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Phenylalanine induced biosynthesis of flavonoids from cell suspension *culture of Lantana camara* L.

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ABSTRACT

Plant cells, tissues, and organs have become abundant sources of secondary metabolites responsible for the antimicrobial defense mechanisms of plants. However, large-scale production of these metabolites has been limited by environmental factors and anthropogenic activities that disrupt ecosystems. Plant tissue culture has emerged as a fundamental approach for enhancing secondary metabolite production. This study aimed to produce flavonoids using phenylalanine as a precursor in the cell suspension culture of *Lantana camara*. Callus cells were initiated from leaf explants of *L. camara* in MS basal medium supplemented with 30 g/L sucrose, 0.5 mg/L BAP, 1 mg/L NAA, and 8 g/L agar. The cell suspension culture was established by transferring the resulting callus into liquid medium of similar composition for two weeks, with varying concentrations of phenylalanine precursor. The total flavonoid content (TFC) of two-week-old suspension cells was compared with that of the control and a four-year-old plant. The results showed that treatment with 4 mg/L phenylalanine produced the highest flavonoid content (6.633 mg RE/g DW). At $p \leq 0.05$, there were significant differences in TFC among phenylalanine concentrations of 0.5, 1, 2, 4, 6, 8, and 10 mg/L. Progressive and proportional increases in TFC were observed from 0.5 to 4 mg/L, while concentrations above 4 mg/L resulted in a decline in flavonoid accumulation. For phenylalanine concentrations of 12, 14, and 16 mg/L, no significant increases in TFC ($p \leq 0.05$, 95% CI) were observed compared with the control. These findings demonstrate a significant advantage of biosynthesizing flavonoids using phenylalanine as a precursor in *L. camara* cell cultures over conventional natural production methods.

Keywords: Bioproduction, total flavonoid content (TFC), phenylalanine, secondary metabolites, callus cell suspension, *Lantana camara*.

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INTRODUCTION

The plant kingdom, comprising about 250,000 species, is a repository of hundreds of thousands of low-molecular-weight, structurally complex chemical compounds known as secondary metabolites (Sandra and Anabela, 2018). These high-value metabolites are biosynthesized through complex pathways and serve as ecological mediators between plants and their environment. They also possess intrinsic biological activities that affect other organisms. Secondary metabolites are often accumulated in specific tissues and structures such as vacuoles, specialized glands, and trichomes. Their production is influenced by multiple factors, including soil type, climatic conditions, seasonal variations in plant collection, and the developmental stage of the plant, as some compounds are synthesized only at specific growth phases (Noor et al., 2017; Sandra and Anabela, 2018).

Lantana camara is a shrub native to Africa and the Americas but has been widely cultivated as an ornamental plant in other regions. It is recognized as one of the world's important medicinal plants, with reported biological activities such as anti-protozoal, anti-inflammatory, antibacterial, antioxidant, and anticancer

properties. Phytochemical analysis has revealed that the leaves, stems, and roots of *L. camara* contain tannins, catechins, saponins, steroids, alkaloids, phenols, anthraquinones, proteins, triterpenoids, flavonoids, glycosides, and reducing sugars, many of which are responsible for its diverse pharmacological effects (El-Sayed et al., 2017).

Elicitors are compounds that stimulate physiological responses in plants. These stimuli may be exogenous, originating from pathogens (exogenous elicitors), or endogenous, produced by plants in response to pathogen action (endogenous elicitors) (Radman et al., 2003). The use of elicitors has become an important strategy for triggering biosynthetic pathways and enhancing the production of secondary metabolites of commercial importance (Chong et al., 2005; Smetanska et al., 2008; Sharma et al., 2011; Hussain et al., 2012), thereby improving production efficiency and reducing costs (Miao et al., 2000; Zhang and Wu, 2003). Secondary metabolites are often released as part of plant defense responses, which are activated by elicitors that act as signaling compounds (Patel and Krishnamurthy, 2013). Artificial elicitors such as copper sulfate ($CuSO_4$) and silver nitrate ($AgNO_3$) have been shown to increase flavonoid production in the suspension cultures of *Digitalis lanata* (Bota and Deliu, 2011) and *Ononis arvensis* (Tumova and Polivkova, 2006).

Over the past decades, significant efforts have been devoted to the extraction, structural elucidation, and evaluation of the biological activities of plant secondary metabolites. Considerable progress has also been made in their production through plant tissue culture techniques, which offer advantages over conventional production systems (Sandra and Anabela, 2018). However, there is no documented literature on the use of phenylalanine as a precursor for flavonoid accumulation in cell suspension cultures of *L. camara*. Therefore, the present study investigates the enhancement of flavonoid production from *L. camara* via in vitro culture using phenylalanine as a precursor through the phenylpropanoid pathway.

MATERIALS AND METHODS

Establishment of callus culture

Leaves from a four-year-old *Lantana camara* plant grown at the Federal College of Forestry were used as explants (Figure 1). Fresh leaves were thoroughly washed under running tap water and rinsed three times with distilled water. They were then soaked in 70% ethanol containing two drops of Tween-80, shaken vigorously for 3 minutes, and rinsed four times with sterile distilled water. To further disinfect, leaves were treated with 1% sodium hypochlorite for 10 minutes with intermittent shaking, followed by four rinses with sterile distilled water under aseptic conditions in a laminar flow cabinet.

Figure 1. *Lantanna camara* ex-plant.

Leaf segments of approximately 2 mm² were inoculated on Murashige and Skoog (MS) basal medium supplemented with 0.5 mg/L BAP and 1.0 mg/L NAA. The medium was fortified with 1 g/L activated charcoal. Cultures were incubated at 25 ± 2 °C, 70 ± 10% relative humidity, under an 8 h light/16 h dark photoperiod for 28 days. Subculturing on MS medium supplemented with 0.5 mg/L BAP and 1.0 mg/L NAA for 14 days was performed to obtain friable callus suitable for suspension culture.

Establishment of cell suspension culture

Suspension cultures were initiated by transferring friable callus into liquid MS medium containing 0.5 mg/L BAP and 1.0 mg/L NAA. To investigate the effect of precursor concentration, the medium was supplemented with L-phenylalanine at concentrations of 0.5, 1, 2, 4, 6, 8, 10, 12, 14, and 16 mg/L, designated as S1–S10, respectively. MS medium without phenylalanine served as the control. The suspension cultures were maintained on a rotary shaker at 90 rpm, 30 °C, for 14 days with periodic monitoring. After incubation, cultures were harvested, dried in an oven at 60 °C, and used for flavonoid estimation.

Preparation of standard calibration curve of rutin

A standard calibration curve of rutin was prepared using absolute methanol as the blank. A stock solution was obtained by dissolving rutin in methanol to yield a concentration of 100 µg/mL. Serial dilutions were performed to obtain working solutions of 10, 20, 30, 40, 50, 60, 70, and 80 µg/mL. One milliliter of each working solution was placed in test tubes, with 1 mL methanol serving as the blank. To each tube, 4 mL methanol and 0.3 mL of 5% sodium nitrite were added. After 5 minutes, 0.3 mL of 10% aluminum chloride solution was added, followed by 2 mL of 1 M sodium hydroxide at the 6th

minute. The solutions were mixed thoroughly using a vortex mixer, and absorbance was measured at 510 nm using a UV–Visible spectrophotometer (Metertech SP-8001, Taiwan). A calibration curve was constructed by plotting absorbance against concentration.

Plant, callus, and cell extraction

Flavonoid extraction was performed following the method of Zainol et al. (2019). Approximately 0.5 g of dried leaf powder (from a four-year-old plant), dried callus, and 14-day-old suspension cells of *L. camara* were homogenized with 20 mL methanol using a ceramic mortar and pestle. The homogenate was transferred into centrifuge tubes and centrifuged at 4000 × g for 10 minutes. The resulting supernatant was collected for further analysis.

Estimation of total flavonoid content (TFC) using rutin as standard

The TFC was determined using the aluminum chloride colorimetric method as described by Zainol et al. (2019). One milliliter of sample extract was mixed with 4 mL methanol. For the blank, 1 mL methanol was treated similarly. To each tube, 0.3 mL of 5% sodium nitrite was added. After 5 minutes, 0.3 mL of 10% aluminum chloride was added, followed by 2 mL of 1 M sodium hydroxide at the 6th minute. The mixture was then adjusted to a final volume of 10 mL with 2.4 mL methanol. The samples were vortexed thoroughly, and absorbance was measured at 510 nm. Results were expressed as mg rutin equivalents (RE) per g dry weight (DW) sample.

Statistical analysis

Data were subjected to two-way analysis of variance (ANOVA) using GraphPad Prism version 8.2. Statistical significance was determined at $p \leq 0.05$.

RESULTS

Standard calibration curve of rutin

A standard calibration curve of rutin was obtained by plotting absorbance at 510 nm against concentration (µg/mL) (Table 1). A line of best fit was generated, yielding the regression equation Y = 0.0005X + 0.0617 with a coefficient of determination (R^2 = 0.9912). The slope of the curve was 0.0005 and the y-intercept was 0.0617 (Figure 2). This calibration curve was subsequently used to determine the flavonoid concentrations in the study samples.

Table 1. Concentration (µg/mL) of rutin as standard.

Concentration (ug/mL)	Absorbance (510nm)
80	0.104±0.001
70	0.098±0.001
60	0.095±0.001
50	0.090±0.001
40	0.082±0.001
30	0.076±0.001
20	0.072±0.001
10	0.068±0.001

Means ±SD (n=3 observation).

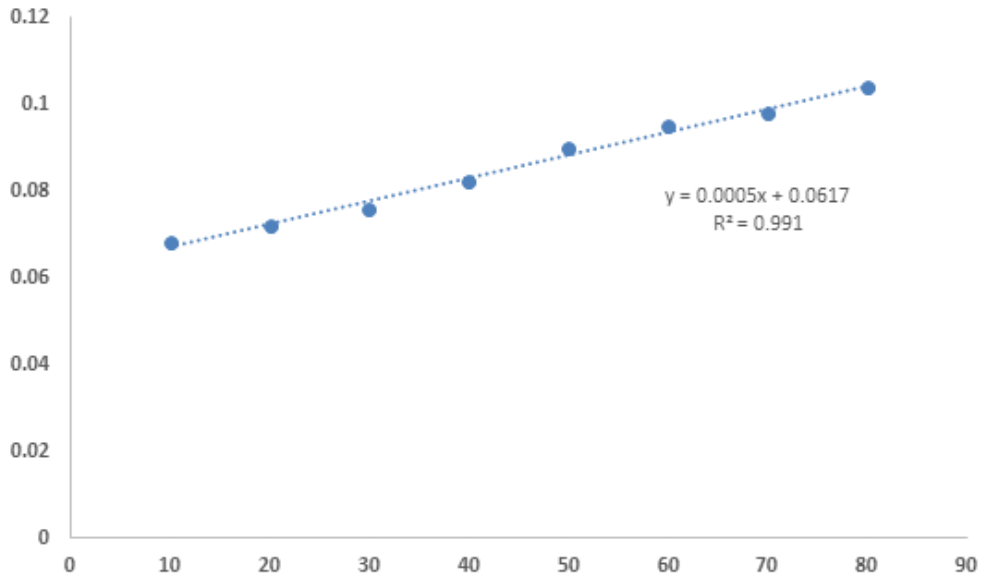

Figure 2. Standard calibration curve of standard flavanoid rutin.

Total flavonoid content of leaf, callus, and cell suspensions of *Lantana camara*

The total flavonoid content (TFC) of leaf, callus, and cell suspension cultures of *L. camara* is summarized in Table 2. The leaf sample exhibited the highest flavonoid content (54.687 ± 0.123 mg RE/g DW), a 7-week-old callus culture contained 9.753 ± 0.047 mg RE/g DW (Figure 3). Among the cell suspension cultures, the highest TFC was recorded at 4 mg/L phenylalanine, with a value of 6.633 ± 0.081 mg RE/g DW, representing a 42% increase compared to the control. The culture supplemented with 6 mg/L phenylalanine showed 6.427 ± 0.047 mg RE/g DW, corresponding to a 39.8% increase relative to the control.

Other formulations also demonstrated increases in TFC relative to the control (Figure 4):

0.5 mg/L: 5.673 ± 0.047 mg RE/g DW (31.9%)
1 mg/L: 4.500 ± 0.081 mg RE/g DW (14.2%)
2 mg/L: 4.447 ± 0.123 mg RE/g DW (13.1%)
8 mg/L: 4.180 ± 0.081 mg RE/g DW (7.6%)
10 mg/L: 4.127 ± 0.047 mg RE/g DW (6.4%)
12 mg/L: 3.993 ± 0.123 mg RE/g DW (3.3%)

At higher concentrations, a decline in TFC was observed:

14 mg/L: 3.860 ± 0.081 mg RE/g DW (0.08% decrease compared with control)
16 mg/L: 3.833 ± 0.047 mg RE/g DW (0.7% decrease compared with control).

At $p \leq 0.05$, there were significant differences in TFC among treatments. Values are presented as mean ± standard error of mean (SEM). Ranking was conducted across treatments, and values with the same superscript are not significantly different.

Table 2. Concentration (μg/mL) of total flavonoid content.

Absorbance (510nm)	TFC (mg/g)
0.113± 0.001	4.127±0.046
0.117±0.002	4.447±0.122
0.133±0.001	5.673±0.046
0.145±0.001	6.633±0.080
0.142±0.001	6.420±0.080
0.118±0.001	4.500±0.080
0.114±0.001	4.180±0.080
0.112±0.001	3.993±0.122
0.110±0.001	3.860±0.080
0.110±0.001	3.833±0.046
0.184±0.002	9.753±0.046
0.745±0.002	54.687±0.122
0.110±0.002	3.863±0.006

Mean ± SD (n=3 observation).

Figure 3. Callus cell of *Lantana camara* growing on MS medium.

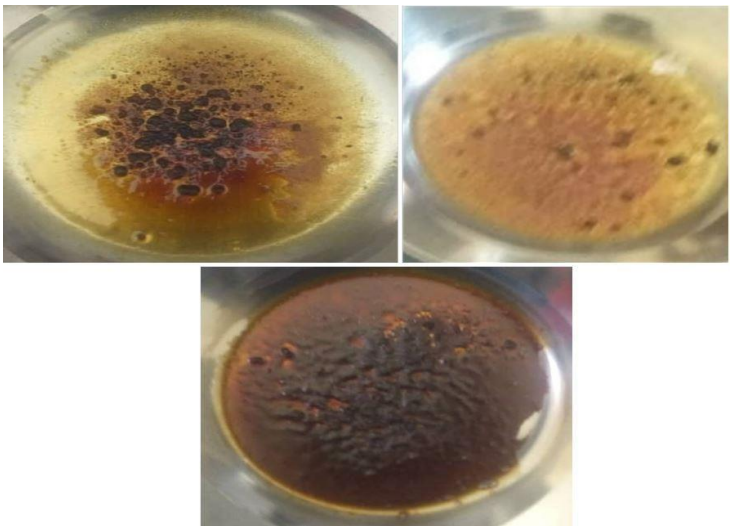

Figure 4. Total flavonoid content based on different phenyl alanine concentration as recovered from suspension culture of *L antana* camara callus tissue.

DISCUSSION

Callus induction and the health condition of callus are critical stages in the accumulation of bioactive compounds in suspension media. A good combination of the two commonly used plant hormones in plant tissue culture (auxin and cytokinin) is also a major determinant of successful callus initiation and proliferation (Jiayu et al., 2018). In this study, young leaves of *L. camara* produced the best callus induction when cultured on media supplemented with 0.5 mg/L BAP + 1 mg/L NAA. This observation is consistent with the findings of Moitreyee et al. (2013), who reported that combining NAA and BAP in a 2:1 ratio enhanced callus growth rate and health. Therefore, the combination of 0.5 mg/L BAP + 1 mg/L NAA was selected as the optimal formulation for subsequent sub-culturing and suspension culture. An appropriate hormone combination and concentration not only promote healthy callus formation but also accelerate induction and biomass growth. Similarly, Stefan et al. (2016) documented an increase of more than 64% in fresh biomass yield when optimal hormonal balance was maintained.

Another important factor in optimizing cell suspension culture for secondary metabolite production is the initial inoculum size, which strongly influences both biomass growth and metabolite accumulation.

Flavonoid biosynthesis occurs through the phenylpropanoid pathway. The first step is catalyzed by phenylalanine ammonia-lyase (PAL), which converts L-phenylalanine into trans-cinnamic acid. Subsequently, cinnamate-4-hydroxylase (C4H) converts trans-cinnamic acid into p-coumaric acid, and 4-coumarate-CoA ligase (4CL) activates it into coumaroyl-CoA. Chalcone synthase then catalyzes the condensation of coumaroyl-CoA with malonyl-CoA to produce chalcone, the central scaffold for flavonoid biosynthesis (Baranski and Scott, 2016).

The influence of phenylalanine as a precursor on flavonoid accumulation in *L. camara* suspension culture is summarized in Table 3. The results revealed that supplementation with 4 mg/L phenylalanine yielded the highest flavonoid content (6.633 mg RE/g DW), representing a 41.76% increase over the control. These findings align with Masoumian et al. (2011), who reported that concentrations above 6 mg/L were unsuitable for flavonoid accumulation. Gueven and Knorr (2011) also noted that excess precursor supply can be toxic to cultures if applied at inappropriate concentrations or stages. In agreement with these reports, this study observed a sharp decline in flavonoid accumulation at higher precursor levels, with 16 mg/L phenylalanine yielding the lowest content (3.833 mg RE/g DW), followed closely by 14 mg/L. Suat and Maziah (2013) similarly reported no significant increases in flavonoid accumulation at higher precursor concentrations, attributing the decline to feedback inhibition once phenylalanine exceeded 5 mg/L.

Table 3. Total Flavonoid Content (TFC) and percentage (%) increase in TFC as compared to the control sample.

Sample	TFC (mg RE/gDW sample)	% increase
S1	4.127 ± 0.046^{e}	6.4
S2	4.447 ± 0.122^{d}	13.1
S3	5.673 ± 0.046^{c}	31.9
S4	6.633 ± 0.080^{b}	42
S5	6.420 ± 0.080^{b}	39.8
S6	4.500 ± 0.080^{d}	14.2
S7	4.180 ± 0.080^{e}	7.6
S8	3.993 ± 0.122^{f}	3.3
S9	3.860 ± 0.080^{f}	-0.08
S10	3.833 ± 0.046^{f}	-0.7
Callus	9.753 ± 0.046^{b}	60.39
Leaf	54.687 ± 0.122^{a}	92.94
Control	3.863 ± 0.006^{f}	0
L.S.D	0.13	

At lower concentrations, flavonoid accumulation was modest but still higher than the control. For instance, at 0.5 mg/L phenylalanine, flavonoid content was 4.127 mg RE/g DW (6.4% above control), increasing to 4.447 mg RE/g DW (13.1% above control) at 1 mg/L, and further to 5.673 mg RE/g DW (31.9% above control) at 2 mg/L. These results demonstrate a concentration-dependent trend: flavonoid accumulation increased progressively with precursor concentration until the optimum (4 mg/L), beyond which accumulation sharply declined. These observations corroborate earlier findings by Suat and Maziah (2013) and Masoumian et al. (2011).

The total flavonoid content of callus cells derived from leaves also followed previously reported trends

(Masoumian et al., 2011; Vijendren et al., 2015). The relatively high flavonoid accumulation in the callus is likely due to its longer culture duration (seven weeks) compared to the shorter-term cell suspension (two weeks). Mortada et al. (2017) reported a total flavonoid content of 63.767 ± 1.122 mg RE/g in *L. camara* leaves, which is not significantly different from the present study's finding of 54.687 ± 0.122 mg RE/g DW.

Overall, this research demonstrates that *L. camara* cell suspension culture is a viable system for flavonoid production via the phenylpropanoid pathway. Callus induction from leaf explants was successfully achieved, and the best hormonal combination for callus formation was identified. Suspension cultures supplemented with phenylalanine precursors revealed a clear concentration-dependent effect, with flavonoid accumulation peaking at 4 mg/L before declining at higher concentrations.

CONCLUSION

This study demonstrated that successful callus induction and suspension culture of *Lantana camara* can serve as an efficient platform for flavonoid production through the phenylpropanoid pathway. The optimal hormonal combination for callus induction was identified as 0.5 mg/L BAP + 1 mg/L NAA, which produced vigorous and healthy callus, confirming the importance of auxin–cytokinin balance for callus proliferation and biomass growth.

Suspension cultures supplemented with phenylalanine precursors showed a concentration-dependent trend in flavonoid accumulation, with the highest content (6.633 mg RE/g DW, a 41.76% increase over control) obtained at 4 mg/L phenylalanine. Concentrations above this threshold caused a significant decline in flavonoid levels due to possible feedback inhibition or toxicity.

Therefore, this research establishes *L. camara* cell suspension culture as a promising approach for commercial flavonoid production, provided that precursor concentrations and hormonal formulations are carefully optimized.

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