Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2017, 9(1):168-173

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Identification and Quantification of Embelin by Validated HPTLC Method and Confirmation by LC-MS from Mangrove Plant *Aegiceras corniculatum* L

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ABSTRACT

Aegiceras corniculatum L. (Myrsinaceae) is a medicinally important mangrove occurring along the west coast of Maharashtra. A simple, sensitive, selective, precise, reliable and robust high-performance thin-layer chromatographic (HPTLC) method for analysis of embelin has been validated for the determination of embelin from methanolic extracts of stem, leaves and fruit part of Aegiceras corniculatum L. embelin was quantified from these extracts using the solvent system of chloroform: ethyl acetate: acetic acid (5:4:1 v/v/v). This simple and precise method gave good resolution for embelin from other constituents of extracts at Rf 0.58. Further confirmation was done by LC-MS analysis in which embelin was observed at 295.1904 m/z ratio and retention time (RT) was 11.546 minute. We are reporting quantification of embelin from different plant parts for the first time.

Keywords: *Aegiceras corniculatum;* Embelin; HPTLC; LCMS; Myrsinaceae

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INTRODUCTION

Aegiceras corniculatum L. (black mangrove or river mangrove), is an endangered medicinally important, evergreen small tree belonging to the family Myrisanceae. It is woody plant reaching up to a height of 4m and is distributed throughout shorelines of India, Sri Lanka, south China, Myanmar, Thailand, Vietnam, Peninsular Malaysia, Singapore, throughout Indonesia, south Philippines, Papua New Guinea, Solomon Islands and to northern and western Australia. This species is vulnerable to the west coast of Maharashtra. Chemical constituents of stem and leaves were studied by phytochemical analysis indicates the presence of amino acids, benzoquinone, tannins, coumarins, flavonoids, saponins, polyphenols, steroids, quinones phenolic acid, triterpenes and hydroquinone derivatives [1] [2]. From stem part, 2-methoxy-3-nonylresorcinol, 5-*O*-ethylembelin, 2-*O*-acetyl-5-*O*-methylembelin, 3,7-dihydroxy-2,5-diundecylnaphthoquinone,2,7-dihydroxy-8-methoxy-3,6-diundecyldibenzofuran-1,4-dione, 2, 8-dihydroxy-7-methoxy-3,9-diundecyl dibenzofuran-1,4-dione, 10-hydroxy-4-*O*-methyl-2,11-diundecyl gomphilactone, 5-*O*-methylembelin, 3-undecylresorcinol, 2-dehydroxy-5-*O*-methylembelin compounds isolated [3].

Embelin (2, 5-dihydroxy-3-undecyl-1, 4-benzoquinone) has been reported from *Embelia ribes* plant. It has a wide spectrum of biological activities, including antioxidant, antitumor, anti-inflammatory, analgesic, anthelmintic, antifertility antimicrobial and antimalarial [4, 5]. Literature survey revealed that no method has been reported for presence and quantification of embelin from different parts of the plant like stems, leaves and fruits methanolic extracts of *A. corniculatum*. Reports of qualitative and quantitative standardization of embelin by HPTLC from *Embelia ribes* are available but not from this plant [6, 7, 8]. Hence the HPTLC method has been developed in the present work for quantitation of embelin from the methanolic extracts of dried stems, leaves and fruits of *A. corniculatum*. The method was found suitable for rapid screening of plant material for their quantitative assessment and can be performed without any special sample pre-treatment.

High-Performance Thin-Layer Chromatography (HPTLC) is the most advanced form of TLC and routine analytical technique due to the use of chromatographic layers of utmost separation efficiency and precise sample application, standardized reproducible chromatogram development and software controlled evaluation. It has reliability in the quantification of analytes at the micro and even in nanogram levels. The major advantage of HPTLC is its low operating cost and a reduced analysis time of high sample throughput.

MATERIALS AND METHODS

Source of plant material

The plant material of *Aegiceras corniculatum* was collected from Ratnagiri, Maharashtra India. The sample was deposited at BSI (Botanical Survey of India), Western regional Centre, Pune and authenticated as BSI/WRC/Cert./2015/ and collection number DS 01.

Chemicals

Embelin (Purity: 97%w/w) was purchased from Sigma, Germany. Solvents used were of Merck analytical grade. Pre-coated silica gel 60 F254 HPTLC aluminum plates (10 × 10 cm, 0.2 mm thick) were obtained from E. Merck Ltd. (Mumbai, India)

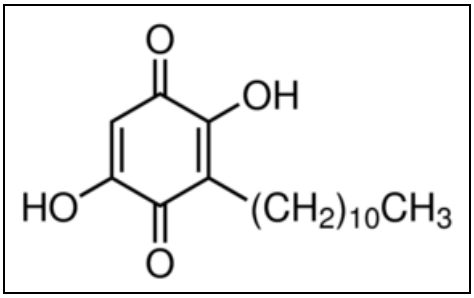

Figure 1: Chemical structure of embelin

Extraction

Plant material of stem, leaves and fruits of the natural population were dried, powdered and weighed. 50 g of each were taken and refluxed with methanol at 60°C for 6 h in a soxhlet extractor. The extracts were filtered and concentrated under reduced pressure in a rotary evaporator. The concentrated crude extracts were then dissolved in methanol to make the concentration of 1mg/ml.

HPTLC analysis

Samples were applied on 10x10 cm pre-coated silica gel 60 F_{254}TLC plates (E. Merck) in the concentration range of 20-100µg/ml as sharp bands using Camag Linomat 5 sample applicator with the aid of Hamilton 100µl syringe under a nitrogen stream. The plate was dried and developed in flat bottom twin trough chamber (20×10×4cm). The mobile phase i.e. chloroform: ethyl acetate: acetic acid in the proportion of 5:4:1 v/v/v was prepared in Camag twin trough chamber by mixing and the chamber was saturated for 5 minutes. The TLC plate was dried in air with the help of dryer and the densitometric scan was performed at 254 nm with Camag TLC scanner III and peak areas were recorded. Calibration curves were constructed by plotting average peak area versus concentrations

Preparation of standard embelin and calibration curve

1mg/ml stock solution of standard embelin in methanol was prepared. From this stock solution, standard solutions of 2µg/µl to10µg/µl were used. For the preparation of calibration curve, 2-10µl of standard solutions of embelin was applied in triplicate on a TLC plate. The plate was developed in a solvent system chloroform: ethyl acetate: acetic acid (5:4:1 v/v/v) at 25 ±2°C temperature up to a distance of 90cm. After development, the plate was dried in air and scanned at 254nm. The peak areas were recorded. Calibration curves of embelin were prepared by plotting peak area vs. concentration.

HPTLC analysis of plant extract

Samples of methanolic extract of *Aegiceras corniculatum* L were filtered through 0.45*µ*m filter, and HPTLC was performed under the conditions optimized for the reference compound. The amount of embelin in plant extract was quantified using calibration curve plotted with embelin.

Validation of the method

The proposed method was validated according to the recommendations laid down in the International Conference on Harmonization (ICH) guidelines [9, 10]

Linearity

Linearity was studied by least square linear regression analysis of peak areas obtained after application of different concentrations of standard embelin solution in ranges 2µg/µl to 10µg/µl.

Precision

Instrumental precision was verified by repeated scanning (n= 7) of the same spot of embelin and was expressed as coefficient of variance (%CV) of the peak areas. The precision of the method was studied by analyzing a standard solution of embelin (10 µg/spot) on the same day for intraday precision. The study was also repeated on different days with the freshly prepared sample to calculate interday precision.

Repeatability

The repeatability was affirmed by analyzing 2, 4, 6, 8, 10 µg/spot of a standard solution of embelin and plant extracts after application on the HPTLC plate (n=7) by expressing it as % CV.

Robustness

Different composition of mobile phases like chloroform: ethyl acetate: acetic acid (4.5: 3.5: 0.5 v/v/v), (4: 3: 1 v/ v/v), (5: 3: 0.5 v/v/v) etc., were tried and the amount of mobile phase, temperature and relative humidity varied in the range of ±5%. The robustness of the method was studied for three different concentration levels of standard embelin like 2, 4, 6, 8, 10 µg/spot.

Specificity

The specificity of the method was ascertained by determining the peak purity of the component by overlaying the fluorescence spectra of embelin in the sample extract with the spectra of reference standard embelin at the start, middle, and end positions of the bands**.**

Limit of detection and limit of quantification

LOD means the lowest concentrations of embelin that can be detected, whereas the LOQ represents the lowest concentrations of embelin that can be determined.

For the evaluation of limit of detection and limit of quantification different concentrations of the standard solutions of embelin were applied and determined on the basis of signal to noise ratio by following equations:

$$LOD = 3.3 \times \text{Standard Deviation/Slope of the calibration curve}$$

$$LOQ = 10 \times \text{Standard Deviation /Slope of the calibration curve}$$

LC-MS analysis

Agilent 1260 binary LC System with Agilent Zorbax Extend C18 RRHT column (50x2.1mm, 1.8µm) was used for analysis. Methanolic extracts of stem, leaves and fruits of A. corniculatum were analyzed by LC-MS for confirmation of the presence of embelin. All MS acquisitions were performed in the positive electrospray ionization mode. The capillary voltage, cone voltage, fragmentor voltage were 4 kV, 45V and 170V, respectively. The gas temperature was set at 325°C. Data was acquired at a scan rate of 3Hz in the mass range 100-100 m/z. Further data was analyzed with Mass hunter qualitative software and METLIN database. 6540 ultra-high definition accurate mass QTOF LC/MS system was used. Injection volume was 1µl and run time was 30 min with the flow rate of 0.3 ml/ min.

RESULTS

The HPTLC densitometric technique is developed and validated for the determination of embelin from methanolic extracts of stem, leaves and fruits part of *A. corniculatum*. The mixtures of several mobile phases were tried to

separate spot of embelin from other spots and got a stable peak from all the test samples. The solvent system chloroform: ethyl acetate: acetic acid (5:4:1 v/v/v) was selected for estimation of embelin, which gave good resolution at Rf value 0.58 identical to test samples (Fig 3). To obtain the fingerprints of sample, densitogram of samples were overlaid with the densitogram of standard embelin as shown in (Fig 4) which clearly indicated common peak in the sample. The wavelength of 254nm was used for quantification of the sample. Linear regression data showed a good linear relationship over the concentration range of 2-10µg. The correlation coefficient R^2 was 0.9920 (y = 422.0x + 2141). The precision and the repeatability at different concentration levels reflect the robustness of the method. Intraday precision and interday precision was found to be 1.5761(%CV) and 2.3191(% CV), respectively. The repeatability of the test samples of stem 3.2582(%CV), leaves 5.466(%CV), fruits 4.7404(%CV) and of the standard embelin 0.6708(%CV) was found. The standard deviation of peak areas was calculated for each condition and % RSD was found to be less than 2%. These low values of % RSD are indicative of the robustness of the method. The LOD and LOQ were obtained with the signal to noise ratio of 3 and 10. The LOD and LOQ were found to be 0.645 µg and 1.957 µg/spot for embelin, which indicates that this method exhibited a good sensitivity for the quantification of embelin. The method was found to be simple, precise, specific and sensitive and can be used for the quantification of embelin from plant parts of *A. corniculatum.*

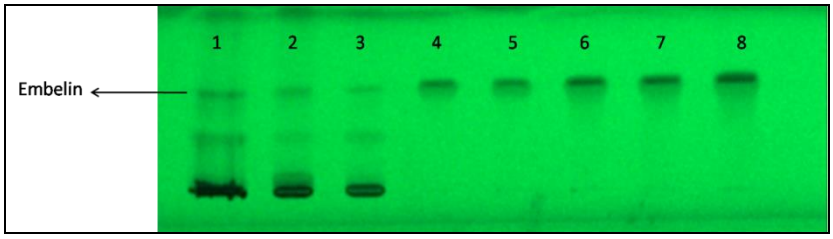

Figure 2: HPTLC profile at 254 nm of methanolic plant extract and standard embelin. Track 1: Stem, Track 2: Leaves, Track 3: Fruit, Track 4-8: Standard embelin concentration of 2 to 10 µg/µl

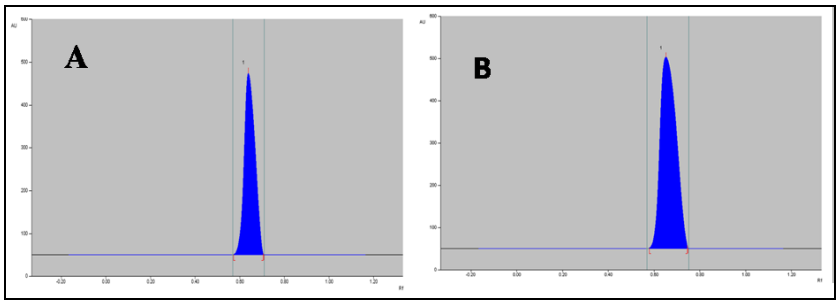

Figure 3: HPTLC peak display of standard embelin (A) and comparative peak in extracts of *A. corniculatum* (B) at 254 nm

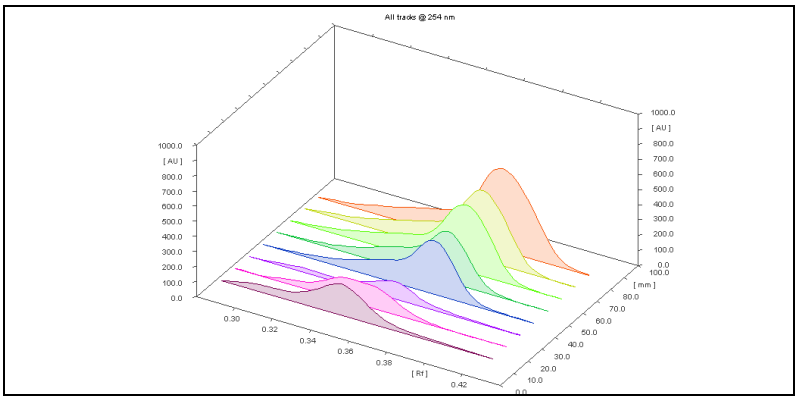

Figure 4: Densitogram - 3D View of embelin in standard and sample. Track 1: Stem, Track 2: Leaves, Track 3: Fruit, Track 4-8: Standard embelin concentration of 2 to 10 µg/µl

Table 1: Validation parameters: Method validation parameters for the quantitation of embelin by proposed HPTLC method

Parameter	Embelin
Linear range	2-10 µg
Precision	10
Intraday Precision(%CV)	1.5761
Intraday Precision(%CV)	2.3191
Repeatability(%CV)	
Embelin	0.6708
Stem	3.2582
Leave	5.4669
Fruit	4.7404
Robustness	Robust
Specificity	Specific Rf 0.58
LOD()	0.6459
LOQ()	1.9574
Solvent system	chloroform: Ethyl acetate: Acetic Acid (5:4:1v/v/v)

Further confirmation of the presence of embelin was done by LC-MS analysis. LC profile of methanolic extracts of stem leaves and fruits of *A. corniculatum* showed the presence of embelin. Confirmation was done by METLIN database. (Table 2, Figure 5 and 6)

Table 2: LC-MS data

Name of compound	Embelin
Observed m/z	295.1904
Observed RT	11.546
observed mass	294.183
DB formula	C17H26O4
DB mass	294.1831
DB mass Error (ppm)	0.39
Target Formula	C17H26O4

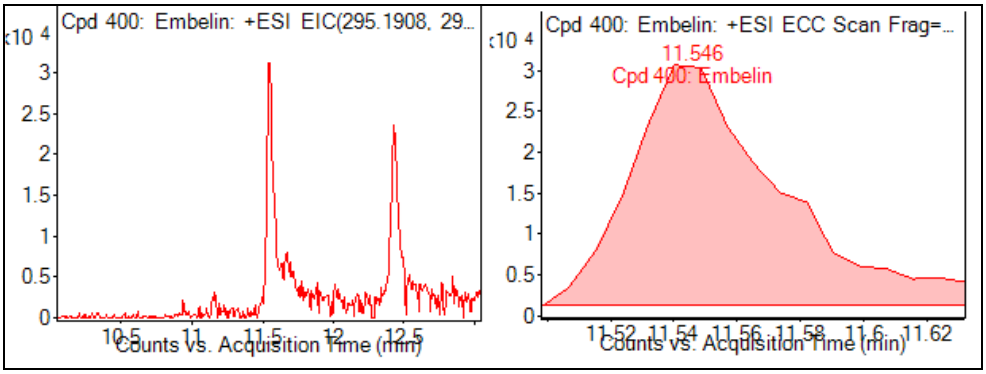

Figure 5: Embelin chromatogram

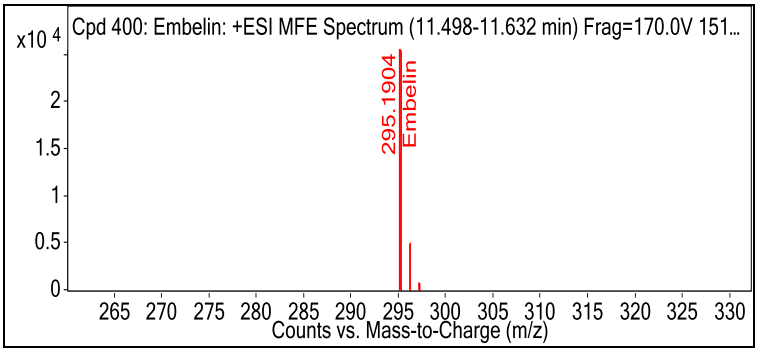

Figure 6: MS zoom Spectra of embelin

Estimation of embelin in methanolic extract of stem leaves and fruits of *Aegiceras corniculatum*

The optimized solvent system was used for the estimation of the embelin in these extracts. There was no interference in analysis from other components present in the extracts. The resolution was good and components were observed at different Rf value. Contents were calculated in % of embelin present in test sample (Table 3)

Table 3: Content of embelin quantitated by HPTLC densitometric method from different plant parts of *A. corniculatum*

Plant part	Content of embelin(%)(w/w)
Stem	0.2847±0.003
Leave	0.2355±0.005
Fruit	0.2495±0.004

(Data represented statistically, mean of three replicate ± standard error, n=7)

DISCUSSION

Embelin is a novel XIAP inhibitor that exhibits various medicinal effects including anti-inflammatory and anti-cancer activities. Embelin induces apoptosis in human glioma cells through inactivating NF-κB which is a crucial transcription factor associated with several human diseases including cancer and also controls multiple genes involved in tumor progression such as cell proliferation and cell survival [11]. Therefore, identification of embelin from various plant parts of natural population of *A. corniculatum* L as a potential candidate and is significantly important. Identification and characterization of embelin from different plant parts like stems, leaves and fruits of *A. corniculatum* confirms it as the novel source of embelin other than *Embelia ribes*. It is the first report of quantification of embelin from different plant parts of *A. corniculatum* L.

CONCLUSION

This HPTLC method is fast, simple, precise, specific and accurate. Validation of the method analysis proved that method is repeatable and selective for determination of embelin from *A. corniculatum* which is endemic to the west coast of Maharashtra.

ACKNOWLEDGMENTS

Authors would like to acknowledge financial support given by Bhabha Atomic Research centre and Savitribai Phule Pune University.

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