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The Effect of *Eurycoma longifolia* Root Methanolic Extract on Gamma Globin Gene Expression "In Vitro Study".

Background: treatment of β haemoglobinopathies by induction of fetal Haemoglobin (HbF) has been used for the last few decades. The toxicity and low efficacy of currently used compound have led to screen for new HbF inducing agents. *Eurycoma longifolia* (*E. longifolia*) has been used traditionally to treat various diseases. However, no study has been conducted to investigate its potency toward induction of HbF. **Objective:** this study aimed to investigate the effect of partially purified sub-fraction (F2) derived from resin chromatography of the crude methanolic extract of *E. longifolia* roots on γ globin gene expression using the human erythroleukaemic K562 cell line model. **Methods:** K562 **cells** were maintained with and without F2, at different doses and time points. Total RNA was extracted from treated and untreated cells and reverse transcriptase PCR (RT-PCR) was conducted to semiquatitate the expression of γ globin gene. **Results:** the finding showed that, F2 has no HbF inducing activity as indicated by the non remarkable difference in the expression of γ globin gene in the treated and untreated K562 cells. **Conclusion:** The protocol developed by this study can be used for screening for HbF inducers.

Keywords: Beta haemoglobinopathies, Fetal haemoglobin, *E. longifolia*, Reverse Transcriptase PCR.

INTRODUCTION

Beta haemoglobinopathies are by far the most common monogenic disorders in human and becoming a growing health care problem and cause considerable morbidity and mortality worldwide {1}. The underlying pathophysiology of β-thalassaemia relates to a quantitative deficiency of functional β globin chains, which leads to an imbalanced globin chain production and an excess of α globin chains and subsequently ineffective erythropoiesis {2}. On the other hand, sickle cell disease (SCD) is due to the amino acid substitution of valine for glutamic acid at position 6 of the β globin chain, which results in the polymerization of sickle haemoglobin (HbS) upon deoxygenation, leading to deformed dense red blood cells {2, 3}. Elevated HbF has been demonstrated to be beneficial in SCD and β-thalassaemia because the increase in levels of HbF inhibits HbS polymerization and also corrects α / non α chains imbalance {4}. For this reason, the induction of high HbF is currently becoming a therapeutic approach in sickle cell and β-thalassaemia diseases {4}. Several studies have successfully induced HbF by various chemical compounds such as hydroxyurea, butyrate, butyrate derivatives, decitabine {5, 6} and others. However, the less efficacy and high toxicity of such compound bring challenge in the treatment of these diseases. Therefore, Screening for less toxic and more effective HbF inducer is of great importance. Natural products might be the solution. *Eurycoma longifolia* (*E. longifolia*) or Tongkat Ali is a slender, evergreen flowering tree from the Simaroubaceae family commonly found in Southeast Asia {7}. Its root organic extracts are rich in many bioactive compounds and have been widely investigated for its antimalarial, aphrodisiac, anti-diabetic, antimicrobial, anti-pyretic and anticancer activities {7}. So far, no study has been conducted to investigate *E. longifolia* extracts on induction of HbF. Therefore, this study aimed to investigate, in vitro, HbF inducing activity of partially purified sub-fractions of *E. longifolia* root methanolic extract using K-562 cells line model.

MATERIALS AND METHODS

2.1. Plant material

The plant and root samples of *E. longifolia* Jack were identified by Professor Ahmad Latif from Universiti Kebangsaan Malaysia (UKM). The plant samples were collected in 2003 from Perak, Malaysia. A voucher specimen (No. 785-117) was deposited in Penang Botanical Garden, Penang, Malaysia. The partially purified sub-fraction (F2) was obtained by resin chromatography of the crude methanolic extract of *E. longifolia* roots as previously reported by Teh, (2010) {8}. Before the treatment, F2 was dissolved in water and then was sterilized using 0.2µm filtration units (Sartorius, Germany).

2.2. Cells and culture medium
Human erythroleukaemic cell line K-562 was purchased from the American Type Culture Collection (ATCC). K-562 cells were maintained in RPMI 1640 medium (Sigma) containing 10% fetal bovine serum (Sigma), 2 mM L-glutamine, and 100 U/ml penicillin and streptomycin (Hyclon). The cells were cultured at 37 °C in a humidified incubator containing 5% CO_2.

2.3. Cell treatment and RNA isolation
K-562 cells were cultured in RPMI medium in the presence and absence of F2 at various concentrations (25, 50, 75 and 100 µg/ml for various time points (8, 16, 24 and 48 h). K-562 cells treated with hydroxyurea (50 µM) were acted as positive control. After each indicated time, cells were harvested via centrifugation at 1000 rpm for 5 minutes. The supernatant was carefully removed and the RNA was isolated from cell pellets using TRI reagent (Molecular Research Centre INC, USA).The concentration and integrity of total RNA were assessed using NanoVue spectrophotometer (GE Life Sciences, Sweden).

2.4. Reverse transcriptase PCR
One microgram of total RNA was treated for 30 min at 37°C with 1 U RNase-free DNase (Promega, Madison, USA) to remove genomic DNA. After denaturation for 10 min at 65 °C, cDNA was synthesized for 1 h at 37 °C by adding 200 U M-MLV reverse transcriptase (Promega, Madison, USA) and 50 pmol oligo (dT) primers (Promega, Madison, USA). One fifth of cDNA was used as the template for the multiplex PCR using upstream and downstream primers for γ globin gene and β actin gene (reference gene). PCR reaction was carried out in total volume of 50 µl containing: 4 µl of cDNA synthesis reaction, 20 µl of 2 X PCR master mixes (Fermentas, USA), 1 µl of (2.7µM) of each primer (Sigma) and 22 µl of DNase free water. The amplification conditions were initial denaturation at 95 ^{0}C for 2 min followed by denaturation at 95 ^{0}C for 1 min, annealing at 55 ^{0}C for 50 seconds, extension at 72 ^{0}C for seconds 35 cycles with final extension at 72 ^{0}C for 7 min. Eight µl of final product from each reaction was electrophoresed in 1.5 % agarose gel containing ethidium bromide (0.1 µg/ml) at 70 volts for 80 min and photographed under UV illumination.

RESULTS
The multiplex PCR reactions for γ globin and β actin genes were successfully optimized. The products of PCR amplification and the primers sequences used in the amplification reactions are shown in Table (I). As shown in Figure (1), the expression of γ globin gene shows no significant difference in the F2-treated and untreated K562 cells at the indicated time points. Hydroxyurea-treated cells show a remarkable difference as indicated by the thick and brighter bands.

DISCUSSION
Reactivation of HbF expression using pharmacological agents represents a potential strategy for the therapy of β-thalassaemia and SCD {5}. 5-azacytidine, hydroxyurea, erythropoietin, butyrate derivatives and combinations of these drugs have been shown to possess γ-globin chain–inducing activity {10}. This study showed that the F2 extract has no potential inducing activity of gamma globin genes. The expression was not affected by the increase in the concentration of the extract or time duration. On the other hand, hydroxyurea showed a remarkable increase in the expression of gamma globin genes which is consistent with the results from other studies {5, 10}. Several studies used quantitative real time PCR (qRT-PCR) to assess the potency of various compound to induce γ-globin gene expression {11, 12}. Using qRT-PCR is expensive and requires expensive machine. This study provides an optimized, semiquatitative RT-PCR and sheep protocol which may be used for screening for HbF inducers using other natural products or synthetic compounds. In conclusion the study showed that the F2 extract has no HbF inducing activity. However the developed multiplex PCR can be used to screen for HbF inducing agents from other sources.

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Table I. Primers sequence and PCR product sizes.

Gene	Sequence (5' – 3')	Product size (bp)
γ globin	FP: TCACAGAGGAGGACAAGGCT *	280
	RP: TCACAGAGGAGGACAAGGCT	
β actin	FP: CGTACCACTGGCATCGTGAT**	459
	RP: GTGTTGGCGTACAGGTCTTTG	

**The primers were designed by authors.*
***Published by (Lockwood CJ et al., 2006) {9}.*

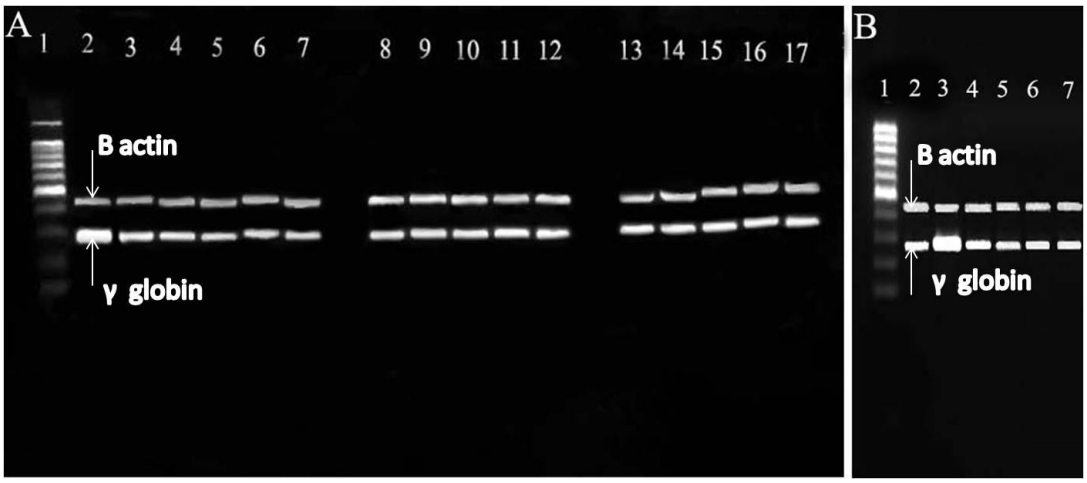

Figure 1. Expression of γ-globin gene in K562 cells maintained with and without F2 extract. K562 cells treated with 50 µM hydroxyurea acted as positive control. Total RNA was isolated from treated and untreated K562 cells, converted to cDNA and subsequently was subjected to reverse transcriptase PCR. Ethidium bromide stained gels show two types of PCR products: γ-globin gene (lower bands) and β actin gene (upper bands).
Lane A1 and B1: 100 bp DNA ladder, Lane A2 and B3: hydroxyurea; Lane A3, A8, A13 and B2: untreated cells (negative control); Lane A4-7: F2 (25, 50, 75 and 100 µg/ml respectively) at 8 h; A9 – 12: F2 (25, 50, 75 and 100 µg/ml respectively) at 16 h; A14 – 17: (25, 50, 75 and 100 µg/ml respectively) at 24 h; B4 – 7: F2 (25, 50, 75 and 100 µg/ml respectively) at 48 h.