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Estimation of Secondary Metabolites from Methanolic Extract of Treated and Non-Treated Callus of *Withania Somnifera* using Thin Layer Chromatography and High Performance Liquid Chromatography

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***Abstract*: *Withania somnifera* (Ashwagandha-Medicinal plant) is one of the most reputed medicinal plants. It is an important source of drugs of traditional system of medicine. The secondary metabolites have a lot of economic importance in the plant breeding, plant defense, pollination, ecological effects and others. The present study has been undertaken to estimate the secondary metabolites (phenolic compounds) from methanolic extracts of UV-B treated and non-treated eight weeks old callus of *Withania somnifera* leaf explants. Qualitative SMs profiling was done through TLC while quantification was carried out through HPLC. TLC and HPLC results showed that the major secondary metabolites were detected in UV-B treated callus extract as compared to the non-treated callus extract. UV-B radiation (for short period) enhanced the production of Secondary metabolites in *Withania somnifera* callus.**

***Keywords*: High Presser Liquid Chromatography, Secondary metabolites, Thin Layer Chromatography, UV radiation, *Withania somnifera*.**

1. Introduction

Withania somnifera is an important medicinal plant, commonly known as Ashwagandha, winter cherry and Indian ginseng, belonging to family Solanaceae and is used in Indian, Unani and African traditional systems of medicine. The species name somnifera means “sleep-inducing” in Latin and the name of Ashwagandha is combination of the word ashwa, meaning horse and gandha, means smell, referring to a strong horse-like odor from the roots of this plant. The plant has been used as an aphrodisiac, anti-inflammatory agent, liver tonic, astringent and more recently to treat bronchitis, asthma, emaciation, ulcers, senile dementia and insomnia. *Withania somnifera* young leaves are enriched in the desired constituents of secondary metabolites. This species may find important application in medicinal treatment with its high withanolides content (Bashir, S.H. 2013, Sangwan, R. S. et al., 2007 and Gaurav, N. et al., 2016). In India, a large number of people depend on traditional medicinal plants for its efficiency, safety and to meet health care needs. Secondary metabolites of medicinal plants have a large use in modern and traditional medicine. It is the sources of important drugs and herbal plant materials containing antioxidant effective in lowering the occurrence of various diseases (Harika et al., 2017). There are ten species under this genus of which three species Withania somnifera, Withania coagulans and Withania obtusifolia are found in India. Among the worldwide list Withania somnifera, Withania coagulans, *Withania adunensis*, *Withania riebeckii* are examples of known important species of this genus which grow in different parts of the world (Savai, et al., 2013). A metabolic profiling of crude extract of leaves and root of *Withania somnifera* has a total of 62 different major and minor primary and secondary metabolites from leaves and 48 from roots. Out of these, 29 metabolites were common to both leaves and root but quantitatively different, which include fatty acids, organic acids, amino acids, sugars, flavones, and sterol derivatives (Gupta et al., 2018). *Withania somnifera* is a very important source of secondary metabolites, withanolides (including withanolide A and withaferin A). Withanolide A is an important secondary metabolite in Withania somnifera, which is having a high medicinal value and possesses potent anti-tumor and antioxidant properties (Praveen N. et al, 2010). Withaferin A is having a hepato protective effect in Diethyl nitrosamine (DEN) induced animals. Withaferin A is a potent free radical scavenger and is known to modulate the activities of antioxidant enzymes due to their interaction with various biomolecules. Withaferin A (WFA) can act with promising antioxidant properties and as a anti-metastatic agent, it can act as a targeted therapy for liver cancer and may be important for cancer prevention (S. Murugan et al., 2015).

The biologically active major chemical constituents of this plant are 12 alkaloids, 35 withanolides, several sitoindosides, flavonoids and steroidal compounds, saponins, glucosides,

starch reducing sugar, a variety of amino acids including aspartic acid, proline, tyrosine, alanine, Glycine, glutamic acid, tryptophan and high amount of iron are also present. Withanolides are mainly localized in the leaves and roots and their concentration usually ranges from 0.001 to 0.5 dry weights. It is a group of C-28-steroidal lactones and C-9 side chain built on an ergostane structure in which C-22 and C-26 are oxidized to from a six membered lactone ring. The basic structure is designated as the 'withanolide skeleton' it may be defined as a 22-hydroxyergostan-26-oic acid-26, 22-lactone (Jain R. et al., 2012; Mundkinajeddu, D. et al., 2014; Adhikari, S. R. and B. Pant, 2013).

Cell and organ culture have been used for production of secondary metabolites; however, yield of with anolide metabolites have been low. Attempts to increase levels of secondary metabolites in cell and organ cultures should be pursued as these metabolites can be produced in shorter periods of time. Among various plant cell or organ culture system, hairy root culture is one of the valuable tools for the biosynthesis of secondary metabolites, metabolic engineering studies, and production of root-derived compounds (Shivanadhan G. et al., 2012). Over the past several years, tissue culture technology has been exploited as an efficient and useful tool for production of commercially important metabolites, biotransformation of intermediates in to pharmaceutically important products and genetic enhancement of medicinal plants (Arya D. et al., 2013).

Secondary compounds responsible for different biological properties. Hence, increasing levels of secondary metabolites in UV-B exposed plants play an important role in plant function. The common response to UV-B stress in plant consequently activation of the genes of phenyl propanoid pathway (aromatic amino acids- phenylalanine and tyrosine) producing phenolic compounds. These phenolic compounds can mitigate the UV-Induced damage by protecting the photosynthetic pathway and cellular components. Increase in concentration of UV-B absorbing phenolic acid and flavonoids compounds are the most consistent response of Plants to UV-B supplementation. Phenolic compounds may have a multifunction role in UV-B defense (Kshama Rai and S.B. Agrawal, 2017).

2. Materials and methods

Different phytochemical tests were conducted for the qualitative and quantitative analysis of secondary metabolites (SMs). Analysis was done using Thin Layer Chromatography (TLC) and High Performance Liquid Chromatography (HPLC) methods.

A. Plant materials

Ten callus culture groups were made on the basis of different combinations and concentrations of PGRs and each group have two UV-treated and non-treated sets. In both sets MS media was supplemented with different concentrations of PGRs. The non-treated sets of each group was maintained for 8 weeks without UV radiation treatment but in treated sets the callus was maintained for 7 weeks without UV radiations and in the 8th week UV radiation were given for a period of 2 hours / day. Among all groups the best response were found in non- treated set of callus in MS media supplemented with, 4 mg/L NAA, 4 mg/L 2,4-D and 10 ml/L coconut water. The UV treated set of callus in MS media supplemented with 4 mg/L NAA, 2 mg/L 2,4-D and 10 ml/L CW was found to be the best responsive so both sets (Treated and non-treated callus) were taken for phytochemical studies.

B. Preparation of callus extracts (sample)

Extraction is the separation of bioactive compounds from plants tissues using selective solvent in standard extraction procedures. The basic principle is to grind the plant material (dry or wet) finer, which increases the surface area for extraction thereby increasing the rate of extraction and solvent to sample ratio of 10:1 (v/w) solvent to dry weight ratio has been used (Tiwari P. et al., 2011). Eight weeks old callus was transferred to 10 ml separate volumetric flask and dissolved in methanol. The solution was grinded for 20 min and filtered through Whatman filter paper no. 41 and the filtrate was used in TLC and HPLC for secondary metabolites analysis.

C. Thin layer chromatography

1) TLC Principle

TLC is a chromatographic technique which is used for the separation of mixture of compounds. The Separation of compounds is based on the differential adsorption as well as partitioning of analysis between the liquid stationary phase and mobile solvent phase.

2) Mobile phase

Mobile phase consists of chloroform: Ethyl acetate in the 60:40 ratio in SS-I and chloroform: Acetone: Formic acid in the 75:16.5:8.5 ratio in SS-II, both mobile phase were used for the separation of the phenolic compounds (flavonoid tannin) (Kathiresan, Prabhu et al., 2011 and Mohammed, S.S.A. et al., 2003).

3) Stationary phase

Stationary phase was done using TLC plate coated with silica gel, which are commercially available 60 F254 (Merck silica gel 60 F254 plate).

4) Procedure

A TLC plate coated with silica gel G was taken and gently draw a straight line across the TLC plate approximately 1 cm from the bottom. Methanolic callus extract was loaded on the TLC plate (silica gel-G) as a single spot at the center of the TLC plates and put in TLC chamber, covered with a lid. The sample was run until the solvent front reaches the top-end border of the plates. The developed plate was taken out from the chamber and the solvent front was marked and allowed to air dry at room temperature for few minutes. The TLC plates were observed under UV light. The spots were visualized in the UV inspection cabinet (254 nm to 365 nm) and the distance of separated spots were marked and separated distance was measured. The Rf value calculated by following formulas (Biradar, S. R. & B. D.

Table 1
Chromatographic properties of the flavonoids of Withania somnifera callus in SS-I phase

TLC Solvent system - Chloroform: Ethyl acetate (60:40)				
Non-treated sample			Treated sample	
S.N.	Fluorescence in UV light	Rf value of WS-1 C	Fluorescence in UV light	Rf value of WS 1UV
1.	Pale Purple	0.369	Pale Purple	0.373
2.	Dark Purple	0.469	Pale Purple	0.582
3.	Dark Purple	0.772	Pale Purple	0.746
4.	Yellow	0.909	Pale brown	0.835
5.			Pale Purple	0.895

Rachetti, 2013, Asante, I. K. et al., 2016).

$$\text{Rf value} = \frac{\text{Distance travelled by the solute}}{\text{Distance travelled by solvent}}$$

D. High performance liquid chromatography

1) Basic principle

HPLC stands for High performance liquid chromatography (sometimes also referred to as High Pressure Liquid Chromatography). HPLC is a chromatographic technique used in analytical chemistry and analytical recent biochemistry to separate a mixture of compounds for the purpose to identify, quantify and purify the individual specific components of the complex mixture. Separation is based on the polar (hydrophilic) or non-polar (hydrophobic) tendency of analytic between two liquid phases.

2) HPLC equipment

HPLC estimation of SMs was performed on Water modular HPLC system, equipped with UV detector. For estimation, Thermo C18 (Dimension 250 x 4.6) RP column, pump 715, software Data Ace, Injector and the mobile phase with the mixture of Acetonitrile: Methanol (50:50) at a flow rate of 1.0 ml/min and the column temperature at 25° C was used. The wavelength of UV detector was set at 254 nm. The HPLC was run and sample detected at 254 nm. The sample injection volume was 20µl and total 20 minutes run time.

3) Preparation of standard stock solution

10 mg of tannic acid was weighed accurately and transferred to separate 10ml volumetric flask, and the volume was adjusted to the mark with the methanol to give a stock solution of 1000 ppm.

4) Preparation of working standard solution (mobile phase)

From stock solutions of tannic acid 1 ml was taken and diluted up to 10 ml with this solution, 1.0, 1.5ml solution was transferred to 10 ml volumetric flasks and make up the volume up to 100 ml with methanol, gives standard drug solution of 10 µg/ ml concentration. The Methanolic extract of *Withania somnifera* leaf explants callus was prepared and subjected to HPLC with Acetonitrile: Methanol (50: 50) as mobile phase and the peak for SMs was obtained in retention time

3. Results and discussion

A. Qualitative estimation of phenolic compounds by TLC

TLC of MeOH callus extract, WS1-C (non-treated) showed five fractions with Rf values that ranged between 0.106 to 0.909 and individually 0.106, 0.369, 0.469, 0.772, and 0.909 respectively on the silica gel TLC plate in SS-1 phase. WS1-UV (treated callus) also showed five fractions with Rf values that ranged between 0.373 to 0.89 and individually 0.373, 0.582, 0.746, 0.835 and 0.89 respectively on the silica gel coated TLC plate in SS-I mobile phase (photo plate 1 A&B and Table 1). Rf values of all samples were identified of flavonoid component series. The characteristics of chromatograms of the individual sample may be due to the presence of the following phenolic compounds: Rf 0.74 amenoflavone, Rf 0.830 rutin, Rf 0.835 rutin. Dark purple color spots indicate the presence of flavonoids.

Fig. 1. TLC plate flavonoids extract from *Withania somnifera* callus in SS-I phase, compounds separated on silica gel G precoated plates under UV light

Fig. 2. TLC plate flavonoids extract from *Withania somnifera* callus in SS-2 phase, compounds separated on silica gel G precoated plates under UV light

Table 2
Chromatographic properties of the flavonoids of Withania somnifera callus in SS-II phase

TLC Solvent system - Chloroform: Ethyl acetate (60:40)				
Non-treated sample			Treated sample	
S.N.	Fluorescence in UV light	Rf value of WS-1 C	Fluorescence in UV light	Rf value of WS-1UV
1.	Pale green	0.295	Purple	0.281
2.	Purple	0.746	Purple	0.366
3.	Purple	0.830	Purple	0.549
4.	Pale green	0.873	Purple	0.591
5.	Purple	0.929	Purple	0.659
6.			Purple	0.788
7.			Purple	0.859
8.			Green	0.943

Table 3
Results for HPLC analysis of Withania somnifera non-treated and treated callus extract

Non-treated callus extract				Treated callus extract		
Peak Number	Rt. (min)	Area	Area %	Rt. (min)	Area	Area %
1.	2.029	73.066	33.973	2.066	174.07	94.940
2.	2.863	142.008	66.027	2.066	0	0
				2.468	9.348	5.096
Total		215.074	100		183.418	100

B. Quantitative estimation of phenolic acid by HPLC

In the present study a methods was developed by using HPLC for quantitative estimation of tannins in Methanolic extracts of *Withania somnifera* callus using tannic acid as standard and Methanol: Acetonitrile (50:50) with pH 4.5 as mobile phase. A representative chromatogram of tannic acid has been given in Fig. 1.

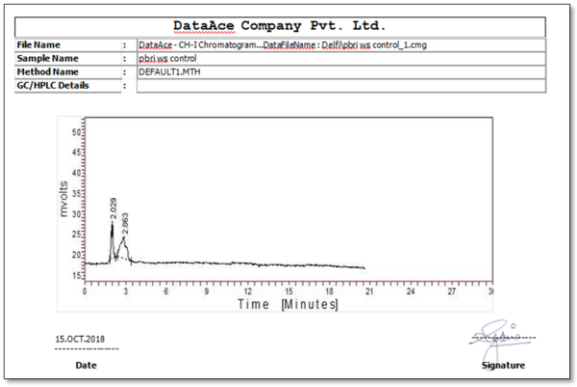

Fig. 3. HPLC (a)

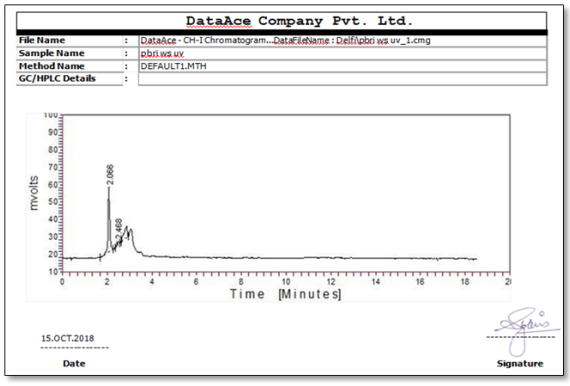

Fig. 4. HPLC (b)

The Chloroform: Acetone: Formic acid (75:16.5:8.5) the S-2 mobile phase (solvents system) was found to possess the highest number of spots and hence most of the compounds are extracted in MeOH solvents. Methanol extract of this plants generally possess phenolics, which are reported by different researchers as antimicrobial compounds (Valarmathy, et al., 2010). The UV-treated samples were compared with non-treated samples of *Withania somnifera* showed that the number of spots of UV-treated callus extract were higher than non-treated callus extract.

The UV-treated samples of *Withania somnifera* were compared with control samples showed that the number of peaks of UV-treated samples were higher than control samples. It means UV-B light (for short period) can enhance the productivity of compounds in the plants. UV-B induction of these secondary biosynthetic pathways and the UV-B induction regulatory genes are likely to be used in combination with new high-throughput strategies to identify the most preferred route correlating with desired metabolite biosynthesis in different plants under UV-B exposure experiments. Kumari Rima et al., (2013), Eichholz I. et al., (2011), Johnson C. B. et al., (1999).

4. Conclusion

Secondary metabolites (SMs) have a lot of economic importance in the plant breeding, plant defense, pollination, ecological effects and others. Plants produce a high diversity of natural products (SMs) with specific function in the protection against predators and microbial pathogens on the basis of their toxic nature and repellence to herbivores and microbes and some of which also involved in defense against abiotic stress (e.g. UV exposure) and also important for the communication of the plants with other organisms and are insignificant for growth and developmental process. Plant SMs possess biological activities, which are important for human life and

health. There are benefits of plant SMs and their use in traditional and modern medicine, food industry, perfumery and cosmetic. The MeOH extract of *Withania somnifera* callus showed maximum fractions with Rf value in Mobile phase SS-II as compared to the Mobile phase SS-I. In UV treated callus extract found maximum fraction with Rf values in SS-II phase as compared to the non-treated callus extract. These studies indicate that the UV treated callus extract with SS-II phase gave best response for analysis of phenolic compounds. The UV treated callus extract detected number of peaks higher than non-treated callus extract. UV-B light (for short period) could enhance the number of peaks, it was shown by UV treated sample as compared to the non-treated sample. UV treated sample of callus extract exhibited increased number of peaks as compared to the non-treated sample of callus extract. These results showed that the PTC with UV supplementation has possibility for commercial and medicinal exploitation of secondary metabolites. Previous studies have indicated that increased UV-B radiation had positive, neutral and negative effect on plant growth, chlorophyll content, cell size, and growth index and callus induction rate. Exposure of UV-B radiation for short period on callus causes increased stress on the callus, and then callus produce SMs as defense bio-chemicals. Therefore, UV-B treatment may be beneficial for the SMs production, however exposure of UV-B radiation for long period may be harmful for callus, its cause death of the cells.

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