



RESEARCH ARTICLE

GC-MS analysis of *Hemidesmus indicus* (L.) R. Br. roots and quantification of 2-hydroxy-4-methoxy benzaldehyde through RP-HPLC

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ABSTRACT

Hemidesmus indicus is a commercially important medicinal crop that catches the attention of both pharmaceuticals as well as the food and flavoring industry. The current investigation intends to reveal the possibilities associated with the morphologically distinct *H. indicus* plants. The GC-MS analysis carried out with broad-leafed *H. indicus* root extracts resulted in the identification of 40 various compounds. Among all the compounds 2-hydroxy-4-methoxy benzaldehyde (MBALD) was found to be the major chemical entity. Further, RP-HPLC analysis was carried out to decipher the amount of MBALD present in roots of two morphologically distinct *H. indicus* plants were found to be 0.1827 and 0.1537 mg/gram of tissue of slender and long leafed and broad-leafed plants, respectively.

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INTRODUCTION

Hemidesmus indicus (L.) R. Br. belongs to the *Apocynaceae* family and is a perennial twining semi-erect woody shrub, has been represented by 264 vernacular names across the world and is popularly known as Indian sarsaparilla (Kher *et al.*, 2020). Indian sarsaparilla differs from true sarsaparilla (*Smilax febrifuga*) and exists as *Hemidesmus indicus* var. *indicus* and *Hemidesmus indicus* var. *pubescens*. This medicinal plant is majorly found in South Asian countries, specifically India, Pakistan, Sri Lanka, Bangladesh, Maldives (George *et al.*, 2008).

The ethnobotanical knowledge about *H. indicus* has been serving people for a long ago, and its mention in Ayurveda under the name of Anantmoool can also be seen among the Rasayana plants (Ved and Goraya 2007). Due to the presence of an aromatic compound, 2-hydroxy-4-methoxy benzaldehyde (MBALD), the roots of this plant have been attributed with a strong smell, which makes it an ingredient in sherbet or flavored sweet drinks and bakery products (Patnaik and Debata, 1996). The roots of these plants make it more commercially valuable and medicinally useful as they possess highly desirable therapeutic properties (Kawlni *et al.* 2017). The root decoction of this plant have shown anti-cancerous activity against colorectal cancer (Turrini *et al.*, 2018) and breast cancer (Suryavanshi *et al.*, 2019).

This study briefly describes the extraction methodologies used in the preparation of solvent root extracts, reviews the utility of Gas chromatography-mass spectrometry for the analysis of compounds present in the *H. indicus* and the procedure for quantification of the commercially valuable compound, MBALD.

MATERIAL AND METHODS

Gas chromatography-Mass spectroscopy analysis

Collection of plant sample: Broad-leafed *H. indicus* plants were collected from the foot-hills of Rajapalayam (9°43'13.9"N, 77°44'10.3"E) and were maintained at the greenhouse, TNAU, Coimbatore. The plants were kept at acclimatizing conditions for normal growth. Two-month-old transferred plants were uprooted, roots were separated and washed under running tap water, and kept for drying until further use.

Preparation of solvent root extract: For the preparation of solvent extract from the roots of *H. indicus*, modified method of Alade and Irobi (1993) was used. The dried roots were ground to a fine powder. Ten gram of root powder was soaked in methanol (100%) for overnight and was filtered through Whatman filter paper No. 1. The filtrate was concentrated using a Soxhlet extractor and was used for GC-MS analysis.

GC-MS condition: One ml of methanolic root

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extract was injected for GC-MS analysis using Perkin Elmer GC-MS Clarus® SQ 8 equipped with DB-5MS (Agilent, USA) capillary standard non-polar column (0.25mm OD x 0.25 µm ID x 30 m length). The instrument was set to an initial temperature of 40 °C and the injection port temperature was ensured at 220 °C, interface temperature set 250 °C, source kept at 220 °C, oven temperature-programmed as 75 °C for 2 min, 150 °C @ 10 °C/min, up to 250 °C at 10 °C per min. The GC conditions were: 1:12 split, helium carrier at 20 psi. The MS conditions were: positive ion mode, electron impact spectra at 70 eV. The mass spectral scan range was set at 50 to 600 Da.

Reverse-phase high-performance liquid chromatography

Explant material: In the current investigation, 2 morphologically different *H. indicus* plants slender and long leafed (SLL) and broad-leafed (BL) (figure 1 and 2, respectively) were taken for the quantification of 2-hydroxy-4-methoxy benzaldehyde from the roots. The dried roots of two morphologically different plants were obtained from Rajapalayam (9°43'13.9"N, 77°44'10.3"E). The roots were finely powdered and kept separately in airtight containers.

Reagents and Chemicals: HPLC grade Methanol and Trifluoroacetic acid (TFA) were used during the study and other reagents were prepared using Milli Q water.

Sample extract preparation: For the preparation of methanolic root extract protocol by Sircar, Dey, and Mitra (2007) was followed. One gram of powder was extracted with 20 mL of aqueous methanol (50:50, v/v). The extract was incubated for 2 days with continuous shaking and was subjected to centrifugation at 10,000 rpm for 10 minutes. The supernatant taken was first filtered with Whatman filter paper No. 1 and further filtered through a 0.22 µm filter. 20µl of the sample was injected into the HPLC system.

Solvent preparation: HPLC grade (98%) 2-hydroxy-4-methoxy benzaldehyde standard was used for the study. 1 mg of standard MBALD purchased from Sigma-Aldrich (Catalogue No.160695, Molecular Weight 152.15 and PubChem Substance ID 24849887) was added 1mL of aqueous methanol (50:50, v/v) to prepare a 1000 ppm standard stock solution. The working standard solution was prepared with a concentration of 10 ppm by dilution of standard stock solution with aqueous methanol (50:50, v/v). Isocratic solvent mixture (mobile phase) was prepared by adding 1 mM aqueous TFA and methanol in a 70:30 ratio. All stock solutions were stored at 4°C.

HPLC Condition: Shimadzu HPLC system

equipped UV-Vis detector was used for RP-HPLC analysis. The separation of compounds was achieved by the C18 reversed-phase column (INNO column, 5µm, 120Å, 4.6×250mm). Shimadzu CLASS-VPTM software was used for data acquisition, processing, and reporting on the Windows XP platform. The wavelength was set to 280 nm for monitoring chromatograms. The mobile phase was maintained at a flow rate of 1 ml min⁻¹. The sample was identified based on the comparison of retention time with those of the standards with keeping the same conditions.

Quantification of MBALD was done by using the retention time and peak area obtained from the chromatogram. The formula for calculating the percentage of 2-hydroxy-4-methoxy benzaldehyde in the dried root extract is as follows:

$$\frac{\text{Peak area of Sample}}{\text{Peak area of Standard}} \times \frac{\text{Concentration of Standard}}{\text{Concentration of Sample}} \times \text{Purity of Standard (\%)}$$

RESULTS AND DISCUSSION

Phytochemical analysis through GC-MS

The gas chromatogram obtained after GC-MS analysis defined the relative concentrations of different compounds being eluted as the function of retention time. The peak heights indicated the relative concentrations of various compounds present in the methanolic extract, whereas the mass spectrometer analysed the compounds and identifies the nature and structure of the compounds based on the time of their elution (Krishnamoorthy and Subramaniam, 2014).

The methanolic root extract of broad-leafed *H. indicus* had shown the presence of a variety of compounds as compared to that of published in earlier literature of *H. indicus*. A total of 40 compounds were identified (figure 3) in the GC-MS analysis and the identification of the compounds in the extract were based on the peak area which represented the percentage of that particular compound which was deciphered from the chromatogram and molecular weight of the compound. The list of all the compounds is given in table 1. Out of all the compounds, it was found that the major volatile compound, 2-hydroxy-4-methoxy benzaldehyde (peak area 11.071%) was present in abundance forming the major entity. Due to the presence of MBALD in large proportion, it could be utilized in various sectors like pharmaceuticals, food industry, anti-microbial formulations, etc.

Anti-bacterial and anti-fungal activity of MBALD was deduced by Mehmood, Dixit, and Singh (2016) who reported the hexane extract prepared from the roots *H. indicus* to be effective with the maximum zone of inhibition (MIC) of 22 mm against *Staphylococcus aureus* and the MIC for *Candida*

Table 1. Chemical constituents present in the methanolic extract of broad-leaved *Hemidesmus indicus*

Peak	Retention time	Area	Area %	Compound
1	3.213	6,992,248.5	0.864	Decane, 4-methyl
2	3.669	3,183,574.0	0.393	Undecane
3	3.809	3,117,720.5	0.385	Melezitose
4	4.139	3,276,007.5	0.405	Undecane
5	5.764	4,431,423.5	0.547	Dodecane
6	6.435	3,287,432.5	0.406	Benzene, 1,3-bis(1,1-dimethylethyl)-
7	7.555	89,619,072.0	11.071	Benzaldehyde, 2-hydroxy-4-methoxy-
8	7.885	2,587,966.5	0.320	Arsine, oxophenyl-
9	8.486	6,022,166.0	0.744	Tetradecane
10	9.201	25,824,714.0	3.190	Guanosine
11	9.456	9,164,683.0	1.132	4-Methyl(trimethylene)silyloxyoctane
12	9.566	6,292,480.5	0.777	Tridecane, 2-methyl-
13	9.671	2,534,709.0	0.313	Dodecane, 5,8-diethyl-
14	9.746	2,271,470.5	0.281	Undecane, 6-ethyl-
15	9.821	5,062,362.5	0.625	2,4-Di-tert-butylphenol
16	10.026	3,298,825.8	0.408	Methyl-4-O-acetyl-2,3,6-tri-O-ethyl-à-d-galactopyranoside
17	10.191	2,665,982.8	0.319	Dodecane, 2,6,10-trimethyl-
18	10.366	2,400,660.5	0.297	Benzaldehyde, 3,4-dimethoxy-, methylmonoacetal
19	10.676	4,810,796.5	0.594	2H-1-Benzopyran-3,4-diol, 2-(3,4-dimethoxyphenyl) 3,4-dihydro-6-methyl-, (2à,3à,4à)-
20	11.397	2,413,610.8	0.298	Hexadecane
21	18.680	41,335,456.0	5.106	n-Hexadecanoic acid
22	18.875	3,974,250.0	0.491	Octadecanoic acid, 4-hydroxybutyl ester
23	21.776	35,683,764.0	4.408	9,12-Octadecadienoic acid (Z, Z)-
24	21.941	222,793,568.0	27.522	9-Octadecenoic acid, (E)-
25	22.351	83,508,704.0	10.316	Octadecanoic acid
26	22.891	27,773,410.0	3.431	trans-13-Octadecenoic acid
27	23.401	19,335,120.0	2.388	9,12-Octadecadienoic acid (Z, Z)-
28	23.806	7,194,610.5	0.889	Chromone, 5-hydroxy-6,7,8-trimethoxy-2,3-dimethyl-
29	24.047	2,900,577.2	0.358	17-Pentatriacontene
30	24.162	4,038,792.5	0.499	Cyclopropanoic acid, 2-[(2-pentylcyclopropyl) methyl]-, methyl-ester
31	24.292	2,521,489.8	0.311	Dasycarpidan-1-methanol, acetate (ester)
32	24.532	2,842,063.0	0.351	2,3-Dihydroxypropyl elaidate
33	24.692	2,906,400.5	0.359	Octadecane, 3-ethyl-5-(2-ethylbutyl)-
34	24.832	3,326,499.8	0.411	10-Acetoxy-2-hydroxy-1,2,6a,6b,9,9,12a-heptamethyl- 1,3,4,5,6,6a,6b,7,8,8a,9,10,11,12,12a,12b,13,1 4b-octadecahydro-2H-picene-4carboxylic acid, methyl ester
35	25.137	6,330,487.0	0.782	Oxiraneoctanoic acid, 3-octyl-, cis-
36	25.277	2,491,672.5	0.308	4-Piperidineacetic acid, 1-acetyl-5-ethyl-2-[3-(2- hydroxyethyl)-1Hindol-2-yl]-à-methyl-, methyl ester
37	25.782	3,111,850.0	0.384	Octadecane, 3-ethyl-5-(2-ethylbutyl)-
38	27.583	8,563,851.0	1.058	Squalene
39	28.288	3,233,305.0	0.399	Diisooctyl phthalate
40	28.373	2,643,616.8	0.327	Oleyl oleate

albicans to be 18 mm with zero cytotoxicity. MBALD is a non-toxic aromatic benzaldehyde that confers antimicrobial, anti-aflatoxigenic potency, antioxidant properties (Harohally et al., 2017). It has good water

solubility and is a potent tyrosinase inhibitor (Ley and Bertram 2001). The Hepatoma HepG2 cells on incubation with root decoction of *H. indicus* in the dose range of 0-50 mg/ml caused significant

inhibition on the growth of the Hep2G cell line, making it amenable for its further use in cancer studies (Thabrew *et al.*, 2005).



Figure 1. Slender and long leafed plant of *H. indicus*

Quantification of MBALD through RP-HPLC

The percentage of MBALD present in morphologically distinct 2 plants of *H. indicus* (SLL and BL) was quantified through HPLC using methanolic root extract.



Figure 2. Broad-leaved plant of *H. indicus*

In the analysis, the retention time for the standard 2-hydroxy-4-methoxy benzaldehyde at 10 ppm was recorded as 54.400 min. at 280 nm. The retention time for the compound MBALD present in

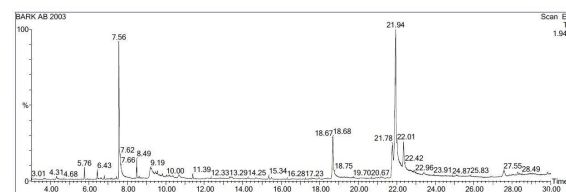
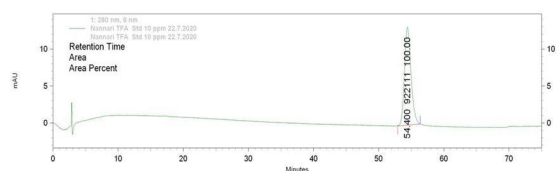


Figure 3. Chromatogram (GC/MS) of the methanolic extract of *Hemidesmus indicus*

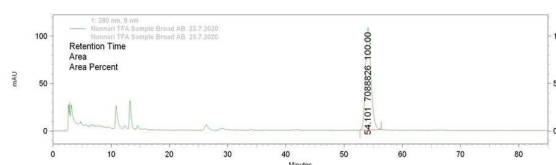
the root extract of SLL in the HPLC chromatogram was observed to be 54.336 and 54.101 in the case of BL. The identity of the sample was confirmed by comparing the chromatograms of both standard



I: 280 nm, 8 nm					
PK #	Retention Time	Area	Area %	Height	Height %
1	54.400	922111	100.000	13228	100.000
Totals		922111	100.000	13228	100.000

Figure 4. Chromatogram (HPLC) of standard (10ppm) 2-hydroxy-4-methoxy benzaldehyde

and sample. The chromatograms of standard, BL, and, SLL are shown in figure (Figures 4, 5, and 6, respectively). Quantification of MBALD was done by using the formula mentioned in materials and methods. It was found that the methanolic root extract of slender and long leafed (SLL) and broad-leaved (BL) *Hemidesmus indicus* plants contains

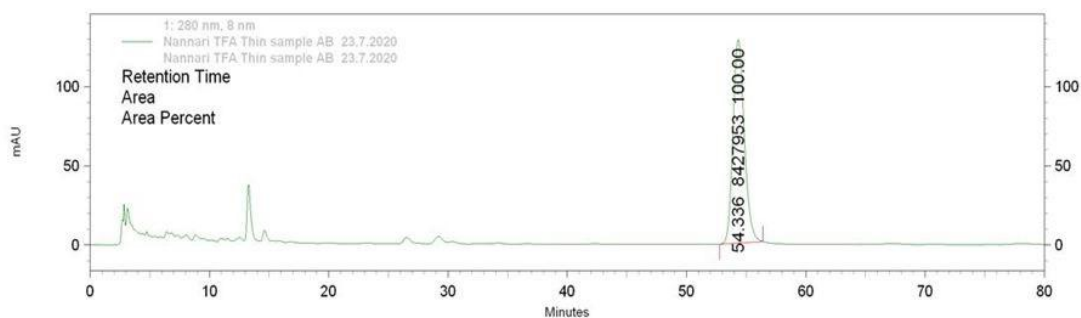


I: 280 nm, 8 nm					
PK #	Retention Time	Area	Area %	Height	Height %
1	54.101	7088826	100.000	107693	100.000
Totals		7088826	100.000	107693	100.000

Figure 5. Chromatogram (HPLC) of broad-leaved sample of *Hemidesmus indicus*

0.1827 and 0.1537 mg of 2-hydroxy-4-methoxy benzaldehyde per gram of tissue.

As the compound 2-hydroxy-4-methoxy benzaldehyde is an isomer of vanillin and is an aromatic compound, the demand for its supply in the food and flavoring industry is high (Rathi *et al.*, 2017). In an earlier report, the quantity of MBALD



1: 280 nm, 8 nm					
PK #	Retention Time	Area	Area %	Height	Height %
1	54.336	8427953	100.000	128347	100.000
Totals		8427953	100.000	128347	100.000

Figure 6. Chromatogram (HPLC) of slender and long leafed sample of *Hemidesmus indicus*

was found to be 0.2638 mg /gram of tissue (Prathibha Devi *et al.*, 2016), which seems more as compared to the values obtained in the current investigation. Methanolic root extracts of seven ecotypes showed variation in HPLC analysis and 2-hydroxy 4-methoxy-benzaldehyde concentration recorded was higher in ecotype 6 and lesser in ecotype 3 (Rathi *et al.*, 2017). The variations in the accumulation of a particular compound in the specific part depend on various reasons such as genotype, plant physiology, climate, environmental conditions, and pathogens, and in some cases, the secondary metabolites are only produced during certain developmental stages (Gonçalves and Romano, 2018). Perhaps, comparatively, the SLL type *H. indicus* was found more promising for extracting MBALD as a source for commercial production.

CONCLUSION

From the current investigation, it could be concluded that *Hemidesmus indicus* contains pharmaceutically important and industrially desirable compounds, which makes it stand in the frontiers of medicinally important plants. 2-hydroxy-4-Methoxybenzaldehyde is an astounding food flavoring metabolite widely used as flavors for food preparation, besides known to inhibit the activity of the enzyme acetyl choline esterase, hence can be used for curing neurodegenerative disorders like Alzheimer's disease. The exact role of MBALD in cancer studies needs to be determined to reveal its effects on the mechanism of growth inhibition of cancer cells, which might be used as natural and low-cost drugs to fight cancer with minimal or no side effects. Further, other compounds present in the roots of *H. indicus* serve wide spectra of utilities, which need to be highlighted in future

studies. Moreover, to meet the growing demand of MBALD, more studies on the secondary metabolite production is need of an hour.

Ethics statement

No specific permits were required for the described studies because no human or animal subjects were involved in this research.

Originality and plagiarism

The submitted article is original and has not been submitted to any other journal.

Consent for publication

All the authors agreed to publish the content.

Competing interests

There was no conflict of interest in the publication of this content.

Data availability

All the data of this manuscript are included in the document. No separate external data source is required. If anything is required from the document, certainly, this will be extended by communicating with the corresponding author through corresponding official mail.

Author contributions

Research grant - DBT; Idea conceptualization - RG; Experiments - BAA; Guidance - RG, SV, JS; Writing-original draft - BAA; Writing- reviewing & editing- BAA, RG

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