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Research Article

Cytotoxicity of Ethanol Extracts of *in vivo*, *in vitro* and Biotized Grown Plants of *Vernonia divergens* on EAC Cell Lines

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ABSTRACT

The present study was attempted to enhance the anticancer activities in *in vitro* grown medicinal plant, *Vernonia divergens*, generated under the stress of a fungus, *Piriformospora indica* and to evaluate the effect of such biotization against EAC cell lines using standard MTT assay. The effect of adenine sulphate in addition to the phytohormones was assessed for micropropagation of *V. divergens* from shoot tip explants. The regenerated plantlets were biotized with a fungus, *P. indica* through soil drenching method. After acclimatization of 30 days, the ethanol extracts of these regenerants were screened for their inhibitory action against EAC mouse cell lines at different concentrations for 72 hours of treatment and compared with that of the inhibitory action of both *in vivo* and *in vitro* (non-biotized) generated plants. The combination of adenine sulphate and phytohormones sufficiently increased the number of shoots and roots per explants. Biotization of micropropagated plantlets not only enhanced the survival rate and growth of root, shoot and leaf of the regenerated plants but it also increased the anticancer potentiality. The level of inhibition of ethanol extract against EAC cell lines of these regenerated plants was found to be low as compared with that of the natural garden plants but the stress of biotization with this fungus helped the regenerants to regain the inhibitory action against the cell lines. Thus, biotization with the fungus, *P. indica* is vital not only in the growth promotion of plants but also demonstrated enhanced anticancer potentialities in micropropagated plants, grown under stress.

Key Words: Anticancer, Biotization, Micropropagation, *Vernonia divergens*, *Piriformospora indica*

INTRODUCTION

Pharmaceutically important phytochemicals which are claimed to be in practice as anticancer drugs are routinely derived from medicinal plants. The synthesis of these compounds depends on the growth rate of the plants[1]. However, the geographical and environmental conditions as well as the genetic make-up of the plants largely contribute in the production and accumulation of these chemicals. Stereo-specificity of these compounds may be another major impediment for the chemical synthesis of these drugs in plants[2]. At this juncture, plant tissue culture technique can be an alternative to overcome these problems for the synthesis of specific compounds. The present work was an attempt to study the anticancer activity of ethanol extracts from *Vernonia divergens*, a medicinal plant employing cell and tissue culture techniques. One part of the study was devoted to the regeneration of *in vitro* plantlets through tissue culture and its biotization with a fungus, *Piriformospora indica* during growth under stress. The other part deals with the study of the effect of the stress due to biotization on anticancer activity of ethanol extracts of *in vivo* grown garden plant as well as *in vitro* generated non-biotized and biotized plantlets.

In recent years tissue culture techniques has been widely exploited especially in the micropropagation of endangered medicinal plants[3]. However, mortality rate of *in vitro* generated plants on transfer in soil has been the matter of concern for industries because survival of these *in vitro* plants as compared to *in vivo* plants remains questionable under adverse environmental stresses. The mutual beneficial relationship with useful microorganisms like bacteria including actinomycetes and fungi, which are otherwise absent under *in vitro* conditions could be one of the reasons[4]. The colonization of bacteria into the plants improves plant performance under stress environment and also enhances yield [5]. Studies with different crops, vegetables and medicinal plants have shown plant-microbe interactions [6,7].

It is an established fact that rate of translocation of nutrients becomes high during plant-microbes interaction. Microbes around plant root system are treated as plant growth promoting rhizobacteria (PGPR). These arbuscular mycorrhizal (AM) fungi and PGPRs have increased the growth and biomass of different plants [8]. *P. indica*, a root endosymbiotic fungus, having property of AM fungi, increases plant growth and overall biomass by translocating phosphorus to the host by an energy dependent process [9].

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Fig.1: Biotization of micropropagated Vernonia divergens plant
a: In vivo grown V divergens in SAP garden of DRS Department of Botany, B R A Bihar University, Muzaffarpur
b: In vitro multiplied shoot clump before subculturing
c: In vitro shoot multiplication after subculturing
d: In vitro rooting in shoots during subculturing
e: Piriformospora indica grown on PDA medium
f: Micropropagated biotized plantlets
g: Biotized roots after maceration showing P. indica infection
h: 30 day old plantlet of non-biotized and biotized V. divergens on transfer in tray beds
i: 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		Shoot formation	No. of shoots	Height of shoots
BAP	NAA	[%]	[Explants^{-1}]	[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).

Table 2: Extent of colonization after 30 days time intervals in acclimatizer and its effect on survival of micropropagated plantlets of *V. divergens.*

Treatments	Colonization of *P. indica* (%)		Plant Survival (%)	
	30 days	90 days	30 days	90 days
Control	-	-	66.0 ± 1.28	68.0 ± 0.88
P. indica	58.0 ± 1.34	78.0 ± 0.88	86.0 ± 1.66	100 ± 0.00

± = S D, n = 3,

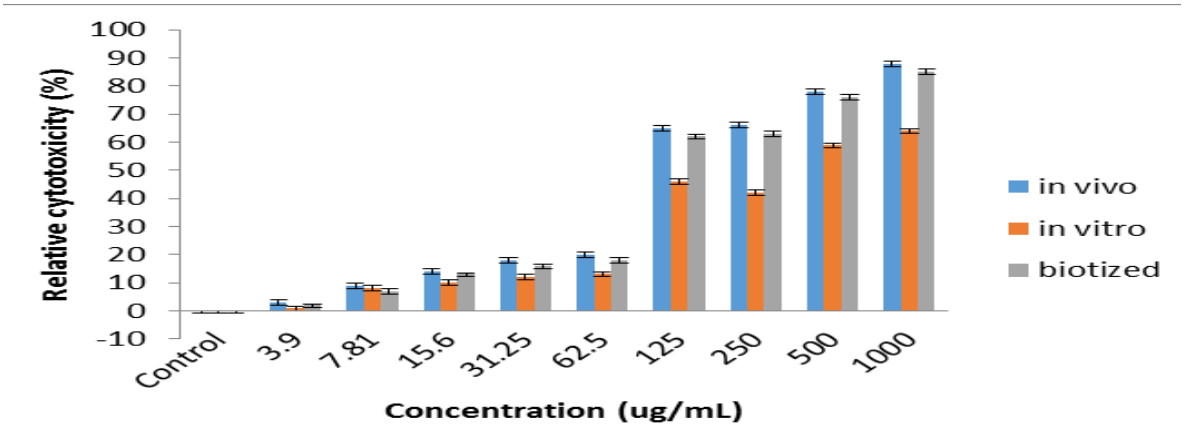

Fig. 2: Effect of different concentration of ethanol extracts of in vivo ( ), in vitro ( ) and in vitro biotized plants ( ) against EAC cell line growth inhibition (Relative Cell Cytotoxicity) in MTT assay.

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.

RESULTS

Micropropagation of the plant was achieved through shoot tips as explants, which were excised from two year old plant of *Vernonia divergens* (Figure 1a) on MS medium supplemented with plant growth regulators at different concentrations. BAP (3mg/L) with adenine sulphate (25 mg/L) was effective for shoot multiplication but the combination with NAA (1mg/L) gave better results. Data revealed that the three good concentrations of BAP supplemented with NAA and same concentration of adenine sulphate were standardized for excellent multiplication of shoots. These concentrations were: 1mg/L BAP + 0.5 mg/L NAA, 2mg/L BAP + 1.0 mg/L

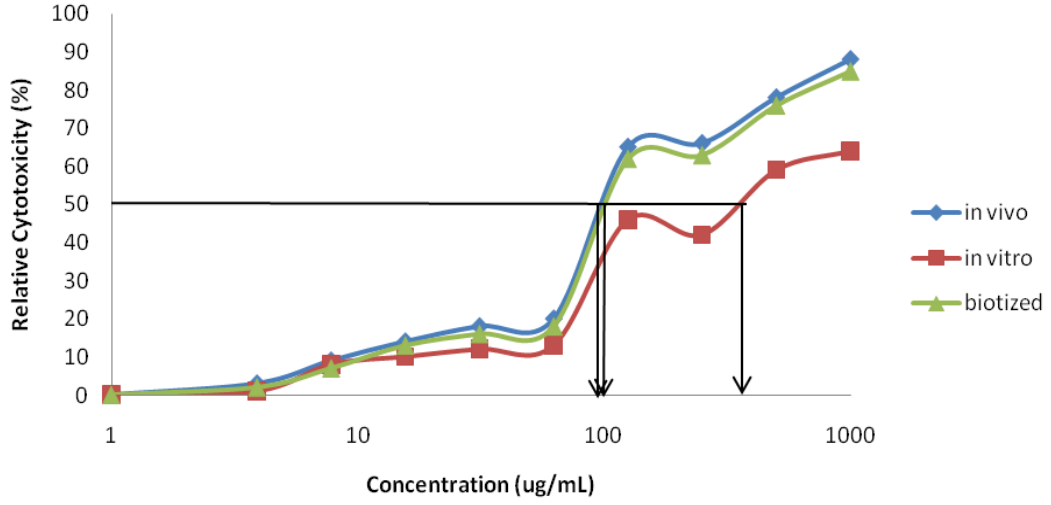

Fig. 3: Relative Cell Cytotoxicity of ethanol extracts of in in vivo ( ), in vitro ( ) and in vitro biotized plants ( ) against EAC cell line: The IC_{50} values were extrapolated from the cytotoxicity curve at 50% of inhibition of EAC cell line after 72 hours of extract treatment.

NAA and 3.0 mg/L BAP +1.5mg/L NAA. Among these, 2mg/L BAP +1mg/L NAA along with adenine sulphate (25 mg/L) was responsible for rapid and heavy multiplication of shoots and the best morphogenic response was obtained (25-30 per explants) (Figure 1b; Table 1). Micro-plants were separated and sub-cultured on different
concentrations of IBA (Figure 1c). Attempts to develop rooting in the regenerated shoots were tried successfully by supplementing IBA (2 mg/L) (Figure 1d). With the help of *P. indica* (Figure 1e), the micropropagated plantlets were biotized and after 30 days, they were examined for colonization (Figure 1f). .

Histological studies of the roots of *V. divergens* colonized by *P. indica* showed inter- and intra-cellular spread of hyphae and the formation of chlamydospores. The colonization was studied on the basis of presence of hyphae and pear shaped chlamydospores in the cortical cells of stained roots (Figure 1g). Survival rate after 30 days of acclimatization in soil was found to be maximum with *P. indica* biotization. By soil drenching method it was observed that the per cent colonization ranged from 58.0 to 78.0 within 30 days to 90 days of transplantation and per cent plant survival ranged from 86.0 to 100 in this period as compared to the control plants (non-biotized) which ranged from 66.0 to 68.0 (Figure 1h, Table 2). It established the impact of colonization since all the treated plants transferred after one month under stress of *P. indica* of growth were survived (Figure 1i).

Significant increase in length and dry weight of root and shoot as well as number of lateral roots was observed due to colonization as compared to the uninoculated control plants. An increase in shoot length and shoot dry weight was recorded *in vitro* plants biotized with *P. Indica* after 30 d of growth in humidified acclimatized room. Leaf of *V. divergens* deserved special attention since it was frequently used by diabetic patients. Therefore, it was conceived to observe the effect of *P. indica* on the length and no. of leaves of micropropagated plants. The length of leaf was 5.6 cm as compared to 3.5 cm in control. Similarly the number of leaves was 7.00 per biotized plant as compared to 4.25 in control (Table 3).

The concentrated ethanol extracts from three sources, viz., *in vivo* natural, garden plants, *in vitro* generated plantlets (non-biotized) and *in vitro* generated biotized plants were
screened for *in vitro* percentage cytotoxicity at 72 hours of treatment. The profile of EAC cell growth inhibition after being treated with plant extracts of three sources mentioned above could be observed (Figure 2). It was found that all the three extracts showed slight cytotoxicity at the concentration below 125 µg/mL. 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 as reported previously [15]. 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 (Figure 2), which were allowed to grow under stress of colonization of the fungus, *P. indica.* The trend was also found to be similar in the IC_{50} value in *in vivo* and *in vitro* biotized plants; it was 100 and 95.5 µg/ml, respectively, whereas the IC_{50} value was raised to 660.6 µg/ml (Figure 3) in *in vitro* non-biotized plant extract. The ethanol extracts significantly ($p < 0.05$) reduced the viability of EAC cells after incubation for 72 hours in a manner that was directly related to the concentrations (0 to 1000 µg/ml) of the extracts in all the three sources.

DISCUSSION

The present work is an attempt to study the anticancer activity of secondary metabolites from selected medicinal plants employing cell and tissue culture techniques. 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 [21]. Generally BAP is considered as most suitable hormone for increasing the large scale multiplication and micropropagation of various plant species [22]. Addition of adenine sulphate resulted into highest percentage of multiple shoot regeneration [23].

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

Table 3: Effect of *P. indica* on root, shoot and leaf of micropropagated *V. divergens* after 30 d of transplantation under humidified acclimatized room condition.

Treatments	Root		Shoot		Leaf	
	length (cm/plant)	dry wt (g/plant)	length (cm/plant)	dry wt (g/plant)	Length (cm)	Number
Control	7.4± 0.38	0.05± 0.05	4.0± 0.72	1.10± 0.44	3.5± 0.65	4.25± 0.46
P. indica	10.5± 0.88	0.18± 0.05	8.3± 1.00	2.30± 0.85	5.6± 1.25	7.00± 0.55

± = S D, n = 3

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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REFERENCES

1. Patrick GL. Anticancer agents: An introduction to medical chemistry. 4th ed., Oxford University Press Inc, New York, 2009, p.519-578.
2. Tan G, Gyllenhaal C, Soejarto DD. Biodiversity as a source of anticancer drugs. *Curr Drug Targets* 2006;7:265-277
3. Chaturavedi HC, Jain N, Kidwai NR. Cloning of medicinal plants through tissue culture: A review. *Indian J Exp Biol* 2007;45:937-948.
4. Gosal SK, Chandi GK, Gosal SS, Varma A. Biotization of micropropagated *Aloe vera* with *Pseudomonas fluorescens* and *Piriformospora indica*. *J Med Arom Pl Sci* 2010; 32:91-96.
5. Gosal SK, Karlupia A, Gosal SS, Chhibba IM, Varma A. Biotization with *Piriformospora indica* and *Pseudomonas fluorescens* improves survival rate, nutrient acquisition, field performance and saponin content of micropropagated *Chlorophytum* sp. *Indian J Biotechnol* 2010; 9:289-297.
6. Nawak J. Review benefits of *in vitro* "biotization" of plant tissue cultures with microbial inoculants. *In vitro Cell Dev Biol Plant* 1998; 34:122-130.
7. Das A, Kamal S, Shakil NA, Sherameti I, Oelmuller R, Dua M, Tutija N, Johri AK, Varma A. The root endophyte fungus *Piriformospora indica* leads to early flowering, higher biomass and altered secondary metabolites of medicinal plant, *Coleus forskohlii*. *Plant Signal Behav* 2012; 7:103-112.
8. Anantha Naik T, Earanna N. Influence of *Azobacter chroococcum* strains of different agroclimatic zones of Karnatka on *Adhetoda vasica* Nees. Karnatka *J Agric Sci* 2006; 20:613-615.
9. Yadav V, Kumar M, Deep DK, Kumar H, Sharma R, Tripathi T. A phosphate transporter from the root endophytic fungus *Piriformospora indica* a role in the phosphate transport to the hot plant. *J Biol Chem* 2010; 285:26532-26544.
10. Varma A, Sahay NS, Butehorn B, Franken P. *Piriformospora indica*: a cultivable plant growth promoting root endophyte with similarities to *Arbuscular mycorrhizal* fungi. *Appl Environ Microbiol* 1999; 65:2741-2744.
11. Verma S, Varma A, Rexer KH, Hassel A, Kost G, Sarbhoy A, Bisen P, Buetehorn B, Franken P. *Piriformaspora indica* gen et sp nov. a new root colonizing fungus. *Mycologia* 1998; 90:896-893.
12. Sahay NS, Varma A. *Piriformospora indica*: a new biological hardening tool for micropropagated plants. *FEMS Microbial Letters* 1999; 18:297-302.
13. Ozaslan M, Karagoz ID, Kalender ME, Kilic IH, Sari I, Karagoz A. *In vivo* antitumoral effect of *Plantago major* L. extract on Balb/C mouse with Ehrlich ascites tumor. *Am J Chin Med* 2007; 35:841-851.
14. Ozaslan M, Karagoz ID, , Kilic IH, Cengiz B, Kalender ME,Guldur ME, Karagoz A, Zumrutdal ME. Effect of *Plantago major* sap on Ehrlich ascites tumors in mice. *Afr J Biotechnol* 2009; 8:955-959.
15. Kumar S, Prakash A, Sinha K, Verma AK. Screening and evaluation of *in vivo* and *in vitro* generated insulin plant (*Vernonia divergens*) for antimicrobial and anticancer activities. *World Acad Sci Eng Technol* 2012; 70:769-773.
16. Kumar S, Prakash A, Vohra, R, Verma AK. Evaluation of anticancer activities of ethanolic extracts of *in vivo* and *in vitro* grown *Coleus forskohlii* Briq. *In: Perspective in Cytology and Genetic.* Eds: Giri AK, Mukherjee A, Bhattacharjee P, 2011; 15:19-30.
17. Saluja MS, Sangameswaran B, Hura IS, Sharma A, Gupta SK, Chaturvedi M. *In vitro* cytotoxicity activity of leaves of *Madhuca longifolia* against Ehrlich Ascites Carcinoma (EAC) cell lines. *Int J Drug Discov Herbal Res* 2011; 1:55-57.

18. Ozaslan M, Karagoz ID, Kilic IH, Guldur ME. Ehrlich ascites carcinoma. Afr J Biotechnol 2011; 10:2375 - 2378.
19. Murashige T, Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiol plant* 1963; 15:473-497.
20. Mosmann T. Rapid calorimetric assay for cellular growth and survival, application to proliferation and cytotoxicity assay, *J Immuno Meth* 1983; 65:55-63
21. Reddy PS, Rodrigues P, Rajasekhran R. Shoot organogenesis and mass propagation of *Coleus forskohlii* from leaf derived callus. *Plant Cell Tissue Organ Cult* 2001; 66: 183-188.
22. Shrivastava S, Banerjee M. *In vitro* clonal propagation of physic nut (*Jatropha curcas* L.): Influence of additives. *Int Integrative Biol* 2008; 3:73-79.
23. Nandgopal S, Kumari BDR. Adenine sulphate induced high frequency shoot organogenesis in callus and *in vitro* flowering of *Cichorium intybus* L. cv. Focus- a potent medicinal plant. *Acta agriculturae slovenica* 2006; 87:415-425.
24. Hazarika BN. Acclimatization of tissue –culture plants. *Curr Sci* 2003; 85:1704-12.
25. Akhir NAM, Chua LS, Majid FAA, Sarmidi MR. Cytotoxicity of aqueous and ethanolic extracts of *Ficus deltoidea* on human ovarian carcinoma cell line. *Br J Med Med Res* 2011; 1:397-409.
26. Ajith TA, Janardhanan KK. Cytotoxic and antitumor activities of a polypore macrofungus, *Phellinus rimosus* Pilat. *J Ethnopharmacol* 2003; 84:157-162.
27. Salau AK, Yakubu MT, Oladiji AT. Cytotoxic activity of aqueous extracts of *Anogeissus leiocarpus* and *Terminalia avicennioides* root barks against Ehrlich Ascites Carconoma cells. *Indian J Pharmacol*ogy 2013; 45:381-385