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Qualitative phytochemical and histological evaluation of stem of *Premna integrifolia* Linn. (Verbenaceae): Standardization of one of traditional 'Dashmula'- Agnimanth.

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ABSTRACT

P. integrifolia Synonym(s): *Premna serratifolia L* . (Verbenaceae) deciduous shrub a member of Brihatpanchamula groups of dashmula, known as Agni-manth, in Sanskrit; malbau in Malaysia, Common beach plant distributed along the coasts and islands of tropical and subtropical Asia, Africa, Australia and the Pacific. It contains sterols, flavonoids apigenin, luteolin and alkaloids-Premnine, Ganiarine and Ganikarine.. Advances in microscope technology like Scanning Electron Microscope have increased the accuracy and capabilities of microscopy as a mean of botanical identification. The objective of this work to provide set of SEM histological characters that may serve as standard for identification. That helps for establishment of botanical biomarkers based on the major microscopic features observed in *P.integrifolia* stem. Secondly physicochemical parameter like moisture content, ash value, extractive value, crude fibre content were evaluated. Phytochemical screening of extracts **PI-AK** (chloroform extract of *P.integrifolia*) and **PI-ET**(Ethyl acetate extract of *P.integrifolia*) were performed. HPTLC fingerprinting of PI-ET extract marked qualitatively with Apigenin and Luteolin simultaneously as standard at Rf 0.39 and 0.29 respectively in mobile phase Toluene: Ethyl acetate :Formic acid (6:4:0.15),scanned at 367nm. Premnine HCl was used for biomarker for fingerprinting of PI-AK extract at Rf 0.59 at 283nm. Stability of PI-ET in solution and over plate with time was checked shows persistent chromatogram. Stability during chromatography checked with 2D chromatogram shows all zone in diagonal line. This research work focused for Apigenin , Luteolin bioflavonoid and Premnine HCl alkaloid as biomarker for standardization of *P.integrifolia* as one of 'Dashmul'.

Keywords: *Premna integrifolia*, Apigenin , Luteolin, Dashmula, , Premnine HCl

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INTRODUCTION

Standardization of herbal formulations is essential to assess quality of drugs based on the concentration of their active principles, physical, chemical, Phyto-chemical, and in-vitro, in-vivo parameters. The quality assessment of herbal formulations is important to justify their acceptability& safety. One of the major problems faced in standardization due to lack of unique quality control parameters for herbal medicines and their formulations.[1]

Dashamula is an ayurvedic formulation made up of ten herbs. The name Dashamula translates to 'The ten roots' and it is a potent ayurvedic formula. It contains ten roots in equal proportion under category as 'Brihat Panchmoola' means big tree five roots and 'Laghu Panchmoola' means small herbals five roots. Brihat Panchmoola includes; *Aegle marmelos, Premna integrifolia, Oroxylum indicum, Stereospermum suveolens, Gmelia arborea* While Laghu panchmoola includes ; *Solanum indicum, Solanum xanthocarpum ,Uraria picta, Desmodium gangeticum and Tribulus terrestris.* [3]

It is particularly beneficial to disorders of the nervous system, lungs , inflammation in the pelvic and sacral areas, expectorant, nervine, analgesic and febrifuge actions, most nervine disorders associated with weakness, debility and pain. It is particularly effective in tremors, sciatica and parkinsons, dry coughs associated with respiratory weakness, potent antioxidant property and used as uterine tonic and detoxifier. [5]

P. integrifolia Synonym(s): *Premna serratifolia L., P. obtusifolia R.* Br.,Large ,thorny, deciduous shrub a member of Brihatpanchamula groups of dashmula, known as Agni-manth, Girikarnika , Ananta in Sanskrit; Chamari, Kharanarvel ,Arni in marathi; Agetha in Hindi ; Gineri in Nepal; Toung -than-gyee in Burmish ;,malbau in Malay;Common beach plant distributed along the coasts and islands of tropical and subtropical Asia, Africa, Australia and the Pacific. Leaves are elliptic, obtuse, dentate, flowers small, greenish yellow with disagreeable odour. The chief active principles are three alkaloids: Premnine, Ganiarine and Ganikarine._The leaves and roots have cardiotonic , astringent, anti-inflammatory, antibacterial, anti- hypoglyceamic, anticoagulant , anti-arthritics and cardio protective properties[15,16,17,18] and are used in cardiac disorder, cough, leprosy, skin disease, constipation, fever, diabetes, obesity, stomach-ache and tumour. [3,9,10] *P. integrifolia* is very important plant during Vedic period where in its stem and sticks were used to produce fire. [2]

As per literature ,three novel diterpenoids reported from the root bark of *P. integrifolia* namely 1β,3α,8β-trihydroxy-pimara-15-ene (**1**), 6α,11,12,16-tetrahydroxy-7-oxo-abieta-8,11,13-triene (**2**) and 2α,19-dihydroxy-pimara-7,15-diene (**3**). 1,3-Dihydroxy and 2-hydroxy diterpenes.[13] , a verbascoside iridoid glucoside from leaves,[11] two alkaloids, Premnine and Ganiarine, have been

isolated from it and their physical and chemical properties described.[12] Isolation and evaluation of flavonoid ie. Luteolin 7-0-methyl ether and apigenin 5, 7-0-dimethyl ether from *P. serratifolia's* leaves . [4]

Present study was under taken for standardization of *Premna integrifolia* as raw material, ingredient of various Ayurvedic and herbal formulation. The study was focused over stem part for histological parameter using trinocular and Scanning Electron microscopy and HPTLC for fingerprinting of flavonoids in ethyl acetate extract and alkaloid in chloroform extract. Literature study do not show biomarking of it with apigenin, luteolin bioflavonoid and Premnine HCl alkaloid. Therefore, selected for fingerprinting of this drug. Simultaneously stability study of extract was done.

MATERIALS AND METHOD

Stem of *P.integrifolia* , phloroglucinol, Conc. HCl, Glycerine, Soda lime, Sulphuric acid.

Analytical Reference Compound and chemicals:

Apigenin, (5mg,A3145-5mg ,Sigma Aldrich), Luteolin (L9283-10 mg, Sigma Aldrich), Analytical grade solvents: Pet. Ether (60-80^0 c), chloroform, ethanol, ethyl acetate, Toluene, formic acid(Merck), Natural product reagent (2-aminoethyl diphenylborinate) (D9754- 5gm, Sigma Aldrich),Premnine HCl (isolated marker as per Literature [12]).

Silica Gel G TLC plate (Merck).

Instrument:

Trinocular microscope-Olympus CX21i, Scanning Electron microscope- PhilipXL-30 SEM, Sputter coater Sc 764,

HPTLC Instrument:

CAMAG Linomet Syringe V , CAMAG TLC scanner V,CAMAG Digistore-Reprostar 3 ,CAMAG Twin trough Chamber ,Win CATS version 1.4.2. Software (CAMAG ,Switzerland),UV chamber (CAMAG, Switzerland),Pre coated silica gel 60 F_{254} aluminium plates(0.2mm thick, Merck, Germany)

Authentication, Collection, Drying and Sizing:

P. integrifolia shrub was identified, flowering branch collected from Igatpuri forest of Nashik District and its herbarium was prepared. It was deposited to Botanical survey Of India, Pune for authentication purpose. Powder was prepared from entire dried stem refer Plate No.1.a.,1.b.,1.c.

Plate No. : 1.a, 1.b., 1.c.: Flowering branch, shrub, and herbarium of *P.integrifolia* Lin.

Physical parameter Evaluation:

Moisture content, Total ash value, acid insoluble ash value, water soluble ash value, sulphated ash value, Extractive values: alcohol soluble, water soluble, Crude fibre content determination by Dutch method, were performed over powder of Stem of *P.integrifolia* . (as per WHO guideline: WHO Library cataloguing in publication Data -1998: Quality control methods for medicinal plant materials, [21,22,23]) Values were recorded and compared with standard value of The Ayurvedic Pharmacopoeia. as per Table 1.

Table 1 : Physical parameter [21,22,23] of stem Powder of *P.integrifolia*

Sr No.	Physical Parameter	% value Obtained	Value as per The Ayurvedic Pharmacopoeia[19]
1	Moisture content	6 % w/w	--
2	Total ash value	5.5 % w/w	NMT 6 %
3	Sulphated ash value	6 % w/w	--
4	Acid insoluble ash value	1.0 % w/w	NMT 1 %
5	Water soluble ash value	1.5 % w/w	---
6	Alcohol soluble extractive value	8 % w/w	NLT 2 %
7	Water soluble extractive value	12 % w/w	NLT 5 %
8	Crude fibre content	35.5 % w/w	--

Microscopical Evaluation:

Histology of stem and powder of *P. integrifolia* through trinocular microscope and Scanning Electron Microscope were studied as follow- Transverse section of lower portion of stem was taken, cleared with clearing agent ie. sodium hypochlorite solution, mounted in glycerine water over glass slide and examined under trinocular microscope according to the standard method given in the textbook of pharmacognosy by TE. Wallis 1967.

Microchemical test Transverse section of stem have been treated with different reagents for the microchemical tests of cell wall content like lignin and cutin and for ergastic cell content like starch and calcium oxalate crystals.

Powder Characteristic : Slide Preparation- Fine powder of stem was boiled with soda lime solution for 10 minutes. It was filtered, washed with water and mounted in glycerine water ,stained with phloroglucinol and conc. HCl and observed under microscope. Photographs are shown in Plate no.2a. to 2.h.

Scanning Electron Microscope (SEM) - powder preparation :

Dried powder in thin layer was mounted on the aluminium holder stud using double sticky carbon tape. Stud with sample coated with gold particle using sputter coater SC 764 for about 20 min. to charge conductive. Further stubs were dried in oven at 60^0c for 3hours.Dried stubs were loaded to SEM holder. Vacuum was generated as $<5 \times 10^{-5}$ Pa. Tri-dimensional images of tissues were scan at different magnification mode. Photographs are shown in Plate No. 2i to 2.l.

Extraction:

Accurately weighed 200gm of stem powder of P. integrifolia charged in Soxhlet assembly and extracted exhaustively with alcohol. Alcoholic extract conc. over rotary evaporator, dried, divided into two part.

Flavonoid Extraction:

First alcoholic part made hydro alcoholic and fractionated with Pet. ether ($60\text{-}80^0$c), chloroform and ethyl acetate consecutively. Ethyl acetate extract concentrated over rotary evaporator, dried, yield noted and designated as PI-ET.

Alkaloid extraction:

Second alcoholic part stirred with 1% warm HCl for 2 hour on mechanical stirrer. Filtered. And fractionated with chloroform to remove coloring matter. Chloroform extract washed with 1 % HCl and washing returned to acidic alcoholic fraction. pH of acidic alcoholic fraction now adjusted to 9 with dilute ammonia solution and exhaustively extracted with chloroform. Chloroform extract

concentrated, dried and dissolved in few ml of 1% alcoholic HCl, allow to stand for 2 hours and then dried, yield noted and designated as PI-AK.

Phytochemical screening by chemical test and TLC: [24]

PI-ET, PI-AK were reconstituted with respective solvents and subjected for phytochemical screening using chemical tests for identification of class of compounds. As per Table 2.

Table no. 2 : Preliminary phytochemical screening of PI-ET and PI-AK [24]

Parameter	PI-AK	PI-ET
Colour	orange brown	Dark brown
Consistency	Solid	Solid
% yield w/w	0.0375	0.155
Chemical test		
Glycosides	-	+++
Carbohydrate	-	-
Steroids/terpene	-	-
Flavonoids	-	+++
Tannins	-	+
Alkaloids	+++	-

PI-AK : *P. integrifolia* chloroform extract,
PI-ET : *P. integrifolia* ethyl acetate Extract,
+++= Positive, -Negative

HPTLC fingerprinting of PI-ET along with Apigenin and Luteolin as biomarker :

Stationary phase: silica gel G 254 (Merck)

Mobile Phase: Toluene: Ethyl acetate: formic acid (6:4:0.15)

Saturation Time: 10 min

Scanning of densitometer: At 347 nm

Sample: PI-ET in methanol

Standard: Apigenin and Luteolin

(Designated as :AS, and LS respectively 50 ppm each separately in methanol)

Sample application: on Track -1, 2,3 : AS (2,3,4 µl); Track-4,5,6: PI-ET (1,1.5,3.0 µl); Track 7,8,9 : LS (2,3,4 µl) Chromatogram scanned over TLC scanner densitometer, recorded as Figure : 1.a.,1.b.

HPTLC fingerprinting of PI-AK along with Premnine HCl as biomarker :

Stationary phase : silica gel G 254 (Merck)

Mobile Phase : Toluene : Acetone: Diethylamine (7:2:1)

Saturation Time : 10 min

Scanning of densitometer : At 283 nm

Sample : PI-AK in methanol

Standard : Premnine HCl (Designated as :Pr-S 50 ppm in methanol)

Sample application: on Track -1,2 : PI-Ak (5,5 µl); Track-3: Pr-S (3.0 µl

Chromatogram scanned over TLC scanner densitometer, recorded as Figure. : 2.a, 2.b.

Stability Of PI-ET in solution, on plate : [20]

Track no. 1: Sample PI-ET (applied)5 µl 3 hours prior to chromatography

Track no. 2, 4 (Twice) :Sample PI-ET 5 µl fresh applied prior to chromatography

Track no. 3: Sample PI-ET 5 µl prepared 3 hours prior to chromatography (in solution)

Chromatogram scanned and recorded as Figure: 3.a., 3.b., 3.c.

Stability of PI-ET during chromatography: [20]

Track no.: 1- Applied as spot: 5 µl

Developed as 2 D chromatogram: 1 D chromatogram first developed, dried recorded and than turned 90 ^{o} and developed again. Chromatogram obtained as Figure 3.d.

Result and discussion:

Authenticated herbarium was certified as *P.integrifolia* (L.) DC. And voucher no. deposited as BSI/WRC/Tech. 2014/DVR-1-Plate No.: 1.c.

As per The Ayurvedic pharmacopoeia Of India physico chemical values found to be in limit as per Table 1. Transverse section of stem shows typical histological parameter such as --It shows central zone made up of large pith parenchyma, radiated with uni and biseriate medullary rays travels through concentric vascular bundle ,Xylem towards centre of pith and phloem outer side. Xylem patch that covers major area of stem and shows lignified vessels, parenchyma and fibres. Phloem shows nonlignified parenchyma, sieve tubes and companion cells and few scattered solitary lignified fibers. Cortex zone shows few layers of ground parenchyma and typical discontinuous patches of lignified cortical stone cells. Cork is made up of five to seven layers of tangentially elongated tightly packed parenchyma filled with brownish tannin content.

Powder characteristics of stem of *P. integrifolia (through trinacular and SEM)* .It shows fragments of cork and cortical parenchyma with ergastic content, lignified pitted vessels , lignified elongated solitary fibres with thick lumen .

SEM Images of powder shows elongated fibers, broken fragments of vessels, ie. Starch grain (5-8-10 µ in dia.) and prismatic calcium oxalate crystals (10-20 µ in dia.) present in parenchyma and broken fibers.

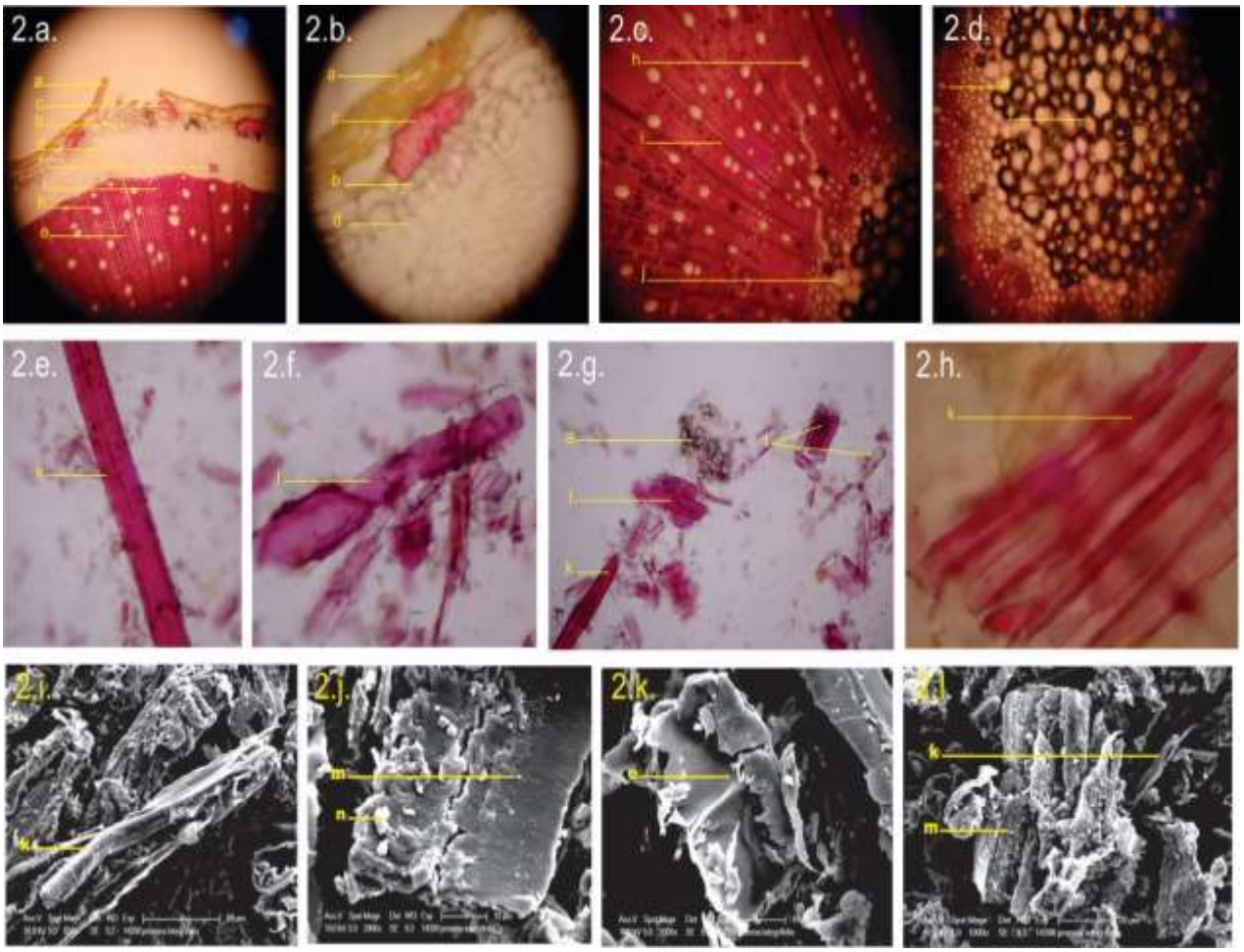

Plate No. : 2.a, 2.b., 2.c., 2.d. - 10x-T.S. Of Stem of *P.integrifolia*, 2.e., 2.f., 2.g., 2.h.-10x-powder characteristics of Stem of *P.integrifolia*, 2.i., 2.j., 2.k., 2.l.- SEM images of powder of Stem of *P.integrifolia*

Labels : a-cork, b-cortex, c-cortical stone cells, d-phloem , e- xylem parenchyma, f- Phloem fibre, h- xylem vessels i- medullary rays, j- pith ,k- solitary fibres-pitted vessels, m-prismatic calcium oxalate crystal, n- starch grain, l- pitted xylem vessel, m-fibres, n- parenchyma.

PI-AK and PI-ET extracts screened for phytochemical test shows presence of alkaloid, glycosides ,tannin and flavonoids as per Table 2.

PI-ET was fingerprinted for marking of bioflavonoid such as Apigenin and Luteolin, were selected as marker as reported in constituents, being flavonoids better extracted out in ethyl acetate extract and being most potential bioflavonoids useful for cancers of the breast, digestive tract, skin, prostate and certain haematological malignancies and protective in other diseases that are affected by oxidative process, such as cardiovascular and neurological disorders.[7,8] Therefore, HPTLC fingerprinting of PI-ET along with Apigenin and Luteolin as biomarker were carried out. It shows better resolution at Rf 0.39 and 0.29 for Apigenin and Luteolin respectively as shown in Plate no. 1.a., 1.b.

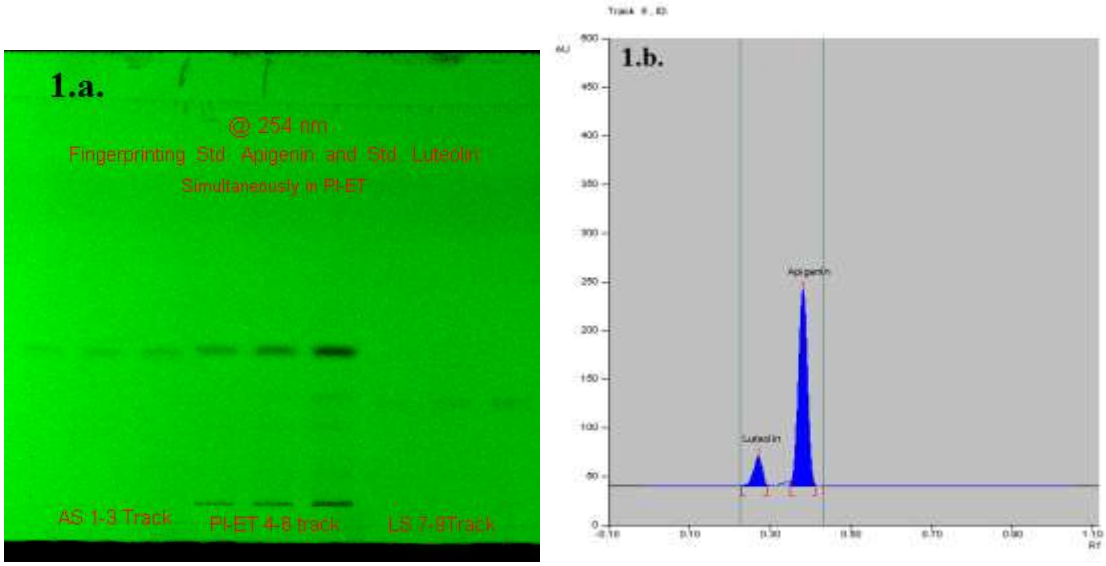

Figure 1a. and 1.b : Resolution chromatogram of PI-Et with marking for Apigenin and Luteolin.

PI-AK was screened for marking of alkaloid Premnine HCl (isolated marker as per Literature [12]) , being alkaloid extracted out in chloroform extract and pharmacologically shows raises blood pressure by contraction of blood vessels, reduces force of contraction of heart and dilates eye pupil [12] .Therefore selected as bioactive marker. Under said chromatographic condition PI-AK shows better resolution at RF-0.59 ,and upon derivatization with Dragendorff's shows orange red colour that even proves in specificity as per Plate No.2.a,2.b.

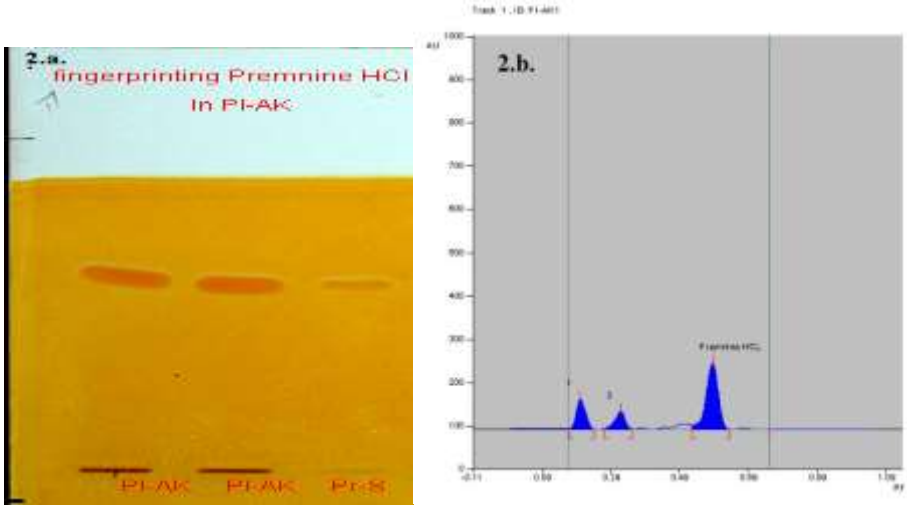

Figure 2a. and 2.b : Resolution chromatogram of PI-AK with marking for Premnine HCl

Stability study of PI-ET done with application of analyte with time interval shows no change in chromatogram of all 4 tracks designed for 3 hours stability in solution and 3 hours on the plate prior to chromatography as per Figure 3.a.,3.b.,3.c.

Stability of analyte during chromatography was studied with 2 D development found acceptable as all zones are located on the diagonal connecting the application position with the intersection of the two solvent front as per Figure 3.d.

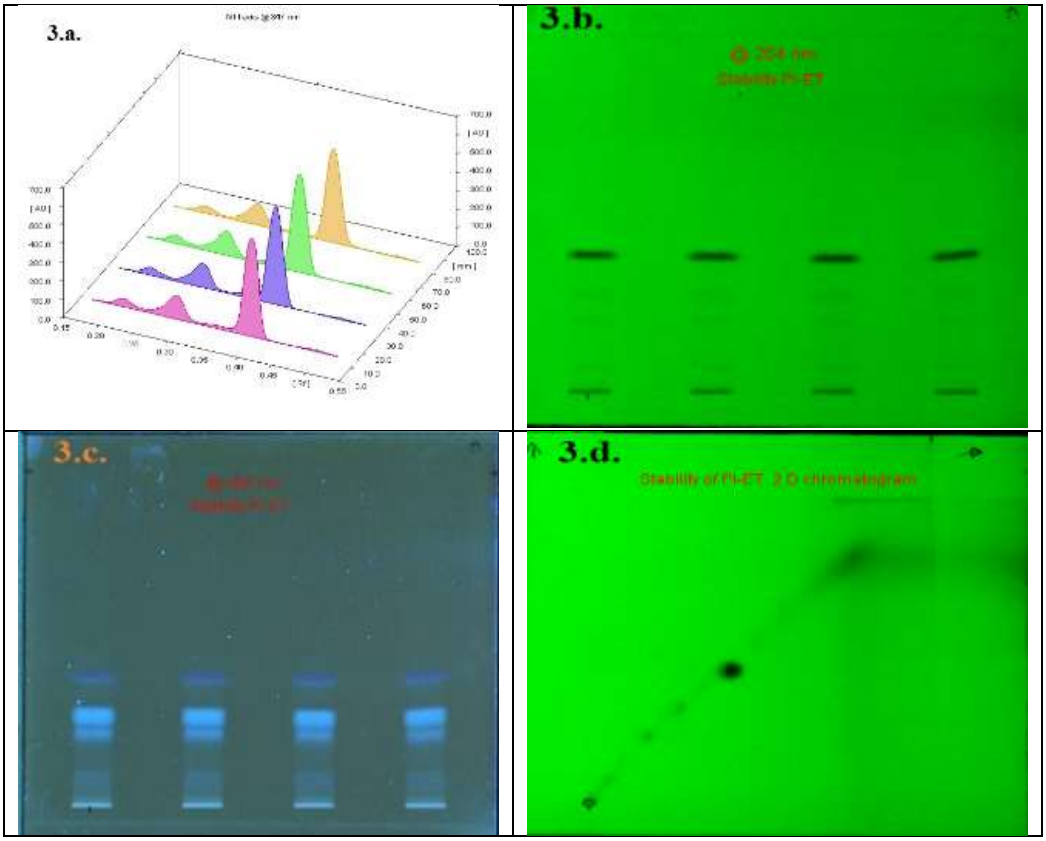

Figure: 3. a. -3DChromatogram for stability Study Of PI-ET in solution, over plate and during chromatogram, 3.b. -chromatogram @254nm, 3.c.chromatogram@366nm, 3.d.-with 2 D development.

CONCLUSION:

Phytochemical screening shows presence of alkaloids, glycosides and flavonoids in respective extracts. PI-ET was marked qualitatively with bioflavonoid Apigenin and Luteolin as standard at Rf 0.39 and .049 respectively. PI-AK was marked qualitatively with bioactive alkaloid Premnine HCl as standard at Rf 0.59. Stability of PI-ET in solution and over plate with time was checked shows persistent chromatogram. Stability during chromatography checked with 2D chromatogram shows all zone in diagonal line. Apigenin , Luteolin and Premnine HCl can be used as biomarker for standardization of *P.integrifolia* .

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