**Synopsis of the thesis on**

# Regeneration and Secondary Metabolite Studies in *Hemidesmus indicus* (L.) R. Br.

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# INTRODUCTION

*Hemidesmus indicus* (L.) R. Br. (Asclepiadaceae) is a slender, laticiferous, semi erect shrub and is used traditionally used in treatment of syphilis, loss of appetite and rheumatic fever (Nadkarni, 1989), rat bite poisoning and scorpion sting (Sharma et al., 1979). The major chemical constituents of the plant are lupeol, vanillin, rutin, 2-hydroxy-4-methoxybenzaldehyde and β-sitosterol (Chatterjee and Bhattacharya, 1955; Subramanian and Nair, 1968; Gupta et al., 1992; Nagarajan and Rao, 2003). Lupeol is a pentacyclic triterpenoid, which is commonly present in many cereals, fruits, vegetables and is also present in some medicinal plants including *H. indicus* (Moreau et al., 2002; Saleem, 2009). It has antioxidant, anti-inflammatory and anti-cancerous properties (Lee et al., 2007; Saleem et al., 2004). Whereas rutin is a flavonoid, which is also present in many plants and it has antioxidant (Sharma et al., 2013), anticancerous (Perk et al., 2014), anti-inflammatory and antidiabetic properties (Lee and Jeune, 2013). Due to high medicinal value, *H. indicus* is indiscriminately collected from the natural habitats and is now a rare species (Sreekumar et al., 2000). This creates gap between demand and supply of the raw material, which can be filled by plant tissue culture technique (Baskaran et al., 2015).

*In vitro* regeneration can be achieved using differet explant but amongst them leaf (Raghu et al., 2006; Joshi et al., 2010) and nodal explants (Baskaran and Jayabalan, 2005; Sujatha and Kumari, 2007) are commonly used to establish shoot cultures. Plant cell and tissue culture can be used as an alternative source for the production important metabolites (Vanisree and Tsay, 2004). However, many a times somaclonal variation can be occurred which affects the biosynthetic potential of *in vitro* grown plants (Mohanty et al., 2011). Hence an assessment of genetic stability of *in vitro* regenerated plants is necessary and this can be done through chemical, biochemical or molecular characterization (Xiaoqiang and Gang, 2006; Singh et al., 2010). Chemical integrity can be assessed using different chromatographic techniques but amongst them high performance thin layer chromatography (HPTLC) has advantages like, more samples can be analyzed simultaneously with less solvent, and is fast and economical (Gan and Ye, 2006; Srivastava, 2011; Siddiqui et al., 2017).

However, many a times *in vitro* cultures produces lower yield of metabolites which can be overcome by feeding the cultures with elicitors which helps in enhancement of metabolites (Zhao et al., 2005). Elicitors at appropriate concentrations might act as signalling molecules which could be perceived by a receptor present on plasma membrane and thus

initiate the signal transduction network involving regulation of gene expression responsible for biosynthesis of compounds (Zhao et al., 2005). This technique has been proved to be an effective for enhancing the production of different secondary metabolite groups like alkaloids, terpenoids, flavonoids, coumarins and phenolic compounds (Brader et al., 2001; Wang et al., 2001). There are many types of elicitors but amongst them, yeast extract and methyl jasmonate are commonly used for elicitation of secondary metabolites (Chen et al., 2001; Yang et al., 2008; Belhadj et al., 2008; Diwan and Malpathak, 2011; Thaweesak et al., 2011).

Keeping this in mind, present study focused on regeneration potency of leaf and nodal explants of *H. indicus* to generate shoots, which can be used as an alternative to wild plants and also help in conservation of this important medicinal plant. Also the shoots developed through *in vitro* technique were assessed for their biosynthetic potential as well as they were subjected to elicitor treatment. At last the gene expression studies will be done using molecular biology tool.

The objectives of this study were the following:

## OBJECTIVES

- Development of shoot cultures from leaf and nodal explants
- Isolation and quantification of lupeol and rutin from *in vivo* and *in vitro* shoots
- Elicitation and quantification of lupeol and rutin in shoot cultures
- Identification of expressed genes using molecular tool

## MATERIALS AND METHODS

**Establishment of Shoot Cultures**

**Plant Material:** Leaves and Nodes

**Methodology:**

Healthy twigs were collected from the Botanical Garden and Arboretum of The M.S. University of Baroda

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Leaves and nodes were excised and kept in running tap water for 1 h

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After 1 h, they were washed thoroughly using labolene

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The detergent was removed using tap water and finally rinsed with D/W

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Leaves and nodes were surface sterilized using bavistin (0.01%) and $HgCl_2$ (0.1%)

↓

Leaves (1 $cm^2$) containing midrib and nodes (2-3 cm) were inoculated in Murashige and Skoog's (1962) medium (MS) fortified with sucrose (3%) and different plant growth regulators (PGRs)

**PGR combinations used for leaf explant-**

- **Control:**
  - ✓ Basal MS medium without PGRs
- **Individual cytokinins:**
  - ✓ BA (5-30 µM)
  - ✓ Kn (5-30 µM)
  - ✓ TDZ (0.1, 0.15, 0.2 µM)
- **Individual auxins:**
  - ✓ IAA (0.1-2 µM)
  - ✓ NAA (0.1-2 µM)
  - ✓ 2, 4-D (0.1-2 µM)
- **Combination of cytokinins:**
  - ✓ BA (5-30 µM) + Kn (5-30 µM)
- **Combination of cytokinins with auxins:**
  - ✓ BA (5-30 µM) + IAA (0.1-2 µM)
  - ✓ BA (5-30 µM) + NAA (0.1-2 µM)
  - ✓ Kn (5-30 µM) + IAA (0.1-2 µM)
  - ✓ Kn (5-30 µM) + NAA (0.1-2 µM)

**PGR combinations used for nodal explant-**

- **Control:**
  - ✓ Basal MS medium without PGRs
- **Individual cytokinins:**
  - ✓ BA (5-20 µM)
  - ✓ Kn (5-20 µM)
- **Individual auxins:**

- ✓ IAA (0.5-2 µM)
- ✓ NAA (0.5-2 µM)

➢ **Combination of cytokinins:**

- ✓ BA (5-20 µM) + Kn (5-20 µM)

➢ **Combination of cytokinins with auxins:**

- ✓ BA (5-20 µM) + IAA (0.5-2 µM)
- ✓ BA (5-20 µM) + NAA (0.5-2 µM)
- ✓ Kn (5-20 µM) + IAA (0.5-2 µM)
- ✓ Kn (5-20 µM) + NAA (0.5-2 µM)

**<u>Rooting</u>**

**Methodology:**

Healthy *in vitro* shoots (> 3 cm) were harvested and leaves from last 2-3 nodes were removed

↓

Shoots were then treated with bavistin (0.01%)

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Shoots were placed in different strengths of MS liquid medium fortified with PGRs

**Media:**

➢ MS / ½MS / ¼MS basal medium-Control

➢ MS / ½MS / ¼MS medium fortified with IBA (1-25 µM)

➢ MS / ½MS / ¼MS medium fortified with NAA (1-25 µM)

**<u>Chemical Profiling of Shoot Cultures</u>**

**Methodology:**

1 g dried powder of *in vivo* and *in vitro* shoots were taken

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Hexane (10 ml) was added and kept in water bath at 50 ºC for 10 min

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The extract was filtered and the procedure was repeated twice using same

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The filtrate was collected in evaporation dish and the same procedure was repeated using ethyl acetate and methanol

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The extracts were kept overnight at room temperature for evaporation and next day the marc was reconstituted to 1 ml using the respective solvents and analyzed using HPTLC

- **Mobile phases:**
  - Hexane extract- Toluene : Methanol (9:1 v/v)
  - Ethyl acetate extract- Toluene : Ethyl acetate : Formic acid (6.5:2.5:1 v/v)
  - Methanol extract- Toluene : Ethyl acetate : Formic acid (6.8:2.5:0.7 v/v)
- **Derivatizing agent:** Anisaldehyde-sulfuric acid
- **Plate analysis:** 525 nm using CAMAG TLC scanner 3 linked to winCATS software

**Quantification of Lupeol and Rutin**

*In vivo* as well as *in vitro* shoots of *H. indicus* were extracted using hexane (for lupeol) and ethanol (for rutin) as procedure given above. For lupeol quantification Toluene : Methanol (9:1 v/v) and for rutin, Ethyl acetate : Butanol : Formic acid : $H_2O$ (5.2:3:1:0.8) was used as mobile phase. The plates were derivatized and scanned at 525 nm for lupeol quantification, whereas for rutin quantification the plates were scanned at 254 nm.

**Elicitation of Shoot Cultures**

Eight weeks old shoots from MS + sucrose (3%) + BA (10 µM) + Kn (5 µM) medium were used for elicitation experiment. After eight weeks the shoots were inoculated in liquid medium of same PGR concentrations, and were harvested after 1, 2, 3 and 4 weeks which were control for the study.

For elicitation using methyl jasmonate, 50 and 100 µM concentrations were added in liquid medium and eight weeks old shoots were subcultured in that. For yeast extract elicitation, different concentrations (25, 50, 100 and 200 mg/l) were used in the medium. The shoots were harvested at same intervals and were dried, powdered and extracted using solvents as mentioned above.

## RESULTS

**Establishment of Shoot Cultures**

**Leaf explant**

Basal MS medium when tried, it failed to induce any morphogenic response in leaf explants of *H. indicus*. When basal medium was fortified with different concentrations of

cytokinins and auxins individually and in combinations induced non-regenerative callus in most of the combinations. Only the medium fortified with BA (20 and 25 µM) + IAA (0.1-2 µM) induced a morphogenic nodular callus. In these medium, shoot bud regeneration was initiated within three weeks which subsequently developed into shoots during fourth week. They proliferated and elongated with simultaneously branching till eight weeks. Optimum regeneration i.e. 19.67 ± 0.81 shoots/explant (100% response) was achieved in medium fortified with BA (20 µM) + IAA (1 µM).

**Nodal explant**

Nodal explants of *H. indicus* when inoculated in basal MS medium, it regenerated only 0.58 ± 0.14 shoots. To achieve multiple shoot formation, the basal MS medium was fortified with different concentrations and combinations of PGRs. Amongst all the combinations tried, combinations of BA (5-20 µM) with Kn (5-20 µM) evoked better response. In these combinations, bud break was observed within a week which elongated and formed shoots during second week along with bud break from the opposite node. These shoots elongated during third week and multiple shoot formation with simultaneous branching was observed in the fourth week. After four weeks, cluster of shoots was transferred to the same combination which helped in further multiplication and elongation of shoots. At the end of eight weeks, all the combinations formed healthy shoots with optimum number of shoots 11.00 ± 0.24 was observed in medium supplemented with BA (10 µM) and Kn (5 µM).

**Rooting**

Elongated shoots were then transferred to liquid MS medium (full, half and quarter strengths) fortified with different concentrations (1-25 µM) of IBA and NAA. Basal MS medium (control) and medium with lower concentrations of IBA (1-6 µM) failed to induce roots within four weeks. Increasing the IBA concentrations (8-25 µM) evoked rooting in shoots, however variation was observed amongst them. Better rooting was observed in quarter strength of MS medium and optimum rooting (100%) with 8.83 ± 0.28 roots/shoot was observed in medium fortified with IBA (20 µM) within four weeks. When NAA was used, meagre response was observed in almost all the combinations tried. Maximum 3.42 ± 0.55 roots (91.67%) were formed within four weeks in full MS medium fortified with NAA (8 µM). Rooted plantlets were transferred to a thermocol cup containing cocopeat:sand (1:1) and covered with polythene bag for hardening under lab conditions.

**Chemical Profiling of Shoot Cultures**

In the present study, *in vitro* shoots from MS + sucrose (3%) + BA (10 µM) + Kn (5 µM) and MS + sucrose (3%) + IAA (2 µM) were harvested and subjected to chemical profiling using HPTLC. All the extracts were subjected to chromatographic separation on TLC plates and similar profiles were observed in both *in vivo* and *in vitro* shoots (eight and sixteen weeks old) for all the extracts after derivatization. Densitometry analysis after derivatization showed variation in peak number and areas between the *in vivo* and *in vitro* shoots derived from medium fortified with auxin. Whereas profiling of *in vitro* shoots from optimized (cytokinin based) medium was almost similar to the profiling of *in vivo*.

**Lupeol and Rutin Quantification**

HPTLC densitometic analysis of hexane extract revealed 0.185 ± 0.00 mg/g lupeol quantity in *in vivo* shoots, whereas in eight weeks old shoots it was 0.187 ± 0.01 mg/g. Whereas when ethanol extract was analyzed, only extract of *in vivo* shoots showed presence of rutin. Spectral analysis for rutin from *in vivo* shoots will be confirmed.

**Elicitation of Shoot Cultures**

- **Yeast extract**

Dried biomass (1 g) was extracted using hexane and after development and derivatization, band of lupeol was observed in all the samples. Densitometric analysis revealed that lupeol content varies according to time and amount of yeast extract concentration in the medium; and 1-2 fold increase in lupeol content was observed in treated cultures.

When ethanol extract was analyzed, bands were present in all the samples at similar Rf as standard and when densitometric analysis revealed peak similar to peak of standard sample. But when spectral analysis was done, the spectra did not match to the standard spectra of rutin. Hence the band observed at 254 nm at similar Rf as standard was not rutin and it was absent in all the elicited samples.

- **Methyl jasmonate**

When methyl jasmonate was added in liquid medium, the shoots turned brown within 1 week of culture in the concentrations tried. Therefore further quantitative analysis for lupeol and rutin was not possible.

Further investigation on gene expression analysis is being carried out for isolation of mRNA from elicited shoots and further gene expression analysis will be done using molecular tool.

## DISCUSSION

In the first experiment, leaves were used to establish shoot cultures and regeneration was achieved in medium fortified with cytokinin and auxin. Cytokinin to auxin ratio is known to influence the regenerative modes (direct or indirect) and it also regulates the type of organ differentiation (Skoog and Miller, 1957). In our study, MS medium fortified with BA + IAA evoked an organogenic response from leaf explant and similar hormone combination was reported to be optimum for regeneration in plants like *Brunfelsia calycina* (Liberman et al., 2010), *Withania somnifera* (Dewir et al., 2010) and *Ajuga bracteosa* (Kaul et al., 2013). Earlier study on leaf explants of *H. indicus* by Sreekumar et al. (2000) is contrary to our findings, they reported regeneration of 2.75 shoots in BA (2.22 µM) + NAA (1.07 µM). Another study on *H. indicus* by Shanmugapriya and Shivakumar (2011) reported callus formation in presence of individual cytokinin (BAP) and auxins (2,4-D, 2,4,5-T, IBA, NAA, IAA), and is similar to findings of the present study.

In next set of experiment, nodes were utilized for shoot culture establishment and medium fortified with BA (10 µM) and Kn (5 µM) was proved to be optimum for regeneration. This is may be due to the fact that cytokinins are known to release bud dormancy and promote axillary bud outgrowth (Shimizu-Sato et al., 2009; Yaish et al., 2010). The combination of BA and Kn is also reported for optimum regeneration in plant species like *Artemisia vulgaris* (Sujatha and Kumari, 2007), *Piper longum* (Soniya and Das, 2002), *Eclipta alba* (Baskaran and Jayabalan, 2005) and *Swertia chirata* (Balaraju et al., 2009).

Shoots developed through *in vitro* regeneration were then utilized for rooting studies. For this, different strengths of medium were tried as rooting is known to affected by nutrient salts of the medium (Murashige, 1979). Half or quarter strength of MS medium has proved to be beneficial for rooting of shoots (Skirvin and Chu, 1979; Garland and Stoltz, 1981; Zimmerman and Broome, 1981). In our study optimum rooting was observed in ¼MS medium fortified with IBA, which is similar to reports for *Crotalaria verrucosa* (Hussain et al., 2008), *Stevia rebaudiana* (Patel and Shah, 2009), *Acmella calva* (Amudha and Shanthi, 2011) and *Camellia sinensis* (Bidarigh and Azarpuor, 2013).

After shoot culture establishment, *in vitro* shoots were analyzed for their biosynthetic potential using HPTLC fingerprinting. Similarly, in plants like *Asparagus adscendens* (Mehta and Subramanian, 2005), *Celastrus paniculatus* Willd. (Martin et al., 2006), *Bacopa monnieri* (Patni et al., 2010) and *Withania somnifera* (Shetty and Chandra, 2012), HPTLC has been used to compare the fingerprints of wild and *in vitro* shoots. When lupeol was quantified in shoot cultures, high lupeol content was observed in medium fortified cytokinins as compared to *in vivo* shoots. The reason is may be due to the fact that cytokinins are known to express/up-regulate genes involved in many secondary metabolite biosynthetic pathways by repressing certain macronutrient transporters (Sakakibara et al., 2006). Coste et al. (2011) have reported that medium fortified with combination of cytokinins (BA and Kn), increased the synthesis of hypericin and pseudohypericin in cultures of *Hypericum hirsutum* (L.) and *Hypericum maculatum* Crantz. In shoot cultures of *H. indicus*, high lupeol content was obtained in *in vitro* shoots as compared to wild shoots, similarly Jain et al. (2011) have also reported high withanolide content in *in vitro* regenerated leaves as compared to wild leaves of *Withania coagulans* (Stocks) Dunal. PGRs are known to affect the secondary metabolite synthesis under *in vitro* conditions (Dornenburg and Knorr, 1995; Lee et al., 2011). Misra et al. (2005) also reported that PGRs affected lupeol synthesis in *in vitro* shoots of *H. indicus* (L.) R. Br.

## CONCLUSIONS

In the present study, shoot cultures were established using leaf and nodal explants of *H. indicus*. However when leaves were used as an explant, it regenerated shoots via indirect organogenesis which can be a source of somaclonal variants. When nodal explants were used for shoot culture establishment, direct organogenesis was observed which ensured clonal multiplication. Generation of true-to-type plants is desirable for conservation and also it can become an alternate to wild plant for secondary metabolite production. Hence nodal cultures were further taken up for further secondary metabolite studies. HPTLC fingerprinting of *in vivo* and *in vitro* shoots revealed that profiling is almost similar for both the samples. Lupeol quantification revealed that the medium fortified with cytokinins (BA and Kn) played a vital role in shoot regeneration as well as metabolites synthesis *in vitro*. At last, the elicitation studies revealed that lupeol content can be increased in shoot cultures of *H. indicus* using yeast extract as an elicitor.

As *in vitro* shoots are true-to-type in nature hence this protocol has a potential for mass cultivation as well as beneficial for harnessing important metabolites from cultures as an alternative to wild shoots. This will help to decrease the threat on natural population of the species and aid in conservation.

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