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ORIGINAL ARTICLE

Chromatographic Investigation of Phytoconstituents in Milk Yam (*Ipomoea digitata* L.) Tubers

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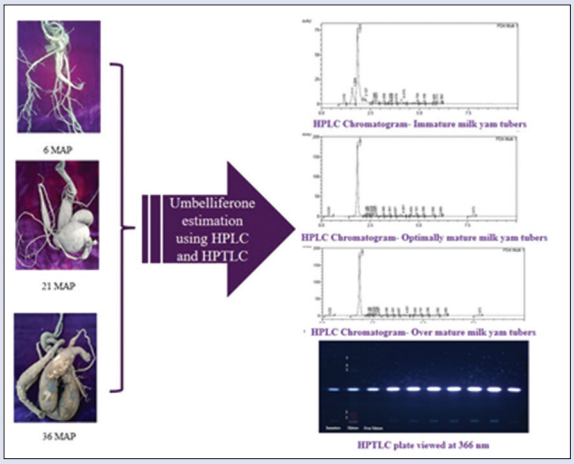
ABSTRACT

Background: Milk yam commonly called Ksheervidhari is a perennial climber; its tubers are an integral crude drug in different Ayurvedic formulations as well as folkloric medicine. It is used as antidiabetic, restorative, carminative, expectorant, galactagogue, stomachic, and appetizer. **Objective:** The present study is done with the aim of identifying the maturity stage of milk yam tuber containing maximum concentration of umbelliferone – a coumarin present in the tubers. **Materials and Methods:** Umbelliferone present in immature tubers (6 months after planting), overmature tubers (36 months after planting), and tubers at optimum maturity (21 months after planting) was assessed using high-performance liquid chromatography (HPLC) and high-performance thin-layer chromatography (HPTLC) analysis. **Results:** HPLC and HPTLC techniques revealed the presence of umbelliferone and it was quantified to be higher in over mature tubers (0.44 and 0.42 µg/g, respectively), followed by optimally mature (0.31 and 0.22 µg/g, respectively) and immature tubers (0.20 and <0.11 µg/g, respectively). **Conclusion:** The study reveals that developmental stages affect umbelliferone concentration in milk yam tubers and it increased as the plant ages.

Key words: Development stages, high-performance liquid chromatography, high-performance thin-layer chromatography, phytochemical, umbelliferone

SUMMARY

- The ideal maturity stage for milk yam tubers with maximum phytochemical constituents is found out by chromatographic techniques. Concentration of umbelliferone present in immature (6 MAP), optimally mature (21 MAP) and overmature (36 MAP) tubers of milk yam was assessed using HPLC and HPTLC techniques. Umbelliferone content is higher in overmature tubers followed by optimally mature tubers. Hence, it is ideal to harvest the tubers from 21 months after planting onwards for maximum phytochemical constituents.



Abbreviations used: HPLC-High Performance Liquid chromatography; HPTLC- High Performance Thin Layer Chromatography; MAP- Months After Planting

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INTRODUCTION

Milk yam (*Ipomoea digitata* L.) is an underutilized medicinal plant having therapeutic as well as nutraceutical potential. *I. digitata* L. (Convolvulaceae) is a type of morning glory plant having chromosome number, $2n = 30$.[1] It is known as Milk Yam in English, Ksheervidari in Sanskrit, and Vidaarikand in Hindi.[2] Several ayurvedic drugs and nutraeutical products such as Chyavanaprasam contain Ksheervidhari.

Alkaloids, glycosides, phytosterols, flavonoids, proteins, gums, and mucilage were present in methanol and water extracts, whereas fats, oils, and resins in petroleum ether and chloroform extracts of milk yam.[3] Ayurvedic Pharmacopoea of India has mentioned different active constituents present in its tubers.[4] Investigations on phytochemical constituents of milk yam tubers were done by several scholars and reported the presence of several bioactive compounds.[5-8]

High-performance liquid chromatography (HPLC) and high-performance thin-layer chromatography (HPTLC) techniques for assessing the variation in phytoconstituents of milk yam were used by Khan, *et al.*[9] Chromatographic techniques were standardized for analyzing the amount of umbelliferone, another important phytoconstituent in its tuber. Percentage recovery of umbelliferone by HPLC and HPTLC was 97.90% and 98.90%, respectively.[10] They reported that both HPLC and HPTLC had the same efficiency and sensitivity for determining umbelliferone from dried tuber powder.

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Umbelliferone is an anticoagulant and anti-HIV in action.[11] Sulaiman *et al*. had isolated two compounds from the acetone fraction of methanol extract using reverse phase HPLC and through liquid chromatography (LC-MS) one of the compounds were fragmented and identified as Caffeoyl glucose ($C_{15}H_{18}O_9$) and another one (compound-2) was not able to be fragmented.[12]

The tubers contain 7-hydroxy-6-methoxycoumarin commonly called Scopoletin which possess several pharmacological properties including inhibitory action on prostrate cancer proliferation.[11] Ethyl acetate:methanol:water:ammonia (13:5:1.8:0.2) solvent system was used to isolate and quantify scopoletin in the methanolic extract of milk yam tubers by Karthik and Padma.[3] TLC fingerprint of the methanolic extract revealed the presence of scopoletin (R_f value 0.56) and it was quantified as 0.029%–0.034% using HPLC and HPTLC methods. Structure elucidation of Scopoletin and β-sitosterol glucoside in the methanolic extract of milk yam tubers was done using Nuclear Magnetic Resonance (NMR) spectroscopy.[13] Studies on the ideal maturity stage for harvesting milk yam tubers for maximum phytochemical potential are limiting; hence, in the present study, investigation of ideal maturity stage for harvesting milk yam tubers containing maximum concentration of umbelliferone is done using chromatographic techniques.

MATERIALS AND METHODS

Plant material collection

A local ecotype of *Ipomoea digitata* L. was collected and rooted cuttings were raised in the Instructional Farm, College of Agriculture, Vellayani (80 25' 46" N latitude and 760 59' 24" E longitude). The Instructional Farm lies in Region-II (Zone-III) of the agro-climatic zones of Kerala and this region experiences a warm humid tropical climate. A herbarium of *I. digitata* L. was prepared in duplicate and submitted to the internationally recognized Janaki Ammal Herbarium (Accession No.-23207) and authenticated from CSIR-Indian Institute of Integrative Medicine, Jammu.

Healthy rooted cuttings of milk yam were raised in potting mixture (soil, sand, and farm yard manure in equal proportions) filled in polybags (40 cm × 24 cm × 24 cm and 600 gauge thickness). and were arranged in the experimental plot by trailing the vines in pandal. For a period of two years at periodic intervals milk yam tubers were collected by depotting the polybags and these were subjected to chromatographic analysis. Quantitative phytochemical analysis had already proved that milk yam tubers harvested by 21 MAP contain maximum secondary metabolites and it was identified as the optimum maturity stage.[14]

Analytical methods

Chromatographic analysis

In the present study, concentration of umbelliferone (a coumarin) present in the tubers with optimum maturity (21 months after planting [MAP]) was compared with immature tubers (6 MAP) and overmature tubers (36 MAP) of milk yam through HPLC and HPTLC analysis.[10]

Research materials

Umbelliferone reference standard (99% purity) was purchased from Sigma-Aldrich Chemie GmbH (Aldrich Division; Steinheim, Federal Republic of Germany).

Toluene (99% purity), isopropanol (99% purity), methanol (99% purity), and ammonia solution (95% purity) were purchased from Spectrochem Pvt. Ltd., Mumbai, Maharashtra, India.

Acetonitrile (99.80% purity) was purchased from Merck, India.

All solvents were of HPLC grade and distilled water was purified using Sartorius (Arium 61315, made in USA) water purification unit.

Tuber extract preparation

Methanolic extract of milk yam tuber powder was obtained by collecting the filtrate (Whatman No. 41 filter paper) of one gram powder added in 10 ml methanol, shaken in a rotary evaporator (30 rpm) over night at room temperature.

Umbelliferone stock solution preparation

Umbelliferone stock solution (1000 µg/ml) was prepared by mixing 10 mg umbelliferone reference standard and 5 ml methanol by thoroughly shaking it in a volumetric flask (10 ml) and was made up to the mark using methanol.

High-performance liquid chromatography estimation of umbelliferone (µg/g)

Mobile phase

Mobile phase used for the detection of umbelliferone was a blend of distilled water and acetonitrile (77:23), sonicated for 10 min.

Working standard solution of umbelliferone

Working standard solutions (0.10–20 µg/ml) were prepared by obtaining aliquots (0.01–2 ml) from stock solution of umbelliferone and each of its volume made up to 10 ml using mobile phase.

High-performance liquid chromatography operating system conditions

HPLC system (Chemito LC 6600) equipped with an isocratic HPLC pump containing a Kromasil C_{18} reverse phase column [250 mm x 4.6 mm internal diameter, 5 µm] with a flow rate of 1 ml/ min was used. The system contains a Rheodyne injector (20 µl loop) and an ultraviolet (UV)- visible detector [Shimadzu UV-1650 PC]. The developed data and chromatograms were recorded by means of Iris 32 Chromatographic software.

High-performance thin-layer chromatography estimation of umbelliferone (µg/g)

Mobile phase

Mobile used in the system is a combination of solvents - Toluene, isopropanol and ammonia in the ratio of 8:2:0.10; sonicated for 10 minutes.

Working standard solution of umbelliferone: Working standard solution (1–30 µg/ml) was prepared by obtaining the aliquots (0.01–0.30 ml) from the stock solution of umbelliferone and each of its volume was made up to 10 ml using methanol.

High-performance thin-layer chromatography operating system conditions

In HPTLC system a precoated silica gel aluminium plate (60 F_{254}; size: 20 x 10 cm [E. Merck, Darrmstadt, Germany supplied by Anchrom Technologists, Mumbai] over which samples were spotted as bands (size) using Camag Linomat IV sample applicator (Muttenz, Switzerland). Loaded with samples in a Camag microliter syringe (Hamilton, Bonaduz, Switzerland). Twin trough glass chamber (size: 20 cm × 10 cm; [Camag, Muttenz, Switzerland]) saturated with mobile phase was used as the linear ascending chamber. The chamber saturation time for mobile phase was optimized as 20 min at room temperature. The length of chromatogram run was 80 mm. Camag TLC scanner III in the reflectance-fluorescence mode at 254 nm as well as 366 nm was used for densitometric scanning and was operated by CATS software (V 3. Camag).

Table 1: High-performance liquid chromatography quantification of umbelliferone in milk yam (*Ipomoea digitata* L.) tubers at different maturity stages

Milk yam tuber sample	Retention time (min)	Area (mV.s)	Area (per cent)	Amount (µg/g)
Standard	1.88	1565828	100.00	-
Immature tuber	1.83	630183	72.29	0.20
Optimally mature tuber	1.84	972683	87.48	0.31
Overmature tuber	1.84	1363514	93.19	0.44

Table 2: High-performance thin-layer chromatography quantification of umbelliferone in milk yam (*Ipomoea digitata* L.) tubers at different maturity stages

Milk yam tuber sample	Retention time (min)	Area (AU)	Area (per cent)	Amount (µg/g)
Standard	0.48	-	-	-
Immature tuber	0.47	7770.70	100.00	<0.11
Optimally mature tuber	0.47	10319.60	100.00	0.22
Overmature tuber	0.48	12436.30	100.00	0.42

RESULTS

Umbelliferone concentration in milk yam tubers of different maturity stages viz., immature, mature, and overmature tubers differed customarily in mature (36 MAP) tubers were done by HPLC and HPTLC analytical tools and the results are presented in Tables 1 and 2, respectively.

Umbelliferone contained in immature, mature, and overmature tubers of milk yam consistently differed when analyzed by HPLC technique and the data are tabulated in Table 1. The presence of umbelliferone in immature, mature, and overmature tubers of milk yam were confirmed by comparing the retention time of standard umbelliferone (1.88 min) with methanolic extracts of the tubers (1.83, 1.84, and 1.84 min, respectively). The HPLC assay results indicated that immature, mature, and overmature tubers of milk yam contained 0.20, 0.31, and 0.44 µg/g umbelliferone, respectively.

HPTLC assay and the data are tabulated in Table 2. In HPTLC analysis, the retention time of standard umbelliferone was 0.48 min. Retention time recorded by immature, mature, and overmature tubers of milk yam (0.47, 0.47, and 0.48 min, respectively) were comparable to that of the standard umbelliferone. Umbelliferone present in immature, mature, and overmature tubers of milk yam was quantified as <0.11, 0.22, and 0.42 µg/g, respectively.

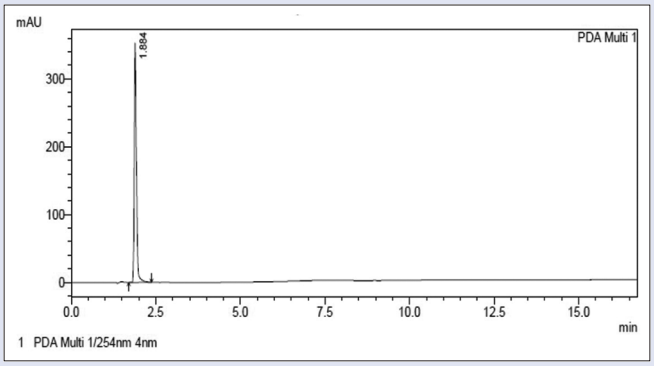

Figure 1: High-performance liquid chromatography of reference standard umbelliferone

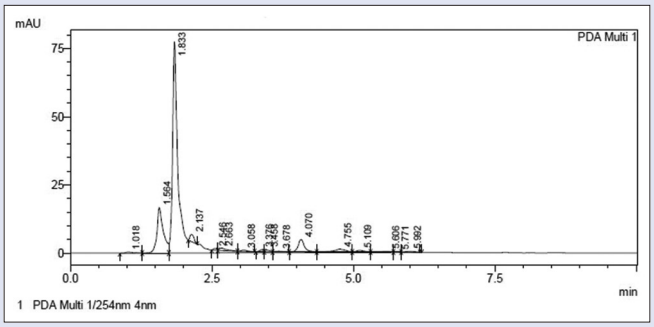

Figure 2: High-performance liquid chromatography – umbelliferone in immature milk yam tubers

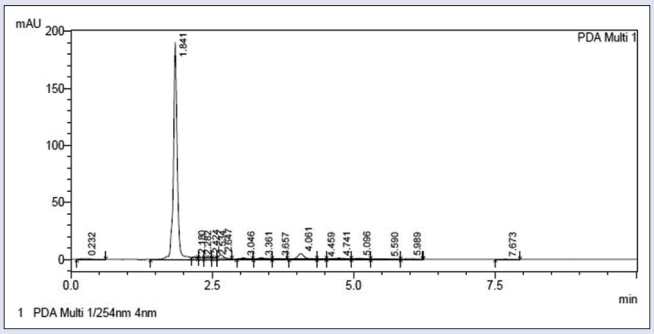

Figure 3: High-performance liquid chromatography – umbelliferone in optimally mature milk yam tubers

DISCUSSION

Umbelliferone is a phenolic compound, a coumarin derivative, chemically it is 7-hydroxycoumarin and benzopyrone in nature.[15] Quantification of umbelliferone from sevral plant sources using chromatographic tools had already been executed by different scholars.[16-19] In the present study, reference standard umbelliferone concentration was compared with the umbelliferone content of milk yam tubers at different maturity. Malik *et al*. documented the use of umbelliferone in the synthesis of anticancer drugs.[20] Umbelliferone was reported to be stable all along the postharvest and processing operations and hence could be used as an ideal marker for quality determination of crude drugs as well as pharmaceutical products.[21]

HPLC chromatogram of reference standard umbelliferone [Figure 1] and methanolic extract of milk yam tubers at different maturity stages indicated the presence of umbelliferone in all the tuber samples [Figures 2-4.]. Umbelliferone concentration was higher in overmature tubers (0.44 µg/g), followed by optimally mature (0.31 µg/g) and immature ones (0.20 µg/g). HPTLC plates spotted with methanolic extract of milk yam tubers and standard umbelliferone at different concentrations viewed under 254 nm and 366 nm also revealed the presence of umbelliferone in all the tuber samples and are shown in Figures 5 and 6, respectively. Umbelliferone exhibited fluorescence when viewed under 366 nm as mentioned by Mazimba.[22]

Apart from umbelliferone, Figure 6 displayed five more bands of phytochemicals within optimally mature milk yam tuber sample which could rationalize that milk yam tubers of 21 MAP are rich in phytochemicals. UV spectra of reference standard umbelliferone [Figure 7] and all the tuber samples overlapped each other [Figure 8] which indicates the presence of umbelliferone in immature, optimally mature and over mature milk yam tubers. Moreover, it is clear from the three

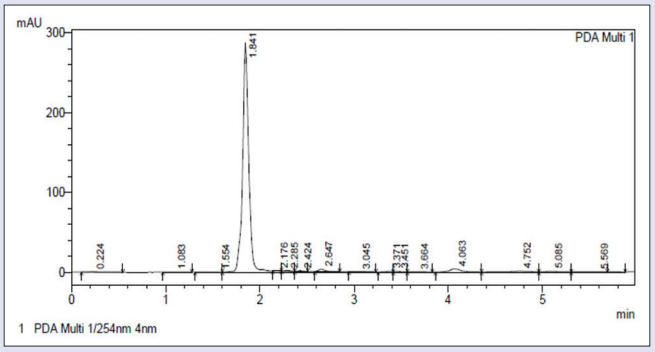

Figure 4: High-performance liquid chromatography – umbelliferone in overmature milk yam tubers

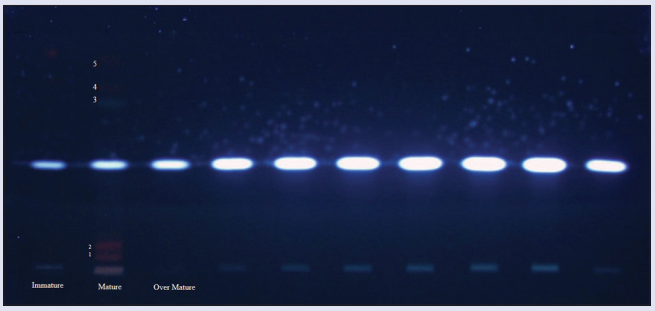

Figure 6: High-performance thin-layer chromatography-366 nm

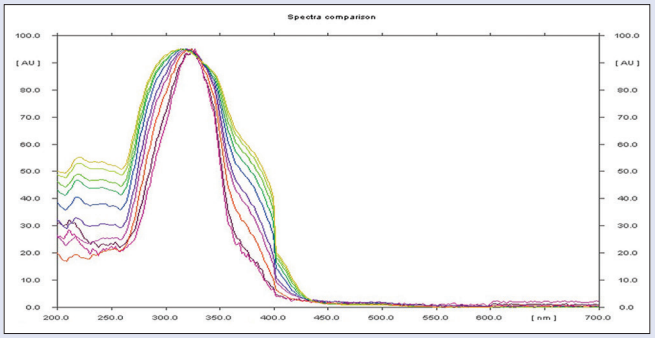

Figure 8: Ultraviolet spectrum – milk yam tubers of different maturity

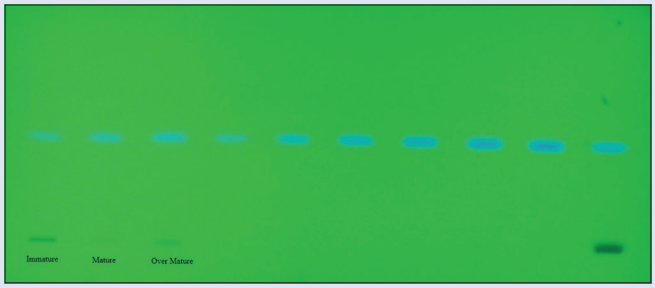

Figure 5: HPTLC 254 nm

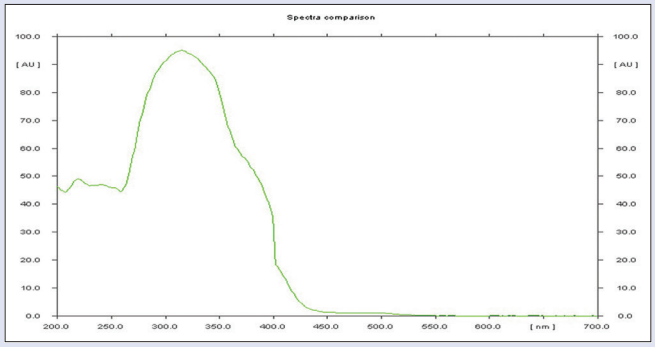

Figure 7: Reference standard umbelliferone – ultraviolet spectrum

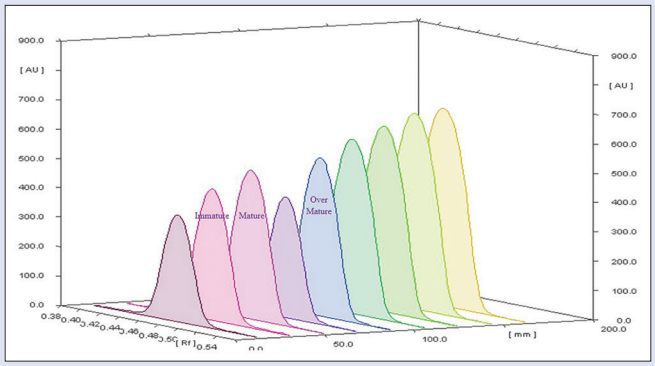

Figure 9: High-performance thin-layer chromatography – three-dimensional chromatogram peaks

dimensional view of umbelliferone peaks [Figure 9] that developmental stages affect umbelliferone concentration, in consequence of higher quantity of umbelliferone (0.42 µg/g) recorded by overmature tubers followed by optimally mature (0.22 µg/g) and immature ones (<0.11 µg/g).

Umbelliferone content recorded by milk yam tubers in the present study is in contrast with the findings of Dighe and Adhyapak, which might be ascribed to the highly mature wild milk yam tubers used, topography variation, climate, etc.[10] Effect of season, maturity, and growth phase on specific phytoconstituents such as morphine, vasicine, and lupeol had been verified using chromatographic tools.[23-25] The umbelliferone concentration estimated herewith satisfied the standard put forward by the Food Safety and Standards Authority of India, on maximum permissible limit of coumarins (not more than 0.30%) in food products.[26]

CONCLUSION

Chromatographic analysis of milk yam tubers having different maturity stages for the concentration of umbelliferone revealed that tubers of 36 months maturity had maximum umbelliferone concentration. Moreover, it was identified that tubers of 21 months maturity contain more phytochemicals than the overmature tubers. Hence, tubers can be collected for medicinal use from 21 months after planting onward.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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