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HPLC AND ESI-MS ANALYSIS OF VANILLIN ANALOGUE 2-HYDROXY--4-METHOXY BENZALDEHYDE IN SWALLOW ROOT – THE INFLUENCE OF HABITAT HETEROGENEITY ON ANTIOXIDANT POTENTIAL

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ABSTRACT

Decalepis hamiltonii Wight & Arn., is a plant species that is endemic to southern parts of India. The aim of this study is to explore the influence of habitat heterogeneity on total phenolics, flavonoids, flavor compound 2-hydroxy-4-methoxy benzaldehyde (2H4MB) and antioxidant potential of tubers. The flavor metabolite 2H4MB was quantified by HPLC using isocratic solvent system (methanol : acetonitrile : water : acetic acid 47 : 10 : 42 : 1) that indicates obvious difference in 2H4MB content of tubers with a maximum of 96.4 ±2.6 and 92.6 ±1.2 mg 100 g^{-1} dry weight basis (DW) in samples from B.R. Hills and Mysore area of Karnataka, followed by samples from Tirumalai Hills and Kurnool from Andhra Pradesh (89.02 ±0.9 mg 100 g^{-1} DW), Tamil Nadu (81.6 ±2.4 mg 100 g^{-1} DW) and Kerala (80.18 ±1.1 mg 100 g^{-1} DW) of tubers. There was variation in total phenolics, total flavonoids and 2H4MB content of root samples collected from different habitats. Also significant variation in free radical scavenging potential of methanol root extracts was noticed, which is directly proportional to the phenolics, and flavonoids content. Overall, there was 10–16% difference in content of 2H4MB in *D. hamiltonii* tubers that were collected from different natural habitats, and this habitat heterogeneity has to be considered vital, while using such tubers for edible purposes and food formulations.

Key words: habitat heterogeneity, root tubers, dichloromethane, 2-hydroxy-4-methoxy benzaldehyde

INTRODUCTION

Synthetic chemicals possessing antioxidant and antimicrobial potential are nowadays discouraged as they are shown to have harmful side effects [Carocho et al. 2014]. The preservative effect of many plant species advocates the existence of antimicrobial and antioxidant constituents in their parts [Pillai and Ramaswamy 2012] and there is a great impetus to promote such natural alternatives for wide range of applications. *Decalepis hamiltonii* Wight & Arn. commonly known as swallow root is a climbing shrub belonging to the family *Periploaceace* (an offshoot of the *Asclepiadaceae*). This plant grows largely in moist and scrub jungles of southern parts of Deccan Peninsula and the Western Ghats of India at an altitude of 300–1200 meters, and considered highly endemic [Wealth of India 2003]. *D. hamiltonii* (swallow root) grows in rocky slopes and crevices of dry and moist deciduous forests of Karnataka (Bellary, Kolar Tumkur, Hassan, Mysore), Andhra Pradesh (Nellore, Cuddapah, Kurnool, Chittoor,

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Anantapur districts) and in Tamil Nadu (Nilgiri, Coimbatore, Chengalpattu, Dharampuri), and also in some parts of Kerala [Wealth of India 2003]. Four of the five species of *Decalepis* are endemic to the Eastern and Western Ghats of peninsular India with the exception, *D. khasiana* [Sharma and Shahzad 2012]. In southern parts of India, *D. hamiltonii* is familiar with various vernacular names viz., Maredu Kommulu or Nannari kommulu or Madina Kommulu (Telugu), Magaliberu (Kannada) and Magali kizhangu (Tamil). The tubers of this plant find use for culinary and medicinal purposes [Jacob 1937]. An analogue of vanillin, chemically known as 2-hydroxy-4-methoxy benzaldehyde (2H4MB) is the key metabolite important for aromatic flavor of its tubers [Thornell et al. 2000, Giridhar et al. 2004]. Destructive harvesting of this plant's tubers for small economic gains by tribal communities deliberated it as an endangered plant [Vedavaty 2004]. Nannari is famous drink made from the roots and stems of either sarsaparilla or swallow root in both Rayalaseema and coastal districts of A.P., and this syrup costs Rs 60–75 per 1000 ml (made from 100 g dry roots) in local market. It is very good for health, especially in summer as it cools the body. Prior research is available in terms of biochemical research that oriented to assess the potential of *D. hamiltonii* tubers for their antimicrobial properties, insecticide potential and as a food preservative [as revised by Pradeep et al. 2016], and also for medicinal [Shefali et al. 2009], antidiabetic potential [Ragini et al. 2010], phytochemical analysis and traditional uses along with sustainable production systems for its conservation through biotechnology intervention [Giridhar et al. 2004, 2005, Gururaj et al. 2004]. Habitat heterogeneity is one of the important extrinsic factor that influences the plant growth and development and also affects the nutrients profile of plant organs [Hutchings and John 2004, Solomon Raju and Venkata Ramana 2011]. The plants tolerant to various ecological factors under diverse habitats are sources of secondary metabolites and may be highly applicable in pharmaceutical and food industries [Zlatic and Stankovic 2017]. Especially in plants wherein, organized cultivation is lacking due to their unexplored or endangered or less known nature; information on habitat heterogeneity will be helpful to identify the plants with superior physical traits. Also it helps to collect plant material with high content of desired metabolites of medicinal value. In fact, the flavor rich root extracts of *D. hamiltonii* are documented under [EAF 2015]. Under this context, identification of root specific metabolites such as 2H4MB [Giridhar et al. 2005] including wide range of bioactive substances [Swapna and Srinivasa Rao 2013] in tubers of *D. hamiltonii* is important, especially, its content in tubers that collected from different habitats is having vital significance. Accordingly, the aim is to find out the influence of habitat heterogeneity on *D. hamiltonii* root specific flavor metabolite, total phenolics and flavonoids.

MATERIALS AND METHODS

Tubers from intact plants of *D. hamiltonii* Wight & Arn., at their natural habitat from diverse ecological areas of Andhra Pradesh (Tirupathi: 13.6500°N, 79.4200°E, Kurnool: 15.8300°N, 78.0500°E), Tamil Nadu (Trichy: 10.8050°N, 78.6856°E), Karnataka (BR Hills: 11.9939°N, 77.1406°E, Mysore: 12.9702°N, 77.5603°E) and Kerala (Idukki: 9.8500°N, 76.9400°E) states were harvested. The collected tubers with 4.0 cm diameter in size were used for flavor metabolite analysis and also for analysis of phenolics, flavonoids, and DPPH assay based on established procedures as described in this section. Initially, tubers were first rinsed with water to remove soil particles and then used. Then tuber's outer skin was peeled off and the inner hard core (medulla) was removed and fleshy portion was cut into small pieces of 0.5–1.5 cm length eventually were dried in oven at 55°C for 48 h to obtain uniformly dried powder that was used for further respective analysis.

500 mg of root powder was weighed and extracted with 10 ml of methanol (80%). The residue was centrifuged at 10,000 rpm for 20 min and pellet was re-extracted with 5 ml methanol (80%). The supernatants were pooled and evaporated to dryness. The extract was pipetted out into a test tube and used for further analysis of total phenolics and total flavonoids. Total phenolic contents of the extracts were evaluated using the Folin-Ciocalteu technique [Hamouz et al. 2006]. 0.1 to 1 ml of working stand-

ard (gallic acid 0.1 mg ml^{-1}) was taken for preparing standard curve. The volume in all the tubes was made up to 3 ml with distilled water. 0.5 ml of Folin--Ciocalteu reagent was added into each test tube and incubated for 3 min, 2 ml of 20% Na_2CO_3 solution was added to each tube. The tubes were vortexed and placed in boiling water bath for exactly 1 min. The contents were cooled and the absorbance was measured at 650 nm and the amount of phenolics present in the samples was obtained by plotting against the standard graph. The total phenolic content was expressed in terms of gallic acid equivalent (GAE) (mg g^{-1} DW extract). Also, the contents of flavonoids in the examined plant extracts were determined using spectrophotometric method [Nataraj et al. 2009]. To prepare standard curve, 0.1 to 1 ml of working standard (quercetin 0.1 mg ml^{-1}) was taken. The volume in all tubes was made up to 4 ml with distilled water. Sodium nitrate (0.3 ml) was added into each test tube and incubated for 5 min at room temperature, then 0.3 ml of 10% $AlCl_3$ solution was added to each tube and incubated for 5 min. Finally, 2 ml of 1M NaOH was added to the tubes and the absorbance was measured at 510 nm and the amount of flavonoid present in the samples was obtained by plotting against the standard graph. Based on the measured absorbance, the concentration of flavonoids was read (mg ml^{-1}) on the calibration line; then, the contents of flavonoids in extracts were expressed in terms of quercetin equivalent (QE) (mg g^{-1} DW extract). Values are reported as means of three separate experiments. All the reagents and chemical were obtained from Himedia Laboratories, Mumbai, India.

To quantify 2H4MB, initially root extracts were prepared. The cut pieces of fleshy part of root were subjected to steam distillation, wherein the steam was generated in a separate flask and allowed to purge into the sample, which was kept under aqueous medium. The generated volatiles, along with the steam condensate, were collected and extracted with dichloromethane (500 ml × 3). The combined extracts were dried over anhydrous sodium sulfate and concentrated using rotavapor to a final volume of 5 ml. All of the samples were filtered through a membrane filter (0.22 µm) before HPLC runs. HPLC separation was conducted using a Shimadzu, CR-20A and the column was waters SunFire C_{18} (250 × 4.6 mm and 5 µm diameter). The mobile phase was isocratic solvent system: methanol : acetonitrile : water : acetic acid (47 : 10 : 42 : 1). The flow rate was maintained at 1 ml min^{-1} and the detection wavelength was 280 nm. Spectra were acquired from 200 to 400 nm with the highest scanning resolution allowed by the acquisition software (1 nm). Identification of the compounds was carried out by comparing their retention times and spectra to those of standard 2-hydroxy-4--methoxy benzaldehyde. Identified peaks were then confirmed by spiking samples with standard mixtures. Soon after the eluted fraction was determined to identify and substantiate by electrospray ionization mass spectrometry (ESI-MS) unit model QTof Ultima Waters Corporation, Micro Mass UK, Manchester. The mass spectral data were accompanied by a Mass Lynux 4.0 SP_4 data acquisition system. The ionization mode was -ve. The interface and the Mass Selective Detector (MSD) parameters were as follows. The source capillary was set to 3.5 kV, with a temperature of 80°C. The cone temperature was operated at 100°C, and the dissolvation temperature at 30°C. The cone was operated at 100°C, dry gas (N_2)-dissolvation gas 500 l h^{-1}, cone voltage 35 V, dissolvation temperature at 150°C, collision temperature 10 kV, TOF-9.10, connected on scan range m/z 100–1000 and data type accurate mass developed new method for the separation and identification of 2H4MB [Pradeep et al. 2017]. The calculation of 2H4MB was based on relative peak area of chromatogram of both standard and samples. HPLC-MS grade acetonitrile and methanol were supplied by Merck (Darmstadt, Germany), acetic acid was supplied from Sigma-Aldrich (St. Louis, MO, USA). The water used in the analysis was obtained from a Milli-Q water purification system manufactured by Millipore (Bedford, MA). All solvents used as the mobile phase were previously filtered and degassed prior to the use. The experiment was repeated twice with three replicates of roots for extraction and analysis.

Free radical scavenging potential of methanol and water extracts from roots of *D. hamiltonii* was determined by the DPPH radical-scavenging using a method [Yamaguchi et al. 1998]. Ascorbic acid (10–100 µg) was taken as the standard. Sample of

different concentrations (1.25–10 mg) was taken in different test tubes. The volume of samples was made up with methanol in such a way that the volume was equal to that of the extracts with the highest concentration. Accordingly, the total volume in each tube was made up to 2 ml with a uniform volume of 0.1 mol l^{-1} DPPH reagent. The contents of the test tubes were thoroughly mixed and a reaction time of 15 min was allowed. The absorbance was measured at 517 nm with methanol as blank; percentage (%) of inhibition or percentage (%) of scavenging activity is calculated using formula:

DPPH scavenging activity (%) = (OD control – OD sample) / OD control × 100

All values presented are mean ±SE of three analytical replicates. Data were subjected to one-way ANOVA followed by post hoc Duncan's Multiple Range Test (DMRT) using SPSS 17 software (SPSS Inc., Chicago, IL, USA) for determining significant differences. A difference was considered significant when $p < 0.01$.

RESULTS AND DISCUSSION

Phenolic and flavonoid compounds are reported to contribute to the antioxidant activity of plant extracts [Rice-Evans et al. 1996]. The total phenolics content was found to be ranging from 35.577 ±0.95 to 39.429 ±1.05 mg g^{-1} DW GAE in different samples. Similarly, flavonoids were found to be ranging from 24.757 ±0.73 to 27.842 ±1.02 mg g^{-1} DW. Maximum and minimum contents of total phenolics and flavonoids content was recorded in root samples from B.R. Hills (Karnataka) and Palakkad (Kerala) respectively (Tab. 1). There was no significant difference noticed for both phenolics and flavonoids content in both the samples that were collected from A.P. Wide difference for free radical inhibition potential (EC_{50}) of root extracts from different samples was evident. Samples from Karnataka exhibited more antioxidant potential with 0.21 ±0.01 – 0.25 ±0.025 mg ml^{-1} of sample extract to achieve (EC_{50}) for DPPH inhibition (Tab. 1). In general, the methanolic extracts contain more phenolics and flavonoids as demonstrated earlier in roots of *Onobrychis armena* [Kubra Karakoca et al. 2015]. Phenolic composition of plant extracts is affected by different factors such as plant variety and climate, etc. [Tomsone et al. 2012]. Moreover, habitat heterogeneity (samples collected at different natural habitats in our study) also contributes. This can somewhat explain the wide range of variation in total phenolics content values obtained from different samples, which used the same evaluation methods [Kubra Karakoca et al. 2015]. Similar observations for methanol extracts from roots of *Onobrychis armena* [Kubra Karakoca et al. 2015] and leaves of *Basella* species [Kumar et al. 2015] were reported. Reactive oxygen species (ROS) are reported to be causative agents for bronchopulmonary dysplasia, intraventricular hemorrhage, and necrotizing entero-

Table 1. Total phenolics, flavonoid and antioxidant capacities of *Decalepis hamiltonii* root extracts

Samples	Total phenolic content (mg g^{-1} DW)	Total flavonoids (mg g^{-1} DW)	DPPH EC_{50} mg ml^{-1})
BR Hills (Karnataka)	39.429 ±1.05^{a}	27.842 ±1.02^{a}	0.21 ±0.01^{a}
Mysore (Karnataka)	38.539 ±1.11^{b}	27.443 ±1.14^{b}	0.25 ±0.025^{b}
Tirumala Hills (A.P)	38.258 ±1.2^{b}	26.959 ±0.8^{b}	0.32 ±0.015^{b}
Kurnool (A.P)	37.854 ±1.02^{b}	26.261 ±0.7^{b}	0.36 ±0.021^{c}
Tiruchi (Tamilnadu)	36.587 ±1.02^{c}	25.020 ±0.9^{c}	0.43 ±0.016^{c}
Palakkad (Kerala)	35.577 ±0.95^{c}	24.757 ±0.73^{c}	0.49 ±0.01^{d}

Values are represented as mean ±SE of three replicates. Significance was tested by Duncan Multiple Range Test at $p < 0.01$, and values with same superscript were found not significantly different from each other

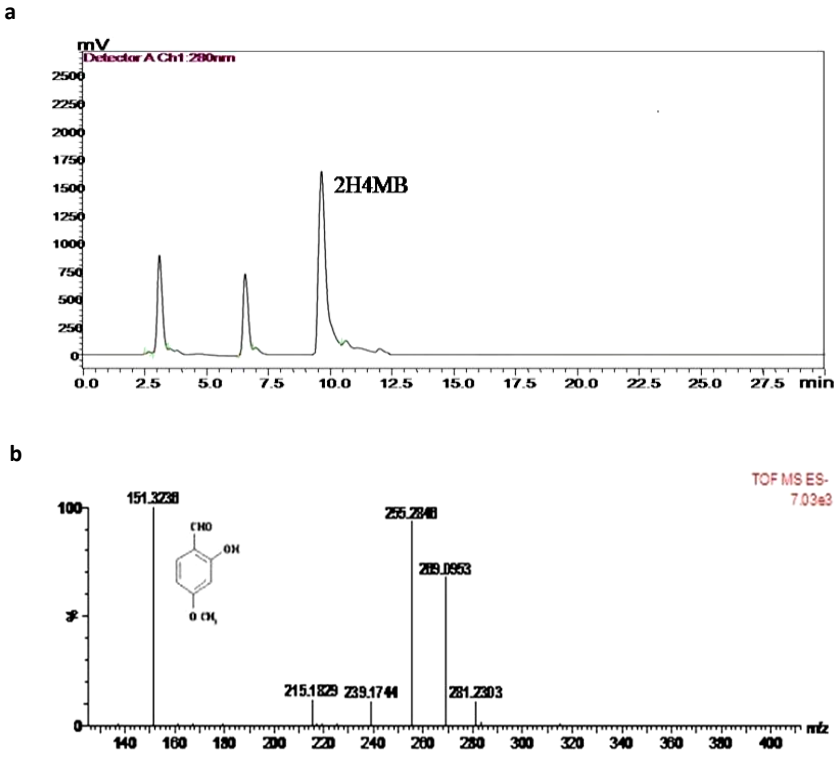

Figure 1. Peak assignment of 2H4MB in DCM extract from tubers of *D. hamiltonii* a) HPLC chromatogram (ret. time: 9.75'), b) ESI-MS in negative ionization mode based on molecular weight $[M-H]^-$ − m/z (151.3238)

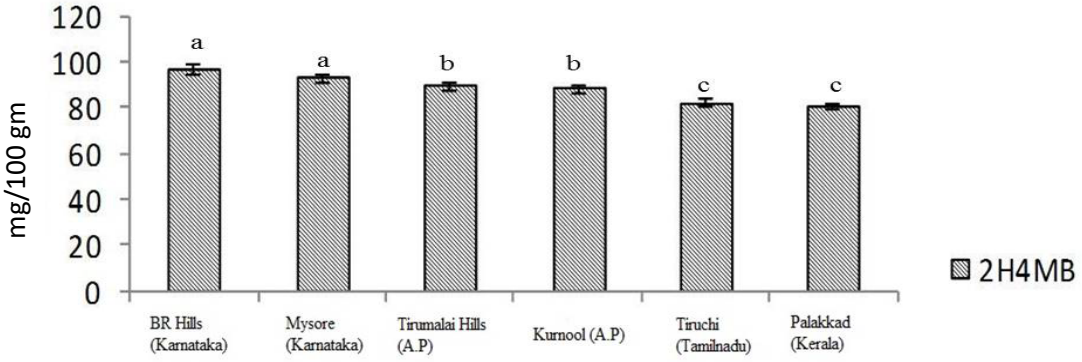

Figure 2. The content of 2-hydroxy-4-methoxy benzaldehyde in tubers of *D. hamiltonii* samples. Values are represented as mean ±SE of three replicates. Significance was tested by Duncan Multiple Range Test at $p < 0.01$, and values with same superscript were found not significantly different from each other

colitis etc., [Sen et al. 2010]. Under this context, methanol extracts of roots of *D. hamiltonii* are important in view of their DPPH scavenging potential.

HPLC analysis of tuber extracts of *D. hamiltonii* revealed the presence of 2H4MB as the major compound in the extracts are summarized in Figures 1 and 2. Though some minor unknown chromatographic peaks were found (which are intermediates of phenylpropanoid pathway), the same were ignored as they were beyond the scope of this study. However in most instances, flavor compound was identified on the basis of MS fragmentation data coupled with characterization in previously published studies [Pradeep et al. 2017]. The influence of habitat was obvious on 2-hydroxy-4-methoxy benzaldehyde content of tubers with a maximum of 96.4 ±2.6 and 92.6 ±1.2 mg 100 g^{-1} DW in samples from B.R. Hills and Mysore area of Karnataka (Fig. 2) followed by samples from Tirumalai Hills and Kurnool from A.P. (89.02 ±0.9 mg 100 g^{-1} DW), Tamil Nadu (81.6 ±2.4 mg 100 g^{-1} DW) and Kerala (80.18 ±1.1 mg 100 g^{-1} DW) of tubers. The characterization of 2H4MB in tubers of *D. hamiltonii* by HPLC and GC was demonstrated earlier [Giridhar et al. 2004, Sharma and Shahzad 2012] followed by NMR confirmation [Harish et al. 2005].

In this study, the trend was the same for both phenolics and flavonoids content. DPPH radical scavenging potential is also concomitant with the same trend. This is attributed to the cumulative effect of phenolics and flavonoids content of roots. As the influence of altitude, at which plants grown had profound influence on plant growth and quality of economically important parts of plants as evidenced from various plants [Sridevi and Giridhar 2015], the same may be true with *D. hamiltonii* root specific flavor metabolite 2H4MB. In general, tuberous roots store energy as starch and these are viewed mainly as sources of carbohydrate energy when used as a food staple. They are, however, also valuable sources of minerals, vitamins, and other bioactive substances and antioxidants. In addition, there is a trend toward using them to produce processed products for both human consumption and industrial use, though the same did not happen for *D. hamiltonii* except its use in making pickles, health drinks and flavored milk which is an unorganized sector, especially in local markets of southern states of India. In a recent communication the effective use of flavor rich tuber extracts of *D. hamiltonii* in the preparation of flavored-health drink was demonstrated [Solomon Raju and Venkata Ramana 2011]. The chemical ecology of a plant species population is characterized by heterogeneity among various habitats that support the population. This could be due to the ability of individual plants to respond to environmental heterogeneity as shown in *Quercus robur* leaf phenolics [Covelo and Gallardo 2001]. Marler [2007] explained how the habitat heterogeneity influences *Cycas micronesica* seed chemistry. Apart from this, soil characteristics influence the quantitative variability of secondary metabolites. In a recent study, Zlatic and Stankovic [2017] demonstrated variability of secondary metabolites viz., phenolics, flavonoids and also antioxidant activity in *Cichorium intybus* from different habitats.

Extrinsic factors such as conditions of cultivation, growing, location, soil nutrients influence the metabolites and nutrient profile of tubers as demonstrated well in tuber crops [Hutchings and John 2004, Hamouz et al. 2006, Nataraj et al. 2009]. Similarly habitat heterogeneity influences root growth and root-shoot partitioning [Hutchings and John 2004]. In the present study as well, all such ecological factors could substantiate the importance of habitat heterogeneity, as it possibly modulate biosynthesis of secondary metabolites and related antioxidant activity of tuber extracts of *D. hamiltonii*. The plants tolerant to various ecological factors under diverse habitats are sources of secondary metabolites and may be highly applicable in pharmaceutical and food industries [Zlatic and Stankovic 2017].

CONCLUSION

In the present study, there was moderate to significant variation in the quantity of phenolics and flavonoids and their activity, along with 10–16% difference in content of 2H4MB in *D. hamiltonii* tubers that were collected from different natural habitats. These plants may adapt to various ecological conditions in the habitat by means of synthesis of the regulation and accumulation of secondary metabolites

such as phenolics, flavonoids and also flavor metabolite 2H4MB and this habitat heterogeneity has to be considered as vital, while using such tubers for edible purposes. In addition, there is a need to further develop sustainable methods to strengthen the inextricable relationship between natural habitats and ethnic communities through traditional environmental and ecological knowledge data base.

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