Antidiabetic Activities of Natural Products of Some Medicinal Plants of Muzaffarpur District

Rajeev Kumar, Dr. Santosh Kumar
University Department of Botany,
Babasaheb Bhimrao Ambedkar Bihar University Muzaffarpur, Bihar, India.

Abstract: - The present study of medicinal plants, widely present in different localities of Muzaffarpur district selected for the present investigation were *Tinospora cordifolia, Vernonia divergens* and *Mucuna pruriens.* The idea was to screen the natural products i.e. bio-ingredients of these plants in terms of antidiabetic activities. Since the pharmaceutical industries stand for over- exploitation of medicinal plants resulting into their possible depletion, thus, an effective alternation for conservation and multiplication of these plants is also required in order to fulfil the present aim of investigation. A more penetrating comparative account in antidiabetic activities was studied by comparing the *in vivo* and *in vitro* extracts on different parameters, such study of *in vivo* and *in vitro* extracts of these plants may lead to understand comparative efficacy of phytochemicals study. Thus, *in vitro* extract production required the protocol to generate micropropagation of plantlets because of their annual propagation i.e. non-availability of the plants throughout the period of research activities.

Keywords: Antidiabetic, Micropropagation, *Tinospora cordifolia*, Vernonia, Medicinal Plants, Muzaffarpur.

I.ITRODUCTION

The medicinal plants, widely present in different localities of Muzaffarpur district selected for the present investigation were *Tinospora cordifolia*, *Vernonia divergens* and *Mucuna pruriens*. The idea was to screen the natural products i.e. bio-ingredients of these plants in terms of antibacterial, antidiabetic and anticancer activities. Since the pharmaceutical industries stand for over- exploitation of medicinal plants resulting into their possible depletion, thus, an effective alternation for conservation and multiplication of these plants is also required in order to fulfil the present aim of investigation. A more penetrating comparative account in antibacterial, antidiabetic and anticancer activities was studied by comparing the *in vivo* and *in vitro* extracts on different parameters, such study of *in vivo* and *in vitro* extracts of these plants may lead to understand comparative efficacy of phytochemicals from both sources on the cytotoxicity assay especially in cancer study. Thus, *in vitro* extract production required the protocol to generate micropropagation of plantlets because of their annual propagation i.e. non-availability of the plants throughout the period of research activities.

Medicinal plants are the richest bio-resource of natural products for traditional system of medicine, food supplement, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs. Natural products (secondary metabolites) have been the most successful source of potential drug as lead compounds for biological activity (Mishra and Tiwari, 2011). Besides, aromatic plants are also the source of fragrances, flours, cosmeceuticals, health beverages and chemical terpenes. Thus, Medicinal and aromatic plants (MAP) are on priority in trading-list of many developing countries. India, with more than 80% of world's biodiversity, including plant genetic diversity with medicinal properties, holds tremendous potential in occupying a global significant position in market for medicinal plants based herbal formulations and medicines as natural products. The first step in the value addition of bio-resources is the production of herbal drug preparations (i.e. extracts), using a variety of method from simple traditional technologies to advanced extraction techniques. Extraction is the separation of medicinally active portions of plant tissues using selective solvents through standard procedures. Such extraction techniques separate the soluble plant metabolites and leave behind the insoluble cellular mass. The products so obtained from plants are relatively complex mixture of metabolites, in liquid or semisolid state or removing the solvent in dry powder form and are intended for oral or external use. These include classes of preparations known as decoctions, infusions, fluid extracts, tinctures or powdered extracts. Such preparations have been popularly called galenicals, named after Galen, the secondary century Greek physician. These products contain complex mixture of many medicinal plants metabolites, such as alkaloids, glycosides, terpenoids, flavinoids and ligans. In order to be used as a modern drug, an extract may be further processed through various techniques of fractionation to isolate individual chemical entities.

The industrial processing of natural products starts with the extraction of active components using various technologies. The general techniques of medicinal plant extraction include maceration, infusion, percolation, digestion, decoction, hot continuous extraction (Soxhlet apparatus), aqueous alcoholic extraction, microwave assisted extraction, ultrasound extraction (sonication), supercritical fluid extraction and phytonic extraction (with hydroflurocarbon solvents). For aromatic plants hydro-distillation techniques (water distillation, steam distillation) hydrolytic maceration followed by distillation expression and enfleurage (cold fat extraction) may be employed.



Fig.1: Biotization of micropropagated Vernonia divergens plant

1. In vivo grown V divergens in SAP garden of DRS Department of Botany, B R A Bihar University, Muzaffarpur
2. In vitro multiplied shoot clump before subculturing
3. In vitro shoot multiplication after subculturing
4. In vitro rooting in shoots during subculturing
5. Piriformospora indica grown on PDA medium
6. Micropropagated biotized plantlets
7. Biotized roots after maceration showing P. indica infection
8. 30-day old plantlet of non-biotized and biotized V. divergens on transfer in tray beds
9. Biotized plantlets of V. divergens on being shifted into highly humidified acclimatized chamber.

Vernonia divergens is an important medicinal plant which does not produce viable seeds and is propagated vegetatively. This plant, commonly known as insulin plant, is a potent sugar killer and is used as an excellent

Table 1: Effect of Adenine sulphate (25 mg/L) in combination with BAP and NAA on multiplication of shoots from
shoot tip explants of *Vernonia divergens* after 25 days of MS medium containing 0.8 % agar and 5 % sucrose.

Growth regulators BAP	NAA	Shoot formation [%]	No. of shoots [Explants^{-1}]	Height of shoots [cm]
-	-	45±1.00	1.32±0.10	1±0.01
1	-	62±1.00	5.75±0.13	1.00±03
2	-	70±1.50	7.63±0.25	1.25±0.01
3	-	76±1.75	10.25±0.65	1.95±0.05
1	0.5	100±0.00	20.00±0.080	3.00±0.01
2	1.0	100±0.00	25.00±1.04	4.00±0.00
3	1.5	100±0.00	15.00±0.54	3.25±0.01

Data represent the mean of 20 cultures, ± Standard Deviation (SD), where n = 3

medicine for diabetes mellitus. The plant could be micropropagated very successfully at combinations of different phytohormones. But the rate of successful transplantation of *in vitro* generated plantlets has been made to increase by a recently developed technique of biotization. It has improved the transplantation process in tobacco, cassava and many other crops with active cultures of AM fungi [10]. Recently *Piriformospora indica* too has been established to have the potentiality in inducing biotization in several crop plants during *in vitro* condition [11]. The suitability for *P. indica* as a biotizing agent for the hardening of tissue culture raised plants has been proved [12]. Considering the property of biotization in
P. indica, attempts have been extended to biotize the *in vitro* generated plantlets of insulin plant, *V. divergens* to bring hardiness with this novel fungus in order to compete under adverse environmental conditions. Earlier, some of the plant extracts were reported to be effective against Ehrlich Ascites Carcinoma (EAC) cell lines [13, 14]. We, too, screened the different dilutions of ethanol extract of both *in vitro* generated plantlets and *in vivo* naturally grown garden plants of *V. divergens* [15] and *Coleus forskohlii* [16] for *in vitro* percentage cytotoxicity at different time periods. The inhibition level against EAC cell lines was found to be higher in ethanol extract of naturally grown plants as compared to that of the *in vitro* generated plants. In the present study, cytotoxicity or cell viability assay using EAC cell lines *in vitro* [17, 18] was carried out for investigating anticancer potentiality in the putative biomolecules present in ethanol extract of *V. divergens* especially after biotization with *P. indica.*

MATERIALS AND METHODS

Plant and microbial materials: Shoot tips (2-3 cm) as explants were excised from two-year-old *V. divergens* plant maintained in the SAP garden of DRS Department of Botany, B R A Bihar University, Muzaffarpur. 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 [19] containing 0.8% agar, 3% sucrose and supplemented concentrations (1.0, 2.0 and 3.0 mg/L) of 6-benzylaminopurine (BAP),
(0.5, 1.0 and 1.5 mg/L) of α-naphthalene acetic acid (NAA) and (25 mg/L) of adenine sulphate (ADS). The pH of the medium was adjusted to 5.8 before addition of agar and autoclaving at 121^0C. The cultures were maintained at 25 ± 2^0C. Multiplication of shoots from the shoot tips once established on MS medium supplemented with different hormones at desirable concentrations were rooted on MS medium supplemented with IBA (1.0, 2.0 and 3.0 mg/L). Thus, obtained complete plantlets were put for hardening in tray-beds in the acclimatizer room once they were biotized with the fungus, *P. indica.*
Microbial Cultures: The fungus, *Piriformospora indica* (DSM 11827) used in the present study was grown on Potato Dextrose Broth at 28 ± 2^0C for 10-12 d. It was affectionately gifted to our laboratory by Prof Ajit Varma, Amity Institute of Microbial Technology, Amity University, Noida, U P, through one of the co-authors (K S).
Biotization: The regenerated plants, raised through tissue culture were placed under stress by permitting biotization in polythene bags (one plantlet per bag), each containing
200 g of vermiculite and soil (1:1). Biotization of *P. indica* was carried out using broth culture containing $3X10^4$ spores (chlamydospores) along with heavy amount of mycelia per polythene bag. The procedure involved the soil drenching method in which the inoculum was made available in the vicinity of plant roots. These biotized plantlets were then transferred in tray-beds with virgin soil in acclimatizer room at 28 ± 2^0C for 30 days with 14/10 hours light/dark conditions and 60-70% relative humidity.
Colonization: Plantlets were microscopically examined for *P. indica* colonization by staining the roots with cotton blue and lacto phenol. Population count of *P. indica* was noted by using standard serial dilution pour plate method in presence of an antibiotic (streptomycin 50 µg/mL).

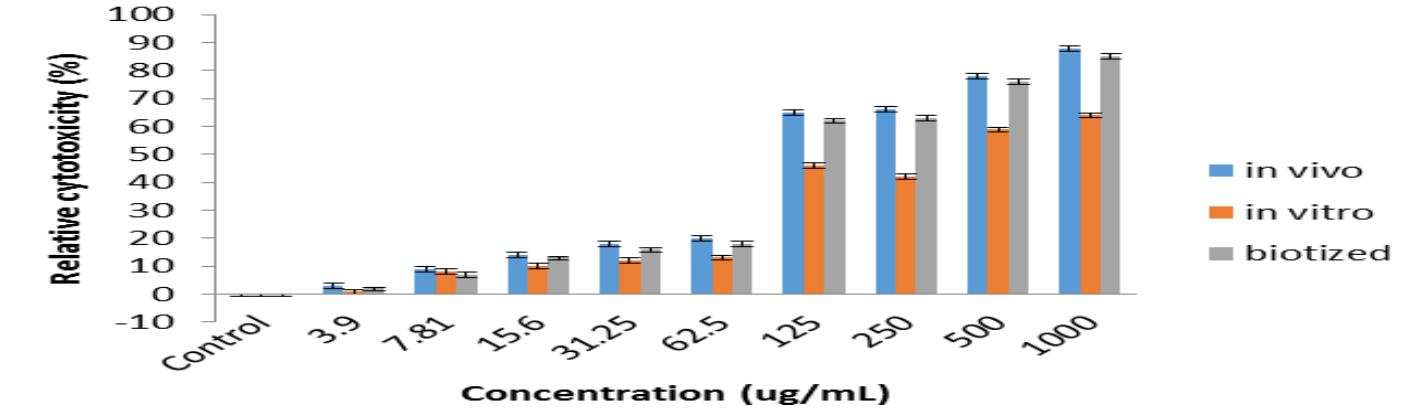

Fig. 2: Effect of different concentration of ethanol extracts of in vivo in vitro and in vitro biotized plants against EAC cell line growth inhibition (Relative Cell Cytotoxicity) in MTT assay.

Plant Biomass: Roots and shoots were washed in tap water and then in 0.1% HCl to remove adhering soil particles. Dry weights were recorded by drying root and shoot samples in an oven at 70^0C for 2 days. Root and shoot length were also measured. Various parameters including per cent survival, per cent colonization of *P. indica*, population count of biotized roots, root-shoot length and dry weight as well as the length and number of leaves were recorded after 30 d of transplantation under humidified acclimatized room condition.

MTT assay: The cytotoxicity assay was done through quantitative determination of viable cells as prescribed by previous protocol [20]. EAC cells ($5X10^3$ per well) were cultured on a flat bottomed 96-well plate. After 24 hrs, 48 hrs and 72 hrs of incubation (at 37°C, 5% CO_2), 20 µl of MTT stock solution was added to each well of the assay plate, which was again incubated for four hours at 37°C. The incubation of stock solution confirmed the formation of formazan crystals by the reduction of tetrazolium salt by the mitochondria of living cells. The medium was removed and 150 µl of DMSO was added to each well to dissolve the MTT metabolic product. The plates were read in ELISA plate reader at the wavelength of 540 nm. Untreated cells are used as a control of viability (100%) and the results are expressed as % viability relative to the control.

Statistical Analysis: The results were expressed as mean
± SD. The data were analysed using one-way analysis of variance (ANOVA) and the value ($p<0.05$) was considered as statistically significant.

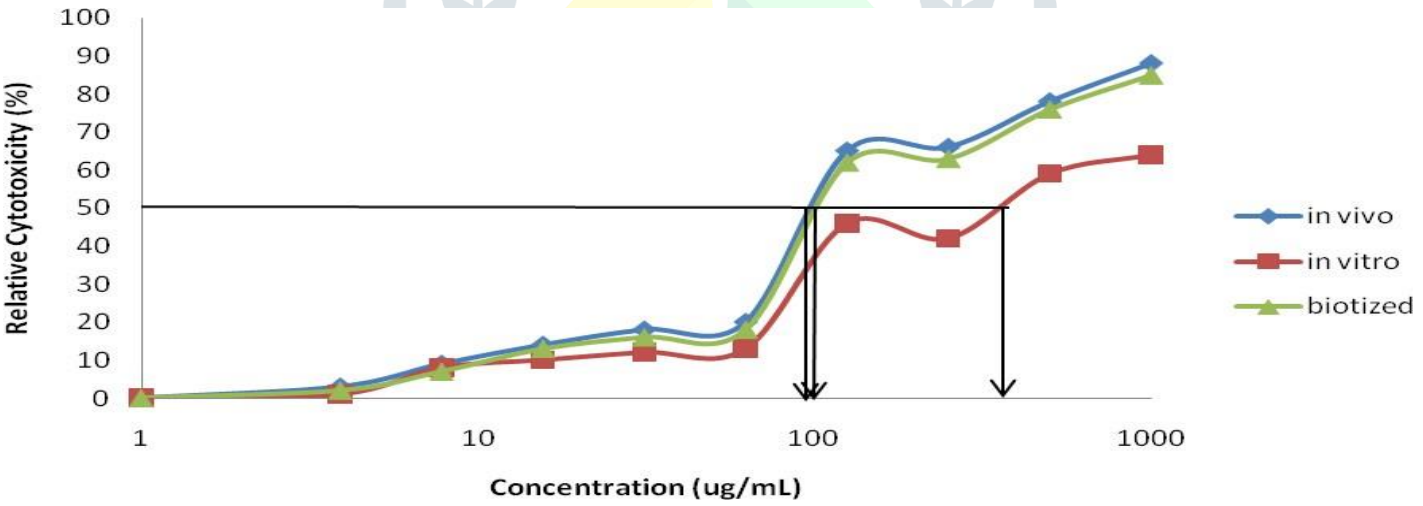

Fig. 3: Relative Cell Cytotoxicity of ethanol extracts of in in vivo, in vitro and in vitro biotized plants against EAC cell line: The IC_{50} values were extrapolated from the cytotoxicity curve at 50% of inhibition of EAC cell line after 72 hours of extract treatment.

RESULTS

The medicinal plants, widely present in different localities of Muzaffarpur district selected for the present investigation were *Tinospora cordifolia*, *Vernonia divergens* and *Mucuna pruriens*. The idea was to screen the natural products i.e. bio-ingredients of these plants in terms of antidiabetic activities. Since the pharmaceutical industries stand for over-exploitation of medicinal plants resulting into their possible depletion, thus, an effective alternation for conservation and multiplication of these plants is also required in order to fulfil the present aim of investigation. A more penetrating comparative account in antibacterial, antidiabetic and anticancer activities was studied by comparing the *in vivo* and *in vitro* extracts on different parameters, such study of *in vivo* and *in vitro* extracts of these plants may lead to understand

comparative efficacy of phytochemicals from both sources on the cytotoxicity assay especially in cancer study. Thus, *in vitro* extract production required the protocol to generate micropropagation of plantlets because of their annual propagation i.e. non-availability of the plants throughout the period of research activities.

The explants of *Tinospora cordifolia, V. divergens* and *M. pruriens* such as nodal segment, shoot tip and/or leaf were made available in order to obtain enhanced multiple shoot formation and establishment of regenerants in *in vitro* condition. The explants of these plants were cultured on MS medium, containing 3% sucrose (as carbon source), 0.8% agar (as gelling agent), supplemented with auxins (2,4-D, IAA, IBA, NAA) and cytokinin (BAP, Kinetin, 2iP) either separately or in combination in the range of 0.5 – 2.0 µg ml^{-1}. The pH (5.8) of the medium and temperature (25±2°C) were maintained under lab conditions. Cultures were illuminated with white fluoresces tubes. Subcultures were carried out once in 3rd week and 20 replicates in each experiment were maintained.

The shoot, thus obtained, to the rooting medium comprising MS medium supplemented IBA (0.5–2.0 µg ml^{-1}) for obtaining *in vitro* rooting in each plantlet Rooted plantlets were removed from the medium and exposed under running water washing for 10 -15 minutes before planting into sterilized polythene bags containing sand with vermiculite in 1:1 ratio. These bags were kept in highly humidified (80%) acclimatized room for hardening for 30 days at 28±2°C and then they were transferred in the field.

Sufficient amount of extracts of both sources, obtained separately in ethanol solvent with the help of Soxhlet apparatus and Rotatory evaporator, were screened for bioactive compounds. There was marked difference in *in vivo* and *in vitro* samples. Alkaloids, phenols, flavonoids and saponins showed higher degree (+++) of precipitation in eathanol extract of *in vivo* plants but it was found to be moderate (++) in in vitro ethanol extract of both sources. But the quantity of alkaloids was found to be just moderate (++) in ethanol extract of *in vivo* generated plantlets whereas phenols and flavonoids were almost negligible.

The *in vivo* and *in vitro* extracts of *T cordifolia, V divergens* and *M pruriens* were examined for its potential antibacterial activity. The ethanol extract (500 µg ml^{-1}) was found to be more penetrating in producing the zone of inhibition. The ethanol extracts of *T cordifolia* showed highest inhibitory activity against *Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae* and *Proteus vulgaris* as compound to *Streptococcus pyrogens* and *Pseudomonas aeruginosa*, whereas the antibacterial activities in ethanol extracts of both *V divergens* and *M pruriens* were found to be moderate in the production of zone of inhibition. The potentiality of antibacterial activities in these samples, on comparison, was found in decreasing order i.e. *T cordifolia* > *V divergens* > *M pruriens*. Thus, the present investigation clearly proved that the ingredients in *in vivo* samples were more pronounced than that of an *in vitro* sample of all the plants under investigation.

The animal system as for example rats were required for investigation on antidiabetic properties of ethanol extracts. First rats were made to suffer with diabetes by inducing alloxan, which could raise the blood glucose level more than 200 - 220 mg 100 ml^{-1} in the experimental rats. The blood glucose was found increased in alloxan diabetic rats as compared to normal rats. But administration of extracts of the medicinal plants from both *in vivo and in vitro* extracts of *T cordifolia, V divergens* and *M pruriens*, had significant decreasing effect on the diabetic rats in blood glucose level. The *in vivo* extract of *V divergens* was found to be more effective in antidiabetic activities than that of *T cordifolia* and *M pruriens*. The decreasing effect could be depicted as *V divergens* > T cordifolia > M pruriens, since the decreasing level in *V divergens* was in the range from 210 to 120 µg ml^{-1}, in *T cordifolia* from 205 to 135 µg ml^{-1}, in *M pruriens* 200 to 170 µg ml^{-1}. Surprisingly the *in vitro* samples of these medicinal plants reacted in different way. The antidiabetic activities were found to be less inhibitory (almost 20%) so far blood glucose level was measured in rats to compare the efficacy of the ingradients present in ethanol extracts.

The cytotoxicity effect was assayed against SiHa and $HepG_2$ cells at the varying concentration of plant extracts of both *in vivo* and *in vitro* samples of the experimental plants, *V divergens* and *M pruriens* by MTT assay. Parcentage cytotoxicity was expressed as IC_{50}. During the investigation, SiHa cells were treated with ethanol extracts and the DNA was directly extracted and was placed on agarose gel electrophoresis. DNA fragmentation was seen as a smear DNA on the gel under Gel. DNA smear was present in extract treatment at 500 mg l^{-1} against SiHa cells. These results confirmed that the bioingrdients present in ethanol extracts could induce apoptosis of HepG2 and SiHa cells.

Since it was invariably observed that the ethanol activities in in vitro samples of these medicinal plants were less by a degree of 20%, it was thought to grow these plants through tissue culture under stress of a fungus, *Piriformospora indica.* Thus, micropropagated micro-plants with roots were biotized by *P indica* by soil drenching method, whose presence was verified histologically. The colonization of *P indica* was studied on the basis of presence of hyphae and pear shaped chlamydospores in the cortical cells of stained roots. It established the impact of colonization since all the treated plants transferred after one month under stress of *P. indica* of growth were found to have survived. Significant increase in length and dry weight of root and shoot as well as number of lateral roots and leaves was observed due to colonization as compared to the uninoculated control plants.

The concentrated ethanol extracts from three sources, viz., *in vivo* natural garden plants, *in vitro* generated plantlets (non-biotized) and *in vitro* generated biotized plantlets of *Vernonia divergens* were screened for *in vitro* percentage cytotoxicity at 72 hours of treatment. At higher concentrations (125, 250, 500 and 1000 µg/mL), the *in vivo* plant extract was inhibitory on EAC mouse cell lines, the percent inhibitions were 65, 66, 78 and 88%, respectively whereas there was 20% less inhibition in ethanol extract of *in vitro* plantlets. However, the level of inhibition in EAC cell lines was almost restored (62, 63, 76 and 85 µg/ml) in *in vitro* generated biotized and acclimatized plantlets, which were allowed to grow under stress of colonization of the fungus, *P. indica.* On extrapolation, the trend was also found to be almost similar in the IC_{50} value in *in vivo* and *in vitro* biotized plants; it was 100 and 95.5 µg ml^{-1}, respectively, whereas the IC_{50} value was recorded at 660.6 µg ml^{-1} in *in vitro* non-biotized plant extract. In this investigation biotization with the fungus, *P. indica* was found

vital not only in the growth promotion of plants but also in enhancing the anticancer potentialities in micropropagated plants, grown under stress.

DISCUSSION

The present work is an attempt to study the antidiabetic activities of secondary metabolites, isolated from selected medicinal plants employing cell and tissue culture techniques. Due to ever growing demand of medicinal plants especially during off season, the tissue culture techniques have been employed for rapid and mass-multiplication and conservation. Plant micropropagation via direct shoots regeneration allowed large scale multiplication of plantlets *in vitro* by preventing clonal variation as opposed to regenerated from calli alone, which often leads to somaclonal variation (Reddy et al., 2001). In general, micropropagated plants, exhibit high mortality rates upon their transfer to soil. Even 5% mortality causes a huge loss during commercial plants production. The humidified acclimatized room and field possess relatively lower humidity, higher light intensity and septic environment that are stressful to micropropagated plants as compared to *in vitro* conditions [24]. The benefit of any micropropagation system can be fully realized only by the successful transfer of plantlets from tissue culture vessels to the ambient conditions found *ex vitro*.

Biotization of micropropagated *V. divergens* with *P. indica* increased resistance of plants from stresses at the time of transplantation, thus protected micropropagated young plantlets from 'transplantation shock'. Plant growth and biomass is greatly influenced by nutrients and environmental conditions [5]. *P. indica* too helped in nutrient uptake by extending its hyphae in the rhizospheric region where even finest roots cannot reach. Thus, role of this probiotic fungus in nutrient uptake and growth of micropropagated *V. divergens* was found to be effective studies in field conditions.

Biological assay such as tumor cell cytotoxicity is a sensitive method to scrutinize the anticancer principle present using EAC cell lines *in vitro* [17]. The significant ($p < 0.05$) decrease in the cancer cell viability with increasing dose and time indicates that the ethanol extracts are cytotoxic even in cultured plant tissues, if exploited [16]. It measures cell membrane integrity by determining mitochondrial activity through enzymatic reaction on the reduction of MTT to formazan [25]. The above results confirm the production of secondary metabolites with anticancer properties [17], even during the morphological differentiation at low concentration [16], but it can be enhanced by new biotechnological interventions such as fungal mediated cultures [26]. *Vernonia divergens*, growing in the field conditions (*in vivo*) acquires the biochemical pathway to produce anticancer compounds. These compounds may be altered during *in vitro* conditions of tissue culture but under stress of the colonization of a fungus even in *in vitro* condition, the pathway to produce the anticancer compounds may be restored. The present findings support the contention that plants under stress of any external agents, either heavy metals or microbes start producing secondary metabolites with medicinal properties. These medicinal compounds have been found to be clinically active against various types of cancer cells. Further research in this area may lead to better treatment of cancer. Phytochemical analyses of the *in vivo* and *in vitro* derived plantlets confirmed the presence of alkaloid, phenols, tennins, flavanoids, glycosides and steroids (Unpublished observations). Pharmacognostical studies and evaluation of total phenolic and flavanoid contents [27] of this tradionally used antidiabetic plant species is in progress.

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