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A comparative study on the *in vitro* effect of treatment time of ems on secondary metabolite production in *Andrographis paniculata* (BURM.F.) Nees

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Abstract
Effect of 0.01% of EMS on secondary metabolite production in *Andrographis paniculata* was conducted in this study. Calli were derived from leaf explants inoculated on Murashige and Skoog's medium supplemented with 2 mg/l NAA, 1 mg/l kinetin and 50 mg/l phenylalanine. In 0.01% EMS treatment for 5 h resulted in a maximum amount of fresh weight (1.55±0.06 g) and dry weight (0.12± 0.01g) of callus and also the callus response (73.33). Treatment of 0.01% EMS also resulted in 6 - fold increase in andrographolide production when compared to control. The study also showed that concentration and treatment time of EMS influenced the secondary metabolite production in the treated samples.

Keywords: *Andrographis paniculata,* andrographolide, callus induction frequency, EMS, phenylalanine

1. Introduction
Andrographis paniculata (Burm. F.) Nees of the family Acanthaceae is one of the most popular medicinal plants widely distributed in India, China, Sri Lanka, Taiwan and other southeast Asian countries. It is commonly used for the treatment of common cold, diarrhea, fever, respiratory tract infections (Negi *et al.*, 2008; Sareer *et al.*, 2014; Wang *et al*., 2014) [1-3] and possess numerous therapeutic potentials including antimalarial (Mishra *et al*. 2011) [4], antioxidant (Lin *et al.* 2009) [5], antibacterial (Burm *et al.* 2010) [6] and anticancer activity (Subramanian *et al*. 2012) [7].
This herb has many vernacular names -- Kalmegh in Bengali, Kiriyath in Malayalam, Nilavembu in Telugu etc. and is commonly known as bhui-neem, because of its bitter taste as that of neem. This plant has pharmaceutically important compounds such as diterpenoids, flavonoids, and polyphenols (Chao & Lin 2010) [8].
Irradiation and chemical mutagenesis have long been used to develop mutant plants for breeding propose (Stadler 1928, Oehlkers 1943) [9, 10]. The use of chemical mutagens is a very popular way to induce mutation which includes ethyl methane sulphonate (EMS), colchicine, polyethylene glycol, sodium azide, 2,4- dichlorophenoxy acetic acid and acridine orange etc. EMS has become one of the best effective, reliable, powerful and frequently used chemical mutagens in plants (Brockman *et al.*, 1984) [11]. The present study deals with the effect of 0.01% EMS on callus initiation and then to isolate and quantify the andrographolide content present in *in vitro* callus culture of control and 0.01% EMS treated plants.

2. Materials and Methods
Healthy growing young branches with 4 to 5 nodes were collected from S.D College, Alappuzha, Kerala, India. Collected healthy shoots were brought to the laboratory by wrapping with a wet muslin cloth. A voucher specimen has been deposited in Kerala University Botanical Herbarium (KUBH 6031).
One week old seedlings were used for the study. The cotton swab method adopted was that of Biswas & Bhattacharya (1971) [12]. A cotton swab dipped in 0.01% EMS solution for 1h, 5h was applied to the apical vegetative bud and EMS solutions were frequently added to the cotton swab by a dropper. The third leaf was taken from healthy seedlings swabbed with 70% alcohol soaked cotton and then were washed in running tap water for 20 minutes followed by washing with 2 drops of labolene for 5 min. After washing with distilled water they were brought it to laminar air flow. The explants were treated with 70% ethyl alcohol for 30 seconds for surface sterilization and rinsed in sterile double distilled water. 0.1% concentration of mercuric chloride with different time durations were used and finally

standardized the optimum concentration for sterilization. 0.1 % mercuric chloride treatment for 6 min was found to be the optimum treatment time for surface sterilization. The various concentrations of auxins alone (0.5,1,2 mg/l NAA, 0.5, 1, 2 mg/l 2,4-D) as well as the combined effect of auxin and cytokinin (NAA (0.5,1,2 mg/l) and kinetin (1mg/l) were tried in this experiments. Proliferated calli from the control and treated samples were grown on the MS medium containing 2 mg/l NAA, 2 mg/l Kinetin and 50 mg/l Phenylalanine were used for the analysis of andrographolide after 70 days by HPLC method.

Procedure of HPLC

Chromatographic conditions

Mobilephase

1) Dissolve 0.14 gm of anhydrous potassium dihydrogen orthophosphate (KH2PO4) in 900 ml of HPLC grade water and add 0.5 ml of orthophosphoric acid. Make upto 1000 ml with water, filter through 0.45 membrane and Degas in a Sonicator for 3 minutes. (Solvent A) 2) Acetonitrile (Solvent B)

Standard preparation

20.0 mg andrographolide was weighed to a 100 ml volumetric flask. 50 ml of HPLC grade methanol was added. Sonicated for 5-10 minutes and warmed on a water bath at 60-70ºC for 5 minutes. Cooled to room temperature and volume was made up to 100 ml with methanol.

Sample preparation

1000 mg of given material were weighed in clean, dried 250 ml beaker, 50 ml of methanol was added into a 250 ml beaker and refluxed for 10 minutes, cool and sonicate for 6 minutes. Cool and transfer to 50 ml volumetric flask, repeat the above step for another 2 times and volume was made up to 50 ml with methanol.

3. Results and Discussions

Explants inoculated into the MS full strength medium supplemented with 2 mg/l 2,4-D showed the high amount of fresh weight (0.5900±0.011) of the callus. But the effect of NAA at the concentration of 0.5 mg/l produced low percentage response of callus (40) fresh weight (0.36) and dry weight (0.026±0.0033) of callus. The combinations of NAA and kinetin at 2 mg/l and 1 mg/l combinations noticed the maximum fresh weight (0.59±0.00 gm) and dry weight (0.036+0.0033) of the callus. Different concentrations of phenylalanine (25,50,75,100 mg/l) were used here. Full strength MS medium supplemented with 2mg/l NAA, 1mg/l kinetin and 50 mg/l phenylalanine showed good result in callus fresh weight (0.6103+0.02) and largest amount of dry weight (0.0533+0.0033) formed in this concentration. Explants treated with 0.01% EMS for 5 h produced calli in MS medium fortified with 2 mg/l NAA, 1 mg/l Kinetin and 50 mg/l phenylalanine. 0.01% EMS treatment produced a higher amount of fresh weight, dry weight and percentage of the response of the callus induction than that of control. Control calli produced high amount of fresh weight (0.61g) and dry weight (0.04433 g) in MS medium containing 2 mg/l NAA, 1 mg/l Kinetin and 50 mg/l phenylalanine. The maximum amount of fresh weight (1.55±0.06 g) and dry weight (0.12± 0.01g) of callus and callus response (73.33 %) were found in 0.01% EMS treatment for 5 hours. In this concentration callus initiation started within 11-13 d of inoculation. The sample treated with 0.01% EMS for 1 hour showed a decrease in fresh weight (1.13 ± 0.02), dry weight (0.85 ± 0.002) and callus response (66.6%) compared to 5 hours treatment time of 0.01% of EMS. (Fig 1-3).

Table 3.1: Effect of concentration of EMS on callus induction

Concentration s of EMS (%)	Time (Hours)	Fresh Weight (gm)	Dry Weight (gm)	% of Response
Control		0.6133±0.02142^{e}	0.04433±0.005239^{e}	56.667±3.333^{d}
0.01	1	1.1300±0.0282^{d}	0.8567±0.002963^{d}	66.6867±3.3333^{bcd}
	5	1.5567±0.06069^{bc}	0.1293±0.01081^{c}	73.333±3.333^{b}

Fig 1: Control

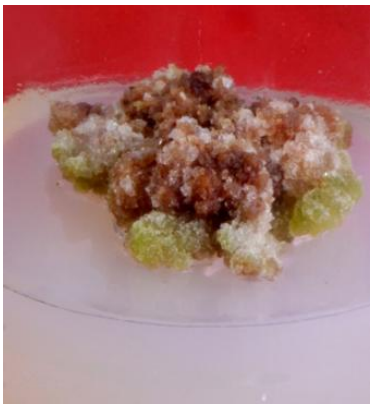
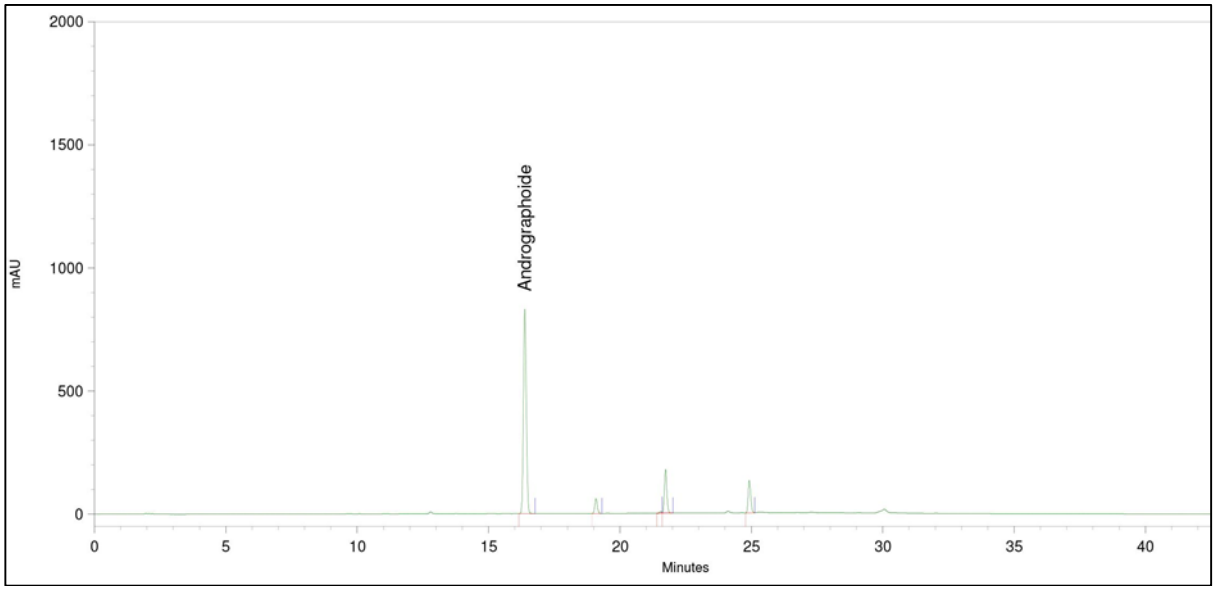


Fig 2: 0.01% EMS for 1 hour

Fig 3: 0.01% of EMS for 5 hour

The calli proliferated in a significant manner and the production of andrographolide was analyzed after 70 d. Control calli produced 0.1mg/g andrographolide. The andrographolide production was 0.6 mg/g in the calli originated from the explant treated with 0.01% EMS for 1 h. The same concentration for 5 h treatment of explant produced calli with the same amount of andrographolide 0.6 mg/g. Treatment of 0.01% EMS for 1 h and 5 h positively influenced the production of andrographolide. The culturing of the calli in this concentration produced notable amount of 0.6mg/g andrographolide as compared to the control calli (0.1mg/g). 0.01% EMS had a significant effect on andrographolide production in callus.

HPLC chromatograms of andrographolide a. standard; b. Control; c. 0.01% EMS for 1 hour; d: 0.01% EMS for 5 hours;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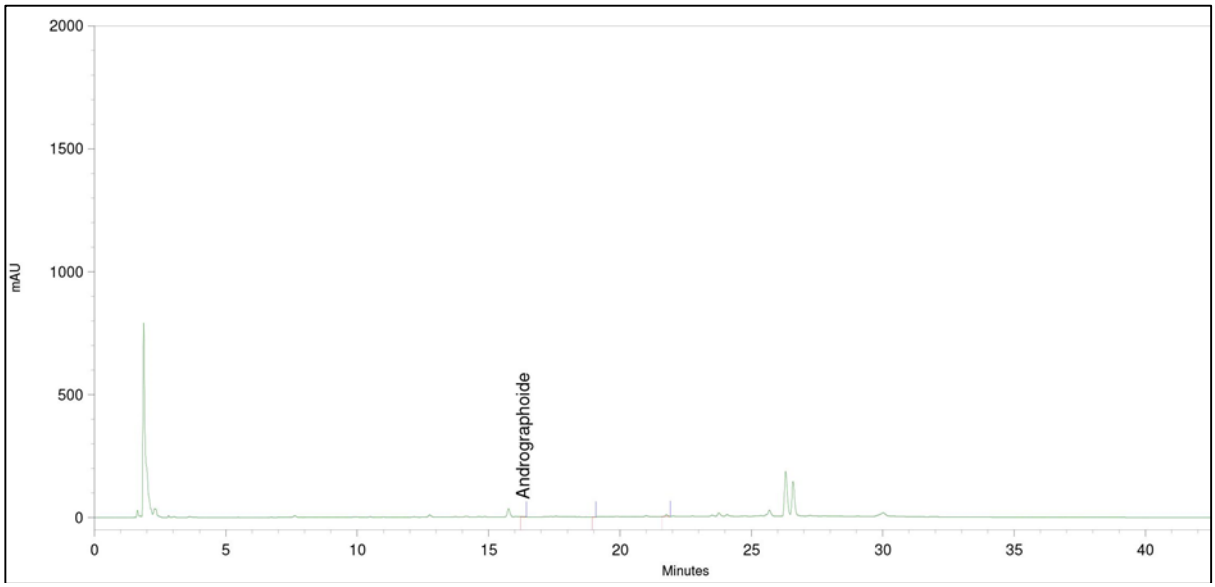
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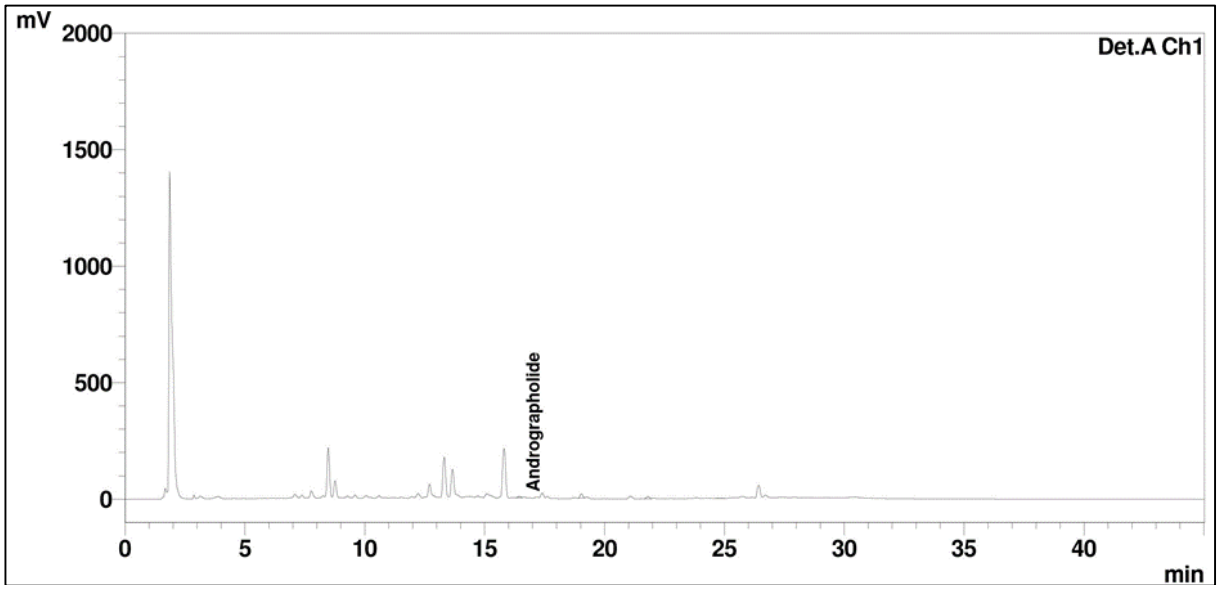
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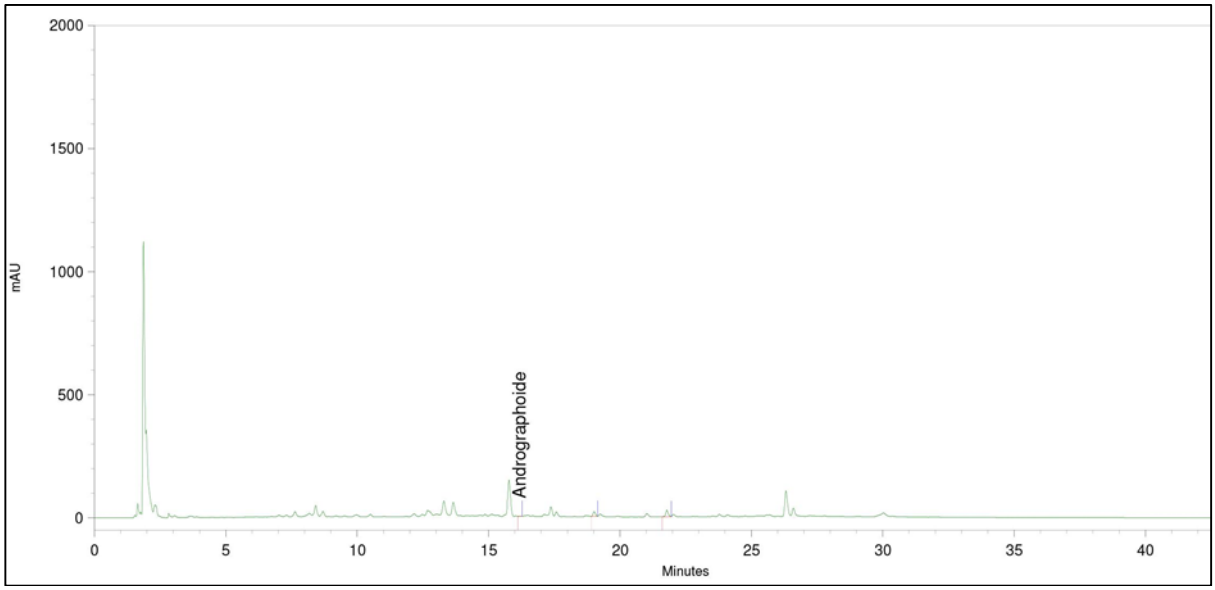

D

By HPLC analysis, control calli contain same amount of andrographolide (0.01% w/w), neo andrographolide (0.01% w/w), 14- deoxy 11-12, didehydro andrographolide (0.01% w/w) and andrographonin (0.002 %w/w) Table 2. The sample treated with 0.01% EMS for 1 hour showed a maximum amount of andrographolide (0.06% w/w) and andrographonin (0.004% w/w).

Table 3.2: Amount of secondary metabolites present in control calli and treated calli.

Secondary metabolites	Control (%w/w)	Sample treated with 0.01% EMS for 1hour (%w/w)	Sample treated with 0.01% EMS for 5 hours (%w/w)
Andrographoilde	0.01	0.06	0.06
Neo Andrographolide	0.01	0.01	0.01
14-Deoxy-11,12-dide hydro andrographoilde	0.01	0.01	0.01
Andrographonin	0.002	0.004	0.002

In this study, among different media used here, full MS medium produced better results. Maximum callus initiation was found in full MS medium fortified with 2 mg/l NAA, 1 mg/l kinetin and 50 mg/l phenylalanine. Kataky & Handique (2010) [13] reported that MS medium was the best suitable medium as compared to other culture media viz., B5 and Nitsch's media.

In the present study, maximum andrographolide was produced in the treated samples of 0.01% EMS and a 6 – fold increase in andrographolide production than the control. Cheng Xiongying *et al*. (1987) [14] reported In rice cultivars, a decreased callus induction percentages were observed in treatments with gamma-rays, EMS and sodium azide. But, up to 3-fold increase in callus growth rate was recorded after treatments with these three mutagenic agents.

4. Conclusion

The effect of EMS treatment on fresh weight, dry weight, callus induction frequency and secondary metabolite production on *in vitro* callus were recorded in this study. Lower concentration of EMS (0.01%) for 1 hour was more effective in the secondary metabolite production than the lower concentration of EMS for 5 hours.

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