



STEROIDS – CHEMICAL CONSTITUENTS OF *PHYLLANTHUS*

FRATERNUS WEBSTER THROUGH TLC AND HPTLC

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ABSTRACT:

Phyllanthus fraternus Webster (Bhoi amla) is important medicinal plant. Plant tissue culture is very useful to increase the production of secondary metabolites. Callus was obtained on MS supplemented with 1.5 mg/l IBA and 1.5 mg/l BAP. Chromatography is the second important technique for analysis and isolation of these herbal drugs. Qualitative and quantitative analysis was done through TLC and HPTLC. TLC of *in vitro* suggested total 11 unidentified steroids, diosgenin, cholesterol, stigmasterol, β -sitosterol were analysed from *in vitro* (8 week old callus) *P. fraternus*. HPTLC of *in vitro* materials was done and scanned at different wavelengths (200 nm, 254 nm, 366 nm). Calibration spectrum, R_f values and amount of particular spot were recorded at particular wavelength through scanning. Total 11 steroids at 200 nm, 7 at 254 nm, 6 at 580 nm were scanned from *in vitro* (8 week old callus) *P. fraternus*. Absorption spectra of total 7 steroids at 200 nm were obtained from *P. fraternus*. Some of these are confirmed while others are unidentified.

KEY WORD: Steroids, *Phyllanthus fraternus*, TLC, HPTLC.

INTRODUCTION:

Plants are very important commercial source of chemical compounds including primary and secondary metabolites. Ayurveda practitioner employ these plant to cure swelling, poultices lesions, tubercular ulcers, scabies, ophthalmia, muscular pain, dropsy, rheumatism, diabetes and even cancer. These properties are mainly because of its primary and secondary metabolites of drugs. This drug is naturally present in the parts like leaf, stem, root, seed or some times in the whole plant. These parts can be artificially cultured on the media and can get maximum amount of drugs. For it, Murashige and Skoog's, 1962 (MS) medium is used as a basal nutrient medium. Different types of growth promoters like 2, 4-D, IAA, IBA, NAA, Kinetin, BAP are used to prepare hormonal medium. Even it can be prepared with alone auxin or cytokinin or in combination of both (Vasil, 1984). Amount of drugs, which are artificially

produced in 8 week old callus can be compared with naturally occur in plant (whole plant). Plant tissue culture approach has been found to be advantageous as it provides a continuous and reliable source of artificial product year around without the destruction of entire plant. With the help of tissue culture, high quantities of desired compounds can be obtained.

According to Staba (1980), steroids are divided into different four types (1) sterols are alcoholic and 'ol' suffix (β -sitosterol, stigmasterol) (2) sterolin are glycosides of sterol and insoluble in water (cholesterol, estrones) (3) steroidal saponins have 'nin' suffix (diosgenin) (4) nitrogen containing steroidal saponins. Saponins may be either triterpenoid glycoside or glucoside of steroids with spiroketal side chain. It is of four types (1) Triterpenoid saponins (panaxadiol, panaxatriol and oleonic acid) (2) Steroidal saponins (diosgenin, tigogenin, prototokoronin, tokorogenin, yamogenin, gitogenin, manogenin) (3) Nitrogen containing saponins (steroidal alkaloid and solasodine) (4) Cardenolide saponins. Terpenoids and steroids are biosynthesised from isoprene or isopentane (C_5) units evolving from mevalonic acid (C_6). The term 'terpene' refers specifically to hydrocarbon compound. (1) Monoterpenoid compounds may be acyclic, monocyclic (pulegone), bicyclic (menthofuran) or involved in the formation of furanochromones, irridoids, cannabinoids or pyrethrins. (2) Sesquiterpenoids are often found as constituents of steam distillable volatile oil and may be acyclic (fanesol) and cyclic (bisabolene) (3) Diterpenes are formed from four isoprene units. They may be acyclic (phytol) or cyclic (gibberellic acids, abietic acid, gossypol, stevioside) (4) Tetraterpenes are formed from the fusion of two diterpene and normally trans as symmetrical. They are cyclic, monocyclic or bicyclic compounds (carotene, xanthophylls) (5) Triterpenoids may be acyclic (squalene), tricyclic or more complex (β -amyrin, cholesterol). They may occur free or as glucoside (saponins). Plant steroids are most often from cycloartenol and occasionally from lanosterol. Steroids are similar to the triterpenoids (C_{23} to C_{29}) but with only two methyl groups attached to the ring system. The C_{24} ethyl group is derived from successive additions of the methyl group methonine. Steroids are often present in tissue culture as phytosterols (β -sitosterol, stigmasterol and campesterol) with either sitosterol or stigmasterol being the dominant sterol (eg. 24-methylene cholesterol and 24-ethylidene cholesterol in *Withania somnifera*). The growth regulators kinetin and IAA stimulated β -sitosterol and stigmasterol. The two most commonly available steroids are cholesterol and β -sitosterol (Dand, 1970). The sitosterol is a mixture of α , β and γ -sitosterols. Approximately two third of the raw material for chemical synthesis of the steroid hormones produced has depended on diosgenin obtained from the plant *Dioscorea* root (Capsi et al., 1974; Curtin, 1983; Prasad and Ammal, 1983). Besides

diosgenin, other plant sterols such as sitosterol, stigmasterol, campesterol solasodine, hecogenin are considered highly promising precursors.

Hence, the present investigation was taken up for qualitative and quantitative analysis of *in vitro* produced steroids through TLC and HPTLC.

PLANT MATERIAL:

Scientific name : *Phyllanthus fraternus* Webster (Kurup et al., 1979; Kumar and Bendre, 1986)

Synonym : *P. niruri* L. (Sutaria, 1958)

Family : Euphorbiaceae

Common names : Bhumiamalki, Bhoiamli

Chemical constituents:

Alkaloids : Phyllanthin, Hypophyllanthin, Nirphyllin, Phyllnirurin, Phyllanthol, Phyllanthenol, Rhamnopyrenoside, Phyllanthenone, Lintetralin, Astragalin, Cymene, Niranthin, Nirtetralin, Niruriside, Phyllochrysin, 4-Methoxy-Securinine, 4-Methoxy-Nirsecurinine, Limonene, Niruretin, Nirurin, Phyllochrysin

Steroids : β -Sitosterol, Cholesterol

Flavonoids : FG₁, FG₂, Quercetin, Quercetin heteroside, Quercetol, Quercitrin, 3, 4, 5-Trimethoxy flavonone, 3, 5, 7-Trihydroxy flavonol

Other compounds : Estradiol, Carilagin, Ellagic acid, Gallic acid, Rutin, Gernanine, Rutinoside, Lupa, Lupeol, Methyl salicylate,

Saponins : Triacontanal, Triacontanol

Medicinal properties and uses: This plant is stomachic, carminative, diuretic, febrifuge, cooling and astringent. It has anti-dysentric, anti-hepatotoxic and anti-inflammatory, anti-septic, anti-spasmodic, anti-viral, anti-dote to snake bite activities. Whole plant is used in dyspepsia, vertigo, malaria, diabetes, menorrhagia, sores, chronic dysentery, tubercular ulcers, wound, bruises, scabies, ringworm, dropsical infection, gonorrhoea, genito-urinary disorders, jaundice, indigestion, intermittent fever, anemia, cough, gout, urinary disease, dermatosis, miscarriage, abdomen tumour, vaginitis and skin eruption. Leaves are used in scabies, bruises, wound, poultice lesions, swelling, ulcer, spleen and liver disorders and problem of joints. Bark is purgative. Stem is used in ophthalmia.

MATERIALS AND METHODS:

(A) TISSUE CULTURE

1. Preparation of nutrient medium
 - Preparation of stock solutions
 - Mix the solutions and stir it
 - Dissolve sugar and add agar-agar
 - Heat the above solution till agar-agar dissolve
 - Add stock solutions of PGRs and make up the volume
 - Adjust pH with HCl or NaOH to 6.8
 - Pour the medium into culture vials
 - Plug the vials with non-absorbent cotton
 - Autoclave for 15 minutes at 120^o C and cool it
2. Inoculation and Culturing of the explants
 - Explants should be washed in soap water, tap water and finally rinsed with distilled water
 - Under aseptic condition treatment with 0.1% HgCl₂ is given for few minutes and finally rinsed with DW
 - Explants are introduced into the vial over a flame to avoid microbial contamination
 - Immediately plug the vial
3. Maintaining the culture vials under controlled light and temperature conditions
4. Periodic observations of the cultured explants
5. Maintaining the culture through subculture
6. Conclude and make inferences about the experiment conducted / field trial of *in vitro* raised plantlets

(B) THIN LAYER CHROMATOGRAPHY (TLC)

The operation performed in TLC is essentially the same as in paper chromatography. This technique involves several steps:

1. Preparing thin layer (e.g. silica gel G.)
2. Choice of solid for support (e.g. glass plate)
3. Sample application

Extraction of steroids: (Tomita et al., 1970)

Procedure: 100 mg dried and powdered callus were defatted and extracted with 100-150 ml petroleum ether (60-80^oC B.P.) for 2-4 hours in soxhlet apparatus. Petroleum ether extract was

hydrolysed with 5% hydrochloric acid (v/v) in 70% ethanol (v/v) (5 ml HCl in 95 ml 70% ethanol) for four hours on water bath (70-80°C). The hydrolysates (floated residual mass) were extracted with 50 ml ethyl acetate. This step was done for 2-3 times till the residual mass gave colourless solution. All ethyl acetate fractions were collected together and dried *in vacuo*. 2-4 ml Ethyl acetate or chloroform was added to the residue and used for further analysis of steroids.

Sample extract: Residue was redissolved in ethyl acetate or chloroform.

4. Choice of solvent (mobile phase)

Hexane : Acetone (8:2) for steroids

5. Development of chromatograph

6. Detecting or spraying reagent

50% H₂SO₄ (50 ml concentrated H₂SO₄ was diluted up to 100 ml with DW) for steroids

7. Identification and calculation of R_f value

R_f = $\frac{\text{Distance traveled by sample}}{\text{Distance traveled by solvent}}$

Distance traveled by solvent

(C) HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY (HPTLC) (Sethi, 1996)

- Formulation : Herbal drugs
- Classification : Alkaloids / Steroids / Flavonoids
- Dosage form : Liquid
- Sample preparation

Extraction of steroids: (Tomita et al., 1970)

Procedure: 100 mg dried and powdered callus were defatted and extracted with 100-150 ml petroleum ether (60-80°C B.P.) for 2-4 hours in soxhlet apparatus. Petroleum ether extract was hydrolysed with 5% hydrochloric acid (v/v) in 70% ethanol (v/v) (5 ml HCl in 95 ml 70% ethanol) for four hours on water bath (70-80°C). The hydrolysates (floated residual mass) were extracted with 50 ml ethyl acetate. This step was done for 2-3 times till the residual mass gave colourless solution. All ethyl acetate fractions were collected together and dried *in vacuo*. 2-4 ml Ethyl acetate or chloroform was added to the residue and used for further analysis of steroids.

Sample extract: Residue was redissolved in ethyl acetate or chloroform.

➤ Conditions of Chromatography:

1. Test plate : HPTLC precoated plate, silica gel 60 F₂₅₄ – aluminium (Merk)
2. Format : 10 X 10 cm²
3. Thickness : 250 μm

4. Spotting volume : 2 µl, 4 µl, 6 µl, 8 µl (Linomat IV)
 5. Separation technique : Ascending
 6. Development chamber : Twin-trough glass chamber (10 X 10 cm²) (Camag) (saturate for 10 minutes prior to development)
 7. Mobile phase : Hexane : Acetone (8:2) for steroids
 8. Spraying reagent : 50% H₂SO₄ (50 ml concentrated H₂SO₄ was diluted up to 100 ml with DW) for steroids
 9. Relative Humidity : 52%
 10. Temperature : 24° C
 11. Migration distance : 80 mm
 12. Migration time : (30 minutes)
 13. Detection : UV
- Densitometric Scanning (Camag Scanner III)
1. Wavelength and Mode
 - a. 200 / 254 nm - Absorbance / Reflectance
 - b. 366 nm - Florescence / Reflectance
 2. Slit Dimension : 6 X 0.45 mm

RESULTS AND DISCUSSION:

Table (1): Tissue culture results of *Phyllanthus fraternus*

Explants	Level of Auxins		Level of Cytokinins		Callus initiation (In week)				Characters
	Name	Concentration (mg/l)	Name	Concentration (mg/l)	1	2	3	4	
Nodal region with leaf	IBA	0.5	BAP	0.5	-	-	-	+	Yellow green coloured, Compact, Shiny and nodular callus
		1		1	-	-	+	+	
		1.5		1.5	+	++	+++	+++	
		2		2	-	++	++	+++	
Leaf	2,4-D	1	KIN	1	-	-	-	+	Less growth of callus and brown leachate of phenols
		2		2	-	-	+	+	

Table (2): TLC results of *Phyllanthus fraternus* alkaloids from 8 week old callus

Materials	Compounds	Spray treatment		Iodine treatment	
		Rf	Colour	Rf	Colour
Callus	Unidentified 1	-	-	0.26	Brown
	Unidentified 2	-	-	0.30	Brown
	Unidentified 3	-	-	0.33	Brown
	Unidentified 4	-	-	0.35	Brown
	Unidentified 5	-	-	0.40	Dark brown
	Unidentified 6	-	-	0.46	Dark brown
	Unidentified 7	0.50	Blue green	0.51	Dark brown
	Unidentified 8	0.53	Blue green	0.54	Dark brown
	Diosgenin	0.57	Blue green	0.57	Dark brown
	Cholesterol	0.61	Purple	0.62	Yellow brown
	Unidentified 11	0.65	Purple	0.65	Brown
	Unidentified 12	-	-	0.70	Yellow brown
	Unidentified 13	-	-	0.85	Yellow brown
	Stigmasterol	0.88	Blue	0.88	Brown
	β -sitosterol	0.95	Violet	0.95	Brown

Table (3): HPTLC results of Steroids of 8 week old callus of *Phyllanthus fraternus*

Secondary metabolite	Spot number	Scanning at different wavelengths					
		200 nm		254 nm		366 nm	
		Rf	Area %	Rf	Area %	Rf	Area %
Steroids	Spot 1	-	-	0.05	0.90	-	-
	Spot 2	-	-	-	-	0.07	2.32
	Spot 3	0.08	1.72	0.08	3.68	-	-
	Spot 4	0.14	3.80	0.14	2.47	0.13	42.93
	Spot 5	0.20	30.48	0.20	35.94	0.21	45.91
	Spot 6	0.24	30.39	0.24	35.83	-	-
	Spot 7	0.27	16.82	0.27	16.75	-	-
	Spot 8	0.32	8.45	0.32	4.43	-	-
	Spot 9	0.38	2.43	-	-	-	-
	Spot 10	0.49	2.06	-	-	0.49	4.55
	Spot 11	0.65	0.52	-	-	-	-
	Spot 12	-	-	-	-	0.70	2.59
	Spot 13	0.81	2.48	-	-	0.82	1.68
	Spot 14	0.91	0.85	-	-	-	-
Total		11		7		6	

Plant tissue culture or the aseptic culture of cells, tissues and organs is an important tool in both basic and applied studies. Callus was obtained on MS medium supplemented with combinations of IBA + BAP and 2,4-D + KIN in *Withania somnifera*. The satisfactory result was obtained on MS + 1.5 mg/l IBA + 1.5 mg/l BAP (Table-1). Chromatography is the second important technique for analysis and isolation of these herbal

drugs. Qualitative and quantitative analysis was done through TLC and HPTLC. TLC of *in vitro* results suggested total 11 unidentified steroids, diosgenin, cholesterol, stigmasterol, β -sitosterol were analysed from *in vitro* (8 week old callus) *P. fraternus*. Some of secondary metabolites were identified with iodine treatment only (**Table-2**). HPTLC results suggested that total 11 steroids at 200 nm, 7 at 254 nm, 6 at 366 nm were scanned from *in vitro* (8 week old callus) *P. fraternus* (**Table-3, Figure-1, 2, 3, Photo-1, 2, 3**). Absorption spectra of total 7 steroids at 200 nm were obtained from *P. fraternus*. Tempesta and Corley, 1988 have identified phyllanthimide alkaloid from *P. sellowianus*. Miguel et al., 1996 have identified geraniin and furosin from *P. sellowianus*. Filho et al., 1998 have identified from *P. sellowianus* roots. Sittie et al., 1998 have identified alkamides from *P. fraternus*. Ahmad and Alam, 2003 have listed constituents of *P. amarus*. Trivedi et al., 1996; Shah et al., 2000; Ravishankara et al., 2001 have estimated secondary metabolites from *P. niruri*, *Cassia species* and *Cinchona species*. Saxena et al (2012) had deeply studied few medicinal plants and its chemical constituents with the help of TLC and HPTLC at Botany department, Gujarat University, Ahmedabad, Gujarat.

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Figure 1: quantitative estimation at 200 nm through HPTLC

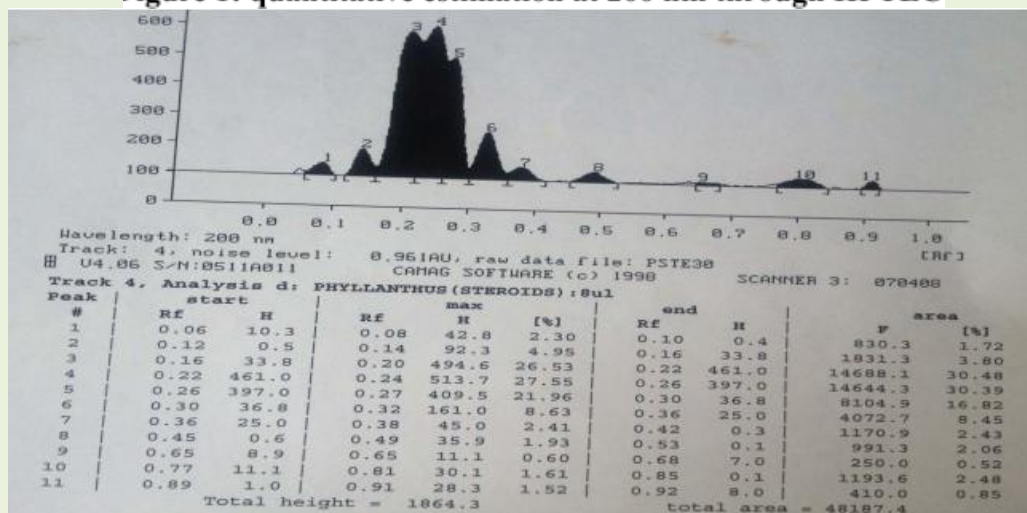


Figure 2: quantitative estimation at 254 nm through HPTLC

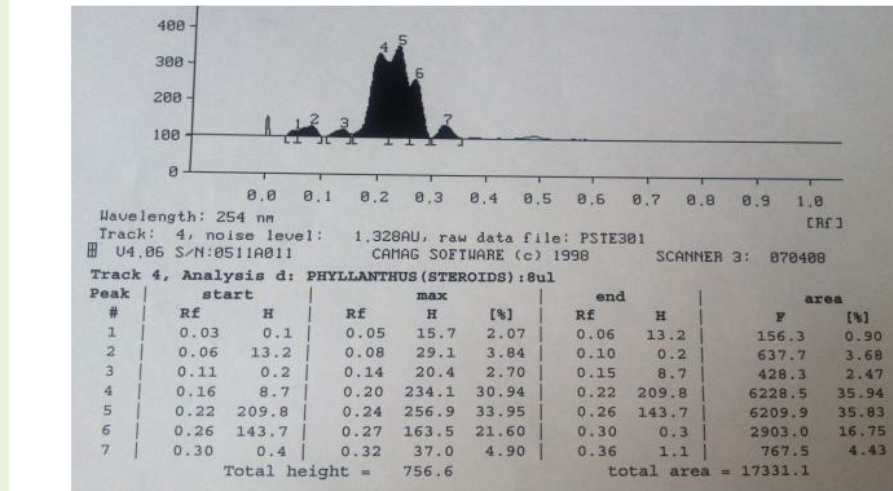


Figure 3: quantitative estimation at 366 nm through HPTLC

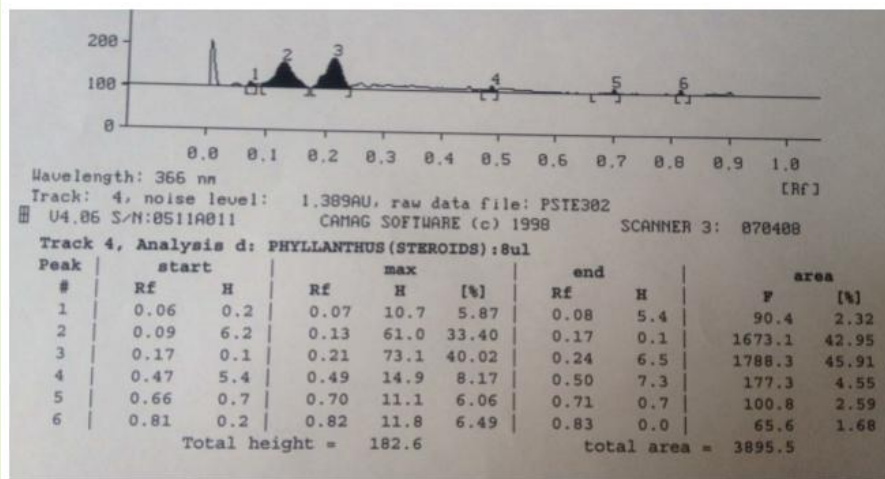


Photo 1: HPTLC photo under fluorescent light

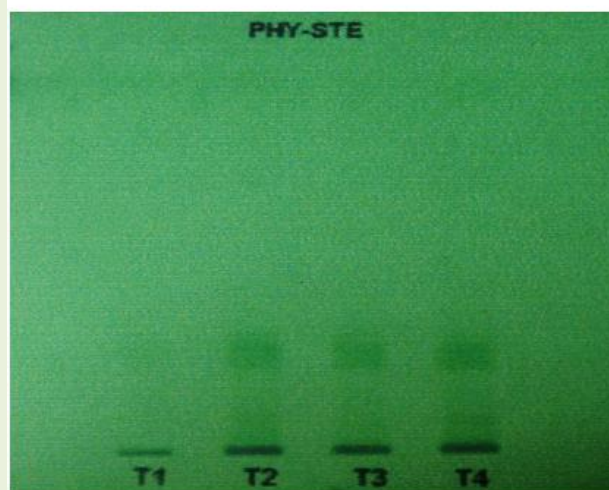


Photo 2: HPTLC photo under UV light

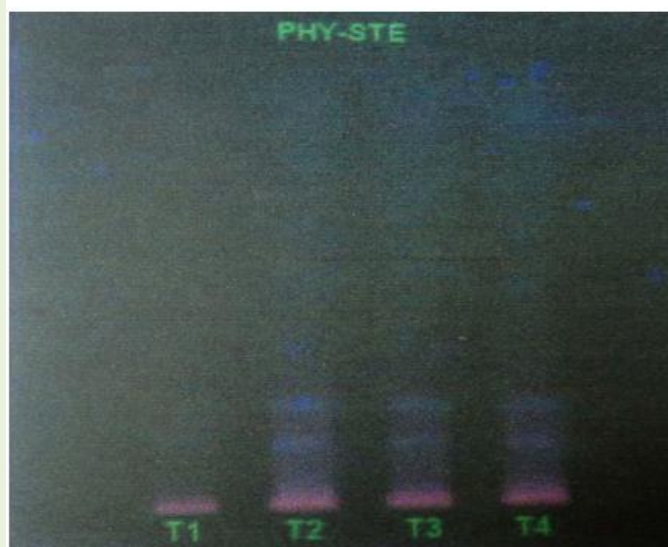


Photo 3: HPTLC photo under Visible light

