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EFFECT OF PRECURSORS ON OLEANOLIC ACID PRODUCTION IN CALLUS CULTURES OF *LANTANA AMARA*

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ABSTRACT

Oleanolic acid is an important pentacyclictriterpenoid saponin exists widely in plants in the form of free acid (or) aglycone. Oleanolic acid is traditionally used in Asian medicine as anti-inflammatory, anti-hyperlipidemic and hepatoprotective substance. Triterpene precursors such as squalene, Farnesyl pyrophosphate (FPP), Isopentenyl pyrophosphate (IPP) are involved in the triterpene biosynthetic pathway. In the present study, the effect of squalene, IPP, FPP (1-5 mg/l) were studied on OA production in *Lantana camara* callus cultures, developed using root explant. Oleanolic acid content in squalene treated callus was determined by RPHPLC. From the resultsit was evident that squalene (3 mg/l) was found to be more effective on oleanolic acid production in callus cultures of *Lantana camara* compared to control callus. Squalene precursor fed callus also produced the highest biomass compared to the callus treated with the other precursors.

Keywords: *Lantana camara*, precursors, callus cultures, Oleanolic acid

INTRODUCTION

Lantana is mostly native to subtropical and tropical Asia and Africa. It grows under varied climatic conditions. Lantana is rich in essential oils. *Lantana camara* plant extracts used in folk medicine for the treatment of cancers, chicken pox, measles, asthma, ulcers, swelling, eczema, tumors, rheumatism, and malaria. Root decoction was used to treat stomach ache and vomiting in infants **[1]**.

Triterpenes and flavones are the common secondary metabolites in *Lantana camara (verbenaceae) and* roots are rich in Oleanolicacid. Oleanolic acid (3β-hydroxy-olea-12-en-28-oic acid) is a pentacyclic-triterpenoid compound **Figure 1**. It is Exists widely in the form of a glycones of triterpenoid saponins **[2]**.

It possessesPharmacological activities like Anti inflammatory, Anti-hyperlipidemic, Anti-viral, Anti-tumor, Hepatoprotective.

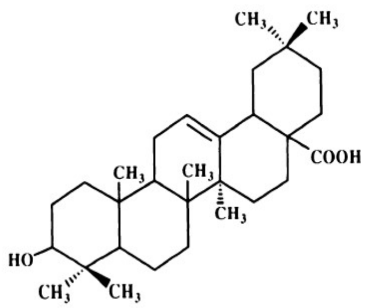

Figure 1: Chemical structure of Oleanolic acid

In Plant tissue culture various techniques are used to produce secondary metabolites. Callus culture is one of the techniques through which secondary metabolites are produced without scarifying the entire plant. One of the strategies in enhancing the natural product accumulation is feeding plant cell cultures with commercially available or easily extractable metabolic precursors. Precursor feeding had been an obvious and popular approach to increase secondary metabolite production in plant cell cultures. The concept is based upon the idea that any compound, which is an intermediate in or at the beginning of a secondary biosynthetic route, stands a good chance of increasing the yield of the final product. Precursor feeding is most likely to be adopted in inducing high levels of secondary product formation in intrinsically low producing cultures. Addition of known precursors in a pathway may enhance the production of secondary metabolites in plant cell cultures if endogenous level of these precursors is a limiting factor **[3]**.

With the aim of increasing the biomass and oleanolic acid yields in callus cultures of lantana camara, the effects of different triterpenes precursors namely squalene, Isopentenyl pyrophosphate (IPP), Farnesyl pyrophosphate (FPP)) were investigated in this study.in triterpenes biosynthetic pathway squalene is the immediate precursor while Isopentenyl pyrophosphate (IPP) and Farnesyl pyrophosphate (FPP) acts as the intermediate in the pathway **[4]**.

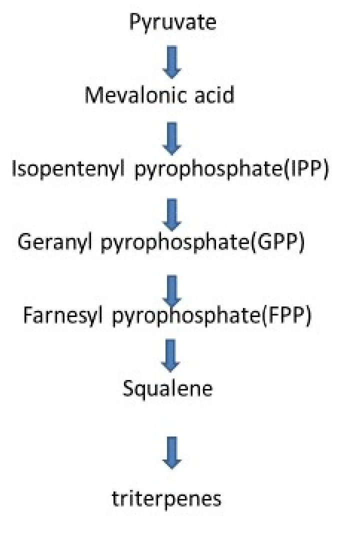

Figure 2: ***Pathway of terpenes biosynthesis***

MATERIALS AND METHODS

1. Plant Materials:

The *lantana camara* plants used in this study were obtained from Agriculture University, Hyderabad. The mature intact plants (four weeks old) were used in the studies for the determination of triterpenes content. To obtain clean samples, freshly collected samples were rinsed with slow running tap water and then separated into its distinctive parts, namely leaf, petiole, stolon and roots.

2. Initiation of in vitro culture and culture conditions:

For the initiation of callus culture, the fresh roots collected were washed thoroughly with water then Dipped in to 5%(v/v) Teepol for 10 min then washed with Distilled water, immersed in 70% (v/v) ethanol for 60 sec, in 5%(v/v) sodium hypochlorite for 20 min, Washed three times with Distilled water. Surface sterilized explants incised into small pieces were aseptically cultured in the MS medium containing 3%(w/v) sucrose supplemented with 2,4-D(1mg/l) and BA(0.5mg/l) at pH 5.8 and solidified with of Agar-Agar (11% w/v), and autoclaved. Then incubated at 25±2 ˚C in dark. Callus obtained was carefully separated from explant source and transferred into the same culture media. After several subcultures, the callus was used as the inoculums for the treatments. The dry weight (DW), triterpene content of each callus cultures was measured in four weeks.

3. Preparation of triterpene precursors:

The triterpenes precursors namely squalene, Isopentenyl pyrophosphate (IPP), Farnesyl pyrophosphate (FPP) studied were microfilter sterilized using 2um Whatman membrane because most of the precursors heat sensitive. Hence, autoclave process inappropriate. The supplementation of all the sterile precursors was carried out at the time of inoculation of callus and cells.

Squalene was dissolved in distilled water prior to microfilter sterilization. Few drops of ethanol were added to help in dissolving the squalene. The Isopentenyl pyrophosphate (IPP) and Farnesyl pyrophosphate (FPP) that separately dissolved in methanol and aqueous NH_4OH at the ratio of 7:3 with the stock concentration of 1mg/ml.

4. Extraction and analysis:

The *Lantana camara* samples were oven dried at 30c before been ground with motor and pestle. The dried, finally powdered tissue soaked in methanol for 48 hr, after which the cells were sonicated for 40 minutes at 30% amplitude with pulsar 5 sec on/off and was continued for next 20 minute at the same amplitude with pulsar 3 sec on/off. After filtration, methanol extract was centrifuged at 10,000 rpm, for 10 min, and the resulting supernatant was pooled, filtered anddried in a rotary evaporator at 40ºC.

5. Reverse Phase High Performance Liquid Chromatography of oleanolic acid:

Quantitative estimation of oleanolic acid was carried out on HPLC system using an, LC-20AD (Shimadzu corporation, Kyoto, Japan), equipped with SPD-20A, UV detector set at wavelength 210 nm and a 20 µl injection loop. Separation was performed on a C_{18} column (250 x 4.6 mm, 5µm). The solvent gradient used in this study was formed with the solvent Methanol: water (80:20 v/v). The Flow rate was 1ml/min. Identification of OA was done by comparing its retention time with that of the standard oleanolic acid compound.

Statistical analysis

RESULTS AND DISCUSSION

Root explants were sterilized and cultured on MS medium supplemented with 2,4-D(1mg/l)+BA(0.5 mg/l), 3% sucrose and incubated at 25±2˚C in Dark. After four weeks of culture, callus was observed directly on the cut surface and callus was friable, Brownish yellow color. Effect of different precursors like squalene, IPP, Farnesyl pyrophosphate (FPP) were studied on biomass production and oleanolic acid content in callus cultures of *Lantana camara* in the present experiment.

The experiment was studied on squalene at the concentrations 1-5 mg/l in the cultures. From all the concentrations examined squalene at 3 mg/l managed to produce growth higher than control **(Figure 2)**. These calli achieved the highest dry weight of 0.25±0.02gDW/culture after four weeks of culture **(Table 1)**. The dry weight attained is increased compared with the control i.e. 0.23±0.03 g DW/culture. The data obtained also revealed that there was no significant difference in terms of the biomass production between control calli and the calli treated with 1,2,4, 5, mg/l of squalene.

The presence of squalene at 3 mg/l managed to increase oleanolic acid production 1.291±0.05 mg/g DW which was higher than that detected in the control 0.763±0.01 mg/g DW **(Table 2)**. At the same concentration at 3 mg/l achieved highest biomass production and enhanced oleanolic acid content. The effectiveness of oleanolic acid accumulation in callus cultures of *lantana camara* could be due to limiting factor of flux where by exogenous supply of a biosynthetic precursors to the culture medium might improve oleanolic acid accumulation if the endogenous level of these precursors is a limiting factor of the flux. Besides, squalene is very close to the end of the triterpenes biosynthetic pathway and it can give rise and converted to the final product more easily.

similarly, in the treatment using Isopentenyl pyrophosphate (IPP) at the concentrations 1-5 mg/l in the cultures. These calli achieved the highest dry weight of 0.23±0.02gDW/culture after four weeks of culture **(Table 1)**. The dry weight attained is a constant compared to the control. The data obtained also revealed that there was no significant difference in terms of the biomass production between control calli and the calli treated with 1, 2, 4, 5, mg/l of Isopentenyl pyrophosphate (IPP).

The presence of Isopentenyl pyrophosphate (IPP) at 1 mg/l managed to increase oleanolic acid production 0.435±0.44mg/l which no significant difference than that detected in the control **(Table 2)**.

However, oleanolic acid production was not triggered in any of the Isopentenyl pyrophosphate (IPP) concentrations tested. This could be due to the absence of some enzyme activities that responsible for the biosynthesis of oleanolic acid in the callus cultures of lantana camara. Besides, limitations of the effect of the Isopentenyl pyrophosphate (IPP) on oleanolic acid production may be resulted from competition by other synthetic pathways for the same chemical as Isopentenyl pyrophosphate (IPP) is also the intermediate precursor for the biosynthesis of some other secondary metabolites.

From all the FPP concentrations tested (1-5 mg/l), it was found that at 2 mg/l managed to produced stable growth and enhancing oleanolic acid production in callus cultures 0.847±0.54 mg/l compared with the control. oleanolic acid content in the meantime was not significantly altered by treatment using any FPP concentrations compared to the control. This study also revealed that the early intermediates in triterpenes biosynthesis are not preferred for stimulating triterpenes production in cultured cells. Farnesyl pyrophosphate (FPP) used in this early intermediate and it did not significantly increase the oleanolic acid accumulation compared to the later intermediate, sqalene. One possible reason for the failure of oleanolic acid stimulation could be that the early precursor is a distant precursor, which might be channelled to other pathways to form the other related compounds.

Table 1: ***Effect of precursors on biomass production of callus cultures***

Precursors	1mg/l DW(g)	2mg/l DW(g)	3mg/l DW(g)	4mg/l DW(g)	5mg/l DW(g)	Control DW(g)
squalene	0.23±0.06	0.24±0.05	0.25±0.02	0.04±0.01	0.05±0.05	0.23±0.03
FPP	0.19±0.06	0.23±0.03	0.15 ±0.02	0.11±0.034	0.05±0.04	0.23±0.03
IPP	0.20±0.03	0.21±0.03	0.23±0.02	0.15±0.03	0.11±0.04	0.23±0.03

The values were expressed as mean of triplicates ± SE

Table 2: ***Effect of precursor on oleanolic acid production***

Conc of Precursor (mg/l) Squalene/FPP/ IPP	Oleanolic acid mg/g DW (Squalene)	Oleanolic acid mg/g DW (FPP)	Oleanolic acid mg/g DW (IPP)
1	0.624±0.14	0.698±0.21	0.435±0.44
2	0.908±0.12	0.847±0.54	0.312±0.06
3	1.291±0.05	0.759±0.64	0.100±0.05
4	0.898±0.05	0.365±0.07	0.089±0.06
5	0.508±0.08	0.327±0.04	0.054±0.01
control	0.763±0.01	0.763±0.01	0.763±0.01

The values were expressed as mean of triplicates ± SE

Figure 2: Biomass production squalene at 3mg/l

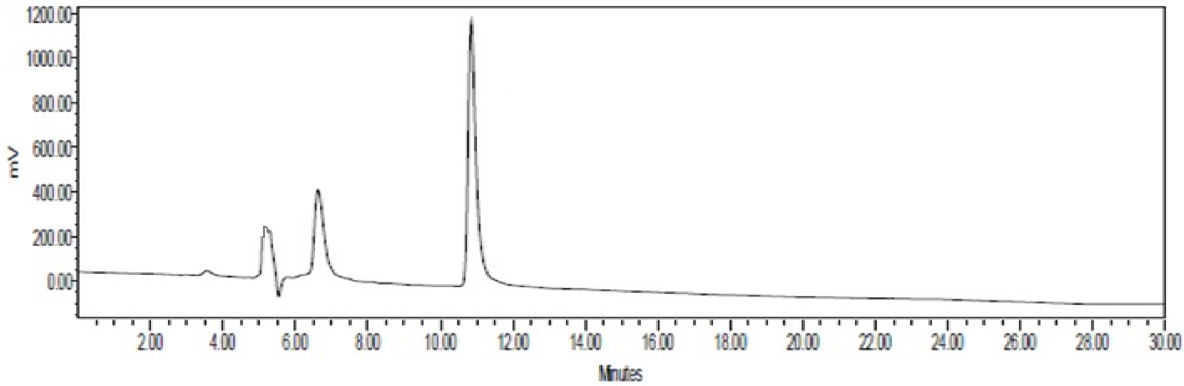

Figure 3: HPLC chromatogram of oleanolic acid (Test sample)

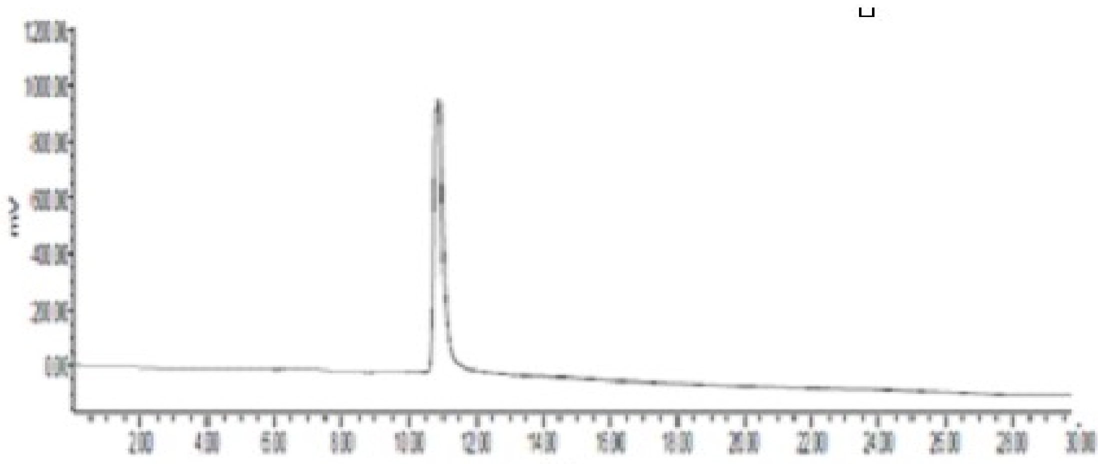

Figure 4: HPLC chromatogram of oleanolic acid (standard)

CONCLUSION

In the present study, the effect of different precursors like squalene, Isopentenyl pyrophosphate (IPP), Farnesyl pyrophosphate (FPP) with varying concentrations on oleanolic acid production by *Lantana camara* callus cultures were examined. The observations supported the enhanced production of oleanolic acidin callus culture of *Lantana camara*using squalene at (3mg/ml) as precursor.

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