RESEARCH ARTICLE

The Effect of Precursors and Elicitors on the Production of Oleanolic Acid in Suspension Culture of *Lantana camara*

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ABSTRACT

Oleanolic acid (OA) is an important pentacyclic triterpenoid. Traditionally used by Asian medicine, OA for anti-inflammatory, anti-hyperlipidemic, and hepatoprotective. This study used triterpene precursors at 1 to 5 mg/L and elicitors in different concentrations at 2, 5, 10 mg/L to enhance OA production in *Lantana camara* suspension cultures. Treated cells were evaluated for their OA contents by using HPLC and compared with the nontreated cells as a control. The results showed that FPP at 2 mg/L influenced the biomass and OA production more over the phytohormone combination, other precursors, and elicitors in Suspension cultures of *L. camara*.

Keywords: Elicitors, *Lantana camara*, Oleanolic acid, Precursors, Suspension culture.

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INTRODUCTION

Public interest has been increasing in therapeutically active phytochemicals, as a promising alternative to synthetic drugs, for treating hard-to-cure ailments, due to the side effects of Allopathy medicines. In addition, globally, there is a great demand for secondary metabolites in the nutraceutical, pharmaceutical and cosmetic industries. Oleanolic acid (OA), (3β-hydroxy Olean-12-en-28-oic acid), a pentacyclic triterpenoid. is a bioactive marker present in many edible plants and medicinal plants. Ginseng roots and olive plants (*olea europaea*) are rich in OA. The compound was named after the oleaceae family, as it is mainly found in these plants. The molecule has been isolated from more than 1600 plant species. OA has multiple therapeutic benefits such as anti-inflammatory, antitumor, anticancer, antimicrobial and anti-hyperlipidemic. *Lantana camara (Verbenaceae)*, a well-known decorative flowering weed, has varied therapeutic applications such as anti cancer, antipyretic, anti-asthmatic, antihypertensive, anti-rheumatic, anti-viral, and anti-leprotic. The leaf extract is reported as antimicrobial, fungicidal, insecticidal, and nematocidal and the root extract is used to treat malaria, rheumatism, and skin rashes.[1-5] The highest concentration of OA in roots of *Lantana camara*.[6]

Secondary metabolites are plant products that do not have any direct function in plants. In many plants, secondary metabolites are produced in very low concentrations. The secondary metabolite production in plants is influenced by soil type, temperature, humidity, rainfall, altitude, nutrients, and plant age. Callus, suspension, and hairy root culture are the most widely used tissue culture techniques. Suspension culture is the growth of homogenous individual plant cells in a liquid nutrient medium. Suspension culture is generally initiated by inoculating an established callus tissue into a liquid nutrient medium that is agitated continuously and the cell suspension culture forms within 4 to 6 weeks. Suspension culture is convenient for a continuous supply of secondary metabolites in large quantities.[7,8] Precursor feeding induces high levels of secondary product formation in intrinsically low-producing cultures. Terpenoids, the major constituents of secondary metabolites, are biosynthesized from acetyl-CoA and are generally insoluble in water. Squalene, IPP, and FPP act as precursors in triterpenoid biogenesis.[9] Elicitors also known as signalling molecules, promote secondary metabolite production in plants by modulating gene expression.[10] These arethe mediator compounds used to promote secondary metabolite formation in cell cultures. Elicitation results in improved secondary metabolite concentration in plant cell culture in less time. Also induces enzymes involved in the biosynthetic pathway, and favours the excretion of metabolites into the medium. Methyl jasmonate and salicylic acid are the key elicitors produced in response to various stress conditions which promote secondary metabolite production.[11,12]

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The aim of the study to increase the biomass and OA yields in suspension cultures of *L. camara* by using different triterpenes precursors and elicitors.

MATERIALS AND METHODS

Chemicals

Teepol, sodium hypochlorite, ethanol, methanol, chloroform, HPLC grade ethyl acetate & methanol, and water. All the chemicals procured were of analytical grade from SD Fine Chem, Hyderabad. 2, 4-D(2,4-dichloro phenoxy acetic acid), BAP (6-Benzyl amino purine), squalene, FPP, IPP, methyl Jasmonate, salicylic acid from HIMEDIA, Hyderabad.

METHODOLOGY

Preparation of Stock Solutions of Plant Growth Regulators

In 100 mL stock solutions containing (1-mg/mL) 2,4-D/BAP were prepared separately. Auxins were dissolved in a few drops of ethanol, warmed, and diluted with double distilled water to the required volume. Cytokinin was dissolved in a few drops of 1N HCL, warmed, and gradually diluted to the required volume using double distilled water and stored at 4°C (Evan *et al*., 1981).

Preparation of Precursor and Elicitor Stock Solutions (1-mg/mL)

Squalene was dissolved in distilled water with a few drops of ethanol to facilitate its solubility. The IPP and FPP stock solutions were prepared by separately dissolving them in methanol and aqueous NH_4OH in 7:3. The prepared precursors and elicitors were sterilized by membrane filtering through microfilter 0.2 µm polyether sulfone membrane (Whatman) and were refrigerated. The precursors were added to the medium during inoculation.

Effect of Precursors and Elicitors on Cell Biomass and OA Production

Precursors and Elicitors were added to the suspension culture containing MS media with 2,4-D(1 mg/L) and BAP (0.5 mg/L). The concentrations of precursors tried on cell biomass and OA production were squalene (1 to 5 mg/L), FPP (1 to 5 mg/L) and IPP (1 to 5 mg/L). The concentrations of abiotic elicitors tested on Cell biomass and OA production were methyl Jasmonate (2, 5, 10 mg/L) and salicylic acid (2, 5, 10 mg/L).

Addition of Precursors, Elicitors to the Medium

The supplementation of all precursors such as squalene, FPP, IPP (1 to 5 mg/L), and elicitors like methyl Jasmonate, salicylic Acid (2, 5, 10 µg/L) at the time of inoculation of cells.

Establishment of Suspension Culture

L. camara suspension culture was initiated by preparing 50 mL MS liquid medium in 150 mL erlenmeyer flask and supplemented with 2,4-D and BAP (1,0.5 mg/L)/precursors/ elicitors and the pH of the media adjusted to 5.8 ± 0.2 with (0.1N NaOH), containing 3% sucrose as a carbon source by varying the media components. To this media, 0.2 gm of fresh friable callus was transferred as initial inoculum and kept continuous agitation by using an orbital shaker at 120 rpm and incubated at a temperature 25 ± 2°C in the dark for 3 weeks. The cells were subcultured for every 3 weeks.

Cells Fresh and Dry Weight Estimation

The suspended cells were separated from the medium by filtration using whatman no.1 filter paper and weighed as fresh weight. After drying them at 60°C for 24 hours, the dry weight of the cells was recorded (Tables 1-5).

Isolation of OA from the Suspension Culture

The suspension-dried cell mass was soaked in methanol for 48 hours and sonicated at 30% amplitude with pulsar 5 seconds on/off for 40 minutes and continued at the same amplitude with pulsar 3 sec on/off for the next 20 minutes. The filtered methanol extract was centrifuged for 10 minutes at 10,000 rpm, and the resulting supernatants were filtered and dried in a rotary vacuum evaporator at a temperature 40°C. By using ethyl acetate, the methanol extract was fractionated. The ethyl acetate fraction was further dried in a rotary vacuum evaporator under reduced pressure at a temperature 40°C before the estimation of OA (Priyanka Srivastava, 2010).

Thin Layer Chromatography (TLC) of OA

The ethyl acetate dried extract was applied as a spot on the TLC plate. Separation was carried out in a glass tank (TLC chamber) with mobile phase of chloroform/methanol (24:1). The plates were exposed to iodine vapours and observed under visible light. The Rf value of the distinct spots observed were calculated.

Estimation of OA by RP HPLC

The vacuum-dried ethyl acetate extract was dissolved in methanol and analyzed by RP HPLC using Shimadzu LC-20AD

Table 1: Result of squalene on cell biomass and OA production

Squalene (mg/L)	*Fresh weight. (g/flask)*	*Dry weight. (g/flask)*	*OA concentration (mg/g DW)*
1	0.55 ± 0.01	0.29 ± 0.01	1.35 ± 0.06
2	0.75 ± 0.02	0.38 ± 0.01	2.54 ± 0.01
3	0.67 ± 0.03	0.32 ± 0.02	1.99 ± 0.02
4	0.52 ± 0.05	0.25 ± 0.08	1.45 ± 0.01
5	0.50 ± 0.08	0.21 ± 0.04	1.32 ± 0.02
Control	0.54 ± 0.05	0.27 ± 0.03	1.33 ± 0.04

Values expressed as mean ± SD of triplicate results

Table 2: Effect of FPP on cell biomass and OA production

FPP (mg/L)	*Fresh weight. (g/flask)*	*Dry weight. (g/flask)*	*OA concentration (mg/g DW)*
1	0.67 ± 0.09	0.35 ± 0.02	1.76 ± 0.04
2	0.82 ± 0.05	0.42 ± 0.04	2.97 ± 0.01
3	0.77 ± 0.01	0.38 ± 0.05	1.82 ± 0.02
4	0.62 ± 0.05	0.34 ± 0.01	1.72 ± 0.03
5	0.52 ± 0.03	0.23 ± 0.05	1.24 ± 0.01
Control	0.54 ± 0.05	0.27 ± 0.03	1.33 ± 0.04

values expressed as mean ± SD of triplicate results

Table 3: Result of IPP on cell biomass and OA production

IPP (mg/L)	*Fresh weight (g/flask)*	*Dry weight (g/flask)*	*OA concentration (mg/g DW)*
1	0.53 ± 0.01	0.26 ± 0.04	1.32 ± 0.01
2	0.55 ± 0.08	0.28 ± 0.03	1.36 ± 0.05
3	0.42 ± 0.03	0.20 ± 0.04	1.08 ± 0.04
4	0.38 ± 0.05	0.18 ± 0.01	0.97 ± 0.04
5	0.41 ± 0.01	0.18 ± 0.02	0.99 ± 0.06
Control	0.54 ± 0.05	0.27 ± 0.03	1.33 ± 0.04

Values are expressed as mean ± SD of triplicate results

Table 4: Effect of Methyl Jasmonate on Cell Biomass and OA production

Methyl Jasmonate (µg/L)	*Fresh wt (g/flask)*	*Dry wt (g/flask)*	*OA concentration (mg/g DW)*
2	0.65 ± 0.01	0.37 ± 0.09	1.83 ± 0.04
5	0.57 ± 0.03	0.29 ± 0.06	1.43 ± 0.03
10	0.48 ± 0.03	0.20 ± 0.05	1.23 ± 0.02
Control	0.54 ± 0.05	0.27 ± 0.03	1.33 ± 0.04

values are expressed as mean ± SD of triplicate results

Table 5: Effect of Salicylic Acid on Cell Biomass and OA production

Salicylic Acid (µg/L)	*Fresh wt (g/flask)*	*Dry wt (g/flask)*	*OA concentration (mg/g DW)*
2	0.58 ± 0.06	0.32 ± 0.02	1.35 ± 0.06
5	0.48 ± 0.01	0.25 ± 0.04	1.02 ± 0.04
10	0.39 ± 0.05	0.22 ± 0.02	0.73 ± 0.03
Control	0.54 ± 0.05	0.27 ± 0.03	1.33 ± 0.04

values are expressed as mean ± SD of triplicate results

system, on a C18 column (250 mm x 4.6 mm, 5 µm), UV detector at 210 nm. The Samples were eluted with Methanol: Water (80:20 v/v) as mobile phase, methanol as a diluent, at a 1-mL/min flow rate under isocratic conditions. 20 µL of each solution of OA isolated was used for quantitative analysis. OA was detected by comparing retention times. Quantifications were performed in triplicate independent experiments (Tables 1-5). A calibration curve was established with a standard sample of OA for the quantification of OA. The stock solution OA was prepared by using methanol at 1-mg/mL. The stock solution of OA prepared was diluted with methanol to prepare 50, 100, 200, 300, and 400 µg/mL of OA. From each concentration, 20 µL was used to plot the standard curve of OA.[7]

Statistical Analysis

The values were presented as mean ± standard deviation (SD). SPSS for windows version 10.0 computer statistical program was using for statistical analysis.

RESULTS AND DISCUSSION

The present study studied the effect of precursors and elicitors on the growth of the cell biomass and production of OA in Suspension culture. Suspension culture of *L. camara* was established by inoculating callus cells into Murashig skoog liquid medium with 2,4-D and BAP (1.0.5 mg/L)/precursors/elicitors.

After three weeks of incubation of suspension culture added with 2,4- D 1 and BAP 0.5 mg/L biomass (0.27 ± 0.03 g) and OA accumulated was (1.33 ± 0.04 mg/g). The results were in accordance with Zhongping *et al.*, 2013 study on Murashig Skoog medium with phytohormones 2,4-D and BAP produced high content of OA in the Suspension culture of cyclocaryapaliurus and polyscias fruticose.

The influence of precursors, squalene, FPP, IPP (1 to 5 mg/L), Elicitors methyl jasmonate, salicylic Acid (2, 5, 10 µg/L) on OA production and biomass in Suspension culture of *L. camara* were studied. The produced OA from the suspension culture was isolated and identified by using TLC. Rf factor of OA was found to be 0.5 same as that of standard OA. RP quantified the produced OA- HPLC using methanol: Water (80:20 v/v) as the mobile phase, λ_{max} at 210 nm.

Squalene, a 1–2 mg/L precursor, promoted biomass and OA production and decreased gradually to 5 mg/L. Squalene showed highest biomass 0.38 ± 0.01 g and OA 2.54 ± 0.01 at 2 FPP from 1 to 2 mg/L, the biomass gradually increased after decreased up to 5 mg/L. FPP exhibited highest biomass 0.42 ± 0.04 g and OA production 2.97 ± 0.01 mg/g at 2 mg/L. Precursor, IPP from increased biomass and OA production gradually from 1 to 2 mg/L and then decreased up to 5 IPP at 2 mg/L produced highest biomass 0.28 ± 0.03 g and highest OA 1.36 ± 0.05 mg/g. The three precursors from 1 to 2 mg/L upon supplementation to MS media resulted in enhanced biomass and OA production thereafter up to 5 mg/L decreased the biomass and OA production, reflecting that 2 mg/L was the ideal concentration of precursor for enhanced biomass and OA production. Among the precursors tried FPP at 2 mg/L had more impact on biomass 0.42 ± 0.04 g and OA production 2.97 ± 0.01 mg/g.

Elicitor, methyl jasmonate at 2 µg/L resulted in highest biomass (0.37 ± 0.09 g) and OA (1.83 ± 0.04 mg/g) but from 5–10 µg/L cell biomass and OA production gradually decreased. Similar results were observed in J. Zhao *et al.*, 2013 and J. S. Norrizah *et al.*, 2012 study on *Gentiana straminea*, Hedyotis corymbose wherein enhanced the accumulation of OA in the presence of methyl jasmonate. Salicylic acid at 2 µg/L as elicitor produced highest biomass 0.32 ± 0.02 g and the highest OA 1.35 ± 0.06 mg/g but with addition of salicylic acid from 5–10 µg/L the biomass and OA production were gradually decreased. The results are in accordance with Swati Sharan, *et al.* 2021 study on the suspension culture of *Ocimum tenuiflorum* wherein methyl jasmonate as an elicitor enhanced the accumulation of OA compared to salicylic acid.

Both the elicitors, methyl jasmonate and salicylic acid at 2 µg/L resulted in highest biomass (0.37 ± 0.09 g) and OA (1.83 ± 0.04 mg/g) and biomass 0.32 ± 0.02 g and OA 1.35 ± 0.06 mg/g production respectively indicating that 2 µg/L is the suitable concentration of the elicitor for biomass as well as OA production. Methyl jasmonate had more effect on biomass and OA production than salicylic acid. Among the phytohormone combination, precursors and elicitors studied on biomass and

OA production the influence of phytohormone combination was found to be the least suggesting that phytohormones have less role in biomass production and OA accumulation in suspension culture of *L. camara*.

CONCLUSION

The present study, suspension culture of *L. camara* established by using callus culture of *L. camara* as inoculum on MS liquid medium. The effect of phytohormone combination (2,4-D (1-mg/L) + BAP (0.5 mg/L)/precursors/elicitors study on biomass and production of OA resulted in highest biomass 0.42 ± 0.04 g and highest OA 2.97 ± 0.01 mg/g with FPP at 2 mg/L as precursor reflecting that precursor FPP at 2 mg/L g influenced the biomass and OA production more over the phytohormone combination, other precursors and elicitors studied.

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