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QUANTIFICATION OF β-SITOSTEROL FROM FIELD GROWN PLANTS AND CALLUS OF *CRATAEVA TAPIA* L.

Priyanka Sharma[*1], Avinash Patil[1] and Darshana Patil [2]

Department of Botany [1] Birla College, Kalyan, Mumbai, Maharashtra, India.
Smt. C.H.M. College [2], Ulhasnagar, Mumbai, Maharashtra, India.

Key words:

Crataeva tapia L., HPTLC, β-sitosterol, callus

Correspondence to Author:
Priyanka Sharma

Department of Botany
Birla College, Kalyan- 421304.
M.S., India.

Email: priyanka.s1909@gmail.com

ABSTRACT: *Crataeva tapia* L. (Family- Capparaceae) is an important medicinal tree, commonly called as 'Varuna'. The plant has been reported to possess several medicinal properties. Propagation of *Crataeva tapia* L. is mostly by seeds, but germination percentage is very low mainly due to presence of hard seed coat. Conservation and propagation of species using biotechnological tools like plant tissue culture are relevant when natural propagation is hampered. Plant cell culture systems have potential for commercial exploitation of secondary metabolites. These secondary metabolites can then be analyzed using chromatographic techniques. In the present study, a simple, precise, rapid and selective High Performance Thin Layer Chromatography (HPTLC) method was developed and validated for detection and quantification of phytochemical marker β-sitosterol in leaf, bark, leaf callus and stem callus of *Crataeva tapia* L. as per ICH guidelines.

INTRODUCTION: Medicinal plants are the most important source of life saving drugs for the majority of the world's population [1]. There is great demand for herbal medicine in the developed as well as developing countries like India, because of their wide biological activities, higher safety of margin and low costs than the synthetic drugs [2].

Though India has rich biodiversity and one among the twelve mega diversity centers, the growing demand is putting a heavy strain on the existing resources causing a number of species to be either threatened or endangered. While over 800 species are used in production by industry, less than 20 species of plants are under commercial cultivation. This poses a definite threat to the genetic stocks and to the diversity of medicinal plants [3].

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Plants produce a wide and diverse assortment of organic compounds, the great majority of which do not appear to participate directly in growth and development. These substances, referred to as secondary metabolites, often are differentially distributed among limited taxonomic groups within plant kingdom [4]. The secondary metabolites are known to play a major role in the adaptation of plants to their environment and also represent an important source of pharmaceuticals [5]. Currently many of these secondary metabolites are extracted from the naturally grown whole plants [6].

Crataeva tapia Linn. ssp. *odora* (Jacob.) Almedia (*Crataeva adansonii* subsp. *odora* (Buch.-Ham.) Jacobs {Syn,: *Crataeva tapia* Linn. ssp. *odora* (Jacob.) Almedia} belonging to family Capparaceae is a much branched deciduous tree, commonly called as 'Varuna' [7]. The plant parts (leaf and bark) have been reported to possess several medicinal properties which include anti-inflammatory [8], anti-oxidant [9], anti-arthritic [10], anti-fertility [11], anti-mycotic [12], anti- diabetic [13], anti-

bacterial [14], anti-diarrhoeal [15], wound healing [16], anti-helminthic [17], urolithic property [18], nephrolithic property [19], hepatoprotective and cardio protective activity [20].

With the increase in the demand for the crude drugs, the plants are being overexploited, threatening the survival of many rare species. Also, many medicinal plant species are disappearing at an alarming rate due to rapid agricultural, urban development, uncontrolled deforestation and indiscriminate collection [21]. Large scale plant tissue culture is found to be an attractive alternative approach to traditional methods of plantation as it offers controlled supply of secondary metabolites independent of plant availability [22]. Callus is the most suitable material for increasing secondary metabolite production [23]. The callus tissues can be extracted by suitable solvents to isolate secondary metabolites [24].

These secondary metabolites can further be screened for detection of major phytoconstituents by different chromatographic techniques. TLC and HPTLC are flexible and cost effective techniques presenting the advantage of the simultaneous detection possibilities, including the great variety of post-chromatographic derivatization reagents [25]. Phytosterols are a subgroup of steroids, an important class of bioorganic molecules, widespread in plants and have similarity to cholesterol in structure. β-sitosterol belonging to this group possesses anti-inflammatory [26, 27], hypocholesterolemic [28, 29] anti-helminthic [30], immuno modulatory [31], benign prostatic hyperplasia [32], antioxidant [33] and anti diabetic [34, 35, 36] activities.

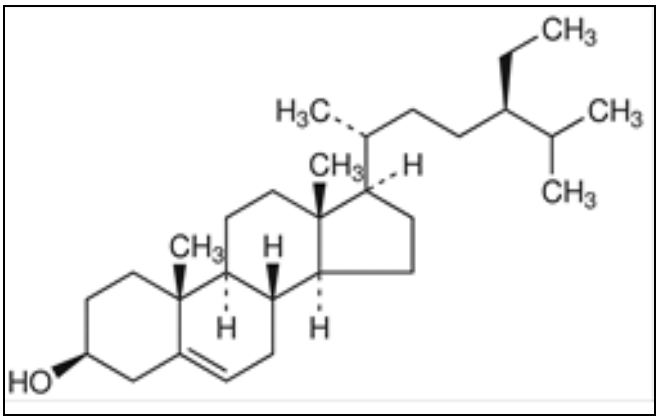

FIG. 1: STRUCTURE OF β-SITOSTEROL

Therefore, the present study deals with development and validation of simple, sensitive and accurate HPTLC method for quantification of β-sitosterol in methanolic extract of leaf, bark, leaf callus and stem callus.

MATERIAL AND METHODS:

Collection of plant material:

The flowering twig of *Crataeva tapia* L. was collected from Kalyan, M.S., India. Herbarium was prepared and authenticated from Blatter herbarium, St. Xavier's College, Mumbai, M.S., India. Leaf and bark were collected and dried in oven at 40 ± 2^0C for a week. After drying, the plant material was homogenized to fine powder, sieved through 100 microns mesh and stored till further use.

Establishment of callus culture:

Leaf and stem from field grown plants of *Crataeva tapia* L. (Kalyan) were selected for *in vitro* culture studies. The explants were treated with 1% Sodium hypochlorite (NaOCl, v/v); 1 minute for leaf and 3 minutes for stem. The explants were then rinsed with autoclaved distilled water thrice. The explants were further treated with 0.5% Mercuric chloride ($HgCl_2$, w/v); 3 minutes for leaf and 5 minutes for stem and rinsed thoroughly with sterile distilled water thrice. Both the explants were trimmed from edges and inoculated on Murashige and Skoog medium (Murashige and Skoog, 1962) fortified with fortified with various concentrations of 2,4-D, BAP, IAA, NAA, singly and combination of BAP and 2,4-D with and without 10% Coconut Milk (CM).

After inoculation, the culture tubes were first placed in dark for one week and then transferred to light intensity of 2000- 3000 lux with 16 hours photoperiod and 8 hours dark period at 25 ± 2^0C. Periodic observations were made and the results were documented and analyzed. The callus was sub cultured at regular interval in respective medium for further growth.

The calli obtained were subjected to drying in hot air oven at 50^0C for 3-4 days. After drying, callus was powdered using mortar and pestle and kept in an air-tight bottle and stored at room temperature.

Reference standard and Reagents:

Reference standard β-sitosterol (≥95% purity) was procured from Sigma-Aldrich. Toluene, Methanol, Anisaldehyde and Sulphuric acid used in the present work were procured from S. D. Fine Chemicals, Mumbai, India.

Preparation of standard β-sitosterol solution:

The stock solution of standard β-sitosterol (500µg/ml) was prepared in methanol. For calibration curve, 500 µg/ml of standard β-sitosterol was further diluted with methanol to give concentration range of 0.4-3.5 µg/ml.

Preparation of Sample extract:

1g of powdered material (leaf, bark, leaf callus and stem callus) was weighed and phytoconstituents were extracted in 10 ml methanol by vortexing for 1-2 minutes, left to stand overnight at R.T. (26 ± 2^0C). The extracts were filtered through Whatmann filter paper No. 41 (E. Merck, Mumbai, India) and the filtrate was used for quantification.

HPTLC Instrumentation:

Chromatographic conditions:

10 µl of extracts were loaded on precoated silica plates (silica gel 60 F_{254} - Merck) as bands of 8 mm width using CAMAG LINOMAT 5 applicator at distance of 14 mm from the edge of the plate. The plate was developed to a distance of 85 mm in a CAMAG twin trough chamber (20 X 10 cm) presaturated for 25 minutes with mobile phase, Toluene: Methanol: Glacial acetic acid (9:1:0.1 v/v). The chromatographic conditions had been previously optimized to achieve best resolution and peak shape.

The plates were further derivatized using Anisaldehyde-sulphuric acid and heated at 105^0C for 5 minutes, visualized using CAMAG TLC visualizer and scanned densitometrically at 520 nm in absorbance mode with tungsten lamp using CAMAG TLC Scanner 3 in conjunction with winCATS software.

Method Validation:

The proposed HPTLC method was validated according to the International Conference on Harmonization guidelines [37 - 43].

1. **Instrumental Precision:**

Instrumental precision of the method was checked by repeated scanning (n=12) of same spot of β-sitosterol (250 µg/ml) and results were expressed as % RSD.

2.

3. **Interday and Intraday Precision:**

Interday and intraday precision was studied by analyzing aliquots of standard β-sitosterol (250µg/ml) on same day (intraday) and on different days (interday) in triplicates. The results were expressed as % RSD.

4.

5. **Linearity:**

Stock solution of 500 µg/ml of β-sitosterol was diluted with methanol to give a range of 0.4 µg/ml to 3.5µg/ml. Each of these concentrations was loaded, plates were developed and detector response for different concentration was measured. Graph of peak area against concentration of β-sitosterol was plotted.

6.

7. **Limit of Detection (LOD) and Limit of Quantification (LOQ):**

For LOD and LOQ determinations, different concentrations of standard solution were applied along with methanol as blank and determined on the basis of signal to noise ratio. LOD was considered as 3:1 and LOQ as 10:1.

8.

9. **Ruggedness:**

Ruggedness of the method was checked by introducing changes in chromatographic parameters, such as mobile phase composition, spotting volume to determine their influence on Rf.

Accuracy

The accuracy of the method was assessed by performing recovery studies at three different levels (80, 100 and 120% by spiking β-sitosterol).

RESULTS AND DISCUSSION:

In the present study, β-sitosterol has been quantified from *Crataeva tapia* L. extracts (leaf, bark, leaf callus and stem callus) by HPTLC densitometric method as per the ICH guidelines. It was found that β-sitosterol resolved well using the solvent system of Toluene: Methanol: Glacial acetic acid (9:1:0.1v/v) (Plate No: 1.1, 1.2, 2.1 and 2.2).

Method Validation:

Instrumental precision:

Instrumental precision was checked by repeated scanning of the same band of β-sitosterol for twelve times and the results are expressed as % RSD (**Table 1).**

Intraday and Interday Precision:

Standard β-sitosterol was spotted both at intra-day (spotting three times within 24h) and inter-day (spotting three times within 3 days intervals) to check the precision. The results are expressed as % RSD (**Table 1**). The results indicate that the method is precise and reproducible.

Linearity:

A good linearity was achieved in the concentration range (0.4 - 3.5 µg/ml) used for β-sitosterol in leaf, bark, leaf callus and stem callus. The densitograms were recorded and the peak areas of β-sitosterol for each applied concentration were noted. LOD and LOQ were found to be 0.09µg/ml and 0.28 µg/ml respectively (**Table 1**).

Ruggedness:

The method was found rugged for the parameters like change in mobile phase composition, change in spotting volume and detection wavelength. No significant changes in R_f or response to β-sitosterol was observed, indicating the ruggedness of the method (**Table 1**).

Accuracy:

The accuracy of the method was established by performing recovery experiments, using the standard addition method, at different levels (80%, 100% and 120%) of 250 ppm standard β-sitosterol solution. Percentage recoveries for β-sitosterol are given in **Table 2**.

Quantification of β-sitosterol in Leaf, Bark, Leaf Callus and Stem Callus:

The presence of β-sitosterol was confirmed by comparing Retention factor (Rf) and color of the sample with that of standard solution of β-sitosterol. The concentrations of β-sitosterol in various samples are depicted in **Table 3**. Amount of β-sitosterol in leaf and bark extracts was found to be 1.7 mg/g and 0.79 mg/g respectively.

TABLE 1: QUANTIFICATION PARAMETERS FOR β-SITOSTEROL

S.No.	Parameters	β-sitosterol
1.	Linearity range (µg/ml)	0.4-3.5
2.	LOD (µg/ml)	0.09
3.	LOQ (µg/ml)	0.28
4.	Instrument precision (% RSD, n=12)	0.26
5.	Intraday precision (% RSD, n= 3)	0.72
6.	Interday precision (% RSD, n= 3)	1.4
7.	Ruggedness	Rugged

TABLE 2: RECOVERY STUDIES OF β-SITOSTEROL AT 80%, 100% AND 120% ADDITION

S.No	Samples	Amount of β-sitosterol present in sample (µg)	Amount of β-sitosterol added (µg)	Recovery (%)	Average of β-sitosterol found (µg)
1.	Leaf	170.00	1	99.45	
2.			1.25	103.26	100.89±1.7
3.			1.5	99.96	
4.	Bark	39.64	1	97.83	
5.			1.25	97.24	98.36±1.2
6.			1.5	100.02	
7.	Leaf callus (MS + 2.5	119.15	1	104.06	99.87±3.4
8.	mg/l 2,4-D)		1.25	95.63	
9.			1.5	99.91	
10.	Stem callus (MS +	340.80	1	98.71	
11.	2.5 mg/l 2,4-D + 10%		1.25	100.37	100.72±1.8
12.	CM)		1.5	103.07	

TABLE 3: QUANTIFICATION OF β-SITOSTEROL IN LEAF, BARK, LEAF CALLUS AND STEM CALLUS

S.No.	Extracts	Content of β-sitosterol (mg/g)
1.	Leaf	1.70±0.02
2.	Bark	0.79±0.01
3.	MS + 2.5 mg/l 2,4-D leaf callus	1.19±0.02
4.	MS + 2.5 mg/l BAP leaf callus	0.98±0.09
5.	MS + 0.5 mg/l BAP + 2.5 mg/l 2,4-D leaf callus	1.17±0.14
6.	MS + 2.5 mg/l IAA leaf callus	0.75±0.14
7.	MS + 2.0 mg/l NAA leaf callus	0.61±0.06
8.	MS + 0.5 mg/l 2,4-D + 10% CM leaf callus	0.74±0.04
9.	MS + 2.5 mg/l BAP + 10% CM leaf callus	0.36±0.04
10.	MS + 0.5 mg/l BAP + 2.5 mg/l 2,4-D + 10% CM leaf callus	0.52±0.13
11.	MS + 2.0 mg/l IAA + 10% CM leaf callus	0.86±0.11
12.	MS + 2.0 mg/l NAA + 10% CM leaf callus	1.23±0.12
13.	MS + 0.5 mg/l 2,4-D stem callus	0.71±0.04
14.	MS + 2.5 mg/l BAP stem callus	0.42±0.04
15.	MS + 0.5 mg/l BAP + 2.5 mg/l 2,4-D stem callus	0.88±0.06
16.	MS + 0.5 mg/l IAA stem callus	1.61±0.06
17.	MS + 2.5 mg/l NAA stem callus	2.68±0.05
18.	MS + 2.5 mg/l 2,4-D + 10% CM stem callus	3.41±0.12
19.	MS + 2.5 mg/l BAP + 10% CM stem callus	1.11±0.10
20.	MS + 0.5 mg/l BAP + 2.5 mg/l 2,4-D + 10% CM stem callus	1.51±0.10
21.	MS + 2.0 mg/l IAA + 10% CM stem callus	1.03±0.01
22.	MS + 1.5 mg/l NAA + 10% CM stem callus	0.85±0.06

Observation in visible light at 520 nm.

Amount of β-sitosterol present in leaf calli obtained on MS medium fortified with various concentrations of PGR's and 10% CM *viz.*, 2.5 mg/l 2,4-D, 2.5 mg/l BAP, 0.5 mg/l BAP + 2.5 mg/l 2,4-D, 2.5 mg/l IAA, 2.0 mg/l NAA, 0.5 mg/l 2,4-D + 10% CM, 2.5 mg/l BAP + 10% CM, 0.5 mg/l BAP + 2.5 mg/l 2,4-D + 10% CM, 2.0 mg/l IAA + 10% CM and 2.0 mg/l NAA + 10% CM was found to be 1.19 mg/g, 0.98 mg/g, 1.17 mg/g, 0.75 mg/g, 0.61 mg/g, 0.74 mg/g, 0.36 mg/g, 0.52 mg/g, 0.86 mg/g and 1.23 mg/g respectively.

Amount of β-sitosterol in stem calli obtained on MS medium fortified with various concentrations of PGR's and 10% CM *viz.*, 0.5 mg/l 2,4-D, 2.5 mg/l BAP, 0.5 mg/l BAP + 2.5 mg/l 2,4-D, 0.5 mg/l IAA, 2.5 mg/l NAA, 2.5 mg/l 2,4-D + 10% CM, 2.5 mg/l BAP + 10% CM, 0.5 mg/l BAP + 2.5 mg/l 2,4-D + 10% CM, 2.0 mg/l IAA + 10% CM and 1.5 mg/l NAA + 10% CM was found to be 0.71 mg/g, 0.42 mg/g, 0.88 mg/g, 1.61 mg/g, 2.68 mg/g, 3.41 mg/g, 1.11 mg/g, 1.51 mg/g, 1.03 mg/g and 0.85 mg/g respectively.

Highest concentration of β-sitosterol (3.41 mg/g) was detected in stem callus obtained on MS medium fortified with 2.5 mg/l 2,4-D along with 10 % CM.

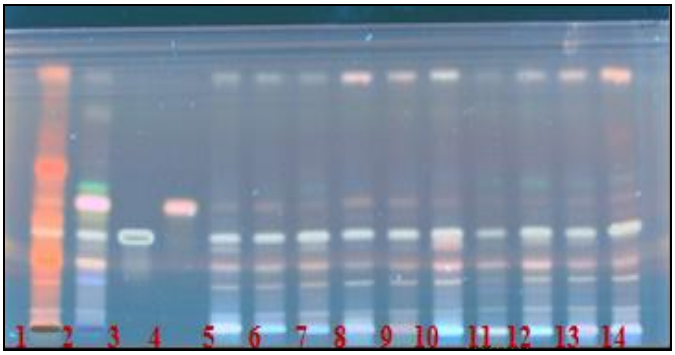

PLATE 1.1: HPTLC FINGERPRINT OF LEAF, BARK AND LEAF CALLUS (MS MEDIUM) OF *CRATAEVA TAPIA* L. UNDER UV LIGHT (366 nm)

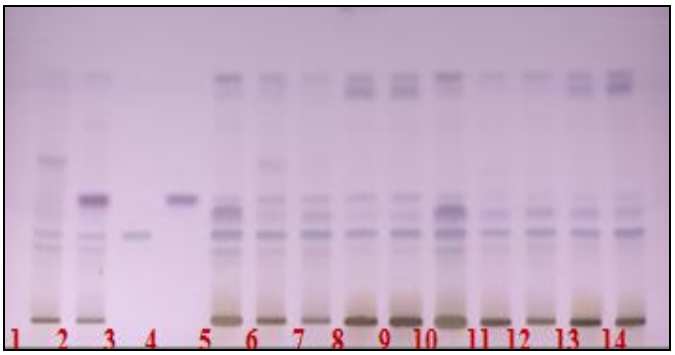

PLATE 1.2: HPTLC FINGERPRINT OF LEAF, BARK AND LEAF CALLUS (MS MEDIUM) OF *CRATAEVA TAPIA* L. UNDER VISIBLE LIGHT

1: Leaf extract; 2: Bark extract; 3: Standard β-sitosterol, 4: Standard Lupeol, 5: 2.5 mg/l 2,4-D; 6: 2.5 mg/l BAP; 7: 2.5 mg/l BAP + 0.5 mg/l 2,4-D; 8: 2.5 mg/l IAA; 9: 2.0 mg/l NAA;10: 0.5 mg/l 2,4-D + 10% CM leaf callus; 11: 2.5 mg/l BAP + 10% CM; 12: 2.5 mg/l 2,4-D + 0.5 mg/l BAP + 10% CM; 13: 2.0 mg/l IAA + 10% CM; 14: 2.0 mg/l NAA + 10% CM

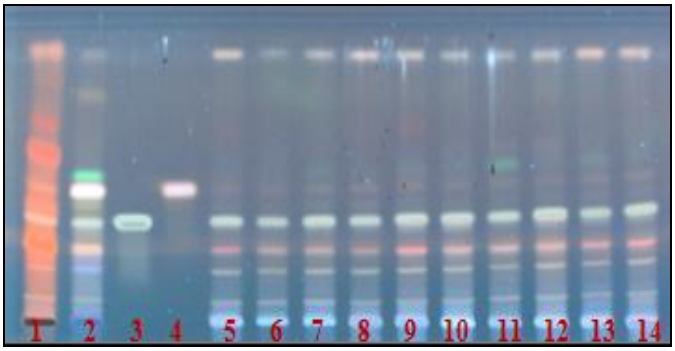

PLATE 2.1: HPTLC FINGERPRINT OF LEAF, BARK AND STEM CALLUS (MS MEDIUM) OF *CRATAEVA TAPIA* L. UNDER UV LIGHT (366 nm)

PLATE 2.2: HPTLC FINGERPRINT OF LEAF, BARK AND STEM CALLUS (MS MEDIUM) OF *CRATAEVA TAPIA* L. UNDER VISIBLE LIGHT

1: Leaf extract; 2: Bark extract; 3: Standard β-sitosterol, 4: Standard Lupeol, 5: 0.5 mg/l 2,4-D; 6: 2.5 mg/l BAP; 7: 0.5 mg/l BAP + 2.5 mg/l 2,4-D; 8: 0.5 mg/l IAA; 9: 2.5 mg/l NAA; 10: 2.5 mg/l 2,4-D + 10% CM; 11: 2.5 mg/l BAP + 10% CM; 12: 2.5 mg/l 2,4-D + 0.5 mg/l BAP + 10% CM; 13: 2.0 mg/l IAA + 10% CM; 14: NAA + 10% CM

Enhancement or production of secondary metabolites using Plant Tissue Cultures can be obtained by inducing callus culture. Callus formation is governed by the source of explants, nutritional composition of the medium and environmental factors [44]. An important milestone in the callus culture is the role of auxin and cytokinin interaction [45, 46]. To maximize the formation of secondary metabolites in Plant Tissue Cultures, it is desirable to initiate callus from the plant part that is known to be a high producer [24]. Callus cells are totipotent [47, 48] and have been widely used in both basic research and industrial applications [49, 50].

Plant Tissue Culture techniques offer the rare opportunity to tailor the chemical profile of a secondary metabolite, by manipulation of the chemical or physical microenvironment, to produce a compound of potentially more value for human use [51]. Plant Tissue Cultures have several advantages because the secondary metabolites are produced in cells, tissues or organs that are cultivated under sterile conditions [52, 4]. The principle advantage of this technique is that it may provide continuous, reliable source of plant secondary metabolites in *in vitro* plant tissues from which these metabolites can be extracted [44]. The utilization of plant tissue culture for the production of secondary metabolites of commercial interest has gained increasing attention over past decades [45].

In the present work, β-sitosterol was detected and quantified in leaf, bark, leaf callus and stem callus of *Crataeva tapia* L. Highest concentration of β-sitosterol (3.41 mg/g) was detected in stem callus on MS medium fortified with 2.5 mg/l 2,4-D along with 10 % CM as compared to leaf (1.7 mg/g) and bark (0.79 mg/g). β-sitosterol was found at higher concentration in callus culture which can serve as an alternative for secondary metabolite production thus helping in conservation of *Crataeva tapia* L.

CONCLUSION: β-sitosterol was detected and quantified in leaf, bark, leaf and stem callus of *Crataeva tapia* L. which can serve as an alternative for secondary metabolite production thus helping in conservation of the plant. Plant Tissue Culture systems could be used for the large scale culturing of plant cells from which secondary metabolites can be extracted and it can provide a continuous, reliable source of natural products.

In the present study, an efficient HPTLC method with simple sample preparation protocol was developed and validated for detection and quantification of an important phytosterol; β-sitosterol from the methanolic extract (leaf, bark, leaf callus and stem callus) of *Crataeva tapia* L.

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