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Standardization of callus induction protocol and effect of hormone concentration on synthesis of Andrographolide from *Andrographis paniculata*

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Abstract
Andrographis paniculata is commonly known as Kalmegh or Kirayat. Belongs to Acanthaceae family and annual herbaceous plant found in Asian countries like India and Sri Lanka. It has high market demand because of its various medicinal uses like treating dysentery, cholera, diabetes, influenza, etc. Andrographolide is major secondary metabolite which is present in *Andrographis paniculata.* It shows medicinal properties like Anti-Cancer, Anti-inflammatory, Anti-Venom, etc. Andrographolide is majorly found in leaves of *Andrographis paniculata*. In this present study we have standardize protocol for callus induction by using growth hormones like BAP, NAA and 2,4-D with different combinations. Excellent quality of callus were used for HPLC analysis. Results revealed that as concentration of hormone enhances content of Andrographolide also enhances and its vice versa. Major content was found 2.36% and lowest was 0.92%. Percentage of content was calculated with the help of formula.

Keywords: *Andrographis paniculata*, Andrographolide, Growth regulators, HPLC, etc.

Introduction
Ayurvedic medicines have got enormous applications in Cosmetic, Agriculture, Pharma and Food industry. The herbal preparations have contributed more specifically in these medicines. *Andrographis paniculata* belongs to Acanthaceae family. This plant is commonly known as Kalmegh or Kirayat. It is also known as "King of Bitters" due to its extreme bitter taste [7]. *Andrographis paniculata* has very useful medicinal values like treating dysentery, cholera, diabetes, consumption, influenza, bronchitis, swelling, itches and piles, this species got tremendous demand. Mostly roots and leaves of plants are used for medicinal purpose.
Andrographis paniculata contains Secondary metabolites like carvacrol, eugenol, myristic acid, hentriacontane, tritriacontane, oroxylon A and diterpenoids like andrograpanin, andropanoside, Andrographolide and neoAndrographolide, terpenoids, flavanoids, saponins [1]. From which Andrographolide is the major content present in leaves of *Andrographis paniculata* (2-4% of whole plant) [6]. It is insoluble in water but soluble in acetone, chloroform, methanol, etc. It shows Anti-cancer activity against cancer cells at G_0/G_1 phase. Andrographolide shows various medicinal properties like Anti-cancer, Anti-venom, Anti-inflammatory, Anti-HIV, Anti-oxidant [3]. In this present study Andrographolide is isolated from callus because vegetative propagation of this plant is very slow to meet the demands of pharmaceutical industries. By using different growth regulators in callus helps to detect Andrographolide content.
Detection of Andrographolide is possible by using HPLC, HPTLC, etc. techniques. Present study is designed because of medicinal importance of Andrographolide [2].

Materials and Methods
Sterilization of explants for callus induction: Leaves of plants were used as explants for callus induction. Leaves were cutted into disc and washed 2 times under tap water then 2-3 times with sterile distilled water. Explants were washed with Tween-20 for 10 minutes then again washed with distilled water. They were soaked in antifungal agent Bavistin (0.1%) for 5-10 minutes and again washed 2-3 times with distilled water. Sterilization was done with HgCl2 (0.1%) for 2 minutes and then with 70% Ethanol for 30 seconds. Washed with sterile distilled water 3-4 times until residues completely wiped out [4].

Media preparation for callus induction: After sterilization of explants were inoculated aseptically on nutrient media containing growth hormones with combinations like 2,4-D (1.0 mg/L and 1.5 mg/L), 2,4-D+BAP (0.5+1.0 mg/L, 1.0+1.5 mg/L, 1.5+2.0 mg/L, 2.0+2.5 mg/L), 2,4-D+NAA (1.0+1.5 mg/L, 1.5+2.0 mg/L, 2.0+2.5 mg/L, 2.5+3.0 mg/L).

Sample preparation for HPLC: 30-35 days old cultured callus was aseptically weighed upto 0.7 gm. Callus was finely crushed in 80% Ethanol in microcentrifuge tube and then sample was used for HPLC analysis [4].

HPLC analysis and Quantification of Andrographolide: HPLC was carried out in RAP analysis, Nashik. Mobile phase consisted Methanol: Water (80:20) for detection of Andrographolide. Standard for Andrographolide was used with 98% purity. Running of sample was done at 223 nm. 20µl sample was injected and flow rate was 0.8ml/min. Sample was running for 6.64 minutes. Percentage of content was calculated by using formula.

Results and Discussion

In this study protocol for callus induction was standardize by using different combinations of growth regulators. Results revealed upto 80% of callus induction. Induction started after 15 days (Fig. 1). Parameters for quality of callus was consider on the basis of physical appearance, quantity and contamination of callus. Poor quality of callus was seen in hormones containing 2,4-D (1.0 mg/L and 1.5 mg/L), 2,4-D+BAP (1.5+2.0 mg/L), 2,4-D+NAA (1.0+1.5 mg/L) (Fig. 2). Good quality of callus found in 2,4-D+BAP (2.0+2.5 mg/L) and 2, 4-D+NAA (2.5+3.0 mg/L) (Fig. 3). Excellent quality of callus found in 2,4-D+BAP (0.5+1.0 mg/L and 1.0+1.5 mg/L) and 2,4-D+NAA (1.5+2.0 mg/L and 2.0+2.5 mg/L) (Fig. 4). Excellent quality of callus was used for HPLC analysis.

Callus was induced by culturing leaf discs on Murashige and Skoog (MS) medium with different concentration of 2, 4-D and combination of 2, 4-D + Kinetin, 2, 4-D+NAA and BAP+NAA. Best callus induction were obtained at lower concentration of 2, 4-D (0.5 and 1.0 mg/l), combination of 2, 4-D+NAA (1.0+1.0mg/l) 2, 4-D + Kin (1.0+0.5mg/l) and BAP + NAA (1.0+1.0mg/l) [9].

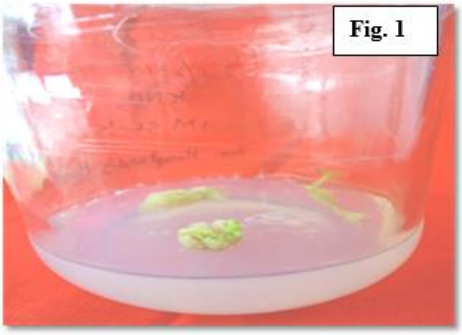
Most of callus amount in 2, 4-D combined with NAA and BAP. Maximum callusing frequency was 92.02% in 2, 4-D combined with NAA. It was observed that leaf response towards callogenesis was with 2, 4-D combined with NAA. The callus was found green in colour and compact in texture [5].

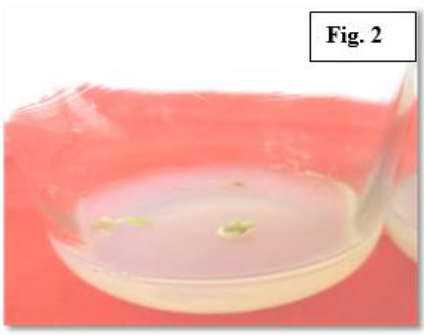
In the present study standard of Andrographolide took 3.8 minutes as retention time and peak area was 6576054. Similarly remaining 4 samples took 3.8 minutes as retention time with different peak areas because of different hormone concentration in callus sample. Content was calculated with the help of formula [8].

$$\%\ \text{Content of Andrographolide} = \frac{\text{Standard area}}{\text{Sample area}} \times \frac{\text{Sample weight}}{\text{Sample dilution}} \times \frac{\text{Standard weight}}{\text{Standard dilution}} \times \%\text{Purity of the standard}$$

Content of Andrographolide observed lowest i.e. 0.92% at low concentration of growth hormone 2,4-D+BAP (0.5+1.0 mg/L). Highest content i.e. 2.36% observed at 2, 4-D+NAA (2.0+2.5 mg/L).

Whole plant material at different stages, from 30 days of plantation up to maturity of the crop was studied. Chromatogram scanned at 250 nm. The results observed under UV light showed a good separation for all compounds. The Rf for Andrographolide was found to be 0.31. The average Andrographolide content varied from 0.42% to 2.02% in the sample studied [8].





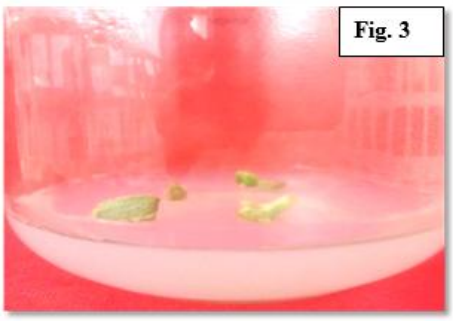
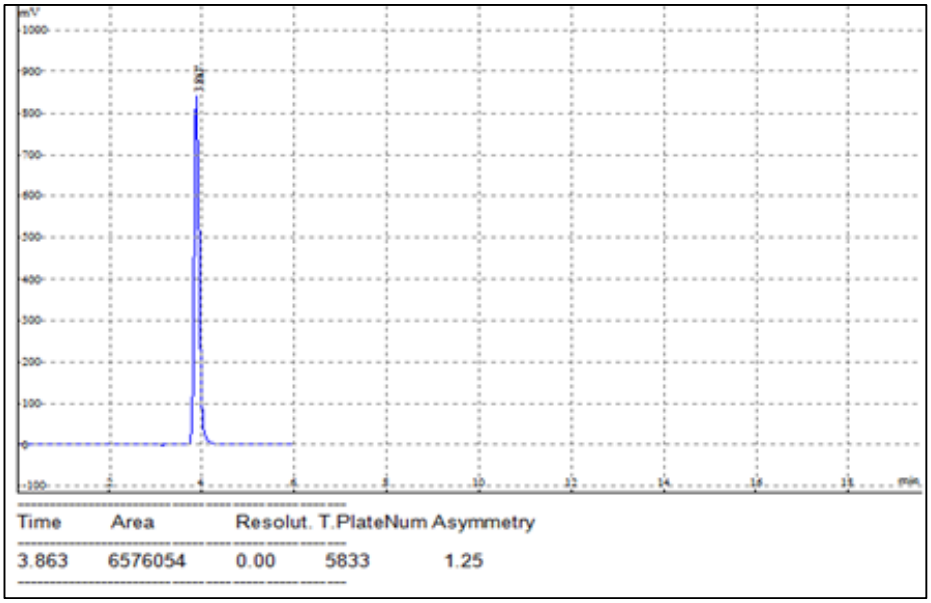
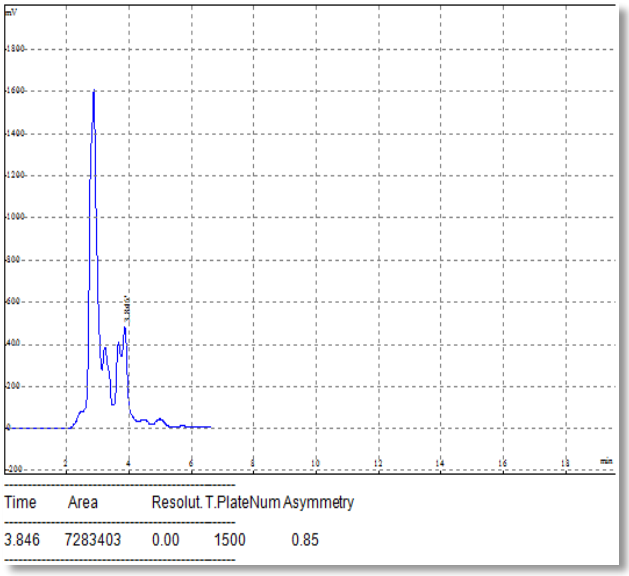


Fig 1: Callus initiation started after 15 days in full MS media.
Fig 2: Poor quality of callus seen in 2, 4-D (1.0 mg/L) after 30 days.
Fig 3: Good quality of callus observed in 2, 4-D+BAP (2.0+2.5 mg/L) after 30 days.
Fig 4: Excellent quality of callus observed in 2, 4-D+NAA (1.5+2.0 mg/L) after 30 days.



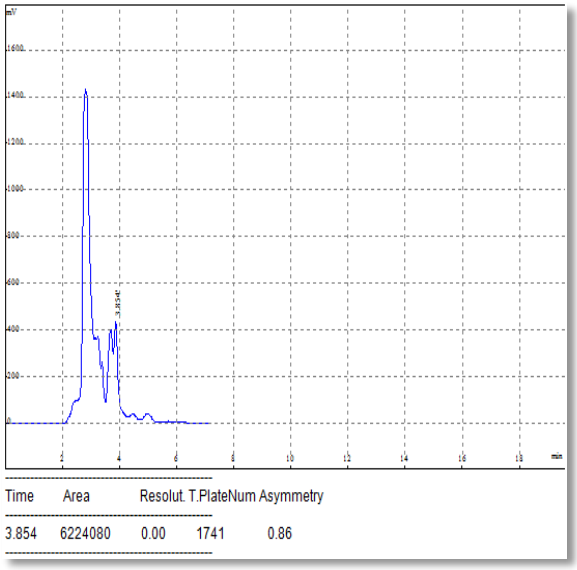
Time	Area	Resolut.	T.PlateNum	Asymmetry
3.863	6576054	0.00	5833	1.25

Fig 5: Standard of Andrographolide



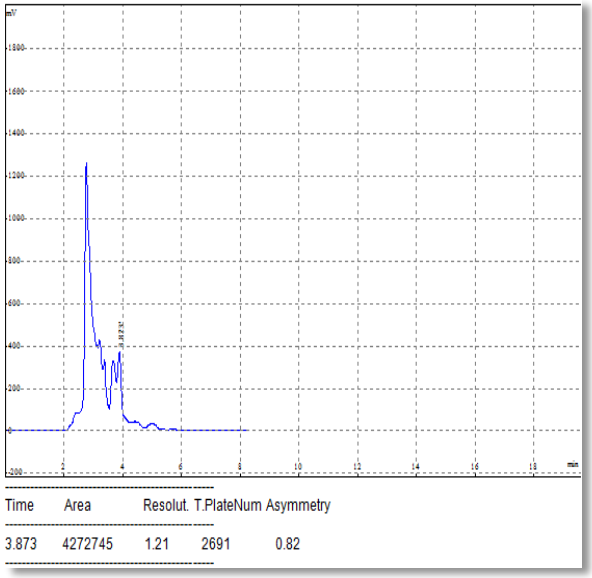
Time	Area	Resolut.	T.PlateNum	Asymmetry
3.846	7283403	0.00	1500	0.85

Fig 6: Sample 1:- 2,4-D+BAP (0.5+1.0 mg/L)



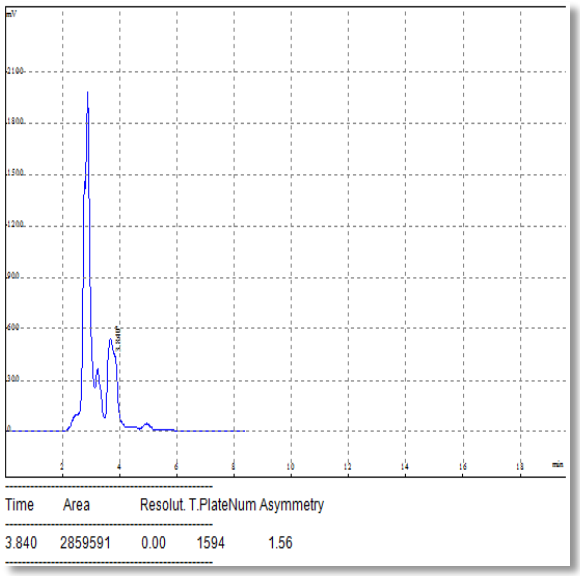
Time	Area	Resolut.	T.PlateNum	Asymmetry
3.854	6224080	0.00	1741	0.86

Fig 7: Sample 2:- 2,4-D+BAP (1.0+1.5 mg/L)



Time	Area	Resolut.	T.PlateNum	Asymmetry
3.873	4272745	1.21	2691	0.82

Fig 8: Sample 3:- 2,4-D+NAA (1.5+2.0 mg/L)



Time	Area	Resolut.	T.PlateNum	Asymmetry
3.840	2859591	0.00	1594	1.56

Fig 9: Sample 4:- 2,4-D+NAA (2.0+2.5 mg/L)

Table 1: Growth hormones concentration for callus induction

S. No.	Hormone	Concentrations (mg/L)	Remarks
1	2,4-D	1.0	+
		1.5	+
2	2,4-D+BAP	0.5+1.0	+++
		1.0+1.5	+++
		1.5+2.0	+
		2.0+2.5	++
3	2,4-D+NAA	1.0+1.5	+
		1.5+2.0	+++
		2.0+2.5	+++
		2.5+3.0	++

Quality of Callus, +: Poor Callus, ++: Good Callus, +++: Excellent Callus

Table 2: Content of Andrographolide

S. No.	Sample Name	Growth Hormone	Hormone Concentration (mg/L)	Content of Andrographolide (%)
1.	Sample 1	2,4-D+BAP	0.5+1.0	0.92
2.	Sample 2		1.0+1.5	1.08
3.	Sample 3	2,4-D+NAA	1.5+2.0	1.58
4.	Sample 4		2.0+2.5	2.36

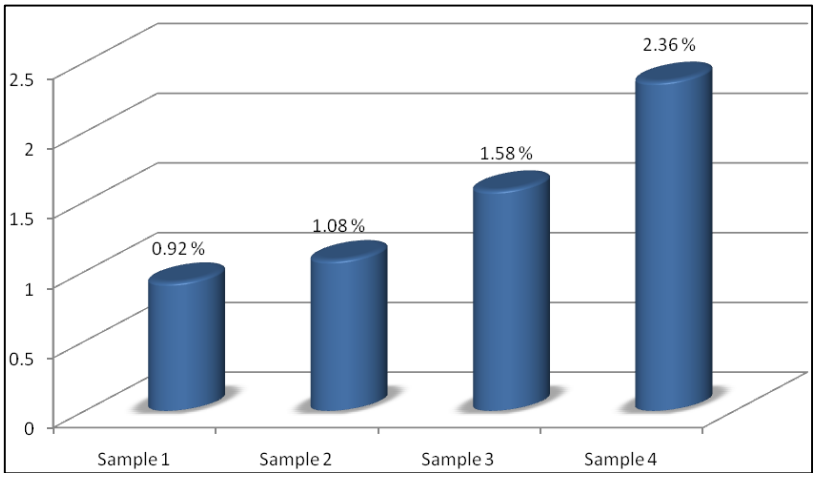


Fig 10: Andrographolide content

Conclusion

From present study it is proved that *in-vitro* propagation of *Andrographis paniculata* gives more quantity of Andrographolide. Best results of callus initiation are possible in MS media containing 2, 4-D with auxin and cytokinins with different combinations. Concentration of growth hormones enhances then content of Andrographolide also enhances. Specially auxins helps to induce content of Andrographolide.

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