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Screening and Evaluation of *in vivo* and *in vitro* Generated Insulin Plant (*Vernonia divergens*) for Antimicrobial and Anticancer Activities

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Abstract*—Vernonia divergens* Benth., commonly known as "Insulin Plant" (Fam: Asteraceae) is a potent sugar killer. Locally the leaves of the plant, boiled in water are successfully administered to a large number of diabetic patients. The present study evaluates the putative anti-diabetic ingredients, isolated from the *in vivo* and *in vitro* grown plantlets of *V. divergens* for their antimicrobial and anticancer activities. Sterilized explants of nodal segments were cultured on MS (Musashige and Skoog, 1962) medium in presence of different combinations of hormones. Multiple shoots along with bunch of roots were regenerated at 1mg l^{-1} BAP and 0.5 mg l^{-1} NAA. Micro-plantlets were separated and sub-cultured on the double strength (2X) of the above combination of hormones leading to increased length of roots and shoots. These plantlets were successfully transferred to soil and survived well in nature. The ethanol extract of plantlets from both *in vivo* & *in vitro* sources were prepared in soxhlet extractor and then concentrated to dryness under reduced pressure in rotary evaporator. Thus obtainedconcentrated extracts showed significant inhibitory activity against gram negative bacteria like *Escherichia coli* and *Pseudomonas aeruginosa* but no inhibition was found against gram positive bacteria. Further, these ethanol extracts were screened for *in vitro* percentage cytotoxicity at different time periods (24 h, 48 h and 72 h) of different dilutions. The *in vivo* plant extract inhibited the growth of EAC mouse cell lines in the range of 65, 66, 78, and 88% at 100, 50, 25 & 12.5µg mL^{-1} but at 72 h of treatment. In case of the extract of *in vitro* origin, the inhibition was found against EAC cell lines even at 48h. During spectrophotometric scanning, the extracts exhibited different maxima (λ) - four peaks in *in vitro* extracts as against single in *in vivo* preparation suggesting the possible change in the nature of ingredients during micropropagation through tissue culture techniques.

***Keywords*—**Anti-cancer, Anti-microbial, EAC mouse cell, Tissue culture, *Vernonia divergens.*

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I. Introduction

NATURE has been a good source of medicinal plants for thousands of years and an impressive number of modern drugs have been isolated from them. Various medicinal plants have been used for years in daily life to treat diseases all over the world. The use of traditional plant extracts as well as other alternative forms of medicinal treatments have been getting momentum since the 1990s [1]. *Vernonia divergens* Benth (Fam.: Asteraceae), commonly known as insulin plant, is a potent sugar killer and is used as an excellent medicine for diabetes mellitus. India is known as a diabetogenic country. Diabetes is one of the most common diseases in human population. In many developing countries of the world, interest in native plant remedies has continued to increase as a result of the growing awareness of the importance of medicinal plants in health care delivery [2}. This interest now extends to many urban and developed communities including parts of Europe and America[3, 4]. In Nigeria and many other poor African countries, the belief in traditional herbal medicine is strong [5, 6]. Natural products are generally known to be efficacious with minimal side effects, but some natural compounds have been reported to act as mutagens and/or carcinogens. These genotoxic insults often lead to cancer, Alzheimer's disease and other chronic degenerative diseases, such as atherosclerosis and heart diseases, which are the leading cause of death in human and animal populations. Plant derived compounds are a major area of interest for safer and more effective antimicrobial and anticancer agents.

The present work is targeted to screen and evaluate the *in vivo* and *in vitro* generated plantlets, *Vernonia divergens* for antimicrobial and anticancer activity.

II. Materials & Methods

The plant, *Vernonia divergens*, grown in the SAP garden of DRS Department of Botany, B.R.A. Bihar University, Muzaffarpur, established under UGC- SAP scheme was used as experimental material. Nodal segments (2-3 cm) as explants were excised from young plants. The explants were washed with 5% (v/v) teepol solution for 10 min, surface sterilized with 0.2% $HgCl_2$ for 2-3 min and rinsed 3-4 times with sterile double distilled water. Explants cultured with solid MS medium [7] containing 0.8% agar, 3% sucrose an supplemented with different concentrations of auxin and cytokinin. The pH of each medium was adjusted to 5.8 before

the addition of agar and autoclaving at 121^0C. The cultures were maintained at 25 ± 2^0C. The ethanol extract of plantlets, generated both *in vivo* and *in vitro* was prepared by percolating the dried plant material (100g) with 95% ethanol in soxhlet extractor and then concentrated to dryness under reduced pressure in rotary evaporator. Dose dependant cytotoxicity was observed with the *in vivo* and *in vitro* plant extract inhibiting the growth of EAC (Ehlrich's Ascitic Carcinoma) mouse cell lines. Spectrophotometric scanning method was used to observe the peaks in *in vitro* extracts against *in vivo* and possible changes in the nature of ingredients during micro propagation. Two test organisms namely one gram negative *Escherichia coli* and *Pseudomonas aeroginosa* and two gram positive bacteria like *Staphylococcus aureus* and *Strptomyces* spp were obtained from Microbial Culture. These cultures were obtained from Micobial Type Culture Collection Institute of Microbial Technology (MTCC, IMT), Chandigarh.

A. In Vitro Culture of Vernonia Divergens

Multiple shoots were regenerated on MS medium supplemented with different concentrations of BAP and NAA. Observations were recorded after interval of 4 weeks. All the cultures were grown under a photoperiod of 16 hrs (illuminated by 40 watt cool-white fluorescent tubes, 1200 lux). Rooted shoots from 4 week old cultures were transferred to soil with vermiculite in 1:1 ratio. For hardening, plants were taken out form the tubes and washed with distilled water to remove the agar medium and were transferred to sterilized tray beds. They were then shifted to highly humidified acclimatized room for hardening for 30 days. Later on they were planted in the field.

B. Preparation of Eethanol Extract of Leaf of Vernonia Divergens

Plantlets of *V. divergens*, generated through both *in vivo* and *in vitro* systems were shade-dried and pulverized. The powder was treated with petroleum ether for dewaxing and removal of chlorophyll. Later, it was packed (2 g) in a Soxhlet apparatus and subjected to hot continuous percolation for 8 h by using 250 ml of ethanol (95% v/v) as solvent. The extract was concentrated to dryness under reduced pressure in rotary evaporator and dried in a desiccator.

C. In Vitro Cytotoxicity Assay

Cytotoxicity assay was carried out in accordance with previously published protocol [8]. EAC (Ehlric's Ascitic Carcinoma) cells (5×10^3 cells/well) were cultured on a flat-bottomed 96 well plate. After 48 hours incubation, 2µl of stock solution was added to each well of the assay plate, which was then incubated for 2 hours at 37^0C. After incubation, the formazan crystals formed by the reduction of tetrazolium salt by the mitochondria of living cells, were dissolved in DMSO. The plates were read in ELISA plate reader at wavelength of 540 nm.

D.Antimicrobial Activity (Agar well diffusion method)

The extracts form leaf, were studied for antimicrobial activity. A loop full of standard strains were inoculated in 30mL nutrient broth in a conical flask and incubated for 24 hr to activate the strains. In agar well diffusion method [9] the media and test bacteria cultures were poured into Petri dishes. The test strain, 0.25mL was inoculated into the media. Care was taken to ensure the proper homogenization. The experiment was performed under strict aseptic condition. After the medium was solidified, a well was made in the plates with sterile bore (6mm). The extracted compound (0.05mL & 0.1mL) was introduced into the well and the plates were inoculated at 37^0C for 24 hr. All samples were tested in triplicates. Microbial growth was determined by measuring the diameter of the zone of inhibition against control i. e. without test compounds. The results were observed as zone of inhibition in mm.

III. Results and Discussion

Plant micropropagation via direct shoots regeneration allows large scale multiplication of plantlets *in* vitro by preventing clonal variation as opposed to regenerated from calli alone, which often leads to somaclonal variation [10]. In this study, nodal segments and were excised from 1 year old plant of *Vernonia divergens* (Fig.1) and were tested for their response to plant growth regulators at different concentrations (Fig. 2)

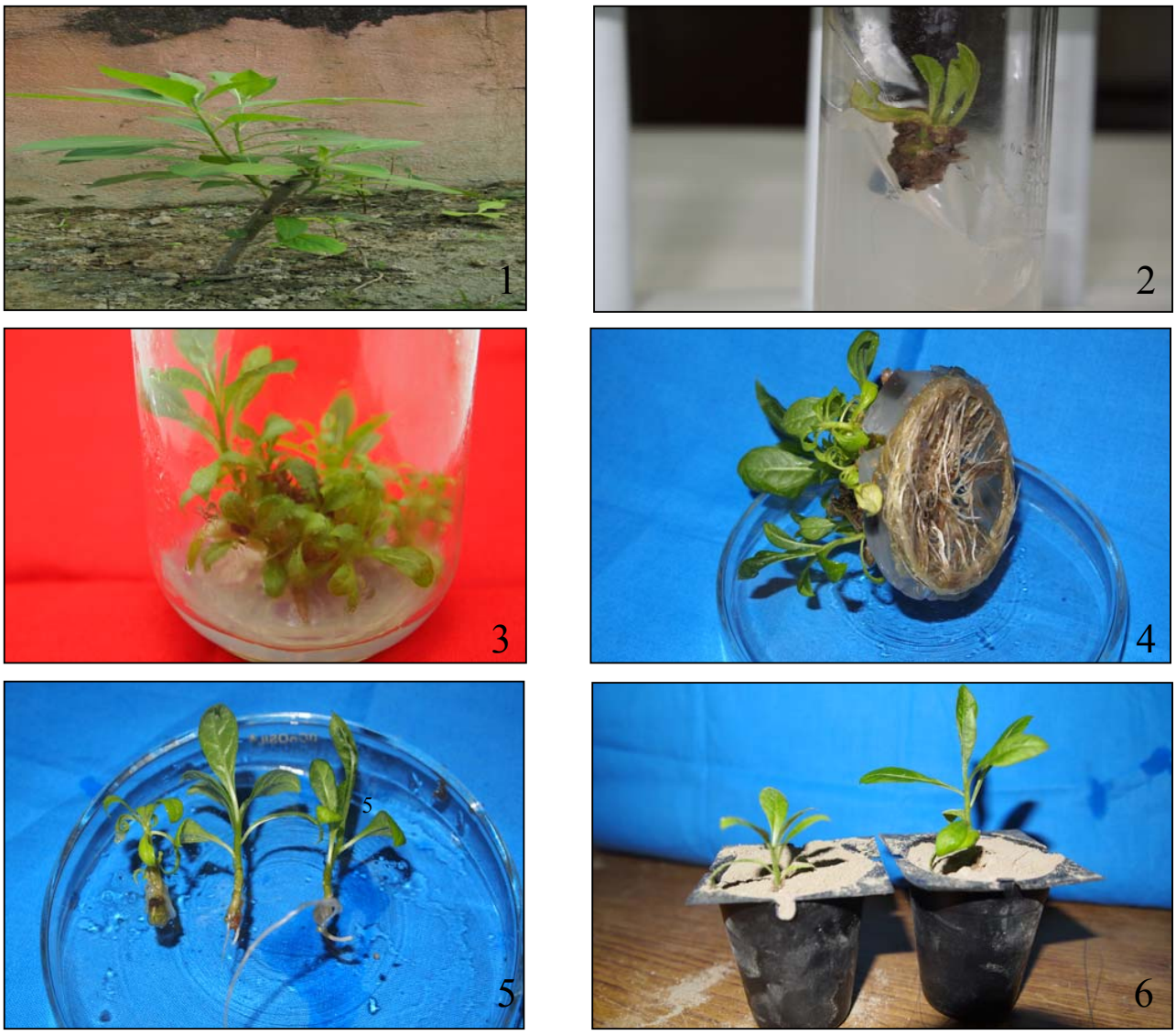

Fig. 1 *Vernonia divergens* Benth. In SAP Garden of DRS Department of Botany, B R A Bihar Univ., Muzaffarpur.

Fig. 2 Development of shoot from nodal explants on MS basal medium; mark green & healthy shoot: 25 days old culture.

Fig. 3 Nodal explant showing devt of direct multiple shoots on MS+ 1mg L^{-1} BAP +0.5 mg L^{-1} NAA.

Fig. 4 Culture showing rooting in bunch on MS + 1mg L^{-1} BAP + 0.5 mg L^{-1}NAA Showing separated microplantlets.

Fig. 5 Showing separated microplantlets.

Fig. 6 30 days old plantlet of *Vernonia divergens* on transfer to two separated tray bags.

Multiple shoots along with bunch of roots were regenerated directly from nodal segments at 1mg l^{-1} BAP and 0.5 mgl^{-1} NAA and the best morphogenic response (4 to 5 per explants) was observed (Fig. 3 &. 4, Table. I). Generally the usage of BAP is considered as most suitable hormone for accentuating the large scale multiplication and micropropagation of various plant species [11]. Micro-plantlets were separated (Fig. 5) and sub-cultured on the double strength (2x) of the above combination of hormones leading to increased length of roots and shoots. Rooting was achieved in bunch of multiple shoot at the same combination of hormone. These plantlets were successfully transferred to soil and survived well in nature (Fig. 6).

The concentrated ethanol extracts were screened for *in vitro* percentage cytotoxicity at different time periods (24 h , 48 h , and 72h) of different dilutions [12]. The *in vivo* plant extract inhibited the growth of EAC mouse cell lines in the range of 65, 66, 78, and 88% at 100, 50, 25 & 12.5µg mL^{-1} but at 72 h of treatment. On the other hand, the extract of *in vitro* origin, the inhibition was found against EAC cell lines even at 48h (Fig.7). However, the inhibitory action was less by 20% in all treatments in case of the ethanol extract of *in vitro* derived plantlets [13]. Therefore, further experiments have been designed to develop *in vitro* plantlets under stress of salt.

ECA Mouse Cell Lines: *Vernonia* Cytotoxicity Aassay

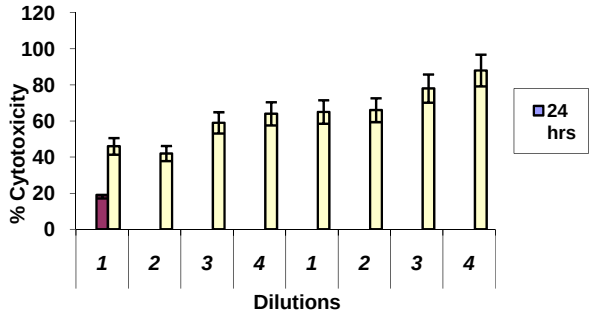

Fig. 7 Growth inhibitory effect of the *in vivo* & *in vitro* ethanol plant extracts against EACMouse cell line

The antimicrobial activity of *in vivo* and *in vitro* extracts of *V. divergens* were tested against *Escherichia coli* and *Pseudomonas aeruginosa* but no inhibition was found against gram positive bacteria like *Staphylococcus aureus* and *Streptomyces* spp. 0.1ml concentration of *in vitro* extracts were found to be effective against both the above gram negative bacteria. Thus it could be of paramount importance since gram negative bacteria were found to be more prone to cause health problems in human population [14]. On spectrophotometric scanning within the visible range (400-700 nm), the result suggested the different nature of the ingredients of ethanol extracts; there were 4 peaks in *in vitro* extract as compared to only one in *in vivo* extract of *V. divergens* (Fig. 8 & 9).

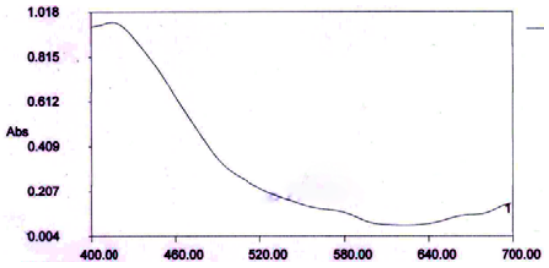

Fig. 8 Spectrophotometric scan of the ethanol extract of *in vivo* grown plants with single peak(λ) at 416.8

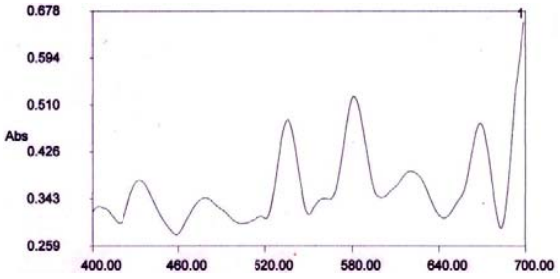

Fig. 9 Spectrophotometric scan of the ethanol extract of *in vitro* grown plants with with four peaks (λ) at 433.6, 478.0, 535.6, and 580.0 of *Vernonia divergens.*

TABLE I
EFFECT OF BAP IN COMBINATION WITH NAA ON SHOOT FORMATION FROM NODAL SEGMENTS OF *VERNONIA DIVERGENS* AFTER 25 DAYS OF MS MEDIUM CONTAINING 0.8% AGAR AND 5% SUCROSE

Growth regulators (mgl^{-1})		Shoot formation No. of shoots [%]	[explants^{-1}]	Ht.of shoots [cm]
BAP	NAA			
-	-	45 ±1.00	1.32±0.10	1±0.01
1	-	48 ±1.00	1.65±0.12	0.98±0.03
2	-	55 ±1.00	2.25±0.13	1.12±0.04
3	-	50 ±1.50	1.95±0.12	1.00±0.03
1	0.5	100 ±0.00	4.49±.0.09	3.00±0.01
1	0.7	100 ±0.00	4.20±0.10	2.5±0.09
1	0.9	100 ±0.00	4.10±0.08	2.3±0.07
2	0.5	95 ±0.00	3.58±0.05	3.23±0.01
2	1.0	97 ±1.00	3.65±0.07	3.25±0.02
2	1.5	93 ±1.00	3.42±0.03	3.21±0.03

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REFERENCES

[1] M. M. Cowman, "Plant products as antimicrobial agents," *Clinical Microbial Review,* vol, 12, pp. 564-582, 1999.
[2] S. S. Gyang, D. D. Nyam and E. N. Sokomba, "Hypoglycaemic activity of *Vernonia amygdalina* (chloroform extract) in normoglycaemic and alloxan-induced hyperglycaemic rats," *Journal of Pharmacy and Bioresources,* vol. 1, pp. 61-66, 2004.
[3] G. V. Gill, S. Redmond, F. Garrott, R. Paisey, "Diabetes and alternative medicine: a case for concern," *Diabetic Medicine,* vol. 22, pp. 210-213, 1994.
[4] G. P. Lease and G. Williams, "Complementary and conventional medicine in clinical diabetes," *International diabetes digest*, vol. 6, pp. 675-676, 1994.
[5] P. G. G. Odeigah, I. A. Taiwo, E. O. Akomolafe and D. O. Durojaiye, "Hypoglycaemic actions of medicinal plants with tolbutamide in the albino rat," *Diabetes international, vol.* 9, pp. 71-73. 1999.
[6] I. A. Taiwo, P. G. C. Odeigah, L. A. Ogunkanmi, "The glycemic effects of *Vernonia amygdalina and V. tenoreana* with tolbutamide in rats and the implications for the treatment of diabetes mellitus," *Journal of Scientific Research and Development,* vol. 11, pp. 122-130, 2008.
[7] T. Murashige and F. A. Skoog, "Revised medium for rapid growth and bioassays with tobacco tissue culture," *Physiol Plantarum,* vol. 15, pp. 473-497, 1962.
[8] T. Mosmann, "Rapid colorimetric as say for cellular growth and survival, application to proliferation and cytotoxicity assays," *Journal of Immunoogical Methods*, vol. 65, pp. 55-63, 1983
[9] C. Parez, M. Paul and P. Bazerque, "Antibiotic assay by agar well diffusion method," *Acta. Biol. Med. Exp*, vol. 15, 113-115, 1990.
[10] P. S. Reddy, P. Rodrigues and R. Rajasekharan, "Shoot organogenesis and mass propagation of *Coleus forskohlii* from leaf derived callus," *Plant Cell Tissue Organ Culture,* vol. 66: 183-188, 2001
[11] S. Shrivastana and M. Banerjee, "*In vitro* clonal propagation of physic nut (*Jatropha curcas L.*), Influence of additives," *Int Integrative Biol.* Vol. 3,73-79, 2008
[12] S. Kumar, A. Prakash, R. Vohra and A. K.. Verma, "Evaluation of anticancer activities of ethanolic extracts of *in vivo* and *in vitro* grown *Coleus forskohlii* Berq." *Perspective in Cytology and Genetics,* vol. 15, pp. 19-30, 2011.
[13] V. Sharma, S. A. Mallick and A. K. Tiku, "Anticancer activity of devil tree (*Alstonia scholaris* Linn.) leaves on human cancer cell lines," *Indian Journal of Agricultural Biochemistry,* vol. 23, pp. 63-65, 2010.
[14] Kandukuri and M. A. Singara, "Antimicrobial activity of five flower extracts against three pathogenic bacteria,' *Nature Environment and Pollution Technology,* vol. 9, pp. 73-76, 2010