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QUANTIFICATION OF STIGMASTEROL UNDER *IN VIVO* AND *IN VITRO* PLANT EXTRACTS OF CHLOROPHYTUM SPS

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ABSTRACT

In the present work, a unique attempt has been made to extract from *in vivo* and *in vitro* plant parts along with callus and undergone HPTLC (High Performance Thin Layer Chromatography) analysis to quantify stigmasterol from *Chlorophytum borivilianum* Santapau & R. R. Fern and *Chlorophytum tuberosum* (Roxb.) Baker. Crude extract of both these plant (*in vivo* as well as *in vitro*) were isolated with polar solvent n-hexane. Stigmasterol was quantified by HPTLC (toluene: methanol 9.5:0.5 [v/v] as mobile phase) following ICH (International Council for Harmonisation) guide lines at R_f (Retention factor) 0.34 which gave single peak at 540nm after derivatization. Method validation carried by applying samples on precoated silica gel 60 F_{254} TLC (Thin Layer Chromatography) plates and found out the precision, accuracy, specificity, reproducibility along with linearity. Standard stigmasterol was applied in the range of 4-9 µg/fractions. The intra-day precision value appeared to be 24.11% RSD (Relative standard deviation) concomitantly 24.77% RSD inter-day precision, that makes the method precise and reproducible. The limit of detection (LOD) - 4µg and Limit of Quantification - 12µg values for both the plant varieties observed to be accurate. The percentage recovery at values of three different levels was profoundly found to be more than 90%.This method clarified the knowledge of availability of stigmasterol in both plant varieties which would be beneficial for pharmaceutical industries.

KEYWORDS: **Stigmasterol, Chlorophytum, HPTLC, Quantification**

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INTRODUCTION

Description of plants

Chlorophytum sps. (Family Liliaceae) is commonly known as 'safed musli' famous for its adaptogenic and immunomodulatory properties; useful in curing sterility which leads to enhancement in male potency. The anti-tumorous activity has been shown by roots as it is prevalent with saponins[1,2] . Worldwide in excess of 175 species of Chlorophytum have been described. Several species were earlier recognized such as *C. borivilianum*, *C. arundinaceum* and *C. tuberosum* etc. Safed musli is prominently called as 'wonder drug'. As it possesses high therapeutic importance, these tubers were mostly part of preparations for Ayurvedic preparations[3]. *Chlorphytum borivilianum* Santapau & R. R. Fern reported having maximum yield and saponin content (2-17% in safedmusli) among these plants[4,5] . The roots of *C. boriavilinum* (Roxb.) Baker contains amino acids, alkaloids, vitamins, saponins, steroids, resins, and phenol[6].

Stigmasterol

Earlier reports suggest that roots mostly contain phytosterols like stigmasterol, β-sitosterol, diosgenin and sarasapogenin[7]. Among phytosterol, stigmasterol found mostly in many species (human chondrocytes, mouse chondrocytes etc.) as anti-hypercholesterolemic, anti-osteoarthritic, anti-inflammatory and laxative[8–10]. The literature review provides that human intake of stigmasterol enhances in the reduction of low-density lipoprotein levels in total serum cholesterol leads to reduce the risk of coronary heart disease[11]. Stigmasterol has also been used in hair treatment and involves in nullifying cobra and viper venoms[12].

HPTLC related

Among the analytical methods HPTLC has cost effective, accuracy, simple to use, speed and reproducible[13–15]. HPTLC can also be better used for quantitative analysis along with ascreening of plant products[16] and typically used for quality control valuation and food investigation of certain medicinal herbs like *Leptadenia reticulata*[6]. Keeping the above aspects in mind, a unique method for rapid screening and quantitative analysis were carried on *in vivo*and*in vitro*plant extracts of *Chlorophtyum*sps, like *borivilianum* and *tuberosum*. Earlier some separation techniques such as GC-MS,HPLC and RP-HPLC has been advised for separation techniques for some medicinal plants derived compounds[17–18].HPTLC acts as a surrogate chromatographic model for estimating partitioning properties in support of the environmental strategy, combinatorial chemistry and health-related issues[19].

MATERIALS AND METHODS

Plant material

The explants (*Chlorophytum borivilianum* Santapau & R. R. Fern and *Chlorophytum tuberosum* (Roxb.) Baker were procured from Medicinal and Aromatic Research Centre, Anand Agriculture University, Anand Gujarat. The tuber segments were carried for tissue culture and produced *in vitro* plants. HPTLC analysis was carried on those *in vivo* and *in vitro* plant parts by stigmasterol as standard.

Chemicals

The marker stigmasterol (Purity: 97% w/w) was procured from Natural Remedies Pvt. Ltd, Bangalore, India. HPTLC grade chemicals were purchased from Merck chemicals, Mumbai, India.

Chromatographic Conditions

Aluminium sheet (precoated silica gel) – 60 F254 TLC plates (E. Merck)-0.25mm thickness TLC plates- 20 x 10cm.

HPTLC instrument particulars

CAMAG Linomat 5 Automatic Sample Spotter (Camag Muttenz, Switzerland) with a100ml syringe (from Hamilton) along with CAMAG glass twin trough chamber (20 x 10 cm); CAMAG TLC Scanner 4, winCATS planar chromatography Manager; temperature (25±2^{0}C), Relative humidity of 40%.

Sample preparation

The *in vivo* and *in vitro* plant parts (*Chlorophytum borivilianum* Santapau & R. R. Fern [CB] roots- CBR, CB leaves –CBL, CB *in vitro* roots- CBIR, CB *in vitro* leaves- CBIL, CB *in vitro* callus- CBIC, and *Chlorophytum tuberosum* (Roxb.) Baker [CT] roots-CTR, CT leaves-CTL, CT *in vitro* roots-CTIR, CT *in vitro* leaves- CTIL, CT *in vitro* callus- CTIC) were dried in an oven at 90^{0}C for 3hr with a gap of 1 day for 3 consecutive days. Each dried samples were ground in a blender. 2 gm from each of the powder was mixed with n-hexane for extraction (3 x 25ml). Each flask was placed on a shaker for 3hrs at 110 rpm. After incubation for overnight at room temperature all the extracts were filtered through Whatman No.1 filter paper and undergone vacuum evaporation to get a solid extract. 0.1gm of this extract was mixed thoroughly in 1ml n-hexane and used for quantification of stigmasterol.

Standard solution of stigmasterol

1mg of stigmasterol was mixed properly with 1ml of chloroform to make it 1mg/ml concentration.

Solvent system

Toluene:methanol (9.5:0.5 v/v) prepared for quantification of stigmasterol in each of the extracted samples.

Procedure

5µl of the samples were applied along with standard in HPTLC sample applicator and after drying it with a hairdryer dipped it into amobile phase which was presaturated for 20 minutes. After development and derivatization with anisaldehyde, chromatograms were captured with CAMAG-automatic image developer at 540nm (Fig. I A, B and IIA, B). The peak area of the stigmasterol amount was measured with purity of the marker 100%.

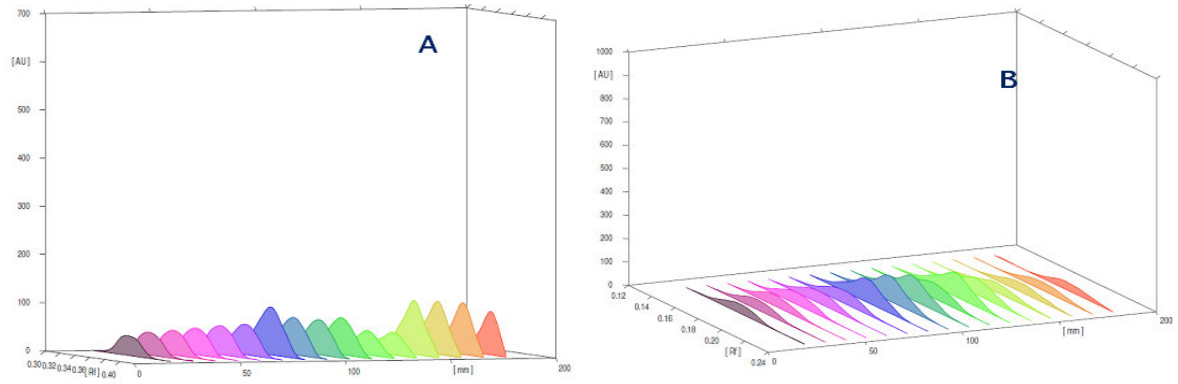

Figure 1
All tracks at 540 nm Linearity of Chlorophytum borivilianum (A) and Chlorophytum tuberosum (B) extracts along with stigmasterol

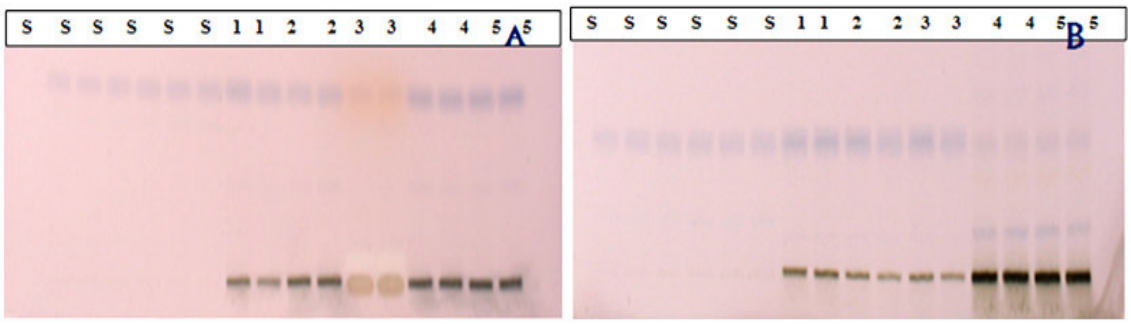

Figure II
(A & B) TLC profile of the samples at 540nm remission; S-Stigmasterol, 1.CBR, 2. CBL, 3. CBIR, 4. CBIL, 5. CBIC, 6. CTR, 7. CTL, 8. CTIR, 9. CTIL and 10. CTIC.

Validation of method
As per ICH guidelines, linearity, LOD, LOQ, accuracy (percentage recovery), intra-day & inter-day precision along with reproducibility and quantification was made.

Quantification of stigmasterol
Standard stigmasterol was applied in the range of 4-9µg/fractions on 6 levels along with 5µl volumes in duplicates of each sample were applied to quantification and linearity curve was measured.

RESULTS AND DISCUSSION

An initial study of stigmasterol was identified with mobile phase toluene: methanol (9.5:0.5 v/v); the best ideal mobile phase for this study with a R_f value of 0.34.The Fig II A and B gives us a clear indication of the separated compounds in each *in vivo* and *in vitro* samples. The linearity curves were obtained in both *Chlorophytum borivilianum* Santapau & R. R. Fernand *Chlorophytum tuberosum* (Roxb.) Baker *in vivo* and *in vitro* samples (Fig. III A & III-B), with an r value of 0.994 in *Chlorophytum borivilianum* (regression equation Y=484.8+123.8*X) as compared to 0.991 for *Chlorophytum tubersoum* (regression equation Y=511.4+90.45*X) (Table I). The RSD of 24.11% and 24.77% for *Chlorophytum borivilianum* Santapau & R. R. Fern and *Chlorophytum tuberosum* (Roxb.) Baker respectively (Table II) makes the method more precise. The quantification of stigmasterol in each sample revealed that both CTR and CTIR having more of this i.e., 18µg as compared to CBIL with a second highest value of 17.26 µg stigmasterol (Table III). The least value of 4.31µg suggests that this might be due to the lack of production of secondary metabolites during the synthesis of stigmasterol under *in vitro* conditions; concomitantly, the other samples shows good enough stigmasterol. As per the ICH guidelines, reproducibility has benn checked to make the instrument as well as the reference compound stigmasterol precise (Fig IV).

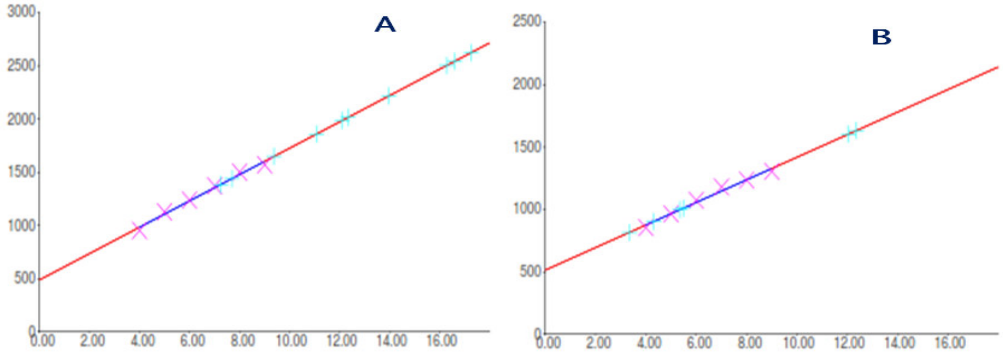

Figure III
Calibration curve of Chlorophytum boriavilinum (A) and Chlorophytum tuberosum (B) Extracts along with stigmasterol as standard

Table I
Calibration curves, limit of detection (LOD) and quantification (LOQ) of compounds under study

Compound	Sample	Rf	Linearity range (µg/spot)	Equation	r	LOD (µg/spot)	LOQ (µg/spot)
Stigmasterol	*C. borivilianum*	0.36	4-9	Y=484.8+123.8*X	0.994	4	12
	C. tuberosum	0.36	4-9	Y=511.4+90.45*X	0.991	4	12

Table II
Intra-day and inter-day precision of all samples with standard stigmasterol (* % R.S.D; mean-n=3)

Compound	Precision	Mean	CV (%)	RSD (%)
Stigmasterol	Intra-day	n= 5	1.884	24.11
	Inter-day	n= 5	1.744	24.77

Table III
Results of Quantification

Sr No	Sample Name	Amount in µg
1	CBR	16.6
2	CBL	12.34
3	CBIR	7.691
4	**CBIL**	**17.26**
5	CBIC	13.97
6	**CTR**	**18.0**
7	CTL	12.06
8	**CTIR**	**18.0**
9	CTIL	5.49
10	**CTIC**	**4.31**

The instruments precision was measured by scanning the same spot with 7µl of stigmasterol 5 times (%CV=0.64) (Fig. IV). The accuracy (recovery percentage) was determined at three different levels (80, 100 and 120%) by spiking known amount of stigmasterol with each sample. The recovery percentage estimated was 94.8, 94 and 90% at 80, 100 and 120% level, which makes it more accurate (Table IV).The robustness and ruggedness were measured by changing the mobile phase by 1 to 2%. It was found to be precise.

Figure IV
Reproducible curve of stigmasterol (540nm)

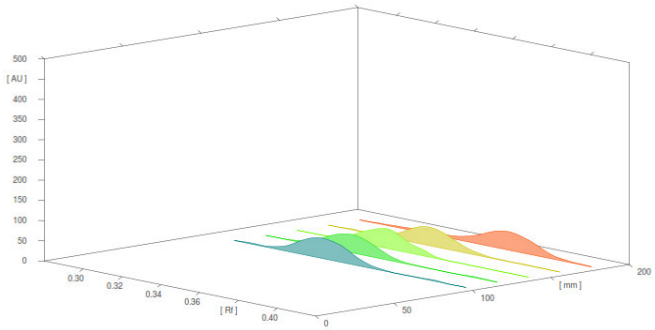

Table IV
Results for recovery percentage

Compound	Spiked amount (µg)	Recovery Percentage
Stigmasterol	2.4	94.8
	3.0	94.0
	3.6	90.0

Earlier studies on stigmasterol have shown its influence on eliciting gene expression in Phytophthora as sterol carrier proteins in the environment[20]. The steroid drugs were of high value used as progestational, adrenocortical, estrogenic and contraceptive agents, was mostly derived from 4-androstene-dione (AD) and 1,4 androsta-diene-3,17-dione (ADD)[21]. There was also difficulties in the analysis of phytosterol and its oxidation during GC[22].Our present study suggests more than 90% recovery which can be considered as one of the efficient efforts for analysis.

CONCLUSION

This present study provides a confirmation of availability of stigmasterol *in vitro* and *in vivo* parts of both the varieties. The current scenario of production of stigmasterol has been improved with the introduction of this study, which directly reflects on market value.Thus our present study creates a new avenue for production and quantification of stigmasterol. The study can be extended by undertaking certain LC-MS and NMR study. The present scenario of herbal medicines gives huge impact on pharmaceutical industry. Simultaneously, the market price of drugs is also a headache for the government, as government has to avail the drugs to the poor people. For the above aspects, the current study give an impact on the production of the stigmasterol simultaneously from the different sources makes it cheaper. Therefore, the future prospects of the stigmasterol may be better with the production with less price.

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CONFLICT OF INTEREST

Conflict of interest declared none.

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