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Changes in secondary metabolites during growth and development of milk yam (*Ipomoea digitata* L.) tubers

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Abstract
Milk yam is a medicinal plant having therapeutic as well as nutraceutical potential. This study was aimed on quality evaluation of milk yam tubers during its different growth stages so as to assess its optimum maturity and harvesting stage. Milk yam vine cuttings having two nodes were raised in polybags and depotted carefully after tuber formation at tri-monthly intervals for a period of two years and subjected to quantitative phytochemical analysis. Tubers harvested at twenty one months after planting recorded optimally high alkaloids (0.83 mg AE g^{-1}), saponins (9.50 mg DE g^{-1}) and flavonoids (0.54 mg QE g^{-1}); moderate glycosides (1.40 µg g^{-1}) and phytosterols (1.18 mg g^{-1}). Hence, twenty one months after planting is identified as optimum maturity stage for harvesting the tubers with better quality.

Keywords: Harvest, maturity, nutraceutical, phytochemical, quantitative

Introduction

Ipomoea digitata L. commonly known as milk yam is a type of morning glory plant naturalized in many parts of the world belonging to Convolvulaceae. In India, it has been noticed in the moist tropical regions, river banks, marshy areas, coastal regions etc. Milk yam grows as a perennial vine having five to seven lobed leaves, as the species name '*digitata*', suggests with bell shaped graceful pink coloured flowers. It is having a tuberous root system with tubers reaching up to 60 cm length and 30 cm thickness, weighing about five to ten kilogram. Its tubers have been used from ancient Sanskrit times as tonics, alteratives, aphrodisiacs, demulcents, galactagogics and cholagogics.

In India, tubers of milk yam are commonly known as vidari/ *Ksheera vidari* and many of the Ayurvedic industries use it in popular Ayurvedic nutraceutical products. Vidari is an important component of the popular ayurvedic formulation *Chyavanaprasha* and used in more than 45 formulations of Ayurveda viz., *Vidaryadikvatha Churna, Vidaryadi Ghrita, Marma Gutika, Manmathabhra Rasa* etc.

Investigation on phytochemical constituents contained in *I. digitata* L. tubers ensured the presence of constituents like alkaloids, tannins, steroids, gums, glycosides, carbohydrates and saponins [16, 20]. The phytochemical content in milk yam made it as a phytoestrogen source, since its activity is similar to estrogen present in human body, which could justify its use in curing ailments related to female reproductive system[3]. Himalaya the Ayurvedic drug company[14] reported that erganovine, is an alkaloid present in the tubers which able to stop menstrual bleeding.

Maturity of the officinal part is also an important criteria for gaining maximum potency for developing formulations out of it. Only mature (bigger size) tubers of *I. digitata* L. have been used for preparing galactagogues and immune-modulatory herbal medicines by the traditional medical practitioners. Hence, the present study is done with the objectives of finding the maturity stage of milk yam with optimum phytochemical composition so that it can be harvested at its best possible quality.

Materials and Methods

The experiments were conducted at the Instructional Farm, College of Agriculture, Vellayani, Thiruvananthapuram, (8^0 25' 46" N latitude & 76^0 59' 24" E longitude) from September 2015-September 2017. Milk yam vines of a local ecotype collected from the Instructional Farm, College of Agriculture, Vellayani was used for the study. A herbarium of *I. digitata* L. was prepared in duplicate and submitted to the internationally recognized Janaki Ammal Herbarium (RRLH) [Accession No.-23207] and authenticated from CSIR-Indian Institute of Integrative Medicine, Jammu.

Healthy rooted cuttings were raised in polybags (40x24x24 cm and 600 gauge thickness), filled with potting mixture (soil, sand and farm yard manure in equal proportions). The polybags were then placed in the experimental plot and the vines were trailed in pandal. The polybags are depotted carefully after tuber formation (three months after planting) at tri-monthly intervals for a period of two years. The tubers were subjected to quantitative phytochemical analysis for secondary metabolites.

Fresh milk yam tubers were dried and ground into coarse powder. Extracts were prepared by soaking 400 g of tuber powder in a glass container containing 600 ml of suitable solvent for eight days with occasional stirring and the filtrate was collected by filtering it through a clean muslin cloth. The filtrate was vacuum distilled under reduced pressure in order to remove the solvent and again collected in a beaker having a perforated aluminium foil covering on its opening, to facilitate the escape of residual solvent (if any) in the extract. The prepared extract was dried in a desiccator or in oven till constant weight is attained (70 ^{0}C), stored in a cool, dry place and used for phytochemical studies.

Quantitative Screening of Phytochemicals

Quantitative analysis of secondary metabolites viz., alkaloids, glycosides, flavonoids, saponins, phytosterols and resins were done in milk yam tubers from three MAP to 24 MAP at trimonthly intervals.

Total Alkaloids (mg AE g^{-1})

The filtrate obtained after treating hydrochloric acid with milk yam tuber extract dissolved in dimethyl sulphoxide (DMSO) was added with bromocresol green and phosphate buffer. To this mixture chloroform was added and absorbance was read against the reagent blank at 470 nm. A set of reference standard solutions of atropine was prepared in the same manner and the total alkaloid content was expressed as milligram Atropine equivalents gram $^{-1}$ of the sample (mg AE g^{-1}) [35].

Total Flavonoids (mg QE g^{-1})

Flavonoids in ethanolic milk yam tuber extracts was estimated according to the procedure given by Har and Ismail[11]. The extracts were mixed with sodium nitrate, aluminium chloride and sodium hydroxide solutions and recorded the absorbance at 510 nm against a reagent blank. The analysis was performed in triplicates and the total flavonoid content was expressed as mg Quercetin equivalent in one gram sample (mg QE g^{-1}).

Cardiac Glycosides (μg g^{-1})

From the diluted ethanolic milk yam tuber extracts tannins, resins and pigments were precipitated and filtered out by the addition of lead acetate. The excess Pb^{2+} ions were also precipitated from the filtrate by the addition of disodium hydrogen phosphate. An aliquot of this filtrate is treated with same volume of Buljet's reagent for the colour development and the absorbance was recorded at 495 nm. A blank was run using distilled water added with Buljet's reagent. The difference between the intensities of the colours of sample and the blank gives the absorbance and is proportional to the concentration of glycosides [9].

Total Saponins (mg DE g^{-1})

Total saponins were estimated using vanillin-sulphuric acid colourimetric reaction [19]. Diluted aqueous milk yam tuber extracts were treated with vanillin reagent and concentrated sulphuric acid and the absorbance was recorded in 544 nm. Total saponin content was expressed as diosgenin equivalents (mg DE g^{-1} extract) derived from a standard curve.

Total Phytosterols (mg g^{-1})

Chloroform extracts of the milk yam tuber powder were prepared by repeatedly refluxing, filtering and drying it under reduced pressure. Aliquots of the re-suspended residue of the powder using the same solvent (chloroform) were treated with Libermann-Burchard reagent and the absorbance was recorded in 400-900 nm against chloroform as the blank[1]. Total phytosterol content (TPC) was calculated as,

$$TPC = \frac{\text{Concentration of standard x Absorbance of the sample}}{\text{Absorbance of the standard}}$$

Results and Discussion

According to Charaka plant phenology greatly affects the quality and quantity of phytochemicals in the crude drug and also the period of harvesting, as the maximum distribution of active constituents should concur[39]. Extended research is still being carried out to assess the right stage of maturity of different medicinal plants employing several phytochemical evaluation tools[32]. This is a preliminary effort on assessing the right stage of maturity of milk yam tubers by quantitative evaluation of secondary metabolites.

Change in Secondary Metabolites

Concentration of secondary metabolites viz., alkaloids, glycosides, flavonoids, saponins, phytosterols and resins in milk yam tubers were found varying all through the growth stages up to 24 MAP and is shown in Table 1.

Total Alkaloids (mg AE g^{-1})

Alkaloids are phytochemicals which are reported to possess analgesic, antispasmodic, antibacterial, cytotoxic, anti-plasmodial, hallucinogenic and antineoplastic activity [29].

Alkaloids were observed to be higher in vegetative growth phase particularly during third, sixth, 15th and 18th MAP and it was lowered during reproductive growth stages (nine, 12, 21 and 24 MAP) (Table 1). Higher alkaloid content during vegetative phase might have been favoured by lower rainfall too. Investigations on change in alkaloid content during different seasons in several other medicinal plants also revealed higher alkaloid content during summer months [34].

The reduction in alkaloid concentration could be ascribed due to two possibilities either that it got diluted with other components in the tubers [18] or the alkaloid metabolites might have got used for carotenoid biosynthesis [5]. A number of workers had screened out alkaloids from different *Ipomoea* sp. of which *Ipomoea obscura* contained 1.10 mg g^{-1} alkaloids in stem, 3.10 mg g^{-1} in leaves and 4.80 mg g^{-1} in seeds [22].

Cardiac Glycosides (μg g^{-1})

Cardiac glycosides are of wide demand in the field of medicine for treating heart diseases particularly congestive heart failure [23]. Quantitative data obtained after inquisition of cardiac glycosides all along the growth stages of milk yam tubers (Table 1.) affirmed that glycosides were significantly higher, 2.60 μg g^{-1} in immature tubers (three MAP).

Table 1: Phytochemical screening of secondary metabolites in milk yam (*Ipomoea digitata* L.) tubers at different maturity stages

Months After Planting (MAP)	Alkaloids (mg AE g^{-1})	Glycosides (µg g^{-1})	Flavonoids (mg QE g^{-1})	Saponins (mg DE g^{-1})	Phytosterols (mg g^{-1})
T_1 (3MAP)	1.45	2.60	0.43	7.14	0.51
T_2 (6MAP)	1.15	1.90	0.57	6.60	0.68
T_3 (9MAP)	0.92	1.23	0.52	5.43	0.88
T_4 (12MAP)	0.52	1.23	0.46	7.14	0.87
T_5 (15MAP)	1.19	1.63	0.35	6.03	0.98
T_6 (18MAP)	1.35	1.53	0.32	5.90	1.02
T_7 (21MAP)	0.83	1.40	0.54	9.50	1.18
T_8 (24MAP)	0.53	1.73	0.58	9.16	1.45
S.Em ±	0.10	0.04	0.03	0.24	0.03
CD (P=0.05)	0.288	0.132	0.087	0.721	0.077

Trim [40] stated that higher proportion of glycoside accumulation took place in younger organs during elongation stage of cell growth. Inquiry on cardiac glycoside content in *Digitalis lanata* also proved the presence of higher concentration in immature plants (two months old) than the mature ones (five months old) [10]. Thereafter, up to 12 MAP cardiac glycoside content in milk yam tubers was reduced followed by a significant augmentation during 15 MAP (1.63 µg g^{-1}). A congruent trend of reduction (18 and 21 MAP) and augmentation (24 MAP) pursued during rest of growth period. In the current study, during the later stages of tuber growth, cardiac glycosides were found to be higher during 24 MAP (1.73 µg g^{-1}) compared to 21 MAP (1.40 µg g^{-1}). The immediate down pours after a dry period happened (Fig. 1.) during 24 MAP which might have assisted it as reported by Kellerman [17]. Cardiac glycosides of plant-origin are known for exhibiting toxicity in higher doses, and a serum digoxin level of >15 ng ml^{-1} is considered toxic [28]. However, in this study tubers collected at 21 MAP are supposed as optimally mature in terms of cardiac glycoside content.

Total Flavonoids (mg QE g^{-1})

Flavonoids are phenolic compounds known to possess strong antioxidant properties. They are reported to have anti-cancer, antiviral, anti-inflammatory activities also [12]. Flavonoid content in milk yam tubers during its growth period (Table 2.) recorded 0.43 mg QE g^{-1} flavonoids in immature milk yam roots/tubers (three MAP) and it was found to be increasing up to six MAP (0.57 mg QE g^{-1}). Thereafter it decreased till 18 MAP (0.32 mg QE g^{-1}) followed by a hike during 21 and 24 MAP (0.54 and 0.58 mg QE g^{-1} respectively).

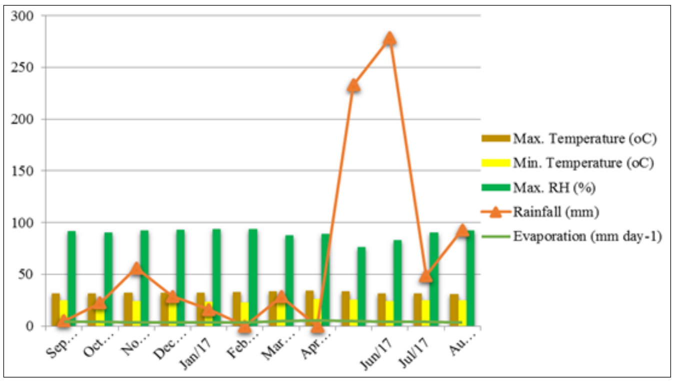

Fig 1: Weather data during Sept. 2016 to Aug. 2017

It could be observed from Fig. 1 that flavonoid content decreased during less rainfall periods (15 and 18 MAP). Such a similar reduction in flavonoids during dry period (February to June) was reported in *Cynara cardunculus* [44]. Enzyme degradation (inactivation) occurred in flavonoid pathway due to the environmental stresses or agricultural practices that might have resulted in the proportionate reduction of flavonoids just as disclosed in potato tubers [45]. Favourable weather conditions with adequate rains during ninth, 21st and 24th MAP might have favoured higher accumulation of flavonoids. Even though sufficient rain was absent during six MAP higher accumulation of flavonoid was observed which might be due to timely adequate irrigation. Sufficient organic manure (vermicompost) addition during these periods might also benefited it. A similar hike in flavonoids was observed in organically grown cassava tubers (39 per cent higher than chemically fertilized) [27]. By analysing the results it is advisable to harvest milk yam tubers during 21 or 24 MAP (0.54 and 0.58 mg QE g^{-1} respectively) during the second year or an early harvest during six or ninth MAP (0.57 and 0.52 mg QE g^{-1} respectively) for higher flavonoid yield is recommended. Vidya [42] had published almost similar amounts of flavonoids for one year (0.18-0.55 mg g^{-1}) and two year (0.23-0.53 mg g^{-1}) old milk yam tubers. Among the under investigated edible tubers like *Leptadenia pyrotechnica* (1.39 mg g^{-1}) [21], *Tacca leontopetaloides* (0.015 mg g^{-1}) [26] etc. milk yam tubers had comparatively higher flavonoid content (0.54-0.58 mg QE g^{-1}).

Total Saponins (mg DE g^{-1})

Saponins are chemically triterpenes and steroidal saponins, both of which worthy to impart astringency and bitter taste at higher concentration [4]. In the past, saponins were considered as anti-nutrients[36] but now, considerable interest has been put forward on exploiting saponin rich plant sources owing to its hypocholestromatic, haemolytic [24], anti-inflammatory and anti-carcinogenic properties [43].

Quantitative phytochemical screening results of immature milk yam tubers (three MAP) revealed higher saponin content (7.14 mg DE g^{-1}) just like alkaloids and glycosides (Table 1.). During the vegetative growth phase (six and nine MAP) it scaled down (6.60 and 5.43 mg DE g^{-1} respectively) and rolled back to 7.14 mg DE g^{-1} by the senescence and regrowth (12 MAP) stage. Scaling down (6.03 and 5.90 mg DE g^{-1} respectively) and rolling back (9.50 mg DE g^{-1}) of saponin content was repeated during the next vegetative (15 and 18 MAP) and senescence (21 MAP) stages respectively. A comparable higher proportion of saponins in early vegetative phase as well as senescence were documented in *Glycyrrhiza glabra* too [13]. Higher proportion of saponins in milk yam tubers during third, 12th, 21st and 24th MAP might have influenced by the rains occurring during that period (Fig. 1.) as reported for Brahmi [30].

Saponins are usually distributed in the aerial parts, which might have remobilized to the storage organs during rains so that it would not wash away [25]. Total saponin yield of milk yam tubers during 12 MAP and 24 MAP corrugates with the findings of Vidya [42] on saponin yield of one year old (5.46-9.33 per cent) and two year old (7.00-9.51 per cent) tubers. Saponin yield of mature milk yam tubers were much lower than that of *Abutilon indicum* (Linn.) (Sweet leaves) a natural source of saponin with 296 mg DE g^{-1} [37] and sweet potatoes, 26.46 mg g^{-1} [2] yet, stayed higher than that of *Dioscorea bulbifera*, 0.14 mg g^{-1} [31]. Decisively for higher saponin yield it is equally good to harvest milk yam tubers during 21 and 24 MAP (9.50 and 9.16 mg DE g^{-1} respectively).

Total Phytosterols (mg g^{-1})

Phytosterols are lipophilic in nature; over 250 sterols were identified from plants [6]. Phytosterols are anti-inflammatory, analgesic, immune-modulators which lower LDL-cholesterol hence protect from cardiovascular diseases, inhibit several cancer cell lines including colon, prostrate, breast etc. [33].

Phytosterol concentration was significantly less during three MAP (0.51 mg g^{-1}) and it exhibited a relatively steady increase all throughout the tuber growth stages (Table 1.). Consequently, highest phytosterol concentration was recorded during 24 MAP (1.45 mg g^{-1}). An equivalent accretion in phytosterol content during growth and development was disclosed by Jayanthy *et al*. [15] in *Desmodium gangeticum*. Jayanthy *et al*. [15] documented least lupeol concentration in young Desmodium plants and the maximum in flowering stage. Moreover, it is seen from Table 1. that the rate of increase in phytosterols was comparatively higher during later stages of tuber growth (15-24 MAP) as well as during nine MAP which might be ascribed to higher temperature prevailed during those periods (Fig. 1). Influence of higher temperature on greater accumulation of cucurbitacins has been reported in *Trichosanthes cucumerina* var. *cucumerina* [8]. Studies proved that stresses like wounds, low or high temperature, oxidative stresses due to air or soil pollution etc. influenced phytosterol concentration in plant roots [38]. The highest phytosterol content observed during later stages might had incited production of fibers as monitored in cotton fiber development [7]. Hence, ideal time for harvesting milk yam tubers with optimum phytosterol concentration could be considered as 21 MAP (1.18 mg g^{-1}) than 24 MAP (1.45 mg g^{-1}). This value is comparable with that of nuts which are the richest phytosterols sources (Cashew nut-1.58 mg g^{-1}; Almond-1.43 mg g^{-1}; Pecan, Pistachio and Walnut-1.08 mg g^{-1}) [41].

Conclusion

Phytochemical screening involving quantitative investigation could judge the ideal time of harvesting milk yam tubers having optimum active components. It could be understood that phytochemical responses revealed a relationship with weather conditions. The ideal maturity of harvesting milk yam tubers were realized as 21 MAP for the reason that alkaloids (0.83 mg AE g^{-1}), glycosides (1.40 µg g^{-1}), flavonoids (0.54 mg QE g^{-1}), saponins (9.50 mg DE g^{-1}) and phytosterols (1.18 mg g^{-1}) in optimum concentration were observed during that maturity stage.

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