Pharmacognostical Standardization of *Withania coagulans* Dunal.

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ABSTRACT

Pharmacognostical standardization of fruits of *Withania coagulans* Dunal. (Solanaceae) has been carried out in the present study. The study includes macroscopical and microscopical evaluation along with estimation of its physicochemical parameters such as ash and extractive values, preliminary phytochemical screening and fluorescence analysis. It also includes quantification of some of the active constituents such as withanolides (withaferin-A) by HPTLC, total phenolic, tannin, flavonoids and flavonols. The present study reveals standardization profile for drug like *Withania coagulans*, which would be of immense value in botanical identification and authentication of plant drug and may help us in preventing its adulteration.

Keywords: *Withania coagulans*: Standardization: Pharmacognosy: HPTLC Quantification: Withaferin-A.
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INTRODUCTION

Pharmacognosy at present has become one of the pillars of areas like pharmacy, medicine, natural product chemistry and many others allowing them to recognize the importance of plants as sources of medicines. This approach has initiated in various active research programmes either to isolate new lead compounds or to produce standardized extracts[1]. For this it is very necessary to evaluate various qualitative and quantitative parameters, which may be helpful in setting standards for particular medicinal plant/parts of the plant. With the help of this standards one can easily identify and characterize an individual drug, which may play a major role in maintaining quality and purity of that particular drug and its formulation and prevent it from being adulterated by drug of same or other genius having low potency[2].

Thus, the present study deals with standardization of one of such medicinal plant i.e. *Withania coagulans* Dunal. (Solanaceae) distributed in the east of the Mediterranean region and extends to South Asia. It shows the presence of esterases, lignan, alkaloids, free amino acids, fatty oils, essential oils and withanolides[3]. The drug has shown to have anti-inflammatory[4], cardio tonic activities[5], hepatoprotective[6], anti-fungal[7], hypoglycemic, free radical scavenging activity[8], hypolipidemic[9] and wound healing activity[10]. Recent investigation has shown that withanolides isolated from the aqueous extract of fruits possessed a good antihyperglycemic and antidyslipidemic activity[11]. Due to its wide therapeutic importance it is worthwhile to obtain various qualitative and quantitative standards of drug to prevent its adulteration.

MATERIAL AND METHODS

Macroscopical and Microscopical studies

The fruits of *Withania coagulans* Dunal. (with persistent calyx and pedicle) were purchased from the local market of Varanasi and were authenticated by Prof. V.K. Joshi, Department of Ayurveda (Banaras Hindu University) and Mr. Narayanappa, Chief botanist TAMPCOL (Chennai). The voucher specimen (SM/WC/02) has been deposited in the department. The fruits were then examined macroscopically with reference to its colour, shape, size, odour and taste etc. Hand section of the petiole, calyx, pericarp and seeds of the overnight soaked fruits

were taken stained and mounted following usual micro techniques[12]. Micro chemical test for cell wall and cell content were performed according to Johansen (1940)[12], Kay (1938)[13] and Trease and Evans (1983)[14]. Schultz's fluid was used to study the characteristics of the individual cells and tissues of pedicel, calyx, pericarp and seeds by mounting with glycerin.

Physicochemical standards

Physicochemical parameters of the powdered drug such as total ash, acid insoluble ash, water and alcohol soluble extractive values were determined according to the procedure mentioned in Indian Pharmacopoeia (1996)[15].

Fluorescence characteristics

Air dried coarse powder of fruits was examined under ultraviolet light according to the method described by Kokaski et al., (1949)[16].

Preliminary phytochemical screening

The fruits of *Withania coagulans* (with persistent calyx and pedicle) were coarsely powdered and extracted with methanol using soxhlet. To the extract water was added (1: 1) and fractionated using water immiscible solvent i.e. chloroform (2: 1) in a separating funnel. Both the fractions chloroform and hydroalcoholic were concentrated and dried in a Rota evaporator initially and then in vacuum desiccator. Preliminary phytochemical screenings of methanolic extract along with chloroform and hydroalcoholic fractions were done for the presence of various phytoconstituents by using standard procedure[17].

Quantitative estimations

Total phenolic and tannin contents were estimated according to the method described by Hagerman et al., (2000b)[18], whereas estimation of total flavonoid and flavonol content were done following the standard procedure proposed by Kumaran & Karunakaran (2007)[19].

Identification and quantification of withaferin-A by TLC and HPTLC method

Identification and quantification of withaferin-A in the two fractions i.e. chloroform and hydroalcoholic along with the parent extract was carried out by following two steps:

1. Confirmative test for the presence of withaferin-A by TLC and preparative TLC: The parent methanolic extract along with its chloroform and hydroalcoholic fractions of fruits were compared with standard withaferin-A (Ranbaxy) by TLC method using toluene: ethyl acetate: formic acid (5: 5: 1) as solvent system and Liebermann-burchard as spraying reagent. Depending upon the result obtained from TLC analysis preparative TLC plate of chloroform fraction was prepared. The wavelength of the compounds obtained as bands were then compared with the wavelength of standard withaferin-A.

2. Quantification of withaferin-A by using HPTLC method: Quantification of withaferin-A was done on both chloroform and hydroalcoholic fraction along with methanolic extract by using solvent system toluene: ethyl acetate: formic acid in the ratio of (5: 5: 1).

Instrumentation

Executed by: anchrom (Mumbai), Applicator: CAMAG Automatic TLC Sampler, Scanner: CAMAG TLC Scanner, Wavelength used: 580nm, Plates used: Silica Merk 60F plates.

RESULTS

Macroscopical description

The fruits are superior, indehiscent, many seeded berry type. It is pedicellate, round to globous in shape, 4–6mm in diameter, yellow to brown in colour and closed in leathery persistent calyx mostly with pedicel. The fruits have an indistinct odour with a slightly bitter taste (Figure. 1).

Figure 1: Fruits of *Withania coagulans*.

Microscopical description

The transverse section of the pedicel shows a single layered epidermis covered with a large number of branched and unbranched trichomes, followed by cortex constituting 6–10 layers of collenchymatous cells. The pericycle shows the presence of pericyclic fibers with intervening parenchymatous cells, whereas the central region represents a continues narrow band of phloem encircling the xylem beneath which is a ring of intraxylary phloem. The centre most region is consist of hollow pith surrounded by parenchymatous cells with a few thick walled lignified fibers towards the intraxylary phloem. Calyx shows a single layer of thin walled cells in upper and lower epidermis with a few branched and more unicellular covering trichomes similar to the pedicel which are present only in the upper epidermis, followed by the presence of few candelabra-type trichomes. The mesophyll is represented by spongy parenchyma traversed by a number of small veins covered with a bundle sheath of thin walled parenchymatous cells (Figure 2).

The transverse section of fruit shows the presence of exocarp which represents a single layer while mesocarp shows a wide zone of parenchymatous cells with strong cellulosic thickening. The endocarp is similar to that of exocarp but at some places the cells are flattened and collapsed. The seeds in transverse section show a single layer of epidermis followed by a layer of highly flattened thin walled sub-epidermal cells. Beneath the sub-epidermis there is a layer of highly lignified palisade-like cells with narrow lumen. The inner epidermis of the seed coat comprise of 1–2 layer of thin-walled parenchymatous cells which 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 (Figure 3).

The powder characteristic of the fruits of *Withania coagulans* is demonstrated in Figure 4-A, which shows a large number of parenchymatous cells of cotyledons (a), fragments of pericarp showing parenchymatous cells (b-c), thick walled endosperm cells showing aluerone grains (d-e), epidermal cells of calyx with unicellular covering trichomes (f) and few xylem vessels with spiral thickening (p). Figure 4-B represents the isolated elements and Table 1 represents the measurements of different cells of fruit of *Withania coagulans* in microns.

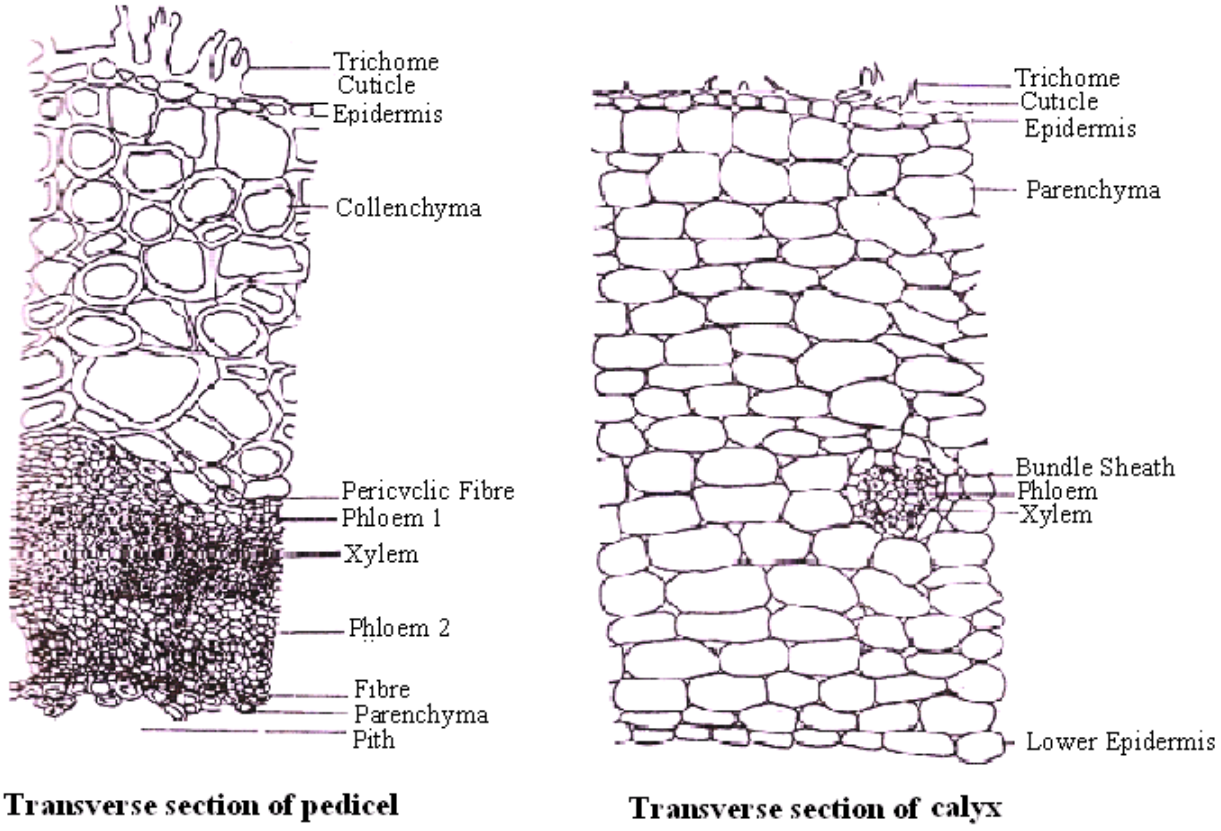

Figure 2: Microscopical characters of pedicel and calyx of fruit of *Withania coagulans*.

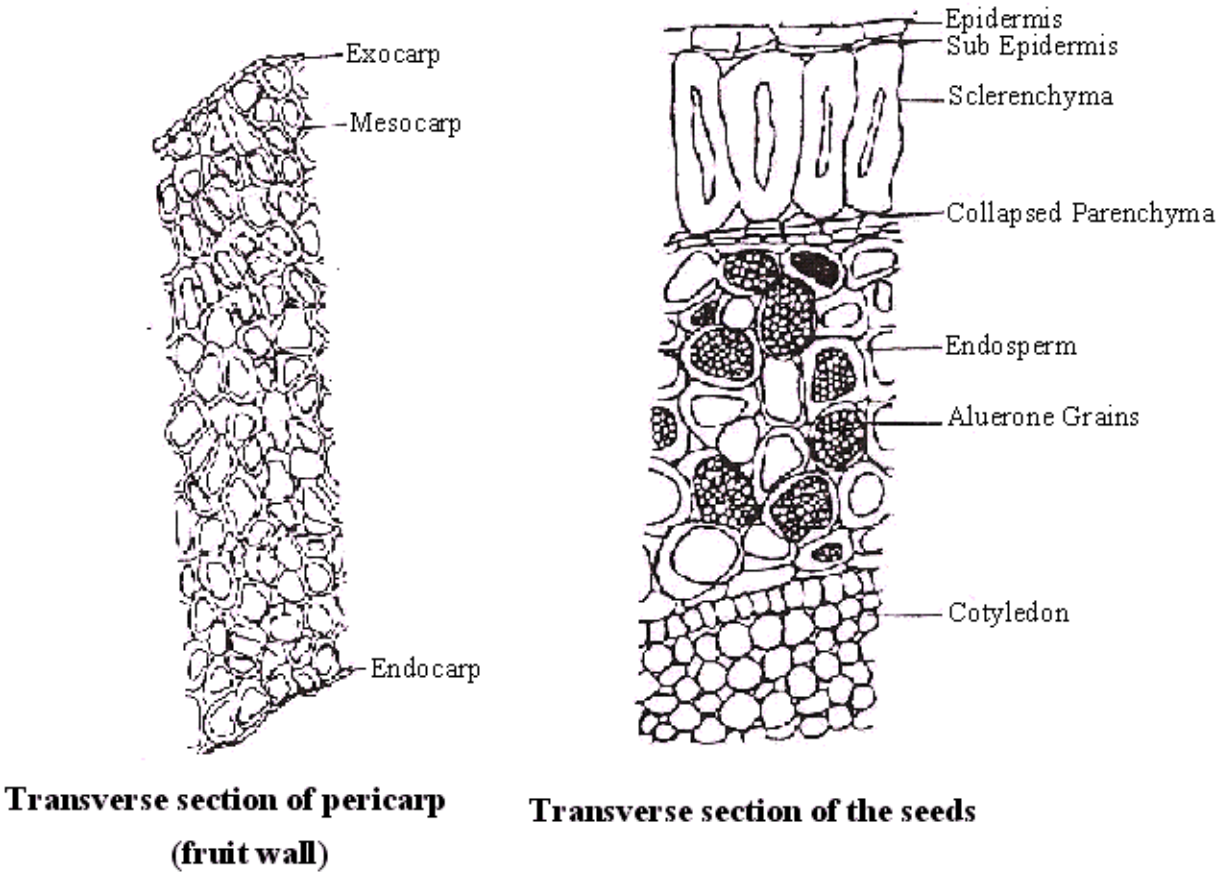

Figure 3: Microscopical characters of pericarp and seed of fruit of *Withania coagulans*.

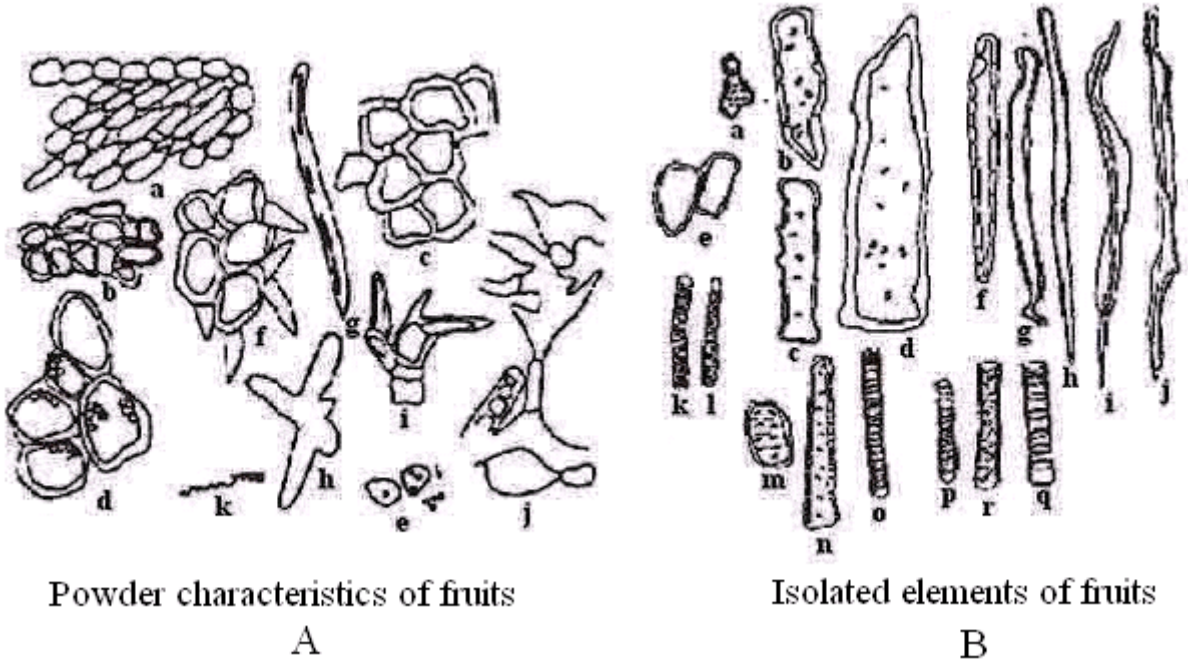

Figure 4: Powder characteristics (A) and Isolated elements (B) of fruits of *Withania coagulans*. Note: In Fig 4 (A) a: Portion of cotyledon, b-c: Pericarp in surface view, d-e: Endosperm cells with aluerone grains, f: Upper epidermis of calyx, g: Fibre, h-j: Trichome, k: Xylem vessel. In Fig 4 (B) a-d: Tracheids, e: Parenchyma, f-j: Fibre, k-r: Tracheidal vessels.

Table 1: Measurements of different cells of fruit of *Withania coagulans* in microns

Different cells of fruits	Measurements in microns
Pedicel:	
Epidermal cells	15–19–23×7–11–15
Spongy parenchyma cells	34–45–56×23–38–45
Xylem	11–15–19×4–8–11
Phloem	8–11–15×4–8–11
Sclerenchymatous cells	26–30–34×15–19–23
Trichomes	113–188–262×23–26–30
Pericarp:	
Parenchymatous cells	19–27–30×11–15–19
Seed:	
Epidermal cells	45–53–60×15–19–23
Scelerenchymatous cells	75–86–98×30–38–41
Endosperm cells	38–45–52×23–26–30
Embryo cells	11–15–23×8–11–15
Calyx:	
Epidermal cells	23–30–38×15–19–23
Spongy parenchyma cells	83–94–101×23–30–38
Vascular bundle	15–19–26×11–15–19

Table 2: Physicochemical standards

Physicochemical standards	%W/W
Total ash	19.0
Acid insoluble ash	12.10
Water soluble extractive value	21.20
Alcohol soluble extractive values	5.10

Values are taken as mean of triplicate.

Physicochemical standards

Various physicochemical standards such as total ash, acid insoluble ash, water soluble extractive and alcohol soluble extractive values are reported in Table 2.

Fluorescence characteristics

Fluorescence analysis helps us in fulfilling the inadequacy of physical and chemical methods for identification of plant drug. Fluorescence analysis of powdered drug on treatment with various reagents was studied under ultraviolet light and their observations are expressed in Table 3.

Preliminary phytochemical screening

Phytochemical screening of hydroalcoholic fraction showed the presence of steroids, alkaloids, phenolic

Table 3: Fluorescence characteristics

Treatment	Fluorescence
Powder as such	Light brown
Powder treated with nitrocellulose in amyl acetate	Dark brown
Powder treated with 1N NaOH in methanol	Reddish green
Powder treated with 1N NaOH in methanol, dried and mounted in nitrocellulose in amyl acetate	Dark green
Powder treated with 1N HCL	Light green
Powder treated with 1N HCL, dried and mounted in nitrocellulose in amyl acetate	Dark green
Powder treated with 1N NaOH in water	Yellowish green
Powder treated with HNO_3 (1:1)	Light green
Powder treated with H_2SO_4 (1:1)	Light green

Table 4: Preliminary phytochemical screening

Test	Methanolic extract	Hydroalcoholic fraction	Chloroform fraction
Steroids	+	+	+
Alkaloids	+	–	+
Tannins	+	+	+
Phenolic	+	+	+
Cardiac glycosides	–	–	–
Anthraquinone glycosides	–	–	–
Saponin	+	+	–
Amino acids	+	+	–
Proteins	+	+	–
Carbohydrate	+	+	–
Organic acids (oxalic acid)	+	+	–

+ indicate presence and – indicate absence.

compounds, tannins, saponins, carbohydrates, proteins, amino acids and organic acids, whereas chloroform fraction showed the presence of mainly steroids and alkaloids (Table 4).

Quantitative estimation

The quantitative estimation of total phenolic, total tannin, total flavonoids and total flavanol content in the parent extract along with the two fractions are given in Table 5. The results showed that the total phenolic content was higher in case of hydroalcoholic fraction as compared to chloroform fraction and was expressed as mg/g equivalent to gallic acid (w/w). The total tannin content in the samples was expressed as mg/g equivalent to tannic acid (w/w) which was observed to be higher in case of chloroform fraction. Total flavonoid and flavanol content as observed in the result was found to be higher in hydroalcoholic fraction as compared to chloroform fraction and was expressed as mg/g equivalent to rutin (w/w).

Identification and quantification of withaferin-A by TLC and HPTLC method

The results obtained from the TLC studies (Figure 5) showed the presence of withaferin-A in both methanolic extract and chloroform fraction (having similar R_f values to that of withaferin-A), whereas it was found absent in hydroalcoholic fraction. Results obtained from the preparative TLC (Table 6) showed five bands among which wavelength of band 4 (209 nm) was found to be similar to that of withaferin-A (208 nm).

Peaks of withaferin-A are expressed in Figure 6 which shows its presence in methanolic extract and chloroform fraction, whereas it was not detected in hydroalcoholic fraction. Their quantification data are given in Table 7.

Table 6: Preparative TLC data for withaferin-A

No of Bands	Wavelength (λ max) (nm)	Absorbance (nm)
Standard withaferin-A	208	2.26
Band 1	(a) 220	1.87
	(b) 266	0.29
Band 2	212.5	2.76
Band 3	(a) 220	1.74
	(b) 280	0.16
Band 4	(a) 280	2.31
	(b) 209	4.00
Band 5	219	1.80

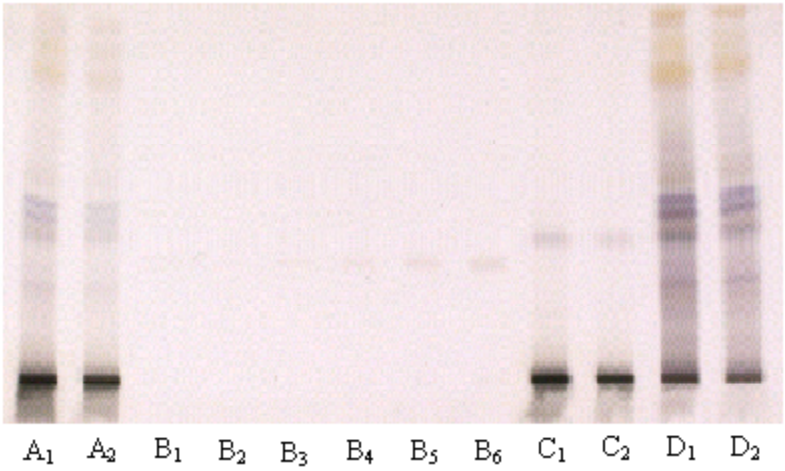

Figure 5: Image of TLC plate at 366nm after derivatization. Note: A_1, A_2 represent methanolic extract, B_1 to B_6 represents standard Withaferin-A, C_1, C_2 represents hydroalcoholic fraction and D_1, D_2 represents chloroform fraction.

Table 5: Quantitative estimation of various phytoconstituents

Phytoconstituents	Methanolic extract	Hydroalcoholic fraction	Chloroform fraction
Total phenolic content mg/g Equivalent to Gallic Acid (w/w)	55.9	43.9	33.1
Total tannin content mg/g Equivalent to Tannic Acid (w/w)	76.6	32.6	48.0
Total flavonoid content mg/g Equivalent to Rutin (w/w)	0.88	0.21	0.07
Total flavonol content mg/g Equivalent to Rutin (w/w)	0.25	0.02	0.013

Values are taken as mean of duplicate.

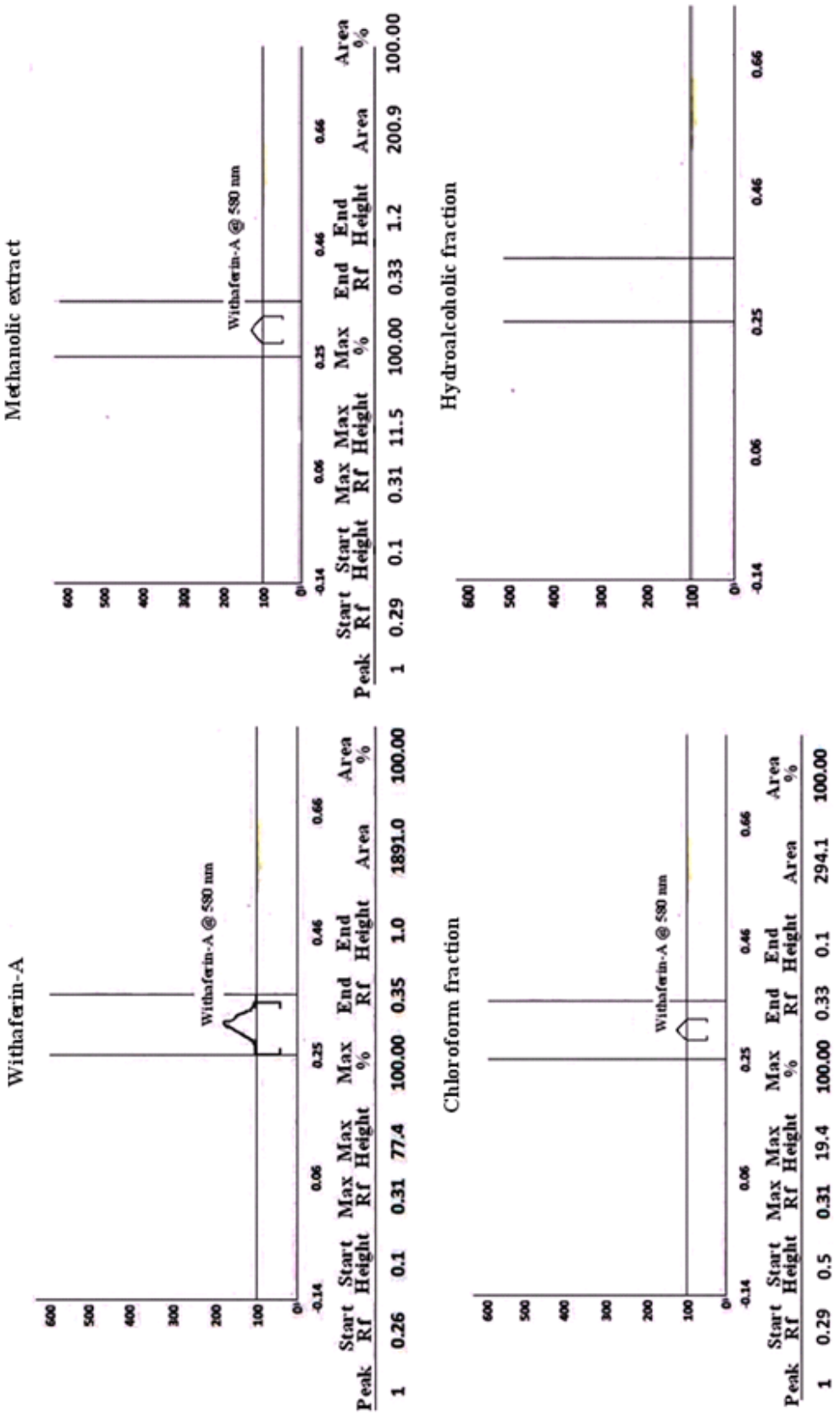

Figure 6: Peak area for withaferin-A

Table 7: Quantification data for withaferin-A by HPTLC

Sample	Concentration	Volume	Results (mg/g of sample)
Withaferin-A	0.2 mg/ml	8 µl	–
Methanolic extract	4.0 mg/ml	10 µl	3.67
Chloroform fraction	10 mg/ml	10 µl	2.10
Hydroalcoholic fraction	9.97 mg/ml	10 µl and 20 µl	Not detected

DISCUSSION

It is assumed that macroscopic and microscopic evaluation of any plant drug are considered to be the primary steps for establishing its quality control profile and according to WHO, botanical standards should be proposed as a protocol for the diagnosis of the herbal drug. The histochemical studies give a preliminary idea about the type of compounds and their accumulation in the plant tissues. Thus, helps us in selecting the particular part or tissue of that plant where the compounds of interest are located[20].

Physicochemical standards such as total ash value helps us in determining both physiological ash (plant tissue) and non-physiological ash (extraneous matter like sand and soil), whereas acid insoluble ash gives an idea about the amount of silica present, especially as sand and siliceous earth. Extractive values help us in determining the amount of active constituents and is done on plant materials for which as yet no suitable chemical or biological assay exists[21]. The phytoconstituents quantified in the present study exhibit great deal of medicinal importance like Phenolic compound as a good anti-oxidants[22], tannins having protein precipitating property[23], whereas flavonoids and flavonols possess a good anti-inflammatory[24, 25] and anti-oxidants activity[26]. The quantified values of the above phytoconstituents can be used as a major tool for obtaining a quality control profile for a drug.

Withanolides i.e. withaferin-A has been previously quantified from the roots and leaves of *Withania coagulans* by HPLC method[27]. In the present study withaferin-A has been quantified from the fruits of *Withania coagulans* with the help of HPTLC method which may act as a chemical marker for standardization of *Withania coagulans*.

The results obtained from the present study may play a major role in setting particular standards for the plant, which might broaden its pharmacognostical, pharmacological, botanical and economical importance. These parameters may also prove beneficial in authentication of the plant. Thus, with the help of these standards we can minimize the adulteration of *Withania coagulans* which will be of great use for the future workers in selecting the correct herbal specimen.

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REFERENCES

1. Ameenah, Gurib-Fakim. Review Medicinal plants: Traditions of yesterday and drugs of tomorrow. *Molecular Aspects of Medicine*. 2006: **27**:1–93.
2. Shanbhag DA, Jayaraman S. Application of HPTLC in standardization of Homoeopathic Mother Tincture. *Pharmacognosy*. 2008: **4**(15):155–159.
3. Kiritikar KR, Basu BD. *Indian Medicinal Plants*. vol. **3**. International Booksellers and Publishers, Dehradun (India):1160–1161 (1999).
4. Budhiraja RD, Sudhir S, Garg KN. Pharmacological investigation on fruits of *Withania coagulans* Dunal. *Planta Medica*. 1977:**32**:154–157.
5. Budhiraja RD, Sudhir S, Garg KN. Cardiovascular effects of a withanolide from *Withania coagulans* Dunal fruits. *Indian journal of physiology and pharmacology*. 1983: **27**(2):129–134.
6. Budhiraja RD, Sudhir S, Craeg FN, Arora B. Protective effect of 3 beta-hydroxy-2, 3 dihydro withanolide F against CCl4 induced hepatotoxicity. *Planta Medica*. 1986: **1**:28–29.
7. Choudhary MI, Dur-e-Shahwar, Parveen Z, Jabbar A, Ali I, Atta-ur-Rahman. Antifungal steroidal lactones from *Withania coagulans* . *Phytochemistry*. 1995: **40**(4):1243–1248.
8. Hemalatha S, Wahi AK, Singh PN, Chansouria JPN. Hypoglycemic activity of *Withania coagulans* Dunal in streptozotocin induced diabetic rats. *Journal of Ethnopharmacology*. 2004: **93**(2–3):261–264.
9. Hemalatha S, Wahi AK, Singh PN, Chansouria JPN. Hypolipidemic activity of aqueous extract of *Withania coagulans* Dunal in albino rats. *Phytotherapy research*. 2006: **20**(7):614–620
10. Hemalatha S, Mishra N, Kumar M, Singh PN, *Chansouria JPN, Mandal V. Wound healing activity of Withania coagulans fruits*. 12th Annual national Convention of Indian Society of Pharmacognosy at Moga, Punjab: OP 13 (2008).
11. Rakesh Mauryaa, Akankshaa, Jayendraa, Amar BS, Arvind KS. Coagulanolide, a withanolide from *Withania coagulans* fruits and antihyperglycemic activity. *Bioorganic & Medicinal Chemistry Letters*. 2008: **18**:6534–6537.
12. Johansen DA. *Plant Microtechnique*, Edition 1: 1940 McGraw Hill Book Co, New York, London:182–203 (1940).
13. Kay CA. *The Microscopical Studies of Drugs*. Edition 1: 1938 Bailiere Tindall and Co, London: **16** (1938).
14. Trease GE, Evans WC. *Pharmacognosy*. Edition 14: 1983 Bailiere Tindall and Co, London (1983).
15. Anonymous. *Pharmacopoeia of India*. Vol.**2**, Edition 4: 1996 Government of India, Ministry of Health, Controller of publication, New Delhi:A-53–55 (1996).

16. Kokaski CJ, Kokaski RJ, Slama FJ. Fluorescence of powdered vegetable drug under ultraviolet radiation. *Journal of American Pharmaceutical Association (Sci. Edn)*. 1949: **47**(10):715–717.
17. Khandelwal KR. *Practical Pharmacognosy, Techniques and Experiments*. Edition 1: 2007. Nirali Prakashan, Pune (India):149–156 (2007).
18. Hagerman A, Harvey-Muller I, Makkar HPS. *Quantification of tannins in tree foliage- a laboratory manual*. Vienna: FAO/IAEA. 2000b:4–7.
19. Kumaran A, Karunakaran J. In vitro antioxidant activities of methanol extracts of five Phyllanthus species from India. *LWT - Food Science and Technology*. 2007: **40**:344–352.
20. Shanmugakumar SD, Amerjothy S, Balakrishna K. Pharmacognostical, Antibacterial and Antifungal potentials of the leaf extracts of *Pithecellobium dulce* Benth. *Pharmacognosy Magazine*. 2006: **2** (7):0973–1296
21. Anonymous. *Quality control methods for medicinal plant materials*, WHO, Geneva, A.I.T.B.S. Publishers & Distributors (Regd.) Delhi-51-:28–30 (2002).
22. Ruch RJ, Cheng SJ, Klaunig JE. Prevention of cytotoxicity and inhibition of intracellular communication by antioxidant catechins isolated from Chinese green tea. *Carcinogenesis* 1989: **10**:1003–1008.
23. Haslam E, Lilley TH, Ya, C, Gaffiney SH, Spencer CM, Martin R, Magnaloto D. Some observations on the role of plant polyphenols in traditional herbal medicines. *Farmaceutischtijdschrift voor Belgie*. 1989: **66**:21–33.
24. Gabor M. *Handbook of experimental pharmacology. Anti-inflammatory drugs*. Springer, New York:68 (1979).
25. Havsteen B. Flavonoids a class of natural products of high pharmacological potency. *Biochemical Pharmacology*. 1983: **32**:1141–1330
26. Crespy V, Morand C, Besson C, Manach C, Demigne C, Remesy C. Quercetin, but not its glycosides, is absorbed from the rat stomach. *Journal of Agricultural and Food Chemistry*. 2002: **50**:618–621.
27. Shaila Dalavayi, Kulkarni S.N, Rajaya Lakshmi Tikala, Itikala S. Determination of Withaferin-A in two Withania species by RP- HPLC method. *Indian Journal of Pharmaceutical Sciences*. 2006: **68** (2):253–256.