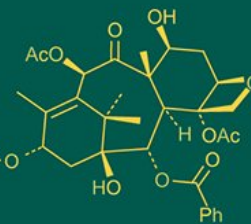
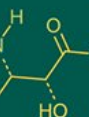
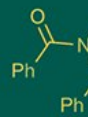


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Urati Mahesh
Ph.D. Scholar, College of
Horticulture,
Anantharajupeta, Dr. YSR
Horticultural University,
Andhra Pradesh, India

KM Yuvaraj
Principal Scientist and Head,
Horticulture Research Station,
Anantharajupeta, Dr. YSR
Horticultural University,
Andhra Pradesh, India

D Divyabharathi
Ph.D. Scholar, College of
Horticulture,
Anantharajupeta, Dr. YSR
Horticultural University,
Andhra Pradesh, India

K Swarajya Lakshmi
The Principal, Horticulture
Polytechnic, Kalikiri, Dr. YSR
Horticultural University,
Andhra Pradesh, India

M Mayilvaganan
Principal Scientist ICAR-
National Research Centre for
Banana, Tiruchirappalli, Tamil
Nadu, India

Corresponding Author:
Urati Mahesh
Ph.D. Scholar, College of
Horticulture,
Anantharajupeta, Dr. YSR
Horticultural University,
Andhra Pradesh, India

Characterization of 2-Hydroxy-4-methoxybenzaldehyde content in tuberous roots of *Decalepis hamiltonii* Wight & Arn. Based on root age and size

Urati Mahesh, KM Yuvaraj, D Divyabharathi, K Swarajya Lakshmi and M Mayilvaganan

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Abstract

Decalepis hamiltonii Wight & Arn., commonly known as swallow root, is an aromatic medicinal plant valued for its characteristic flavour and bioactive constituents. The tuberous roots are particularly rich in 2-hydroxy-4-methoxybenzaldehyde (2H4MB), a characteristic aromatic flavour compound and structural isomer of vanillin. The concentration of 2H4MB may vary with the developmental stage and physical characteristics of tuberous roots. Therefore, the present investigation was undertaken to characterize the 2H4MB content in tuberous roots of *D. hamiltonii* based on root age and size. Four categories of tuberous roots were evaluated, viz., T₁: three-year-old large roots (>3 cm diameter), T₂: three-year-old small roots (<3 cm diameter), T₃: two-year-old large roots (>3 cm diameter) and T₄: two-year-old small roots (<3 cm diameter). The compound was extracted using methanol and quantified by high-performance liquid chromatography (HPLC). Significant variation in 2H4MB content was observed among the root categories. The highest 2H4MB content was recorded in T₁ (4.40 mg g⁻¹), followed by T₂ (3.43 mg g⁻¹), T₃ (2.43 mg g⁻¹) and T₄ (2.24 mg g⁻¹). The difference among treatments was significant at the 5 per cent level, with a critical difference of 0.16 mg g⁻¹. Three-year-old roots recorded higher 2H4MB content than two-year-old roots, while large roots recorded higher values than small roots within the respective age groups. The findings characterize the variation of this important flavour compound according to root age and size and indicate that three-year-old large tuberous roots are a promising raw material category for obtaining higher 2H4MB content. The study provides useful information for raw material selection, harvest standardization and quality-oriented utilization of *D. hamiltonii* tuberous roots.

Keywords: *Decalepis hamiltonii*, swallow root, 2-hydroxy-4-methoxybenzaldehyde, 2H4MB, HPLC, tuberous root, root age, root size, flavour compound

Introduction

Decalepis hamiltonii Wight & Arn., commonly known as swallow root, is an important aromatic and medicinal plant belonging to the family Apocynaceae. The species is endemic to the southern peninsular region of India and is valued primarily for its tuberous roots, which possess a characteristic pleasant aroma and flavour. The roots have traditionally been used in the preparation of beverages, pickles and other food products and have gained considerable attention because of their aromatic and bioactive constituents (Nagarajan and Rao, 2003; Sachan *et al.*, 2006)^[1, 10].

The characteristic aroma of *D. hamiltonii* roots is mainly associated with 2-hydroxy-4-methoxybenzaldehyde (2H4MB), an aromatic aldehyde and structural isomer of vanillin. The compound is considered one of the important flavour constituents of swallow root and contributes significantly to its characteristic sensory properties. Nagarajan and Rao (2003)^[1] reported the occurrence of 2H4MB in the roots of *D. hamiltonii* and *Hemidesmus indicus* and developed a chromatographic method for its determination. The presence of 2H4MB along with other aromatic compounds such as vanillin, p-anisaldehyde and salicylaldehyde has also been reported in methanolic extracts of *D. hamiltonii* roots (Sachan *et al.*, 2006)^[10].

The importance of 2H4MB has generated considerable interest in its quantification and biosynthesis.

Giridhar *et al.* (2005) ^[5] demonstrated the production of 2H4MB in root cultures of *D. hamiltonii*, confirming that the compound is a root-specific flavour constituent. Subsequent studies indicated that the biosynthesis of 2H4MB is associated with the phenylpropanoid pathway. Kamireddy *et al.* (2017) ^[6] reported the involvement of ferulic acid and 4-coumarate-CoA ligase-mediated metabolism in the biosynthesis of 2H4MB in *D. hamiltonii*. These findings indicate that the accumulation of 2H4MB is closely associated with the biochemical development of the tuberous root.

The concentration of secondary metabolites in plant organs can vary considerably with developmental stage. In *D. hamiltonii*, changes in 2H4MB concentration have been observed during tuberous root development. Kundu *et al.* reported differential expression of genes associated with the phenylpropanoid pathway during flavour development in the tuberous roots, suggesting that developmental regulation of metabolic pathways contributes to changes in flavour compound accumulation. Similarly, a proteomic investigation of tuberous root development demonstrated differences in the abundance of proteins associated with flavour development and reported changes in 2H4MB concentration between developmental stages (Kundu *et al.*). Apart from root age, physical characteristics of the tuberous roots may also be useful in identifying raw material with desirable levels of flavour compounds. Root diameter is a readily observable characteristic that can be used during harvesting and post-harvest handling. However, information characterizing 2H4MB concentration simultaneously in relation to root age and size is comparatively limited. Identification of a root category with higher 2H4MB concentration would therefore be useful for selecting quality raw material for flavour-oriented utilization and value addition.

Although previous investigations have established the occurrence, quantification and biosynthetic pathway of 2H4MB in *D. hamiltonii*, systematic characterization of the compound based on different root ages and size categories is still required. Such information would help in identifying suitable harvest material with higher concentration of this characteristic flavour compound.

Therefore, the present investigation was undertaken to characterize the 2-hydroxy-4-methoxybenzaldehyde content in tuberous roots of *Decalepis hamiltonii* Wight & Arn. based on root age and size using high-performance liquid chromatography (HPLC) and to identify the root category possessing the highest concentration of 2H4MB.

2. Materials and Methods

2.1 Experimental material

Fresh tuberous roots of *Decalepis hamiltonii* Wight & Arn. representing different ages and sizes were used for the estimation of 2H4MB. Based on root age and diameter, four root categories were considered.

Treatment	Root age	Root size
T ₁	Three-year-old	Large (>3 cm diameter)
T ₂	Three-year-old	Small (<3 cm diameter)
T ₃	Two-year-old	Large (>3 cm diameter)
T ₄	Two-year-old	Small (<3 cm diameter)

The classification was based on the root age and diameter criteria used in the experiment.

2.2 Sample preparation

Fresh tuberous roots were cleaned thoroughly to remove adhering soil and other foreign material. The cleaned roots were dried at 40 °C until constant weight was attained. The dried roots were ground into a fine powder and used for extraction and HPLC analysis.

2.3 Extraction of 2H4MB

The extraction procedure was carried out based on the protocol adopted from Sircar *et al.* (2007) ^[9]. Approximately 0.5 g of the dried and powdered root sample was accurately weighed and extracted with 10 mL methanol. The sample was subjected to sonication for 30 minutes to facilitate extraction of the target compound.

The extract was filtered through Whatman No. 1 filter paper to remove suspended particles. The filtrate was subsequently passed through a 0.45 µm syringe filter before HPLC injection.

Sircar *et al.* (2007) ^[9] developed and validated a reverse-phase HPLC method for the determination of 2H4MB and 2-hydroxy-4-methoxybenzoic acid in root organs of *Hemidesmus indicus*, providing an analytical basis for chromatographic quantification of 2H4MB in aromatic roots.

2.4 HPLC conditions

The quantitative estimation of 2H4MB was carried out using an HPLC system equipped with a UV-visible detector. A C18 reverse-phase column (250 mm × 4.6 mm, 5 µm particle size) was used for separation.

The mobile phase consisted of methanol and water in a ratio of 70:30 (v/v), delivered at a flow rate of 1.0 mL min⁻¹. The injection volume was 20 µL and the column temperature was maintained at 30 °C. Detection was carried out at 280 nm according to the analytical protocol used in the study.

Important: The uploaded instrument chromatogram records a separate 2H4MB sample set and shows a detector channel labelled 320 nm, with a 10 µL injection volume. Therefore, the wavelength and injection volume should be verified from your original validated instrument method before the manuscript is submitted. The manuscript should report the actual conditions used for quantification.

2.5 Preparation of standard and calibration curve

A standard stock solution of 2H4MB was prepared by dissolving a known quantity of authentic 2H4MB standard in methanol. Serial dilutions were prepared to obtain standard solutions of different concentrations.

The standard solutions were injected into the HPLC system and their corresponding peak areas were recorded. A calibration curve was prepared by plotting peak area against the concentration of 2H4MB standard. The concentration of 2H4MB in the samples was calculated using the resulting calibration equation.

2.6 Quantification of 2H4MB

The sample chromatograms were compared with those obtained for the authentic 2H4MB standard. The concentration of the compound was calculated from the calibration curve and expressed as mg g⁻¹ dry weight of tuberous root.

2.7 Statistical analysis

The data obtained for 2H4MB content were subjected to statistical analysis using the appropriate analysis of variance

procedure. Treatment means were compared at the 5 per cent level of significance. The critical difference (CD), standard error of the mean [SE (m)] and coefficient of variation (CV) were calculated.

3. Results and Discussion

3.1 Characterization of 2H4MB content according to root age and size

Significant variation was observed in 2H4MB concentration among the four categories of *D. hamiltonii* tuberous roots. The concentration ranged from 2.24 to 4.40 mg g⁻¹ dry weight (Table 1).

Table 1: Characterization of 2-hydroxy-4-methoxybenzaldehyde content in tuberous roots of *Decalepis hamiltonii* based on root age and size

Treatment	Root category	2H4MB (mg g ⁻¹)
T ₁	Three-year-old, large roots (>3 cm)	4.40 ^a
T ₂	Three-year-old, small roots (<3 cm)	3.43 ^b
T ₃	Two-year-old, large roots (>3 cm)	2.43 ^c
T ₄	Two-year-old, small roots (<3 cm)	2.24 ^d
CD at 5%		0.16
SE(m)		0.05
CV (%)		3.91

Means followed by different superscript letters differ significantly at the 5% level.

The highest 2H4MB content was recorded in T₁ (4.40 mg g⁻¹), comprising three-year-old large tuberous roots. This was followed by T₂ (3.43 mg g⁻¹), T₃ (2.43 mg g⁻¹) and T₄ (2.24 mg g⁻¹). All four treatments were significantly different from each other, as indicated by the distinct superscript letters.

The 2H4MB concentration in T₁ was approximately 28.3 per cent higher than T₂, 81.1 per cent higher than T₃ and 96.4 per cent higher than T₄. Thus, the highest concentration was associated with the combination of greater root age and larger root diameter.

3.2 Relationship between root maturity and 2H4MB accumulation

The higher concentration of 2H4MB in three-year-old roots suggests that accumulation of this characteristic flavour compound is associated with tuberous root development and maturity. The higher values observed in three-year-old roots compared with two-year-old roots may reflect progressive biochemical development of the storage organ and increased synthesis or accumulation of secondary metabolites.

The present findings are consistent with previous studies demonstrating developmental variation in 2H4MB in *D. hamiltonii*. A proteomic investigation comparing developmental stages reported 2H4MB concentrations of approximately 160 and 510 µg g⁻¹ at two different stages of tuber development, indicating substantial variation in flavour compound accumulation during development.

The expression of phenylpropanoid pathway genes has also been shown to vary during flavour development in *D. hamiltonii* tuberous roots. Such developmental changes in metabolic pathway activity could contribute to differences in the accumulation of 2H4MB observed between younger and older roots.

The present results therefore provide additional evidence that root maturity is an important consideration when selecting *D. hamiltonii* tubers for their 2H4MB content.

3.3 Relationship between root size and 2H4MB concentration

Root size also showed a consistent association with 2H4MB concentration. Among three-year-old roots, large roots (T₁) recorded 4.40 mg g⁻¹ compared with 3.43 mg g⁻¹ in small roots (T₂). Similarly, among two-year-old roots, large roots (T₃) recorded 2.43 mg g⁻¹ compared with 2.24 mg g⁻¹ in small roots (T₄).

The higher concentration observed in large roots may be associated with their greater degree of tuberous development. Larger roots generally represent more advanced development of the storage organ and may consequently possess greater accumulation of characteristic secondary metabolites.

However, the present experiment was designed to characterize root categories rather than independently quantify the statistical main effects of age and diameter. Therefore, the differences should be interpreted as variation among the four predefined root categories, rather than as definitive independent effects of age and root size.

3.4 Comparison with previous reports

The occurrence of 2H4MB as an important characteristic compound of *D. hamiltonii* roots has been established in earlier investigations. Nagarajan and Rao (2003)^[1] reported 2H4MB as a major component of the volatile oils of *D. hamiltonii* and *Hemidesmus indicus*. They also observed variation in the quantity of the compound among fresh and dried roots from different origins.

The present study differs from those investigations in that the root material was classified specifically according to age and diameter, while 2H4MB was expressed on a dry-weight basis following methanolic extraction and HPLC quantification. Therefore, direct numerical comparison with values reported as a percentage of volatile oil is not appropriate. Nevertheless, both studies demonstrate considerable variation in the concentration of this characteristic aromatic compound.

Giridhar *et al.* (2005)^[5] also demonstrated the ability of *D. hamiltonii* root cultures to produce 2H4MB and reported a maximum flavour compound concentration of approximately 40 µg g⁻¹ dry weight under the conditions tested. The substantially higher values observed in the present mature field-grown roots may reflect differences in plant developmental stage, genetic background, growing conditions, extraction procedure and analytical expression. Further, Kamireddy *et al.* (2017)^[6] demonstrated the involvement of the phenylpropanoid pathway in 2H4MB biosynthesis in *D. hamiltonii*, with evidence suggesting that ferulic acid is channelled through 4-coumarate-CoA ligase towards 2H4MB formation. This provides a biochemical basis for considering developmental differences in metabolic activity as one possible explanation for the variation observed among root categories.

3.5 Significance for raw material characterization

The present characterization demonstrates that the physical characteristics of tuberous roots can be associated with their 2H4MB concentration. Among the evaluated categories, three-year-old large roots recorded the highest value of 4.40 mg g⁻¹, indicating their potential as a preferred raw material category when the objective is to obtain roots with higher 2H4MB concentration.

The finding is particularly useful because root age may not always be readily determined during commercial collection,

whereas root diameter can be assessed more easily. A combination of harvest age and root diameter may therefore provide a practical criterion for quality-oriented selection of *D. hamiltonii* roots.

Since 2H4MB is recognized as a characteristic flavour constituent of *D. hamiltonii*, selection of roots with higher concentrations may be useful for subsequent flavour extraction and value-added product development.

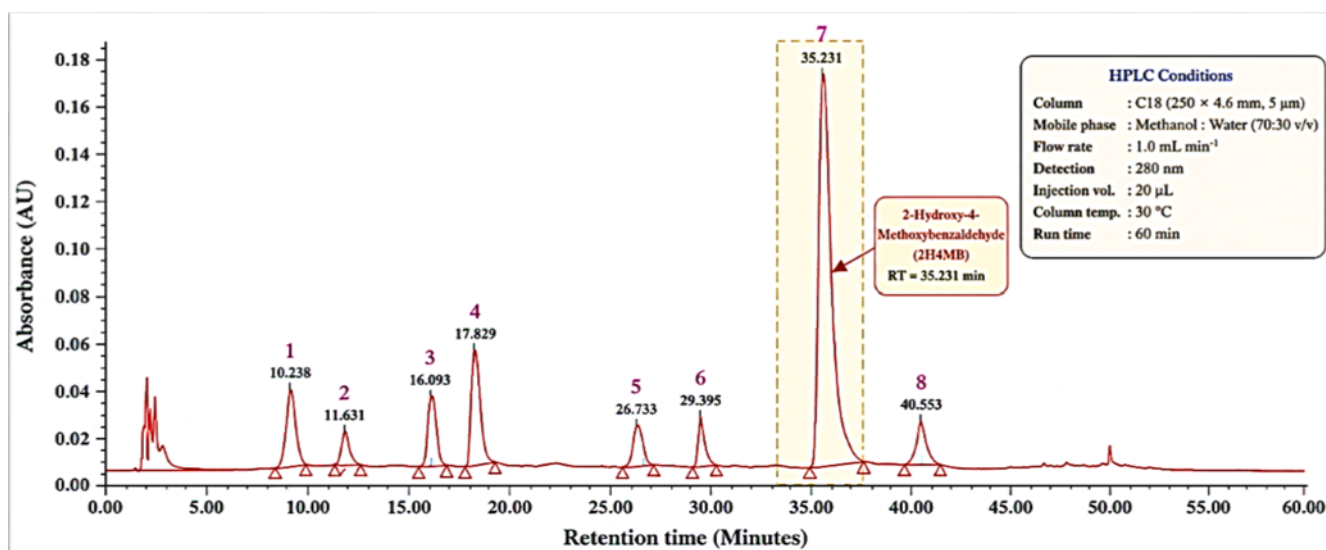


Fig 1: HPLC chromatogram of 2-hydroxy-4-methoxybenzaldehyde (2H4MB) in three-year-old large tuberous roots of *Decalepis hamiltonii* Wight & Arn. (T₁)

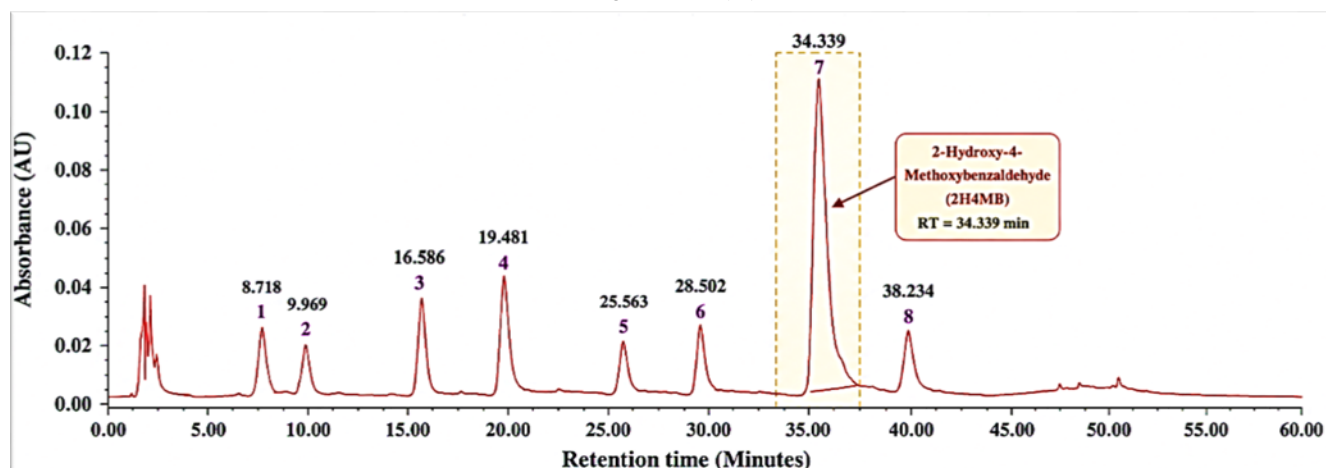


Fig 2: HPLC chromatogram of 2-hydroxy-4-methoxybenzaldehyde (2H4MB) in three-year-old small tuberous roots of *Decalepis hamiltonii* Wight & Arn. (T₂)

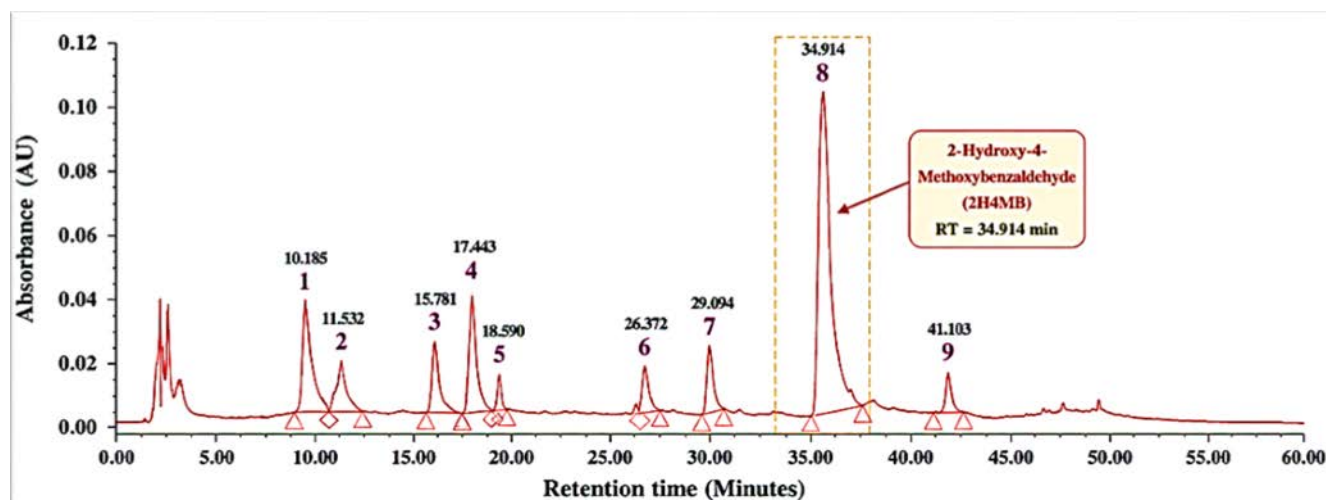


Fig 3: HPLC chromatogram of 2-hydroxy-4-methoxybenzaldehyde (2H4MB) in two-year-old large tuberous roots of *Decalepis hamiltonii* Wight & Arn. (T₃)

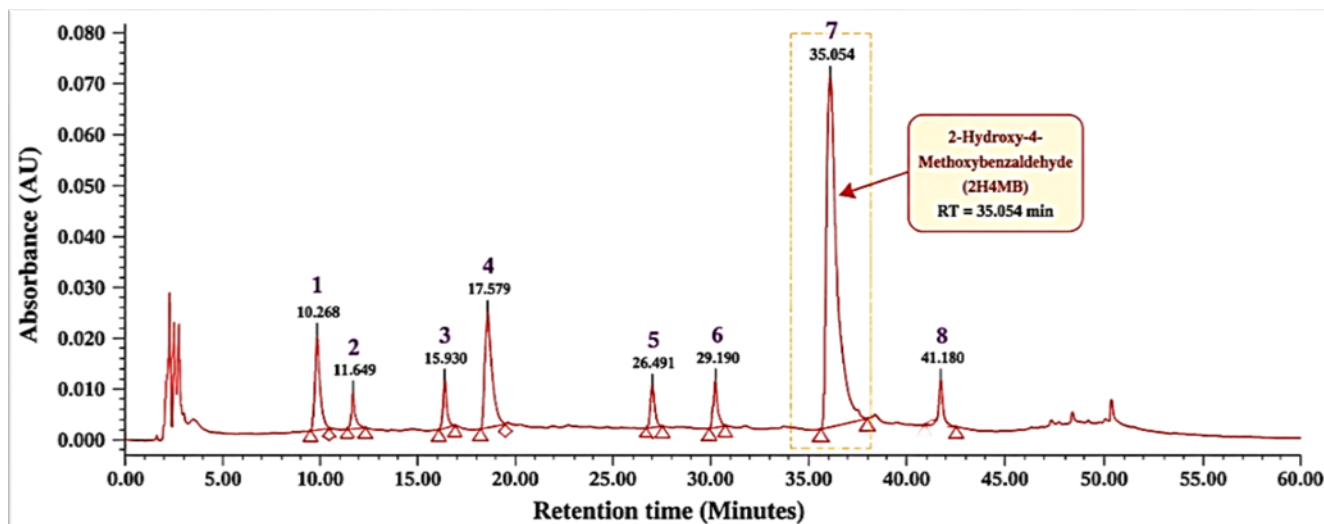


Fig 4: HPLC chromatogram of 2-hydroxy-4-methoxybenzaldehyde (2H4MB) in two-year-old small tuberous roots of *Decalepis hamiltonii* Wight & Arn. (T₄)

4. Conclusion

The present investigation characterized the 2-hydroxy-4-methoxybenzaldehyde content of *Decalepis hamiltonii* tuberous roots according to root age and size. Significant variation was observed among the four root categories, with 2H4MB concentration ranging from 2.24 to 4.40 mg g⁻¹ dry weight.

Three-year-old large roots (>3 cm diameter) recorded the highest concentration of 4.40 mg g⁻¹, followed by three-year-old small roots (3.43 mg g⁻¹), two-year-old large roots (2.43 mg g⁻¹) and two-year-old small roots (2.24 mg g⁻¹). The results indicate that more mature and larger tuberous roots were associated with higher 2H4MB concentration. Among the root categories evaluated, three-year-old large tuberous roots (>3 cm diameter) can be identified as the superior category based on 2H4MB concentration. This information can be useful for quality-oriented raw material selection and future utilization of *D. hamiltonii* roots for flavour extraction and value-added products. Further studies involving a wider range of root ages, diameter classes and genotypes would help establish more precise quality standards for 2H4MB-rich tuberous roots.

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