Screening of Medicinal Plants for Fetal Hemoglobin Inducer as Alternative Treatments in β-Thalassemia

β-Thalassemia caused defect in β-globin chain synthesis leading to accumulation of excess unbound α-globin chains consequently pathologies of red blood cells. Alternative treatment by stimulating γ-globin chain production that assembles with α-globin chains to form fetal hemoglobin (HbF) could reduce excess unbound α-globin chains consequently decreasing clinical severity of β-thalassemia. This study aims to find HbF inducing property from medicinal plant extracts. Human erythroleukemic cell reporter line carrying EGFP under control of γ-globin promoter was used for screening 24 crude extracts from 14 medicinal plants. Three crude extracts from Crinum amabile, Crinum asiaticum var. asiaticum and Olax scandens showed high HbF induction activity (>2-fold increased) and low toxicity (>80% cell viability).

Keywords: β-Thalassemia, Fetal hemoglobin, Medicinal plants

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Introduction

β-Thalassemia is a worldwide common inherited red cell disorder. Each year, it is estimated that 70,000 children are born with various forms of thalassemia and over half of them are affected by severe forms of β-thalassemia (Mettananda, Gibbons and Higgs, 2015). In Thailand, it estimates there are 3-9% β-thalassemia carriers and 30-50% hemoglobin E (HbE) carriers (Chaibunruang et al., 2018). The disease arises from mutations in the β-globin gene on chromosome 11 resulting in the absence or reduce β-globin synthesis (Cao and Galanello, 2010). The continued production of normal level of the α-globin chain leads to the imbalance between α/β-globin chain synthesis causing the accumulation of excess unbound α-globin chains. The insoluble α-globin aggregates in erythroid progenitors leading to increased oxidative stress causing erythroid precursors premature death or ineffective erythropoiesis in bone marrow and shortened red blood cells survival leading to severe anemia and pathological changes of various organs including growth retardation, endocrinopathies, severe bone changes, osteoporosis, hepatosplenomegaly, immune abnormalities, severe infection, pulmonary hypertension, thromboembolic events, heart failure and other complications (Ribeil et al., 2013; Voskou et al., 2015).

β-Thalassemia patients with severe anemia required regular blood transfusions. However, the long-term blood transfusion causes alloimmunization and iron overload. Iron deposit in organs such as liver and heart resulted in organ failure. Iron chelation such as deferiprone is recommended to reduce iron accumulation. Nevertheless, their side effects are agranulocytosis, gastrointestinal symptoms, arthralgia, zinc deficiency and fluctuating transaminase levels (Galanello and Campus, 2009). The only curative treatment is hematopoietic stem cell transplantation (HSCT), which had limitations such as difficult to find HLA-matched between donor and recipient and high treatment cost. While gene therapy is still in infancy stage and very high cost. In addition, both treatments require well clinical management of conventional therapeutics means that patients had no thalassemic face and no complications such as hepatosplenomegaly before HSCT or gene therapy (Cao and Galanello, 2010; Cappellini et al., 2018). Therefore, due to these limitations alternative treatment is needed.

The clinical severity in patients is determined by the excess free α-globin chain pool (Mettananda, Gibbons and Higgs, 2015; Srinoun et al., 2009). The HbF inducer can stimulate γ-globin chain production that bind with free α-globin and form fetal hemoglobin (HbF, α₂γ₂) consequently reducing severity in the patients. Hydroxyurea, the only US FDA-approved HbF inducer, reduces the need for blood transfusions in β-thalassemia patients. However, there are reports about side effects, including cytopenia, hyperpigmentation, weight gain, azoosperma and marked hypomagnesemia (de Dreuzy et al., 2016). Moreover, it also exhibits variable responders with an approximately 20-50% partial responders and non-responders in transfusion dependence β-thalassemia patients. Therefore, novel HbF inducing agents are needed.

Plant constituents from medicinal plants have been reports to induce HbF expression such as flavonoid from Anaxagorea luzonensis (Pabuprapap et al., 2019), curcuminoids from Curcuma longa
(Chaneiam et al., 2013), labdane diterpenes from Curcuma comosa (Chokchaisiri et al., 2010), resveratrol from grape (Fibach et al., 2012) and angelicin from Aegle marmelos (Lampronti et al., 2003). However, these compounds were still low HbF induction efficiency. There is potential that plant constituents from medicinal plant would be novel HbF inducers with low toxicity, which could be uses as alternative treatment for of β-thalassemia patients.

Here, 24 crude extracts from 14 medicinal plants were screened in a K562:Δγ-γ-EGFP reporter cells, a human erythroleukemic cells that carry EGFP under control of γ-globin promoter. Three crude extracts from Crinum amabile, Crinum asiaticum var. asiaticum and Olax scandens showed high HbF induction activity with low toxicity.

Objective of the study

This study aims to identify candidate crude extracts from medicinal plants that have high HbF induction activity and low toxicity in a K562:Δγ-γ-EGFP reporter cell line.

Methodology

The crude extracts from medicinal plants

Sources of the plant materials: Crinum amabile and Crinum asiaticum var. asiaticum were collected from Nonthaburi Province, Thailand; Derris scandens was purchased from Bansamunpaisot herbal store, Bangkok, Thailand; Rhoeo spathacea was collected from Bangkok, Thailand; Stephania pierrei was collected from Prachin Buri Province, Thailand; and Vitex glabrata was collected from Takhli District, Nakon Sawan Province, Thailand. Auricularia auricule, Mimusops elengi, Tourneforlia roxburghii and Volvariella volvacea were collected from Yangon, Myanmar; Byttneria pilosa was collected from Rakhine State, Myanmar; Mucuna puriens was collected from Sagaing Region, Myanmar; Olax scandens and Xylocarpus sp. were collected from Ayeyarwaddy Region, Myanmar.

A. auricule, B. pilosa, M. elengi, M. puriens, O. scandens, R. spathacea, T. roxburghii, V. volvacea and Xylocarpus sp. were pulverized and macerated with methanol and the methanol extracts were obtained. The solvents were evaporated to produce the lyophilized form. C. amabile, C. asiaticum var. asiaticum, S. pierrei and V. glabrata were minced and macerated with methanol. The filtered solution of each extract was evaporated in vacuo to yield the crude methanol extract, which was then suspended in distilled water and sequentially partitioned with n-hexane, ethyl acetate and n-butanol to produce lyophilized crude extracts (see last column of Table 1). D. scandens was pulverized and extracted successively with n-hexane, ethyl acetate and methanol. The solvents were evaporated under reduced pressure for lyophilization. The lyophilized stock was dissolved in 100% dimethyl sulfoxide (DMSO, Sigma) at a concentration of 10 mg/mL, aliquot for single-use and stored at -20°C in the dark. Hydroxyurea (Sigma) was dissolved in sterile distilled water and filtered through a 0.2 μM filter at a
concentration of 10 mg/mL, stored at 4°C. Cisplatin (Pfizer) was dissolved in sterile distilled water and filtered through a 0.2 µM filter at concentration 3.3 mM, stored at 4°C.

A reporter cell line

A reporter cell line, K562::Δγ-γ EGFP cells, containing green fluorescence protein (EGFP) coding sequence in-frame replacement of the Δγ- and γ-globin coding sequence in the human β-globin cluster was used for screening of γ-globin gene inducer (Vadolas et al., 2004). The cells were cultured in SPL lifesiences® T-75 flasks containing RPMI 1640 supplemented with 20% fetal bovine serum (FBS) (Sigma) and 1% penicillin/streptomycin (GIBCO-Invitrogen). K562::Δγ-γ EGFP cells were incubated at 37°C in a 5% CO₂ in air atmosphere and maintained the cell density of the culture by replacement of fresh medium every 3-4 days. Start new cultures at 1 x 10⁵ viable cells/mL. Subculture at 1 x 10⁶ cells/mL.

Treatment for inducing HbF production

K562::Δγ-γ EGFP cells were seeded in 24-well plates at final concentration 1.5x10⁵ cells/mL at final volume 2 mL. Cells were treated with either crude extracts at different concentrations (1, 10, 20, 50 and 100 µg/mL) or hydroxyurea (1, 10, 20, 50 and 100 µg/mL) or cisplatin (10 µM) or DMSO (0.5% v/v) at 37°C in a 5% CO₂ in air atmosphere for 5 days without addition or replacement of medium. Hydroxyurea and cisplatin were used as positive control while DMSO was used as vehicle control. Experiments were done in 3 replications at different passages of the cell lines.

Analysis of EGFP expression and cell viability

The induction of EGFP expression in treated K562::Δγ-γ EGFP cell was measured by FACScan flow cytometer and Cell Quest software (BD Bioscience). The cell was stained with propidium iodide (PI) and immediately analyzed by FACScan flow cytometer, Cell Quest software (BD Bioscience). Data was acquired from 10,000 events of total cells. The mean fluorescent intensity (MFI) of EGFP in crude extracts-treated reporter cells were normalized with MFI of EGFP in DMSO-treated cells as formula;

\[
\text{Fold change of EGFP expression} = \frac{\text{Mean fluorescent intensity of EGFP from crude extract-treated cells}}{\text{Mean fluorescent intensity of EGFP from DMSO-treated cells}}
\]

Statistical analysis

The fold changes of EGFP expression and the percentages of cell viability were shown as mean±SD of three independent measurements and statistically analyzed by paired-T test compared between groups. Values of significance were considered at \( P<0.05 \).
<table>
<thead>
<tr>
<th>Crude extracts code</th>
<th>Common Thai name</th>
<th>Scientific name</th>
<th>Part of plants</th>
<th>Extraction solvent</th>
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<tr>
<td>AA-MeOH</td>
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<td>Auricularia auricule-judae</td>
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<td>Kum-Yan-Kreua</td>
<td>Byttneria pilosa</td>
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<td></td>
<td>Heartwoods</td>
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<tr>
<td>CA(R)-Hexane</td>
<td>Plap-Pleung-Daeng</td>
<td>Crinum amabile</td>
<td>Bulbs</td>
<td>Ethyl acetate</td>
</tr>
<tr>
<td>CA(R)-EtOAc</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
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<td>CA(R)-BuOH</td>
<td></td>
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<td></td>
<td>n-Butanol</td>
</tr>
<tr>
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<td>Plap-Pleung-Khao</td>
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<td>Bulbs</td>
<td>Ethyl acetate</td>
</tr>
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<td>Mimusops elengi</td>
<td>Flowers</td>
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<td>Xylocarpus sp.</td>
<td>Seeds</td>
<td>Methanol</td>
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</table>

Highlight presents crude extracts that were high efficiency to induce HbF production and low toxicity.
Results

Screening for HbF inducing activity from crude extracts of medicinal plants

Twenty-four crude extracts from 14 medicinal plants were screened for HbF inducing activity in K562::Δγ-γ EGFP reporter cells. The medicinal plants, Auricularia auricule-judae, Byttneria pilosa, Crinum amabile, Crinum asiaticum var. asiaticum, Deriis scandens, Mimusops elengi, Mucuna pruriens, Olax scandens, Rhoeo spathacea, Stephania puierei, Tournefortia roxburghii, Vitex glabrata, Volvariella volvacea and Xylocarpus sp. were extracted with n-butanol or ethyl acetate or hexane or methanol (Table 1). Cisplatin and hydroxyurea, well-known HbF inducers, were used as positive controls.

The EGFP expression and cell viability were measured and analyzed by using flow cytometry. The FSH-H/SSC-H analysis of the reporter cell line without debris in region R1 (Figure 1A) and then, collected PI negative cells as % cell viability in region R2 (Figure 1B). Unstained samples were used as a negative control for fixed gate region R2 (Figure 1C). The expression of EGFP in PI negative cells was analyzed in region R3 (Figure 1D).

The effect of crude extracts on the reporter cells was classified into 4 groups according to HbF inducing activity and toxicity as 1) high HbF induction and high toxicity (HH), 2) high HbF induction and low toxicity (HL), 3) low HbF induction and high toxicity (LH) and 4) low HbF induction and low toxicity (LL) (Figure 2). The cut-off of high HbF induction was ≥2-fold increasing of EGFP expression compared to DMSO-treated K562::Δγ-γ EGFP reporter cells while low HbF induction was <2-fold increasing of EGFP expression compared to DMSO-treated K562::Δγ-γ EGFP reporter cells. The cut-off of high toxicity was <80% cell viability measured by PI staining while low toxicity was ≥80% cell viability measured by PI staining.
Figure 2  Classification of 24 crude extracts from 14 medicinal plants according to HbF induction and toxicity. High induction was ≥2-fold increasing of EGFP expression compared to DMSO-treated K562::Δγ-αγ EGFP reporter cells. Low induction was <2-fold increasing of EGFP expression compared to DMSO-treated K562::Δγ-αγ EGFP reporter cells. High toxicity was <80% cell viability measured by PI staining. Low toxicity was ≥80% cell viability measured by PI staining.

Increased EGFP expression in cells treated with 10 µM cisplatin and 50 and 100 µg/mL hydroxyurea was found as positive control (Figure 3A). High toxicity of cisplatin was also observed (78.84±8.3%) (Figure 3B). While hydroxyurea at concentrations 1 to 100 µg/mL had low toxicity. Moreover, there were 3 crude extracts that had high HbF induction activity with low toxicity, the criteria for candidate HbF inducing agents, which were ethyl acetate extract from *Crinum amabile*, ethyl acetate extract from *Crinum asiaticum* var. *asiaticum* and methanol extract from *Olax scandens* (Figure 2). The remained 13 crude extracts had low HbF induction efficiency while 8 crude extracts had high toxicity (Figure 2). The most effective doses that induced the highest EGFP expression in cells treated were 50 µg/mL ethyl acetate extract from *Crinum amabile*, 10 µg/mL ethyl acetate extract from *Crinum asiaticum* var. *asiaticum* and 100 µg/mL methanol extract from *Olax scandens* (2.01±0.16, 2.09±0.22 and 2.82±0.31 folds, respectively) (Figure 3A). The cell viability of cells treated with acetate extract from *Crinum amabile*, ethyl acetate extract from *Crinum asiaticum* var. *asiaticum* and methanol extract from *Olax scandens* at the most effective dose were 84.95±3.55%, 93.21±1.26% and 81.95±5.95%, respectively (Figure 3B).

Discussion and Conclusions

The standard treatments for β-thalassemia nowadays are blood transfusion and iron chelation. The only curative treatment is hematopoietic stem cells transplantation, which has limitations. The pathology of β-thalassemia resulted from the excess unbound α-globin chains and the extent of free α-globin chains determines the clinical severity of thalassemia patients. Reducing the amount of excess unbound α-globin chains by stimulating γ-globin chains production is an alternative way to decrease clinical severity of the patients. The γ-globin chains can combine with the excess unbound α-globin chains to produce HbF and lead to improve the severity in the patients. Hydroxyurea, the only FDA-approved HbF-inducing agent, has side effects such as neutropenia and thrombocytopenia lead to risk
Figure 3  Analysis of EGFP expression and cytotoxicity of K562::Δγ-γ EGFP cells were treated with crude extracts from medicinal plants. The K562::Δγ-γ EGFP cells were untreated (UN) or treated with ethyl acetate extract from Crinum amabile (CA(R)-EtOAc), ethyl acetate extract from Crinum asiaticum var. asiaticum (CA(W)-EtOAc) and methanol extract from Olax scandens (OS-MeOH). Cells were treated with either hydroxyurea (HU, 1, 10, 20, 50 and 100 µg/mL) or cisplatin (CI, 10 µM) as positive controls. As vehicle control, cells were treated with DMSO (DM, 0.5%v/v). The fold changes in EGFP expression were compared with DMSO treated cells. (A) EGFP expression and (B) cell viability of K562::Δγ-γ EGFP cells were analyzed after 5 days of treatment by flow cytometry. The fold-changes were calculated by normalizing with DMSO-treatment. Data presents mean±SD. *Significant difference when compared with DMSO treatment at P < 0.05. **Significant difference when compared with DMSO treatment at P < 0.01. The arrows showed the most effective dose of the crude extracts that induced the highest EGFP expression with low toxicity. CI; cisplatin, DM; DMSO, HU; Hydroxyurea and UN; untreated cell.
of susceptible to infection and bleeding. Moreover, a notable proportion of patients have poor outcomes or no response to hydroxyurea treatment (Testa, 2009). Therefore, better compounds that can induce higher levels of HbF with less or no drug adverse effects are needed. This study is a first step in finding novel HbF inducer drug candidates from medicinal plants for treatment of β-thalassemia patients. Three from 24 crude extracts screened showed high HbF induction activity with low toxicity including ethyl acetate extract from bulbs of *Crinum amabile*, ethyl acetate extract from bulbs of *Crinum asiaticum* var. *asiaticum* and methanol extract from leaves of *Olax scandens*.

*Crinum amabile* or Plap-Pleung-Daeng in Thai and *Crinum asiaticum* var. *asiaticum* or Plap-Pleung-Khao in Thai is in the family Amaryllidaceae. *Crinum* is usually used for medicinal purposes due to its alkaloid constituents (Ilavenil et al., 2011). The alkaloids have various bioactivities, such as antitumor, antibacterial, antifungal and immune stimulation (Sun et al., 2009). Moreover, bulbs extracted from these 2 plants contain flavonoids, tannins and saponins (Diego et al., 2018; Riris, Simorangkir and Silalahi, 2018; Vishnu and Srinivasa, 2017). There have been reported that flavonoid extracted from *Anaxagorea luzonensis* promotes the γ-globin expression (Pabuprapap et al., 2019). However, the mode of action is still unclear.

*Olax scandens* or Parrot olax in Myanmar or Kradeaa, Krathxk, Sak-Krathxk, Krathxk-ma, Nançum, Nangchom and Phak rud in Thai has been reported for use as food and therapeutic purpose. Different parts of the plant were used to relieve fever, constipation and cough. Mouth ulcers can be cured by using freshly young leaves. Fermentation of boiled leaves was applied externally in headaches (Misra and Misra, 2014). However, this study is the first evidence shown that crude extracts from leaves of *Olax scandens* could be used for HbF induction. The phytochemical contents of the leaf extracted from *Olax scandens* consisting of alkaloids, saponin, tannin and triterpenoids (Naik et al., 2015). Cucurbitacin D, a highly oxygenated triterpenoid, has been proposed as p38 pathway activator and stabilization of γ-globin mRNA leading to induces HbF synthesis in K562 cells and human hematopoietic progenitors from β-thalassemia patients (Liu et al., 2010). The crude extract from *O. scandens* might contain compounds that induce HbF production and might contain some toxic substances that effect on cell death. Further studies to identify the active chemical compound in the crude extract and synthesize analogs of the candidate compounds to improve HbF induction with low toxicity are needed.

In conclusion, high throughput screening using K562::Δγ-Δγ EGFP reporter cell lines was simple, rapid and low cost to use for screening of novel HbF inducing agent from medicinal plants. Two crude extracts with high HbF induction and low toxicity were identified. Active compound(s) in the crude extract needed to be determined and validated in primary erythroid progenitor cell cultures obtained from normal volunteers and/or β-thalassemia patients. In addition, structural modification of candidate active compound(s) to improve efficacy is warrant. This study provides the first step in discovery candidate drug for further pre-clinical and preclinical study for treatment of β-thalassemia patients. This also will be increasing the value of medicinal plants and economics in the country.
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References


