"Pharmacognostic authentication and constituents' validation by HPLC for four different plant species of Vidari marketed in India"

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Abstract

The aim of the present study is to use detail diagnostic characters and instrumental validation for authentication of a traditionally important herbal drug known as "Vidari" in local Indian market.
Vidari drug has four different botanical entities namely *Pueraria tuberose* DC (P.t) (family: Leguminosae), *Ipomoea mauritiana* Jacq (I. m) (family: Convolvulaceae), *Adenia hondala* de Wide (A. h) (family: Passifloraceae) and *Cycas circinalis* (C. c) Linn (family: Cycadaceae). In this study morphological, histological characters, proximate analysis viz foreign organic matters, moisture content, ash value, extractive value and preliminary investigations on phytoconstituent were evaluated separately. Furthermore validated HPLC profiles of all the species revealed the variation in peak characterization and content of major constituent i.e. puerarin present in the same. The physicochemical values of all four species were evaluated and revealed within the prescribed limits for *P.t* indicating its authenticity except alcohol and water soluble extractive value for *C. c* (2.5-3.7% & 11.5-18.2%) and water soluble extractive value for *I. m* (11.3-18.5%). The water soluble extractive value (31-41.5%) and total ash content (8-11%) in *P. t* were found maximum. Qualitative screening of phytochemicals indicated the presence of Carbohydrates, Glycosides, Saponins, Phytosterols, Flavanoids, Proteins, Gums & mucilages and absence of Phenolic acids and Tannins in all the species used as *Vidari*. HPLC quantification revealed the presence of puerarin when compared with standard puerarin peak eluted at Rt 18 minutes.

Key words: Authentication, diagnostic characters, proximate analysis, HPLC, Vidari.

Introduction

In modern civilization the complicated health related problems are increased a lot due to food habits and to minimize them people are using allopathic medicines which gives temporary relief. To minimize one cause some other complications are developed due to side effect of the synthetic medicines. Hence plant based medicines are great deal of interest in recent era due to the demand for various medicinal plants in the production of herbal medicines is ever booming with their minimal side effects but the increasing market demand have led to scarcity of some plants. In order to meet the rising demand for the raw drugs, adulteration and substitution have become frequent which in turn results in compromised quality of herbal medicines. Dried plant products sold in the market are generally difficult to identify and at the same time numerous problems are confronted to taxonomists in the

identification of traded herbal drugs[1]. Correct identification of raw drug is the first step in quality control of herbal medicines. Different herbal raw drugs are sold under the same name and the same drug possesses different vernacular names in different regions. These create controversies with respect to the botanical identities of drugs available in the market and very easily adulterated or substituted one with another. Furthermore demand, supply and regional availability are some of the reasons for substitution and adulteration with other species. Hence pharmacognostical standardization of herbal drugs include macroscopic, microscopic, physio-chemical constants and other instrumental analysis of investigated parts are essential in order to validate genuineness of the crude drugs procured from plant, animal and mineral origins[2].

Oflate, Vidari is one such Ayurvedic drug where at least four different botanical entities have been recorded in use by the industry[3]. These include *P.t* (Roxb.ex Willd.) DC (Fabaceae), *I. m.* Jacq (Convolvulaceae), *A. h* (Gaertn) de Wide (Passifloraceae) and *C. c* Linn (Cycadaceae). The substitutes may or may not resemble *Vidari* in terms of morphology, properties or actions. *Vidari* is an ingredient of Chyavanaprasha, one of the top-selling products of Ayurvedic industry[3]. The Ayurvedic Pharmacopoeia of India correlates *Vidari* to *P. t* (Fabaceae) and it is traditionally used for bleeding disorders, decreased seminal quantity, purification of blood, tuberculosis, cough, pain, burning micturition, herpes, rejuvenator, tonic, restorative, aphrodisiac, galactagogue, diuretic, demulcent, haemorrhage, bronchial asthma and urinary disorders[4-6]. Tubers of Vidari are used in more than 45 formulations of Ayurveda like Marmagutika, Nityananda Rasa, Salavaryadi Ghrta, Asvagandhadyarista, Sarasvatarista, Mahavisagarbha Taila[6], Vidaryadi ghrtam, Cyavanaprasam[4], Vidari ghrita, Pugakanda, Vidaryadikwatha churna[7]. Most authors have equated Vidari with *P. t* and *Ksiravidari* with *I. m*[4]. Kerala physicians also accept *I. m*, locally called Palmutukku as the plant source of *Vidari*. Studies on the market samples in Kerala reveal that in addition to *I. m*, the large spherical tubers of *A. h* which is known as Mutukku in vernacular are also sold in the market under the name *Vidari* which may be an adulterant or an unauthorized substitute. A market survey made in Gujarat, Karnataka and Tamil Nadu revealed that the decorticated stem of *C. c*, a gymnosperm is being marketed there as *Vidari*[4]. Traded samples of Vidari collected from Bangalore local market also revealed that the botanical entities were stems of *C. c.* Therefore a great deal of adulteration or substitution is encountered in the commercial markets due to varied geographical locations where these plants grow. Since authentic identity of medicinal species is an important determinant of quality, safety and efficacy of herbal medicines, it is important that the correct botanical identity of the medicinal drug be established. In view of that, the present study was undertaken to investigate the biological and chemical similarities and differences of species used as *Vidari* and compared their phytoconstituents using HPLC instrumentation method for authentication and quality determination.

Materials and Methods

Collection and Authentication of Samples

Dried mature tubers of *P. t, I. m, A. h* and stems of *C. c* used for the study were collected by qualified field botanists from different locations of Belgaum, Bangalore local market (Karnataka), Wayanad, Kozhikode, Idukki (Kerala), Pune, Bheamashanka (Maharashtra) and Tirupati (Andhra Pradesh). The samples were authenticated by qualified plant taxonomists of Herbarium division of FRLHT. All samples were subjected to macroscopical identification. Voucher specimens (Specimen numbers: *P.t*, L/07/02/032; *I. m*, L/07/02/035; *A. h*, L/07/10/027 and *C. c*, L/07/04/011) were preserved at Raw Drug Collection Centre, FRLHT, Bangalore, India.

Pharmacognostic studies:

All the samples are cleaned with running water and removed adhering dust and then dried under shade. Separately powdered with the help of pulvarizer and the powdered were used for further studies. Morphological characters of plants like color, surface texture, taste and odor were examined as per WHO guideline[8, 9]. Free hand sections were taken, cleared with chloral hydrate and treated with phloroglucinal, mounted in glycerin and finally anatomical structures were determined as per the prescribed method[8, 10]. Organoleptic evaluation, behavior of the powder with different chemical reagents, foreign organic matters, moisture content, Ash values, extractive values and preliminary phytochemical analysis[8, 10-12] were determined. Thereafter total alkaloids, saponins, carbohydrates and proteins are estimated as per described standard methods[13, 14].

Phytochemical analysis by HPLC method:

The methanolic extracts of all samples were injected in shimadzu HPLC system consist of LC-10AT VP pump, a rheodyne injector, SPD 10AVP UV-Visible detector and CLASS-VP6 software was used for the analysis. The stationary phase was Merck C 18 (250 x 4.6mm) column with 5µ particle size. The mobile phase, consisting of gradient system of Methanol and Water: Pump A: Water and Pump B: Methanol with elution at 0.001(min) B.Conc.10%, 5.00(min)-20%, 10.00 (min)-30%, 15.00(min)-40%, 20.00(min)-50%, 25.00(min)-70%, 30.00(min)-80%, 40.00(min)-90%, 45.00(min)-100%. The column was equilibrated with the initial solvent ratio for an hour and then pumped at the rate of 1.0ml/min. The injector volume was 20µl and the chromatogram was run for 45min with the detection UV at 254 nm.

Results and Discussion

Morphology

The mature tubers of *P. t*, *I. m*, *A. h* and stems of *C. c* were subjected to morphological study. Morphology of all the species was compared. Photographs are shown in Fig 1 and observations are tabulated in Table.1.

Microscopy

The powders and transverse sections of all the species of *Vidari* were examined for the microscopical characteristics and revealed the different characters for different species viz. **Starch grains:** Among the four species observed, *P. t* has very minute sized simple starch grains. Abundant simple to compound starch grains densely arranged in *I. m* and *A. h* were found to possess numerous, comparatively large sized simple starch grains and very few compound starch grains. Compound starch grains were very prominent in *C. c*. **Calcium oxalate crystals:** Calcium oxalate prismatic crystals were found in *P. t* and *C. c*. but not in *A. h* and *I. m*. **Vessel elements:** *P. t* contains vessel elements with simple and scalar form cross perforation plates and tracheids. *I. m* and *A. h* had pitted vessels and *C. c* had tracheids.

Transverse sections (T.S) of four species used as Vidari

T.S. of *P.t* shows indistinct epidermis, 3-4 layers of cork cells followed by 5-7 layers of parenchymatous cells, cork cambium brown in color and 2 to 3 celled thick. Endodermis well developed, pericyclic fibres followed by two or three layers of stone cells. The medullary rays and phloem cells are filled with starch grains. Xylem consists of vessel elements, tracheids, fibres and parenchyma (Figure-2 a, b).

T.S of *I. m* shows 5-8 layers of thin walled cork cells followed by 8-10 layers of thin walled parenchymal cells filled with starch grains with distinct centric hilum. Secondary phloem consists of companion cells, sieve tube elements and phloem parenchyma, traversed by uni- or biseriate medullary ray; secondary xylem consists of xylem parenchyma, vessels, fibres (Figure-3 a, b).

T.S of *A. h* shows 6-7 layers of thin walled cork cells followed by many layers of cortex of parenchymal cells containing large sized simple starch grains with few compound grains. Vessel elements consist of numerous fibres and parenchyma (Figure- 4a, b).
T.S of *C.c* shows parenchymal cells containing more number of compound starch grains; pitted vessels and tracheids (Figure- 5a, b).

Comparative pharmacognostical studies on all four species used as *Vidari* reveal that they are different in morphology, microscopical features. Ayurvedic Pharmacopoeia of India recognizes *P. t* as authentic *Vidari* species and has provided physicochemical values for the same[6].

Proximate analysis
All the four species used as *Vidari* were subjected to evaluate their foreign organic content, moisture content, total ash, acid insoluble ash, alcohol soluble extractive values and water-soluble extractive values and revealed differences between all the species of *Vidari* but all the experimented values were within the prescribed limits of API for *P.t* indicating its authenticity. The water soluble extractive values of *P. t* (31-41.5%) were found to be more than other species (*I.m* 11.3-18.5%, *A.h* 33.2-39.4% and *C.c* 11.5-18.2%). The water soluble extractive values of *I. m* (11.3-18.5%) and *C. c* (11.5-18.2%) were observed to be almost similar. The alcohol soluble extractive values of *C. c* (2.5-3.7%) were found to be very less compared to other species (*P.t* 10.8-15.6%, *I.m* 12-15% and *A.h* 13-17.8%). The total ash content was found to be maximum in *P.t* (8-11%) and very less in *C.c* (2.5-3.2%). The acid insoluble ash content was found to be maximum in A. h (0.35-0.88%) than other species (*P.t* 0.25-0.35%, *I.m* 0.3-0.5% and *C.c* 0.24-0.35%). All data were depicted in Table-2 and the data have similarities with the literature reported by Khan et al., (2009)[15].

Preliminary phytochemical screening
The preliminary phytochemical investigations of all the samples were performed which show the presence of different types of major secondary metabolites namely carbohydrates, glycosides, saponins, phytosterols, flavonoids, proteins, gums and mucilages which revealed their potent therapeutic activity but all the species are shown negative for tannins. Alkaloids and resins were present in *P. t* and *I. m* but were absent in *A. h* and *C. c* (Table-3) whereas small amount of phenolic compound was present in *I. m* which shown the positive result as described by Sulaiman et al., (2014)[16].

Phytoconstituents like β-sitosterol, stigmasterol, daidzein, puerarin, isoflavone C-glycoside-4^{1},6^{11}-diacetyl-puerarin (root); pterocarpan-tuberosin (roots & tubers) were reported in *P. t*[17-19]. *I. m* was reported to contain Taraxerol, taraxerol acetate, β-sitosterol, scopoletin and 7-O-β-D-glycopyranosyl scopoletin (Scopolin)[20]. Cyanogenetic glycosides, volkensin were reported for *A. lobata* and *A. digitata* but phytoconstituents for *A. h was* not reported in literature. Furthermore, literatures revealed *C. c* leaves contain flavones, seeds contain starch, nuts contain cycasin, non protein amino acids which were similar to this present study[21-23].

Quantitative phytochemical analysis
Drugs were subjected to quantitative phytochemical evaluation in order to find out the total saponins, alkaloids, carbohydrate and total protein content present. The results indicated that total Carbohydrate and alkaloids content were maximum in mature tubers of *I. m* i.e 7.2% and 0.35% respectively but the same content were least in *C. c* (1.9%) and *P.t* (0.27 %) respectively. Furthermore total Saponin and protein content were maximum in *P. t* (11.768% and 8.8% respectively) and least in *C. c* (3.715%) and *A. h* (3.1%) respectively. All the results were correlated and linear regration equation showed y = -0.355x +

7.495, R^2 = 0.0087 for *P t;* y = 0.787x + 3.235, R^2 = 0.0978 for *I. m*; y = -0.514x + 4.88, R^2 = 0.0504 for *A h* and y = -0.005x + 2.2, R^2 = 2E-05 for *C. c.* (Graph-1).

HPLC detection of phytoconstituents present in four species of Vidari

HPLC chromatogram for standard Puerarin revealed the retention time at 18.15 minutes (Graph-2) and based on that all the samples of Vidari were analyzed and data clearly indicated that all the species of Vidari i.e. *P.t, I. m, A. h* and *C. c* also showed peak at 18 min which are almost same elution time as standard apart from two other common peaks at 10.5 and 20.5 minutes (Graphs-3) but the percentage content were varied. The puerarin content was higher of 0.56 % in *P. t* followed by *C. c*, 0.011 %, *I. m*, 0.00043 % and *A. h*, 0.00038% (Table-4).

HPLC profile disclosed that there are significant differences between the species used as *Vidari.*. There was no published literature on the presence of puerarin in any of the three species other than in *P. t*. However peak that corresponded to puerarin in all the four species were studied and the content of puerarin was significantly lower than that present in *P. t.* There was some overlap in these regions and hence further study of putative puerarin-like compound is required.

Conclusion

The present study investigated the biological and chemical similarities and differences of species used as *Vidari* in their phytoconstituents and bioactivities using modern tools like HPLC. In detail macroscopic, microscopic diagnostic characters and different proximate analysis drawn from the present study by using simple techniques helped in authentication of genuine samples of Vidari. This was first such report on the comparative determination of characteristics on "Vidari". Therefore the general phytoconstituents profile as well as bioactivity obtained in the current study revealed the potential guidance from traditional knowledge for developing modern standards for herbal medicines and the need for further more research for substitute and adulterant of raw drugs used in Traditional Medicine industry.

Acknowledgement

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Table 1. Data showing morphological features of all the four species used as *Vidari*

Characteristics	*Pueraria tuberosa*	*Ipomoea mauritiana*	*Adenia hondala*	*Cycas circinalis*
Colour	Outer brown skin with whitish inner part	Outer surface brownish black in colour with white inner portion	Greyish brown bark followed by inner greenish yellow	Milky white in colour with yellowish fibrous elements
Odour	Agreeable	Agreeable	Agreeable	Agreeable
Taste	Sweet	Mucilagenous, Slightly sweet	Slightly astringent, irritating	Tasteless
Form	Variable, oval to spherical, even oblong,	Oblong-ovoid to subcylindrical	Cylindrical, tapering at both	Variable pieces

	globose		ends	
Size(approx)	10-15 cm(dia)	14-18 X 6-9 cm to 20X30 cm	20-24 X 6-8 cm	1-3cm
Other features	Light brown epidermis with transverse warts and ridges.	Periderm is brown thick and slightly reticulate	Tubers are woody in nature	Pith pieces are thick, woody and embedded with yellow fibrous parts

Table 2. Data showing proximate values of moisture content, ash content, extractive values of all four species used as *Vidari*

Standards	***Pueraria tuberosa***	***Ipomea mauritiana***	***Adenia hondala***	***Cycas circinalis***	**Limits for *Pueraria tuberosa* (API Part I, Vol- V, 2006. pp193)**
Foreign Organic Matter (%)	Nil	Nil	Nil	Nil	NMT 2%
Moisture content (%)	8.5 - 9.5	8 - 9.5	3.5-4.5	8.4-10.6	NMT 10%
Total Ash (%)	8 -11	3.3-4.5	3.5-8.06	2.5-3.2	NMT 11%
Acid insoluble Ash (%)	0.25-0.35	0.3-0.5	0.35-0.88	0.24-0.35	NMT 1 %
Alcohol soluble Extractive value (%)	10.8-15.6	12-15	13-17.8	2.5-3.7	NLT 13%
Water soluble Extractive value (%)	31-41.5	11.3-18.5	33.2-39.4	11.5-18.2	NLT 22%

Table 3: Summary of Comparative analysis of phytoconstituents

Phytoconstituents	***Pueraria tuberosa***	***Ipomoea mauritiana***	***Adenia hondala***	***Cycas circinalis***
Alkaloids	+	+	-	-
Carbohydrates	+	+	+	+
Glycosides	+	+	+	+
Saponins	+	+	+	+
Phytosterols	+	+	+	+
Fats & Fixed oils	+	+	+	+
Resins	+	+	-	-

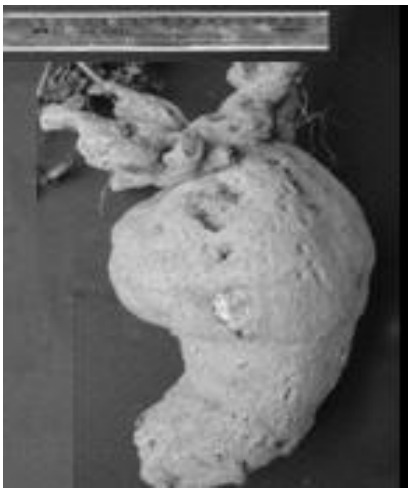
Flavonoids	+	+	+	+
Phenolic acids, Tannins	-	+	-	-
Proteins	+	+	+	+
Gums & mucilagenous	+	+	+	+

Table 4: Summary of major peaks of species used as *Vidari*

Retention time(minutes)	*Puraria tuberosa*	*Ipomoea mauritiana*	*Cycas circinalis*	*Adenia hondala*
~2.8	✗	✓ (7.355%)	✓ (21.1%)	✓ (16.6%)
~10.5	✓ (0.541%)	✓ (1.235%)	✓ (0.797%)	✓ (3.035%)
~11.6	✗	✓ (16.58%)	✗	✗
~14.5	✗	✓ (2.948%)	✗	✓ (3.356%)
~18.0	✓ (73.1%)	✓ (1.478%)	✓ (29.424%)	✓ (0.946%)
~20.5	✓ (9.4%)	✓ (1.548%)	✓ (1.902%)	✓ (0.858%)
~23.0	✓ (1.32%)	✗	✗	✗
~28.5	✓ (1.183%)	✗	✗	✗

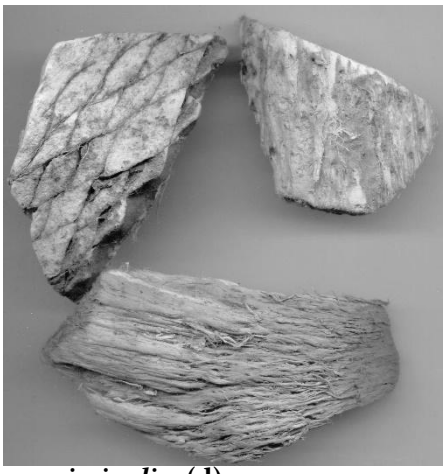
Fig.1 Photographs of all four species used as *Vidari*

***Pueraria tuberosa* 1(a)**



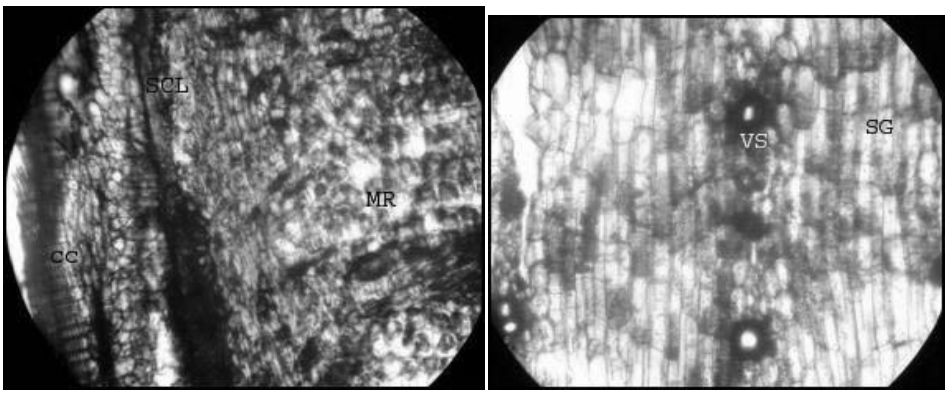
***Ipomoea mauritiana* 1(b)**

Adenia hondala 1(c)



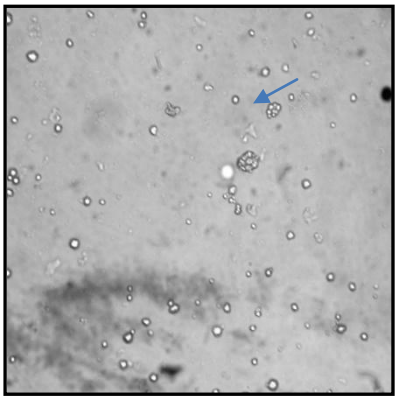
Cycas circinalis 1(d)

Fig. 2(a) T.S.of *Pueraria tuberosa* (Roxb. ex Willd.) DC. – Fabaceae (x100)

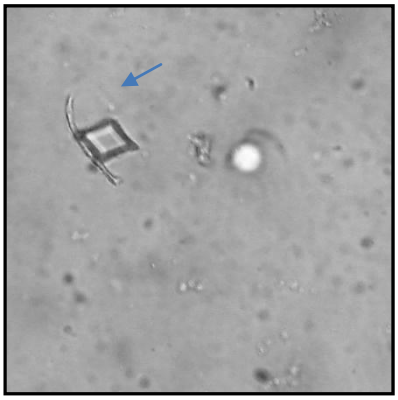


MR=Medullary rays, CC= Cork Cells, SCL= Stone Cell Layer, VS = Vasculature, SG= Starch Grains, PCOC = Prismatic Calcium Oxalate Crystal

2(b) Powder microscopy

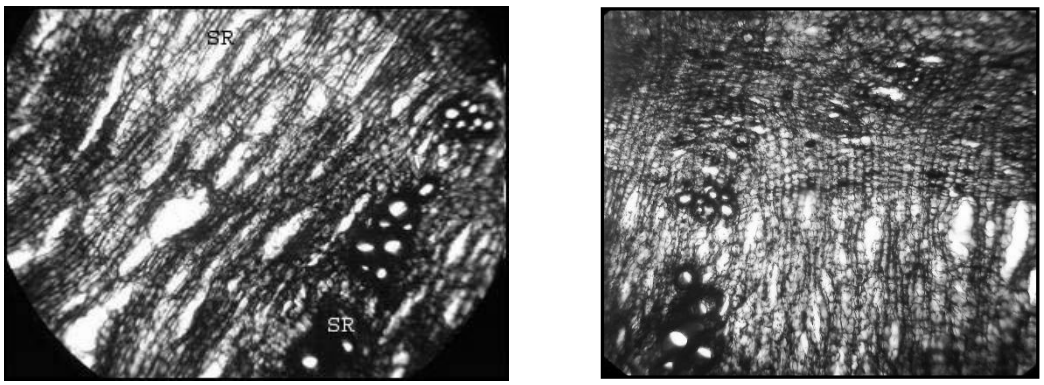


SG= Starch Grains,



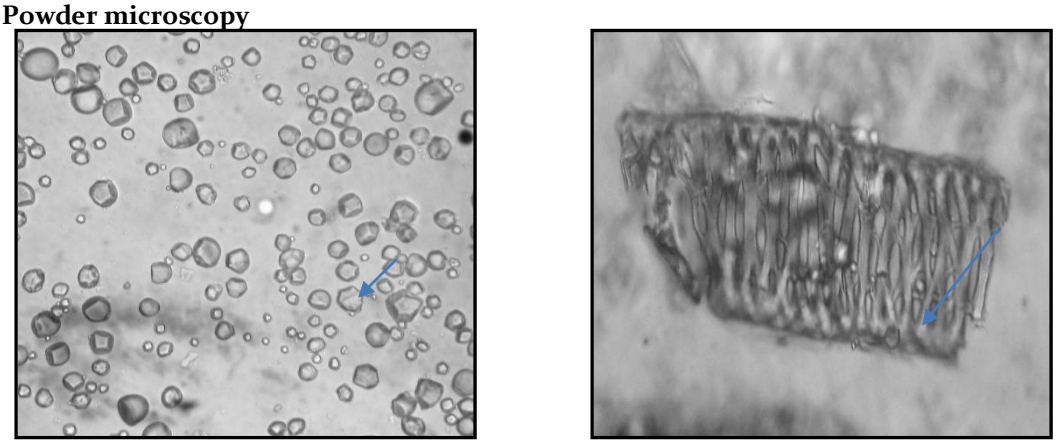
PCOC = Prismatic Calcium Oxalate Crystal

3 (a) T.S. of *Ipomoea mauritiana* Jacq. – Convolvulaceae (x100)



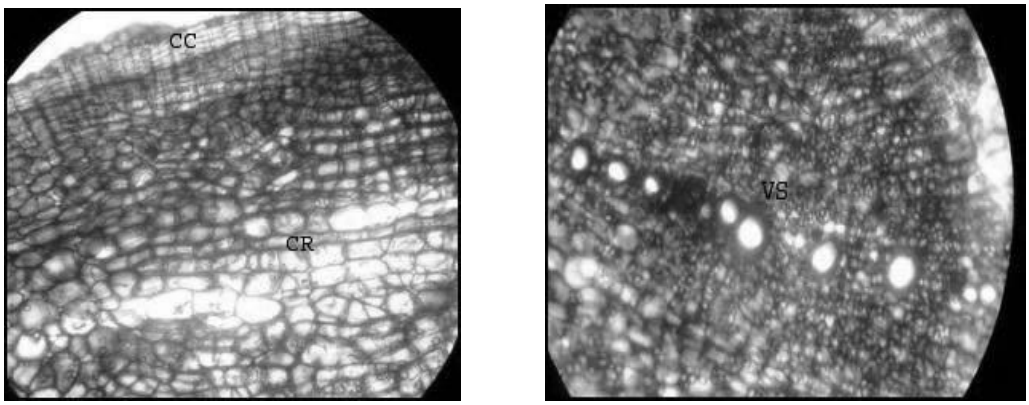
SR=Stelar Region, MR= Medullary rays

3(b)Powder microscopy

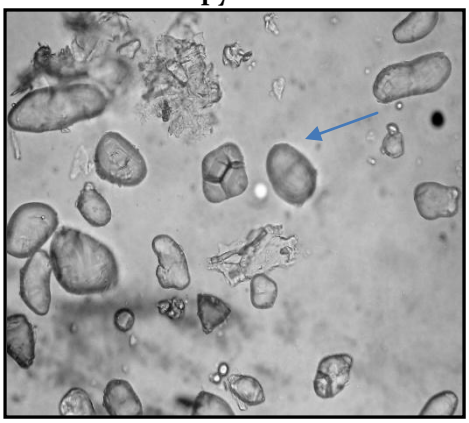


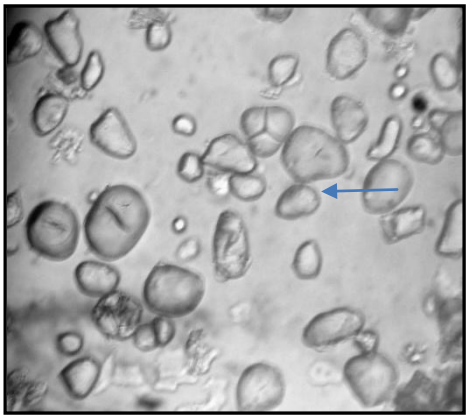
SSG= Simple Starch Grain, **RE= Reticulate Vessel**

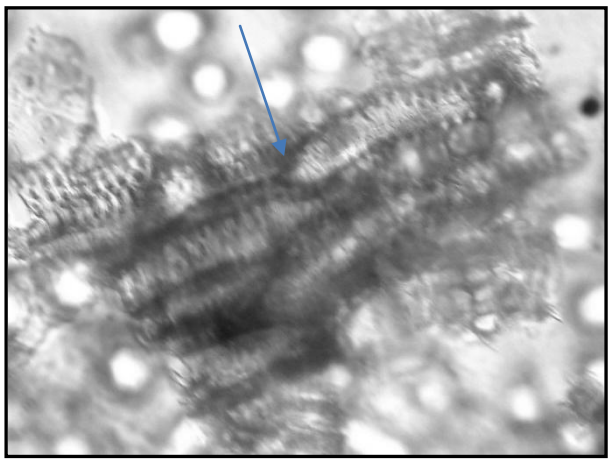
4 (a) T.S. of *Adenia hondala* (Gaertn.) W.J. de Wilde – Passifloraceae (x100)



4 (b) Powder microscopy

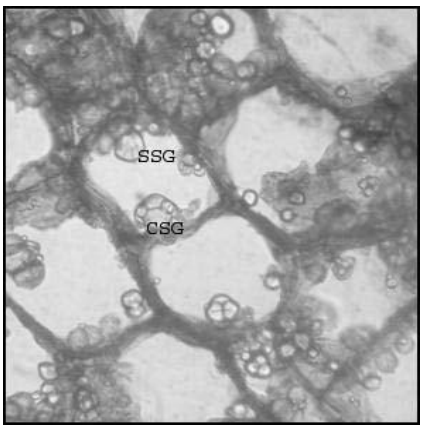




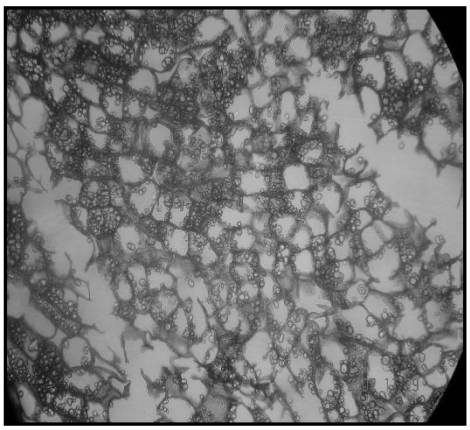


CC=Cork Cells, CR= Cortex, VS= Vasculature, CSG= Compound Starch Grains, SSG= Simple Starch Grains, V= Vessels.

5 (a) T.S. of *Cycas circinalis* L. – Cycadaceae (x100; x400)

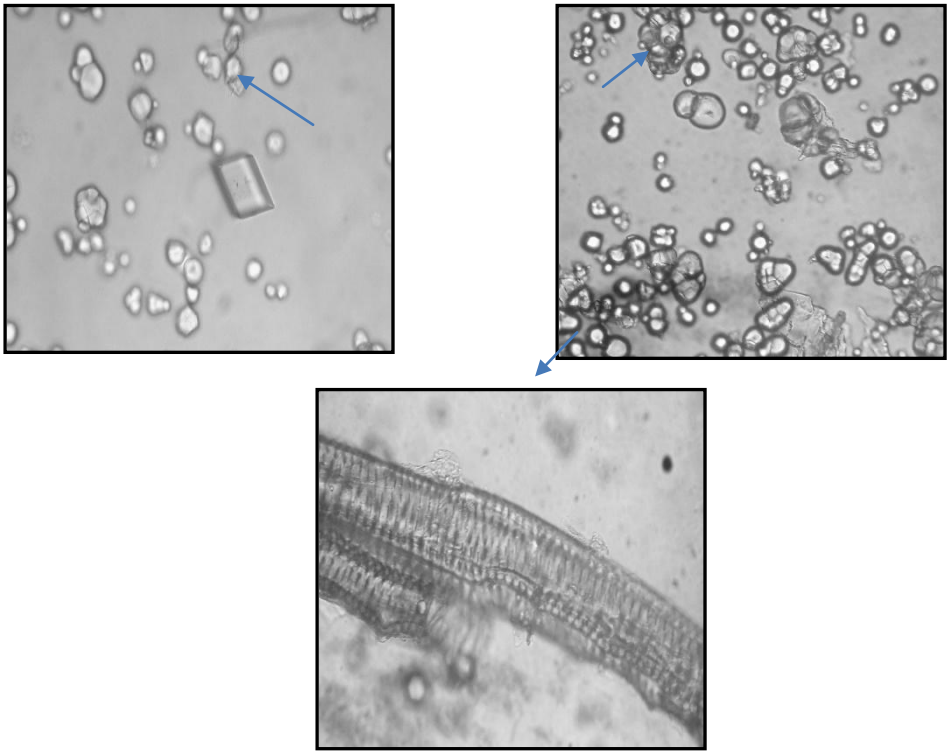


x 400



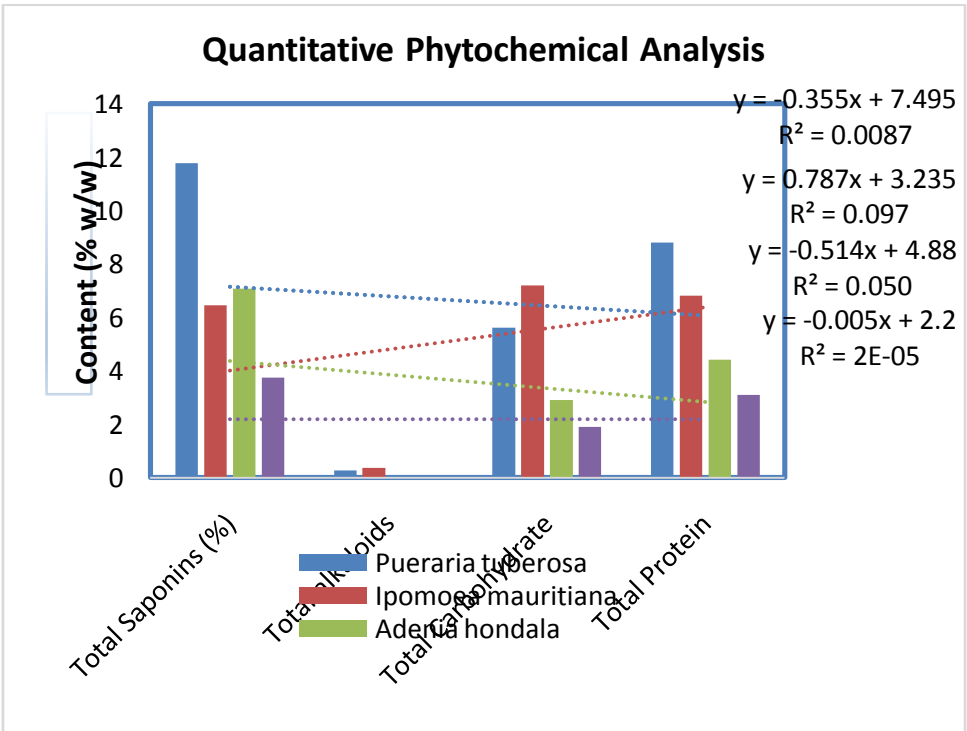
x100

5(b) Powder microscopy

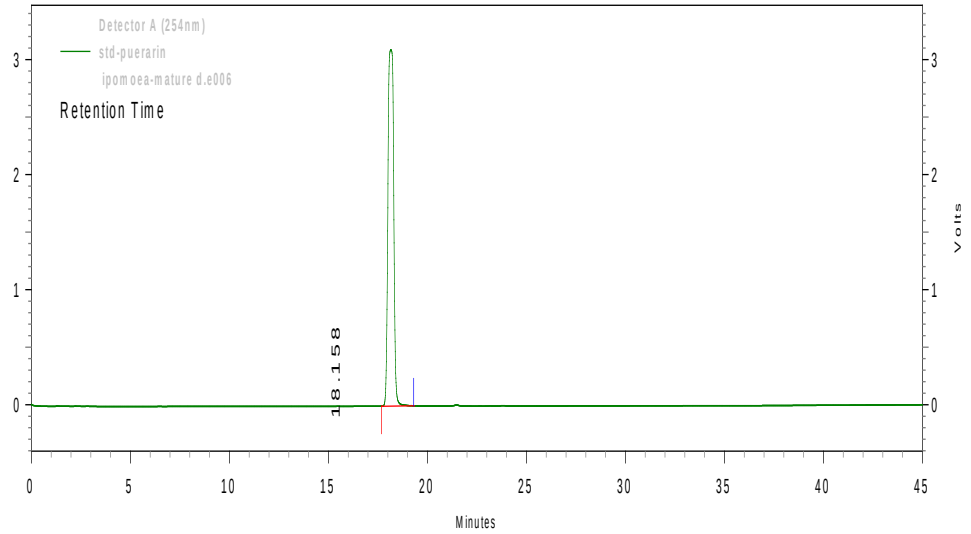


CSG= Compound Starch Grains, SSG= Simple Starch Grains, T= Tracheid

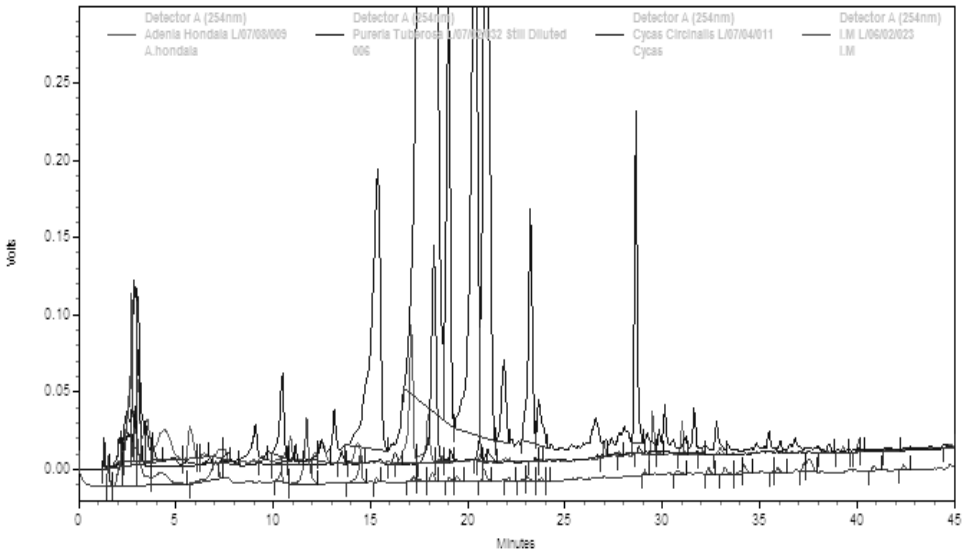
Graph-1: Quantitative analysis for four species of *Vidari*



Graph-2 HPLC chromatogram of standard Puerarin in methanol (1 mg/ml)



Graph-3: HPLC chromatograms of methanolic extracts of mature tubers of *P. t*, *I. m*, *A. h* andstems of *C. c*



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