Int J Pharm Bio Sci 2013 Oct; 4(4): (B) 731 - 740

Research Article **Biotechnology**

International Journal of Pharma and Bio Sciences **ISSN 0975-6299**

CHITOSAN AND *ASPERGILLUS NIGER* MEDIATED ELICITATION OF TOTAL FLAVONOIDS IN SUSPENSION CULTURE OF *ANDROGRAPHIS PANICULATA* (BURM.F.) NEES.

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ABSTRACT

Plant Flavonoids are considered as naturally occurring bioactive compounds. In the present study, chitosan and *Aspergillus niger* elicitors were used as chemical and fungal elicitors, respectively. The cell suspension culture of *Andrographis paniculata* (Burm. f.) Nees was established in 50 ml MS media supplemented with 2,4-D: BAP (1.0:0.5 mg/l) and treated with chitosan and *Aspergillus niger* elicitors to enhance the accumulation of total flavonoids under in vitro condition. Quantitative analysis of total flavonoids accumulation was done by aluminum chloride colorimetric method. The results revealed that chitosan treatment with 20 mg for 24 hrs. duration explored highest elicitation of flavonoid i.e. 3.51 mg/g (2.72 fold) compared to control and rest of the treatments. Biological elicitor, *Aspergillus niger* with 2 ml, 4 days treatment was most promising with induction of 1.39 fold enhancement in flavonoid content (3.37 mg/g) over the control (2.42 mg/g).

KEYWORDS: Chitosan, *Aspergillus niger,* elicitor, flavonoid.

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INTRODUCTION

Andrographis paniculata (Burm.f.) Nees. belongs to family- Acanthaceae. It is commonly known as Kalmegh or King of Bitters. *A. paniculata* for its wide range of bioactive properties is used in Ayurveda, Unani, Siddha and Homeopathy systems of medicines. The bioactive constituents of A. paniculata are diterpene lactones [1]. The other constituents include andrograpanin, deoxyoxoandrographolide [2, 3, 4, 5, 6]; glycosides viz., neoandrographolide and andrographoside [7] and flavonols which include Oroxylin, wogonin, andrographidine A, B, C, D, E and F [8, 9] . *A. paniculata* is reported to possess antipyretic [10], antiviral [11], anti-malarial [12], potential cancer therapeutic agent [13], cardioprotective [14], hepatoprotective [15], hypoglycemic, anti-oxidant [16], immune enhancer [17], sedative [18], thrombolytic [19] and anti HIV [11] properties. Plants show a wide range of morphological and physiological responses to physical and chemical factors known as elicitors. These responses are considered as defense reactions by the plants to ensure their survival, persistence and competitiveness [20]. Plant cell cultures are considered as a promising system for the induction of enhanced production of secondary metabolites with suitable chemical and biological elicitors. Various biotic and abiotic elicitors have been reported to enhance the synthesis of secondary metabolite in plant system [21]. e.g. *Abrus precatorius* Linn.[22], *Ammi majus* L. [23], *Ocimum basilicum* [24] and *Medicago truncatula* [25]. The saponin content in *Vigna radiata* cell suspension was increased when subjected for 0.1% Triton X- 100 for 5 days [26]. Botrytis mycelium homogenate has been reported to enhance sanguinarine in *P. somniferum* cell culture [27]. In the present study *Aspergillus niger* and Chitosan were used as fungal and chemical elicitors, respectively. Chitosan is a structural component of the cell wall of several plant fungal pathogens. The effect of chitosan has been investigated in plant systems like *Nicotiana tabacum* and *Eschscholzia californica* for secondary metabolite production [28]. *Aspergillus niger* is one of the most common contaminant fungus of food. *Aspergillus niger* elicitors have been reported to enhance the production of secondary metabolites in cultured cells of plant system [29]. Some workers have reported enhancement in flavonoid content in different plant systems. Six weeks old suspension culture of *Pluchea lanceolata* (Rasna) showed high accumulation of flavonoid (0.23 mg/g dry wt.) [30]. Similarly, in *Pueraria tuberose* callus culture, the isoflavonoid content is reported to be enhanced up to 0.093%, with increased BAP and sucrose concentrations [31]. Considering the medicinal importance of flavonoids and potentiality of *Aspergillus niger* and Chitosan to enhance secondary metabolite accumulation, the present study was taken up with the objective to evaluate the effect of chemical and fungal elicitors on the enhanced accumulation of flavonoids in the cell suspensions of *Andrographis paniculata.*

MATERIALS AND METHODS

(i) Cell suspension culture and experimental set up

The profuse callus obtained in MS media supplemented with 2, 4-D: BAP (1.0:0.5 mg/l). The callus was cut into small pieces and about 5 g of callus was transferred to 50 ml liquid MS media containing same media composition except agar (Fig.1. a-b). The cell suspension was incubated in gyratory shaker with 110 r.p.m at 25$\pm$1^{o}C under dark condition for 30 days. The cell viability of suspension culture was determined by Guava ViaCount assay using Guava Easy CD4 System [32]. The cell viability of 30 days old suspension culture was noted 86% by Guava ViaCount Assay.

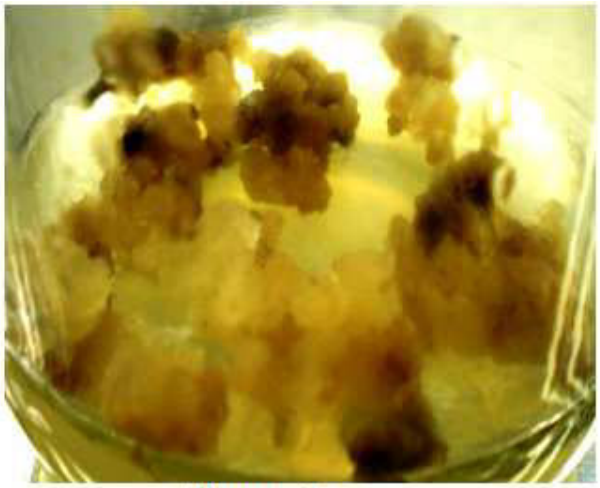


Figure.1. a.
A. paniculata matured callus culture

Figure.1. b.
A. paniculata suspension grown in 50 ml MS medium

(ii) Elicitor preparation

Chitosan elicitor was prepared as dissolving it in 5% (v/v) HCl and pH was adjusted to 5.0 with 1 N NaOH and the final volume was adjusted to 10 mg ml^{-1} [33]. 5 mg, 10 mg and 20 mg of chitosan per 50 ml of *Andrographis paniculata* suspension culture. Each elicitor concentration was subjected to 24 hrs, 48 hrs, 96 hrs treatment duration. *Aspergillus niger* cultures grown in PDB were autoclaved and harvested after three weeks. The fungal mycelia mat was removed and washed with distilled water and dried completely in oven at 40°C. The dried mycelia mat was powdered using mortar and pestle and used as dry cell powder (DCP). One g of DCP was boiled in 100 ml acidified distilled water (pH 2) for 45 minutes. After boiling, the culture was filtered and the final volume was adjusted to 100 ml with distilled water. The pH of the filtrate was adjusted to 5 with 1 N NaOH. This fungal elicitor solution was autoclaved and 1.0 ml, 1.5 ml and 2.0 ml of this fungal elicitor were added in 50 ml of *Andrographis paniculata* suspension culture separately. Each of this elicitor concentration was subjected for 4 days, 7days and 10 days of treatment duration. The polysaccharide content in each *Aspergillus niger* elicitor dose was determined by the

phenol sulfuric acid method using glucose as the standard [34]. The elicitor treatments were given at late exponential phase on 25 days. The cultures were incubated at 25 ± 2°C in gyratory shaker (110 r.p.m.) under dark condition.

(iii) Analytical methods

The cell suspensions were filtered and washed several times with distilled water after elicitors treatment. The filtered cells were dried in oven at 50°C and each powdered sample (150 mg) was extracted in 2 ml of methanol by sonication using Sonics Vibra Cell (VCX 130). This extract was used for determination of total flavonoid content in cell samples by UV- Vis spectrophotometer. The total flavonoid content present in the samples was determined by Aluminum chloride colorimetric method [35]. The results are expressed as mean ± standard deviation of triplicate of experiments.

(iv) HPTLC fingerprinting of flavonoids

The identification of flavonoids was done by High performance Thin layer chromatography The samples were applied on 20X10 cm TLC plates (TLC aluminium sheets silicagel 6600 F 254, E. MERCK KGaA) as 8 mm wide bands using CAMAG Linomat 5 with N_2 flow, 10 mm from the bottom. The TLC plates were developed using CAMAG twin trough glass tank. The mobile phase n-butanol: acetic acid: water (8:2:10) was poured in CAMAG twin trough glass tank and it was kept for 15 min. for saturation. After saturation each plate was placed in CAMAG twin trough glass tank for developing

Figure 2 (a)

HPTLC chromatogram for the detection of flavonoids in Chitosan treated cell suspension culture of Andrographis paniculata.

Image at 366 nm

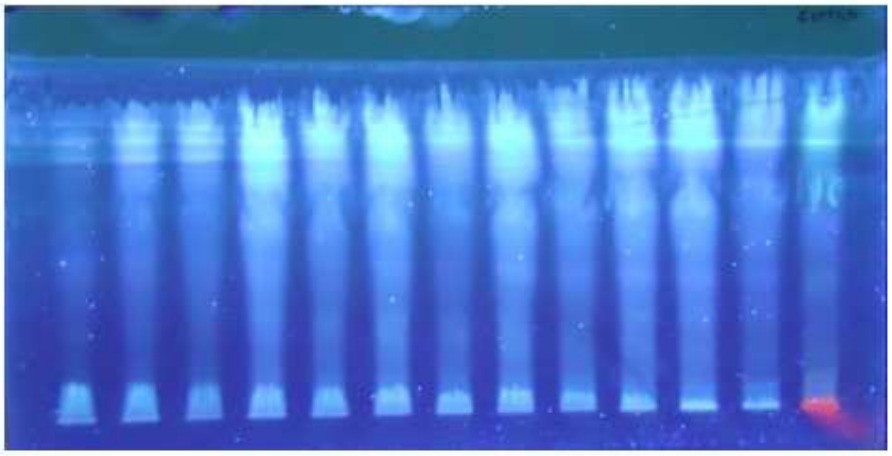
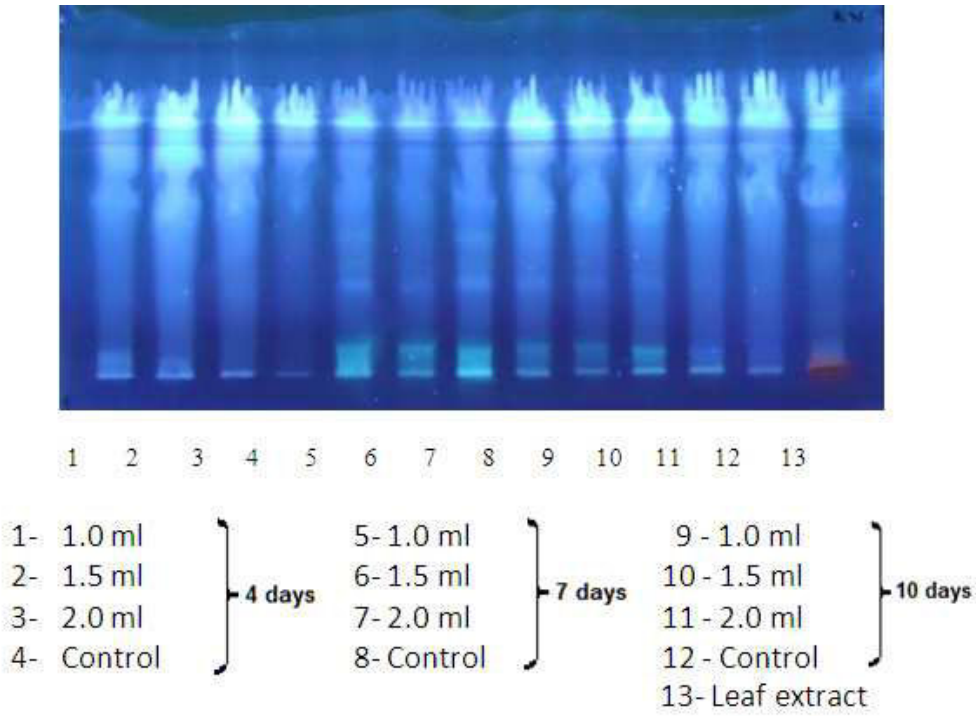


Figure 2(b)
HPTLC chromatogram for the detection of flavonoids in Aspergillus niger elicitor treated cell suspension culture of Andrographis paniculata.
Image at 366 nm



chromatogram. The plates were run up to 8 cm. The HPTLC runs were made in laboratory condition of 25± 2°C and 50% relative humidity. After development, the plates were dried and the spots were observed under UV light at 366 nm for flavonoid detection. The plates were then derivatized with 1% ethanolic $AlCl_3$ and examined under UV-light 254 nm and 365 nm after heating for 10 min on hot plate. The flavonoids were detected as violet spot under UV light at 366 nm (Fig.2. a-b).

RESULTS AND DISCUSSION

Secondary metabolites are produced by the plants when they are under stress conditions like environmental alteration and pathogen attack. Moreover, their production also depends on the developmental. Since ancient time plants have been used as a source of medicine for curing the diseases [36]. Elicitor treatment has been reported to affect the secondary metabolite biosynthesis in plant cell cultures e.g. *Plumbago rosea* [37], *Centella asiatica* [38], *Withania somnifera* [39], *Datura metel* [40] and *Gymnema sylvestre* [41].

1. Effect of Chitosan on accumulation of total flavonoid content

Chitosan has been reported as a potent chemical elicitor for enhancing the secondary metabolites under in vitro condition. In 24 hrs. of treatment duration, the flavonoid content was linearly correlated with the concentrations of chitosan. The cell suspension culture treated with 5 mg of chitosan showed increment in flavonoid production which was 1.92 mg/g dry wt. Similarly on 10 mg and 20 mg of chitosan resulted in 3.22 mg/g dry wt. and 3.51 mg/g dry wt. of flavonoid respectively. All the concentrations of chitosan in 24 hrs. of treatment exposure significantly enhanced the flavonoids production. The highest flavonoid accumulation was found to be in 20 mg treated samples which was 2.72 fold higher (3.51 mg/g dry wt.) than that of control (1.29 mg/g dry wt.).

However, 48 hrs. chitosan treatment indicates gradual decrease in flavonoid accumulation. In lowest concentration (5 mg chitosan), the flavonoid content was measured 3.68 mg/g dry wt. In 10 mg chitosan treated samples, the flavonoid content was 3.22 mg/g dry wt. whereas in 20 mg of chitosan treated samples, the flavonoid content decreased at 1.28 mg/g dry wt. which was even slightly lower to the control samples (1.52 mg/g). The 5 mg chitosan was found to be optimal concentration which resulted in 2.42 fold increase (3.68 mg/g dry wt.) and 10 mg caused 2.11 fold increase (3.22 mg/g dry wt.) in total flavonoid content over control. However, higher concentration of chitosan, 20 mg did not show enhancement. The flavonoid production was increased in all studied concentrations of Chitosan when subjected for 96 hrs. treatment duration. The 5 mg, 10 mg and 20 mg of chitosan resulted in 3.28 mg/g dry wt., 3.23 mg/g dry wt. and 3.14 mg/g dry wt. of flavonoid, respectively. The 5 mg of chitosan was observed to be most favorable for maximum flavonoid production compared to respective control in 96 hrs treatment duration. The flavonoid content at this stage reached 1.34 fold (3.28 mg/g dry wt.) as compared to control (2.43 mg/g dry wt.). Similar results were found when *A. paniculata* Nees. cell suspension was treated with 0.05 mM of salicylic acid for 24 hrs which showed 1.39 fold increase in flavonoid content [36]. Chitosan has been reported to elicit production of paclitaxel (139 µg/g dry wt. vs. 89 µg/g dry wt.) in cell suspension culture of T. x media [42]. Chitosan (200 mg/l) yielded 166.4 mg/l menthol in *Mentha piperata* cell suspension when treated for 12 days which 40 fold increase as compared to control [43]. Chitosan 10 mg/l of concentration has been reported to enhance phenylethanoid glycosides (PeGs) production to 364.6 mg/l which was 3.4 fold higher than that of control in cell suspension culture of *Cistanche deserticola* [44]. It has been reported that the chitosan treatment (150 mg/l) yields 6.71 fold higher plumbagin accumulation compare to control in *Plumbago rosea* L. cell suspension culture [37]. In *Armoracia lapathifolia* hairy root cultures, the total peroxidase activity (POD) was increased about 170% after 48 hrs. of treatment with 100 mg/l of Chitosan [45]. The results obtained indicated that the longer treatment duration is favoring enhancement in flavonoid content (Table-1). The positive variations in the content due to treatment period may be attributed to the differential mode of interaction of elicitor doses that are only turned suitably positive when interact slowly with the elicitor like chitosan.

Table 1
Effect of elicitors on flavonoid synthesis.

Name of elicitors	Treatment durations	Concentrations of elicitor	Total flavonoid content in mg/g	Fold increase
Chitosan	24 hrs	Control	1.29 ± 0.16	
		5 mg	1.92 ± 0.05	1.48
		10 mg	3.22 ± 0.50	2.49
		20 mg	3.51 ± 0.13	2.72
	48 hrs	Control	1.52 ± 0.17	
		5 mg	3.68 ± 0.51	2.42
		10 mg	3.22 ± 0.46	2.11
		20 mg	1.28 ± 0.35	-
	96 hrs	Control	2.43 ± 0.17	
		5 mg	3.28 ± 0.83	1.34
		10 mg	3.23 ± 0.65	1.32
		20 mg	3.14 ± 0.18	1.29
Aspergillus niger	4 days	Control	2.42 ± 0.10	
		1 ml	2.81 ± 0.15	1.16
		1.5 ml	2.73 ± 0.24	1.12
		2 ml	3.37 ± 0.11	1.39
	7 days	Control	2.61 ± 0.20	
		1 ml	2.77 ± 0.18	1.06
		1.5 ml	2.78 ± 0.06	1.06
		2 ml	3.54 ± 0.54	1.35
	10 days	Control	2.79 ± 0.22	
		1 ml	2.37 ± 0.10	-
		1.5 ml	2.23 ± 0.13	-
		2 ml	1.59 ± 0.14	-

2. Effect of Aspergillus niger elicitor on accumulation of total flavonoid content

The impact of fungal elicitor, *A. niger* was also evaluated in studied plant system. Treatment with 1 ml and 1.5 ml of extract for 4 days treatment showed the enhancement in flavonoid accumulation which was 2.81 mg/g dry wt. and 2.73 mg/g dry wt. respectively. The higher concentration, 2 ml of *A. niger* extract showed a significant enhancement in flavonoid content (3.73 mg/g dry wt.) over control (2.42 mg/g dry wt.). In 4 days of treatment duration, although all the concentration responded positively, the maximum flavonoid accumulation was noted in the samples treated with 2 ml of *A. niger* extract with 1.39 fold increase (3.73 mg/g dry wt.) in flavonoid content as compared to control (2.42 mg/g dry wt.) On increasing the treatment duration to 7 days, the total flavonoid content was increased with the increasing concentrations. In 1 ml and 1.5 ml of treated samples, the variations in flavonoid accumulation were not significantly different. The flavonoid content was 2.77 mg/g dry wt. and 2.78 mg/g dry wt. for 1 ml and 1.5 ml of *A. niger* extract treated samples respectively. However, the maximum flavonoid content was reported in 2 ml of *A. niger* extract treated samples which yielded 1.35 fold increase (3.54 mg/g dry wt.) compared to control (2.61 mg/g dry wt.) It is to be noted here that further increase in the treatment duration did not influence the flavonoid production in any of the studied concentrations of *A. niger* extract. In longer treatment duration the treatment with *A. niger* elicitor showed decline in flavonoid accumulation as compared to control (2.79 mg/g dry wt.). In higher concentrations, the flavonoid content decreased significantly. It was 2.37 mg/g dry wt., 2.23 mg/g dry wt. and 1.59 mg/g dry wt. for 1 ml, 1.5 ml and 2 ml of *A. niger* extract respectively. The results obtained

indicate that *A. niger* elicitor with lower doses enhances the product. However, the flavonoid content was declined during extended incubation period (Table-1). The results showed that the amount of flavonoid produced varied with duration of incubation in the presence of elicitors. This reveals that the duration of incubation in the presence of elicitor was rather important with respect to flavonoid production. *A. niger*. elicitor has induced 2-3 fold enhancement in plumbagin production in cell suspension culture of *Plumbago rosea* L. [37]. In *Arnebia euchroma* culture, 2 ml of *A. niger* elicitor enhanced shikonin concentration (61.62 mg/l, 1.54 fold higher than the control) after 8 day of treatment [46]. *Penicillium expansum* elicitor (1.2%) induced 1.59 fold increase in total flavonoid content (2.38 mg/g) in *A. paniculata* cell suspension culture[47]. Analysis done clearly exhibit the impact of chitosan and *A. niger* elicitors on total flavonoid content. The study shows that the elicitation depends on type of elicitor, elicitor dose and treatment duration and type of compound to be elicited. The positive impact is restricted to the extent of acceptance of suitable elicitor dose by experimental system. In the present analysis, the maximum enhancement in total flavonoid content (2.72 fold) was observed in Chitosan treatment (20 mg, 24 hrs.) among all studied elicitors. On the basis of the findings of present work, it can be concluded that chitosan (20 mg, 24 hrs. is most favorable for elicitation of flavonoids in studied plant system, *Andrographis paniculata.* The result obtained on elicitation revealed that the studied elicitor trigger the synthesis of secondary metabolites in *Andrographis paniculata* at *in vitro* level.

CONCLUSION

With various medicinal properties of flavonoids, research going on to enhance the production of flavonoids by means of biotechnological tools. The concept of elicitation of secondary metabolite using elicitors is therefore, gained high importance as it targets straight to the bioactive compounds. Our investigation has shown that the application of Chitosan and *Aspergillus niger* elicitors are potent enough to enhanced the synthesis of flavonoids in cell suspension of *A. paniculata* and the elicitation is dependent phenomenon on the type of elicitor, treatment duration and its doses.

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