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Evaluation of Antimicrobial and Anticancer activities of Methanol Extract of *in vivo* and *in vitro* grown *Bauhinia variegata* L.

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Abstract

The present investigation deals with the biological activities of the extracts of the medicinal plant Bauhinia variegata L.[BV], generated through in vivo and in vitro processes for their antibacterial and anticancer activities. Nodal explants of BV when placed on MS medium fortified with 6-Benzyl amino purine (BAP) at 5µg/ml resulted into multiple shoots. These shoots, on transfer developed bunch of roots in presence of Indole-3-butyric acid (IBA) at 2-4 µg/ml. The methanol extracts of such regenerated in vitro plants along with that of natural in vivo garden plants on comparison were found to be more effective against gram positive bacteria when compared to gram negative bacteria. But the screening of the in vitro cytotoxicity on EAC mouse cell lines responded almost with the same degree of inhibition for the ethanol extract, derived from both in vivo and in vitro sources.

Keywords: Bauhinia variegata, antimicrobial, anticancer activity, in-vitro explants.

Introduction

Bauhinia variegata L. (Fam: Fabaceae), is a popular ornamental plant, native to Southeast Asia that grows in tropical and subtropical climate. It is a medium-sized deciduous tree, found in sub-Himalayan areas[1]. The flower is very beautiful and attractive that the plant could earn local name like kachnar, orchid tree, camel's foot, mountain ebony, etc[2]. The bark of this plant is described as astringent, and alliterative. The root and bark are depurative, anthelminthic and anti-inflammatory, that are useful in diarrhoea, dysentery, skin disease, leprosy, intestinal worms, wounds, ulcer, tumours, etc[3]. The main chemical compounds – different types of flavonoids, were isolated from the bark and stem of the plant[4-5]. A total of six flavonoids have been isolated from non-woody aerial parts of *Bauhinia variegata*[6][BV]. The antimicrobial activities of plant extract can be determined by various methods such as disc diffusion, agar well diffusion and twofold serial dilution techniques[7]. The agar well diffusion technique for screening of the antimicrobial activity of medicinal plant is normally considered[8]. The aim of the present research work is to find out the antibiotic and anticancer properties of the medicinal plant, *Bauhinia variegata* through pharmacological investigation. For this purpose, the methanol extract obtained from the *in vivo* as well as the *in vitro* generated plants have been exposed to determine the efficacy in terms of antibacterial and anticancer activities. Since nearly all of the identified components from plants, which are active against microorganisms, are aromatic or saturated organic compounds, they are often obtained through initial ethanol or methanol extraction[9]. Tissue culture techniques now-a-days provide great advantage in generating the plants under controlled condtions[10]. In *in vitro* plants, the change of the chemical constituents that occurs can help the plants to counter a pathogen attack. Thus, the present work was first targeted to observe the effect of different phytohormones on the induction *in vitro* explants. Further, the methanol extract of these *in vitro* regenerated plantlets along with *in vivo* ones were tested for cytotoxicity on Ehlrich Ascitic Carcinoma [EAC] cell lines[11].

Material and Methods

The plant sample used in the present experiment was collected from the Botanical garden, Sector 40, Noida after sprouting of the fresh leaf in plant (figure 1A). It was officially identified by NBRI, Lucknow. Shoot tip and nodal segments (2-3 cm) as explants were excised from the 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[12] containing 0.8% agar, 3% sucrose and 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 121ºC. The cultures were maintained at 23±2ºC. Sub-culturing of the *in vitro* shoots was carried out at periodical intervals of 4 weeks using MS medium supplemented with BAP and NAA either alone or in combination. The number of shoots produced after subculture divided by number of shoots inoculated was regarded as rate of multiplication. The shoot multiplication experiments have been regularly conducted and maintained for over one year now.

***In vitro* culture of *Bauhinia variegata*:** Multiple shoots were regenerated on MS medium supplemented with different concentrations of BAP (3-7 µg/ml) and NAA/2, 4-D (2-4µg/ml). 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). Plants were taken out from the tubes and washed with distilled water to remove the agar medium, transferred to sterilized tray beds and shifted to highly humidified room for hardening for another 20 days. Rooted shoots from 6-8 week old cultures were transferred to soil with vermiculite in 1:1 ratio. Plants were later planted in the field.

Preparation of ethanol extract of *Bauhinia variegata:* Plantlets of *B. variegata*, generated through both *in vivo* and *in vitro* systems were shade-dried and pulverized. The powder was treated with petroleum ether for de-waxing and removal of chlorophyll. Later, it was packed (2 g) in a Soxhlet apparatus and subjected to hot continuous percolation for 12h, using 250 ml of methanol (95% v/v) as solvent. The extract was concentrated to dryness under reduced pressure in rotary evaporator and dried in a desiccator.

***In-vitro* Cytotoxicity Assay:** Cytotoxicity assay was carried out in accordance with previously published protocol[13]. EAC cells ($5x10^3$ cells/well) were cultured on a flat-bottomed 96 well plate. After 48 hours incubation, 20µl of MTT solution (5mg/ml) was added to each well of the assay plate, which was then incubated for 4 hours at 37ºC. 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[14].

Determination of Antibacterial activity: The antibacterial activity of extracts against the bacterial strains viz., *Escherichia coli* MTCC 64, *Enterobacter aerogenes* MTCC 111, *Klebsiella pneumoniae* MTCC 39, *Pseudomonas aeruginosa* MTCC 424, *Salmonella typhi, Bacillus subtilis* MTCC 121, was tested by agar well diffusion method, and zones of inhibition were measured. Each experiment was performed in triplicate and the average value of inhibition zones and standard deviation were calculated. The zone of inhibition was compared with that of standard Gentamycin concentration of 1mg/100µl[15].

Results and Discussion

Plant tissue culture techniques were employed to develop *in vitro* multiple shoot regeneration through direct organogenesis. Nodal segments of the freshly collected plant, *BV* (figure-1B), were examined for their response to different combinations of phytohormones. MS medium supplemented with various growth regulators like 6-Benzyl amino purine [BAP], Naphthelene acetic acid [NAA] and 2,4-Dichlorophenoxy acetic acid [2,4-D] either singly and/or in combination, at different concentrations was used for shoot regeneration from nodal segments and Indole-3-butyric acid [IBA] for root regeneration (table-1).

Results showed that combination of NAA (3-7 µg/ml) and BAP (2-4 µg/ml) at higher concentrations was not very favorable for shoot regeneration from nodal segments as compared to BAP alone at 5µg/ml. Since the requirement of the experiment was to obtain methanol extract of *in vitro BV* plant, no attempt was taken to develop callus and/or callus mediated plantlets. Emergence of young putative shoot war noticed within a month from the day of inoculation (figure-1B). The growth of shoot was fast because length was distinctly noticeable in the next fortnight (figure-1C).

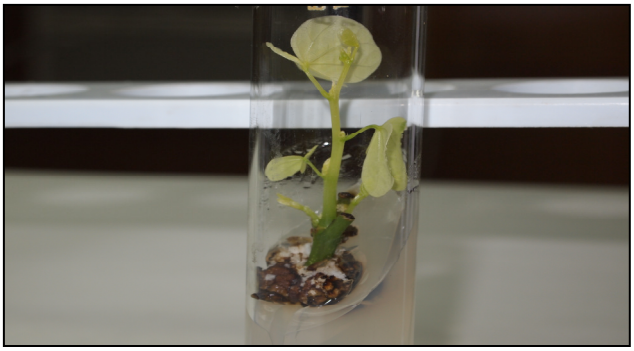
Table-1
Effect of different plant growth regulators (PGRs) on shoot formation and root induction from nodal explants of *Bauhinia variegata* L. after 25 days of culture on MS medium containing 0.8% agar and 3% sucrose

Growth regulators [mg/l]				Shoot formation [%]	No. of shoot(s)/ explant	No. of root/ explant
BAP	**IBA**	**NAA**	**2,4 D**			
1	-	-	-	10.30±0.08	0.68±0.10	-
2	-	-	-	11.87±0.08	0.70±0.14	-
3	-	-	-	41.44±0.50	1.00±0.00	-
4	-	-	-	52.55±0.50	1.65±0.11	-
5	-	-	-	78.12±0.30	4.79±0.08	-
6	-	-	-	71.40±0.80	5.02±0.10	-
7	-	-	-	81.00±0.50	5.91±0.10	-
3	-	2	-	46.00±0.10	1.23±0.20	-
5	-	3	-	48.03±0.20	1.49±0.50	-
7	-	4	-	52.04±0.40	2.03±0.70	-
3	-	-	2	53.07±0.20	1.22±0.40	-
5	-	-	3	55.02±0.65	1.40±0.30	-
7	-	-	4	56.79±0.06	1.9±0.40	-
-	2	-	-	-	-	1.44±0.07
-	3	-	-	-	-	1.49±0.08
-	4	-	-	-	-	1.57±0.08

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. Fully-grown shoots of 3-month old shoots were transferred to half strength MS medium supplemented with 2-4 µg/ml of IBA. Initiation of rooting was noticed within a fortnight (figure-1D) and within one month, bunch of multiple roots appeared on each developing shoots (figure-1E). Such plantlets were either exposed for methanol extraction or successfully transferred to soil, which survived well in nature (figure-1F).

(A)

(B)



(C)

(D)

(E)

(F)

Figure-1

A: *Bauhinia variegata* L. In Botanical Garden, sector 40, Noida, Uttar Pradesh, B: Development of shoot from nodal explants on MS basal medium; mark green & healthyshoot: 25 days old culture, C: Nodal explant showing development of direct multiple shoots on MS+ 5 µg/ml BAP, D: Culture showing emergence of root after transfer on MS basal medium + 3 µg/ml IBA, E: Shoot with bunch of roots after prolonged culture, F*: *Bauhinia variegata* L: Regenerated plantlet coming out after 20 days from fully humidified acclimatized chamber

The methanol extract from both *in vivo* and *in vitro* generated plants of *BV* were tested against a number of microbes, only *Escherichia coli and Pseudomonas aeruginosa* were found to be resistant at a concentration of 50 µg/ml. TLC purification of this extract produced a purple spot of R_f value 0.68 for which antimicrobial activity was determined by the agar well diffusion method. Inhibition zone by aqueous solution of methanol extract from both *in vivo* and *in vitro* generated plantlets (figure 2) clearly gave an edge to the *in vivo* plants so far the antimicrobial activity was concerned. But both types of extract were found to be more effective against gram positive then gram negative bacteria[14]. The decreased level of antibacterial activity in *in vitro* plantlets suggests that some of the chemicals are either lost or may have transformed in other active compounds[16]. Medicinal plants can be poisonous if wrong plant parts or wrong concentrations are used[17]. Herbal medicines are assumed to be harmless, nevertheless, herbal extracts need to be assured for its quality control and efficacy for a particular dose.

Extracts used in our study exhibited variation in its antibacterial property against both gram positive and gram negative bacteria. This antibacterial activity may be attributed to the active compounds that are present in the plant extracts. However, some plant extracts were unable to exhibit antibacterial activity against tested bacterial strains. Bacterial strains are known to have resistant mechanisms, for example, enzymatic inactivation, target site modification and decreased intracellular drug accumulation[18]. The concentrated methanol extract was screened for *in vitro* cytotoxicity at various time points (24h, 48h and 72h) at different dilutions (100, 50, 25 and 12.5 µg/ml). The calculated percent growth inhibition of EAC cells post treatment with 100µg/ml methanol extracts prepared both by *in vivo* and *in vitro* generated plantlets, was surprisingly similar post 72 hrs. The percent growth inhibition was 18, 49 and 63% in case of *in vitro* extract and 32, 42 and 62% *in vivo* plant extract after 24hr, 48hrs and 72 hrs respectively. The response was almost negligible at other concentrations (figure 3).

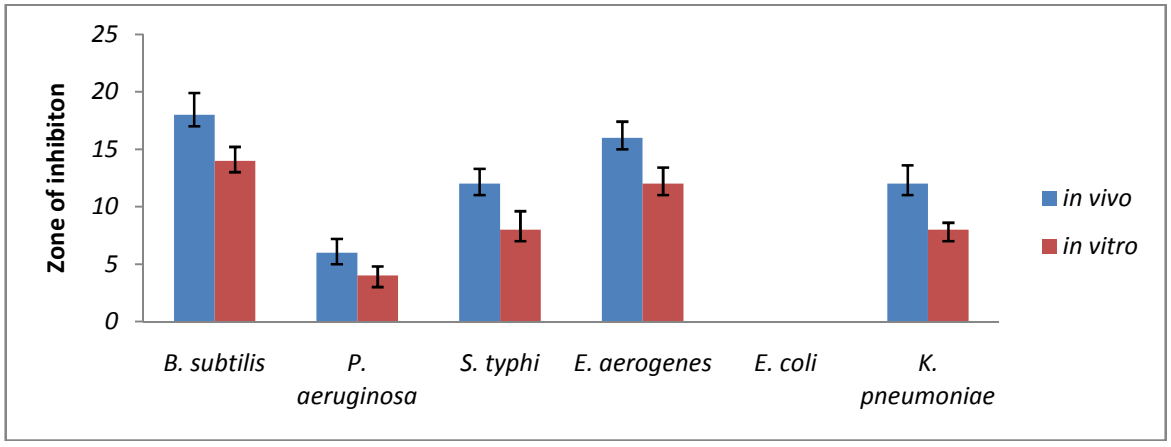


Figure 2
Comparative zone of inhibition of methanol extracts, prepared from *in vivo* and *in vitro* generated plantlets of *B. variegata*

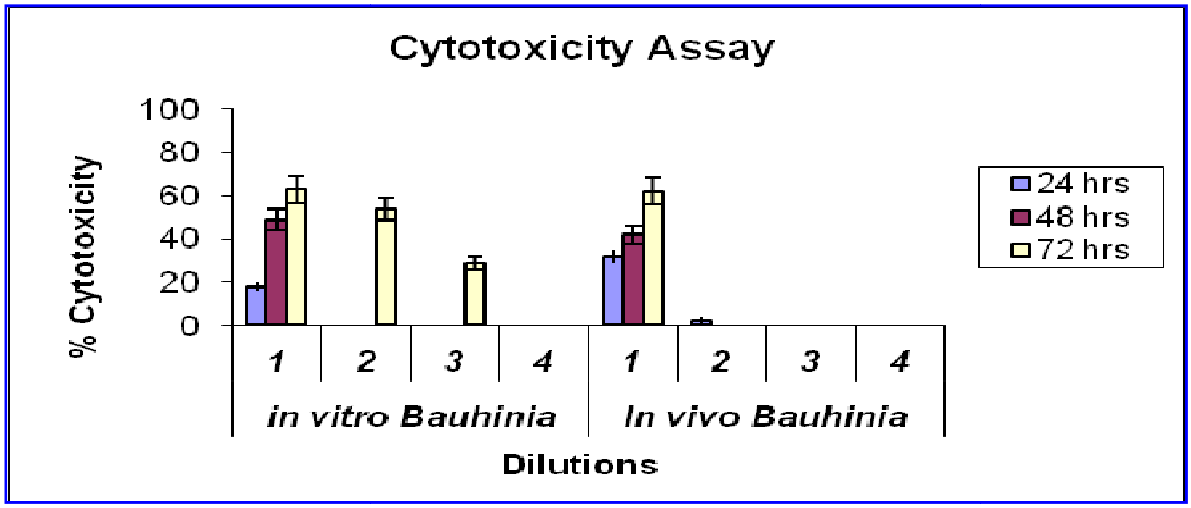


Figure 3
Cytotoxicity assay of methanol extracts from both *in vivo* and *in vitro* grown *Bauhinia varigata* L. at different time periods (1, 2, 3 and 4 represent dilution of extracts at 100, 50, 25 and 12.5 µg/ml)

At a higher concentration (100µg/ml) the methanol extract exhibited anticancer activities whichis in unison with published literature. Ethanol extract was reported to be cytotoxic against human epithelial larynx cancer and human breast cancer (HBL-100)[19] cell line. In essence, the present work revealed that *BV* contains some important chemical constituents that can be exploited in the management of bacterial and cancer treatment[20].

Conclusion

Although *in-vitro* explants and *in-vivo* BV, both exhibited 60% activity which is very encouraging, efforts are on to improve the anticancer activity of the *in-vitro* explants. The presence of biologically active substances such as alkaloids, steroids, triterpenoids and flavonoids in the leaf extracts of BV may be responsible for the antibacterial activity and anticancer activity against the test cultures used as revealed by phytochemical studies of plant extracts[21-22].

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