Iron-Chelating and Anti-Hemolytic Properties of Ethanolic Extract of Lotus (*Nelumbonucifera* Gaertn) Leaves

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Background: *Iron overload is the major consequence of blood transfusion in β-thalassemia patients. Redox iron plays a critical role in the formation of reactive oxygen species and subsequently leads to oxidative stress damage in many cells, especially red blood cells and hepatocytes. Iron deposition in hepatocytes is associated with fibrosis and cirrhosis. Polyphenolic compounds found in natural products are interesting iron chelators and antioxidants.*
Objective: *This study aims to evaluate the iron-chelating properties and free-radical scavenging activities of lotus leaf extract in iron-loaded HepG2 cells.*
Material and Method: *Lotus (Nelumbonucifera Gaertn) leaves were extracted with 80% (v/v) ethanol. The extract was examined for free-radical scavenging activity by using 2, 2-diphenyl-1-picrylhydrazyl assay (DPPH assay); iron-binding and anti-hemolytic activities using spectrophotometrical method. Iron-depriving activity of the extract was determined in iron loaded human hepatocellular (HepG2) cells using fluorescence technique.*
Results: *The lotus extract showed antioxidant and anti-hemolytic activities in a concentration-dependent manner. Furthermore, it was able to bind iron rapidly and was saturated within 10 minute. With 24-hour treatment, this extract dose dependently decreased the level of labile iron pool in iron loaded HepG2 cells.*
Conclusion: *Lotus leaf extract had strong antioxidant activities, iron chelating properties on iron loaded HepG2 cells and anti-hemolytic activity.*

Keywords: *Antioxidant, Iron-chelating, Free radicals, Anti-hemolytic, Labile iron pool, Iron overload*

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Iron is an essential trace element for all life and plays an important role in oxygen sensing and transport, electron transfer, and catalysis[1]. Excessive iron caused catalyzes the production of a variety of reactive oxygen species (ROS), such as superoxide anion, hydrogen peroxide and hydroxyl radicals via Haber-Weiss and Fenton reactions[2]. ROS generation by iron is attributed to the ability of the metal in the redox cycle and can damage a variety of cells and tissues including the heart, liver, pancreas, erythrocytes and endocrine glands, resulting in dysfunctions of the organs[3].

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Multiple blood transfusions are responsible for iron overload in β-thalassemia patients, which this can lead to a high accumulation of intracellular iron and saturation of plasma transferrin. The excess iron appears in successive forms of toxic iron as labile iron pool (LIP) in cytoplasm, non-transferrin bound iron (NTBI) and labile plasma iron (LPI) in serum[4-6]. LIP acts as an intermediate or transitory pool between extracellular iron and the cellular iron associated with proteins. The requirements of iron are regulated and maintained in the cells, but an excess is prevented from developing as it will trigger cellular damage[7]. LIP is a low-molecular-weight pool of weakly chelated iron (ferrous and ferric ion) present in cytoplasm of the cells[8,9] and it can be scavenged by permeated lipophilic iron chelators[8]. Nowadays, three chelators including deferiprone (DFP), deferoxamine (DFO) and deferasirox

(DFX) are in clinical use for the treatment of chronic iron overload[10-13]. Effectiveness, cost, compliance, quality of life and side effects of the chelators are all relevant considerations. Plant polyphenols may be considered as a bifunctional antioxidant and iron-chelating compounds. The compounds can prevent damage from harmful ROS, potentially through radical scavenging, inhibition of ROS generation and iron chelation[14]. The polyphenols, especially, EGCG and ECG that are abundant in green tea have high antioxidant activity as well as iron binding activity[15]. Moreover, flavoniods such as quercetin, luteolin, kaempferol, galangin and chrysincan exhibit antioxidant behavior related to their iron-chelating ability[16].

Lotus (*Nelumbonucifera* Gaertn), an aquatic vegetable, belonging to the Nymphaeaceae family is cultivated in Eastern Asia for food and as a traditional medicinal herb. Almost all the tissues of lotus, including leaves, flower stalks, flower stamens, seeds and rhizomes, are used in traditional medicine[17,18]. Many studies have mentioned their pharmacological and physiological activities, including anti-oxidation[19,20], anti-HIV[21], anti-obesity[22,23] and anti-bacterial activity[24]. Flavonoids and phenolic compounds including quercetin-3-O-beta-D-glucuronide, hyperoside, and isoquercitrin, are abundant in the lotus leaves[19,25]. The antioxidant activity of lotus leaves has been reported, but the iron-chelating activity remains unknown. In this study, first we investigated the phenolic, flavonoid contents and antioxidant activity in the ethanolic extract of lotus leaves. Secondly, iron chelating activity of the extract was elucidated in vitro and in iron-loaded HepG2 cells. Finally, cytotoxicity and anti-oxidative stress activity of lotus leaf extract in HepG2 cells were estimated.

Material and Method

Chemicals

A stock of ferric nitrate (AAS iron reagent, 1,000 ppm, in 0.5% HNO_3; APS Finechem, Seven Hills, Australia) was used as the iron source for the preparations. 3-[N-morpholino] propanesulfonic acid (MOPS), hydrogen peroxide, catechin, ascorbic acid, gallic acid, 3-(4, 5-dimethylthiazolyl-2)-2, 5 diphenyltetrazolium bromide (MTT), and 2, 2'- azo-bis-2- methyl-propanimidamidedihydrochloride (AAPH) were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). Folin Ciocalteu reagent and methanol were purchased from Merck Co. (Germany) Calcein-AM solution (Invitrogen Corporation, CA, USA), and 2', 7'-dichlorodihydrofluorescein diacetate (DCFH-DA) (Sigma-Aldrich, St. Louis, MO, USA) were the fluorescent probes. All other chemicals and reagents used were of AnalaR grade. Lotus leaves (*Nelumbonucifera* Gaertn) were collected in Ubon Rachathani, Thailand.

Lotus leaf extraction

The dried powder lotus leaves (100 g) were mixed with 1,000 ml of ethanol (80% v/v) at room temperature for 24 hour. The supernatant was collected by filtering the mixture through filter paper No. 1. Activated charcoal was added to the filtrate for 10 minute, passed through a filter paper and centrifuged. The supernatant was collected and concentrated by using rotary evaporator further lyophilized (yield = 7.28%). The extracted powder was kept at -20°C until used.

Determination of total phenolic content

The total phenolic content (TPC) of lotus leaves extract was measured using the Folin-Ciocalteau reagent[26]. Briefly, various concentrations of the extract were incubated with Folin-Ciocalteau reagent in dark for 3 minute. The mixture was added to 7.5% sodium carbonate and allowed to stand in dark for 30 minute. Optical density (OD) was read at 765 nm using UV-VIS spectrophotometer. Gallic acid was used as standard and the results were expressed as mg gallic acid equivalent (GAE)/g dry extract.

Determination of total flavonoid content

The aluminum chloride method was used for determination of total flavonoid content (TFC) of lotus leaf extract[27]. The lotus leaf extract was incubated with 5% $NaNO_2$ solution for 5 minute. Then, 10% aluminium chloride solution was added to react. The reaction mixture was treated with 1 mM NaOH solution and finally measured OD at 415 nm against reagent blank. Concentration of flavonoid in the extract was calculated from the calibration curve made from various standard catechin concentrations and expressed as mg catechin equivalent (CAE)/g dry extract.

DPPH radical-scavenging assay

Free-radical scavenging activity of the lotus leaf extract was determined by using DPPH radicals method[28]. The extract solution was incubated with 80 mM methanolic DPPH radical ($DPPH^{\bullet}$) solution (deep purple color) for 30 minute at room temperature. OD of the decolorized $DPPH^{\bullet}$ solution was measured at 517 nm with a spectrophotometer. The percentage of DPPH

radical scavenging activity was calculated by the following equation:

Inhibition (%) = $(OD_{control} - OD_{sample})/OD_{control}$ x 100

The half of the maximal inhibitory concentration (IC_{50}) was defined as the concentration necessary to decrease free radicals by 50% as measured by the OD of $DPPH^{\bullet}$.

Anti-hemolytic activity assay

AAPH generates initiating free radicals, which could attack the RBC membrane to induce lipid peroxidation and eventually cause hemolysis. The hemolysis induced by AAPH provides a good approach for studying the free radical-induced membrane damage[29]. Human RBC suspension (5% hematocrit) was prepared in phosphate buffered saline (PBS) solution, pH 7.4. The cell suspension was pre-incubated with various concentrations of lotus leaf extract at 37°C for 60 minute and subjected to hemolytic activity assay. Then, the treated cells were incubated with AAPH solution (a final concentration of 50 mM) at 37°C for 3 hour. Finally, degree of hemolysis was evaluated by measuring OD at 540 nm. The reaction without the extract was used as control sample. The percentage of anti-hemolysis was calculated from following equation:

% Inhibition = 100 x $(1 - OD_{sample})/OD_{control}$

Iron binding assay

Lotus leaf extract (a final concentration of 1 mg/ml) was incubated with ferric nitrilotriacetate (Fe^{3+}-NTA) solution, pH 7.0 (0-400 µM at final concentrations) at room temperature for 60 minute. OD of the resulting colored complex was measured continuously in the wavelength range of 200-800 nm using a scanning double-beam UV-VIS spectrophotometer. Equivalent concentrations of the extract solution were used as the blank. A stock solution of lotus leaf extract was freshly prepared by dissolving the obtained extract powder in 50 mM MOPS solution, pH 7.0. Working Fe^{3+}-NTA solution was freshly prepared by mixing the stock standard iron solution with nitrilotriacetate (NTA) solution (a molar ratio of Fe^{3+}: NTA = 1:5).

Cytotoxicity test

Cell culture

HepG2 cells were used for biochemical and toxicological study[30]. The cells were maintained in DMEM medium (Gibco™, Life Technologies, USA) supplemented with 10% (v/v) fetal bovine serum (Gibco™, Life Technologies, USA), 100 U/ml penicillin and 100 U/ml streptomycin, and incubated at 37°C under a normal humidified atmosphere (95% air and 5% CO_2).

Cell viability assay

HepG2 cells ($5x10^3$ cells/well) were incubated with the lotus leaf extract (0-400 µg/ml) in 96-well culture plates at 37°C for 24 hour. The MTT test[31] was used to investigate cytotoxic effect of the extract on the cells.

Chelation of intracellular LIP in iron loaded hepatocytes

Firstly, HepG2 cells ($5x10^3$cells/well) were incubated with 0.5 mM ferric ammonium citrate (FAC) solution at 37°C for 24 hour. Secondly, the cells were treated with the lotus extract solutions (0-100 µM) for 24 hour. Thirdly, the treated cells were incubated with 1 µM calcein-AM solution previously prepared in DMEM at 37°C for 15 minute. Finally, fluorescent intensity (FI) was measured with a 96-well spectrofluorometer ($\lambda_{excitation}$ 485 nm, $\lambda_{emission}$ 535 nm)[32]. Amount of LIP was inversely proportional to the measured FI signal[8,33].

Measurement of ROS levels in hepatocytes

DCFH-DA can penetrate into the cells and be hydrolyzed by esterase in viable cells to produce 2', 7'-dichlorofluorescein (DCFH) (reduced form), which produced DCFH will be subsequently oxidized by existing ROS to 2', 7'-dichlorofluorescein (DCF) (oxidized form). Increasing of a green fluorescent signal indicates increased intracellular oxidative stress. HepG2 cells ($5x10^3$ cells/well) were incubated with the lotus extract at 37°C for 24 hour. The treated cells were washed three times with the DMEM medium, incubated with DCFH-DA solution (10 µM) for 30 minute, and challenged with H_2O_2 solution (125 µM) for 15 minute. FI was measured with a 96-well spectrofluorometer ($\lambda_{excitation}$ 485 nm, $\lambda_{emission}$ 535 nm)[34].

Results

Antioxidant content and activity

The mean ± SD amount of TPC and TFC obtained from three independent triplicate experiments were 156.82±2.95 mg GAE/g dry extract and 78.29±9.99 mg CAE/g dry extract, respectively. Apparently, IC_{50} value of the extract was 55.71±4.85 µg/ml and percentage of DPPH radical scavenging activity was increased proportionally to the extract concentration (Fig. 1).

Anti-hemolytic activity

The inhibitory effect of the extract on AAPH

induced lipid peroxidation could diminish RBC hemolysis and consequently lower measured $OD_{415\ nm}$ value. We found that the percentage of anti-hemolytic activity of the extract was increased in a concentration-dependent manner, which it reached 92.95±1.09% when using 100 µg/ml of the extract (Fig. 2).

Iron-binding activity

Spectral analysis demonstrated that the complex resulting from the reaction of ferric ion and the lotus leaf extract constituents exhibited a predominant absorption peak at 300 nm (Fig. 3), which the binding was dependent on the lotus extract concentration. Similar result was found with ferrous ion reaction (Fig. 4).

Toxicity study in HepG2 cells

As shown in Fig. 5, the extract decreased the viability of HepG2 cells in a dose dependent manner. From the graph, IC_{20} and IC_{50} values of the extract examined in HepG2 cells were 50 and 87.5 µg/ml, respectively.

Levels of redox iron and oxidative stress in iron-loaded hepatocytes

Expectedly, treatment with the lotus leaf extract tended to lower level of LIP (representing intracellular chelable redox iron) in the FAC-loaded HepG2 cells in a concentration-dependent manner (Fig. 6). Consistently, the extract also tended to decrease

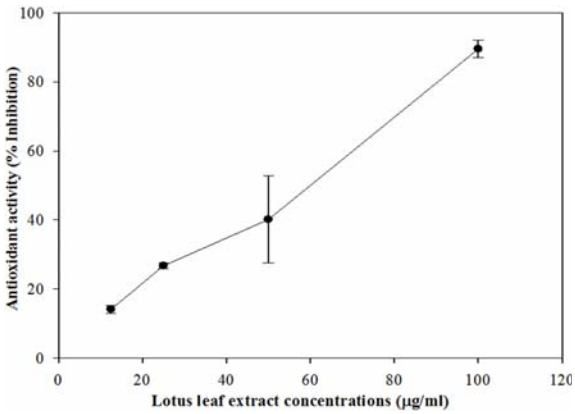

Fig. 1 Antioxidant activity of lotus leaf extract. Data obtained from three independent triplicate experiments are shown as Mean ± SD.

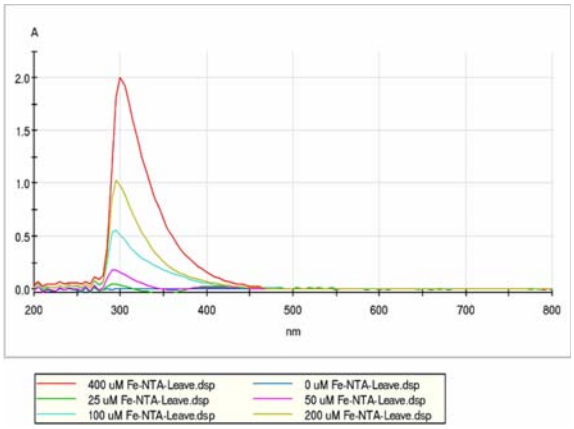

Fig. 3 Spectral analysis of the iron-chelate complex resulting from reaction of Fe-NTA (0-400 µM) and lotus leaf extract (1 mg/ml).

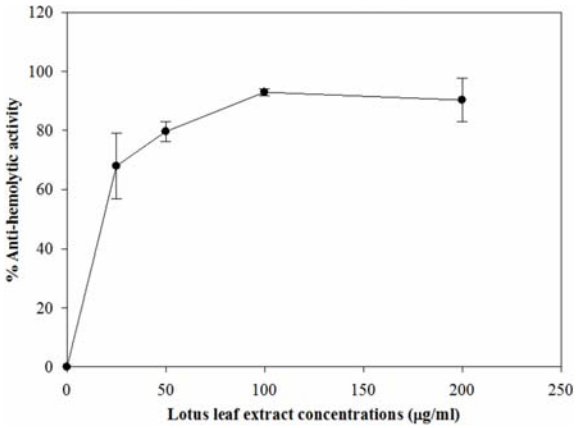

Fig. 2 Percentage of anti-hemolytic activity of lotus leaf extract. Data obtained from three independent triplicate experiments are shown as Mean ± SD.

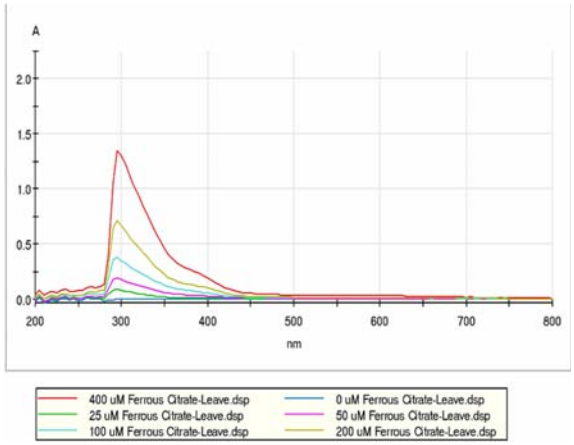

Fig. 4 Spectral analysis of the iron-chelate complex resulting from reaction of FAC (0-400 µM) and lotus leaf extract (1 mg/ml).

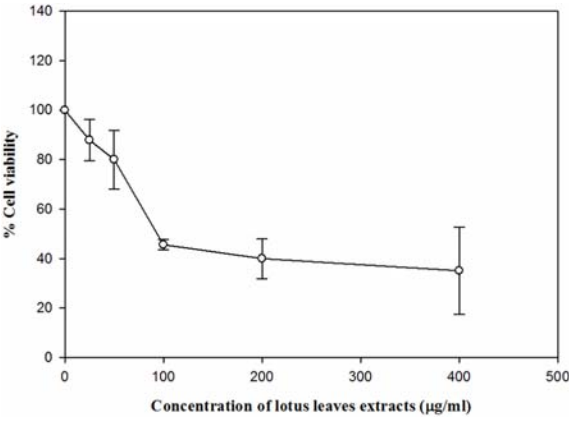

Fig. 5 Toxicity of lotus leaf extract (0-400 µg/ml) in HepG2 cells for 24 hour. Data obtained from three independent triplicate experiments are shown as Mean ± SD.

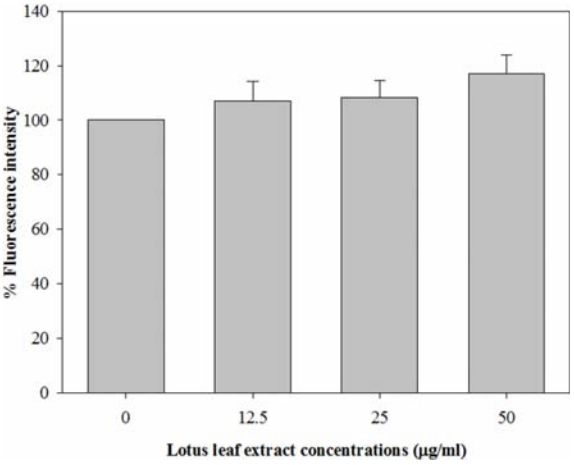

Fig. 6 Dose-response effect of lotus leaf extract on level of LIP in iron-loaded HepG2 cells. Data obtained from three independent triplicate experiments are shown as Mean ± SD.

level of ROS (representing cellular oxidative stress) in the FAC-loaded HepG2 cells in a concentration-dependent manner (Fig. 7). Obviously, treatment with 50 mg/ml lotus leaf extract was the most effective.

Discussion

Recently, phytochemicals such as polyphenolics and flavonoids have become interesting due to the therapeutic potentials of medicinal plants as antioxidants in reducing free-radical induced cell and tissue injury. The mechanism of antioxidant activities refers to both the ability of polyphenol compounds to prevent damage from ROS through radicals scavenging

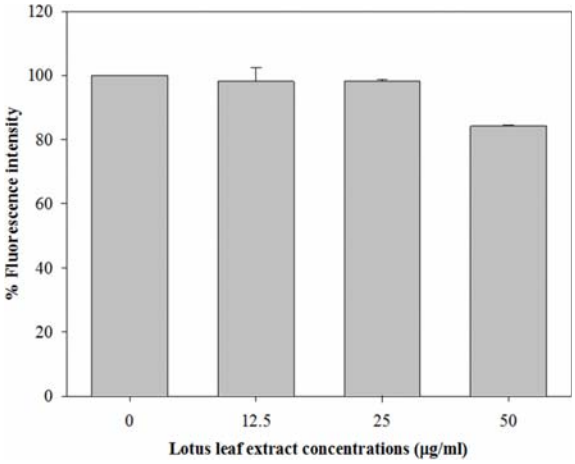

Fig. 7 Dose-response effect of lotus leaf extract on level of ROS in iron-loaded HepG2 cells. Data obtained from three independent triplicate experiments are shown as Mean ± SD.

and iron binding properties.

The lotus plant (*Nelumbonucifera* Gaertn) has a long history of use in traditional medicine such as stamens, seedpods, flowers, rhizomes and leaves[17]. Lotus leaf extract exhibited antioxidant activity, which is partially relevant to flavonoid content. Active compounds in lotus leaves were widely investigated. The phenolic constituents, including quercetin, quercetin-3-O-glucopyranoside, quercetin-3-O-glucuronide, quercetin-3-O-galactopyranoside and kaempferol-3-O-glucopyranoside showed high potent inhibition of LDL oxidation. Whereas, myricetin-3-O-glucopyranoside exerted the high capacity to scavenge DPPH radical[19]. Moreover, lotus leaf extract had a strong protective effect against UVB-induced phototoxicity in the mitochondrial model[35]. The antioxidant activity of plants might be due to their phenolic compounds. Flavonoids are groups of polyphenolic compounds with known properties, which include free-radical scavenging activity[36]. We have tested the biological activities of ethanolic lotus leaf extract. The amounts of phenolic and flavonoid contents were 56.82±2.95 mgGAE/g dry extract and 78.29±9.99 mgCAE/g dry extract, respectively.

The DPPH radical (DPPH$^{\bullet}$) scavenging assay is widely used to screen the antioxidant potential of natural products. A stable DPPH$^{\bullet}$ has a deep violet color. When antioxidant reacts with the radical, the electron is paired off and DPPH solution is decolorized. The scavenging activity of the antioxidant depends on the number of electrons taken up[27]. The strong

scavenging capacity of the extracts of lotus leaves on DPPH$^{\bullet}$ were possibly due to the hydrogen donating ability of the polyphenolic compounds such flavonoids present in the ethanolic extracts. The amount of lotus leaf extract at the concentration of 55.71±4.85 μg/ml could decrease level of DPPH$^{\bullet}$ by 50% (IC_{50}).

There are many pathological conditions associated with free-radical initiated peroxidation of membrane lipid such as hemolytic anemia (such as glucose-6-phosphate dehydrogenase deficiency) and ineffective erythropoiesis (such as β-thalassemia)[37]. Plasma membrane like red blood cell membrane contains a high amount of polyunsaturated fatty acids, which are sensitive to oxidative reaction with molecular oxygen and ferrous ion to be converted to lipid hydroperoxide. Erythrocytes are suitable for investigation of the oxidative damage on the cell membrane. Anti-hemolytic activity of various natural products was estimated by APPH induction in vitro. APPH induced membrane lipid-peroxidation of erythrocytes via the initiation of peroxy radicals[38,39]. Anti-hemolytic effects of lotus leaf extract indicated a strong peroxyl radical scavenging activity. The results indicated the protective effect of the lotus leaf extract on the oxidative damage of cell membrane in a dose dependent manner (0-100 μg/ml). Lotus leaf extract may protect erythrocytes from lipid peroxidation and hemolysis.

Redox species such as hydrogen peroxide can directly inactivate different enzymes, usually by the oxidation of essential thiol groups. It is able to cross cell membranes quickly, and may slowly oxidize a number of cell compounds[27]. The evaluation of intracellular antioxidant activity of the extract was estimated the intracellular oxidation of DCF-DA fluorescence probe. DCF is trapped within the cell after hydrolysis of the diacetate group. Hydrogen peroxide together with persisting oxidants is able to oxidize the trapped DCF, causing an increase of fluorescent intensity signals[40]. The amount of DCF formation is directly proportional to the cellular ROS production. Our result showed potent antioxidant property of the lotus leaf extract in the cells challenged with H_2O_2 when compared to the untreated cells.

Cellular excessive iron leads to toxicity and cell death via Haber-Weiss reactions-based free radical formation and lipid peroxidation, which may come, originate from sources such as regular blood transfusions for β-thalassemia patients[41]. H_2O_2 can react with ferrous ion (Fe^{2+}) to form hydroxyl radicals, and this may be the origin of many of its toxic effects. The ferrous ion increases lipid oxidation through the breakdown of hydrogen and lipid peroxides to reactive free radicals via the Fenton reaction. The iron chelating capacity of a compound is important for reducing the catalysis transition metals in lipid peroxidation[42]. In this method, lotus leaf extract binds with ferric and ferrous ion to form complexes. The complexes could inhibit iron catalyzing ROS generation. Therefore, minimizing ferric and ferrous ions by the antioxidant extracts may afford protection against oxidative damage.

The cryptozoic LIP is a source of chelatable and redox-active iron, which is transitory and serves as a crossroad of cell iron metabolism. In principle, elevated levels of LIP can promote ROS generation. Iron uptake from non-transferrin sources (such as non-transferrin bound iron, NTBI) in plasma compartment can lead to progressive deposition of excess iron in hepatic parenchymal cells and lead to oxidative damage[43-46]. The excess iron deposition is associated with liver fibrosis and cirrhosis[46]. Moreover, the two redox irons have been reported to induce lipid peroxidation in HepG2 cells[48,49]. Since cellular LIP levels are associated with ROS production, LIP is a target for iron chelator-mediated cell protection[49]. Iron overload in HepG2 cells can be initiated by progressive increase of NTBI via transferrin-independent pathways[50]. Thus, hepatocyte cultures are useful for the investigation of the protective and therapeutic effects of iron chelators against iron-induced lipid peroxidation. As evident, a previous study demonstrated that lotus leaf extract could reduce LIP in iron-loaded HepG2 cells. Consistently, flavonoids such as quercetin and its derivatives in lotus leaves exhibited an efficient reduction of cellular iron[19,25,51,52].

In conclusion, the lotus (*Nelumbonucifera* Gaertn) leaf extract is a source of polyphenols and might be used for alleviating or preventing harmfulness in many oxidative stress disorders, especially β-thalassemia patients with iron overload.

What is already known on this topic?

Previous studies, lotus leaves extract have many pharmacological and physiological activities, including anti-oxidation, anti-HIV, anti-obesity and anti-bacterial activity, but the iron-chelating activity remains unknown.

What this study adds?

This study investigated the iron-chelating properties and free-radical scavenging activities of lotus leaf extract in iron-loaded HepG2 cells. The extract had

strong antioxidant activities, iron-chelating properties on iron loaded HepG2 cells and anti-hemolytic activity. It might be used for alleviating or preventing harmfulness in many oxidative stress disorders, especially β-thalassemia patients with iron overload.

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Potential conflicts of interest

None.

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คุณสมบัติในการจับเหล็กและยับยั้งการแตกของเซลล์เม็ดเลือดแดงของสารสกัดจากใบบัว

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ภูมิหลัง: *สาเหตุหลักของการเกิดภาวะเหล็กเกินในผู้ป่วยธาลัสซีเมียชนิดบีต้าคือ การเติมเลือดเป็นประจำ และการดูดซึมธาตุเหล็กที่มากขึ้นที่ลำไส้เนื่องจากการสร้างเม็ดเลือดแดงผิดปกติ ธาตุเหล็กที่มากเกินเป็นสาเหตุหลักทำให้มีการสร้างอนุมูลอิสระจำนวนมากนำไปสู่ภาวะเครียดทำลายเซลล์ต่าง ๆ ในร่างกาย โดยเฉพาะอย่างยิ่งเซลล์เม็ดเลือดแดงและเซลล์ตับ การสะสมธาตุเหล็กปริมาณมากที่เซลล์ตับทำให้เกิดพังผืดและเกิดตับแข็งในที่สุด สารประกอบโฟลีฟีนอลจากพืชเป็นที่สนใจในการพัฒนายาขับเหล็กและยับยั้งการเกิดอนุมูลอิสระ*

วัตถุประสงค์: *ศึกษาคุณสมบัติในการจับธาตุเหล็กและลดอนุมูลอิสระของสารสกัดใบบัวในเซลล์ตับเพาะเลี้ยงที่มีภาวะเหล็กเกิน*

วัสดุและวิธีการ: *สกัดสารสำคัญจากใบบัวโดยสารละลายเอทานอล 80% ทดสอบฤทธิ์ของสารสกัดในการยับยั้งอนุมูลอิสระโดยวิธี DPPH ศึกษาคุณสมบัติในการจับธาตุเหล็กและยับยั้งการแตกของเซลล์เม็ดเลือดแดง โดยเทคนิคสเปกโตรโฟโตเมทรี ทดสอบฤทธิ์ในการลดธาตุเหล็กในเซลล์ตับโดยเทคนิคสเปกโตรฟลูออเมทรี*

ผลการศึกษา: *สารสกัดจากใบบัวมีคุณสมบัติในการยับยั้งอนุมูลอิสระและยับยั้งการแตกของเซลล์เม็ดเลือดแดงในลักษณะขึ้นกับความเข้มข้นที่เพิ่มขึ้น นอกจากนี้ยังมีคุณสมบัติในการจับธาตุเหล็กอย่างรวดเร็วภายใน 10 นาที และลดปริมาณธาตุเหล็กในเซลล์ตับในลักษณะที่ขึ้นกับความเข้มข้นภายใน 24 ชั่วโมง*

สรุป: *สารสกัดใบบัวมีคุณสมบัติในการลดอนุมูลอิสระ ลดธาตุเหล็กในเซลล์ตับเพาะเลี้ยงและยับยั้งการแตกของเซลล์เม็ดเลือดแดง*