GENE EXPRESSION ANALYSIS INVOLVED IN FLAVONOID BIOSYNTHESIS OF *PHYLLANTHUS AMARUS*

PHÂN TÍCH BIỂU HIỆN CÁC GEN LIÊN QUAN ĐẾN QUÁ TRÌNH SINH TỔNG HỢP FLAVONOID Ở CÂY DIỆP HẠ CHÂU

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ABSTRACT

Flavonoids, a diverse group of secondary metabolites, are known for their health-promoting properties, including antioxidant, anti-inflammatory, and anticancer activities. *Phyllanthus amarus* is a medicinal plant with extensive traditional use and it is a rich source of flavonoids. This study aimed to elucidate the gene expression in flavonoid biosynthesis in *P. amarus*. Using quantitative polymerase chain reaction (qPCR), we investigated the expression of Phenylalanine ammonia-lyase (*PAL*), chalcone synthase (*CHS*), and chalcone isomerase (*CHI*), which are key genes associated with flavonoid biosynthesis. Our findings contribute to a deeper understanding of flavonoid biosynthesis in *P. amarus* and provide valuable insights into the molecular mechanisms involved. This knowledge can be leveraged to optimize the production of specific flavonoids with potential health benefits and to develop strategies for enhancing the bioactivity of *P. amarus* as a medicinal plant. This study thus contributes to the expanding field of plant biochemistry and lays the foundation for further research on the biosynthesis and regulation of flavonoids in Phyllanthus amarus.

Keywords: *Phyllanthus amarus, flavonoid biosynthesis, gene expression analysis, CHI, CHS, PAL.*

TÓM TẮT

Flavonoid, một nhóm chất chuyển hóa thứ cấp đa dạng, được biết đến với đặc tính tăng cường sức khỏe, bao gồm các hoạt động chống oxy hóa, chống viêm và chống ung thư. *Phyllanthus amarus* là một cây thuốc được sử dụng phổ biến, rộng rãi, và là một nguồn flavonoid dồi dào. Nghiên cứu này nhằm mục đích làm sáng tỏ sự biểu hiện gen trong quá trình sinh tổng hợp flavonoid ở *P. amarus*. Sử dụng phản ứng chuỗi polymerase định lượng (qPCR), chúng tôi đã nghiên cứu biểu hiện của Phenylalanine amoniac-lyase (*PAL*), chalcone synthase *(CHS*) và chalcone isomerase (*CHI*), là các gen chủ chốt liên quan đến sinh tổng hợp flavonoid. Những phát hiện của chúng tôi góp phần hiểu biết sâu sắc hơn về quá trình sinh tổng hợp flavonoid *ở P. amarus* và cung cấp những hiểu biết có giá trị về các cơ chế phân tử liên quan. Kiến thức này có thể được tận dụng để tối ưu hóa việc sản xuất các flavonoid cụ thể có tiềm năng mang lại lợi ích sức khỏe và phát triển các nhằm tăng cường hoạt tính sinh học của *P. amarus* như một cây thuốc. Do đó, nghiên cứu này góp phần mở rộng lĩnh vực hóa sinh thực vật và đặt nền tảng cho các nghiên cứu sâu hơn về sinh tổng hợp và điều hòa flavonoid ở *Phyllanthus amarus.*

Từ khóa: *Phyllanthus amarus, sinh tổng hợp flavonoid, phân tích biểu hiện gen, CHI, CHS, PAL.*

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1. INTRODUCTION

1.1. General introduction to *Phyllanthus amarus*

Phyllanthus amarus, commonly known as Stonebreaker or Seed-under-leaf, is a tropical medicinal plant that has been widely used in traditional medicine for treating various diseases such as liver disorders, gastrointestinal ailments, and kidney disorders [1]. This leafy herbal plant is found in tropical regions in the

Americas, Africa, India, China, and Southeast Asia, this plant is a small, annual herb that grows to a height of 10 - 60cm with thin branches spread out; each branch has two rows of small, elliptic-oblong leaves of 5 - 10mm long that are arranged alternately and its radial flowers are star-shaped and about 2mm in size [1]. The plant is rich in beneficial compounds including flavonoids, alkaloids, terpenoids, and lignans. Flavonoids in this plant have pharmacological activities such as anti-inflammatory, antioxidant, and anti-cancer properties [2]. This plant has a flavonoid biosynthesis process to be able to prepare flavonoids. However, the regulatory mechanisms underlying the biosynthesis of these flavonoids in *Phyllanthus amarus* are not well understood.

Fig. 1. Phyllanthus amarus whole plant

1.2. General introduction to Flavonoids and their roles

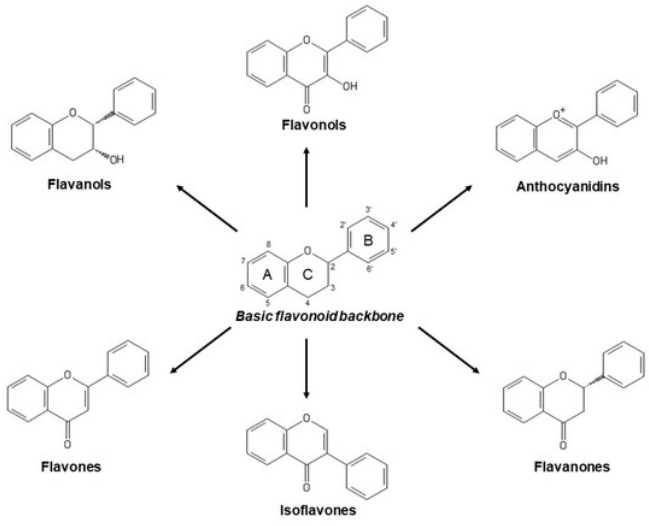

Fig. 2. Basic flavonoid structure and main types of flavonoids [3]

Flavonoids are secondary metabolites found extensively in plants that play various and essential roles in plants, including pollination, pigmentation, UV protection, plant defense, and highly beneficial for human health due to their potent pharmacological effects [3]. They are derived from phenylpropanoid metabolism, which involves the synthesis of several intermediate compounds, including phenylalanine, coumaric acid, and cinnamic acid [4]. These compounds are catalyzed by a series of enzymes to produce the flavonoid backbone, which is structurally composed of two aromatic rings linked by a three-carbon bridge (C6-C3-C6) and functional groups that vary depending on the type of flavonoid (Fig. 2). In this study, we investigate the gene expression patterns and mechanisms involved in the biosynthesis of flavonoids in *Phyllanthus amarus*.

1.3. Flavonoid biosynthesis process in *Phyllanthus amarus*

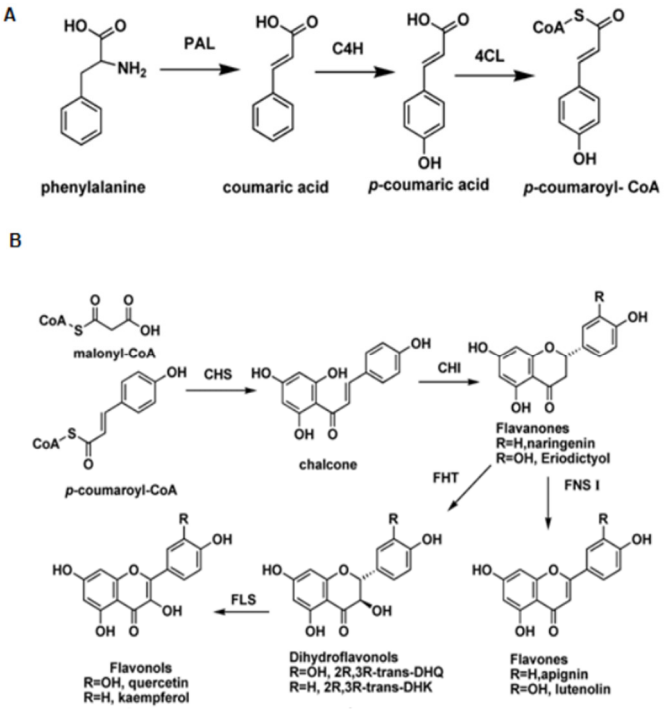

Fig. 3. The biosynthesis pathway of flavonoids. (A) The biosynthesis of p-coumaroyl-CoA; (B) The biosynthesis of different kinds of flavonoids from p-coumaroyl-CoA and malonyl-CoA. PAL: Phenylalanine ammonia-lyase, C4H: Cinnamic acid 4-hydroxylation, 4CL: 4-Coumarate: Coenzyme A ligase, CHS: Chalcone synthase, CHI: Chalcone isomerase, FHT: Flavanone 3β-hydroxylase, FNS I: Flavone synthase I, FLS: Flavonols synthase [5]

Flavonoid biosynthesis is a complex metabolic pathway involving a series of enzymatic reactions. It starts with the conversion of phenylalanine into naringenin chalcone, which serves as the precursor for various classes of flavonoids. Enzymatic modifications of naringenin chalcone lead to the synthesis of different subclasses of flavonoids, such as flavones, flavonols, and

flavanones. Phenylalanine ammonia-lyase (*PAL*), chalcone synthase (*CHS*), and chalcone isomerase (*CHI*) are key enzymes involved in the flavonoid biosynthetic pathway [5] (Fig. 3). *PAL* is the first enzyme in the phenylpropanoid pathway, a precursor to the flavonoid biosynthetic pathway. It catalyzes the deamination of phenylalanine to trans-cinnamic acid, a critical step that links primary and secondary metabolism in plants [6]. *CHS* is considered the gateway to flavonoid biosynthesis. It catalyzes the condensation of one molecule of CoA-ester of cinnamic acid or derivatives with three molecules of malonyl-CoA, yielding a naringenin chalcone [7]. This reaction is the first committed step in flavonoid biosynthesis, leading to the production of a wide range of flavonoids. *CHI* plays a crucial role following the action of *CHS*. It catalyzes the cyclization of chalcones into (2S)-flavanones, a critical step in the flavonoid biosynthetic pathway [8]. This fast and practically irreversible reaction drives the pathway towards flavonoid production.

To compare the performance of these genes, we used 2 other genes Phenyl coumaran benzylic ether reductase (*PCBER*) and pinoresinol reductases (*PrR*), which are in the lignan biosynthesis pathway and have the same initial steps as flavonoid biosynthesis pathway in the phenylpropanoid pathway.

1.4. Significance of studying gene expression related to flavonoid synthesis in *Phyllanthus amarus*

In this analysis, we investigate the molecular mechanisms involved in the biosynthesis of flavonoids in Phyllanthus amarus. Specifically, we will analyze the gene expression patterns of key regulatory genes and transcription factors involved in flavonoid biosynthesis, using quantitative real-time PCR (qPCR) and statistical analyses. By examining the gene expression patterns under different developmental stages and environmental stimuli, we aim to gain insights into the regulatory mechanisms that influence flavonoid biosynthesis in Phyllanthus amarus.

The study of gene expression involved in flavonoid biosynthesis in *Phyllanthus amarus* can provide valuable insights into how these beneficial compounds are produced. This could further our understanding of the plant's medicinal properties and potentially lead to enhanced cultivation methods or new medical treatments. Understanding the genetic basis of flavonoid biosynthesis also opens up possibilities for bioengineering efforts to increase flavonoid production or alter the types of flavonoids produced, with wide-ranging implications for agriculture, nutrition, and medicine.

2. SCIENTIFIC OBJECTIVE

To understand the regulatory mechanisms: Investigate the transcriptional regulation of genes involved in flavonoid biosynthesis in Phyllanthus amarus, aiming to uncover the key regulatory factors and signaling pathways that control the production of specific flavonoids.

To determine gene expression patterns: Quantify the expression levels of specific genes involved in flavonoid biosynthesis under different experimental conditions, such as tissue types, developmental stages, or environmental factors, to identify patterns of gene expression and gain insights into the factors influencing flavonoid production.

3. MATERIAL AND METHOD

3.1. Material

Phyllanthus amarus plants were collected from Thai Nguyen at the age of 4 months. Fully developed leaves were harvested from mature plants for subsequent analysis.

3.2. Extraction

Total RNA was extracted from 5 - 10 grams of leaves and stems which were collected using the phenol/chloroform extraction method. Leaf and stem tissues were ground to a fine powder with 2% PVP in liquid nitrogen before adding 2ml of Extraction Buffer (0.1M Tris.HCl pH 8.0, 1% SDS, 0.05M EDTA pH 8.0; 1% β-mercaptoethanol) warmed to 65°C. Centrifuge at 15000rpm, 4°C, 10 minutes after adding phenol: chloroform acid (1:1) to remove DNA, help stabilize the surface and prevent foaming when mixing. Repeat the previous step with 5M NaOAc and phenol acid: chloroform at a ratio of 3:7. The solution was washed with 100% and 70% ethanol to dissolve and wash away the precipitation. The resulting RNA was treated with DNase to remove genomic DNA contamination. The quality of the extracted RNA was assessed by gel electrophoresis.

3.3. cDNA Synthesis (Reverse Transcription)

Reverse transcription was performed using Revert Aid Reverse Transcriptase KIT (Thermo Fisher Scientific). Purified RNA was reverse transcribed into complementary DNA (cDNA) using random hexamer and reverse transcriptase enzyme (ribolock and revert acid). The cDNA synthesis reaction mixture was incubated at a specified temperature and time according to the kit instructions.

Primer annealing at 25°C in 5 minutes, DNA polymerization at 42°C in 60 minutes, and enzyme deactivation at 70°C in 5 minutes. The synthesized cDNA served as a template for subsequent gene expression analysis.

The verification of cDNA synthesis outcomes was conducted utilizing housekeeping genes and Polymerase Chain Reaction (PCR) methodologies. Post the conversion of the RNA sample into cDNA, a PCR reaction was initiated, employing the synthesized cDNA as a template and primers specific to the housekeeping genes. Upon completion of the PCR procedure, the resultant PCR product was subjected to electrophoresis on an agarose gel to ascertain the presence of bands correlating to a predetermined size.

3.4. Primer Design

Table 1. Primer sequence, Tm, and length of CHI, PCBER, PrR, PAL CHS, CHI

Gene	Primer Sequence (5′ - 3′)	Tm (°C)	Length (bp)
PaCHI	F: ACAGTGACCATGATCTTGCC	55	122
	R: TTGTCAAGAGCTTCGCCTTC		
PCBER	F: TCCAACATAGTTTAAAATAGGAGAG	56	265
	R: TTTGTACCATTTCTGGAGTCAAGA		
PrR	F: AGGTATTTGGACTTTGTCTGGAT	54	250
	R: ATTTCTTCTTGTTAGAGACACTGC		
PAL	F: ATTGCTGTGAAAACCAAATCGAAC	55	273
	R: GAAAACGAGAAGAATGCTAGCAC		
CHS	F: CACAGCGGGTTATGCTATAC	56	156
	R: GTCTATCTGCACTTGGTGG		
CHS13	F: GAAGCGATACATATGCACCTGA	55	180
	R: TGCGTGATCTTTGACTTTGG		
EF 1	F: CACAGCGGGTTATGCTATAC	56	117
	R; GTCTATCTGCACTTGGTGG		

The primer design for *CHI*, *PCBER*, *PrR*, *PAL CHS*, and *CHI* genes is a meticulous process that begins with the identification of the gene sequence. This sequence can be sourced from a reliable database. In this research, the sequence was collected in NCBI, scientific paper, and from some species of the same genus. The next step involves selecting locations on the gene sequence for the forward and reverse primers. These locations should ideally flank the region of the gene that is intended for amplification.

The design of the primer sequences is a critical step that is based on several key criteria. The primers typically be 18 - 24 bases in length, with a G/C content within the range of 40 - 60%. The melting temperature ™ should lie within 50 - 60°C, and the Tm of primer pairs should not deviate by more than 5°C from each other. It is also essential to ensure that the primers do not contain complementary regions to prevent primer-dimer formation.

To ensure specificity, Primer-BLAST has been used to verify that the primers will bind exclusively to the target gene and not to other regions in the genome. Once the primer design had been finalized and deemed satisfactory, it was ordered from the supplier.

3.5. PCR Amplification

Polymerase Chain Reaction (PCR) was employed to amplify the target genes involved in flavonoid biosynthesis. Gene-specific primers were designed using bioinformatics tools and synthesized by a commercial provider. PCR reactions were performed in a thermal cycler, consisting of the cDNA template, gene-specific primers (Table 1), dNTPs, DreamTaq DNA Polymerase, $MgCl_2$, and Buffer. The cycling parameters included denaturation at 95°C for 20 seconds, annealing at 53°C for 20 seconds, and extension at 72°C for a 20s. The PCR products were resolved on agarose gel electrophoresis to visualize the amplified fragments.

3.6. Quantitative Real-time PCR (qPCR)

Quantitative real-time PCR (qPCR) was conducted to quantify the expression levels of target genes accurately. qPCR reactions were carried out using a qPCR kit and a real-time PCR instrument. The reaction mixture contained cDNA, gene-specific primers, fluorescent probes, and the qPCR master mix. The amplification was monitored in real time, and the quantification cycle (Cq) values were recorded. The relative expression levels of the target genes were determined using appropriate reference genes and normalization methods.

To determine qPCR performance, create a standard curve using a series of DNA samples with concentrations ranging from 10^6 to 10^2.

3.7. Statistical Analysis

Statistical analysis was performed using appropriate software packages. The expression data obtained from qPCR experiments were analyzed using the delta-delta Ct method. This method involves calculating the difference in the cycle threshold (Ct) values between the target and reference genes (delta Ct), and then comparing these values between the control and treatment groups (delta-delta Ct). The resulting values can be used to calculate the

fold change in gene expression, providing a measure of the relative expression levels of the target genes.

4. RESULT

4.1. RNA quality and quantity analysis

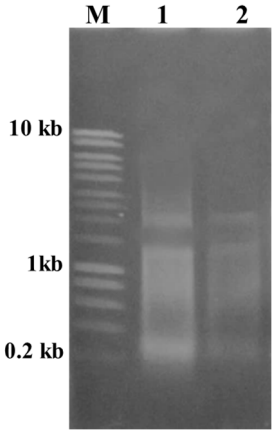

Fig. 4. RNA Samples of *Phyllanthus amarus* leaves (1), and stems (2)

To assess the quality and quantity of total RNA extracted from *P. amarus* leaves and stem, we performed denaturing agarose gel electrophoresis and spectrophotometric measurements. The gel electrophoresis showed two distinct bands corresponding to the 18S and 28S ribosomal RNAs, with no signs of smearing or degradation (Fig. 4). The 28S band was about twice as intense as the 18S band, indicating good RNA integrity. The RNA concentration and purity were determined by measuring the absorbance at 260nm and 280nm using a NanoDrop spectrophotometer. The average RNA concentration of root and leaf samples was 718.6ng/µl and 732.5ng/µl, and the average A260/A280 ratio was 1.93 and 1.9; indicating a high RNA purity.

4.2. Optimal primer design and validation for real-time PCR analysis

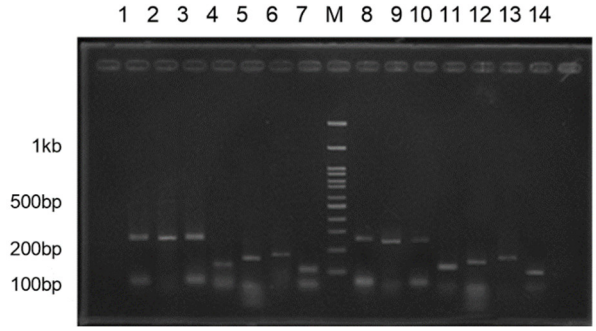

Fig. 5. Optimal real-time PCR results of *Phyllanthus amarus*. Leaf (1-7) and stem (8-14) samples with primers of PAL (1,8), PrR (2,9), PCBER (3,10), CHI (4,11), CHS1 (5,12), CHS13 (6,13), EF1 (7,14)

To validate the primer quality and quantity, we performed gel electrophoresis of the real-time PCR products using Ethidium bromide (EtBr) staining. The gel electrophoresis showed clear and single bands for each gene, with no signs of primer dimers or nonspecific amplification (Fig. 5). The expected sizes of the PCR products were confirmed by comparing them to a DNA ladder. These results demonstrate that the primers designed for *PAL*, *PrR*, *PCBER*, *CHI*, *CHS*1, *CHS*13, and EF1 genes are suitable for real-time PCR analysis of *P. amarus* leaf and stem samples.

4.3. Q-PCR result and analysis

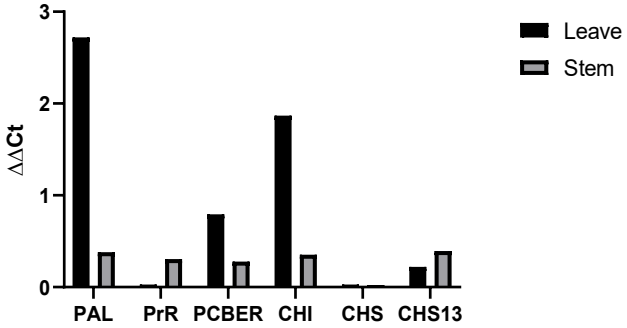

Fig. 6. q-PCR result

The qPCR results showed a significant increase in the expression of *PAL* and *CHI* in the leaves of *Phyllanthus amarus* (Fig. 6). ΔΔCt data generally do not differ too much between tubes with the same sample and primer. The ΔΔCt values were 2.2721 and 1.8687 respectively, which were significantly higher than the ΔΔCt values of 0.38 and 0.353 observed in the stems. The expression of *CHS* in both stems and leaves was not significantly higher than the control, with ΔΔCt values ranging from 0.22 to 0.35. When comparing the two biosynthetic processes of lignan and flavonoid, it can be seen that although the same process is followed by phenylalanine biosynthesis, but expression of gene flavonoid biosynthesis (*CHI* and *CHS*) is generally greater than *PrR* and *PCBER* of lignan.

The findings indicate that flavonoid biosynthesis is more active in *Phyllanthus amarus* leaves compared to lignan biosynthesis, which could explain the high flavonoid content observed in this plant species.

5. DISCUSSION

The results of this study provide valuable insights into the gene expression involved in the flavonoid biosynthesis pathway in Phyllanthus amarus. The quality and quantity of the extracted RNA were confirmed through denaturing agarose gel electrophoresis and spectrophotometric measurements, ensuring the reliability of the subsequent qPCR analysis. The distinct

bands corresponding to 18S and 28S ribosomal RNAs, along with the high RNA purity indicated by the A260/A280 ratio, suggest that the RNA was well-preserved with minimal degradation.

The successful validation of primer quality and quantity further strengthens the reliability of the qPCR results. The clear and single bands for each gene observed in gel electrophoresis indicate specific amplification without primer-dimers or nonspecific amplification.

The qPCR results indicate a significant increase in the expression of Phenylalanine Ammonia-Lyase (*PAL*) and Chalcone Isomerase (*CHI*) in the leaves of Phyllanthus amarus. This suggests that the flavonoid biosynthesis pathway, which these genes are a part of, is active in this tissue. Interestingly, the expression of Chalcone Synthase (*CHS*), another gene involved in this pathway, was not significantly higher than the control in both stems and leaves. This is intriguing because *PAL* and *CHI*, which act upstream and downstream of *CHS* in the pathway, showed high expression. This could suggest a complex regulation of this pathway. It's possible that *CHS* is being tightly regulated to control the flow of metabolites through the flavonoid biosynthesis pathway. The enzyme encoded by *CHS* catalyzes a key step in this pathway, and its activity can have a significant impact on the production of flavonoids. Alternatively, it's also possible that post-transcriptional or post-translational mechanisms are influencing *CHS* activity. For example, the *CHS* protein might be very stable or have a high catalytic activity, meaning that even low levels of gene expression could result in sufficient enzyme activity.

Besides, it was observed that the expression of genes involved in flavonoid biosynthesis (*CHI* and *CHS*) was generally greater than those involved in lignan biosynthesis (*PrR* and *PCBER*). This could suggest a higher metabolic flux towards flavonoid biosynthesis. Metabolic flux refers to the rate at which metabolites flow through a metabolic pathway. A higher expression of genes involved in a particular pathway often indicates a higher metabolic flux through that pathway, as more enzymes are available to catalyze the reactions. In this case, it suggests that *Phyllanthus amarus* might be producing flavonoids at a higher rate than lignans.

The insights gained from this study on the gene expression involved in flavonoid biosynthesis in *Phyllanthus amarus* have significant implications for future scientific research and applications. The differential expression of genes in various tissues of the plant provides a genetic basis for the enhancement of flavonoid content through selective breeding or genetic manipulation techniques. This could lead to the development of *Phyllanthus amarus* strains with increased medicinal properties, thereby expanding its therapeutic potential.

Moreover, the methodologies and findings from this study could be extrapolated to investigate flavonoid biosynthesis in other plant species, potentially leading to the discovery of new phytochemical compounds with medicinal properties. Future research could also delve deeper into understanding the environmental and physiological factors influencing flavonoid biosynthesis, which would contribute to the development of optimized cultivation practices for *Phyllanthus amarus* and similar medicinal plants. This research thus serves as a stepping stone towards the advancement of plant-based medicine and pharmacognosy.

6. CONCLUSION

This study provides a comprehensive analysis of the gene expression involved in the flavonoid biosynthesis pathway in Phyllanthus amarus. The high-quality RNA extracted from the leaves and stems of the plant, along with the successful validation of primer quality, ensured the reliability of the qPCR results.

The significant increase in the expression of *PAL* and *CHI* in the leaves suggests an active flavonoid biosynthesis pathway in this tissue. The comparison between lignan and flavonoid biosynthesis processes revealed a generally higher expression of genes involved in flavonoid biosynthesis, indicating a potential metabolic preference.

These findings enhance our understanding of secondary metabolite biosynthesis in Phyllanthus amarus, a plant known for its rich medicinal properties. However, further studies are needed to fully elucidate these pathways and their complex regulation. This could have significant implications for the pharmacological use of *Phyllanthus amarus* and could potentially lead to new applications in medicine.

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THÔNG TIN TÁC GIẢ

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