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 In 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. 100μL of each extract (1mg/mL) was added to a test tube containing 50μL of the phenol reagent (1 Folin–Ciocalteu assay. 100μL of each extract (1mg/mL) was added to a test tube containing 50μL of the phenol reagent (1

Further 1.85 mL of distilled water was added to the solution and allowed to stand for 3 mins. and then 300μL Na2CO3 (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 using Quercetin as the standard. The results were expressed in terms of Quercetin equivalents (QE) in mg/g plant extract (Kamal et al., 2011).

### DPPH radical scavenging activity

The antioxidant capacity of the *Withania 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 200μL of control i.e. standard gallic acid was added at different concentration (25-250μg/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

\[
\text{percentage inhibition} = \left( \frac{\text{Ab control} - \text{Ab sample}}{\text{Ab control}} \right) \times 100
\]

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), 3mgL 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 regeneration was found in 2 mg/L BAP + 0.5mg/L 2, 4-D (Fig. 1b, c) (Kumar et al., 2011, Velayutham, 2006), whereas,
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)

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% H₂SO₄ 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
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 withanine (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.

### REFERENCES


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

<table>
<thead>
<tr>
<th>Mediumcomposition (mg/L)</th>
<th>Callus induction</th>
<th>Percentage efficiency(%)</th>
<th>Callus texture</th>
</tr>
</thead>
<tbody>
<tr>
<td>R. serpentina</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BAP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>0.0</td>
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<tr>
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<tr>
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<td>50</td>
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<td>14</td>
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</tr>
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</table>

**Table 1** shows the callus induction efficiency at different growth hormonal combinations. The percentage efficiency is calculated based on the callus induction and callus texture is described as Whitish yellow, compact.


