



Research Article

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ESTABLISHMENT OF CALLUS CULTURES OF *ANDROGRAPHIS PANICULATA* FOR THE ASSESSMENT OF ANDROGRAPHOLIDE CONTENT

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ABSTRACT

Andrographolide is the main bioactive component of the medicinal plant, *Andrographis paniculata*. Callus cultures of *Andrographis paniculata* are known to produce only paniculides (sesquiterpene lactones) and not andrographolides (diterpene lactone) that are produced by the intact plant. In this study, Callus cultures of *Andrographis paniculata* were established from leaf explant. Different growth regulators greatly influenced the growth of callus cultures. Callus was established on Murashige and Skoog medium at various concentrations of 2, 4-D & TDZ alone (1.0-2.5 mg l⁻¹) and in combinations (2, 4-D + NAA, 2, 4-D + Kn, BAP+ NAA). Best callus induction was obtained at MS + 2, 4-D + NAA (1.0 +1.0 mg l⁻¹). HPLC of best callus samples were undergone to detect their andrographolide content. The highest amount of andrographolide content (7.64 mg g⁻¹ FW) was observed in callus grown on MS supplemented with 1.0 mg l⁻¹ 2, 4-D + 0.5 mg l⁻¹ NAA followed by 1.0 mg l⁻¹ 2, 4-D + 1.5 mg l⁻¹ Kn and 2.5 mg l⁻¹ BAP + 0.5 mg l⁻¹ NAA. The present study thus indicates that andrographolide production can be induced in the callus of *A. paniculata* by treatment with plant growth hormones, although the content predominates in leaves and stem.

Keywords: callus cultures, Andrographolide, TDZ, and HPLC.

INTRODUCTION

Plants are plugh source of high-priced secondary metabolites which are steadily synthesized during their lifecycle. *Andrographis paniculata* Nees (family Acanthaceae) is one of the most revered medicinal plants distributed in India and south Asian countries. It synthesizes diverse active compounds mainly diterpene lactones, flavonoids and polyphenols¹. However, the prime constituent is andrographolide which is commercially important for having a broad range of pharmacological activities such as anticancer², anti-inflammatory, analgesic and antioxidant³, antimicrobial⁴, hepatoprotective⁵, cardio-protective⁶, antihypertensive⁷, antiviral⁸, antifungal⁹, immune enhancement and anti-HIV activities¹⁰.

Secondary metabolites of medicinal importance are drawing much attention these days owing to world's inclination towards herbal medicines. But the quality and quantity of active substances collected from wild and field grown plants are often fluctuating and heterogeneous depending on environmental conditions. Moreover, their continuous extraction through plants & herbs has left their natural resources deteriorated. In vitro culture of plants can overcome these problems; since the environmental conditions that affect plant metabolism can be strictly regulated. Hence, the practice of tissue culture techniques to produce these compounds under controlled conditions has become a fascinating field of research¹¹. It is promising and can even yield higher amounts in an adapted culture medium.

Various methods have been suggested for quantitative estimation of andrographolide in *Andrographis paniculata*. The gravimetric method described in the Indian pharmacopoeia was found to give high values¹². This is due to some yellow coloring substance other than andrographolide which is also soluble in ethyl acetate. The spectro photometric method proposed by Maiti¹³ suffers from the disadvantage that the red color formed

with the addition of alcoholic potassium hydroxide to the solution of andrographolide is unstable and fades away quickly. Gaid¹⁴ proposed a spectrophotometric method by extracting pure andrographolide from kalmegh measuring its absorbance at 226 nm but the extraction process was very tedious. Bhat¹⁵ suggested a chemical method involving a lactone titration but the method that has been reported is not found to be suitable for detecting minute quantities. High performance liquid chromatographic methods were reported for estimation of andrographolide in *Andrographis paniculata*. Thin layer chromatographic methods were also described for estimation of andrographolide in *Andrographis paniculata* extracts and also performed by using capillary electrophoresis chromatography. The methods described above have several limitations like preparation of samples for estimation of andrographolide. In the present study, accurate, simple, specific and reproducible HPLC method has been used as HPLC is a versatile, robust, and widely used technique for the quantification of natural products.

As production of secondary metabolites in plants by callus cultures and their quantification with the help of HPLC prove to be a significant tool for developing a sustained source of these medicinal components without disturbing the natural flora, efforts were made to establish the callus cultures of *Andrographis* and estimate the andrographolide content by HPLC.

MATERIAL AND METHODS

Plant material and explants preparation: Young leaves of *A. paniculata* Nees were obtained from 1-2 months old plant growing in the Greenhouse, Centre for Plant Biotechnology, Hisar, India. Explants were thoroughly washed under running tap water and with detergent (Laboline,

0.1%, v/v, Hi Media, Mumbai) for 5 min, followed by 0.1% mercuric chloride (Hi Media, Mumbai) wash for 2 min and then four washes of sterile distilled water in the sterile condition.

Establishment of callus culture: For callus induction, the leaves were aseptically cut into small pieces and inoculated on full strength MS medium with different concentrations of 2,4-D & TDZ (0.5 - 2.5 mg l⁻¹) and combinations of 2,4 D (2.0 mg l⁻¹) with BAP, Kn and NAA (0.5, 1.0 mg l⁻¹). The pH of medium was adjusted to 5.8 ± 0.02 and was autoclaved at 121 °C for 15 min. Explants were inoculated in culture bottles and placed in culture room having high light intensity (40 –50 l mol m⁻² s⁻¹ PFD), provided by cool and white fluorescent tubes (Philips, India), at 26 ± 2 °C and 55– 60 % RH.

All tissue culture chemicals used in the studies were of analytical grade and obtained from Hi media Laboratories, Mumbai, India. There were 15 replicates for each treatment and the experiment was repeated thrice. The cultures were observed periodically and the morphological changes were recorded at weekly intervals.

Extraction and analysis of andrographolide in callus samples: Extraction and estimation of andrographolide in the callus samples was carried out following the method of Praveen et. al.¹⁵. The air dried powdered plant samples (500 mg) were extracted by mixture of dichloromethane and methanol (1:1) by cold maceration for 10 min. The extract was filtered and solvent was removed under vacuum. The extract was washed 2 to 3 times with toluene and then dissolved in methanol. 20 µL aliquots of sample were injected in to HPLC. The HPLC analysis was continued for 15 min, since the retention time of the andrographolide was 2.871 ± 0.004 min. Shimadzu Model-LC2010 CHT, Serial No. C21254808828 HPLC instrument was used for the chromatographic separation using C 18 column (250 nm x 4.6 nm). The mobile phase was methanol: water (65: 35) and flow rate was 1 ml min⁻¹. The detector wavelength was 230 nm. Andrographolide standard 99.8 % (product code A009, Lot No. T11B001) was procured from Natural Remedies, Bangalore). The standard Andrographolide took 2.871 minutes as retention time. The total andrographolide content was an average of three replicates.

Calibration curve of standard

10 mg ml⁻¹ Andrographolide standard solution was prepared in methanol (stock solution). Standard working solutions were prepared by diluting standard stock solution with methanol in the concentration range 10-50 µg ml⁻¹.

Statistical analysis

All numerical data were statistically analyzed by ANOVA at 5% significant level. Data presented in tables correspond to the mean values of three replicates.

RESULTS

Establishment of callus cultures: Varied amount of callus with contrasting morphology was formed on MS medium supplemented with various combinations and concentrations of growth hormones. Callus was induced within two weeks after inoculation of explants. All tested hormonal combinations produced callus and showed significant variation (table-1) at 5% level of probability. Though callus induction occurred at all concentrations (0.5- 1.5) of 2, 4-D alone, copious amount was generated by 1.5 mg l⁻¹ 2, 4-D. Higher concentrations of 2, 4- D did not favor the callogenesis leading to remarkable decrease in callusing efficiency. Callus formed at 2, 4 – D alone was green and compact with 85.07 % callusing efficiency.

The most generous amount of callus formation was obtained in four weeks when combination of 1.0 mg l⁻¹ 2, 4-D with 1.0 mg l⁻¹ NAA was tried. The maximum callusing efficiency of 92.02% was obtained at 2, 4 –D combined with NAA. It was observed that the response of leaf explants towards callogenesis was highest in MS + 2, 4-D + NAA. The callus formed was of green color and compact in texture.

Various combinations of 2, 4-D with Kn were also tried to observe the pattern of callus formation. Callus formed on this combination was yellowish in color and friable in texture. MS with 1.5 mg l⁻¹ 2, 4-D +1.0 mg l⁻¹ Kn showed 88.84% of callogenesis. Callusing response was average (70.43%) at the medium containing 2.5 mg l⁻¹ TDZ which produced green and compact callus. The hormonal combinations of BAP (1.0-2.5 mg l⁻¹) + NAA (0.5-2.0 mg l⁻¹) showed poor callusing response ranging from 24.4 – 39.2%.

Table 1: The Effect of Different Concentrations of growth hormones on andrographolide production in callus culture. The mean comparisons were determined by the ANOVA at p < 0.05 level. CD – 1.162, SED – 0.568

MS + Growth Hormone (mg l ⁻¹)					Mean Response % ± S.E.
2,4-D	NAA	KIN	BAP	TDZ	
0.0	0.0	0.0	0.0	0.0	00.00
0.5					74.09 ± 0.1
1.0					79.13 ± 0.4
1.5					85.07 ± 0.3
1.0	0.5				76.18 ± 0.1
1.0	1.0				92.02 ± 0.3
1.5	1.0				56.22 ± 0.3
1.0		0.5			46.29 ± 0.3
1.0		1.0			73.23 ± 0.4
1.5		1.0			88.84 ± 0.3
	0.5		2.5		24.04 ± 0.3
	1.0		2.5		21.77 ± 0.3
	1.0		3.0		39.13 ± 0.3
				1.0	42.73 ± 0.6
				2.0	52.02 ± 0.2
				2.5	70.43 ± 0.4
	1.0			2.0	68.21 ± 0.3

Table 2: The Effect of Different Concentrations of growth hormones on andrographolide production in callus culture. The mean comparisons were determined by ANOVA at p < 0.05 level. C.D. - 0.555, SEd – 0.256

MS + Hormone supplement (mg l ⁻¹)	Andrographolide content (mg g ⁻¹ FW)
2,4-D (1.0)	4.37 ± 0.06
2,4-D (1.5)	5.48 ± 0.3
2, 4-D + NAA (1.0 + 1.0)	8.34 ± 0.3
2, 4-D + NAA (1.0 + 0.5)	6.14 ± 0.08
2, 4-D + Kn (1.0 + 0.5)	5.41 ± 0.2
2, 4-D + Kn (1.5 + 1.0)	5.33 ± 0.7
TDZ (2.5)	3.98 ± 0.06
TDZ + NAA (2.0 + 1.0)	4.44 ± 0.2



Figure 1: Performance of *A. paniculata* explants in different hormonal concentration

- A. Leaf discs inoculated on MS containing different plant growth hormones;
- B. Callus from MS basal medium containing 1.00 mg/L 2, 4-D;
- C. Callus induced from MS basal medium containing 1.00 mg/L 2, 4-D and 1.00 mg/L NAA;
- D. Callus from basal medium containing 1.00 mg/L 2, 4-D and 0.50 mg/L Kinetin;
- E. Callus induced from MS basal medium containing 1.00 mg/L BAP and 1.00mg/L NAA.

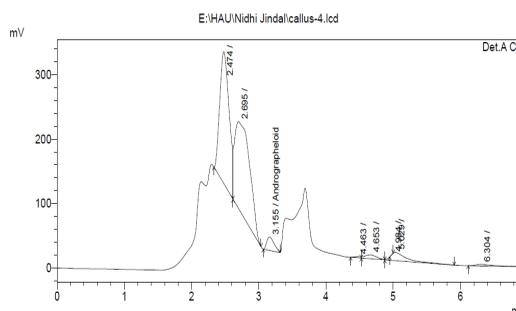
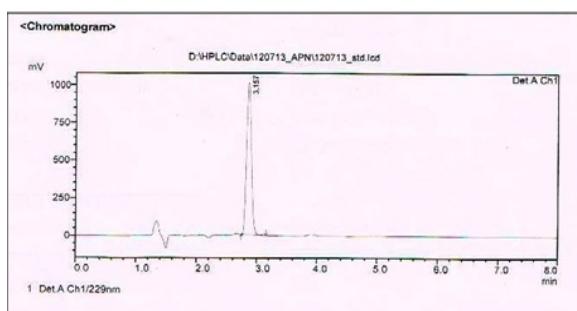


Figure 2: HPLC analysis of Andrographolide content in callus of *A.paniculata*

- a. Standard Andrographolide.
- b. Highest amount of Andrographolide isolated from callus grown on MS + 1.0 mg l⁻¹ 2, 4-D + 1.0 mg l⁻¹ NAA.

Impact of growth hormones on accumulation of andrographolide in callus cultures: Best combinations of growth hormones were tried to achieve in vitro raised callus samples for the estimation of their Andrographolide content. All the callus samples collected within 4-6 weeks undergone for HPLC estimation and were found to contain Andrographolide. Standard Andrographolide gave the peak area of 113903 at 3.157 min. retention time (Figure 2a). Callus grown on MS + 1.0 mg l⁻¹ 2, 4-D + 1.0 mg l⁻¹ NAA was observed to contain highest amount of Andrographolide (8.34 mg g⁻¹ FW) (Figure 2b), followed by (6.14 mg g⁻¹ FW) Andrographolide present in callus grown on 1.0 mg l⁻¹ 2, 4-D + 0.5 mg l⁻¹ NAA. Further, MS with 1.5 mg l⁻¹ 2, 4-D alone and 2,4- D with kn gave comparable amount of Andrographolide (5.41 mg g⁻¹ FW) though the nature of callus on both types were quite different. However, the content was lowest (4.44 mg g⁻¹ FW) in the callus grown on TDZ even in the combination with NAA. The analysis of variance (ANOVA) showed the significant variation between the hormonal treatments at 5% probability level.

DISCUSSION

Utilization of tissue culture techniques for the production of secondary metabolites offers a great alternative to shift the undue pressure from natural flora. Since *Andrographis paniculata* has a wide profile of medicinally important chemical compounds, in vitro production of these metabolites has become focus of attraction since the technique is season independent and time savvy¹⁶⁻¹⁷.

The present study was aimed at the establishment of callus cultures for the production of the primary active compound of this plant i.e. Andrographolide, and to observe the impact of plant growth hormones on andrographolide content evaluating the best source for its in vitro production. MS medium supplemented with 2, 4-D, NAA, BAP and kinetin were utilized by previous researchers for in vitro cultures of several species¹⁸. Hence, several combinations and concentrations of PGRs were used to initiate and establish callus cultures. Growth regulators 2, 4-D, NAA, BAP and kinetin are frequently used to induce callus tissues in many plant species¹⁹.

In case of callus cultures, maximum callusing efficiency was obtained at combination of 2, 4-D + NAA (1.0 + 1.0 mg l⁻¹) on full MS medium followed by 2, 4-D + Kn (1.5 + 1.0 mg l⁻¹). A

distinct feature of this investigation is the induction of highly morphogenic callus by TDZ (1.0-2.5 mg l⁻¹) which showed optimum amount of callus formation with 70.4% response. There have been reports earlier about callogenesis with the help of TDZ²⁰. Poor callus formation was observed on combinations of BAP + NAA with full strength MS medium. As the commercial importance of secondary metabolites has evolved in recent years, it has resulted in a great interest in secondary metabolism, particularly in the possibility of altering the production of bioactive plant metabolites by means of tissue culture technology. Extraction and quantification of secondary metabolites with the help of shoot and callus cultures have been previously discussed^{21,22}.

Therefore, attempts were made to collect best callus samples from in vitro cultures to quantify Andrographolide content by HPLC. Callus grown on MS + 1.0 mg l⁻¹ 2, 4-D + 1.0 mg l⁻¹ NAA produced 8.34 mg g⁻¹ FW Andrographolide which was highest among all the combinations of growth hormones tried. 2, 4-D alone and in combination with Kn yielded similar amount of Andrographolide (5.41 mg g⁻¹ FW) in spite of their morphologically different callus. Though callusing efficiency was considerable (70.43 %) in case of MS media supplemented with TDZ but it yielded very low amount of Andrographolide (4.44 mg g⁻¹ FW).

CONCLUSION

Production of secondary metabolites through tissue cultures has heightened the importance of tissue culture techniques. Callus has the potential to behave like an intact plant. A bit of tissue from a medicinally important plant can be grown in vitro and secondary metabolites or drugs can be directly extracted from the callus tissues without sacrificing the whole plant. The present study demonstrates the role of plant growth hormones for the establishment of callus cultures of *Andrographis paniculata*. Apart from 2, 4-D, NAA, Kn and BAP, the role of TDZ is noticeable for callogenesis. Further, estimation with the help of HPLC confirms the presence of Andrographolide in callus cultures of *Andrographis*. The study illustrates the influence of plant growth hormones on the production of Andrographolide in in vitro cultures. Hence, these results are profitable in producing enhanced amount of Andrographolide to meet the demands of medicinal world.

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