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Biochemical and phytochemical variations during the growth phase of *Withania somnifera* (L.) Dunal

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Abstract
Withanolides are therapeutically important secondary withanolides present in *Withania somnifera.* The present study was conducted to identify the growth stage of the plant that corresponds with the maximum amount of *in vivo* withanolide concentration and to correlate these values with the concentration of other phytochemicals and metabolic products. The plants were grown from seedling stage till 150 days from germination. At an interval of every 30 days the plants were checked for differences in the concentrations of withanolides and various other phytochemical compounds. HPLC analysis revealed a significant increase in the withanolide A and withaferin A at 60 days from germination which showed a strong correlation with the carbohydrate levels of the plant. This data will be useful for commercial extraction of withanolides from *W. somnifera* at higher concentrations.

Keywords: *Withania somnifera*, Withanolides, HPLC, Withanolide A, Withaferin A, Growth Phases, Plant Maturity

Introduction
Withania somnifera (L.) Dunal (Solanaceae) commonly known as Ashwaganda is a small green shrub that naturally grows in drier climates. These plants are commonly found in countries like India, Pakistan, Sri Lanka, Egypt, Congo, Morocco, Baluchistan, Afghanistan, South Africa and Jordan. In India they are found to naturally occur in the Western Ghats and they are extensively cultivated in Madhya Pradesh, Uttar Pradesh, Punjab, Gujarat and Rajasthan [1, 2].

W. somnifera is cultivated for its medicinal properties, due to which it is a common ingredient in many Ayurvedic preparations. Its roots have been used to treat fevers, skin diseases, gynecological disorders, arthritis, rheumatism and inflammations of various body parts [3]. Owing to similarities in its medicinal properties as well as the activity of its biologically active constituents with that of the ginseng it has been given the moniker of Indian ginseng [4].

The biologically active constituents present in *W. somnifera* have been found to be C28 steroidal lactones with ergostane backbone [5]. Chemically they are 22-hydroxy ergostane-26-oic acid 26, 22-d-lactones that are broadly clubbed under the term withanolides [6]. This includes compounds like Withanolide A, Withanolide B, Withanone, Withaferin A. Amongst these Withanolide A and Withaferin A are found in relatively higher concentrations in plants. Withanolide A and Withaferin A have been shown to posses various therapeutic activity against diseases like various forms of cancer, Alzheimer's disease and Parkinson's disease, diabetes etc [7–9]. They also posses anti-inflammatory, antistress, antioxidant, immunomodulatory, hemopetic adaptogen, memory enhancing, antivenom, rejuvenating and antidiuretic properties [10, 11] due to which the plant is currently highly sought after with researchers around the world trying to improve the extraction process and to increase the withanolide concentrations in the plant itself.

For commercial purposes field grown plants are used. The crop requires dry climate during its growth so they are usually grown after the monsoon season during the second half of the year during June or July. Late winter rains are found to be beneficial for their growth and development. Since they do not require continuous irrigation, they can be grown on plots which are unsuitable for the growth of other crops and therefore do not interfere with the cultivation of other economically important plants. Since their pests are rare and they do not require fertilizers the initial cost for production is not that high. The plants start producing flowers and fruits from December onwards and they are harvested at 150 days after sowing, the maturity of the plant is judged by the drying of the leaves of the plants and the ripening of the berries which gives them a red colour [12, 13].

Studies have shown that there are chemotypic variations among the wild varieties and the cultivated varieties that correspond to differences in their phytochemical constituents. The concentration of the various phytochemical constituents seem to depend on a number of factors that include developmental age of the plant when harvested, tissue from which they are extracted, nutrients, temperature, and the geographical location in which they are grown [6].

There have been various studies conducted to understand the metabolic profile of *W. somnifera* and the pathways involved in their biosynthesis. Tissue culture techniques have also been used to increase the concentration of the withanolides present in the plant artificially with the use of various elicitors like jasmonic acid, salycilic acid, polyamines, chitosan etc [3, 11, 14]. changes in concentrations of various plant hormones have also been used to increase the concentration of withanolides in *in vitro* grown plants. The initial concentration and any increases that can be induced artificially in the phytochemical constituents of tissue culture plants depends on the stage of life cycle of the plant from which the explant is taken. Differences in developmental stages can lead to differences in initial with anolide concentrations as plants produce secondary metabolites at different levels during different times. Therefore it is important to know the relative concentration of various withanolides at different stages of the plants life cycle.

There is very little knowledge available in literatures about the differences in with anolide concentrations during the plants growth phases. Therefore the current study has aimed to discern the differences in morphological, biochemical and phytochemical differences in *W. somnifera* from the time of sowing to 150 days after germination when the plant is typically harvested and to correlate the data so as to obtain a reliable time frame of with anolide concentrations in *W. somnifera* during different stages of its growth.

Materials and method

Chemicals and materials

The chemicals used for the experiments were obtained from Himedia, Bombay and the withanolide A and withaferin A standard were obtained from Sigma-Aldrich, Germany. The Sonicator and rotary evaporator used for extract preparation were GT Sonic and Adarsh- Rotary Vaccum Evaporator Quickvap respectively. UV spectroscopic measurements were done using Shimadzu UV-1800 spectrophotometer (Kyoto, Japan). The HPLC analysis was performed using using Skyray instrument, LC-310 Riverse Phase HPLC (RP_HPLC) that was equipped with LC-UV100 detector and Sharpsil-U C18(250(L)x4.6mm ID).

Plant sample

W. somnifera seeds were obtained from University of Agricultural Sciences, GKVK Bangalore and sown in pots in the polyhouse of CHRIST (deemed to be university), Bangalore and raised for 150 days (from germination). Plant samples were collected periodically at an interval of 30 days for the experimental procedures.

Protein estimation

Protein content was estimated using Bradford assay (1976) [15]. Fresh leaves (500 mg) were collected and homogenized in 10mL phosphate buffer (0.05M and pH 7.4). The crude homogenate was centrifuged at 1000 x g for 10 min at 4˚C. From the supernatant 0.2mL was taken and 5mL Bradford reagent was added. A UV–visible spectrophotometer was used to record absorbance at 595 nm. For quantification of the protein content in the plant sample, a standard calibration curve was prepared using Bovine serum albumin. Protein content was defined as a milligram of Bovine serum albumin equivalents per g of leaf sample.

Carbohydrate estimation

Carbohydrate content was estimated using Phenol-Sulfuric acid method [16]. Fresh leaves (100 mg) were taken and homogenized in 5mL of 2.5N HCl and placed in a boiling water bath for 3 h. The cooled crude homogenate was neutralized with sodium carbonate till effervescence ceased and centrifuged at 1000 x g for 10 min. From the supernatant 0.2mL was taken and 1ml phenol [2% (v/v)] was added followed by 5mL of sulfuric acid [96% (v/v)] in a cold water bath. A UV–visible spectrophotometer was used to record absorbance at 490 nm. For quantification of the carbohydrate content in the plant sample, a standard calibration curve was prepared using D-glucose. Carbohydrate content was defined as a milligram of D-glucose equivalents per g of leaf sample.

Extract preparation

The dried plant samples were grind to a fine powder using a grinder. 1g of the powdered plant sample was extracted with 10mL methanol under sonication for 30 minutes. The slurry was then centrifuged at 8000 rpm for 15 minutes. The supernatant was carefully transferred to a round bottom flask and placed in a rotary evaporator apparatus. The methanol was evaporated in the rotary evaporator at 52˚C for 30 minutes. The remaining dried extract was re-dissolved in 5mL methanol and again centrifuged at 8000 rpm for 15 minutes to remove any particulate matter [14].

Total phenolic content

The TPC was measured using Folin-Ciocalteu assay [17]. From the extract 0.2mL was taken to which 0.5mL of the Folin-Ciocalteu reagent (1:1) was added followed by 2mL of sodium bicarbonate solution [20% (w/v)]. The obtained solution was maintained for 2 h in dark at room temperature. A UV–visible spectrophotometer was used to record absorbance at 638 nm. For quantification of the TPC content in the plant sample, a standard calibration curve was prepared using catechol. TPC was defined as a milligram of catechol equivalents (CAE) per g of dry weight.

Total Tannin content

The TTC was measured using Folin-Ciocalteu assay [17]. From the extract 0.2mL was taken to which 0.5mL of the Folin-Ciocalteu reagent (1:9) was added followed by 2mL of sodium bicarbonate solution [20% (w/v)]. The obtained solution was maintained for 2 h in dark at room temperature. A UV–visible spectrophotometer was used to record absorbance at 725 nm. For quantification of the TTC content in the plant sample, a standard calibration curve was prepared using Tannic acid. TTC was defined as a milligram of Tannic acid equivalents (TAE) per g of dry weight.

Total Flavonoid content

TFC were evaluated using a modified aluminium chloride colorimetric method [18]. From the extract 0.1mL was taken to which 0.1mL of the aluminium chloride [10% (w/v)] was added followed by 0.1mL of 1M potassium acetate. The obtained solution was maintained for 40 min at room temperature. A UV–visible spectrophotometer was used to record absorbance at 415 nm. For quantification of the TFC

content in the plant sample, a standard calibration curve was prepared using quercetin. TFC was defined as a milligram of quercetin equivalents (QE) per g of dry weight.

Determination of antioxidant activity

Antioxidant activity was estimated using DPPH scavenging assay [19]. 30µl of the Plant extract was taken and made up to 3ml with methanol. 1 ml of DPPH [0.004% (w/v)] was added in dark condition and followed by incubation for 30 minutes in dark. A UV–visible spectrophotometer was used to record absorbance at 513 nm. The antioxidant activity was calculated by using the formula

Percentage Radical Scavenging Activity = $[(A_0 – A_1)/A_0]$ x 100

Where

A_0 is the absorbance of the blank (methanol with DPPH)

A_1 is the absorbance of the sample (ascorbic acid and the plant samples)

Estimation of Withanolides using HPLC analysis

The HPLC analysis was performed as per the procedure prescribed by Sivanandhan *et al*., (2012) [14]. 1g of the dried and powdered plant sample was extracted with 10mL methanol under sonication for 30 min. The slurry was then centrifuged at 8000 rpm for 15 min. The supernatant was concentrated in rotary evaporator at 52˚C for 30 mi. The remaining dried residue was re-dissolved in 5mL methanol and centrifuged at 8000 rpm for 15 min. The supernatant was then filtered through 0.45 µm membrane and analyzed using a HPLC apparatus.

Results and discussion:

Protein content

The protein content was found to be 0.71 ± 0.0007, 1.59 ± 0.001, 2.54 ± 0.0004, 4.03 ± 0.0008 and 3.84 ± 0.0008 mg/g for 30, 60, 90, 120, 150 days from germination respectively. There is a constant increase in the concentration of protein in the plant samples at each 30 days interval till 120 days from germination. This result is contrary to the results obtained by L. Johnson *et al*., (2010) who reported a decrease in the protein concentration of the leaf during the maturation of the corn ear [20]. In the case of W. *somnifera* it was observed that the total protein content of the leaves increased regardless of the flowering and fruit development.

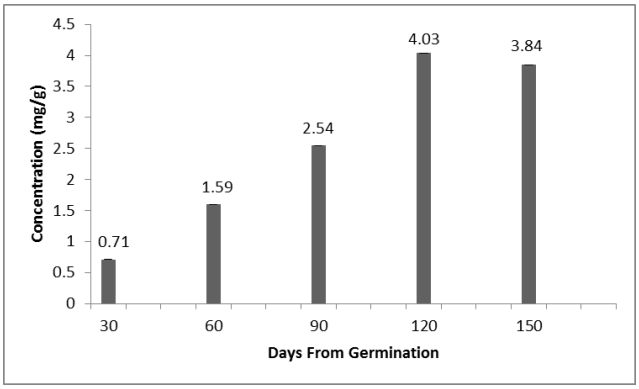


Fig 1: Concentration in mg/g Vs Days from germination of W. somnifera – graph depicting the change in the protein content of the plant through different time periods.

Carbohydrate content

The carbohydrate values obtained were 20.33 ± 0.52, 33.33 ± 0.62, 34.08 ± 0.62, 19.16 ± 0.76 and 24.58 ± 0.76 mg/g for 30, 60, 90, 120, 150 days from germination respectively. The concentration is seen to increase from 30 to 60 days from germination and only a slight increase is seen from 60 days from germination to 90 days from germination (flowering and fruiting starts). The value drops after 90 days at 120 days from germination and again increases slightly at 150. Sulpice *et al*. (2009) discovered a negative correlation between plant growth and starch levels in the leaves of Arabidopsis [21]. This validates the results obtained in the current experiment where a decrease in the plant carbohydrate is observed in correlation to the start of the fruiting stage. This is due to the translocation of sucrose and starch into the fruits and also due to the breakdown of these energy reserves to obtain the energy required by the plants to produce flowers and fruits.

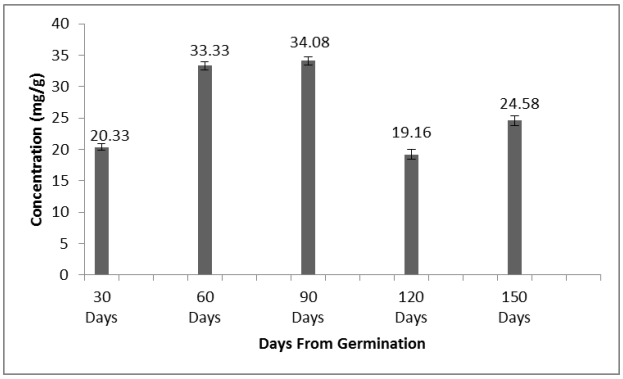


Fig 2: Concentration in mg/g Vs Days from germination of W. somnifera – graph depicting the change in the Carbohydrate content of the plant through different time periods.

Total phenol content

The total phenol values obtained were 2.82 ± 0.13, 1.82 ± 0.02, 2.03 ± 0.02, 1.14 ± 0.07 and 3.03 ± 0.05 mg/g for 30, 60, 90, 120, 150 days from germination respectively. The trend observed is that the value of total phenol is high at 30 days from germination and then drops at 60 days from germination and increases slightly at 90 days from germination at this stage flowering and fruiting also begins. Then the value drops again at 120 days from germination this is followed by a drastic increase in the total phenol value at 150 days from germination. Similar results were also reported by Morello´ *et al*. (2005), who showed that henolics contents decreased as maturation proceeds in olive drupes [22].

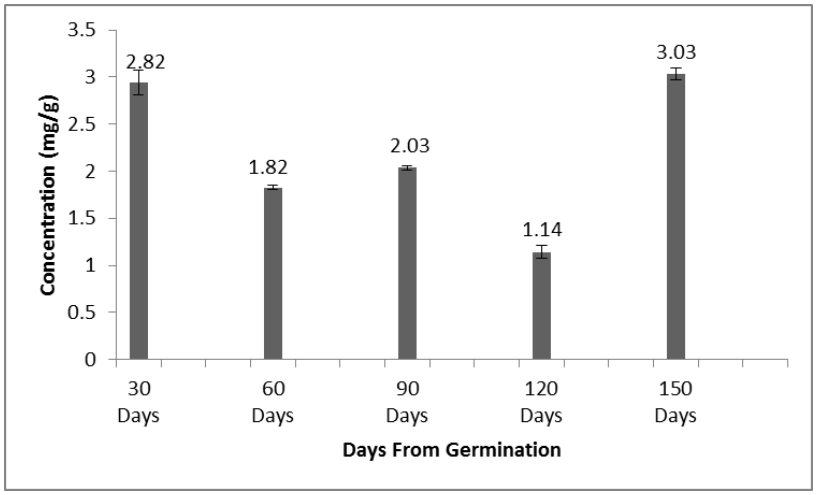


Fig 3: Concentration in mg/g Vs Days from germination of W. somnifera – graph depicting the change in the Total Phenol content of the plant through different time periods.

Total tannin content

The tannin content obtained was 2.31 ± 0.02, 1.74 ± 0.02, 2.30 ± 0.02, 0.93 ± 0.01 and 3.50 ± 0.03 mg/g for 30, 60, 90, 120, 150 days from germination respectively. The trend observed is that the value of tannin content is high at 30 days from germination and then drops at 60 days from germination and increases again at 90 days from germination. Then the value drops again at 120 days from germination. This is followed by a drastic increase in the tannin value at 150 days from germination. It was observed by E. D. Donnelly in 1959 that the amount of tannin is seen to increase with the maturity of the plant which is contrary to the results obtained in this research [23].

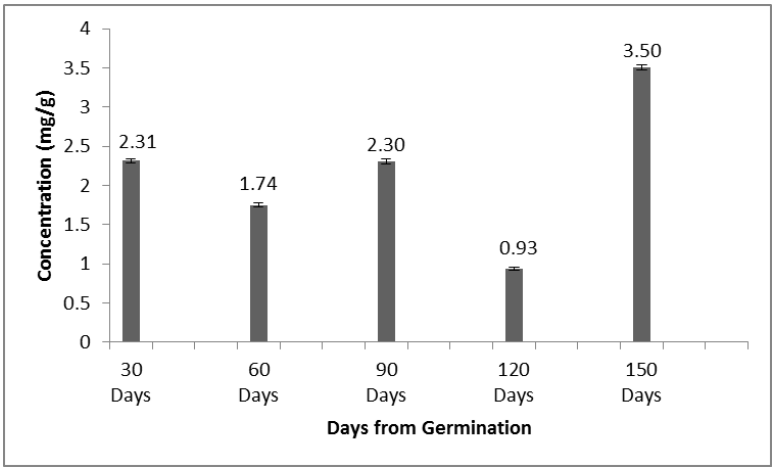


Fig 4: Concentration in mg/g Vs Days from germination of W. somnifera – graph depicting the change in the Tannin content of the plant through different time periods.

Total flavonoids content

The flavonoids content obtained was 5.58 ± 0.02, 1.86 ± 0.005, 2.50 ± 0.02, 1.26 ± 0.005 and 1.75 ± 0.005 mg/g for 30, 60, 90, 120, 150 days from germination respectively. The trend observed is that the value of flavonoids content is high at 30 days from germination and then drops at 60 days from germination and increases again at 90 days from germination. Then the value drops again at 120 days from germination. This is followed by a slight increase in flavonoids value at 150 days from germination.

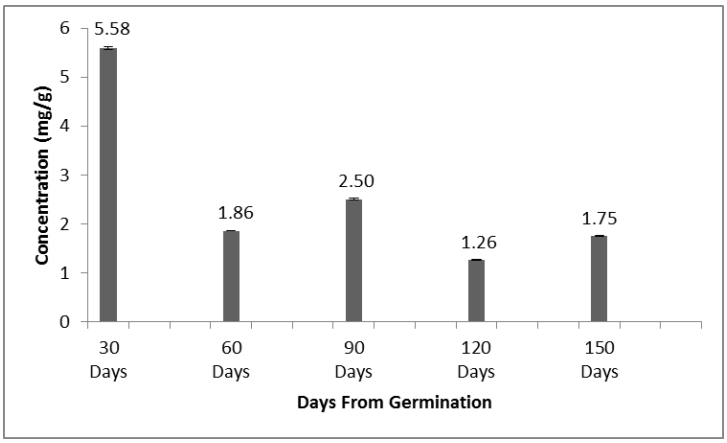


Fig 5: Concentration in mg/g Vs Days from germination of W. somnifera – graph depicting the change in the Flavonoid content of the plant through different time periods.

Antioxidant activity: DPPH scavenging assay

The percentage of antioxidant activity obtained was 80.19 ± 0.56, 70.62 ± 0.68, 95.31 ± 0.49, 66.66 ± 0.37 and 92.07 ± 0.67 for 30, 60, 90, 120, 150 days from germination respectively. The trend observed is that the value of percentage of antioxidant activity is high at 30 days from germination and then drops at 60 days from germination and increases again at 90 days from germination reaching the highest value that is observed in this data. Then the value drops again at 120 days from germination to the lowest value observed. This is followed by an increase in the percentage of antioxidant activity at 150 days from germination. Similar results were reported by Duda-Chodak *et al*. (2011) for their research on apple fruits, they observed that antioxidant and phenols contents decrease as the plant matures. A strong correlation is seen between the total phenolics content and the antioxidant activity. M. Nadeem *et al*., (2018) arrived at a similar conclusion with their work on *Ficus carica* L. [24]

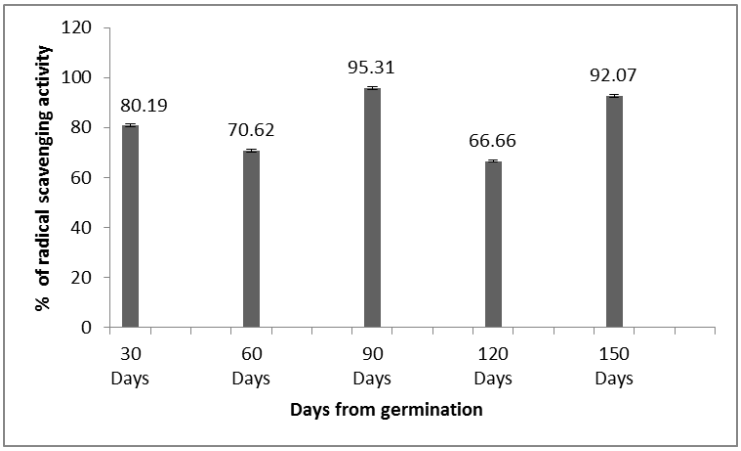
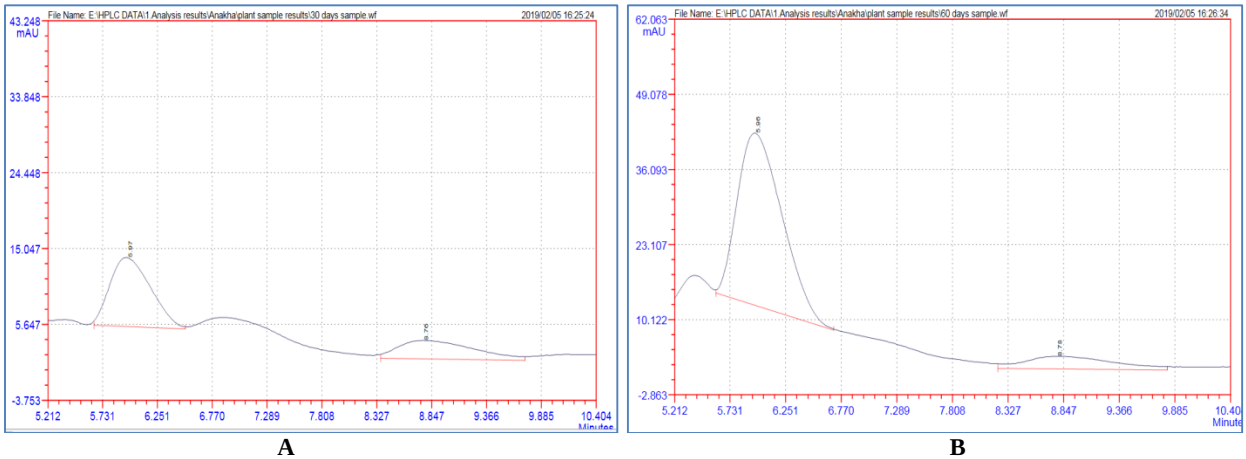
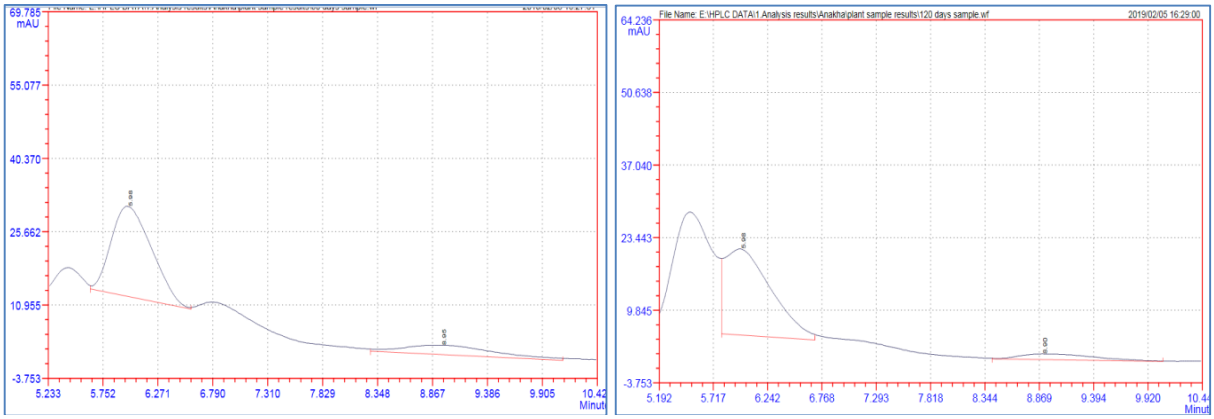


Fig 6: Percentage of Antioxidant Activity Vs Days from germination of W. somnifera – graph depicting the change in the antioxidant activity of the plant through different time periods

HPLC Analysis of *W. somnifera* during different time periods of development



A **B**



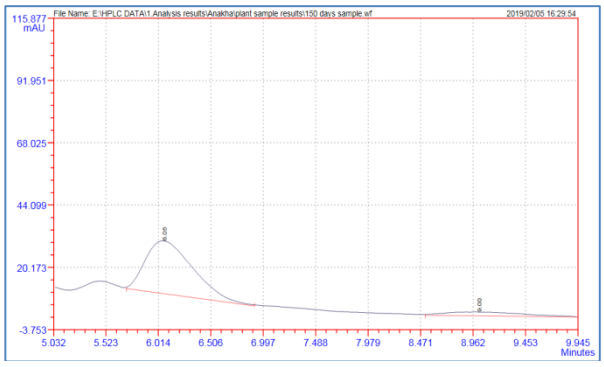


Fig 7: Graph A, B, C, D and E showing the different peaks obtained for withanolide A (8.76) and withaferin A (5.97) present in the extract of W. somnifera for 30, 60, 90, 120 and 150 days old plant respectively

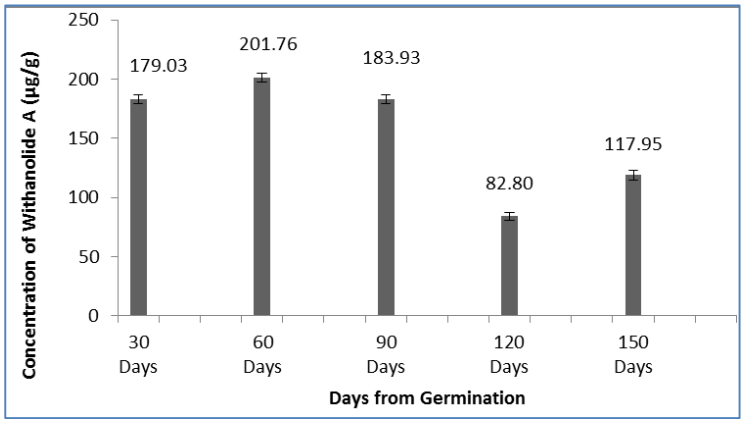


Fig 8: Graph showing the changes in the withanolide A concentration with the change in time period of W. somnifera's growth.

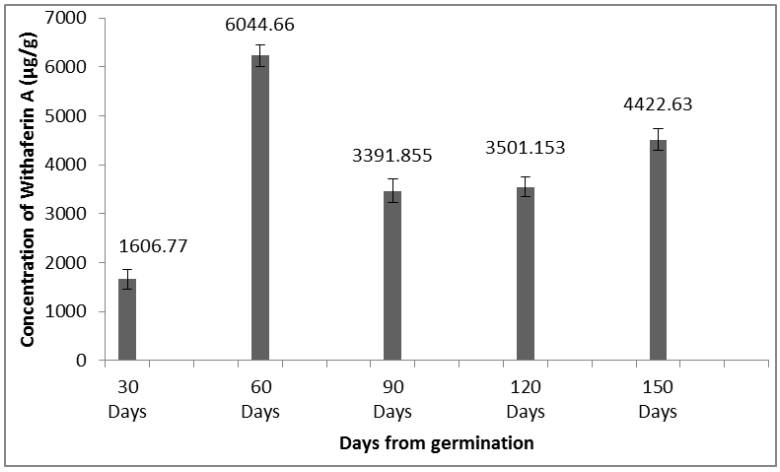


Fig 9: Graph showing the changes in the Withaferin A concentration with the change in time period of W. somnifera's growth.

The concentration of withanolide A was found to be 179.03 ± 3.97, 201.76 ± 3.52, 183.93 ± 3.56, 82.80 ± 3.28 and 117.95 ± 4.06 µg/g for 30, 60, 90, 120 and 150 days from germination respectively. The value increases from 30 days to 60 days and then drops a little. This is followed by a drastic drop at 120 days followed by a moderate increase at 150 days. The highest value obtained is at 60 days and the lowest at 120 days from germination

The concentration of Withaferin A was found to be 1606.77 ± 199.72, 6044.66 ± 228.38, 3391.85 ± 239.92, 3501.15 ± 198.40 and 4222.63 ± 217.37 µg/g for 30, 60, 90, 120 and 150 days from germination respectively. The value increases drastically from 30 days to 60 days and then drops drastically at 90 days. This is followed by a slight increase at 120 days and then again at 150 days. The highest value obtained is at 60 days and the lowest at 30 days from germination.

This indicates that the maximum withanolide concentrations are found in plant stages before flowering and the value decreases at later stages after the production of fruits starts. The values for both withanolide A and withaferin A are similar to the values obtained for carbohydrates indicating that their concentrations may be directly linked. This may be because the primary compounds that take part in their biosynthetic pathway come from the carbohydrates produced by the plants. The carbohydrate reserve present in the plant will primarily be used for the energy required for flowering and the rest translocated into the fruits produced. Since the reproductive stage is an important for the plant it may withhold the use of carbohydrates for production of secondary metabolites till excess carbohydrates have accumulated in the plant once again. Since the carbohydrates are scarce the rate of production of secondary metabolites like withanolides may be reduced. Similar experiment was conducted by N. Dhar *et al*., (2013). They observed that the concentration of withanolide A and withaferin A is the highest at fruit set stages that is the stage when fruits start to form on the plant that is between 90 to 120 days from germination [25].

The HPLC analysis indicated that both withanolide A and withaferin A are present in higher concentration at 60 days from germination just before flowering stage commences. These results can help us conclude that for the purpose of extraction of these withanolides using plants at the stage of 60 days from germination would be economically better. This can result in the extraction of higher concentrations of these valuable compounds than normal. Micropropogation or tissue culturing of these plants too can be done using plants at this stage for higher initial concentration of these withanolides.

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