 300 ng/spot, respectively. The bandwidth was 6 mm and the distance between tracks was 12 mm. The concentration peak was plotted on the basis of concentration of myricetin spotted. Figure 2 shows linear regression line $y = 11.278x + 404.8$. The concentration of 50–300 ng/spot was showed a good linear relationship based on its regression data. The obtained curve was adhered to Beer's law. It was due to the high value of correlation coefficient and SD for intercept value less than 2%.

Estimation of myricetin in the herbal extracts

Myricetin content in herbal extracts was determined by taking 1 g of each sample plant part with 50 ml of selected solvents. The resulting solutions were centrifuged for 15 min at 3000 rpm. The myricetin content was present in the supernatant. The scanning was performed by

Table 1. HPTLC quantification for myricetin content in different extracts of *M. esculenta*

Sample extract	Plant parts/ Active content	Retention time (min)	Peak height	Myricetin content (% w/w)
Ethanol	Root	0.63	43.0	0.1128 ± 0.005
	Leaf	0.68	197.4	0.1763 ± 0.002
	Bark	0.62	21.6	0.1491 ± 0.001
	Standard (Myricetin)	0.61	175.5	-
	Stem	0.68	452.3	0.2159 ± 0.002
	Seed	-	-	-
Methanol	Root	0.70	124.4	0.2183 ± 0.001
	Leaf	0.67	70	0.2291 ± 0.004
	Bark	0.68	92.2	0.2393 ± 0.004
	Standard (Myricetin)	0.68	238.2	-
	Stem	0.68	278.5	0.3139 ± 0.003
	Seed	0.68	101.7	-
Petroleum ether	Root	-	-	-
	Leaf	0.73	125.4	-
	Bark	0.70	411.1	0.0184 ± 0.004
	Standard (Myricetin)	-	-	-
	Stem	-	-	-
	Seed	-	-	-
Chloroform	Root	0.71	132.3	-
	Leaf	0.69	380.1	0.0139 ± 0.003
	Standard (Myricetin)	0.70	203.0	-
	Seed	0.70	145.0	-
Aqueous	Leaf	0.69	159.5	0.2120 ± 0.005
	Bark	0.72	167.6	0.0200 ± 0.007
	Standard (Myricetin)	0.72	296.4	-
	Stem	0.72	166.4	0.2111 ± 0.003

applying the filtered solutions on the TLC plate. This analysis was repeated 6 times to observe any change of interference.¹⁵ The spot at $R_f = 0.67$ was present in the chromatogram of the extracts corresponding to myricetin. Interestingly, no interference was seen in the tested as shown in Figure 3 and Figure 4-8.

RESULTS

HPTLC fingerprinting and chromatography

Different extracts of *M. esculenta* i.e. ethanol, methanol, petroleum ether, chloroform and aqueous extracts were evolved using HPTLC fingerprint technique. HPTLC pre-coated plates

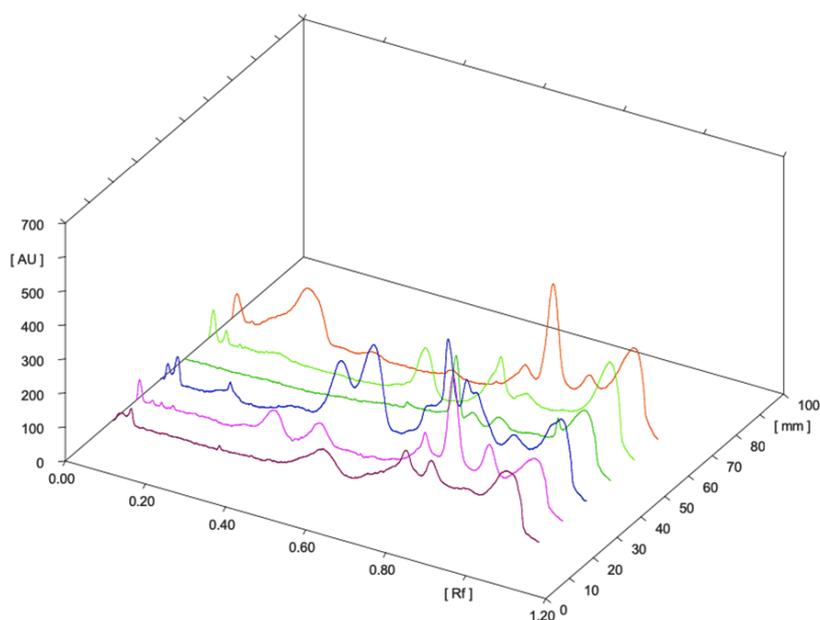


Fig. 3. Densitometric chromatogram of myricetin and methanolic extract of *M. esculenta* (root, leaf, bark, stem and seed) at 366 nm

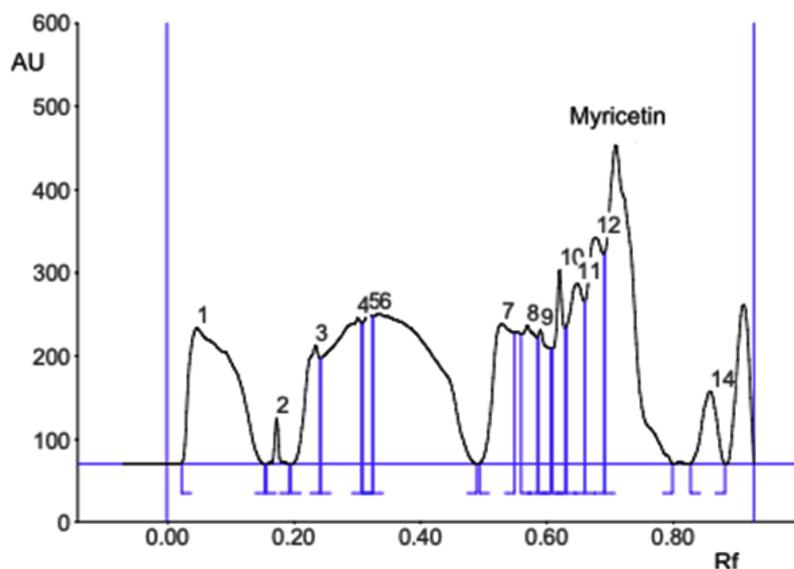


Fig. 4. Densitometric chromatogram of ethanolic extract of stem of *M. esculenta* after derivatization at 366 nm

having silica gel F254 was used to quantify standard myricetin with mobile phase Toluene: EthylAcetate: Formic acid: Methanol (3:3:0.6:0.4).

The Rf value was about 0.67. Figure 3 and Figure 4-8 showing the peak for Rf value of myricetin. It was matched with the Rf value of extract that was about 0.67.

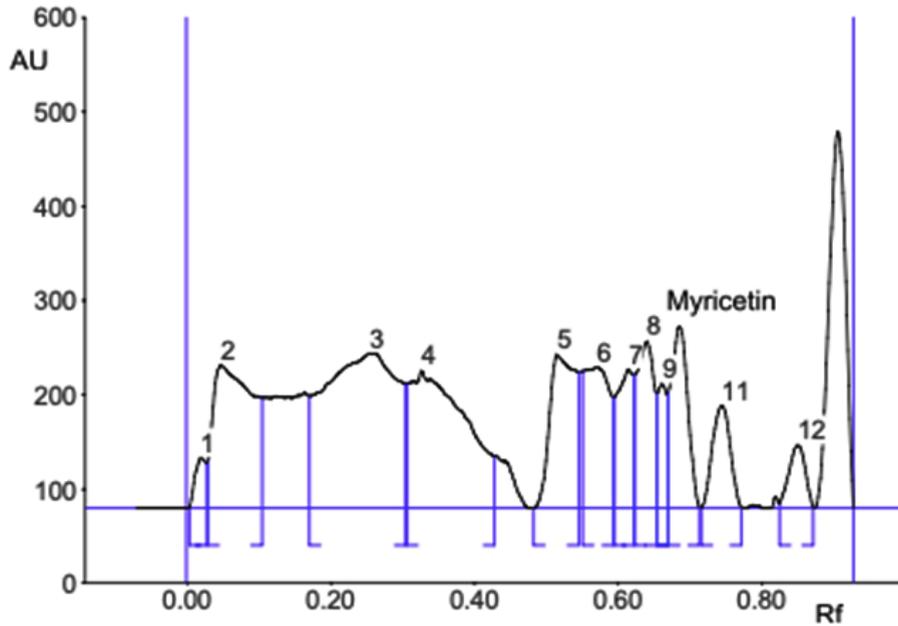


Fig. 5. Densitometric chromatogram of methanolic extract of stem of *M. esculenta* after derivatization at 366 nm

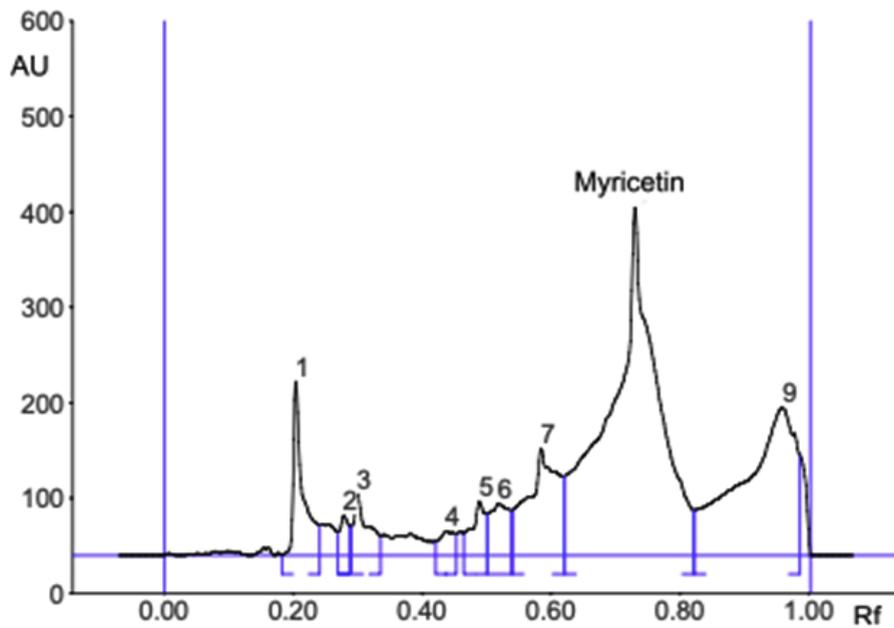


Fig. 6. Densitometric chromatogram of petroleum ether extract of bark of *M. esculenta* after derivatization at 366 nm

Quantification of myricetin using HPTLC

The method we developed was simple and precise to quantify myricetin a marker compound in different solvent extracts of *M. esculenta*. However, TLC procedure was optimized to quantify the herbal extracts. The mobile phase gave sharp,

well-defined and better peak resolution. The chromatogram of the standard compound was used for the identification of myricetin and spot at Rf 0.67. The chamber was saturated to get well-defined spots at room temperature.

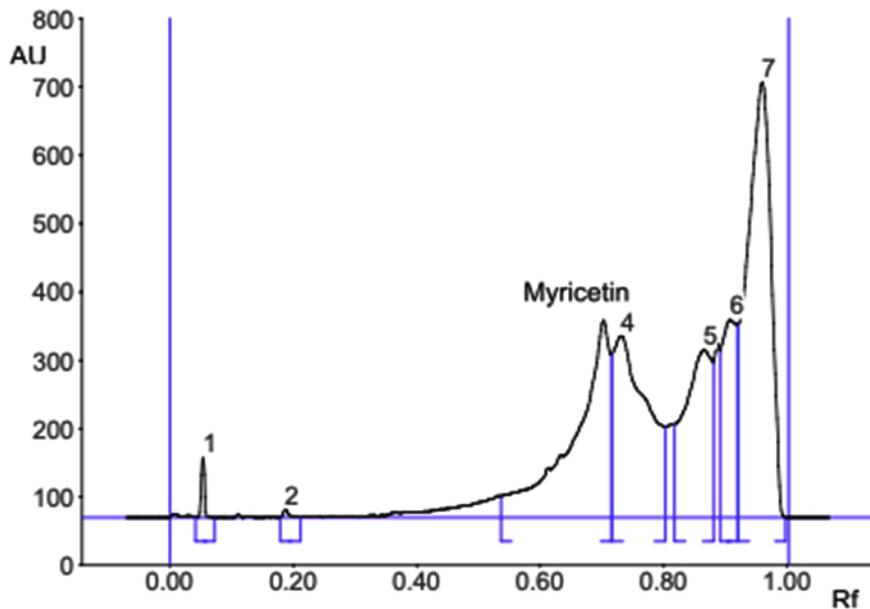


Fig. 7. Densitometric chromatogram of chloroform extract of leaf of *M. esculenta* after derivatization at 366 nm

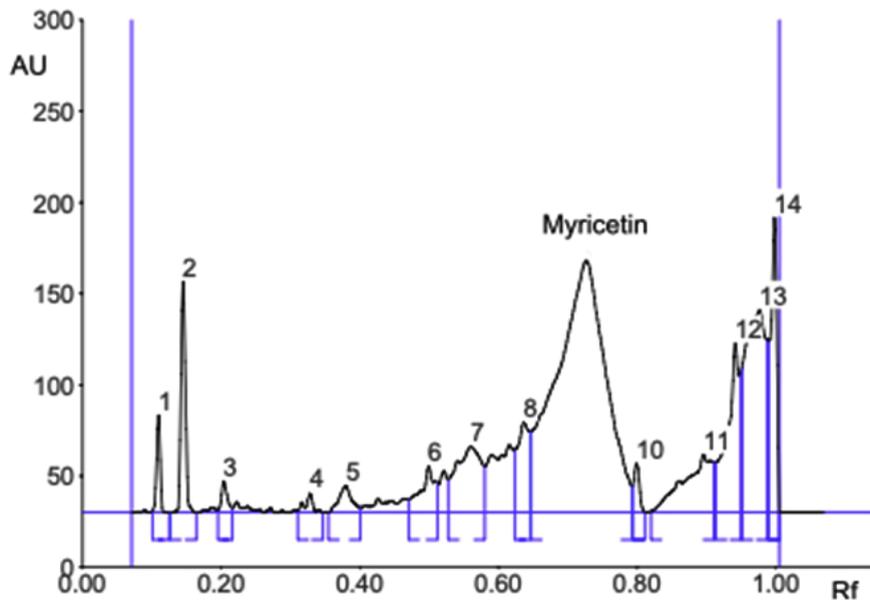


Fig. 8. Densitometric chromatogram of aqueous extract of leaf of *M. esculenta* after derivatization at 366 nm

The derivatization was performed at 366 nm. The identification of myricetin bands obtained in the TLC plate of standard and solvents extracts of different plant parts of *M. esculenta* was confirmed by the chromatogram. It was compared with the retention factor of myricetin from the standard and sample solution. The corresponding peak of myricetin from the herbal extracts had the same retention factor as that from the myricetin standard (Rf 0.67). The linearity range for myricetin was 50–300 ng/spot. It was correlated with correlation coefficient, intercept and the slope 0.9945, 11.278 and 404.8, respectively ($Y = 11.278x + 404.8$).

The content of myricetin quantified was found to be 0.1128 ± 0.005 , 0.1763 ± 0.002 , 0.1491 ± 0.001 and $0.2159 \pm 0.002\%$ w/w, respectively in root, leaf, bark and stem of *M. esculenta* ethanolic extract (Table 1). The content of myricetin in methanolic extract of *M. esculenta* was found to be 0.2183 ± 0.001 , 0.2291 ± 0.004 , 0.2393 ± 0.004 and $0.3139 \pm 0.003\%$ w/w, respectively in root, leaf, bark and stem of parts of the plant. The content of myricetin in petroleum ether extract of *M. esculenta* was found to be $0.0184 \pm 0.004\%$ w/w in the bark of the plant. The content of myricetin in chloroform extract of *M. esculenta* was found to be $0.0139 \pm 0.003\%$ w/w in the leaf of the plant. The content of myricetin in aqueous extract of *M. esculenta* was found to be 0.2120 ± 0.005 , 0.0200 ± 0.007 and $0.2111 \pm 0.003\%$ w/w, respectively in the leaf, bark and stem parts of the plant. The highest myricetin content was found in the order of methanolic stem extract > ethanolic stem extract > aqueous leaf extract > petroleum ether bark extract > chloroform leaf extract of *M. esculenta*.

DISCUSSION

In this study, the quantification of myricetin was performed by developing the HPTLC method on different solvent extracts of *M. esculenta* plant parts. Results showed that the highest quantity of myricetin was found in methanolic and ethanolic extracts of *M. esculenta* stem and aqueous extract of *M. esculenta* leaf. Kamboj and Saluja, 2017 developed the HPTLC method for stigmaterol from stem and leaf of *Bryophyllum pinnatum*.¹⁷ They reported that the highest amount of active constituent was seen methanolic extract

of the plant. Patel *et al.*, 2010 also reported the quantification of active compounds was seen in methanolic extract of the plant.¹⁵ The HPLC-ESI-MS can separate and identify anthocyanins and flavonoids from onion with high efficiency and high quality.¹⁸ The identification and quantification of active compounds responsible for antiplasmodial activity was also reported in different plant parts *viz.* leaf, stem and root of *Cissampelos pareira*.¹⁹ Kumar *et al.*, 2017 reported the presence of gallic acid in *M. esculenta* by HPTLC in the methanolic extract of leaves.²⁰

A comparative study has shown that myricetin present in higher amount in methanolic extract of stem part followed by ethanolic extract of stem part and aqueous extract of leaf part of *M. esculenta*. The quantity of active constituents present in any plant extract affects the activity of plant extract.²¹ Since myricetin is used in the treatment of a variety of diseases, therefore, its quantification may be helpful in the standardization of herbal formulation. Our results were in agreement with the study of Patel *et al.*, 2015 on HPTLC method development for quantification of myricetin in *M. esculenta*.²¹ They reported a similar quantity of myricetin found in the stem bark of the plant. This study further play a significant role in the conservation and sustainable pharmaceutical uses of plant wealth from the Himalayan region. The unorganized activities brings plant species to the stage of extinction. There are different studies involved in the conservation and documentation of medicinal plants from hilly areas.²²⁻³⁰ The quality of nutraceuticals and medicinal substrates are subjects of standardization and copyright.³¹⁻³³

CONCLUSION

The developed HPTLC method for quantification of myricetin present in *M. esculenta* plant was simple, rapid, accurate, precise method. The data present study can be used as a quality control standard. The shape of the peak generated through this method enabled a good resolution of myricetin. No interference with the peak of myricetin was seen, it may be due to recovery (96.77–99.44%) that was close to 100%. The HPTLC images shown in Figures 1 and 3 indicating samples separated without any diffuseness and tailing. It can be concluded from the study that

the proposed HPTLC method is sensitive, simple and economic for routine quality control analysis. It can also be useful for the quantification of myricetin from different parts of *M. esculenta* and for standardization purposes.

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Conflict of interest

The authors declared no conflict of interest.

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