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Phytochemical evaluation and quantification of beta-sitosterol in geographical variation of *Withania coagulans* Dunal by HPTLC analysis

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Abstract

The recent global resurgence of interest in herbal medicines, has led to an increase in demand for herbal drugs and consequently a decline in their quality, particularly due to a lack of adequate evidence proof data for assessing the quality of drug. The dried seeds of *Withania coagulans* Dunal of Solanaceae family, play a major role in indigenous system of medicine for the treatment of ulcers, dyspepsia, rheumatism, dropsy, *etc*. Organoleptic parameters are not much reliable in establishing the standards of herbal drugs for which an attempt was made through analytical analysis, providing a more concrete picture regarding the qualitative and quantitative aspects which were widely accepted in the quality assessment of herbal drugs such as TLC and HPTLC studies. In the present study, β-sitosterol has been quantified in methanol and ethyl acetate extracts of *Withania coagulans* Dunal from different states or regions, showing the variation of β-sitosterol content in the drug due to geographical variation. TLC carried with mobile phase Toluene: Ethyl acetate: Glacial Acetic acid (6:1.5:0.5 (v/v)) on Precoated aluminium silica gel plates (Merck) and densitometric determinations was done at 254 nm. Calibration curve was prepared and the amount of β-sitosterol estimated in the extracts by comparing the respective peak areas with that of the standard. A faster, reliable and sensitive HPTLC method has been developed and validated for the analysis of β-sitosterol in seeds of *Withania coagulans*. Other parameters studied such as phytochemical screening, morphology, heavy metals, aflatoxin contamination and fluorescence behaviour to lay down the standard for the genuine drug.

Key words: Phytochemical screening, Safety evaluation, HPTLC studies, β-sitosterol, Quantification

Introduction

The traditional medicines are increasingly solicited through the traditional practitioners and herbalists in the treatment of infectious diseases. Medicinal plants play a vital role for the development of new drugs. The bioactive extract should be standardized on the basis of active compounds (Mathur and Agrawal, 2011). Herbal drug technology is used for converting botanical materials into medicines, where standardization and quality control with proper integration of modern scientific techniques and traditional knowledge is important. Seeds of *Withania coagulans* Dunal used in Unani System, known as Tukm-e-Hayath which is a small genus of shrubs, popularly known as Indian cheese maker. It is common in Iran, Afghanistan and East India, and also used in folk medicine. It is known differently in various languages such as English - Vegetable rennet; Persian - Arusaka, Paneer-bad; Urdu - Paneerband, Tukme-Hayath; Telugu - Panneru-gadda. It is fairly common in dry hot and stony places up to 1700 m, found in North-west India, Shimla, Punjab, Gujarat, Garhwal

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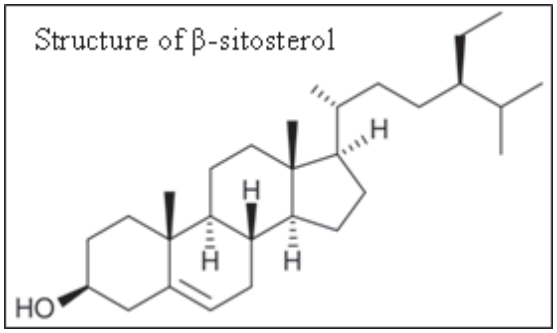
and Kumaon. *Withania coagulans* is commercially important for its ability to coagulate milk, in the treatment of ulcers, rheumatism, dropsy, consumption and sensile debility. The seeds are reported to be sedative, emetic and stomachic, a blood purifier and febrifuge, an alternative, diuretic and bitter tonic in dyspepsia as well as a growth promoter in infants (Watt, 1972; Kirtikar and Basu, 1996; Nadkarni, 1976 and Hemlatha *et al.,* 2004). It is well known in the indigenous system of medicine for the treatment of ulcers, dyspepsia, rheumatism, dropsy, consumption and sensile debility (Hemalatha *et al.,* 2008). In indian sub-continent, the berries are used as a blood purifier. The twigs are chewed for cleaning of teeth and the smoke of the plant is inhaled for relief in toothache (Dymock *et al.,* 1972 and Anonymous, 1969). High percentage of β-sitosterol and Linoleic acid are the factors reported to be responsible for the hypocholesterolemic effect (Anonymous, 1982).

β-sitosterol is a phytosterols which is ubiquitous throughout the plant kingdom and reported to possess broad range of biological activities. β-sitosterol is a main phytosterol, found in numerous plants including rice, wheat, corn, nut, peanut *etc*. β-sitosterol has recorded an amazing health benefits as an hepatoprotective (Shailajan *et al.,* 2005), antioxidant and antipyretic (Ahmed *et al.,* 2001 and Ali, 1967), inflammatory disorders and immunomodulatory (Karl, 1997), anti-inflammatory (Gupta *et al.,* 1980), rheumatoid arthritis (Bouic *et al.*, 1996), colon cancer (Awad *et al.*, 2000a), benign prostatic hypertrophy (Awad *et al.,* 2001 and 2000b), and breast cancer (Awad *et al.,* 2000c). Preliminary phytochemical screening, TLC fingerprinting and co-TLC studies (with â-sitosterol) of seeds of *Withania coagulans* revealed the presence of β-sitosterol and an identical spot as that of standard β-sitosterol was observed. Further, it was confirmed by Rf values comparison and multiwavelength scanning.



Previously, β-sitosterol has been quantified by liquid chromatography and tandem mass spectrometry, using atmospheric pressure photoionization (APPI– LC–MS–MS) (Lembcke, 2005) while there are other reports, using liquid chromatography (LC) with evaporative light scattering detection (ELSD) (Nair and Hoogmartens , 2006), online liquid chromatography- gas chromatography (LC–GC) (Kamm *et al.,* 2002), and gas chromatography (Sorenson *et al.,* 2006). Considering the wide therapeutic applications of β-sitosterol, as well as an alternative quantification technique of marker constituent was generated to ensure identity and quality of the selected plants. This is a sensitive, specific and reproducible HPTLC method for the quantification of β-sitosterol from the seeds of *Withania coagulans* (Misar *et al.,* 2010).

Chemical constituents: Dihydrostigmasterol and β-sitosterol, Withanolides, Esterases, Fatty oil, Essential oil, Triacontane, and Amino acids.

Materials and Methods

Collection of material

Withania coagulans Dunal seeds were procured from the local market of different states or regions *i.e.*, Andhra Pradesh, Bhopal, Delhi, Aligarh and Mumbai and are authenticated with the help of a Botanist, Dr. V.C. Gupta at Central Research Institute of Unani Medicine before carrying out the study.

All solvents were of HPLC grade. β-sitosterol (98% purity) was procured from Sigma-Aldrich, Bangalore for reference standard.

The present investigation includes parameters such as morphology, physicochemical analysis, TLC and HPTLC fingerprint, phytochemical screening, fluorescence study, safety evaluation, quantification of active principle *etc*. Physicochemical parameters were determined according to the methods described in 'The Unani Pharmacopoeia of India' (Anonymous, 2009). Fluorescence analysis was carried out as per the method described by Trease and Evans (1972) and GBC-908 AA model Atomic Absorption Spectrophotometer (AAS) was used to determine the concentration of heavy metals. Microbial load and aflatoxins contamination were analyzed as per the methods described in WHO guidelines (Anonymous, 1998). Phytochemical screening was carried out in methanol and ethyl acetate extracts of drug as per the methods described by Trease and Evans (1972) to know the nature of phytoconstituents present in the drug.

Preparation of the sample drug extract

Five grams of powdered drug each of *Withania coagulans* from different states or regions were macerated in 100 ml of methanol and ethyl acetate separately, in a stoppered 250ml conical flask and was kept for 2 hours while shaking at regular intervals. Later the contents were filtered through Whattmann No. 41 paper and evaporate the solution to 20 ml. The solution, thus, obtained was used as sample for the determination of components.

Preparation of the standard solution of β-sitosterol

Stock solution (100µg/ml) of β-sitosterol was prepared by dissolving with methanol in 10ml standard volumetric flask

and different aliquots were prepared. For standard drug, methanol is used and for sample drug, methanole and ethyl acetate is used to check in which solvent standard concentration is more.

Chromatography

HPTLC was performed on 20 cm × 10 cm Precoated Aluminium Sheets of Silica Gel 60 F_{254} (Merck). Samples solution of about 10µl were applied as 6 mm width bands using automatic TLC applicator system of the DESAGA Sarstedt Gruppe (Germany). A linear ascending development with Toluene: Ethyl acetate: Glacial Acetic acid (6:1.5: 0.5) (v/v) as mobile phase was carried out in a twin trough glass chamber previously saturated with mobile phase vapour for 20 min. at room temperature (25 ± 2°C). The development of solvent distance was 85 mm. After development, plates were airdried. Scanning was performed using densitometer of DESAGA Sarstedt Gruppe (Germany) at 254nm and 366nm wavelength and operated by ProQuant 1.06 version software. The source of radiation was a deuterium lamp emitting a continuous UV spectrum in the range 190-400 nm. The slit dimensions were 4 mm × 6 mm.

Development of HPTLC technique

After the development, TLC plate was then removed dried completely and detected with under UV Cabinet system for detection of spots. Further, it is scanned with the Densitometer CD60 of DESAGA Sarstedt Gruppe system under the UV range of 254nm, and 366nm as shown in the figures. A corresponding densitogram was then obtained in which peaks are appeared for the corresponding spots being detected in the densitometer while scanning and the peaks area under the curve corresponds to the concentration of the component in the sample for the concentration that applied on the TLC plate.

Results and Discussion

Organoleptic characters

The crude drug consists of the seeds of *Withania coagulans* Dunal. of Solanaceae family, having yellow colour.

Morphology of Withania coagulans Dunal

***Macroscopic*:** Seeds: Dark brown, ear shaped, glabrous, pulp brown, having yellow, berry, globose, 1.5-1 cm long, 0.7-1 cm width, seeds oval to rounded, yellowish brown, 41-59 in number, 0.1-0.3cm long, 0.2-0.3cm wide, dotted (Figure 1).

***Microscopic*:** The T. S. section of seeds shows a single layer of epidermis, followed by a layer of highly flattened thin walled sub epidermal cells. Under the sub-epidermis, there is a layer of highly lignified palisade like cells having narrow lumen. The epidermis of the seed coat inner comprise of 1-2 layer of thin-walled parenchymatous cells which are at places are collapsed showing hyaline-like structure. The endosperm is represented by cells showing strong cellulosic thickening filled with aluerone grains without any globoide. The cotyledon shows thin walled radially elongated cells enclosing a wide zone of round to oval to polyhedral parenchymatous cells.

Quantitative estimation of β-sitosterol by HPTLC *analysis*

TLC of the β-sitosterol along with methanol and ethyl acetate extracts of *Withania coagulans* developed with the mobile phase solvent system and Rf values for the β-sitosterol is 0.45±0.02 and the corresponding spot at the same Rf value was obtained in the other drug extracts at 254nm and under UV 366nm of chromatogram is shown in the Figure 4 and an overlay of densitometric scan of *Withania coagulans* Dunal, collected from different regions and β-sitosterol in fluorescence 254nm was shown in Figure 2.

The Rf value 0.45±0.02 of β-sitosterol is in correspndance with all the spots at the same Rf value in other drug extracts and were tabulated with respect to position, area percentage and the β-sitosterol concentration corresponds to each peak in the solution applied on the TLC plate are shown in the Table 7 in drugs extracts of different states or region. Further, in Table 8, amount of β-sitosterol in g% with respect to drug is mentioned. A graph illustrated for the amount of β-sitosterol in g% with respect to *Withania coagulans* Dunal, collected from different states or regions has been depicted in the Figure 3.

Figure 1: Macroscopic feature of seeds of *Withania coagulans* Dunal

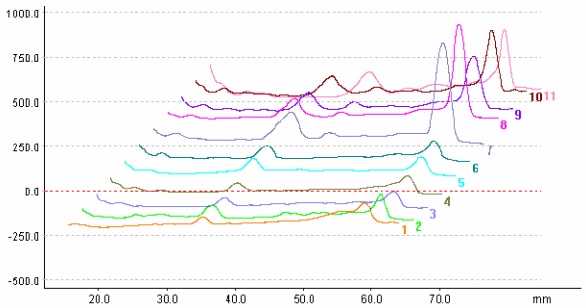


Figure 2: An overlay of densitometric scan of seeds of *Withania coagulans* Dunal collected from different regions and β-sitosterol in fluorescence at 254nm

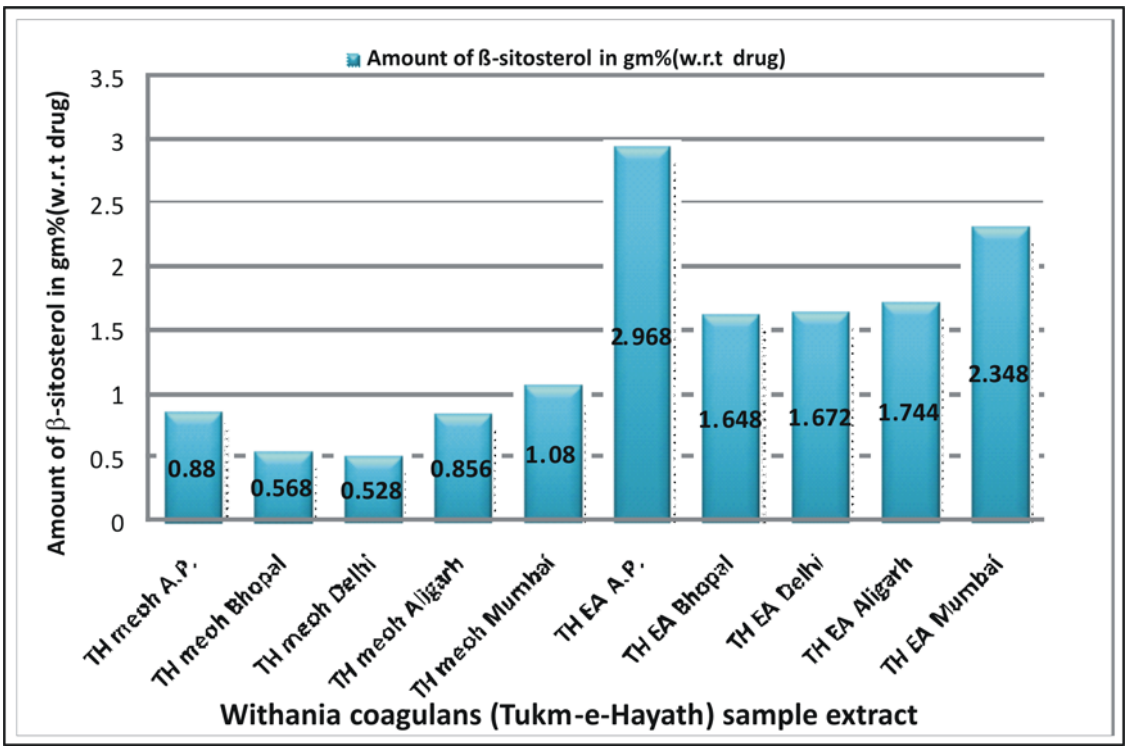


Figure 3: A graph showing the amount of β-sitosterol in g% with respect to *Withania coagulans* Dunal collected from different states or regions

TLC plate of Tukhme-Hayath of different extracts at U.V. 254nm

TLC plate of Tukhme-Hayath of different extracts at U.V. 366nm

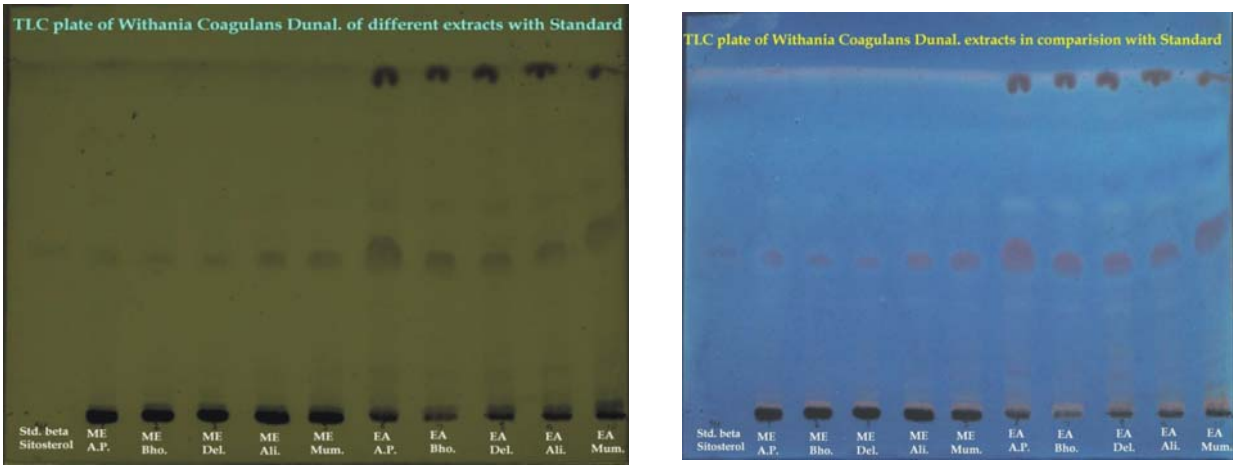


Figure 4: TLC chromatogram of methanol and ethyl acetate seed extracts of *Withania coagulans* Dunal collected from different regions and β-sitosterol at UV 254nm and 366nm

Physicochemical studies

Physicochemical data of *Withania coagulans* such as total ash, acid insoluble ash, water soluble ash, alcohol soluble matter, water soluble matter, pH of 1% and 10% aqueous solution, loss of weight on drying at 105^{0}C and volatile oil are summarized in Table 1.

Table 1: The physicochemical parameters data expressed here as mean values of the three readings calculated

S.No.	Parameter	Results found
1.	**Total ash**	3.95 - 4.803g%
	Acid insoluble ash	0.04 - 0.06 g%
	Water soluble ash	1.58 - 1.93 g%
2.	**Alc. sol .matter**	10.6 - 11.12 g%
	water sol. matter	25.52 - 26.58 g%
3.	**pH values**	
	a. 1% Aqueous solution	4.36 - 4.43
	b. 10% Aqueous solution	4.39 - 4.41
4.	**Loss of weight on drying at 105ºC**	9.17 g%
6.	**Moisture content**	
	(by Karlfischer Titrator Method)	0.3301%
7.	**Volatile oil**	Nil

Phytochemical screening of *Withania coagulans* Dunal was carried out in different solvent such as ethanol, methanol, chloroform, aqueous, ethyl acetate, petroleum ether extracts to know the nature of compounds present. Qualitative tests for phytoconstituents were carried out for alkaloids, carbohydrates, resin, glycosides, phenols, saponins, proteins, starch, steroids, tannins and flavonoids which are tabulated in Table 2.

Table 2: Phytochemical screening of the nature of compounds present in different solvent extracts of *Withania coagulans* Dunal

S.No.	Phytoconstituents	Ethanol	Methanol	Chloroform	Aqueous	Ethyl acetate	Pet ether
1.	Alkaloids	+	+	+	+	+	+
	1. Dragendroff's reagent	+	+	+	+	+	+
	2. Mayer's test	+	+	+	+	+	+
	Carbohydrates 1.Benedict's test	+	+	-	+	+	-
	2. Molisch's test	+	+	-	+	+	-
2.	Resinifiedvolatile oils	-	-	-	-	-	-
3.	Glycosides	+	+	+	+	+	+
4.	Phenols: 1. $FeCl_3$ test	+	+	+	+	+	+
5.	Saponins	+	+	-	+	+	+
6.	Proteins: 1. Millon's test	+	+	-	+	+	+
7.	Starch	+	+	-	+	+	+
8.	Phytosterols(Steroids) 1. Salkowski reactiontest	+	+	+	+	+	+
9.	Tannins: 1. Ferric chloride test:	+	+	+	+	-	+
10.	Flavanoids: 1. Shinoda test	+	+	+	+	+	+

Fluorescence analysis of powdered drug reaction with chemicals ordinary light and UV light were observed and reported in Table 3. Fluorescence analysis of powdered drug extracts in different solvents were observed and reported in Table 4. Heavy metals and aflatoxins contamination along with their permissible limits were given in Tables 5 and Table 6, respectively. β-sitosterol detection in correspondence with all the spots at the same Rf value in other drug extracts were tabulated with respect to position, area percentage and the β-sitosterol concentration corresponds to each peak in the solution applied on the TLC plate are shown in the Table 7 in drugs extracts of different states or region. Further, in Table 8 amount of β-sitosterol in g% with respect to drug is mentioned.

Table 3: Fluorescence analysis of powdered drug

S.No	Reagents	UV light		Visible light
		Short 254nm	Long 366nm	
1.	Powder as such	Brown	Black	Dark brown
2.	Powder treated with 1N NaOH in methanol	Black	Dark brown	Dark brown
3.	Powder treated with 1N NaOH in water	Black	Dark brown	Dark brown
4.	Powder treated with 1N HCl	Black	Light brown	Black
5.	Powder treated with 50% HNO_3 aqueous	Black	Blackish green	Dark brown
6.	Powder treated with 50% H_2SO_4 aqueous	Black	Black	Black
7.	Powder treated with glacial acetic acid	Black	Pale yellow	Dark brown

Table 4: Fluorescence analysis of powdered drug extracts in different solvents

S.No.	Extraction solvent	UV light		Visible light
		Short 254nm	Long 366nm	
1.	Acetone extract	Black	Pale yellow	Yellow
2.	Alcoholic extract	Greenish black	Light green	Light brown
3.	Chloroform extract	Black	Pale yellow	Brown
4.	Petroleum ether extract	Black	Pale yellow	Yellow
5.	Methanol extract	Greenish black	Pale yellow	Dark brown
6.	Ethyl acetate extract	Greenish black	Light blue	Yellow
7.	Aqueous extract	Black	Pale yellow	Dark brown

Table 5: Heavy metal analysis

S.No.	Parameter analyzed	Results	Permissible limits as per WHO
1	Arsenic	Nil	Not more than 3.0 ppm
2	Cadmium	Nil	Not more than 0.3 ppm
3	Lead	Nil	Not more than 10.0 ppm
4	Mercury	Nil	Not more than 1.0 ppm

Table 6: Aflatoxin contamination

S.No	Parameter analyzed	Results	Permissible limits as per WHO
1	B1	Nil	Not more than 0.50 ppm
2	B2	Nil	Not more than 0.10 ppm
3	G1	Nil	Not more than 0.50 ppm
4	G2	Nil	Not more than 0.10 ppm

Table 7: Concentration of β-sitosterol in µg present in different solvent extracts of *Withania coagulans* Dunal collected from different states or regions

Sample Name	Y-Pos	Area (%)	Concentration
TH meoh A.P.	40.8 mm	156.608	22.0 µg
TH meoh Bhopal	40.3 mm	105.676	14.2 µg
TH meoh Delhi	40.0 mm	99.625	13.2 µg
TH meoh Aligarh	40.4 mm	155.310	21.4 µg
TH meoh Mumbai	40.3 mm	169.451	27.0 µg
TH EA A.P.	42.5 mm	346.539	74.2 µg
TH EA Bhopal	40.1 mm	236.844	42.1 µg
TH EA Delhi	40.1 mm	230.391	41.8 µg
TH EA Aligarh	42.1 mm	228.992	43.6 µg
TH EA Mumbai	42.9 mm	291.142	58.7 µg

Table 8: Amount of β-sitosterol in g%(w.r.t drug)present in different solvent extracts of *Withania coagulans* Dunal collected from different states or regions

Sample name	Concentration	Amount of β-sitosterol in gm%(w.r.t drug)
TH meoh A.P.	22.0 µg	0.880 %
TH meoh Bhopal	14.2 µg	0.568 %
TH meoh Delhi	13.2 µg	0.528 %
TH meoh Aligarh	21.4 µg	0.856 %
TH meoh Mumbai	27.0 µg	1.080 %
TH EA A.P.	74.2 µg	2.968 %
TH EA Bhopal	42.1 µg	1.648 %
TH EA Delhi	41.8 µg	1.672 %
TH EA Aligarh	43.6 µg	1.744 %
TH EA Mumbai	58.7 µg	2.348 %

Conclusion

The drug under study was subjected for physicochemical analysis, which is very much supportive in establishing the standard along with the other parameters such as macroscopic, microscopic, fluorescence behavior as reported in the present investigation including heavy metals, aflatoxins contamination found nil, allowing in the permissible limits of WHO. HPTLC studies were thoroughly studied in methanol and ethyl acetate extract and β-sitosterol successfully quantified and observed the variation of content with respect to the drug. Amount of β-sitosterol content found maximum in ethylacetate extract of A.P. with 2.96 g%. On the basis of these data, the drug was broughtup in determining and ascertaining its quality and standardization of drug**.** Thus, the study is likely to help in the quality assurance of drug with HPTLC technique which is cost effective, less time consuming and fast analysis to lead formulation with the more therapeutic efficacy due to chemical constituents present in it.

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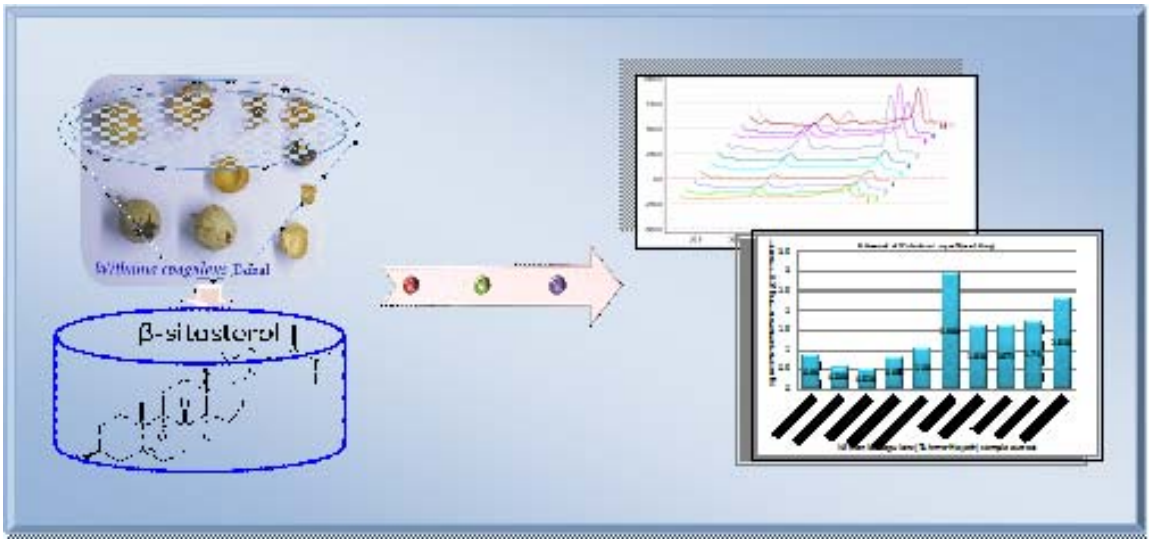
Graphical abstract



- A faster, reliable and sensitive HPTLC method has been developed and validated for the analysis of β-sitosterol in seeds of *Withania coagulans* Dunal.
- Standardization and quantification of β-sitosterol.
- Used as a reference standard in quality control of *Withania coagulans* in marketed samples. Active marker species content can be compared within the market products.

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