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# COMPARISON OF VARIOUS ACTIVE INGREDIENTS BETWEEN *EX-SITU* AND *IN-VITRO* GROWN PLANTS OF *WITHANIA SOMNIFERA* L. AND *CICHORIUM INTYBUS* L.

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**ABSTRACT**
Different plant parts of two traditional medicinal plants viz. *Withania somnifera* L. and *Cichorium intybus* L. were analyzed for their reducing power using Folin-Ciocalteu and Aluminium chloride method respectively. The preliminary phytochemical screenings of various extracts revealed the presence of alkaloids, glycosides, steroids, saponins, tannins and flavonoids and then comparison among these parameters was done in *Ex situ* and *In vitro* cultivated plant. Thin layer chromatography (TLC) of alkaloids, flavonoids and terpenoids were carried out with different solvent systems. Among the samples of *W. somnifera* and *C. intybus* maximum percentage scavenging activity was observed in methanolic leaf extract. The maximum 2, 2-diphenyl-1-picrylhydrazyl (DPPH) activity was found in the leaf extract. The phenolic content in the above plants ranged from 2 to 10 mg GAE/g while the flavonoid from 3 to 12 mg GAE/g. No direct correlation was found between total phenolic content and antioxidant activity. Dissimilar pattern of antioxidant and phytochemical constitutes of *Ex situ* and *In vitro* grown plant was observed.

## INTRODUCTION

Ashwagandha (*Withania somnifera* L.) belongs to the family *Solanaceae* is an important medicinal plant, commonly used as a domestic remedy for several diseases. The pharmacological effect of the roots of *W. somnifera* is attributed to its active ingredients (*withanolides and withaferin)* which have a wide range of therapeutic applications. To date, very limited data exists on phenolic compounds reported in *W. somnifera* leaves, roots and fruits as well as their antioxidant effects to support their traditional claims (Alam *et al.,* 2011). The major biochemical compounds of Indian ginseng are steroidal alkaloids and steroidal lactones.

Chicory *(Cichorium intybus* L.) is a medicinally important plant that belongs to the family *Asteraceae*. Significant increase in secondary metabolite (esculin) was observed in *In vitro* grown plantlets of chicory (Rafsanjani *et al.,* 2011). Secondary metabolites like alkaloids are produced by a large variety of organisms including bacteria, fungi, plants, animals. They often have pharmacological effects and are used for medications as recreational drugs. Flavonoids are widely distributed in plants, fulfilling many functions viz. producing yellow or red/blue pigments in flowers, protection from microbes and insects etc. The strong and growing demand in market for these natural products has focused attention on I*n vitro* plant materials as potential factories for secondary phytochemical products, and has paved the way for new research exploring secondary product expression *in vitro*. The root, stem and leaves of regenerated plants or the induced callus may be used fresh or dried, as raw drugs or different secondary metabolite are extracted from them(Supe *et al*., 2011). Till now very few reports of cell culture which can accumulate alkaloids at level significantly higher than the parent plants. Plants tissue culture may very well contain metabolic pathways that have been modified from that of the plant (Gita *et al.,* 2003).

In the present work, a reproducible method for high frequency callus induction of *C. intybus* and *W. somnifera* was established and DPPH radical scavenging activity, phenolic and flavonoid content of *ex situ* and *in vitro* grown callus have been estimated.

## MATERIALS AND METHODS

Fresh Leaves, stems and buds of *C. intybus* and *W. somnifera* were collected. The explants were surface sterilized using bavistin (0.5%) and streptomycin (0.03%) and savlon followed by washing with mercuric chloride (0.1%) and 70% ethanol (Mohan 2011). The medium used for callus induction was MS medium supplemented with 2, 4-D (0.5-1.0 mg/L) alone or in combination with BAP (0.5-1.0 mg/L). The number of callus and percentage of callus induction frequency was recorded after 35 days. Proliferated callus were sub cultured for shooting (2, 4-D: 0.5-1.0 mg/L + BAP: 0.5-5.0 mg/L) and rooting (2, 4-D: 0.5-10 mg/L + BAP: 0.5-1.0 mg/L).

Phytochemical screening of *In vitro* and *Ex situ* explants was carried out using standard procedures (Edeoga 2005). The plant material was tested for the presence of alkaloids, tannins, saponins, flavonoids, glycosides and other active ingredients present in the plant and fractionated by Thin layer chromatography (Tiwari *et al.* 2011).

**TLC study of various active compounds**

The alkaloids and flavonoids were separated using appropriate solvent systems viz. Toluene: Chloroform: ethanol (8.5:57:14.5), toluene: acetone: formic acid (38:10:5) and benzene: ethyl acetate (9:1) respectively. The separated components were observed in leaves and stems samples under visible light after spraying with appropriate reagents.

**Determination of antioxidant activity**

**Total phenol content**

The extraction of total phenolics was performed using the Folin– Ciocalteu assay. 100$\mu$L of each extract (1mg/mL) was added to a test tube containing 50$\mu$L of the phenol reagent (1 N). Further 1.85 mL of distilled water was added to the solution and allowed to stand for 3 mins. and then 300$\mu$L $Na_2CO_3$ (20% in water v/v) was added and vortexed, and the final volume (4mL) was obtained by adding 1.7mL of distilled water. A reagent blank was prepared using control. The final mixture was vortexed, and then incubated for 1h in the dark at room temperature. The absorbance was measured at 725. Standard curve was prepared using concentrations of gallic acid in methanol: water (50:50 v/v). Total phenolic value was calculated in terms of Gallic acid equivalents (GAE) in mg/g plant extract (Alam *et al.,* 2011, Kamal, 2011).

**Total flavonoids content**

Aluminum chloride colorimetric method was used for flavonoids determination. 0.5mL plant extract (1mg/mL) in methanol was mixed with 1.5 mL of methanol, 0.1mL of 10% aluminum chloride, 0.1mL of 1 M potassium acetate and 2.8mL of distilled water. It was incubated at room temperature for 30 mins. and the absorbance was measured at 415nm. The calibration curve was prepared by using Quercetin as the standard. The results were expressed in terms of Quercetin equivalents (QE) in mg/g plant extract (Kamal, 2011).

**DPPH radical scavenging activity**

The antioxidant capacity of the *W. somnifera and C. intybus* was also studied through the evaluation of the free radical-scavenging effect on the DPPH radical. The free radical scavenging activity was measured in terms of hydrogen donating or radical scavenging ability, using the stable radical, DPPH. 3mL of DPPH (0.1mM) in methanol was prepared and added to 200$\mu$L of control *i.e.* standard gallic acid was added at different concentration (25-250$\mu$g/mL) and test solution. After 30 mins. incubation, absorbance was measured at 517nm. Methanolic, ethanolic and aqueous extracts of the leaves and stems were evaluated and compared with the respective extracts of the callus. Results were expressed in terms of Gallic acid equivalents (GAE) in mg/g plant extract (Ozgen *et al.,* 2004). The results were expressed in terms of percentage inhibition as

[(Ab control-Ab sample)/Ab control] * 100

Where, Ab control = absorbance of control, Ab sample = absorbance of sample.

## RESULTS AND DISCUSSION

Different combinations of 2, 4-D (0.5-2.0mg/L) and BAP (0.5-2.0mg/L) were used for callus induction from leaf and stem explants. 2, 4-D (2.0mg/L) and BAP (1.0mg/L) was found to be optimum for obtaining high frequency of nodulated callus (Fig. 1d) in *C. intybus* (Velayutham *et al.,* 2006)**.** 3mg/L BAP + 0.5mg/L 2, 4 D found to be optimum for shooting (Fig. 1e), whereas, 8 mg/L 2, 4-D was best for rooting. In *W. somnifera* 2, 4-D (0.5mg/L) and BAP (2.0mg/L) was optimum for callusing (Fig. 1a) (Sharma *et al.,* 2010). The response shown by different explants varied widely depending on the concentration of 2, 4-D. The overall callus induction frequency on MS medium varied from 10-50 percent in *Withania* and from 5-30 percent in Chicory (Vermeulen *et al.,* 1993). In Chicory, Maximum shoot regenerating was found in 2 mg/L BAP + 0.5mg/L 2, 4-D (Fig. 1b,c) (Kumar *et al.,* 2011, Velayutham, 2006), whereas,

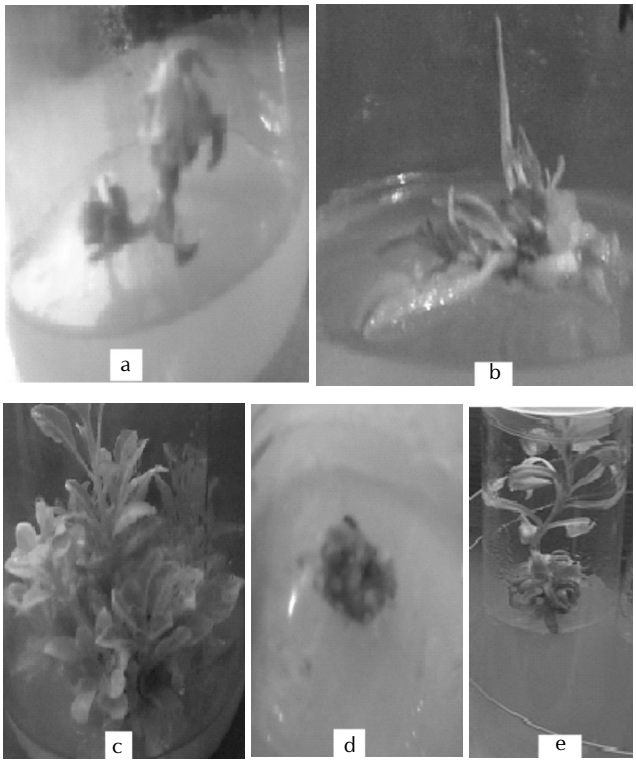


**Figure 1: Plant regeneration in *Withania* (a: callus induction, b: shoot initiation, c: multiple shooting) and in *C. intybus*(d: callus induction e: plantlet formation)**

Maximum rooting was observed in 2.0mg/L 2, 4-D (Arumugam and Gopinath, 2011, Rafsanjani *et al.,* 2011).

Different solvent systems were used for the extraction of active ingredients. Ethanol was found to be optimum for the isolation of flavonoids, whereas, methanol for alkaloids (Tiwari *et al.,* 2011). Aqueous extracts were also prepared to perform and compare the quantitative analysis of the phytochemicals present in *ex situ* and *in vitro* conditions. In both cases, the maximum yield was observed in methanolic extract whereas

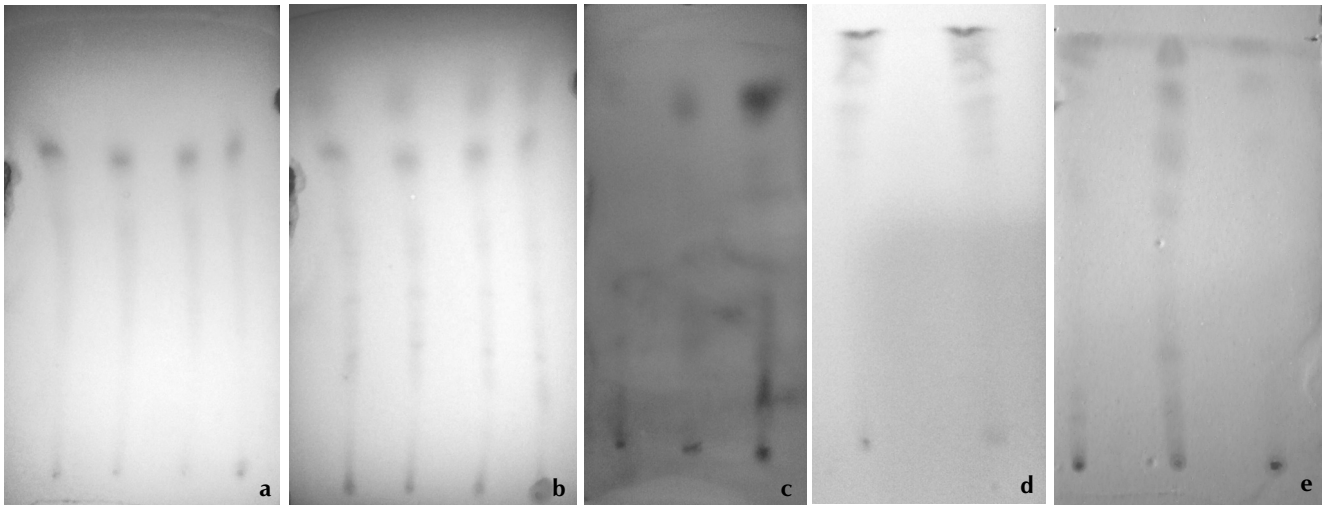


**Figure 2: TLC for active ingradients in leaf and stem of *W.somnifera* (b: flavonoids, d: alkaloids), *C. intybus* (a: flavonoids, c: alkaloids) and *In vitro* grown plants (e: alkaloids)**

minimum was obtained in water extract of callus. This suggests that the leaves of the *ex situ* grown plants contain a significant amount of phytochemicals and could be extracted in methanolic solvent. However, *ex situ* conditions doesn't facilitate the use of water as a good solvent for extraction of the phytochemicals. The water soluble extractive values indicated the presence of tannins and other inorganic components. The alcohol soluble extractive values indicated the presence of polar constituents like phenols, alkaloids, steroids, glycosides and flavonoids. From these observations it may be concluded that *Withania* contains alkaloids and flavonoids in *ex situ* and *in vitro* conditions, whereas, saponins and glycosides were absent in callus. In case of *Cichorium*, terpenoids and flavonoids were present in both plant and callus, while tannins and glycosides were found only in field conditions (Singh *et al.,* 2010). These fluctuations in the concentration and quantities of secondary metabolites is basically due to environmental influences (Supe *et al.,* 2011)

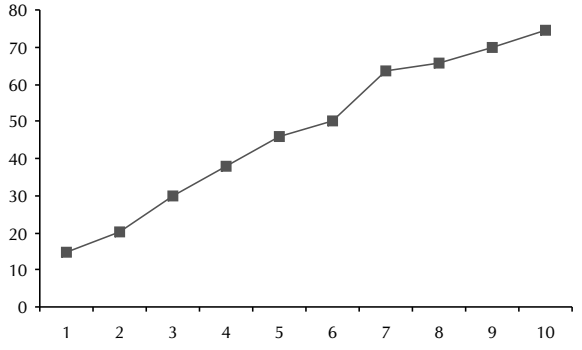


**Figure 3: Graph of % DPPH activity vs. concentration (Mohan *et al.* 2011)**

Thin layer chromatography of *W. somnifera and C. intybus* extracts were performed for identification of different active ingredients. The qualitative testing of active ingredients was done by TLC and same were confirmed by standard Rf values of different active ingredients. Flavonoids were extracted in methanol, whereas, alkaloids were isolated in ethanol (Tiwari *et al.,* 2011). Toluene: acetone: formic acid (38:10:5) gave the best separation of the different components present in the flavonoid extract, followed by development of color in iodine vapors and spraying with sulphuric acid reagent (10% $H_2SO_4$ in methanol). For alkaloids, a number of solvent systems were tried whereas the best results were obtained in Toluene: Chloroform: Ethanol (8.5:57:14.5). For alkaloids, TLC showed 3 spots in *W. somnifera* leaf and stem (Fig. 2d). Two intense spots were confirmed as withanolide (Std Rf 0.65) and

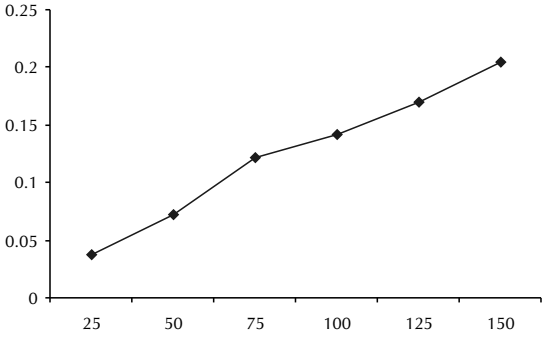


**Figure 4: Standard calibration curve for gallic acid**

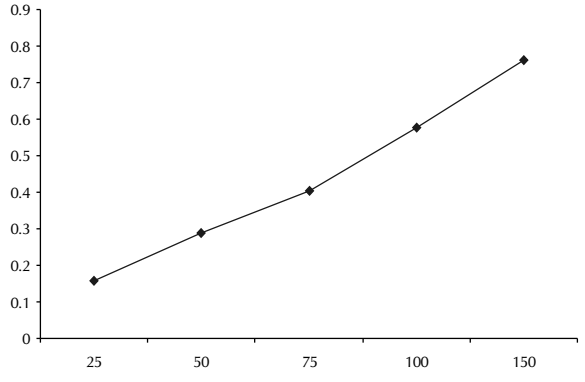


**Figure 5: Standard calibration curve for quercetin**

**Table 1: Callus induction efficiency at different growth hormonal combinations**

| Mediumcomposition (mg/L) | | Callus induction | Percentage efficiency(%) | Callus texture |
|---|---|---|---|---|
| *R. serpentina* | | | | |
| 2, 4-D | BAP | | | |
| 1.5 | 1.0 | - | 0 | - |
| 1.5 | 1.5 | - | 0 | - |
| 2.0 | 0.2 | + | 10 | Light green, friable |
| 2.0 | 0.5 | + + | 30 | Light green, friable |
| 2.0 | 1.0 | + + + | 50 | Light green, friable |
| 2.0 | 1.5 | - | 0 | - |
| - | - | - | - | - |
| *V. minor* | | | | |
| BAP | NAA | | | |
| 14 | 0 | - | - | - |
| 14 | 0.01 | - | 0 | - |
| 14 | 0.1 | - | 0 | - |
| 14 | 0.2 | + | 10 | Light yellow, compact |
| 14 | 1 | - | 0 | - |
| 14 | 2 | + + | 40 | Whitish yellow, compact |
| 14 | 3 | + + + | 60 | Whitish yellow, compact |

withaferin A (Std Rf 0.6) (Sumithradevi 2011). Leaf and stem of *C. intybus* showed 3 different spots (Fig. 2c). The spot with Rf 0.69 was identified to be Lactucopicrin (Std Rf 0.65). Whereas, TLC of flavonoids showed 4 and 3 spots in *W. somnifera* stem and leaf sample respectively (Fig. 2b). Two major spots were confirmed as choline (Std Rf 0.34) and withanone (Std Rf 0.2). Leaf and stem fraction of *C. intybus* showed 3 spots in each (Fig. 2a). Most intense spot was confirmed as lactucin having Std Rf 0.7(Thin Layer Chromatography Atlas). Lesser amount of alkaloids was detected in *In vitro* grown plants ((Fig. 2e).

The antioxidant activity of medicinal plants is mainly related to their bioactive compounds, such as phenolics and flavonoids. There were significant differences in terms of their scavenging abilities present among the leaves and stem samples, expressed as percentage of inhibition on the DPPH radical. Among the three extracts, the least scavenging activity was found in aqueous extract while the maximum activity was found in methanolic extracts in *W. somnifera* and least activity was found in ethanol and maximum in methanolic in case of *C. intybus*. The reasons behind the markedly higher radical scavenging capacity exhibited by the different types of extracts probably lie in their diverse botanical origin. Antioxidant potential of plant extracts is directly related to its phenolic and flavonoids content (Alam *et al.,* 2011).

The results of the DPPH radical scavenging activity of *C. intybus* shows that *ex situ* plants possess very high percentage of antioxidant activity (70.4 %) whereas the *in vitro* grown callus shows least antioxidant activity (30.5 %) (Alam *et al.,* 2011) and in case of Withania the *ex situ* plants possesses 56.3 % of DPPH scavenging activity and *In vitro* has 37.2 % (Fig. 3).

Phenolics are well established to show antioxidant activity and contribute to human health. The content of phenolics was evaluated from the concentration vs. absorbance graph (Fig. 4). The phenolic content in *C. intybus* was least in ethanolic (2.5mg/g) and maximum in methanol (7.8mg/g), whereas, in case of *W. somnifera* least in aqueous (2.8mg/g) and maximum in methanol (8.5mg/g). The corresponding phenolic content of *In vitro* grown callus of *W. somnifera* and *C. intybus* was 6.6mg/g GAE and 7.3mg /g GAE respectively. Flavonoids are well-known antioxidant constituents of plants and possess a broad spectrum of chemical and biological activity, including radical scavenging properties. The total flavonoids content for *W. somnifera* was found to be 11.5mg/g QE in the methanolic leaves, whereas, the total flavonoids content for *C. intybus* was found to be maximum in the methanolic leaves extract 9.9mg/g QE extract(Fig. 5).

The Pharmacognostic characters and phytochemical values reported in this paper could be used as the diagnostic tool for the standardization of the different plant parts of *Withania somnifera* and *Cichorium intybus*. They have a definite role to play in the health care system around the globe. The *In vitro* antioxidant studies provide sound scientific footing to enhance confidence on the traditional claims of *W. somnifera* and *C. intybus*. Hence the plant is a potential source of natural antioxidant which could be useful in physiological and pathological medicine, and of great interest to food manufacturing industries.

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