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COMPARATIVE LC-MS PROFILE OF THE THREE SPECIES *HYPERICUM HOOKERIANUM, HYPERICUM MYSORENSE AND HYPERICUM WIGHTIANUM*

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ABSTRACT

The genus *Hypericum* consists of both advanced and primitive tribes and taxa containing a complex mixture of secondary metabolites many of which are still being investigated. In the present study, we analyzed the methanol extracts of three *Hypericum* species (*H.hookerianum, H.mysorense, and H.wightianum*) of the Palni hills in southern India for the presence of bioactive hypericin and hyperforin and found characteristic discrimination of the three species based on LC-MS profiles. We report for the first time that *H.mysorense* lacks both these metabolites, *H.wightianum* contains only *hypericin* while *H.hookerianum* contains both the metabolites.

KEYWORDS : Hypericum, LC-MS, *H.hookerianum, H.mysorense, and H.wightianum*

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INTRODUCTION

The genus Hypericum (Hypericaceae) consists of 484 species[1] of herbs, shrubs and rarely trees distributed in warm temperate, subtropical and tropical montane forests of the world. Many of them find use in traditional medicine for the treatment of wounds, skin infections, inflammation, eczema, burns, rheumatism, neuralgia, gastroenteritis, ulcers and hysteria but they are best known for their remedial effect on anxiety and depression[2]. Of all, *Hypericum perforatum* is the most popular folk medicine and a commercially recognized species used both as a phytopharmaceutical and a modern medicine[3]. Pharmacological and clinical studies have shown therapeutic superiority of *H. perforatum* extracts over single isolated constituents and bioequivalence of the active extracts with synthetic chemotherapeutics. Anti-depressant applications of *H. perforatum* products such as Psychotonin R, Neuroplant R, and Hyperforat R have become enormously popular in Europe[4]. A standardized extract with a phytochemical composition of the prenylated acyl phloroglucinol derivative, hyperforin and the red pigmented naphthodianthrone, hypericin in 10:1 ratio is a globally marketed phytopharmaceutical of the species. Marketing of St. John's wort products as dietary supplements in Europe and USA and as prescribed medicine in Germany to treat mild to moderate depression is accepted by the industry. Economic evaluation has justified the use of *H. perforatum* as a cost effective alternative to synthetic antidepressants with reduced side effects and improved outcomes[5]. Less than a third of the known species of Hypericum have been surveyed for chemicals[6].The antidepressant activity of St. John's Wort paved the way for phytochemical investigations of other species of hypericum.However, even now Phytochemically, they are complex with at least 8 classes of compounds including hypericins (Hypericin, pseudohypericin), hyperforijns (hyperforin, adhyperforin), flavonoids, xanthones, biflavonoids, tannins and phenolic acids [7]. Hypericin, hyperforin and flavonoids of the shoots and xanthones of the roots putatively belong to the polyketide pathway in which type III polyketidesynthases act as key enzymes[8]. The phytochemistry of *Hypericum* has engaged the attention of scientists mainly for the two marker compounds, hypericin and hyperforin due to their potent biological activities. While hyperforin is present in significant quantity only in *H. perforatum*, others are either deficient in both (*H. gentianoides*) or contain hypericin (*H. montbretti*, *H. maculatum*, *H. triquetrifolium*) only. Hypericin is a known photosensitive antiretroviral and antitumor agent while hyperforin, a major lipophilic chemical component of the herb is bactericidal, anti-cancerous and anti-depressant principle which stabilizes neurotransmission by increasing intracellular concentration Na+ ions and simultaneously inhibiting synaptic reuptake of neurotransmitters by activating TRPC6 channel. Flavonoids in Hypericum may be occur as anti-oxidant, anxiolytic and mildly anti-depressant principles[9]. Porzel et al provided evidence that H. Polyphyllum containing both hypericin and hyperforin might be used as an alternative to St. John's Wort .India harbours 27 species of Hypericum in parts of the Himalayas, Aravallis in Central and Western Ghats in peninsular India [10]. The antidepressant acitivity of St. John's Wort extracts have given the clue to investigate the other species of Hypericum for similar metabolites. But for *H. perforatum,* other species are least investigated for phytochemical constituents and pharmacological activities. *Hypericum hookerianum*, the Hooker's wort is an evergreen shrub of the Khasi and Jaintia hills of the Eastern Himalayas and Nilgiri and Palni hills of the Western Ghats in southern India reported as a folk medicine used the Toda tribe of the Nilgiris for the treatment of skin infections, inflammation and anxiety related problems[11]. This species has been pharmacologically tested to possess wound healing, antioxidant , antitumor , bactericidal , *in vitro* cytotoxic and anti-herpes activities.

The Palni hills is a biodiversity rich wildlife sanctuary harboring such species as *H. mysorense*, *H.wightianum* and *H. japonicum*. Of these, *H. japonicum* is a popular Chinese medicinal herb already investigated as a rich source of flavonoids, phloroglucinols and xanthones and used for the treatment of bacterial diseases and infectious hepatitis. While *H.mysorense* is reported to possess wound healing property in the Ayurvedic system of traditional medicine and anti-HSV-1 and antitumor activities, *H. wightianum* with antidepressant activity is rarely investigated. Largely due to the paucity of phytochemical information, the objective of the present study was to investigate leaf extracts of the three *Hypericum* species of Palni hills (*H. hookerianum, H. mysorense, H.wightianum)* for the presence of the major bioactive metabolites, hypericum and hyperforin using LC-MS analysis. This is the first report documenting distinct qualitative differences between the three species in respect of the presence and absence of these marker chemcials.

MATERIALS AND METHODS

Plant Material

Top shoot cuttings with leaves and flowers of the three Hypericum species (*H.hookerianum*, *H.mysorense* and *H.wightianum)* were collected in July 2014 from plants naturally growing in Pambar, Eettipallam and Perumalthoppu parts of the Kodaikanal hills in the Western Ghats region of southern India and were identified and authenticated by Prof. N.Raaman of the Centre for Advanced Studies in Botany, University of Madras. Leaves were dissected out, washed in tap water, dried in shade and used for the experiments.

Extraction

Dried leaves were pulverized and 250 g of powdered material was extracted with 50 ml of HPLC grade methanol at room temperature for 72 h and filtered, saving the filtrate. The residue was re-extracted quickly once with HPLC methanol and the combined filtrates were reduced to 5 mL using a Remi rotary evaporator, The extract diluted to 10 mL using methanol was filtered through Whatman No. 1 filter paper and concentrated to dryness. The final residue was dissolved in HPLC grade methanol and subjected to LC-MS analysis.

LC-MS analysis

The methanol extracts of different species were analyzed by rapid resolution liquid chromatography with mass selective detection using Shimadzu LC-MS 2020 series liquid chromatography coupled with an electron spray mass spectrometer. Aliquots of the methanolic extracts of each plant were filtered through 0.45 µ Teflon filter, transferred to 2 mL amber HPLC vials and 20 µL aliquots injected for separation using reversed phase RP-C18 column 150 × 2 mm held at 50°C. The mobile phase was (A) 20 mL ammonium acetate; and (B) acetonitrile. The flow rate was 1 mL / min in the following gradient system: 0-10 min, 50% B; 10-25 min, 90% B; 30-35 min, 50% B. The total run time was 35 min. The mass detector conditions were set as follows: ESI negative ionization mode; full scan mode from 50 to m/z; capillary voltage, 4000 V and fragmentor voltage 80 V; ESI temperature, 325°C; gas flow rate, 5 L/min. To increase the sensitivity, lower the noise and simplify the spectra, negative ionization was used. Hypericin and hyperforin standards (Sigma-Aldrich) were used for retention time matching.

Identification of components

Interpretation of mass spectra of LC-MS was done using the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns. The mass spectrum of the unknown component was compared with the spectrum of the known component stored in the NIST library and name, molecular weight and structure of the components of the test materials were ascertained.

RESULTS AND DISCUSSION

Published literature on spectral data and chromatographic elution patterns of phloroglucinols and naphthodianthrones (Fuzzabi et al., 2001; Tolonen et al., 2002;

Wolfender et al., 2003) were used to investigate presence of hypericin and hyperforin in the three Hypericum species. Lc(-)-ESI-MS and LC-UV data were utilized to confirm the identity of both the compounds in the crude methanolic extracts. The methanolic extract of three Hypericum species (*H.hookerianum*, *H.mysorense* and *H.wightianum*) was analyzed for the presence of hypericin and hyperforin using LC-MS and the total run time was 35 minutes. The molecular ions [M-H] ⁻of these two compounds were monitored in the negative ion mode: Hypericin *m*/*z*– 503, and Hyperforin *m*/*z* – 535. The (-)-ESI mass spectra of hypericin (*t*R = 26.51 min and 27.63) from *H. hookerianum* and *H. wightianum* showed a parent molecular ion at *m*/*z* 503 [MH]–(Fig.6 and Fig.9).The UV spectrum of hypericin showed the absorption maxima at 254 nm. The identity of this compound was verified by comparison of its ESI mass spectrum, UV spectrum,and the retention time with an authentic standard of hypericin, where complete matching was observed . In *H. hookerianum*, the (-)-ESI mass spectraof the peaks at *t*R = 31.08 min showed parent molecular ions of 535for [M-H]– (Fig.7), in the negative ionization mode. The UV spectrum showed an absorption maximum at 254 nm, characteristic of phloroglucinols. These mass spectral data suggested that this compound was hyperforin.

Table 1
Retention time of identified active compounds by mass spectrometry

Name of the plant	Mode	Retention time, t_R [min]	UVmax[nm]	$[M-H]^-$, m/z	Active compounds
H.hookerianum	Negative ion mode	31.083	254	535	Hyperforin
		26.517	254	503	Hypericin
H.mysorense	Negative ion mode	-	-	-	-
H.wightianum	Negative ion mode	27.633	254	503	Hypericin

Figure 1
LC - Chromatogram of standard Hypericin

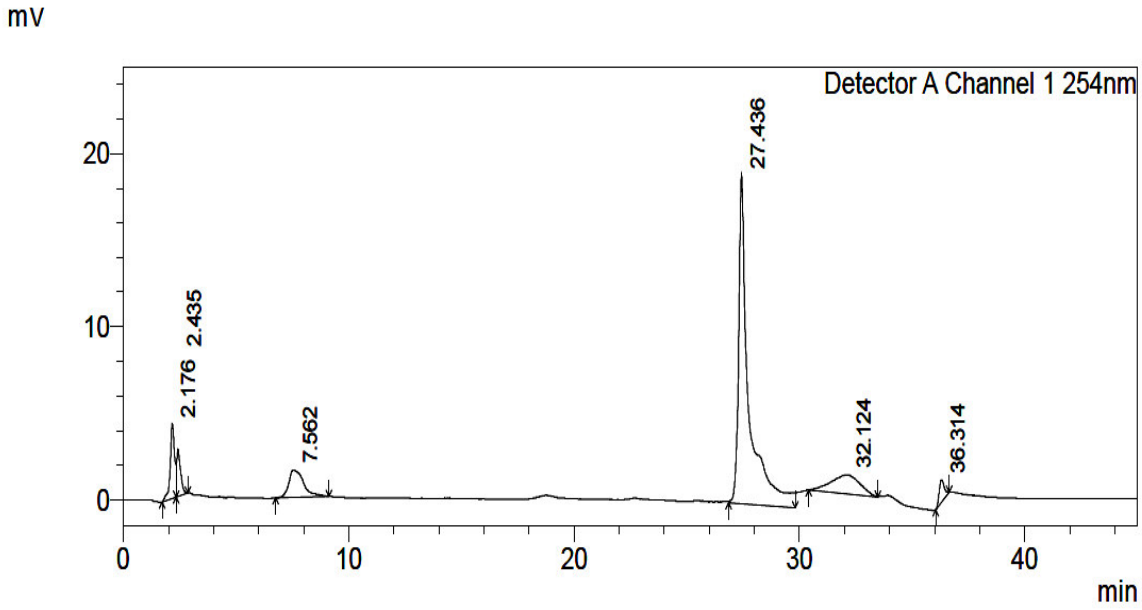


Figure 2
Mass spectrum of standard Hypericin

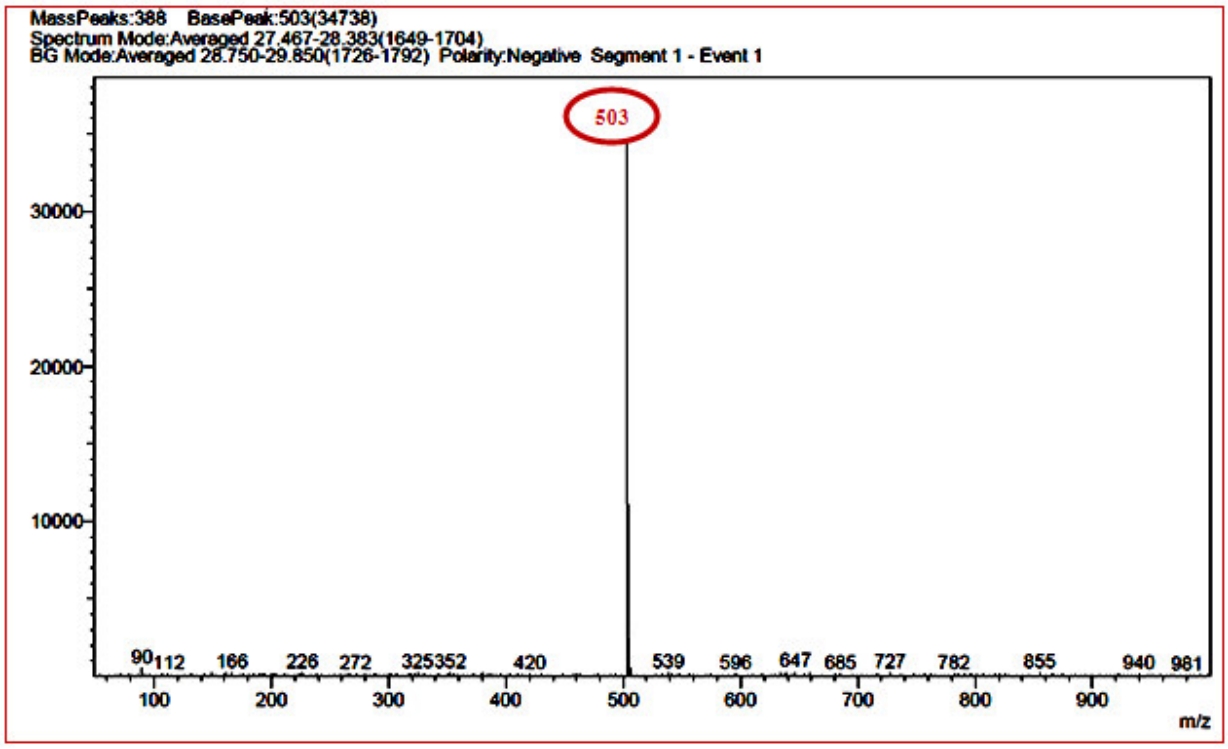


Figure 3
LC - Chromatogram of standard Hyperforin

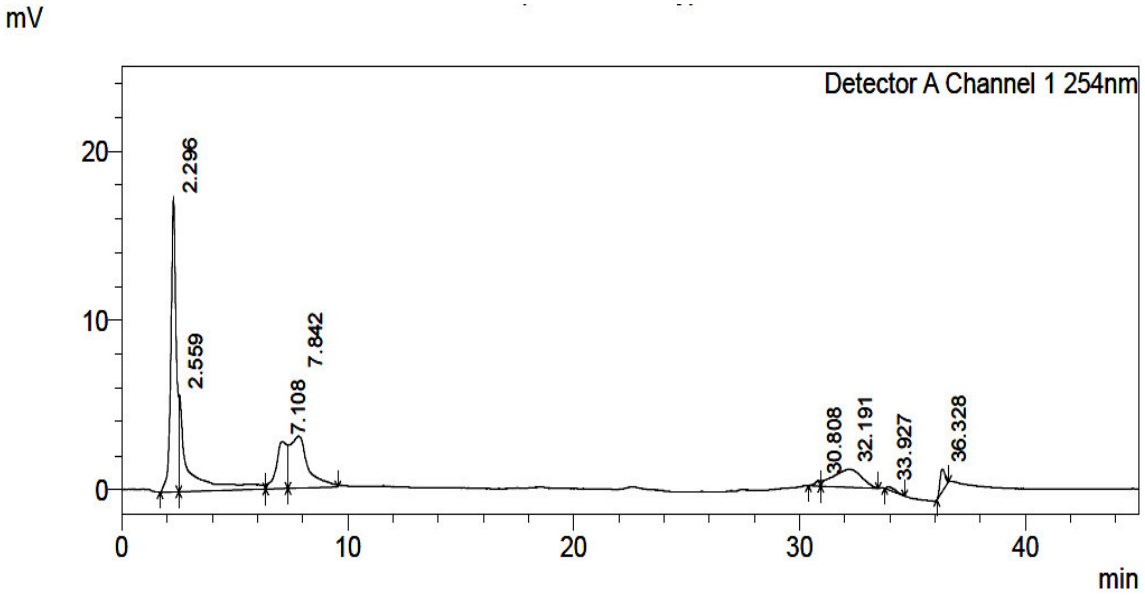


Figure 4
Mass spectrum of standard Hyperforin

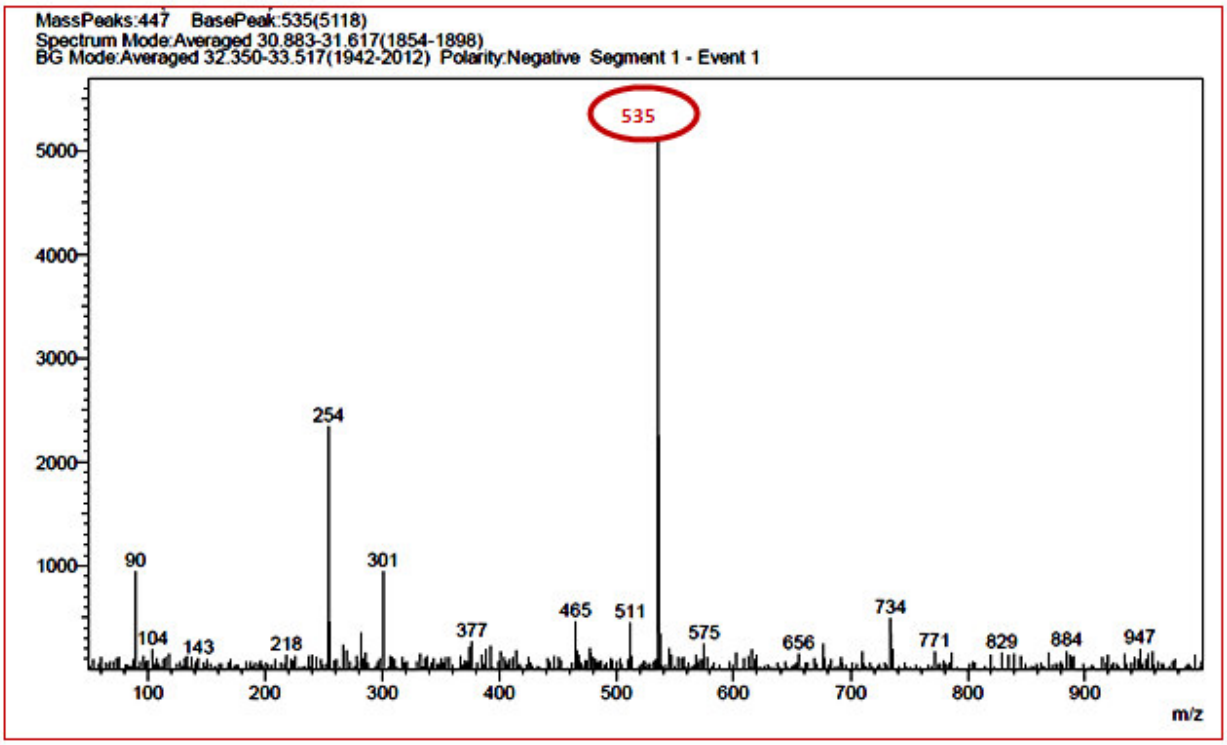


Figure 5
LC-Chromatogram of methanol extract of leaves of H. hookerianum

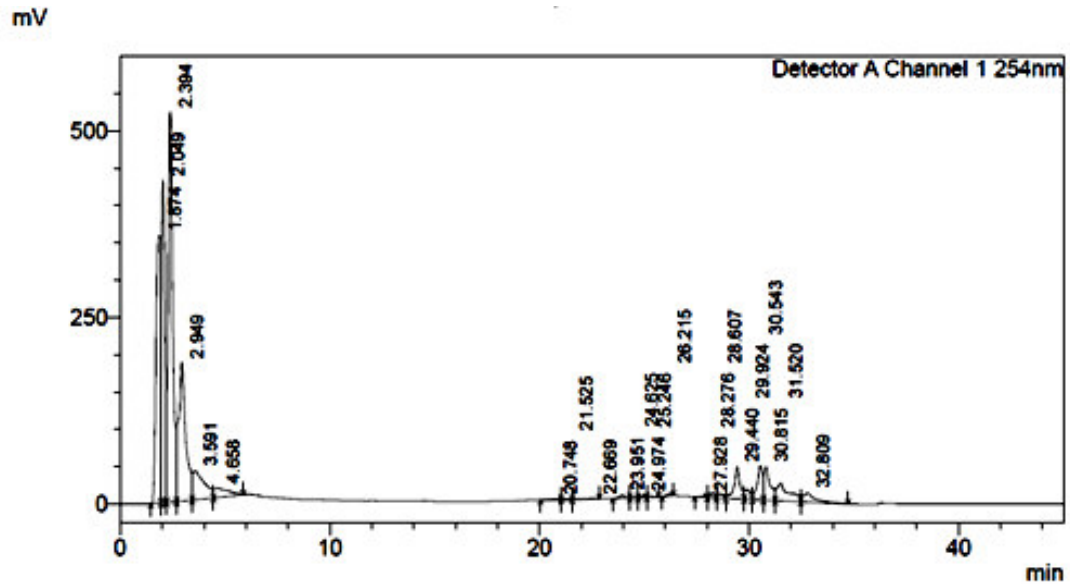


Figure 6
Mass spectrum of methanol extract of leaves of H. hookerianum for Hypericin (503) –negative ion mode

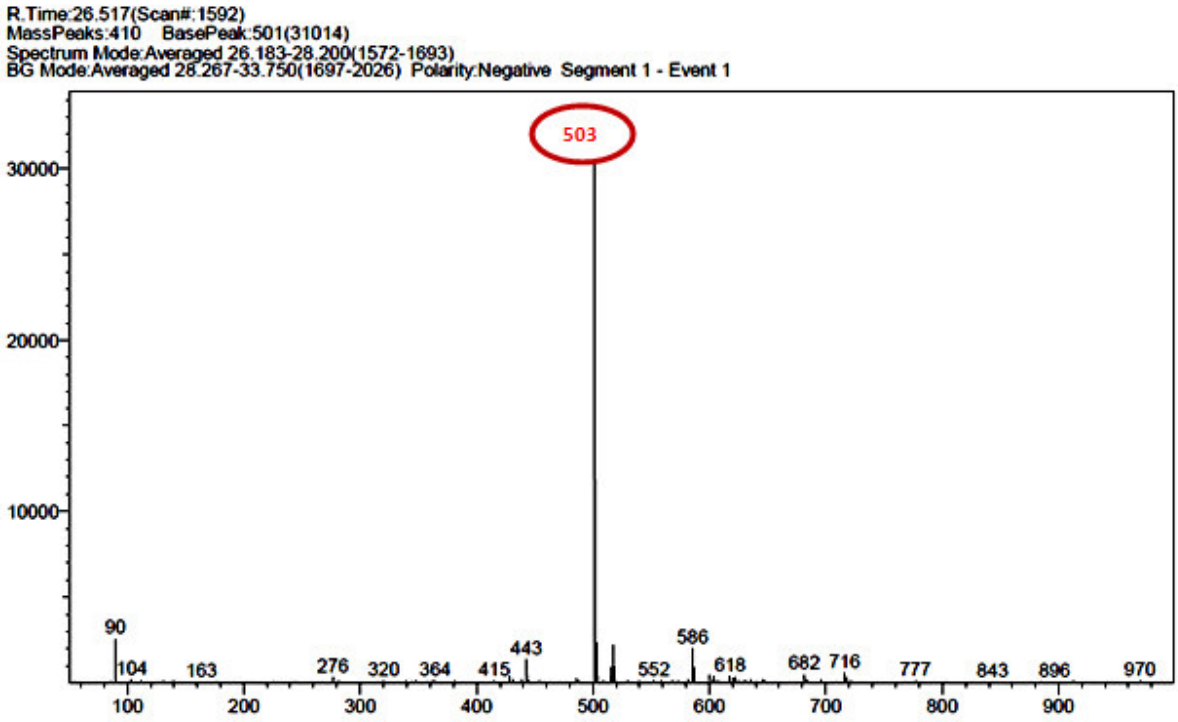


Figure 7
Mass spectrum of methanol extract of leaves of H. hookerianum for Hyperforin (535) –negative ion mode

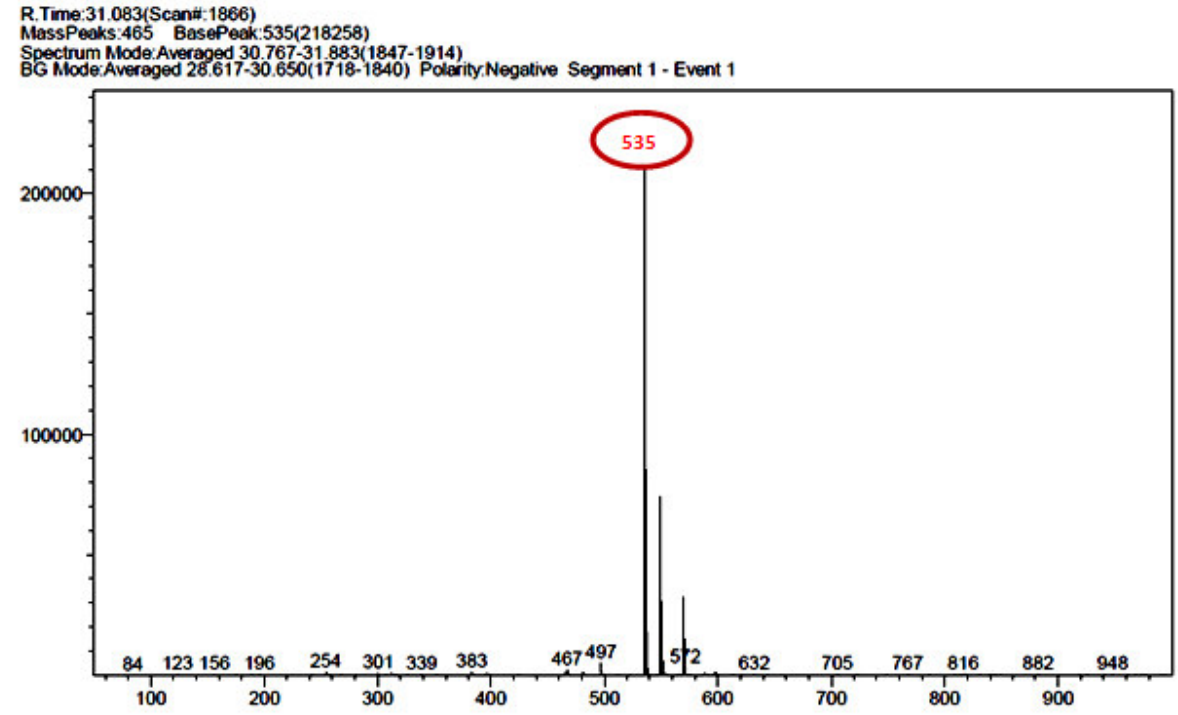


Figure 5
LC-Chromatogram of methanol extract of leaves of H. mysorense

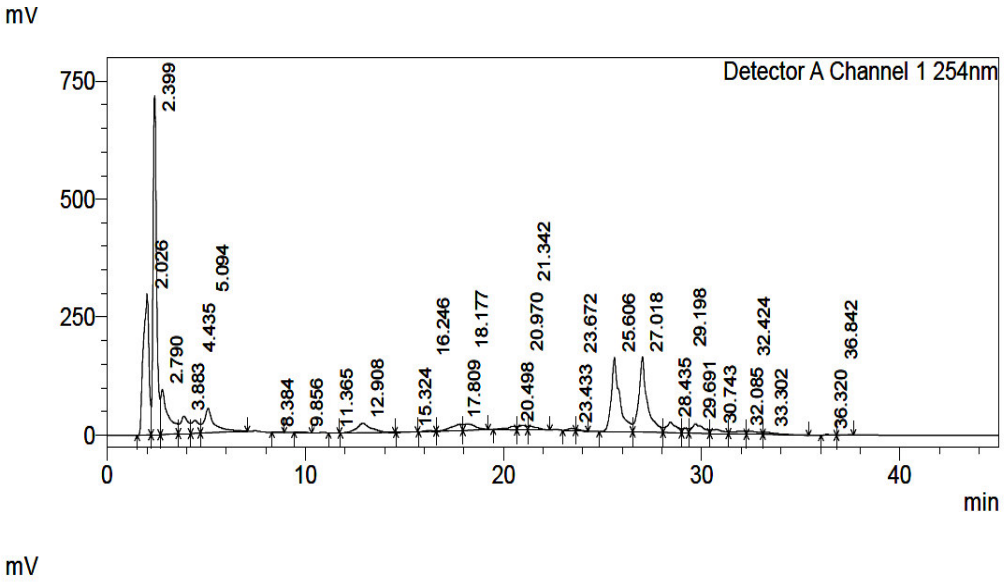


Figure 9
Mass spectrum of methanol extract of leaves of H. mysorense

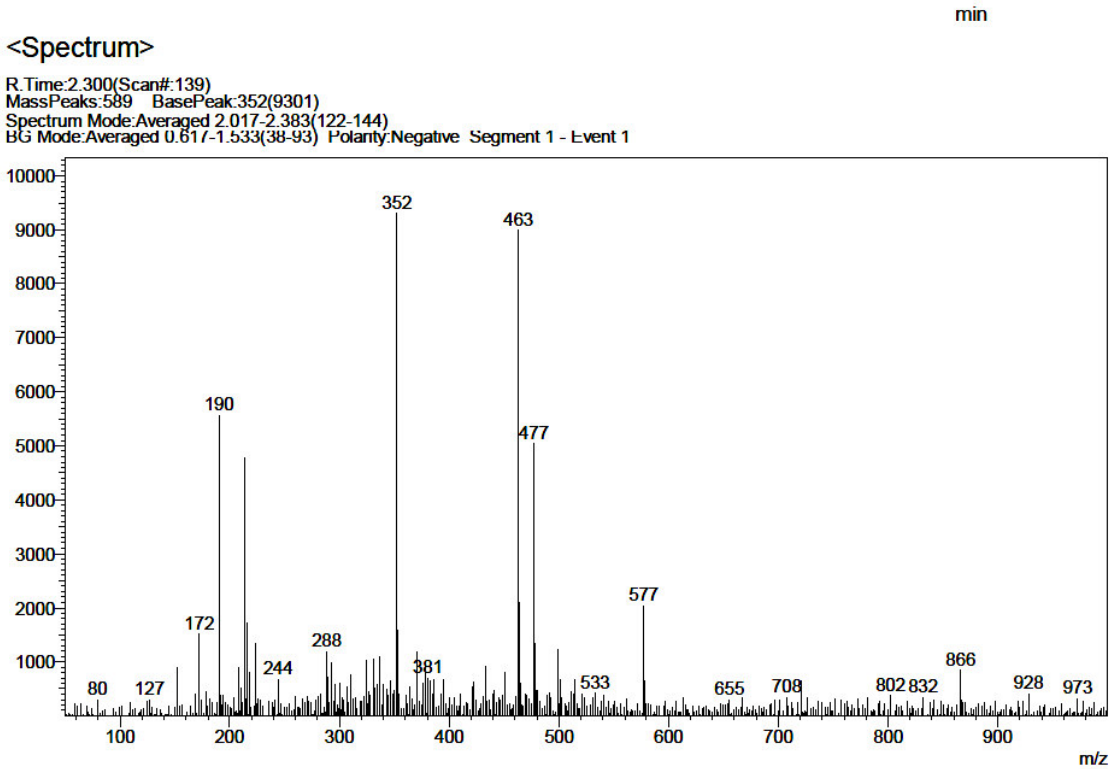


Figure 8
LC-chromatogram of methanol extract of leaves of H. wightianum

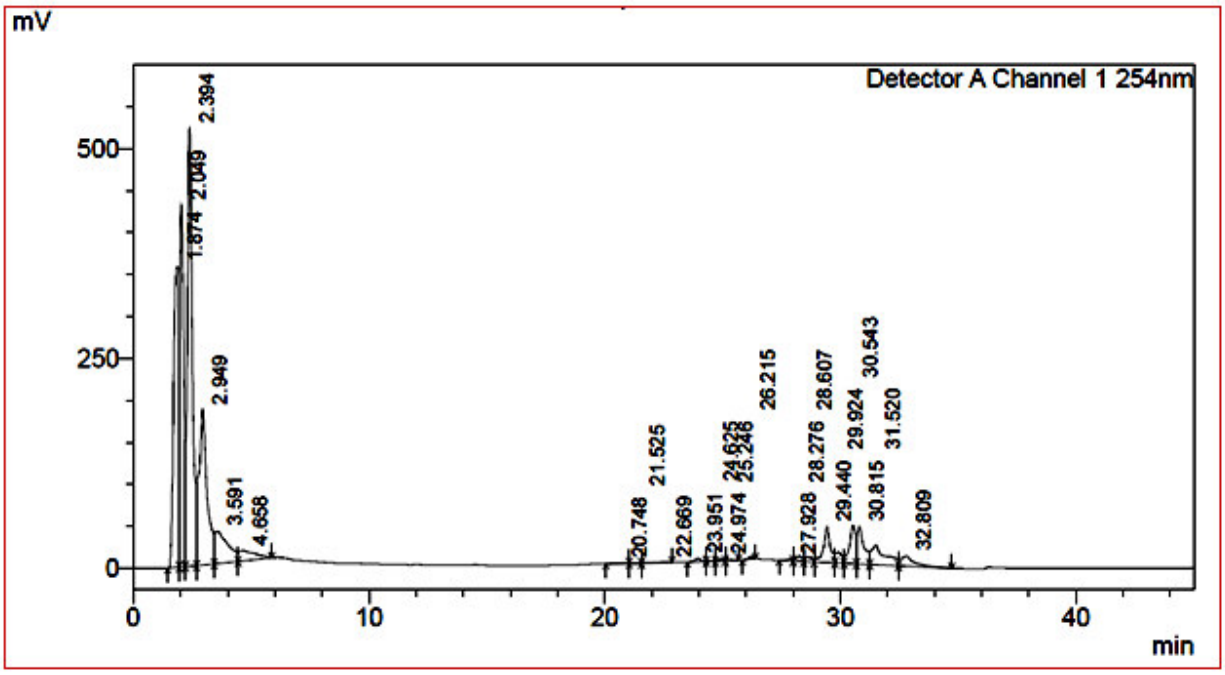
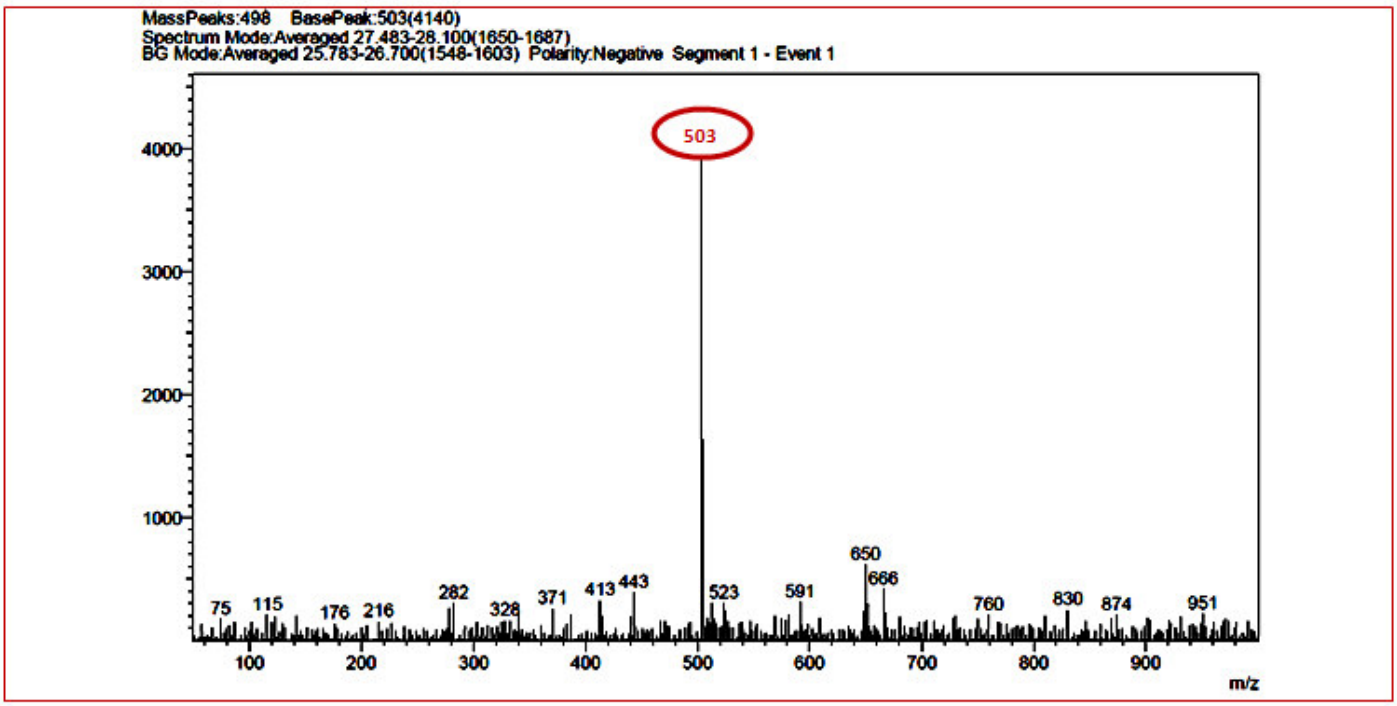


Figure 9
Mass spectrum of methanol extract of leaves of H. wightianum
For Hypericin (503) –negative ion mode



DISCUSSION

The methanolic extract of three Hypericum species (*H.hookerianum*, *H.mysorense* and *H.wightianum*) was analyzed for the presence of hypericin and hyperforin using LC-MS and the total run time was 35 minutes. The molecular ions $[M-H]^-$ of these two compounds were monitored in the negative ion mode: Hypericin *m*/*z*– 503, and Hyperforin *m*/*z* – 535. The (-)-ESI mass spectra of hypericin (*t*R = 26.51 min and 27.63) from *H. hookerianum* and *H. wightianum* showed a parent molecular ion at *m*/*z* 503 [MH]–(Fig.6 and Fig.9).The UV spectrum of hypericin showed the absorption maxima at 254 nm. The identity of this compound was verified by comparison of its ESI mass spectrum, UV spectrum,and the retention time with an authentic standard of hypericin, where complete matching was observed. In *H. hookerianum*, the (-)-ESI mass spectra of the peaks at *t*R = 31.08 min showed parent molecular ions of 535for [M-H]– (Fig.7), in the negative ionization mode. The UV spectrum showed an absorption maximum at 254 nm, characteristic of phloroglucinols. These mass spectral data suggested that this compound was hyperforin (Tolonen *et al*., 2003). The result showed that *Hypericum hookerianum* contains both hypericin and hyperforin whereas *Hypericum wightianum* contain only hypericin and it is important to note that both hypericin and hyperforin were not detected in *Hypericum mysorense*(Table 1).

CONCLUSION

The data presented here in this study have enabled to draw the following conclusions: Nowadays, the interest in study of natural products is growing rapidly, especially as a part of drug discovery programs. The present study revealed that *H.hookerianum* is fairly a rich source of Hypericin and Hyperforin while *H.wightianum* is for Hypericin reported herein for the first time. There is a need to quantify these bioactive compounds further so that *H.hookerianum* and *H.wightianum* as a valuable source of these compounds can be assessed.

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