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High Quality cDNA synthesis and Amplification of Chalcone Synthase Gene (*CHS*) from *Justicia gendarussa* Burm. *F.*

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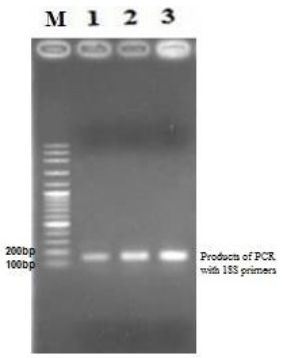
Graphical abstract



Abstract

Chalcone synthase (CHS) (EC 2.3.1.74) catalyzes the first committed step of flavonoid biosynthesis pathway. It combines 4-coumaroyl-CoA with three C2 units from malonyl-CoA to produce naringenin chalcone which transforms to various numbers of flavonoids. In this work, we attempt to isolate the *CHS* gene from Gendarusa, which could serve as a preliminary study to elucidate the *CHS* gene regulation in the flavonoid biosynthetic pathway of Malaysian medicinal plants generally, and in *Justicia gendarussa* Burm. *F.* species specifically. The total RNA was isolated from the mature leaves of *Justicia gendarussa* Burm. *F.* using the modified CTAB method. Extracted RNA, treated with RQ1 RNase-free DNasekit and cDNA synthesis in order to prepare the rich template of DNA for PCR by using M-MLV Reverse Transcriptase kit. High concentration of cDNA and A260/280 ratio reversely transcribed cDNAs guarantee the quality and quantity of synthesized cDNA. The *CHS* gene was amplified using the Phusion DNA Polymeraseand showed the same size of the previously amplified *CHS* gene from Melastoma, 1100bp.

Keywords: Flavonoids; *Justicia gendarussa* Burm. *F.*; Chalcone synthase *(CHS)*

Abstrak

Chalcone synthase (CHS) (EC 2.3.1.74) memangkinkan langkah komited pertama laluan biosintesis flavonoid. Ia menggabungkan 4-coumaroyl-CoA dengan tiga unit C2 dari malonyl-CoA untuk menghasilkan naringenin chalcone yang mengubah untuk pelbagai nombor flavonoid. Dalam karya ini, kita cuba untuk mengasingkan gen CHS dari Gendarusa, yang boleh dijadikan sebagai satu kajian awal untuk menjelaskan peraturan gen CHS dalam laluan biosynthetic flavonoid tumbuhan perubatan Malaysia secara umumnya, dan dalam Justicia gendarussa Burm. F. spesies khusus. Jumlah RNA telah diasingkan daripada daun matang Justicia gendarussa Burm. F. menggunakan kaedah CTAB diubahsuai. Dipetik RNA, dirawat dengan RQ1 RNase bebas DNasekit (Promega) dan sintesis cDNA untuk menyediakan template yang kaya DNA bagi PCR dengan menggunakan M-MLV Songsang transcriptase kit (Promega). Kepekatan yang tinggi dan nisbah cDNA A260/280 reversely cDNAs disalin penerima kualiti dan kuantiti disintesis cDNA. Gen CHS telah dikuatkan menggunakan Polymerase DNA phusion (New England Biolabs) dan menunjukkan saiz yang sama gen dikuatkan sebelum CHS dari Melastoma, 1100bp.

Kata kunci: Flavonoids; *Justicia gendarussa* Burm. *F.*; Chalcone synthase (CHS)

1.0 INTRODUCTION

Justicia gendarussa Burm. *F.* is a well-known medicinal plant originating from China (Ratnasooriya, 2007), which is commonly known as Gendarusa in Malaysia(http://www.plantnames.unimelb.edu.au/Sorting/Justicia.html). It belongs to the Acanthaceae family, and has linear-lanceolate leaves and small white flowers with pink or purple spots inside (Arokiyaraj, 2007). In traditional Indian and Chinese medicine, the leaf of the plant is used to treat ailments such as fever, hemiplegia, rheumatism, arthritis, headache, earache, muscle pain, respiratory disorders, and a digestive trouble (Paval *et al*., 2009).

Previous research (Mustafa *et al*., 2010), has reported that *Justicia gendarussa* Burm. *F.* contained high flavonoid content in different tissues such as leaves, stem and callus as well as a cell suspension (Amid *et al*., 2011). Flavonoids are the most important plant pigment (Morit, 1939) and played a crucial role in UV protection, nitrogen fixation and defending against predators and pathogens (David E. Saslowsky, 2005). *In vitro*

studies of dietary flavonoids showed anti-inflammatory properties by inhibiting the activation of enzymes which are included in the inflammatory process (Kyuichi *et al*., 2005). Recent study also proves the protective effect of Gendarusa on carrageenan-induced inflammation in mice (Kavitha *et al*., 2011).

The enzyme Chalcone synthase (CHS) (EC 2.3.1.74) (Heller W, 1980) catalyzes the first committed step of flavonoid biosynthesis (Richard, 2000). It combines 4-coumaroyl-CoA with three C2 units from malonyl-CoA to produce naringenin chalcone (Fukia *et al*., 1998) which could transform to various numbers of flavonoids (Huang et al., 2000). Several *CHS* genes have been cloned from other plants including Arabidopsis thaliana (CAI30418) (R. L. Feinbaum, 1988), Medicago sativa L. (P30074)(McKhann HI, 1994), Pinus sylvestris (CAA43166)(J. Fliegmann, 1992), Equisetum arvense (Q9MBB1)(Yamazaki Y, 2001), Psilotum nudum (BAA87922)(Yamazaki Y, 2001), Marchantia paleacea (BAD42328)(Harashima *et al*., 2004), and Physcomitrella patens (ABB84527)(Jiang, 2006). However, the enzyme properties and the mechanism of gene regulation of *CHS* from Malaysian medicinal plants are scarcely studied, although the product of *CHS*, flavonoids, have been quantified in crude extracts including in Gendarusa (R.A. Mustafa, 2010). Thus, in this work, we attempt to isolate the *CHS* gene from Gendarusa, which could serve as a preliminary study to elucidate the *CHS* gene regulation in the flavonoid biosynthetic pathway of Malaysian medicinal plants generally, and in *Justicia gendarussa* Burm. *F*. species specifically.

■2.0 EXPERIMENTAL

2.1 Cultivar Selection and Sampling

Plant *of Justicia gendarussa* Burm. *F*. Was grown outdoors at the Faculty of Bioscience and Bioengineering (FBB), under natural light and temperature. Fresh mature leaves were collected and weighed to be used for RNA extraction.

2.2 RNA Extraction

Total RNA extraction from *Justicia gendarussa* Burm. *F*. was done by using a modified CTAB method which is adapted from Japelaghi *et al*., (2011).

2.3 DNase Treatment and cDNA Synthesis

DNase treatment was carried out using RQ1 RNase-free DNasekit (Promega) as described by the manufacturer. In order to synthesis cDNA, M-MLV Reverse Transcriptase kit (Promega) was used as described by the manufacturer.

2.4 Polymerase Chain Reaction

In this study two types of primers were used in PCR reaction; a)18 S primer ;b) CHS degenerate primer.

a) It is crucial to ensure that the RT-PCR worked accordingly and the total RNA was reverse transcripted to cDNA. In order to check the fidelity of this step, a PCR reaction with 18S RNA primers (F-Q18SR 5'-AAA CGG CTA CCA CTC CAA G-3', R-Q18SR 5'-CCT CCA ATG GAT CCT CGT TA-3') was done. PCR reaction was performed in 25 µL reaction volume containing cDNA template, 5X Green GoTaq® Reaction Buffer, 10 µM of each primers, 10 µM dNTPs, enzyme Go Taq®Flexi DNA Polymerase (promega), 2mM $MgCl_2$ and distilled water. PCR condition is as follow: 1 cycle at 94°C (predenaturation) for 2 minutes, 30 cycles at 95°C (denaturation) for 1 minute, 58°C (annealing) for 1 minute, 72°C (extension) for 70 seconds; and ended with 1 cycle at 72°C (final extension) for 10 minutes. The PCR product was run on 1% agarose gel.

b) The polymerase chain reaction (PCR) was carried out by using F-dCHS1 (CCK GAC TAC TAC TTC CGS ATC) primer and R-dCHS1 (CTG TGS AGG ACR ACA GTC TC) primer. PCR reaction was performed in a 20 µL reaction volume using MyCycler Thermal Cycler System (Bio- Rad). The reaction components were prepared with ice and each of the PCR tubes contain cDNA of template, 5Xphusion HF Buffer (New England BioLabs), 10mM dNTPs, 10 µM primers, Phusion DNA Polymerase (New England BioLabs) and distilled water. PCR reaction is as follows: 1 cycle at 98°C (predenaturation) for 30 seconds; 35 cycles at 98°C (denaturation) for 10 seconds, 62°C (annealing) for 30 seconds, and 72°C (extension) for 40 seconds; and ended with 1 cycle at 72°C (final extension) for 5 minutes. All amplified products were run in 1% agarose gel and HiYield Gel/PCR fragment extraction kit (Real Biotech Corporation) was used to purify the band of interest from agarose gel according to the manufacturers' instruction.

■3.0 RESULTS AND DISCUSSION

3.1 Quality and Quantity of Total RNA Extracted from *Justicia gendarussa* Burm. *F*.

Justicia gendarussa Burm. *F*. contains lots of secondary metabolites such as flavonoids which are benzo- γ -pyrone derivatives consisting of phenolic and pyrane rings that tend to contaminate nucleic acids during extraction (Samanta, 2011). To overcome this challenge and extract the total RNA, three separate extractions were carried out from mature leaves of *Justicia gendarussa* Burm. *F*.. Table1 shows the concentration, A260/280 and A260/230 ratios of extracted RNA from three different samples. The acceptable range of this ratio for DNA sample is 1.5~2 and the best amount is about 1.8. This ratio of the extracted samples is more than 2, because of two reason; firstly, the composition of the samples which is mostly RNA, not DNA and secondary, the extraction method which mostly focus on extracting the total amount of RNA.

Table 1 Quantity and Quality of total RNA extracted from three samples of mature leaf of *Justicia gendarussa* Burm. *F*. using modified CTAB method

RNA extract	1	2	3
Concentration (ng/µl)	59.3	218.3	91.5
A260/280	2.17	2.20	2.21
A260/230	1.95	1.87	2.05

3.2 High Quality cDNA Synthesis

As mentioned previously, for cloning purposes, the RNA template used for cDNA synthesis must be DNA-free in order to avoid false amplification. All three RNA samples were treated with RQ1 RNase-free DNasekit (Promega) to remove the high

DNA contamination present. Treated samples were run on 1% agarose gel and compared with the one which has not treated with the RQ1 RNase-free DNasekit (Promega) as a control (Figure 1). Removing the DNA band by treating the samples with RQ1 RNase-free DNasekit (Promega) minimum the risk false amplification happening during the PCR as the result of DNA contamination.

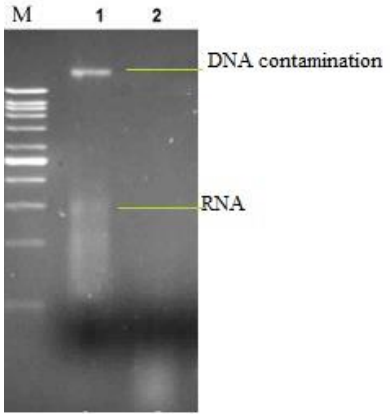


Figure 1 DNase treatment of RNA sample. M: VC 1kb DNA ladder (Vivantis), Lane 1: RNA sample 1 beforeDNase treatment, Lane 2: RNA sample 1 after DNase treatment

3.3 RT-PCR

Subsequently, single stranded DNase-treated RNA was used in RT-PCR as a template to synthesize the double stranded cDNA. The quality of transcribed cDNA was evaluated via PCR amplification using the 18S primers. If the total extracted RNA reverse transcribed correctly to cDNA, the 18S primers should bind to the cDNA and amplify the 18sRNA sequence with the length of ~150bp. These primers specifically bind to the 18S rDNA which was synthesized via RT-PCR. The length of the amplified sequence was ~150bp that prove the fidelity of this process (Figure 2).

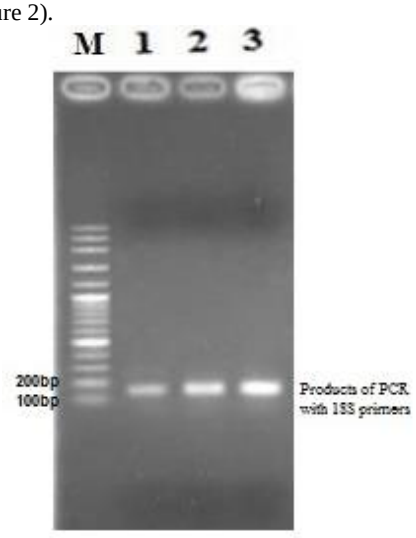


Figure 2 Products of PCR with 18S primers using separate extracted RNA as templates. M: VC100bp Plus Ladder (Vivantis), Lane1: DNase treated RNA sample 1 PCR product with 18S primers, Lane 2: DNase treated RNA sample 2 PCR product with 18S primers, Lane 3: DNase treated RNA sample 3 PCR product with 18S primers

Concentration of synthesized cDNA was assessed with nanodrop spectrophotometer. Table 2, lists the concentration, A260/280 and A260/230 ratios of reversely transcribed cDNAs. High concentration of cDNA samples and acceptable range of A260/280 ratio make these cDNA samples, good candidate to be used as the DNA template in PCR amplification of *CHS* gene.

Table 2 Concentration of three cDNA samples

cDNA sample	1	2	3
Concentration (ng/uL)	2114.7	2573.8	2689.1
A260/280	1.75	1.74	1.77
A260/230	2.22	2.17	2.20

4.3 Amplification of *CHS* Gene from *Justicia gendarussa* Burm. *F.*

PCR amplification of the *CHS* gene was performed by using the reverse transcripted cDNA as a template and the designed degenerate primersIn order to design the degenerate primers, nucleotide sequences of *CHS* in Zea mays (226505455), Arabidopsis thaliana (145357993), Vitis vinifera (225451636), Populus trichocarpa (224072643), Triticum aestivum (33465873) and Ricinus communis (255558527) were retrieved from http://www.ncbi.nlm.nih.gov and aligned (http://multalin.toulouse.inra.fr/multalin). Primers were designed accordingly and the properties of primers were assessed by OligoAnalyzer 3.1 (http://eu.idtdna.com/analyzer/applications/oligoanalyzer/default.aspx) and reported in table 3. All of the primers were obtained from First Base. Suitable GC content of the primers, appropriate annealing temperature with the difference of only 0.3°C and absence of any hair pin or primer dimer suggest these primers as the good ones apply in PCR step.

Table 3 The characteristics of CHS degenerate primers

Primer	Tm (°C)	Size (nucleotide)	GC %
F-dCHS1	54.5	21	50
R-dCHS1	54.8	20	48

A band was successfully amplified with the Phusion DNA Polymerase (Biolabs). This *CHS* gene amplified was approximately 1.1 kb in size (Figure 3) which is comparable to the *CHS* gene from *Melastoma decemfidum Roxb ex.* used as positive control (Hidayah Jamalnasir *et al.*, 2013).

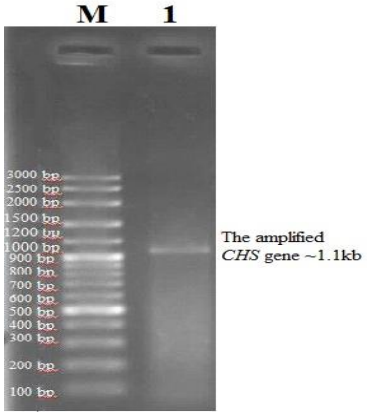


Figure 3 Amplified *CHS* gene with Phusion DNA Polymerase (Biolabs) on 1% agarose gel. The *CHS* gene amplified with the correct size of ~ 1.1 kb. M: VC 100bp Plus Ladder (Vivantis), Lane 1: amplified *CHS* gene

It should be taken into account that Phusion DNA Polymerase (Biolabs) has the proofreading activity (3´ to 5´ exonuclease) in addition to 5´ to 3´ activity of Taq DNA Polymerase (Cline, 1996). This ability of Phusion DNA Polymerase (Biolabs) alongside with 50-fold lower error rate than that of Taq DNA Polymerase make the Phusion DNA Polymerase one of the most accurate thermostable polymerases (Flaman, 1994) which is highly favourable for cloning purposes involving novel species with no database Information.

In this work, the sequencing of the *CHS* gene was not obtained because of the low concentration of PCR product which was much smaller than the one required for sequencing. In order to sequence the *CHS* gene from *Justicia gendarussa* Burm. *F*. more concentrated RNA should be extracted from the plant which could result in more concentrated PCR products. This product could be sequenced. The retrieved sequence will be aligned with the sequence of *CHS* gene from other plants to study the gene.

■4.0 CONCLUSION

In conclusion, high quality RNA was extracted from mature leaves of *Justicia gendarussa* Burm. *F*. using a modified CTAB method. concentration of extracted RNA was in an acceptable range and A260/280 ratio shows the good quality of the RNA with less chemical contamination. Accordingly, high concentrated cDNA with the suitable range of A260/280 ratio prove the purity and quality of this cDNA sample. Comparing the size of the amplified sequence with the one from *Melastoma decemfidum Roxb ex. Jack* indicate that amplified sequenced is possibly the partial *CHS* gene of *Justicia gendarussa* Burm. *F*.. However, this PCR product should be cloned in a bacteria and extracted with the same primers were used in PCR and verified by sequencing. Sequencing and cloning the *CHS* gene are important to study the Flavonoid biosynthesis pathway and manipulate the production of flavonoids.

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References

[1] Amid, A. J. N., Jamal, P., Zain, WNWM. 2011. Observation of Antioxidant Activity of Leaves, Callus and Suspension Culture of Justicia Gendarusa. *Afr J Biotechnol*. 10: 18653–18656.

[2] Arokiyaraj, S. P. K., Agastian, P. Balaraju, K. 2007. Immunosuppressive Effect of Medicinal Plants of Kolli Hills on Mitogen-stimulated Proliferation of the Human Peripheral Blood Mononuclear Cells in Vitro. *Indian Journal of Pharmacology*. 39(4): 180–183.

[3] David, E. Saslowsky, U. W., and Brenda, S. J. Winkel. 2005. Nuclear Localization of Flavonoid Enzymes in Arabidopsis. *The American Society for Biochemistry and Molecular Biology*. 1.

[4] Harashima, S., Takano, H., Ono, K., & Takio, S. 2004. Chalcone synthase-like Gene in the Liverwort, Marchantia Paleacea var. Diptera. [Research Support, Non-U.S. Gov't]. *Plant Cell Rep*. 23(3): 167–173. doi: 10.1007/s00299-004-0808-9.

[5] Heller, W. H. K. 1980. Highly Purified "flavanone synthase" from Parsley Catalyzes the Formation of Naringenin Chalcone. *Arch. Biochem. Biophys*. 200: 617–619.

[6] Hidayah Jamalnasir, A. W., Noor Azmi Shaharuddin and Azman Abd Samad. 2013. Isolation of High Quality RNA from Plant Rich in Flavonoids, Melastoma Decemfidum Roxb ex. Jack. *AJCS*. 7: 911–916.

[7] Huang, Jin-Xia, Q. L.-J., Yang Ji, YIN Hao, GU Hong-Ya. 2000. A Preliminary Study on the Origin and Evolution of Chalcone Synthase (CHS) Gene in Angiosperms. *Acta Botanica Sinica*. 46(1): 10–19.

[8] J. Fliegmann, G. S., S. Schanz, L. Britsch, J. Schröder. 1992. Molecular Analysis of Chalcone and Dihydropinosylvin Synthase from Scots Pine (Pinus Sylvestris), and Differential Regulation of These and Related Enzyme Activities in Stressed Plants. *Plant Mol. Biol*. 18: 489–495.

[9] Jiang, C. S., Clark K., Kim, S. Y., Suh, D. Y. 2006. Cloning and Characterization of Chalcone Synthase from the Moss, Physcomitrella Patens. [Research Support, Non-U.S. Gov't]. *Phytochemistry*. 67(23): 2531–2540. doi: 10.1016/j.phytochem.2006.09.030.

[10] Kavitha, S. K., Viji, V., Kripa, K., & Helen, A. 2011. Protective Effect of Justicia gendarussa Burm.f. on Carrageenan-induced Inflammation. [Research Support, Non-U.S. Gov't]. *J Nat Med*. 65(3-4): 471–479. doi: 10.1007/s11418-011-0524-z.

[11] Kyuichi, K., A. M., Hajime Ohigashi. 2005. Nobiletin, a Citrus Flavonoid, Down-Regulates Matrix Metalloproteinase-7 (matrilysin) Expression in HT-29 Human Colorectal Cancer Cells. *Bioscience, Biotechnology, and Biochemistry*. 69(2): 307–310.

[12] McKhann, HI. H. A. 1994. Isolation of Chalcone Synthase and Chalcone Isomerase cDNAs from Alfalfa (Medicago sativa L.): Highest Transcript Levels Occur in Young Roots and Root Tips. *Plant Mol Biol*. 24(5): 767–769.

[13] Morit, S. 1939. Research for Vitamin P. *Journal of Biochem*. 29(3): 487–501.

[14] Paval, J., Kaitheri, S. K., Potu, B. K., Govindan, S., Kumar, R. S., Narayanan, S. N., & Moorkoth, S. 2009. Anti-arthritic Potential of the Plant Justicia gendarussa Burm F. *Clinics*. 64(4): 357–362. doi: 10.1590/s1807-59322009000400015.

[15] R. A. Mustafa, A. A. H., S. Mohamed and F. Abu Bakar. 2010. Total Phenolic Compounds, Flavonoids, and Radical Scavenging Activity of 21 Selected Tropical Plants. *Journal of Food Science*. 75(1): 28–34.

[16] Ratnasooriya, W. D., S.A. Deraniyala and D.C. Dehigaspitiya. (2007). Antinociceptive Activity and Toxicological Study of Aqueous Extract of Justicia gendarussa Burm. F. in rats. *Phcog Mag*. 3: 145–150.

[17] R. L. Feinbaum, a. F. M. A. 1988. Transcriptional Regulation of the Arabidopsis Thaliana Chalcone Synthase Gene. *Mol Cell Biol*. 8(5): 1985–1992.

[18] Samanta, A., G. D., Sanjoy Kumar Das. 2011. Roles of Flavonoids in Plants. *Int J Pharm Sci Tech*. 6(1–3).

[19] Sylvie Richard, G. L., Robert G. Rutledge and Armand Séguin. 2000. Induction of Chalcone Synthase Expression in White Spruce by Wounding and Jasmonate. *Plant Cell Physiol*. 41(8): 983–987.

[20] Toshio Fukia, B.-S. C., Kanako Maruno, Yukiko Miyakawa, Masataka Konishi and Taro Nomura. 1998. An Isoprenylated Flavanone from Glycyrrhiza Glabra and Rec-Assay of Licorice Phenols. *Phytochemistry*. 49(7): 2005–2013.

[21] Yamazaki, Y., S. D., Sitthithaworn W, Ishiguro K, Kobayashi Y, Shibuya M, Ebizuka Y, Sankawa U. 2001. Diverse Chalcone Synthase Superfamily Enzymes from the Most Primitive Vascular Plant, Psilotum Nudum. *Planta*. 214(1): 75–84.