EMS Induced Mutagenesis in *Poshita* and *Jawahar 22* of *Withania somnifera* (L.) Dunal (Solanaceae)

Ananya Das¹, Animesh K. Datta^{1*}, Arnab Bhattacharya¹,
Anjan Bhattacharyya² and Shyamal Ghose³

¹ Department of Botany, Genetics and Plant Breeding Section, Kalyani University,
Kalyani 741235, West Bengal, India

² Plant Cell and Tissue Culture Technology Laboratory, Department of Genetics, Bidhan
Chandra Krishi Viswavidyalaya, Mohanpur, Nadia West Bengal, India

³ Regional Analytical Laboratory on MAP, Department of Agricultural Chemicals,
Bidhan Chandra Krishi Viswavidyalaya, Mohanpur, Nadia West Bengal, India

Received May 18, 2010; accepted May 24, 2010

Summary Eight macromutants (*albina*, *xantha*, *chloroxantha*, *bushy*, *thick leaf*, *dwarf*, *early flowering* and *synchronous flowering*) were induced in *Poshita* and *Jawahar 22* (recommended varieties) of *Withania somnifera* (L.) Dunal; (Family: Solanaceae) at M₂ following EMS treatments (0.25, 0.50, 1.00% for 2 h and 4 h durations) to dry seeds. *Albina* and *Xantha* in both varieties and *chloroxantha* in *Poshita* were non-viable. Viable mutation frequency was recorded to be 0.00% to 3.00% in *Poshita* and 0.00% to 2.31% in *Jawahar 22*; however, estimated mutation frequency over M₂ population was 2.54%. Meiosis was nearly normal in mutants as control (2n=48), except *chloroxantha* of *Jawahar 22* (22.58II+2.84I/cell at MI, 57.0% cells with 24/24 separation at AI, 39.27% pollen fertility; control: 23.69II+0.62I/cell at MI, 90.0% cells with 24/24 AI separation, 71.05% pollen fertility). The mutant trait(s) were monogenic recessive to normal. Analysis of morphological parameters (root and shoot attributes including seed yield), secondary metabolites (total alkaloid and withanolides; withaferin A and withanolide A by HPLC) and fibre content of the roots (withaferin A was analyzed also from leaf) of M₃ plants revealed that *bushy* and *thick leaf* were noteworthy mutants.

Key words *Withania somnifera*, EMS, Mutagenesis, Cytogenetic analysis, Morphological and biochemical traits, HPLC.

Withania somnifera (L.) Dunal (Family: Solanaceae; common name: Ashwagandha; English name: Winter cherry) is a perennial shrub with immense importance in traditional (Gupta *et al.* 2004, Patra *et al.* 2004) as well as in modern (Kothari *et al.* 2003, Iqbal and Datta 2007a) systems of medicine. The pharmacological significance of the species is due to the presence of alkaloids and withanolides (steroidal lactones) primarily in the roots (Mirjalili *et al.* 2009a); however, the presence of withaferin A is also reported from the leaf (Gupta *et al.* 1996, Das *et al.* 2009). Amongst the withanolides, withaferin A and withanolide A are key components for therapeutic uses as they possess anticancerous (Oh *et al.* 2008), antioxidant (Bhattacharya *et al.* 2001), and neuropharmacological (Sangwan *et al.* 2007) properties apart from other uses.

Although natural variation exists in *W. somnifera* (Singh and Kumar 1998, Mirjalili *et al.* 2009b), it would be worthwhile to raise novel plant types in the species exploring the already existing superior genotypes (*Poshita* and *Jawahar 22*) using the methodology of induced mutagenesis as experimentally induced mutation provides an opportunity to create allelic variation at one or more gene loci of interest, thereby widening the gene pool. The objective of the work is to raise mutant(s) which may meet the demand of the raw product and chemical constituents of active

* Corresponding author, e-mail: dattaanimesh@gmail.com

principles in national and international markets. Report on induced mutagenesis in *W. somnifera* is rather meager (Iqbal and Datta 2007b). The present authors report their endeavours on EMS induced mutagenesis in 2 recommended varieties (*Poshita* and *Jawahar 22*) of *W. somnifera* and this communication sheds some light on mutation frequency, cytomorphological and genetical aspects of the mutants and their chemical analysis.

Materials and methods

Plant material

Mother seed stock of *Poshita* (released from Central Institute of Medicinal and Aromatic Plants, Lucknow, India, Misra *et al.* 2001, moisture content 4.62%, seed size $2.2 \pm 0.46 \times 1.6 \pm 0.32$ mm, 100 seed weight 0.63 ± 0.02 g, seed colour-sunset yellow-code No.-14913 and fruit colour-chrome 11309) and *Jawahar 22* (released from Mandsaur, Madhya Pradesh, India, Nigam *et al.* 1991, moisture content 3.75%, seed size $2.3 \pm 0.24 \text{ mm} \times 1.7 \pm 0.47$ mm, 100 seed weight 0.67 ± 0.02 g, seed colour-sunset yellow 14916 and fruit colour-orange 11261) were used as material source for mutagenic treatment.

Mutagenic treatment and raising M_1 , M_2 , M_3 generations

Seeds of *Poshita* and *Jawahar 22* were treated with different concentrations of ethyl methane sulphonate (EMS-Kodak Chemicals, U.S.A.: 0.25, 0.50 and 1.00% dilutions were made in 0.2 M Phosphate buffer and treatment performed for 2 h and 4 h durations with intermittent shaking at $32.1 \pm 1^\circ\text{C}$ at pH 6.8). The doses were applied after pilot trials. Control (dry seeds imbibed overnight in distilled water) and the treated seeds (thorough wash in running water) of both varieties were placed in petriplates (500 seeds in each lot) lined with moist filter paper and the germinated seedlings were transferred to nursery beds (after 20 d) and subsequently to the field plots (4–5 leaf stage) of the Botany Department, Kalyani University (Latitude $22^\circ 50'$ to $24^\circ 11' \text{N}$, longitude $88^\circ 09'$ to $88^\circ 48' \text{E}$; soil-umber colour-26217, 9.91% sand, 2.89% silt and 87.20% clay; carbon, hydrogen and nitrogen present were 3.25%, 0.56% and 0.76% respectively assessed by CHN analyzer; soil pH 6.87) at a spacing of 30 cm between plants and 45 cm between lines to raise M_1 generation during the months of September to February (2008–2009). Selfed seeds of each surviving M_1 plant (2–3 inflorescences per plants were bagged) were harvested separately and M_2 plants were raised as plant progenies during the months of March and August (2009).

The treated as well as control plant populations were carefully screened for macromutants throughout the growth period in the field and the frequency of the mutants was assessed as per 100 plants (Gaul 1964). Plant progenies were raised at M_3 (September to February 2008–2009) from selfed seeds of M_2 mutants and control lines. Morphometric traits (Table 1) were analyzed from M_3 plants (4 to 10 plants were scored based on availability) on harvest. The seedling colour of chlorophyll mutant (classified after Blixt 1961) and all colour codes were laid with reference to British Atlas of Colour (7th edition 2007). Chlorophyll content (from leaf tissues) of mutant and respective control was quantitatively estimated as per Arnon (1949).

Meiotic analysis

Meiosis was studied in the plant types (control and mutant) at M_3 from flower buds fixed in Carnoy's fixative (72 h with 2 changes at 24 h intervals) and stored in 70% alcohol. PMCs and pollen grains were stained in 2% propionocarmine solution; fully stained pollen grains were considered fertile (Marks 1954). Photomicrographs were taken from temporary squash preparations.

Inheritance of traits

Selfed M₂ seeds of the mutants segregating at M₃, were estimated by using χ^2 test analysis.

Extraction and estimation of secondary metabolites

Total alkaloids, withanolides, withaferin A and withanolide A were extracted and estimated primarily from the roots (only withaferin A was also assessed from the leaf) in control and mutant plant types. Total alkaloids and withanolides were extracted (3 g samples in each case and 3 replicates per plant type) following the methodology reported by Gupta *et al.* (1996) with minor modifications as were suggested by Das *et al.* (2009). The crude extracted withanolide was dissolved in methanol (HPLC grade) and volume was made up to 5 ml. Withaferin A and withanolide A content in plant types were quantified by HPLC (Shimadzu-LC 10AT equipped with Photo diode array detector-SPD-M10A; reverse phase C18 column-Phenomex, size 250×4.60 mm; mobile phase-methanol:water-9:1 v/v; flow rate-0.6 ml/min and the detector was set at 227 nm; injection volume-20 μ l). Quantification was accomplished by using a 5 point calibration curve prepared by diluting the stock solution of the analytical standards in methanol (HPLC grade). The retention time of analytical standards of withaferin A and withanolides A were 5.08 and 5.65 respectively. A good linearity was achieved between the range of 1.0 μ g/ml and 10.0 μ g/ml with a correlation coefficient of 0.995 (withaferin A) and 0.997 (withanolide A). The limit of detection were estimated to be 0.29 μ g/ml and 0.25 μ g/ml for withaferin A and withanolide A respectively based on signal to noise ratio 3:1. Amount of withaferin A and withanolide A in samples were computed from chromatograms using the formula suggested by Scott (1996). Recoveries of both the compounds at different fortification level (5 ppm, 10 ppm, 20 ppm) were determined in triplicate to validate and evaluate the accuracy of the method. The average recoveries were 96.85% for withaferin A and 95.11% for withanolide A.

Total fibre content of sun dried roots (3 replicates in each case) was estimated following Crampton and Maynard (1970).

Results and discussion

A total of 8 different types (*Poshita*-Fig. 1: 5 viable and 3 non-viable; *Jawahar 22* Fig. 2: 6 viable and 2 non-viable) of macromutants were spotted at M₂. EMS induced mutations seem to bring about gross morphological changes in branching pattern (*bushy*-Fig. 3), nature of leaf (*thick leaf*-Fig. 4: leaves are coarse in nature and dark green-12164 compared to normal-green 12163; chlorophyll content- Chlorophyll **a**: 0.39 mg/g of tissue and Chlorophyll **b**: 0.44 mg/g of tissue, compared to normal Chlorophyll **a**: 0.21 mg/g of tissue and Chlorophyll **b**: 0.25 mg/g of tissue), growth habit (*dwarf*: however dwarf mutants of *Jawahar 22* failed to survive beyond 3 to 4 leaf stage in field at M₃), maturity (*early flowering*-plants flowered between 65 and 75 d from germination compared to 95 to 110 d in control plants; *synchronous maturity*-the plants were also early flowering and both mutant types showed early maturity as well) and seedling colour (*albina*, *xantha* and *chloroxantha*). The mutant trait(s) of the viable macromutants were confirmed at M₃. *Albina* (*Poshita*: 0.26%; *Jawahar 22*: 0.26% to 3.33%), *xantha* (*Poshita*: 0.51% to 4.00%; *Jawahar 22*: 0.00% to 1.81%) were non-viable (died at cotyledonary leaf stage) mutants but *chloroxantha* (yellowish green-12160; chlorophyll content-Chlorophyll **a**: 0.18 mg/g of tissue and Chlorophyll **b**: 0.20 mg/g of tissue) was found viable in *Jawahar 22* (0.25%, 2 h-0.51%) and nonviable in *Poshita* (0.50%, 2 h-0.26%).

Total mutation frequency estimated was not dose dependent (*Poshita*: 2.22%–1.00%, 2 h to 4.00%–0.25%, 2 h and 4 h, 0.50%, 4 h; *Jawahar 22*: 1.17%–1.00%, 2 h to 3.33%–1.00%, 4 h). The viable mutation frequency varied from 0.00% (0.25% and 1.00%, 4 h) to 3.00% (0.25%, 2 h) in *Poshita* (800 plants scored) and 0.00% (0.5% and 1.0%, 4 h) to 2.30% (0.25%, 2 h) in *Jawahar 22*

Figs. 1–6. Plant types (1–4) and meiotic configurations (5–6) in *W. somnifera* varieties. 1. Control-*Poshita*. 2. Control-*Jawahar 22*. 3. *Bushy* mutant of *Poshita*. 4. *Thick leaf* mutant of *Jawahar 22*. 5. 24II at MI in control. 6. 15II+18I in *chloroxantha*. Univalents (→) marked. Scale bar=10 μ m.

(1050 plants studied). The spectrum of mutation was relatively higher among treatments in *Poshita* (1 to 7) than *Jawahar 22* (1 to 6). The estimated frequency of mutation in the M_2 population was 2.54% and the mutant types occurred in the following order: *xantha* > *early flowering* > *synchronous flowering* > *albina* > *bushy* > *chloroxantha* = *dwarf* > *thick leaf*. This relatively high frequency of mutation noted may be attributed to low turnover of plants at M_1 and M_2 . In this regard it is worth mentioning that *W. somnifera* seldom occurs in the North Eastern part of India including the West Bengal plains (Singh and Kumar 1998); however, Das *et al.* (2009) made a positive endeavour in the direction of successful cultivation of *W. somnifera* in the region.

Meiotic analysis revealed $2n=48$ (Figs. 5–6) chromosomes (*Poshita*: control-23.67 II+0.67 I/cell-132 cells scored, mutants-23.53 II+0.95 I/cell to 23.87 II+0.26 I/cell-39 to 96 PMC analyzed;

Table 1. Morphological attributes in *W. somnifera* plant types

Plant types	Varieties	Morphological attributes								
		Plant height (cm)	Total branches/ plant	Leaf yield (g)	Total number of berries (at harvest)	Seed yield (g)	Total plant weight (g)	Root length (cm)	Total branches/root	Root weight (g)
Control	P	74.75±1.18	8.47±0.32	5.98±0.08	49.80±0.52	3.43±0.21	45.53±0.46	19.26±0.61	4.27±0.18	5.28±0.50
Thick Leaf	J	69.01±1.78	4.13±0.17	5.78±0.13	38.60±0.48	3.53±0.15	41.71±0.84	17.03±0.17	3.27±0.20	4.87±0.12
	P	71.43±0.85	7.80±0.52	6.32±0.44	49.80±0.45	3.27±0.32	41.59±0.74	16.29±0.15	4.20±0.52	4.95±0.85
Early flowering	J	58.40±0.84	5.10±0.45	5.50±0.25	41.50±0.51	3.20±0.25	38.70±0.66	15.50±0.21	3.50±0.31	4.50±0.51
	P	68.59±0.25	7.80±0.47	5.51±0.52	53.40±0.65	3.52±0.54	40.03±0.32	18.00±0.17	4.40±0.84	5.10±0.26
Synchronous maturity	J	51.20±0.91	4.70±0.51	4.90±0.18	42.00±0.64	3.80±0.38	40.20±0.71	16.20±0.23	3.50±0.25	4.70±0.19
	P	68.53±0.23	6.80±0.54	5.65±0.44	56.40±0.51	3.94±0.63	41.19±0.28	15.94±0.23	4.60±0.71	4.86±0.55
Dwarf	J	53.10±0.65	4.50±0.24	4.60±0.24	44.50±0.68	4.10±0.34	40.70±0.69	14.70±0.21	3.10±0.19	4.20±0.26
	P	15.44±0.86	4.30±0.52	1.78±0.25	11.33±0.45	1.06±0.41	8.13±0.41	4.10±0.32	5.33±0.21	1.34±0.47
Bushy	P	87.00±0.52	12.00±0.52	7.57±0.21	68.71±0.46	5.48±0.19	62.50±0.54	21.09±0.57	5.86±0.20	5.92±0.43
	J	75.50±0.87	7.20±0.33	6.10±0.31	61.00±0.74	5.50±0.61	52.80±1.11	18.30±0.45	5.20±0.43	5.20±0.74
Chloroxantha	J	41.20±0.51	3.20±0.27	1.90±0.34	11.20±0.45	0.80±0.52	6.70±0.74	12.40±0.28	4.10±0.51	1.50±0.65

P-Poshita; J-Jawahar 22.

Jawahar 22: control-23.69 II+0.62 I/cell-136 meiocytes studied, mutants-22.58 II+2.84 I/cell to 24II-43 to 71 cells observed) always at metaphase I (MI). Univalent frequency was found relatively high (Fig. 6) in *chloroxantha* (2.84 I/cell at MI-*Jawahar 22*) and *bushy* (0.95 I/cell at MI-*Poshita*). Predominant chromosomal association noted at MI of the plant types was 24 II formation (*Poshita*: control-84.26%, mutants-74.58% to 87.18%; *Jawahar 22*: control: 86.51%, mutants-51.16% to 100.00%); while, the others ranged from 14 II+20 I (*chloroxantha*-*Jawahar 22*) to 23 II+2 I. Chromosome segregation was equal (24/24) in 87.91 and 90.30% cells of control plants of *Poshita* (364 cells analyzed) and *Jawahar 22* (874 cells scored) respectively at anaphase I (others had 1-4 laggards and bridge formation) but in mutants (*Poshita*-42 to 65 cells scored; *Jawahar 22*-38 to 72 cells assessed) it varied from 57.00% (*chloroxantha*) to 100.00% (*dwarf-Poshita*; *thick leaf*, *early* and *synchronous flowering*-*Jawahar 22*). Pollen fertility was low in *chloroxantha* (39.27%), possibly due to pairing abnormalities, but in other plant types it was more or less comparable (*Poshita*: control-59.96%, mutants-57.23% to 71.21%, 115 to 472 pollens grains scored; *Jawahar 22*: control-71.05%, mutants-71.12% to 73.56%, 87 to 304 pollens grains analyzed).

Segregation patterns were assessed at M₃ of mutants as either 3:1 or 1:1 (*thick leaf*: *Poshita*-normal 14, mutants 13, $\chi^2=0.04$ for 1:1, $p>0.80$, *Jawahar 22*-normal 15, mutants 14, $\chi^2=0.03$ for 1:1, $p>0.80$; *early flowering*: *Poshita*-normal 16, mutants 08, $\chi^2=0.89$ for 3:1, $p>0.3$; *Jawahar 22*-normal 19, mutants 17, $\chi^2=0.11$ for 1:1, $p>0.70$; *synchronous flowering*: *Poshita*-normal 08, mutants 04, $\chi^2=0.44$ for 3:1, $p>0.50$, *Jawahar 22*-normal 10, mutants 09, $\chi^2=0.05$ for 1:1, $p>0.80$;

Table 2. Chemical parameters and crude fiber content in mutant and control plant types of *W. somnifera*

Plant types	Varieties	Chemical attributes					Crude fiber (%)
		Total alkaloid (%)	Total withanolides (%)	Withanolide A (mg/gm)	Withaferin A (mg/gm)	Withaferin A (%) leaf	
Control	P	0.51	0.37	0.62	0.54	1.72	21.60
	J	0.28	0.26	0.58	0.48	1.23	23.50
<i>Thick Leaf</i>	P	0.58	0.39	0.60	0.55	1.84	25.30
	J	0.30	0.28	0.55	0.54	1.26	21.60
<i>Early flowering</i>	P	0.49	0.35	0.61	0.52	1.62	21.70
	J	0.25	0.25	0.52	0.46	1.19	21.50
<i>Synchronous maturity</i>	P	0.46	0.34	0.61	0.50	1.71	24.50
	J	0.25	0.24	0.53	0.44	1.15	22.20
<i>Dwarf</i>	P	0.42	0.29	0.52	0.49	1.54	25.40
	P	0.53	0.36	0.64	0.56	1.74	21.40
<i>Bushy</i>	J	0.30	0.29	0.60	0.51	1.24	20.60
	J	0.31	0.19	0.48	0.39	1.20	22.70
<i>Chloroxantha</i>							

P-Poshita; J-Jawahar 22.

Figs. 7–8. Chromatograms. 7. HPLC profile of *Bushy* mutant of *Poshita*. 8. HPLC profile of *thick leaf* mutant of *Jawahar 22*. Among the assorted peaks, **a**. and **b**. denote peaks for withaferin A and withanolide B respectively.

dwarf: *Poshita*-normal 11, mutants 10, $\chi^2=0.05$ for 1:1, $p>0.80$; *bushy*: *Poshita*-normal 11, mutants 10, $\chi^2=0.05$ for 1:1, $p>0.80$, *Jawahar 22*-normal 16, mutants 15, $\chi^2=0.03$ for 1:1, $p>0.80$; *chloroxantha*: *Jawahar 22*-normal 10, mutants 09, $\chi^2=0.05$ for 1:1, $p>0.80$), thereby indicating possible monogenic recessive inheritance of the mutant trait (s).

Morphological and chemical attributes of M_3 plant types are presented in Tables 1 and 2 respectively. Results indicated that *bushy* mutant plants of both varieties for all traits are superior to control (highest amount of withanolide A-Fig. 7). Leaves of *thick leaf* mutants (*Poshita* and *Jawahar 22*) had a higher amount of withaferin A (Fig. 8), which is promising. Crude fiber content in roots of all mutants of *Jawahar 22* and *bushy* mutant of *Poshita* showed a decreasing trend than the control which is rather desirable.

Thus, the mutants evolved as a consequence of gene mutation seem to be in the direction of the objectives underlined (*bushy*, *thick leaf*). Apart from this, *early flowering* and *synchronous flowering* correspond closely to the ideotypes being looked for in crops. *Dwarf* and *chloroxantha* mutants may also be used as marker(s) traits. Variation released through mutation has widened the gene pool and enriched genetic diversity in the species for exploitation in efficient breeding.

References

- Arnon, D. I. 1949. Copper enzyme in isolated chloroplast. Polyphenol oxidase in *Beta vulgaris*. *Plant Physiol.* **24**: 1–15.
- Bhattacharya, A., Ghosal, S. and Bhattacharya, S. K. 2001. Anti-oxidant effect of *Withania somnifera* glycowithanolides in chronic footshock stress-induced perturbation of oxidative free radical scavenging enzyme and lipid peroxidation in rat frontal cortex and striatum. *J Ethnopharmacol* **74**: 1–6.
- Blixt, S. 1961. Quantitative studies of induced mutations in peas. V. Chlorophyll mutations. *Agric. Hort. Genet. Landskrona* **19**: 402–447.
- Crampton, E. W. and Maynard, L. A. 1970. The relation of cellulose and lignin content to nutritive value of animal feeds. *J. Nutr.* **15**: 383–395.
- Das, A., Datta, A. K., Ghose, S. and Bhattacharyya, A. 2009. Growth and cultivation of two varieties of *Withania somnifera*. *J. Trop. Med. Pl.* **10**: 225–230.
- Gaul, H. 1964. Mutations in plant breeding. *Rad. Bot.* **4**: 155–232.
- Gupta, A. P., Verma, R. K., Misra, H. O. and Gupta M. M. 1996. Quantitative determination of withaferin-A in different plant parts of *Withania somnifera* by TLC densitometry. *J. Medi. Aro. Plant Sci.* **18**: 788–790.
- Gupta, M. L., Misra, A. K. and Khanuja, S. P. S. 2004. Root-rot and wilt: a new disease of ashwagandha (*Withania somnifera*) caused by *Fusarium solani*. *J. Med. Aro. Plant Sci.* **26**: 285–287.
- Iqbal, M. and Datta, A. K. 2007a. Ashwagandha (*Withania somnifera*)—the elixir of life. *Plant Archives* **7**: 449–445.
- and — 2007b. Induced mutagenesis in *Withania somnifera*. *J. Trop. Med. Pl.* **8**: 47–53.
- Kothari S. K., Singh, C. P., Vijay, K. Y. and Singh, K. 2003. Morphology, yield and quality of ashwagandha (*Withania somnifera* L. Dunal) roots and its cultivation economics as influenced by tillage depth and plant population density. *J. Hortic. Sci. Biotechnol.* **78**: 422–425.
- Marks, G. E. 1954. An aceto-carmin glycerol jelly for use in pollen-fertility counts. *Biotech Histochem* **29**: 277–278.
- Mirjalili, M. H., Moyano, E., Bonfill, M., Cusido, R. M. and Palazón, J. 2009a. Steroidal lactones from *Withania somnifera*, an ancient plant for novel medicine. *Molecules* **14**: 2373–2393.
- , Fakhr-Tabatabaei, S. M., Alizadeh, H., Ghassempour, A. and Mirzajani, F. 2009b. Genetic and withaferin A analysis of Iranian natural populations of *Withania somnifera* and *W. coagulans* by RAPD and HPTLC. *Nat Prod Commun* **4**: 337–346.
- Misra, H. O., Sharma, J. R., Lal, R. K., Gupta, M. M., Shukla, N., Singh, N. K. and Singh, N. 2001. Registration of a new variety Poshita of *Withania somnifera*. *J. Medi. Aro. Plant Sci.* **23**: 97–98.
- Nigam, K. B., Ptidar, H., Kandalkar, V. S. and Pthan, M. A. 1991. A new, promising, pre-release variety, 'WS20', of ashwagandha (*Withania somnifera*). *Indian J Agr Sci* **61**: 581–582.
- Oh, J. H., Lee, T. J., Kim, S. H., Choi, Y. H., Lee, S. H., Lee, J. M., Kim, Y. H., Park, J. W. and Kwon, T. K. 2008. Induction of apoptosis by withaferin A in human leukemia U937 cells through down-regulation of Akt phosphorylation. *Apoptosis* **13**: 1494–1504.
- Patra, D. D., Singh, K., Misra, H. O., Gupta, A. K., Singh, J., Singh, S. C. and Khanuja, S. P. S. 2004. Agrotechnologies of Ashwagandha. *J. Medi. Aro. Plant Sci.* **26**: 332–335.
- Sangwan, R. S., Chaurasia, N. D., Lal, P., Misra, L. N., Uniyal, G. C., Tuli, R. and Sangwan, N. S. 2007. Withanolide A biogenesis in *in vitro* shoot culture of Ashwagandha (*Withania somnifera* Dunal), a main medicinal plant in ayurveda. *Chem. Pharm. Bull.* **55**: 1371–1375.
- Scott, R. P. W. 1996. *Chromatographic Detectors: Design, Function, and Operation*. Marcel Dekker Inc., New York.
- Singh, S. and Kumar, S. 1998. Chemical Constituents of Ashwagandha (*W. somnifera*). In: S. Singh and S. Kumar (Eds.). *Withania somnifera* The Indian Ginseng Ashwagandha. Central Institute of Medicinal and Aromatic Plants, Lucknow. p. 131.