Pharmacognostical studies of *in vivo* grown garden plant and *in vitro* generated plantlets from nodal explants of *Justicia adhatoda* L.

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***Abstract: - Justicia adhatoda* L. (Family: Acanthaceae) has been exploited in India because of rich source of polyphenolic compounds which are used in stress related therapy. The Pharmacognostical study includes preliminary phytochemical screening of alkaloids, phenols, flavonoids and saponins which were confirmed in both *in vivo* grown garden plants as well as *in vitro* generated plantlets of *J adhatoda*. Explants inoculated on MS medium supplemented with different combination of auxins and cytokinins were successfully employed; combination of NAA (0.5µg/ml) and BA (2.0µg/ml) for nodal explants was found to be effective concentration to obtain plant regenerants from nodal explants. Rooting in the regenerants was also successfully initiated on transfer into half MS basal medium, supplemented with IBA (1.0 µg/ml). Addition of ADS (0.5µg/ml) in the above combinations of phytohormones induced multiple shoot from nodal explants. After hardening for 35 days in highly humidified acclimatized room, nearly 70 % of rooted plantlets survived in the field. Phytochemical screening revealed the presence of alkaloids, phenolic compounds, flavonoids and saponins in the methanol and aqueous extracts of both *in vivo* grown garden plants and *in vitro* genereated plantlet samples. Further analysis of the extracts on Thin Layer Chromatography and spectrophotometric estimation confirmed the presence of vasicine alkaloids in these extract samples with marked difference in quantity.**

Keywords: Micropropagation; Plant Growth Regulators; Adenine Sulphate; TLC; Alkaloids; Vasicine

I.ITRODUCTION

Justicia adhatoda L., a widely distributed medicinal plant is recommended in the treatment of different ailments like bronchitis, muscle pain, arthritis, ulcer, allergy, asthma, fever jaundice, etc. (Maurya & Singh, 2010; Mandal and Laxminarayna, 2014) The chief alkaloids which were thoroughly investigated were vasicine and vasicinone derived from stem leaf and root of this shrub (Dinesh and Parameswaram, 2009). The vast use of the plant in medicinal purpose may lead to its unavailability in the coming days. Therefore, a natural biotechnological approach may be adapted through tissue culture technique since the propagation is also restricted due to poor seed germination and shoot cuttings for vegetative propagation relied on season (Ahmad & Bansal, 2002). The conventional method of propagation in *J gendarussa* was found to be affected by soil contamination (Thomas and Yoichiro, 2010). Thus, *in vitro* propagation through nodal explants gives a promising idea for plant regeneration (Abhayankar and Reddy, 2007; Khalekuzzaman *et al.,* 2008). Besides comparative account on qualitative and quantitative analysis of alkaloid in these two extract samples, *in vivo* grown garden plants and *in vitro* generated regenerated plantlets, it may also provide insight on the biochemical synthesis of alkaloids.
In modern time plant based natural products continue to give impact on health care (Shabnam Jawed *et al.,* 2002). The role of these active products has been realised as antioxidant, antidiabetes, antibacterial and antiulcer activities (Wong *et al.*, 2006). It was realised to find out the comparative account on the efficacy of alkaloids isolated from both *in vivo* and *in vitro* sources. The difference in findings in the methanol extracts from in vivo and in vitro sources may help to study the required environmental stress during growth. Thus, in the present study the aim was to develop *in vitro* regenerants and the difference, if any in the production of bioactive compounds in the two sources.

Materials and Methods

Plant materials, explants preparation and *in vitro* culture

Healthy, disease free and younger twigs of *J. adhatoda* were brought in the laboratory from the departmental garden. Nodal explants of the plant were washed under running tap followed by their treatment with few drops of twin-20. Further sterilization was carried in 0.1% HgCl2 for 3-5 min, followed by several rinsing with sterile double distilled water. These explants (2-3 cm.) were placed in culture tube containing 15 ml of MS medium (Morashige and Skoog, 1962) supplemented with different hormones (IAA, NAA, 2,4-D, BAP, KN) in various concentration. Resulting regenerants after 35 days old culture containing 2.5-3.5 cm long shoots were periodically subcultured. Adenine Sulphate (0.5 mg/l) was also added in specific experiments. Cultures were maintained at 25±2^{0}C under 2000 lux light intensity with 60% humidity.

Root formation and Hardening

The shootlets, thus obtained, were transferred to MS basal medium with IBA (0.5-2.0mg/l) and NAA (0.5- 2.0mg/l) for root initiation. In the experiment 25 explants were used and after a period of 35 days percentage of rooting, root length and the number of roots per individual shoot were recorded. After 35 days the plantlets were taken out gently and washed with distilled water carefully to remove the medium attached on the roots and emerging shoots. The plantlets were then transferred to pots containing sand, red soil and farmyard manure in 1:1:1 ratio for hardening. These pots were kept in highly humidified acclimatized room at 28 ± 2^0 and humidity of 70-80% for hardening. Again after 35 days of hardening, these rooted plantlets were successfully transferred into field where the survival of plantlets was noticed at about 70%.

Preparation of Methanol and Aqueous Extracts:

The plant parts of *J. adhatoda* generated through *in vivo* as well as *in vitro* system were subjected for methanol and aqueous extraction separately. 30 gms of plant materials from both sources were grinded into powder form in a pre-chilled mortar and pestle. Each plant parts powder of both sources was packed in Soxlet apparatus separately, where 250 ml of either methanol or water were made to percolate for 24 hours. Later on, the extract was concentrated to dryness under reduced pressure in rotatory evaporator and dried in desiccators. Dark green residues were obtained after concentrating the extract under reduced pressure. Thus obtained (aqueous and methanol) extracts were stored in desiccators for further phytochemical investigations.

Phytochemical Screening:

Qualitative chemical tests were designed to detect the presence of alkaloids, phenolic compounds, flavonoids and saponins in the methanol and aqueous extracts of both *in vivo* and *in vitro* samples (Allayie, *et al*., 2016):

Detection of Alkaloids:

Solvent free extracts (20mg) was stirred with few drops of dilute HCl and filtered. The filtrate was tested carefully with various alkaloid reagents as follows.

Mayer's test (Evans, 1997): A few drops Mayer's reagent was added into a few ml of filtrate. A white creamy precipitate indicated the presence of alkaloids.

Mayer's reagent was prepared by dissolving 1.358 gm of Mercuric Chloride in 60 ml of water. Another solution by dissolving 5gm of Potassium Chloride into 10 ml of water was prepared. These two solutions were mixed and made up to 100 ml with water. This was Mayer's reagent.

Dragendroff test (Waldi, 1965): One ml of Dragendroff reagent was added ino a few ml of filtrate. A prominent yellow precipitate indicated the presence of alkaloids.

Dragendroff reagent contained Bismuth Carbonate (5.2g) and Sodium Iodide (4 gm) which were boiled for a few minutes with 50ml glacial acetic acid. After 12 hrs the precipitated sodium acetate crystal was filtered off with the help of glass funnel. 40 ml of clear red brown filtrate was mixed with 160 ml of ethyl acetate and 1ml of water to prepare stock solution. Working solution was prepared by mixing 20 ml of acetic acid into 10 ml of stock solution and made up to100 ml by adding the required amount of water.

Detection of Saponins (Kokate, 2010): The extracts 50 mg of both *in vivo* grown twigs and *in vitro* generated plantlets were diluted with distilled water separately and each was made up to 20 ml. The suspension was shaken in a graduated cylinder for 15 min. A 2-3 cm layer of foam indicated the presence of saponins in the extract.

Detection of Phenolic Compounds (Mace, 1963): The extracts obtained from *in.vivo* grown twigs and *in vitro* generated plantlets; 50 mg each was dissolved in 5 ml of distilled water, a few drops of neutral 5% Ferric Chloride solution was added. A dark green colour indicated the presence of phenolic compounds.

Detection of Flavonoids (Jia *et al*., 1999): Aqueous solution of extract derived from both *in vivo* grown twigs and *in vitro* generated plantlets were treated with 10% Ammonium hydroxide solution Yellow florescence indicated the presence of flavonoids.

Thin Layer Chromatography (TLC):
Silica gel and distilled water were used to prepare a slurry coating materials and plates were coated by using the spreading device, with a layer about 0.30mm thick coated plates were then dried and activated in oven for 30 min. A pencil line is drawn near the bottom and a small drop of Test solutions (methanol and aqueous extracts of *in vivo* and *in vitro* samples*)*

and Standard solution containing vasicine were placed separately on it; and the spots were placed to become dry. Plates were placed in Chromatographic chamber containing the Ethyl acetate: Methanol: Ammonia (100: 13.5: 10). The colour of the spots was noted with Dragendorff's reagent and the Rf values were calculated (Harborne, 1973).

Rf= Distance travelled by the compound /
Distance travelled by solvent

Quantification of total alkaloids

Quantification of total alkaloids in the methanol extract was done by Spectrophotometer (Systronics, India) by measuring at 545 nm against blank. The number of total alkaloids in the methanol sample was calculated using standard curve of vasicine (Soni *et al*., 2008).

Results

Shoot Regeneration

Phytohormones free medium completely failed to regenerates shoot even after 35 days of inoculation. However, Cytokinin induced shoot regeneration up to a satisfactory level; 60% in presence at BA (2µg/ml) and 40% at KN (1.5µg/ml). However, combination of Cytokinin and Auxin gave more satisfactory result 80% in presence of BA (2µg/ml) and NAA (0.5µg/ml) (Table 1).

Table 1: Effect of different concentrations of Cytokinins and Auxins on shoot regeneration of nodal explants of *J. adhatoda*.

Plant growth Regulator (mg/l)				Regeneration (%)	Mean number of shoots/explants	Mean shoot length (cm)
BAP	KN	IAA	NAA			
0.5				20	1.2±0.02	0.6±0.01
1.0				25	1.8±1.06	1.0±0.10
1.5				30	2.0±0.28	1.4±0.00
2.0				40	2.4±0.26	1.4±0.15
2.5				50	2.8±0.20	1.8±0.08
3.0				40	1.0±0.80	0.6±0.50
	0.5			20	1.3±0.30	1.1±0.04
	1.0			30	1.3±0.40	1.5±0.11
	1.5			35	3.8±0.20	1.8±0.10
	2.0			40	4.3±0.30	2.2±0.11
	2.5			50	5.1±0.27	2.8±0.08
	3.0			40	4.8±0.20	2.8±0.00
1.0		0.5		20	2.0±0.00	1.5±0.00
1.5		0.5		20	2.5±0.25	1.8±0.40
2.0		0.5		35	3.8±0.02	1.2±0.02
2.5		0.5		40	3.0±0.00	1.0±0.00
3.0		0.5		50	4.8±0.02	1.0±0.00
1.0			0.5	25	2.5±0.20	1.2±0.20
1.5			0.5	30	3.8±0.40	1.5±0.50
2.0			**0.5**	**65**	**5.5±0.02**	**2.8±0.00**
2.5			0.5	50	4.2±0.05	1.8±0.50
3.0			0.5	30	3.0±0.00	1.4±0.50
	1.0	0.5		20	2.3±0.10	1.4±0.00
	1.5	0.5		30	2.8±0.20	1.2±0.20
	2.0	0.5		30	3.0±0.50	1.0±0.50
	2.5	0.5		50	4.5±0.10	1.2±0.20
	3.0	0.5		40	4.2±0.20	1.4±0.00
	1.0		0.5	30	2.8±0.05	1.2±0.05
	1.5		0.5	30	3.0±0.20	1.2±0.20
	2.0		0.5	40	3.2±0.20	1.2±0.50
	2.5		0.5	45	3.0±0.20	1.2±0.50
	3.0		0.5	40	3.0±0.10	1.0±0.00

Effect of Adenine sulphate on multiplication of shoots

Shoot formation was established from nodal explants at BAP (1.0 mg - 3.0 mg/l) or KN (1.0 - 3.0 mg/l) with NAA (0.25 - 2.0 mg/l) and ADS (0.5mg/l). Maximum number and height of shoot were also achieved at BAP (2mg/l) +NAA (0.5mg/l)

+ ADS (0.5mg/l). Routine sub-culturing of shoots in the similar medium BAP (2.0mg/l) + NAA (0.5mg/l) + ADS(0.5mg/l) in after 4 weeks resulted in high frequency of multiple shoots. The length of shoot proliferation with an average length of 3.2 cm within 45 days was marked with multiple shoots on sub culturing with Adenine Sulphate (0.5µg/ml) (Table 2).

Table 2: Effect of Adenine sulphate (0.5mg/l) with different concentration of growth regulators on shoot regenerants of J. adhatoda.

Growth regulators (mg/l)				Culture developing shoots (%)	*Average No. of multiple shoots	*Average height of shoots (cm)
BAP	KN	NAA	ADS			
			0.0	0	0.0	0.0
			0.5	0	0.0	0.0
1.0		0.25	0.5	33	21.42±0.40	1.50±0.75
1.5		**0.50**	**0.5**	**72**	**55.55±0.52**	**4.25±0.09**
2.0		1.00	0.5	56	31.00±0.52	3.27±0.75
2.5		1.50	0.5	58	36.38±0.67	2.45±0.68
3.0		2.00	0.5	45	32.00±0.69	2.28±0.72
	1.0	0.25	0.5	45	18.45±0.72	2.00±0.24
	1.5	0.50	0.5	60	52.73±0.20	3.85±0.05
	2.0	1.00	0.5	52	32.63±0.70	3.21±0.23
	2.5	1.50	0.5	50	28.74±0.76	2.23±0.46
	3.0	2.00	0.5	32	13.67±0.73	2.25±0.20

Rooting and Hardening

The shoots regenerated from nodal explants were placed on half strength MS medium supplemented with IBA (1.0µg/ml) was found to be most effective since 52% of shoots started forming roots further sub culturing another 28 days grown 2-3 roots. Such rooted plantlets having 2-3 small leaves were successfully acclimatized for 35 days in acclimatizer room with 70 % humidity on vermicelli soil placed in polythene bags. After 35 days these plantlets were transferred to the departmental garden having soil: sand (1:1) ratio were the survival of the regenerated plants was noticed at 70%.

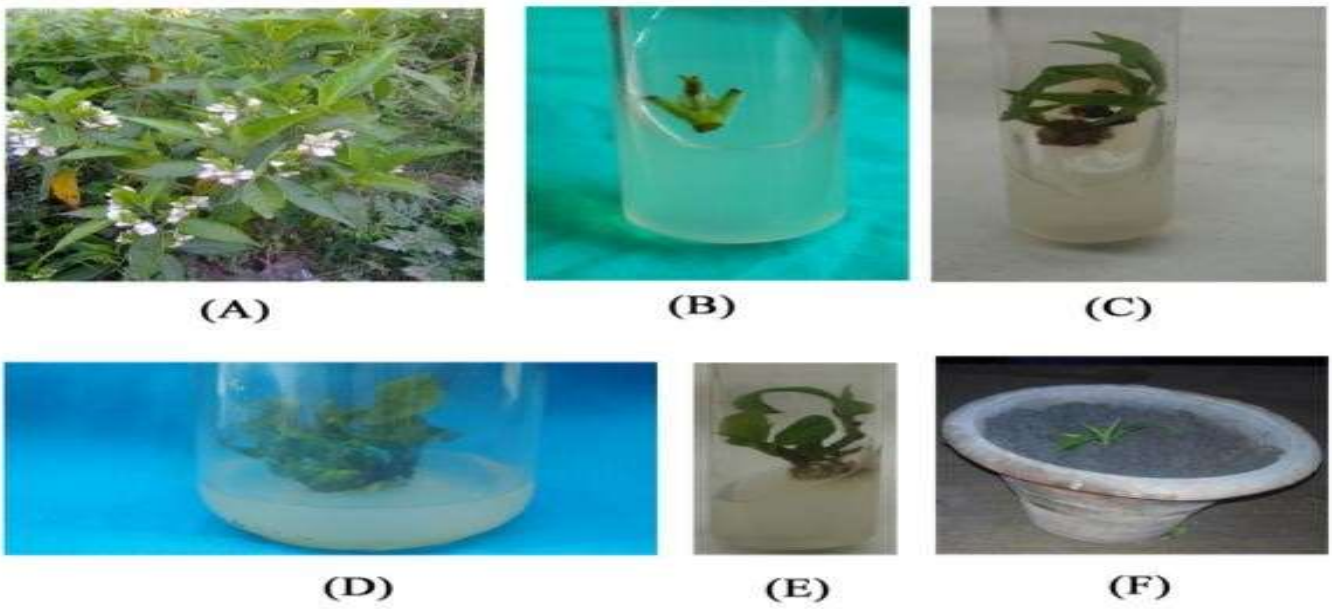

Fig. 1: (A) Garden plant of *J. adhatoda* ; (B) Nodal explants on MS medium with phytohormones; (C) Shoot formation on above medium BAP (2µg/ml) + NAA (0.5µg/ml); (D) Multiple shoot on addition of adenine sulphate (0.5µg/ml); (E) Differentiation of roots on MS medium with IBA (1.0µg/ml); (F) Hardening of *in vitro* regenerated plantlet

Discussion

Medicinal property which conferred therapeutically potential in plants is considered to be due to the presence of secondary metabolites. Thus, investigation for different phytochemicals, viz., alkaloids, phenols, flavonoids and saponins were made basis for preliminary investigation. A comparative study was attempted between *in vivo* grown garden plants and *in vitro* generated plantlets to visualize the effect of environmental factors on the production of bioactive compounds. Earlier,

production of photochemical was analysed in *in vitro* regenerated *Justicia gendarussa* Burm.F. plant. (Bhagya and Chandrashekar, 2012) As a result, established procedures to quantify the bioactive compounds in the extracts prepared from *in vivo* grown plants and *in vitro* generated plantlets were undertaken in methanol and aqueous solvents. The colour and nature of extracts was found to be different in different solvent; ranging from white creamy, dark green to yellow and sticky to dry. Similar colour reaction was reported in the detection of phytochemicals in *in vitro* cultured cells of *Brassica nigra* (Hussain *et al*., 2010), *Mimusops elengi* (Gami *et al*., 2010) and *Ricinus communis* (Khafagi, 2007). The protocols of plant tissue culture were followed in order to generate regenerants and multiple shoots were successfully produced (Mandal and Laxminarayana, 2014). Application of adenine sulphate in the medium generated multiple shoot as was achieved earlier in case of *Petunia alba* (Prakash *et al*., 2013).

Phytochemical screening of *in vivo* and *in vitro* grown plants indicated the presence of most of the secondary metabolites tested in the present investigation. However, phenols and flavonoids were absent in *in vitro* generated plant extracts but alkaloids and saponins were present in both types of extracts, though quantity was always found to be less in *in vitro* generated extracts samples. Thus, quantitative and qualitative estimation indicated the presence of some of the metabolites in both the *in vivo* and *in vitro* samples but at the same time these two samples documented record difference in methanol and aqueous extracts in both quality and quantity. In *Naringi crenulta*, aqueous extract too showed lesser amount for alkaloids (+) but higher amount of phenols (Allayie *et al*., 2016).

Thin layer chromatographic technique is a useful analytical tool for the isolation and identification of organic compounds. The TLC for alkaloids showed better separation in methanol extracts of *in vivo* and *in vitro* samples, however in the later case the band appeared feeble because of the almost half of the amount as compared to former. But the band was almost at par with that of the vasicine, used as control. The Rf value was also found similar which confirmed presence of vasicine in the alkaloids of *J. adhatoda* not only in *in vivo* grown plants but in *in vitro* generated plantlets. Since *in vitro* plants were grown in controlled conditions, the synthesis of alkaloids may not be required to defend the plants as in uncontrolled atmosphere. Thus, it would be a viable opportunity if the plants are allowed to grow in *in vitro* condition but under stress of environmental factors (Kumar *et al*., 2012).

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