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Enhanced production of Andrographolide from cell cultures of *Andrographis paniculata* by the elicitaion with Arachidonic acid

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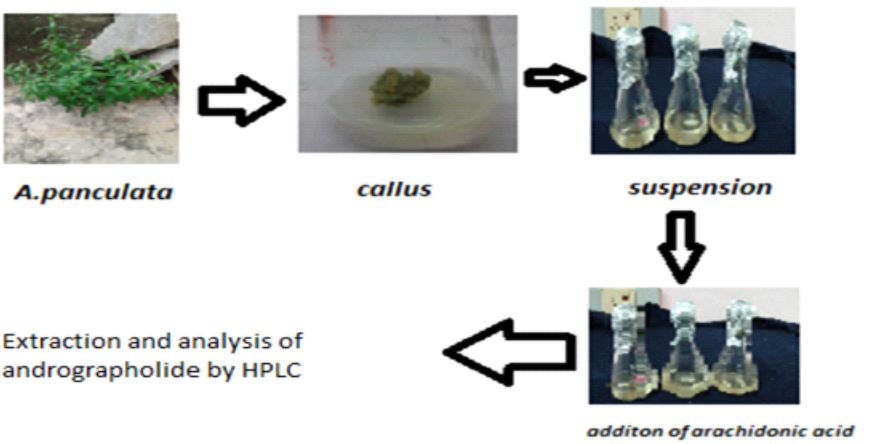
Graphical Abstract



Abstract

Background:Andrographis paniculata (Burm. F.) Wall ex. Nees, belonging to the family *Acanthaceae* produces a group of diterpene lactones, one of which is the pharmaceutically important andrographolide.

Hypothesis: It is known to possess various important biological properties like hepatoptroective, anticancer, anti-HIV, anti inflammatory, etc.

Method: The production of andrographolide in the cell suspension cultures of *Andrographis paniculata* by elicitation was investigated.

Results: Arachidonic acid enhanced the andrographolide content in the suspension cultures of *Andrographis paniculata.* The maximum andrographolide production was found to 109.6 0.62% w/w over the control cell cultures 26.40.79% w/w with the addition of arachidonic acid (100 M) on day-7.

Conclusion: This study could pave the way for establishing a continuous and higher production of andrographolide, which has an enormous pharmacological importance.

Key words: *Andrographis paniculata*, Arachidonic acid**,** suspension cultures, and HPLC

Introduction

Plants produce secondary metabolites as part of the defence mechanism against microbes and higher organisms. These products are of immense use as potential drugs, nutraceuticals and food additives. Though many molecules have been synthetically designed, nature remains the source of highly sophisticated and biologically privileged compounds as they play a key role in increasing the survival fitness of living being (Koehn and Carter 2005). Numerous drugs and drug precursors in the current pharmacopoeia originate from plants. Limited yield of these bioactive compounds present a significant challenge for large-scale drug development. Plant cell and tissue culture systems can act as an alternate platform for large-scale production of bioactive compounds under controlled conditions (Leonard et al 2009).

Andrographis paniculata (Burm. F.) Wall ex. Nees, also called Kalmegh or "King of Bitters" belongs to family Acanthaceae. It is traditionally used as a folklore cardiovascular remedy in India, China, and other Southeast Asian countries. It have been reported as having

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antibacterial,antifungal,antiviral,choleretic,hypoglycemic,hypocholesterolemic,adaptogenic, anti-inflammatory, emollient, astringent, diuretic, carminative, anthelmintic, antipyretic, gastric and liver tonic. Due to its "blood purifying" activity it is recommended for use in cases of leprosy, gonorrhea, scabies, boils, skin eruptions, and chronic and seasonal fever (Sheeja 2006, Shen 2002, Wiart 2005, Handa and Sharma 1990, Chander et al 1995, Trivedi, and Rawal, 2000, Visen et al 2007, Kumar et al 2004, Rajagopal et al 2003). *A. paniculata* contains diterpenes, lactones and flavonoids. Flavonoids mainly exist in the root, but have also been isolated from the leaves. Aerial parts contain alkanes, Ketones, and aldehydes and the bitter principles in the leaves were due to presence of the lactone andrographolide(Calabrese et al 2000).

Cell cultures have a higher rate of metabolism because their initiation leads to faster proliferation of cells and a condensed biosynthetic cycle. Further, plant cell cultures are not restricted by the environmental, ecological and climatic conditions, and cells can thus proliferate at higher growth rates than the whole plant in cultivation. The secondary metabolite content in cell cultures can be further enhanced with elicitor treatment. Several studies have indicated that plant cultures are stimulated by elicitors ensuing rapid accumulation of secondary metabolites. The phyto chemical yield enhancement was observed in the various elicitor-treated medicinal plant cell cultures, e.g. *Abrus precatorius*Linn.,*Silybum marianum* (L.) Gaertn, *Commiphora wightii,Ammi majus L., Ocimum basilicum* and *Medicago truncatula* (Suryakala et al 2012). Hence, the present study was taken up with the objective to evaluate the effect of abiotic elicitor i.e., *Arachidonic acid* on the enhanced accumulation of andrographolide in the cell suspensions of Andrographis *paniculata.*

Materials and methods

Collection of Plant Material

Healthy live plantlets of *Andrographis paniculata* (Burm. F.) Wall ex. Nees, were collected from Padmakshi temple, Hanmkonda, Warangal and authenticated by a (Prof.V.S.Raju, Department of Botany Kakatiya University, Warangal, Telangana. Further planted and maintained in garden of college for further use.

Chemicals and compounds

Murashige and Skoog's (1962) (MS) media and Arachidonic acidwere purchased from HiMedia Pvt. Ltd., India. The chemicals acetonitrile and methanol high performance liquid chromatography (HPLC) grade used for the analysis were purchased from Merck, India. Standard andrographolidewas purchased from Yucca Enterprises, Mumbai, India.

Initiation of Suspension Cultures

Suspension culture was initiated by leaves with phytohormones 2,4D (2mg/l)+ KN(1mg/l) grows on MS Media. Suspension cultures initiated from leaf callus showed good growth after regular subcultures. Suspension culture extract were analyzed for secondary metabolites.

Addition of Arachidonic acid to suspension cultures

The cell culture of *Andrographis paniculata* after 2 passages were cultured onto MS media (25ml of media in each flask) as inoculums into 50 ml flasks under laminar flow. The elicitors of different concentrations of Arachidonic acid (100µM and 500µM), were added to the *A.paniculata* cell cultures, separately on 3rd, 6th, 9th, 12th, 15th and 18th day. For each concentration 3 flasks were used while running suitable control. Then the flasks were incubated in refrigerated shaker incubator at 25±2˚C and 120 rpm for 18 days.

Extraction

The cell cultures, after 18 days of incubation at 25±2˚C and 120rpm with different concentrations Arachidonic acid of and control cultures were withdrawn and contents (cells and medium) of each flask were extracted with HPLC grade Methanol (Suryakala et al 2012).

Determination of Andrographolide

TLC Analysis

Analysis of cell suspension cultures as well as Arachidonic acid treated cultures of *Andrographis paniculata* was carried out by Co-TLC by using pre-coated silica gel G plates of 250 µm layer, UV 254 by co-chromatography with authentic sample Andrographolide. The TLC analysis carried out (Chloroform: Methanol:: 7:3) by trial and error method and detected the spot under UV light (Mohan 2013).

Preparation of standard solutions

Stock solution of Andrographolide (1.0 mg/ml) was prepared in methanol and diluted to a series of appropriate concentrations (50, 100, 150, 200, 250, 300, 1000 µg/ml ofAndrographolide with the same solvent. An aliquot (20 µl) of the diluted solutions was injected into the HPLC for the construction of six point calibration curves.

Preparation of sample solutions

. The dried extract of cell suspension cultures as well as elicitor (Arachidonic acid) treated cultures of *Andrographis paniculata* was (0.5g) was dissolved in methanol. The methanolic extracts were filtered and dried under reduced pressure at 50 ± 5°C. The dried filtrate (10 mg) was re-dissolved in mobile phase (1 ml), filtered through 0.45 µm membrane and an aliquot (20 µl) of the filtrate was injected into HPLC for analysis.

High Performance Liquid Chromatogram analysis

Analysis of cell suspension cultures as well as Arachidonic acid treated cultures of *Andrographis paniculata* was carried

out by HPLC. Analysis was performed on a Shimadzu Ultra Fast Liquid Chromatogram (UFLC) system equipped with binary gradient pump, LC-20AD UV/Visible detector. The chromatographic resolution was achieved on a Luna C-18 (2) column (250 × 4.6 mm, 5 µm particle size) from Phenomenex. The mobile phase used for the analysis was water: acetonitrile: methanol (55:30:15) in isocratic elution with flow rate 1 ml/min at 223 nm detection wavelength (Sharma, and Jha, 2012).

Statistical Analysis

All the experiments were performed in triplicate, and the data were expressed as means± standard deviations. One-way ANOVA analysis followed by the Duncan's test was used to determine significant ($p \leq 0.05$) differences.

Results

TLC and HPLC analysis

The production of andrographolide from callus cultures was analysed by TLC and HPLC analysis. With TLC analysis the R_f values of andrographolide had the same value as the authentic andrographolide; when derivatized with UV light both spots were orange and with a Rf value of 0.45.

Table 1: Growth Kinetics of cell suspension of*A.paniculata* and Production Kinetics of andrographolide

S. No.	Age(days)	Growth Index	Andrographolide(%w/w)
1	3		10.56±1.30
2	6	17.2±0.85	14.7±0.72
3	9	20.46±1.40	14.93±1.55
4	12	30.46±0.58	27.6±1.31
5	15	38.63±1.35	35.4±1.15
6	18	28.36±1.00	24.56±1.04

The HPLC analysis gave the same retention time (7.9 min.) as the standard for aandrographolide peak (Figure-1). The HPLC was used for quantification of andrographolide in callus cultures as well as elicitors treated cultures. The cell cultures of *A.paniculata* shown a maximum growth index of 38.63±1.35 and androgpholide content as 35.4±1.15 % w/w(Table-1 and figure 2-3).

The addition of Arachidonic acid to the suspension cultures of *A.paniculata* enhanced the yield of Andrographolide by 5 times more than to the control cultures (Table-2 and figure- 4). The effect was not significant when Arachidonic acid was added on "3" day it induced the production of Andrographolide cell cultures of *A. paniculata* to a considerable level.

Table 2: Effect of Arachidonic acid (AA) on bioproduction of Andrographolide in suspension cultures of *Andrographis paniculata.*

S. No.	Treatment	Andrographolide (%w/w)
1	AA 100 µM (day-3)	39.4± 0.62
2	AA 100 µM (day-7)	109.6 ±0.62
3	AA 100 µM (day-10)	73.23±1.34
4	AA 500 µM (day-3)	41.56 ± 0.96
5	AA 500 µM (day-7)	71.96 ±1.15
6	AA 500 µM (day-10)	35.5 ±1.57
7	Control (alcohol)	

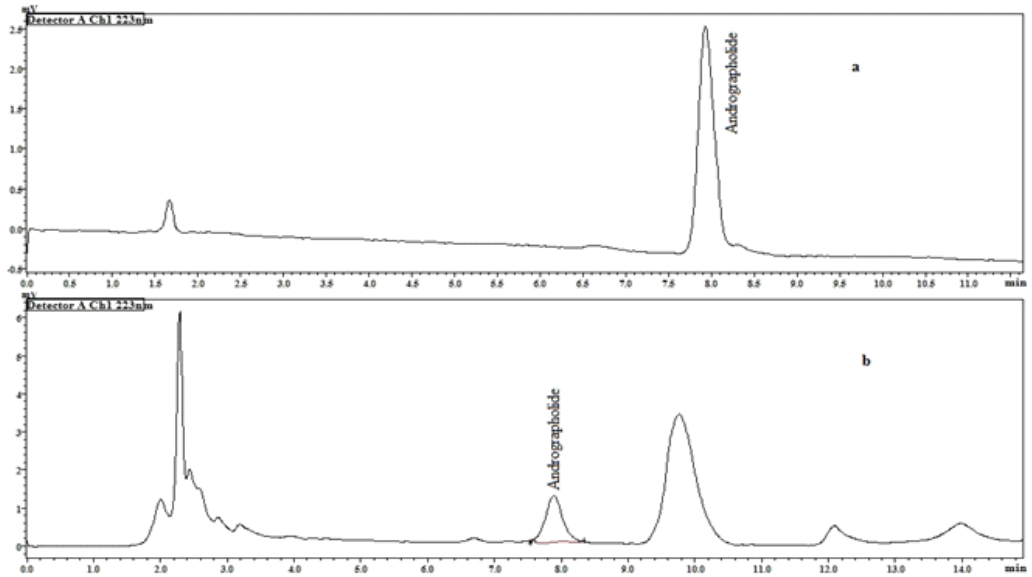

Figure 1.a) Standard chromatogram of Andrographolide b) Chromatogram showing Andrographolide in extracts of callus culture.

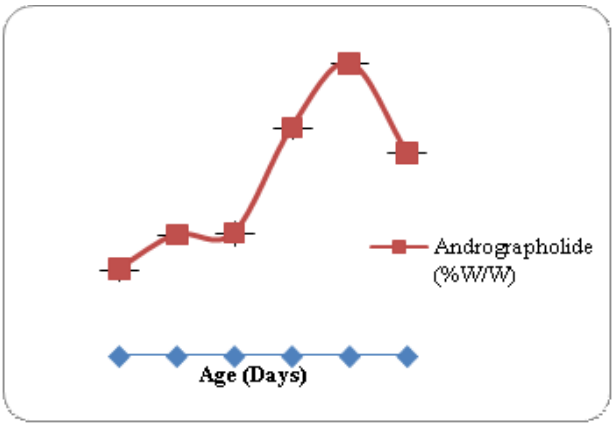

Figure 2: Growth Kinetics of cell suspension of *A.paniculata*

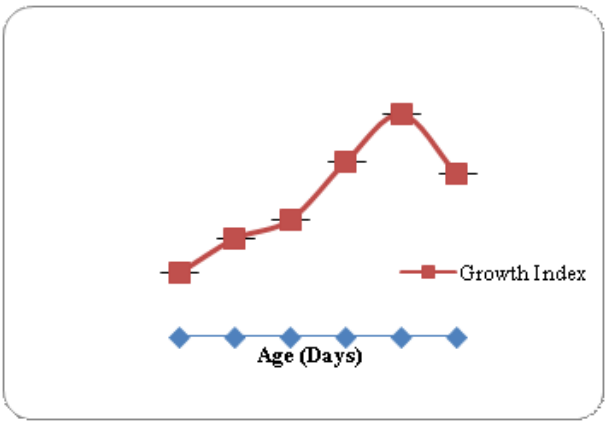

Figure 3: Production Kinetics of andrographolide in cell suspension of *A.paniculata*

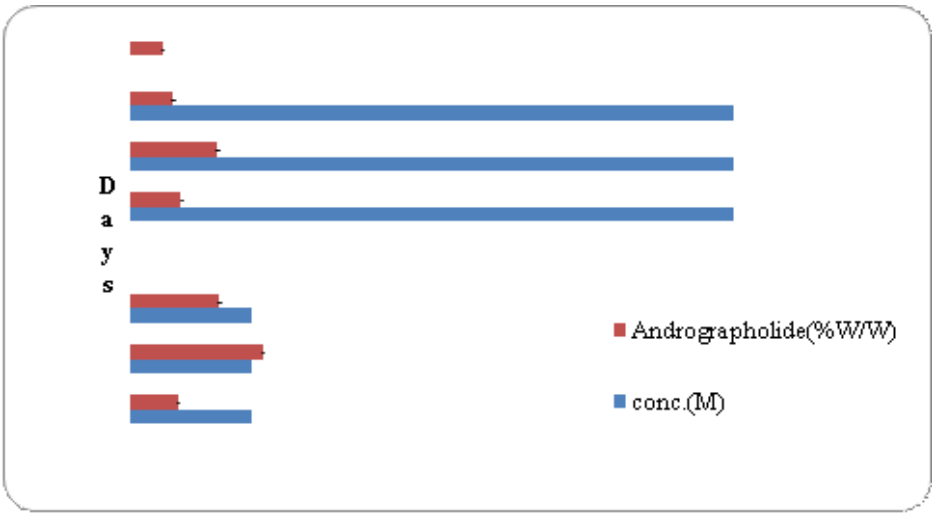

Figure 4: Effect of Arachidonic acid (AA) on bioproduction of Andrographolide in suspension cultures of *Andrographis paniculata*

Discussion

The suspension cultures are known to accumulate considerable amounts of secondary metabolites, which can be further enhanced by elicitation. Elicitor treatment was shown to affect the secondary metabolite biosynthesis in suspensions, root and whole plant cultures with commercial potential. Previous studies of elicitor treatment leading to increase to the secondary metabolite content of the cell suspension cultures of *Plumbago rosea, Centella asiatica, Withania somnifera, Datura metel* and *Gymnema sylvestre(*Suryakala et al 2012, Mohan 2013, Sharma, and Jha, 2012, Ajungla et al 2009, Bhuvaneswari et al 2012).

The present study was carried out to find the influence of Arachidonic acid at different concentrations, on production of Andrographolide in suspension culture of *A. paniculata.* The addition of Arachidonic acid(100 M) on day-3 to the cell cultures of *A. paniculata* enhanced the yield of Andrographolide over the control cultures.There is 5 fold improvement in the production of Andrographolide (109.6 0.62)over the control cell cultures (26.40.79) with the addition of Arachidonic acid (100 M) on day-7.

Maximum andrographolide production was observed on addition of Arachidonic acid on day 7. Addition on day 3 and day 10 did not have significant effect. Further, higher concentration of Arachidonic acid on day 7 also did not have beneficial effect as compared to lower concentration on the same day. However there are several reports on enhanced production of secondary metabolites from tissue cultures of medicinal plants with the addition of Arachidonic acid. The addition of Arachidonic acid significantly improved the production of baccatin-3 and deacetyl baccatin-3 in cell cultures of *Taxus wallichiana* and forskolin in cell cultures of *coleus forskolii*(Meenu et al 2012).

Conclusion

The production of Andrographolide in cell cultures of *Andrographis paniculata* by using Arachidonic acid increase the production five times more than control. The present result also showed significant amount of androgrpgholide (109.6 0.62% mg/gm) compared to intact plant (0.81-1.86% mg/gm). This study could pave the way for establishing a continuous and higher production of andrographolide, which has an enormous pharmacological importance.

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