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Elicitation of flavonoids by Salicylic acid and *Penicillium expansum* in *Andrographis paniculata* (Burm. f.) Nees. cell culture

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Salicylic acid and *Penicillium expansum* extract were used as chemical and fungal elicitors to enhance the synthesis of total flavonoid content in suspension culture of *Andrographis paniculata*. Salicylic acid elicitor was added in 0.05mM, 0.5mM and 1.5mM concentrations to the cell suspension of *Andrographis paniculata* and studied for 24, 48 and 72 hrs. of treatment duration. Similarly, 0.3%, 0.6% and 1.2% of *Penicillium expansum* homogenate was treated with *Andrographis paniculata* suspension culture for 2 days, 5 days and 8 days. All the elicitors administered at late exponential phase (25 days old culture).Our findings revealed that the salicylic acid treatment for 24 hrs with 0.05 mM concentration of treatment duration showed 1.39 fold increment in total flavonoid content (1.72 mg/g) whereas *Penicillium expansum* elicitor (1.2%, 2 days) indicates 1.59 fold enhancement (2.38 mg/g) as compared to control (1.49 mg/g). This concentration and incubation period was superior to other studied concentrations and incubation periods for *P. expansum* elicitor. Among all the studied elicitors, *Penicillium expansum* (1.2%, 2 days) was found to be superior over Salicylic acid (0.05 mM, 24 hrs).

Keywords: *Andrographis paniculata*; flavonoid; elicitation; Salicylic acid; *Penicillium expansum*

Plant tissue culture has proven as an important technique for improving the utility sources and naturally occurring active metabolite content in medicinally important plant system. This is a highly appreciable and acceptable biotechnological concept that contributes largely in exploring and conserving the natural sources of herbal medicines to achieve high product recovery.

The secondary metabolites with high medicinal properties produced by plant cell culture attracted R & D sectors of many pharmaceutical companies. In 1984, Shikonin was the first product which was produced from cell cultures of *Lithospermum erythrorhizon* in 750 L bioreactors by Mitsui Petrochemical Industries, Japan. The cost of

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Shikonin at that time (1983) was approximately $ 4,000 Kg^{-1}(Curtin 1983). Taxol, an anticancer drug produced from cell cultures of *Taxus* plant has been successfully industrialized for commercial purpose. Ajmalicine from *Catharanthus roseus,* Caffeine from *Coffea Arabica,* Ginsenoside from *Panax ginseg,* Serpentine from *Catharanthus roseus* and Vomilenine from *Rauwolfia serpentine* , L-Dopa from *Mucuna pruriense*, Vinca alkaloids from *Catharanthus roseus*, Berberine from *Coptis japonica*, diosgenin from *Dioscorea deltoidea* and capsaicin from *Capsicum frutescens* and many more has been successfully produced *from* cell cultures worked out (Masanaru 1994 , Vanisree et.al 2004 and Bhojwani and Razdan, 1996).

Different strategies for the metabolic engineering are worked out by the scientists for qualitative and quantitative improvement of biologically active compounds. These strategies includes use of suitably conditioned bioreactor, chemical and biological elicitors, increasing the cell permeability and alteration in media composition (Brodelius *et al.* 1989; Mendhulkar and Moinuddin 2009).

Andrographis paniculata Nees. commonly known as 'Kalmegh' belongs to the family Acanthaceae and it is a well known medicinal plant. The plant is recommended for its drug utility in Indian Pharmacopoeia and widely used in Ayurveda, Unani, Siddha and Homeopathy systems of medicines. The plant is reported to possess terpenoids and flavonoids. The major terpenoids viz. 14-deoxy-11-oxoandrographolide, 14-deoxy-11, 12-didehydroandrographolide and 14-deoxyandrographolide andrographolide are the active constituents of this plant. The plant is reported to possess liver stimulant properties, astringent, anodyne, tonic and alexipharmic properties, and is useful in dysentery, cholera, diabetes, consumption, influenza, bronchitis, swellings and itches, piles and gonorrhea (Zhao and Frang 1991). The most significant pharmaceutical properties of this plant are anticancerous (Kumar *et al.* 2004) and anti-HIV (Calabrese *et al.* 2000).

In the present work, the elicitation of flavonoids in *Andrographis paniculata* (Burm.f.) Nees. was experimentally worked out using a phenolic phytohormone, Salicylic acid and a blue-colored mold plant pathogen, *Penicillium expansum.* Taguchi *et al.* 2001 has reported that Salicylic acid (SA) induces gene regulation related to the biosynthesis of secondary metabolites in plants. *Penicillium expansum* elicitor has been used to enhance the production of plant secondary metabolites at *in vitro* level (Buitelaar *et al.* 1992).

Materials and methods

Cell culture and cell viability

The cell suspension culture was raised by transferring calli into liquid MS media supplemented with 2, 4-D: BAP (1.0:0.5) hormonal combination and concentration except agar (Fig. 1). The cell suspension was incubated in gyratory shaker with 110 r.p.m. at 25 $\pm$2 ^{o}C under dark condition for 30 days (Fig.2). The cell viability was tested by Guava ViaCount assay using Guava Easy CD4 System. For cell viability assay, 190µl of via count solution (viability counting solution) was added in 10 µl of suspension culture and incubated for 5 min at room temperature. The analysis was performed using Cytosoft software for Guava Via Count The results were recorded as viable cell count, total cell count (both in number of cells/ml) and the percent viability (Guava ViaCount Reagent, 2006).

Preparation of chemical elicitor, Salicylic acid

100 mg of salicylic acid dissolved in 10 ml distilled water and was autoclaved at 15 psi for 20 min. 0.05 mM, 0.5 mM and 1.5 mM

concentrations of SA were added into 25 days old cell suspension culture of *Andrographis paniculata*. These cultures were incubated at 25 $\pm$2 ^{o}C in gyratory shaker (110 r.p.m.) under dark condition. Each concentration was studied for 24, 48 and 72 hrs. of treatment duration. All the experiments were done in triplicate.

Preparation of fungal elicitor, *Penicillium expansum*

Penicillium expansum culture grown in 250 ml flasks containing 100 ml of the PDB medium were harvested when the culture attained stationary phase after four weeks. The flask containing fungal cultures along with PDB medium was autoclaved, separated and washed several times with demineralized water. The mycelial residue was resuspended in demineralized water and homogenized. This homogenate was autoclaved prior to use and applied in the concentrations, 0.3%, 0.6% and 1.2% in 50 ml of *Andrographis paniculata* cell suspension cultures separately. Each treatment was subjected for 2 days, 5 days and 8 days treatment duration. The polysaccharide content in *Penicillium expansum* homogenate was determined by the phenol sulfuric acid method using glucose as the standard (Sadasivam and Manickam, 2005).

Analytical methods

The cell suspensions after elicitor treatment were filtered and washed several times with distilled water. The filtered cells were dried in oven at 50^{o}C to a constant weight and powdered using mortar and pestle. Each powdered sample (150 mg) was sonicated using 2 mm probe for 10 min with pulse rate operating at 10 sec. on and 2 sec. off, amplitude 20% in 1.5 ml of methanol using Sonics Vibra Cell (VCX 130) instrument. The extract was centrifuged at 5000 rpm for 5 min. after sonication. The supernatant was transferred into 2 ml eppendorf tubes and the final volume was adjusted to 2 ml with methanol (HPLC grade). This extract was used for quantification of total flavonoid content in cell samples by UV- Vis spectrophotometer.

Identification and quantitative analyses of flavonoids

The identification of flavonoids in the samples was carried out by HPTLC analysis. The TLC plates were observed under UV light at 366 nm. All the studied samples were tested for the presence of flavonoids. On spraying the TLC plates with 1% ethanolic $AlCl_3$ reagent on TLC plates, the flavonoid compounds were clearly evident as bright violet bands in all the samples (Plate 1 and 2) and the total flavonoid content in the samples was done by Aluminum chloride colorimetric method as described by Chang *et al.*, (2002).

Results

Cell viability

The Guava Easy CD4 System showed 86% cell viability of 30 days old cell suspension culture of *Andrographis paniculata*.

Effect of salicylic acid on total flavonoid content

In 24 hrs. treatment duration, 0.05 mM of Salicylic acid showed enhancement in total flavonoid content which was 1.72 mg/g dry wt. (Table-1). It indicates 1.39 fold increase in the flavonoid content which was the highest for this incubation period. The 0.5 mM SA showed 1.37 fold increase (1.69 mg/g dry wt.) whereas 1.5 mM indicates 1.06 mg/g dry wt. flavonoid content which was lower than that of control (1.23 mg/g dry wt.). The variations in total flavonoid content were evident with 0.05 mM and 0.5 mM of SA treatment only for 24 hrs. treated samples. However, these variations were not remarkable.

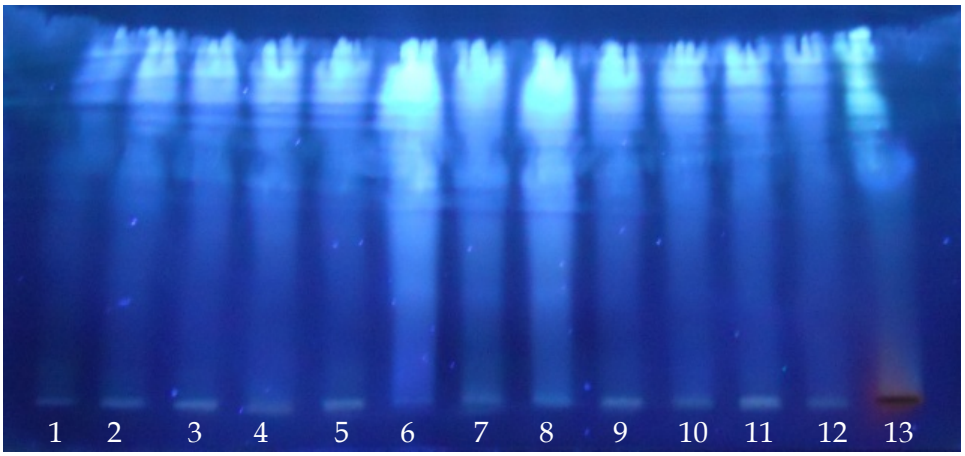

Plate 1: HPTLC chromatogram for the detection of flavonoids in salicylic acid treated cell suspension culture of *Andrographis paniculata*. Image at 366 nm

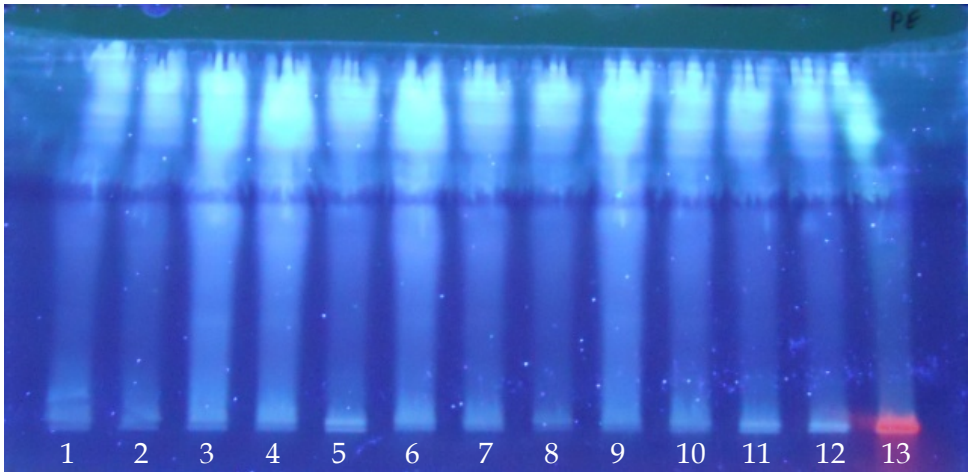

Plate 2: HPTLC chromatogram for the detection of flavonoids in *P. expansum* elicitor treated cell suspension culture of *Andrographis paniculata*. Image at 366 nm

Table 1-Effect of elicitors on flavonoid synthesis

Name of elicitors	Treatment durations	Concentrations of elicitor	Total flavonoid content in mg/g	Fold increase
Salicylic acid	24 hrs	Control	1.23 ± 0.12	
		0.05 mM	1.72 ± 0.29	1.39
		0.5 mM	1.69 ± 0.13	1.37
		1.5 mM	1.06 ± 0.20	0.86
	48 hrs	Control	1.50 ± 0.06	
		0.05 mM	1.61 ± 0.08	1.07
		0.5 mM	1.68 ± 0.11	1.12
		1.5 mM	1.71 ± 0.08	1.14
	72 hrs	Control	1.69 ± 0.10	
		0.05 mM	2.14 ± 0.02	1.26
		0.5 mM	2.12 ± 0.12	1.25
		1.5 mM	2.02 ± 0.20	1.19
Penicillium expansum	2 days	Control	1.49 ± 0.06	
		0.3%	1.56 ± 0.04	1.04
		0.6%	1.69 ± 0.07	1.13
		1.2%	2.38 ± 0.16	1.59
	5 days	Control	2.48 ± 0.20	
		0.3%	2.60 ± 0.07	1.04
		0.6%	2.36 ± 0.09	-
		1.2%	2.27 ± 0.10	-
	8 days	Control	2.66 ± 0.08	
		0.3%	1.53 ± 0.20	-
		0.6%	1.76 ± 0.03	-
		1.2%	1.65 ± 0.02	-

In 48 hrs of treatment exposure the results obtained were in contrast with the results of 24 hrs. treatment duration. In this treatment, increased concentration of SA contributes total flavonoid accumulation. All the concentrations of SA yields enhanced flavonoid content. However, the optimal concentration for flavonoid production in 48 hrs. of treatment exposure was 1.5 mM which resulted in 1.14 fold increase (1.71 mg/g dry wt.) as compared to control (1.50 mg/g).

When 0.05 mM, 0.5 mM and 1.5 mM concentrations of SA were added in the cell suspension cultures of *A. paniculata,* a significant improvement was observed in flavonoid production for 72 hrs. of treatment duration. All the concentrations of SA in 72 hrs. of treatment duration proved to be effective for flavonoid production. The total flavonoid content was estimated as 2.14 mg/g dry wt. in 0.05 mM, 2.12 mg/g dry wt. in 0.5 mM and 2.02 mg/g dry wt. in 1.5 mM

of SA treated samples. The results obtained in 72 hrs. of treatment duration represent increased flavonoid content as compared to 24 and 48 hrs. of treatment duration (Fig. 3).

Fig.1: 10-12 weeks old matured callus

Fig. 2: *Andrographis paniculata* suspension culture grown in tissue culture bottles containing 50 ml of liquid MS medium.

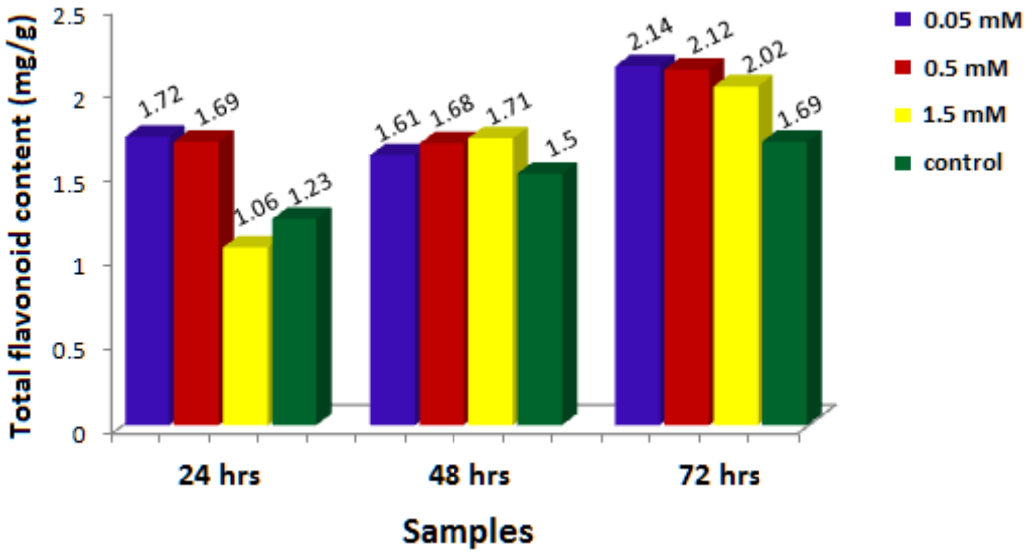

Fig.3: Effect of salicylic acid on total flavonoid content in cell suspension culture of *Andrographis paniculata.*

Estimation of total carbohydrate content by phenol sulphuric acid method

Phenol sulphuric acid method was used to determine the dose concentration of each elicitor, the total carbohydrate content present in each elicitor revealed 4.08 mg, 8.16 mg and 16.32 mg of fungal polysaccharide l^{-1} in 0.3%, 0.6% and 1.2% of the homogenate of *Penicillium expansum* respectively.

Effect of *Penicillium expansum* elicitor on total flavonoid content:

In 2 days treatment duration with *Penicillium expansum* elicitor, increasing concentrations of the elicitor stimulated the

flavonoid accumulation. The flavonoid content was measured as 1.56 mg/g dry wt. and 1.69 mg/g dry wt. for 0.3% and 0.6% concentration of *P. expansum* elicitor, respectively. The 1.2% concentration represented most effective results (2.38 mg/g dry wt.) compared to other concentrations and the enhancement in flavonoid content was 1.59 fold higher than control (1.49 mg/g dry wt., Table-1.).

When the treatment duration was increased to 5 days, only 0.3% of *P.expansum* elicitor showed marginal improvement in flavonoid content which was 2.60 mg/g dry wt. and no other concentrations influenced flavonoid accumulation. Further increasing the treatment duration to 8 days did not influence flavonoid production with any concentrations of *P. expansum* elicitor (Fig. 4).

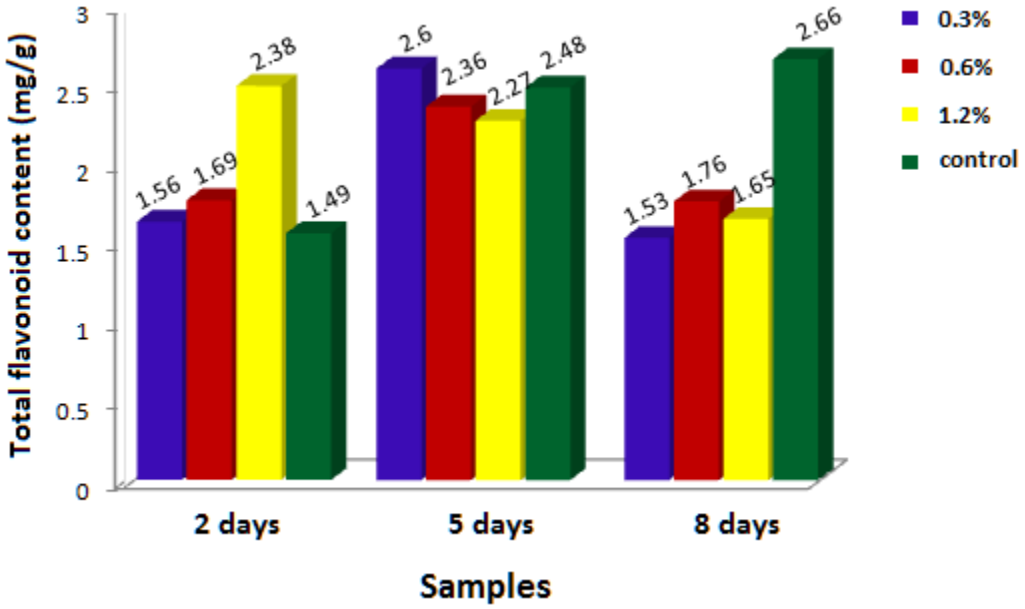

Fig. 4: Effect of *Penicillium expansum* elicitor on total flavonoid content in cell suspension culture of *Andrographis paniculata.*

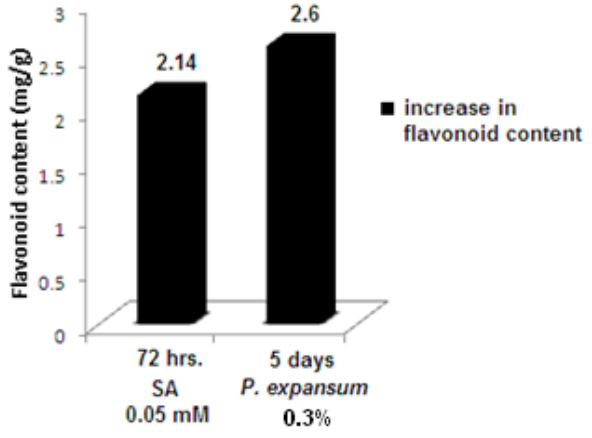

Fig. 5: Comparison of flavonoid enhancement

Discussion

The results obtained in SA treated samples indicate that 0.05 mM as the most effective concentration of SA and 72 hrs. of treatment duration is best treatment duration among all the studied treatments.

The finding of the present study is in agreement with the fact that the stationary phase of suspension culture shows higher accumulation of flavonoids (Satio and Mizukami, 2002). In *Pueraria tuberose* cell suspension culture, 20 µM of SA was found to be most favorable in isoflavonoid production for 48 hrs. of treatment duration

(Goyal and Ramawat, 2008). The alkaloid production in hairy root cultures of *Brugmansia candida* was enhanced after 24 hrs. of treatment with 0.01 mM of SA (Pitta *et al.* 2000).

Penicillium expansum elicitor (1.2%, 2 days) showed 1.59 fold enhancement in flavonoid content over control was superior to other studied concentrations and incubation periods for *P. expansum* elicitor. The results obtained for *P. expansum* elicitor suggest that the incubation period for elicitation using is a crucial factor than the elicitor concentration. In *Penicillium expansum* elicitor treated samples, longer treatment duration did not favor any elicitor concentration for elicitation of flavonoids.

Maojun *et al.* (2006) reported enhanced puerarin content using *Penicillium citrinum* in *Pueraria thomsonii* cell suspension culture. The fungal elicitors from *P. expansum* (1.5 %, v/v) showed 15 % increase in thiophene accumulation in hairy root cultures of *Tagetus patula.* (Buitelaar *et al.* 1992). Savitha *et al.* (2006) reported 2.2 fold enhancement in betalain production in 7 days of elicitation with *Penicillium notatum* (0.25 % concentration) in hairy root cultures of *Beta vulgaris*.

The comparative enhancement in flavonoid content by chemical and fungal elicitors was the point of interest in present investigation. The results obtained reveal that SA (0.05 mM) concentration is most effective in eliciting flavonoid production (2.14 mg/g dry wt.) when treated for 72 hrs. whereas the optimum elicitor concentration and treatment duration of *Penicillium expansum* was recorded 0.3% and 5 days respectively which yielded 2.6 mg/g of flavonoid and this flavonoid content was more than the SA treated samples (Fig.5).

The present investigation in the medicinal plant, *Andrographis paniculata* has provided an insight to understand the precise response of this particular plant system to the chemical and fungal elicitors under *in vitro* condition for elicitation.

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References

Bhojwani SS and Razdan MK. 1996. Plant Tissue Culture: Theory and Practice, a Revised Edition. Elsevier, Netherland, pp.539.

Brodelius P, Funk C, Haner A and Villegas M. 1989. A procedure for the determination of optimal chitosan concentrations for elicitation of cultured plant cells. Phytochemistry, 28: 2651-2654.

Buitelaar RM, Cesário MT and Tramper J. 1992. Elicitation of thiophene production by hairy roots of *Tagetes patula*. Enzyme and Microbial Technology, 14: 2-7.

Calabrese C, Berman SH, Babish JG, Ma X, Shinto L, Dorr M, Wells K, Wenner CA and Standish LJ. 2000. A phase I trial of andrographolide in HIV positive patients and normal volunteers. Bastyr University Research Institute, Bastyr University, Washington 98028, USA. Phytother Res. 14: 5, 333-338.

Chang CC, Yang MH, Wen HM and Chern JC. 2002. Estimation of total flavonoid content in propolis by two complementary colorimetric methods. Journal of Food and Drug Analysis, 10: 3, 178-182.

Curtin ME. 1983. Harvesting profitable products from plant tissue culture. Biol. Tech., 1:649- 657.

Goyal S and Ramawat KG. 2008. Increased isoflavonoid accumulation in cell suspension cultures of *Pueraria tuberose* by elicitors. Indian J. of Biotech., 7: 378-382.

Kumar RA, Sridevi K, Kumar NV, Nanduri S and Rajagopal S. 2004. Anticancer and immunostimulatory compounds from

Andrographis paniculata. J Ethnopharmacol, 92:291-295.

Maojun XU, Jufang D and Muyuan Z. 2006. Nitric oxide mediates the fungal elicitor-induced puerarin biosynthesis in *Pueraria thomsonii* Benth. suspension cells through a salicylic acid (SA)-dependent and a jasmonic acid (JA)-dependent signal pathway. Science in China Series C: Life Sciences, 49:4, 379-389.

Masanaru M. 1994. Plant tissue culture an alternative for production of useful metabolites. Rome.FAO Bulletin no. 108.

Mendhulkar VD, Moinuddin M Ali and Raut RW. 2009. Saponin estimation in *Vigna radiata* cell culture treated with cell permeabilizing agent. Triton X-100. Advances in Plant Sciences. 22: I, 1-5.

Pitta-Alvarez SI, Spollansky TC and Giulietti AM. 2000. The influence of different biotic and abiotic elicitors on the production and profile of tropane alkaloids in hairy root cultures of *Brugmansia candida*. Enzyme Microb Technol. Feb. 1; 26(2-4):252-258.

Satio K and Mizukami H. 2002. Plant cell cultures as producers of secondary compounds, in Plant biotechnology and transgenic plants, edited by C. Kirsi Majra and H. B. Wolfang (Marcel Dekker Inc., New York), 77-108.

Savitha BC, Thimmaraju R, Bhagyalakshmi N and Ravishankar GA. 2006. Different biotic and abiotic elicitors influence betalain production in hairy root cultures of *Beta vulgaris* in shake-flask and bioreactor. Process Biochemistry, 41:1, 50-60.

Taguchi G, Yazawa T, Hayashida N and Okazaki M. 2001. Molecular cloning and heterologous expression of novel glucosyltransferases from tobacco cultured cells that have broad substrate specificity and are induced by salicylic acid and auxin. Eur J Biochem., 268: 4086-4094.

Vanisree M, Lee CY, Lo SF, Nalawade SM, Lin CY and Tsay HS. 2004. Studies on the production of some important secondary metabolites from medicinal plants by plant tissue culture. Bot. Bull. Acad. Sin., 45: 1-22.

Zhao HY and Frang WY. 1991. Antithrombotic effect of *Andrographis paniculata* Nees in preventing myocardial infarction. Chi Med J (Engl). 104:770-775.