ABSTRACT:

Withania somnifera Dunal (Ashwagandha) is important medicinal plant. Plant tissue culture is very useful to increase the production of secondary metabolites. Callus was obtained on MS supplemented with 2 mg/l 2, 4-D and 0.2 mg/l KIN. Chromatography is the second important technique for analysis and isolation of these herbal drugs. Qualitative and quantitative analysis was done through TLC and HPTLC. TLC of in vitro suggested 5 unidentified steroids and Diosgenin, cholesterol, Stigmasterol, β-sitosterol were obtained in in vitro materials. HPTLC of in vitro materials was done and scanned at different wavelengths (200 nm, 254 nm, 366 nm). Calibration spectrum, Rf values and amount of particular spot were recorded at particular wavelength through scanning. Total 3 steroids at 200 nm, 1 at 254 nm, 5 at 580 nm were analyzed. Absorption spectra of each substance were also prepared at 200 nm. Some of these are confirmed while others are unidentified.

KEY WORD: Steroids, Withania Somnifera, TLC, HPTLC.

INTRODUCTION:

Plants are very important commercial source of chemical compounds including primary and secondary metabolites. Ayurveda practitioner employ these plant to cure swelling, poultices lesions, tubercular ulcers, scabies, ophthalmia, muscular pain, dropsy, rheumatism, diabetes and even cancer. These properties are mainly because of its primary and secondary metabolites of drugs. This drug is naturally present in the parts like leaf, stem, root, seed or some times in the whole plant. These parts can be artificially cultured on the media and can get maximum amount of drugs. For it, Murashige and Skoog's, 1962 (MS) medium is used as a basal nutrient medium. Different types of growth promoters like 2, 4-D, IAA, IBA, NAA, Kinetin, BAP are used to prepare hormonal medium. Even it can be prepared with alone auxin or cytokinin or in combination of both (Vasil, 1984). Amount of drugs, which are artificially produced in 8 week old callus can be compared with naturally occur in plant (whole plant). Plant tissue
culture approach has been found to be advantageous as it provides a continuous and reliable source of artificial product year around without the destruction of entire plant. With the help of tissue culture, high quantities of desired compounds can be obtained.

According to Staba (1980), steroids are divided into different four types (1) sterols are alcoholic and ‘ol’ suffix (β-sitosterol, stigmasterol) (2) sterolin are glycosides of sterol and insoluble in water (cholesterol, estrones) (3) steroidal saponins have ‘nin’ suffix (diosgenin) (4) nitrogen containing steroidal saponins. Saponins may be either triterpenoid glycoside or glucoside of steroids with spiroketal side chain. It is of four types (1) Triterpenoid saponins (panaxadiol, panaxatriol and oleonolic acid) (2) Steroidal saponins (diosgenin, tigogenin, prototokoronin, tokorogenin, yamogenin, gitogenin, manogenin) (3) Nitrogen containing saponins (steroidal alkaloid and solasodine) (4) Cardenolide saponins. Terpenoids and steroids are biosynthesised from isoprene or isopentane (C₅) units evolving from mevalonic acid (C₆).

The term ‘terpene’ refers specifically to hydrocarbon compound. (1) Monoterpenoid compounds may be acyclic, monocyclic (pulegone), bicyclic (menthofuran) or involved in the formation of furanochromones, irridoids, cannabinoids or pyrethrins. (2) Sesquiterpenoids are often found as constituents of steam distillable volatile oil and may be acyclic (fanesol) and cyclic (bisabolene) (3) Diterpenes are formed from four isoprene units. They may be acyclic (phytol) or cyclic (gibberellic acids, abietic acid, gossypol, stevioside) (4) Tetraterpenes are formed from the fusion of two diterpene and normally trans as symmetrical. They are cyclic, monocyclic or bicyclic compounds (carotene, xanthophylls) (5) Triterpenoids may be acyclic (squalene), tricyclic or more complex (β-amyrin, cholesterol). They may occur free or as glucoside (saponins). Plant steroids are most often from cycloartenol and occasionally from lanosterol. Steroids are similar to the triterpenoids (C₂₃ to C₂₉) but with only two methyl groups attached to the ring system. The C₂₄ ethyl group is derived from succisive additions of the methyl group methonine. Steroids are often present in tissue culture as phytosterols (β-sitosterol, stigmasterol and campesterol) with either sitosterol or stigmasterol being the dominant sterol (eg. 24-methylene cholesterol and 24-ethyliene cholesterol in Withania somnifera). The growth regulators kinetin and IAA stimulated β-sitosterol and stigmasterol. The two most commonly available steroids are cholesterol and β-sitosterol (Dand, 1970). The sitosterol is a mixture of α, β and γ-sitosterols. Approximately two third of the raw material for chemical synthesis of the steroid hormones produced has depended on diosgenin obtained from the plant Dioscorea root (Capsi et al., 1974; Curtin, 1983; Prasad and Ammal, 1983). Besides diosgenin, other plant sterols such as sitosterol, stigmasterol, campesterol solasodine, hecogenin are considered highly promising precursors.
Hence, the present investigation was taken up for qualitative and quantitative analysis of in vitro produced steroids through TLC and HPTLC.

**PLANT MATERIAL:**

**Scientific name**: *Withania somnifera* Dunal

**Family**: Solanaceae

**Common names**: Asandh, Asagandh, Ashwagandha (Nadkarni, 1954)

**Known chemical constituents:**

Alkaloids: Withanine, Withaninine, Somniferine, Tropeltigloate, Somniferinine, Somnininie, Nicotine, Visamine, Withasomine

Salts: Cuscohygrine, Anahygrine, Tropine, Pseudotropine, Anaferine

Steroidal Lactones: Withaferin-A, Withanone, WS-1, Withanolide E C_{28}H_{38}O_{7}, Withanolide F C_{28}H_{38}O_{6}, Withanolide G C_{28}H_{36}O_{4}, Withanolide H C_{28}H_{36}O_{5}, Withanolide I C_{28}H_{36}O_{5}, Withanolide J C_{28}H_{36}O_{5}, Withanolide K C_{28}H_{36}O_{5}, Withanolide L C_{28}H_{36}O_{5}, Withanolide M C_{28}H_{36}O_{6}

Nitrogen containing compounds: Withanol C_{25}H_{34}O_{5}, Somnisol C_{32}H_{46}O, Somnitol C_{33}H_{46}O_{7}

Steroids: Cholesterol, β-sitosterol, Stigmasterol, Diosgenin, Stigmastadien, Sitoinosides VII, Sitoinosides VIII, Sitoinosides IX, Sitoinosides X

Flavonoids: Kaempferol, Quercetin

**Medicinal properties and uses:** Leaves and roots of this plant are abortifacient, aphrodisiac, diuretic, nerveine tonic, alterative, narcotic, sedative, astringent, growth promoter and anthelmintic. It has anti-arthritic, anti-bacterial, anti-dote for scorpion sting, anti-stress, anti-tumour and anti-cancer activities. It is used in toning of uterus, consumption, dropsy, leucoderma, impotence, rheumatism, debility from old age, ulcer, sexual and genital weakness, assumption, rheumatic swelling, loss of memory, loss of muscular energy, spermatorrhoea, syphilis, sterility of women, blood discharge, leucorrhoea, anemia with emaciation, nervous exhaustion, multiple sclerosis, neoplasia, cancer and fatigue. Fruits and seeds are diuretic and used in coagulation of milk (Nadkarni, 1954)

**METHODOLOGY:**

**A) TISUE CULTURE**

1. Preparation of nutrient medium
Preparation of stock solutions
- Mix the solutions and stir it
- Dissolve sugar and add agar-agar
- Heat the above solution till agar-agar dissolve
- Add stock solutions of PGRs and make up the volume
- Adjust pH with HCl or NaOH to 6.8
- Pour the medium into culture vials
- Plug the vials with non-absorbent cotton
- Autoclave for 15 minutes at 120°C and cool it

1. Inoculation and Culturing of the explants
   - Explants should be washed in soap water, tap water and finally rinsed with distilled water
   - Under aseptic condition treatment with 0.1% HgCl₂ is given for few minutes and finally rinsed with DW
   - Explants are introduced into the vial over a flame to avoid microbial contamination
   - Immediately plug the vial

2. Maintaining the culture vials under controlled light and temperature conditions
3. Periodic observations of the cultured explants
4. Maintaining the culture through subculture
5. Conclude and make inferences about the experiment conducted / field trial of in vitro raised plantlets

(B) THIN LAYER CHROMATOGRAPHY (TLC)
The operation performed in TLC is essentially the same as in paper chromatography. This technique involves several steps:
1. Preparing thin layer (e.g. silica gel G.)
2. Choice of solid for support (e.g. glass plate)
3. Sample application

**Extraction of steroids:** (Tomita et al., 1970)

**Procedure:** 100 mg dried and powdered callus were defatted and extracted with 100-150 ml petroleum ether (60-80°C B.P.) for 2-4 hours in soxhlet apparatus. Petroleum ether extract was hydrolysed with 5% hydrochloric acid (v/v) in 70% ethanol (v/v) (5 ml HCl in 95 ml 70% ethanol) for four hours on water bath (70-80°C). The hydrolysates (floated residual mass) were extracted with 50
ml ethyl acetate. This step was done for 2-3 times till the residual mass gave colourless solution. All ethyl acetate fractions were collected together and dried in vacuo. 2-4 ml Ethyl acetate or chloroform was added to the residue and used for further analysis of steroids.

**Sample extract:** Residue was redissolved in ethyl acetate or chloroform.

4. Choice of solvent (mobile phase)
   Hexane : Acetone (8:2) for steroids

5. Development of chromatograph

6. Detecting or spraying reagent
   50% H$_2$SO$_4$ (50 ml concentrated H$_2$SO$_4$ was diluted up to 100 ml with DW) for steroids

7. Identification and calculation of Rf value
   \[ Rf = \frac{\text{Distance traveled by sample}}{\text{Distance traveled by solvent}} \]

(C) HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY (HPTLC) *(Sethi, 1996)*

- **Formulation:** Herbal drugs
- **Classification:** Alkaloids / Steroids / Flavonoids
- **Dosage form:** Liquid
- **Sample preparation**

**Extraction of steroids:** *(Tomita et al., 1970)*

**Procedure:** 100 mg dried and powdered callus were defatted and extracted with 100-150 ml petroleum ether (60-80°C B.P.) for 2-4 hours in soxhlet apparatus. Petroleum ether extract was hydrolysed with 5% hydrochloric acid (v/v) in 70% ethanol (v/v) (5 ml HCl in 95 ml 70% ethanol) for four hours on water bath (70-80°C). The hydrolysates (floated residual mass) were extracted with 50 ml ethyl acetate. This step was done for 2-3 times till the residual mass gave colourless solution. All ethyl acetate fractions were collected together and dried in vacuo. 2-4 ml Ethyl acetate or chloroform was added to the residue and used for further analysis of steroids.

**Sample extract:** Residue was redissolved in ethyl acetate or chloroform.

- **Conditions of Chromatography:**
  1. **Test plate:** HPTLC precoated plate, silica gel 60 F$_{254}$ – aluminium (Merk)
  2. **Format:** 10 X 10 cm$^2$
  3. **Thickness:** 250 µm
4. Spotting volume: 2 µl, 4 µl, 6 µl, 8 µl (Linomat IV)
5. Separation technique: Ascending
6. Development chamber: Twin-trough glass chamber (10 X 10 cm²) (Camag) (saturate for 10 minutes prior to development)
7. Mobile phase: Hexane: Acetone (8:2) for steroids
8. Spraying reagent: 50% H₂SO₄ (50 ml concentrated H₂SO₄ was diluted up to 100 ml with DW) for steroids
9. Relative Humidity: 52%
10. Temperature: 24° C
11. Migration distance: 80 mm
12. Migration time: (30 minutes)
13. Detection: UV

➢ Densitometric Scanning (Camag Scanner III)
   1. Wavelength and Mode
      a. 200 / 254 nm - Absorbance / Reflectance
      b. 366 nm – Florescence / Reflectance
   2. Slit Dimension: 6 X 0.45 mm

RESULT:

Table (1): Tissue culture results of *Withania somnifera*

<table>
<thead>
<tr>
<th>Explants</th>
<th>Level of Auxin</th>
<th>Level of cytokinin</th>
<th>Callus initiation (in weeks)</th>
<th>Characters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Name</td>
<td>Concentration (mg/l)</td>
<td>Name</td>
<td>Concentration (mg/l)</td>
</tr>
<tr>
<td>Nodal region with leaf</td>
<td>2,4-D</td>
<td>1</td>
<td>BAP 2</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>Leaf</td>
<td>2,4-D</td>
<td>2.0</td>
<td>KIN 0.5</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.0</td>
<td></td>
<td>0.25</td>
</tr>
</tbody>
</table>
Table (2): TLC results of steroids of 8 week old callus of *Withania somnifera*

<table>
<thead>
<tr>
<th>Materials</th>
<th>Compounds</th>
<th>Spray treatment</th>
<th>Iodine treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Callus</td>
<td></td>
<td>Rf</td>
<td>Rf</td>
</tr>
<tr>
<td></td>
<td>Unidentified 1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Unidentified 2</td>
<td>0.10</td>
<td>Yellow</td>
</tr>
<tr>
<td></td>
<td>Unidentified 3</td>
<td>0.11</td>
<td>Light green</td>
</tr>
<tr>
<td></td>
<td>Unidentified 4</td>
<td>0.19</td>
<td>Green</td>
</tr>
<tr>
<td></td>
<td>Unidentified 5</td>
<td>-</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td>Unidentified 6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Unidentified 7</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Unidentified 8</td>
<td>-</td>
<td>-</td>
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<tr>
<td></td>
<td>Unidentified 9</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Diosgenin</td>
<td>0.57</td>
<td>Green</td>
</tr>
<tr>
<td></td>
<td>Cholesterol</td>
<td>0.61</td>
<td>Purple</td>
</tr>
<tr>
<td></td>
<td>Unidentified 10</td>
<td>-</td>
<td>-</td>
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<tr>
<td></td>
<td>Unidentified 11</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Stigmasterol</td>
<td>0.91</td>
<td>Blue</td>
</tr>
<tr>
<td></td>
<td>β-sitosterol</td>
<td>0.95</td>
<td>Violet</td>
</tr>
</tbody>
</table>

Table (3): HPTLC results of Steroids of 8 week old callus of *Withania somnifera*

<table>
<thead>
<tr>
<th>Secondary metabolite</th>
<th>Spot number</th>
<th>Scanning at different wavelengths</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>200 nm</td>
</tr>
<tr>
<td>Steroids</td>
<td>Spot 1</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>Spot 2</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Spot 3</td>
<td>0.29</td>
</tr>
<tr>
<td></td>
<td>Spot 4</td>
<td>0.37</td>
</tr>
<tr>
<td></td>
<td>Spot 5</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Spot 6</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>3</td>
</tr>
</tbody>
</table>

**DISCUSSIONS:**

Plant tissue culture or the aseptic culture of cells, tissues and organs is an important tool in both basic and applied studies. Callus was obtain on MS medium supplemented with combinations of 2,4-D + BAP and 2,4-D + KIN in *Withania somnifera*. The satisfactory result was obtained on MS + 2mg/l 2,4-D + 0.25mg/l KIN (Table-1). TLC results suggested that Total 5 unidentified steroids, diosgenin, cholesterol, stigmasterol, β-sitosterol in callus (Table-2) were analysed from *W. somnifera*. Some of secondary metabolites were identified with iodine.
treatment only. HPTLC results suggested that In vitro materials (8 week old callus) of W. somnifera were scanned at different wavelengths (200 nm, 254 nm, 366 nm). Calibration spectrum, Rf values and maximum absorbance at particular wavelength of each substance were recorded through scanning. Total 3 steroids at 200 nm, 1 at 254 nm, 5 at 580 nm were scanned from in vitro (8 week old callus) W. somnifera. Absorption spectra of total 2 steroids at 190 nm were obtained from W. somnifera (Table-3, Figure 1, 2, 3, Photo-1, 2, 3, 4). Kundu et al., 1979 have given the chemistry of withanolides. Moza and Singh, 1967 have estimated total alkaloids. Gupta et al., 1996 have used TLC densitometry for withaferin-A. Bhar and Hansel, 1982; Vitali et al., 1996; Bhattacharya et al., 1997 showed immuno-modulatory and anti-oxidant activities through steroidal lactones – withanolides of W. somnifera. Asthana and Raina, 1989 have given the pharmacology of W. somnifera. Saxena et al (2012) had deeply studied few medicinal plants and its chemical constituents with the help of TLC and HPTLC at Botany department, Gujarat University, Ahmedabad, Gujarat.

REFERENCES:


**Figure 1**: quantitative estimation at 200 nm through HPTLC
Figure 2: quantitative estimation at 254 nm through HPTLC

![Image of HPTLC analysis at 254 nm]

Figure 3: quantitative estimation at 580 nm through HPTLC

![Image of HPTLC analysis at 580 nm]
Photo 1: HPTLC photo under fluorescent light

Photo 2: HPTLC photo under UV light without stain
Photo 3: HPTLC photo under UV light with stain

Photo 4: HPTLC photo under visible light