Influence of mycorrhizal infection on with aferin A and with anolide A & D production in root and leaf of *Withania somnifera* (L.) Dunal

Parihar Pratibha

Department of Biotechnology, Genetics and Bioinformatics,
Natubhai V. Patel College of
Pure and Applied Sciences, Sardar Patel University, Vallabh Vidyanagar-388120, Gujarat, India.

Abstract

The withanolides including withaferin A and withanolide A and D present in the root and leaf extracts of *Withania somnifera* (L.) Dunal were analyzed by TLC with solvent system chloroform: methanol (9:1) showed clear and well resolved spots using methanolic leaf and root extract of Ashwagandha. The results confirmed the presence of high concentration of withaferin A in the leaf and withanolide A in the root with Rf value of 0.32 and 0.48, respectively. Quantitative and qualitative analysis was further performed by HPLC with a solvent system ethanol: water (1:1) and result showed that mycorrhiza inoculation increased the total withanolide production by 42% in leaf and 51% in the root. Thus, AM symbiosis can significantly increase the content of some secondary metabolites in Ashwagandha.

keywords: *Withania somnifera, withaferin A, withanolide A, HPLC*

Introduction

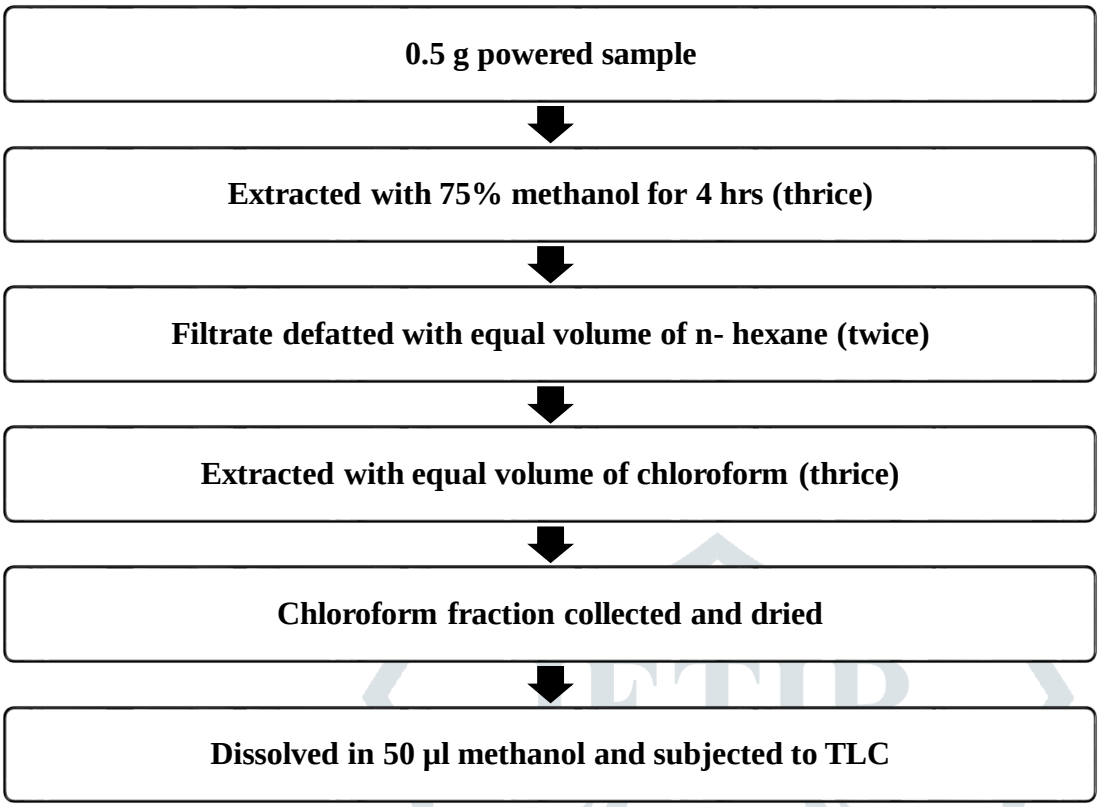
Plant secondary metabolites can boost the immune system, protect the body from free radicals and kill pathogenic germs. Therefore, it is required to preserve the knowledge of medicinal plants, herbs, spices and herbal remedies; which are received from past generations for posterity. Symbiotic fungi usually perform compatible and friendly interactions with host plants, which contribute to growth promotion and secondary metabolites accumulation simultaneously, such as alkaloids and terpenoids with pharmacological characteristics. In this association, Arbuscular Mycorrhizal Fungi (AMF) improves uptake of soil nutrients such as P, N, Zn^{2+}, $Cu^{2+}$and K^{+}; thereby influencing plant growth and survival, plant nutrition, tolerance to water stress and adverse environmental conditions (Smith and Read, 1997).

Ratti and Upadhyay (2012) screened different germplasms of Ashwagandha collected from different geographical regions. They observed extreme variability in their morphological, biochemical characters and percentage colonization on association with AM fungi and withanolide content. They concluded that percent root colonization was highest in Lucknow region (56%) followed by Madhya Pradesh region (52%), while it was lowest in Punjab region (30%).

Materials and Methods

In the present investigation, plants were raised from the seed material of the genotype JA 20 (Jawahar Asgandh 20), a cultivated variety of Ashwagandha developed by Jawahar Lal Nehru Krishi Vishwa vidhalaya Mandsore, Madhya Pradesh, India and mycorrhizal spores used were *Glomus mosseae*, purchased from "The Energy and Resource Institute TERI, New Delhi".

Fresh leaves and roots were collected from five month old plants during mature stage. Leaves and roots were shade dried and ground to fine powder using mortar and pestle. Methanolic extract was prepared from leaf and root samples. The samples were fractionated with Thin Layer Chromatography (TLC).



Preparation of TLC Plates

The glass plates (20 x 10 cm) were washed and made grease free with acetone. These glass plates were then coated with silica gel G_{254} (0.2–0.3 mm thick) prepared by dissolving 5g /20ml distilled water. The plates were dried at room temperature followed by activating at 100ºC for 30 minutes and cooled to room temperature.

Development of Thin layer chromatogram

Methanolic extracts (1µl) prepared using leaf and root samples were separately loaded about 1cm from the edge of the plate with the help of capillary chromatograms were developed in a number of solvent systems **(Table a)**. The solvent system with best resolution of various components for leaf and root extracts was SS-5.

Table a: Solvent systems used for TLC analysis of leaf and root extracts in *Withania somnifera* (L.) Dunal

Solvent system code	Solvent system	Ratio
SS-1	EtOAc: MeOH	5:5
SS-2	$CHCl_3$: EtOAc	1:1
SS-3	EtOAc	(100%)
SS-4	Hexane : EtOAc	7:3
SS-5	$CHCl_3$: MeOH	9:1
SS-6	$CHCl_3$: EtOAc	1:1

Visualization and detection of R_f value

After air drying, the TLC plates were sprayed uniformly with 5% methanolic H_2SO_4 and heated in a hot air oven at 100ºC for 10 min. The image of the chromatogram thus developed was recorded immediately. The Relative front (R_f)

value of each of the spots was computed as ratio of distance traveled by the spot to the distance traveled by the developing solvent on the chromatogram.

Sulphuric Acid Reagent

It was prepared by adding 5 ml of sulphuric acid in aliquots to 95 ml cold methanol while cooling with ice carefully.

$$\text{Relative front } (R_f) = \frac{\text{Distance traveled by sample}}{\text{Distance traveled by solvent}}$$

High Performance Liquid Chromatography (HPLC)

HPLC is considered as the most sensitive and reliable method for analysis. Shade dried root and leaf samples (1 g each) from the selected plant were powdered and percolated separately four times with ethanol: water (1:1) at room temperature (25 ± 2 ºC) for 6 hours each at stirring. The extracts were filtered through Whatman No. 1 filter paper to remove debris and the solvent was evaporated under vacuum at 50 ± 5 ºC in a rotavapor. The dried samples were weighed and dissolved in a known volume of HPLC grade methanol (1 mg/1 ml, w/v) for quantitative analysis. Standards (1.2 mg/ml) were prepared in HPLC-grade methanol. Analysis was performed with HPLC system equipped with 515 quaternary gradient pump, 717 Rheodyne injector, 2996 PDA detector and Empower software (version 3.0). The samples used for HPLC were filtered through 0.45 M filter (Millipore, Bedford).

The extracts were injected (20µl) in a C-18 (4.6 mm × 250 mm) column. The fractions eluted with MeOH–H_2O (60:40) at a flow rate of 0.7 ml min^{-1} were analyzed at 30 ºC to provide efficiency to the peaks and the UV chromatograms were monitored at 237 nm. The identity of withanolides was confirmed by three standards. The peaks were identified based on retention time with comparison to retention time of the standard marker compounds. Quantification of withanolides was carried out by using the peak area of the sample chromatogram in the regression equation of the calibration curve for each withanolides.

Column: C_{18} Phenomenex (250 x 4.60mm) - 5µ
Mobile phase (Gradient): Acetonitrile (B): Water(A)

Time (min)	Function	Value
0.01	B. Conc	20.0
22.00	B.Conc	85.0
27.00	B.Conc	85.0
28.00	B.Conc	20.0
32.00	STOP	

Flow rate: 1.6 ml/min
Wavelength : 215nm
Injection loop capacity: 20µl
Concentration of Samples: 1mg/ml of standard and isolated compound.

$$Response\ Factor = \frac{Peak\ Area}{Standard\ Amount} = Analyte$$

$$Amount\ of\ Analyte = \frac{Peak\ Area}{Response\ Factor} = Sample\ Amount$$

RESULTS AND DISCUSSION

Thin layer chromatography was opted as an effective, economical, simple and preferred method to study chemical fingerprint or chemoprofile in Ashwagandha. Mathew and his coworkers (2005) have developed TLC fingerprints of ten important crude drugs obtained from different plant parts *viz.* roots (*Plumbago indica*, *Rauvolfia serpentina*), shoots

(*Centellaa siatica*, *Andrographis paniculata*), tubers (*Holostemma adakodein*, *Curculigo orchioides*) and bark (*Saraca asoca*) and clearly demonstrated the suitability of the fingerprints in identification, detection of adulterants and differentiation of closely related species.

In the present study, phytochemicals extracted from leaves and roots of mature plants of selected genotype of *Withania somnifera* (L.) Dunal were analyzed using TLC. Both control and mycorrhiza inoculated samples were simultaneously analyzed. Out of six different combinations of solvents examined **(Table a)**, the solvent system SS-5 having composition of chloroform: methanol (9:1) was found to be the best in giving clear and well resolved spots for methanolic extact of leaf and root of Ashwagandha.

The leaf sample resolved into nine spots in SS-5 solvent system with Rf values 0.07, 0.12, 0.17, **0.32, 0.48**, 0.64, 0.73, 0.8 and 0.98; while the root sample resolved in five spots with Rf values 0.16, 0.29, **0.48**, 0.58 and 0.93; which coincided with standard samples of withaferin A and withanolide A (Rf value of **0.32** and **0.48**, respectively) **(Figure a & b)**.

Quantification of six major bioactive withanolides from roots and leaves of Ashwagandha through HPLC revealed a significant chemical variability. Kumar et al. (2007) also reported a wide range of variations in withanolide accumulation and morphological characters among the populations and in different plant parts of *W. somnifera*. Highly significant ($P < 0.05$) qualitative and quantitative differences were observed between control and mycorrhizal associated samples of leaf and root tissues. The high degree of variation in the quality and quantity of active principle in different organs was also reported in the previous studies (Cirak et al., 2007, 2012; Kumar et al., 2007, 2011; Kitchlu et al., 2011; Katoch et al., 2012). The representative HPLC chromatograms of standard marker compounds **(Figure c)** and the leaf and root extracts were developed **(Figure d & e)**. The total withanolide percentage (w/w) in the control and mycorrhizal inoculated leaf samples were 0.85 and 1.21 respectively whereas in roots control sample, withanolide content was 0.17 and it was 0.28 in root inoculated sample.

The results confirmed the presence of high concentration of withaferin A in the leaf and withanolide A in the root. Mycorrhiza inoculation increased the total withanolide production by 42% in leaf and 51% in the root **(Figure f)**. That means, AM symbiosis can significantly increase the content of some secondary metabolites in Ashwagandha.

Withanolide accumulation correlated positively with developmental stages and highest content of these withanolides was found at maturity in both roots and leaves. This indicated that medicinal plants should be harvested at maturity stage for maximum economic benefit (Mir et al., 2014) in terms of withanolide content. Yaseen et al. (2011) reported that vesicular infection was high at flowering stage, reaching to maximum at fruiting stage in Ashwagandha. Thus, confirming the role of mycorrhiza in increasing the contents of secondary metabolites (Zeyad et al., 1999).

Raveesha et al. (2014) reported that quantitative analysis of withanolides and steroidal lactones in the Ashwagandha, revealed an increased in primary metabolites like proteins, carbohydrates, amino acids and secondary metabolite like phenolics.

Kapoor et al., (2004) also obtained similar results for the accumulation of essential oil in fennel. However, Nell et al., (2010) observed either unchanged or significantly decreased content of sesquiterpenic acid in different genotypes of *V. officinalis*, when plants were treated with P. Therefore, the effects of P on secondary metabolites may vary among different medicinal plants.

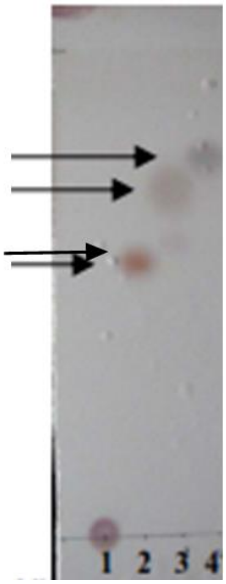


Fig. a: TLC profile of standard samples: lane 1- Withanoside IV, lane 2 - Withaferin A, lane 3 –Withanone , lane 4 -Withanolide A (Arrows indicates the position of spots resolved on chromatography)

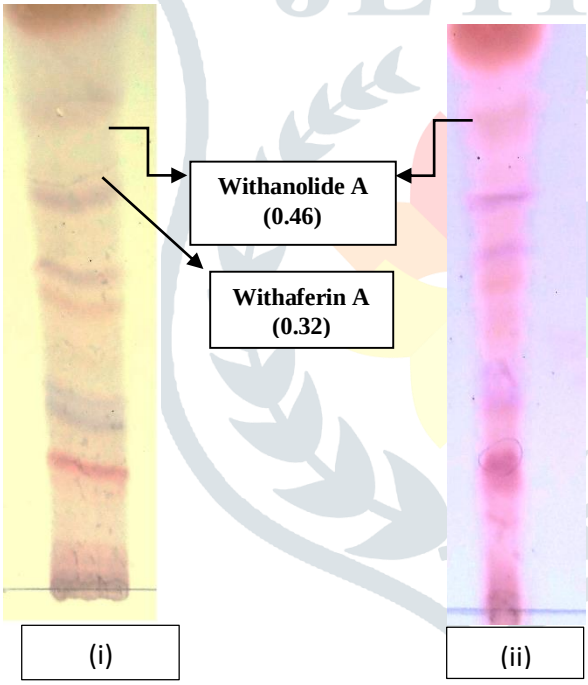


Fig. b (i): Withanolide A and Withaferin A isolated from the leaf of genotype JA 20

Fig. b (ii): WithanolideA isolated from the root of genotype JA 20

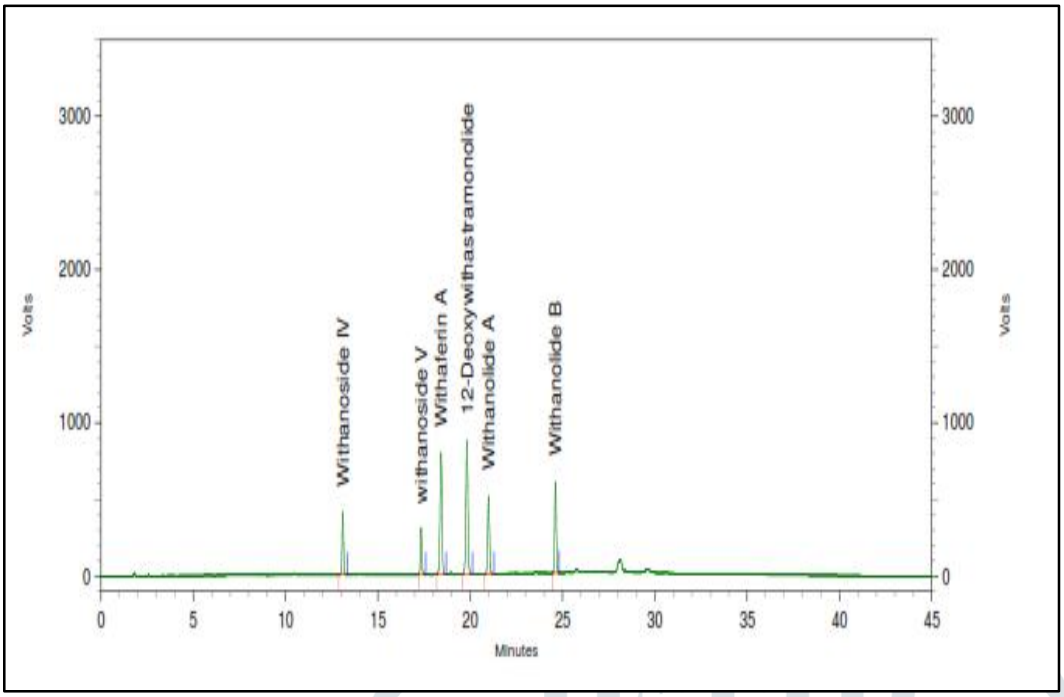


Fig. c: HPLC chromatogram of standard markers compound

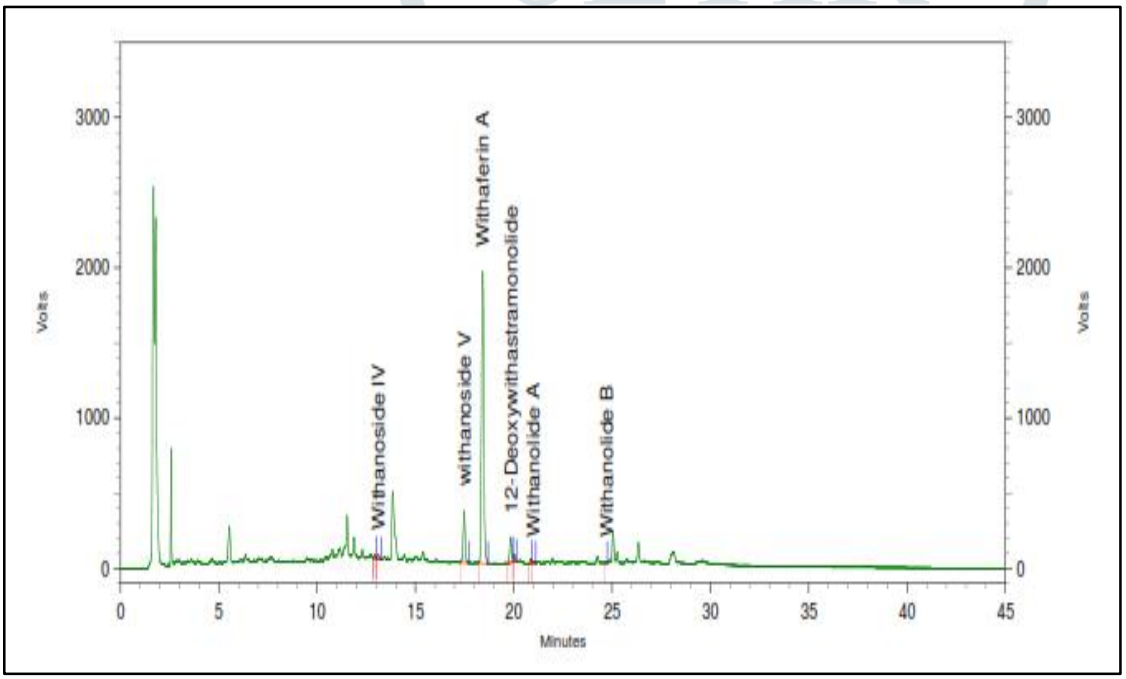


Fig. d: HPLC chromatogram of control (without mycorrhiza) leaf sample of genotype JA 20

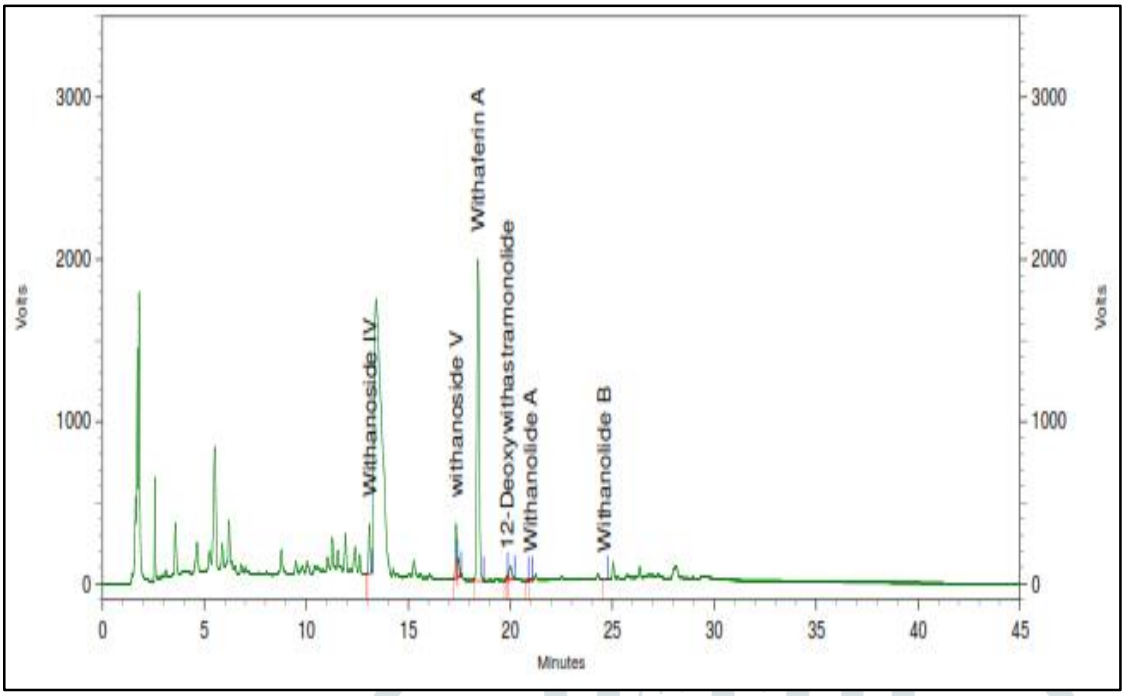


Fig. e: HPLC chromatogram of mycorrhiza inoculated leaf sample of genotype JA 20

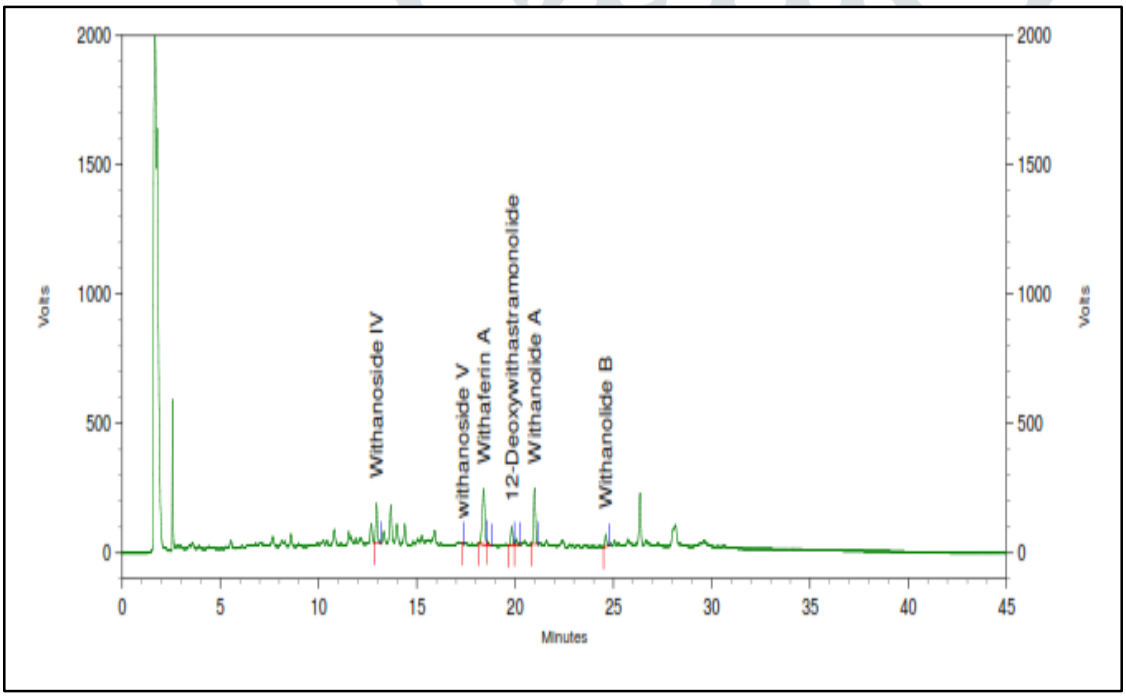


Fig. f: HPLC chromatogram of control (without mycorrhiza) root sample of genotype JA 20

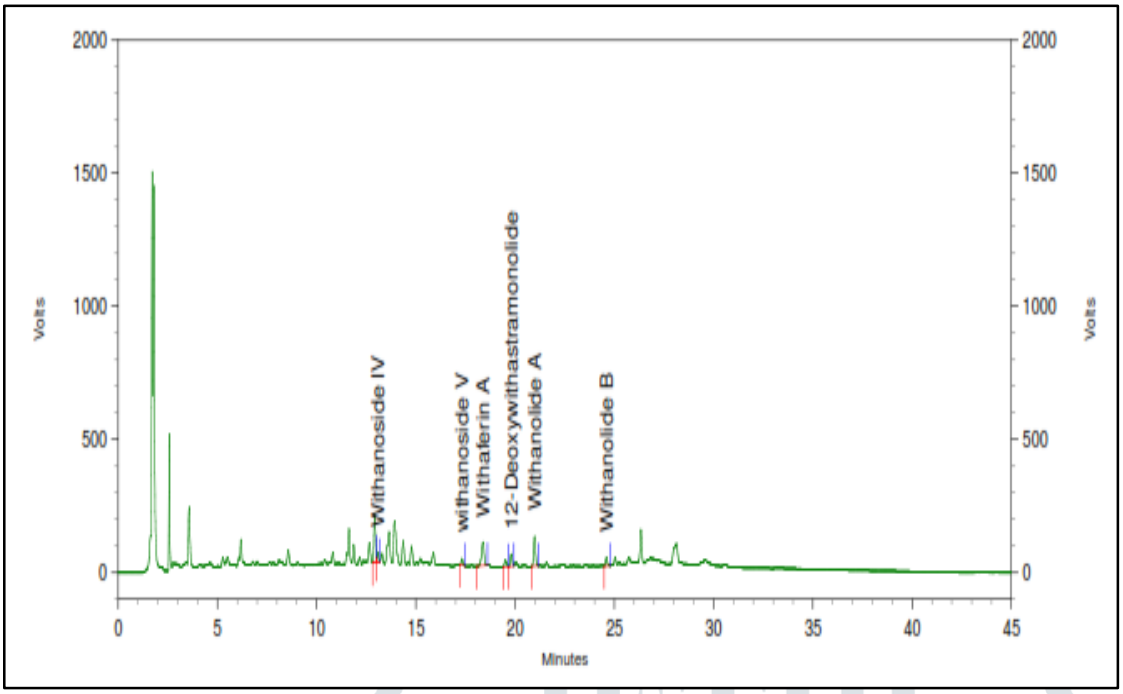


Fig. g: HPLC chromatogram of mycorrhiza inoculated root sample of genotype JA 20

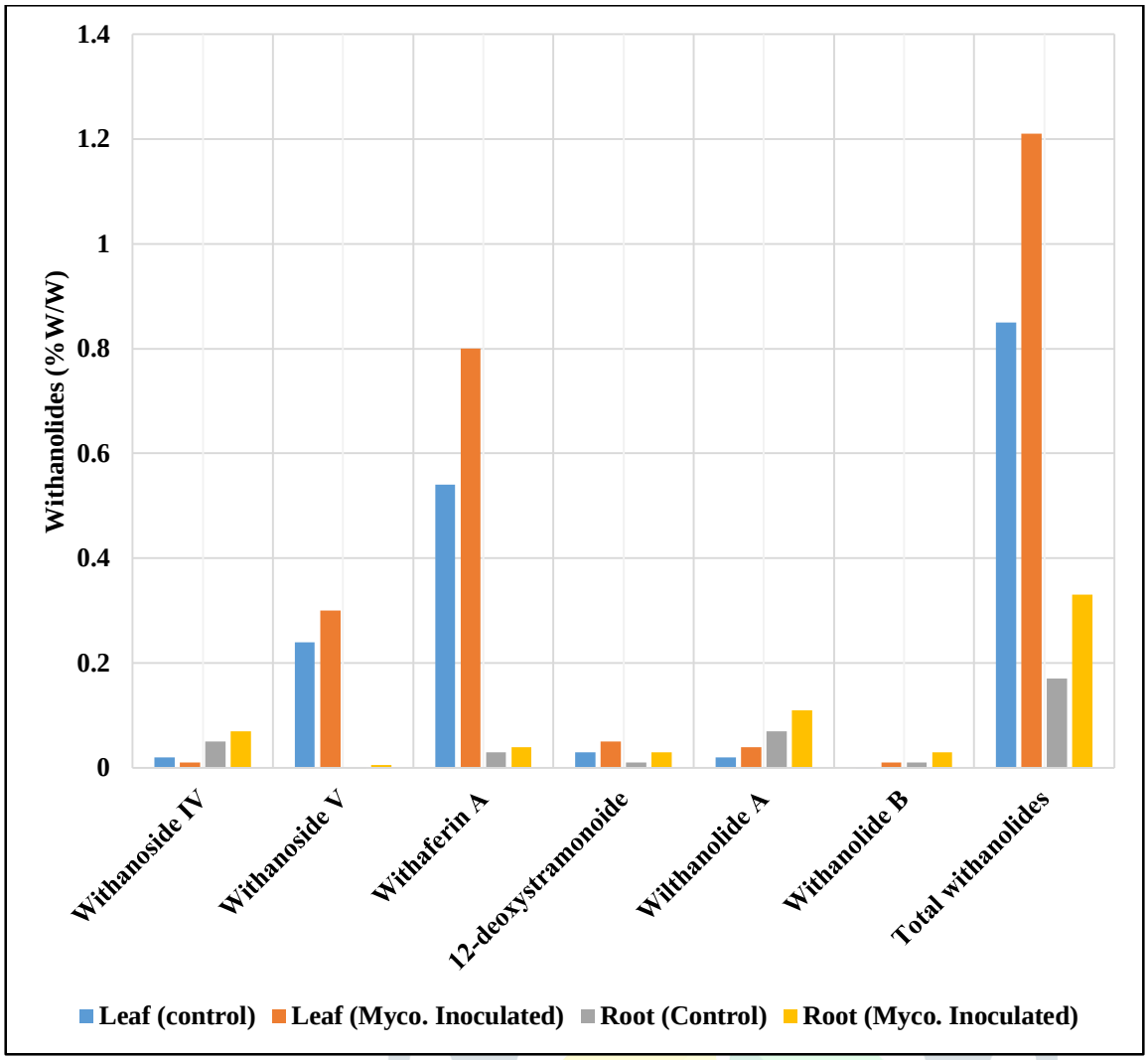


Fig. h: Withanolides (mg % w/w) in control and mycorrhiza inoculated leaf and root samples of genotype JA 20

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