นิพนธ์ต้นฉบับ

Bioactivities of *Leucaena leucocephala* Young Leaf Crude Extract

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Abstract

Leucaena leucocephala (*krathin* in Thai) has been used in foods and traditional medicines for a long time. This study aimed to assess *L. leucocephala* young leaf extract for determining its total phenolic content, investigate the inhibition of Heinz body formation, hemolytic effect and protection of G6PD-deficient human erythrocytes against oxidative damage, antimicrobial activity, and cytotoxicity on leukemic cell lines. The results demonstrated that the total phenolic content of *L. leucocephala* young leaf extract was 325.6 ± 0.7 mg gallic acid equivalent per gram of dried extract (mg GAE/g extract). The extract could inhibit the Heinz body formation induced by acetylphenylhydrazine (APH) and prevent hemolysis damage induced by hydrogen peroxide in a concentration-dependent manner. The highest inhibition of Heinz body formation and protection values were about 12.8% and 4.3%, respectively, at the concentration 1,000 g/mL for G6PD-deficient erythrocytes. Regarding toxicity, KG-1a cell was more sensitive to the extract than Molt4 cell. The IC20 of extract on KG-1a cell was 79.2 ± 0.5 μg/mL, but the extract had no antimicrobial effect on bacteria from clinical samples. It is thus suggested that further in vivo studies should conducted on the effect of *L. leucocephala* young leaf extract to determine its non-toxic therapeutic doses for preventing oxidative stress and hemolysis in G6PD-deficient erythrocytes.

Key words: *Leucaena leucocephala*, G6PD-deficiency, antioxidative activity, Heinz bodies' formation, leukemia

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การศึกษาฤทธิ์ทางชีวภาพของสารสกัดหยาบจากใบอ่อนกระถิน

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บทคัดย่อ

กระถิน (*Leucaena leucocephala*) เป็นสมุนไพรพื้นบ้านที่มีการนำมาใช้ในการรักษาโรคและนำมาประกอบอาหาร การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาคุณสมบัติของสารสกัดจากใบอ่อนของกระถิน ครอบคลุมในส่วนของปริมาณสารฟีนอลที่เป็นองค์ประกอบ คุณสมบัติในการยับยั้งการสร้าง Heinz body ผลที่มีต่อการแตกของเม็ดเลือดแดง และการป้องกันการแตกของเม็ดเลือดแดงของผู้ที่มีภาวะพร่องเอนไซม์ G6PD ในภาวะที่ถูกกระตุ้นด้วยสารอนุมูลอิสระ ตลอดจนคุณสมบัติในการยับยั้งการเจริญเติบโตของแบคทีเรียและความเป็นพิษต่อเซลล์มะเร็งเม็ดเลือดขาวเพาะเลี้ยง ผลการศึกษาพบว่า สารสกัดจากใบอ่อนของกระถินมีปริมาณของสารฟีนอล 325.6 ± 0.7 มิลลิกรัมสมมูลกรดแกลลิคต่อกรัมสารสกัด นอกจากนี้ ผลของสารสกัดยังสามารถยับยั้งการสร้าง Heinz body ในภาวะที่ถูกกระตุ้นด้วยสาร acetylphenylhydrazine (APH) และสามารถป้องกันการแตกของเม็ดเลือดแดงได้ในภาวะที่มีการกระตุ้นด้วยสารไฮโดรเจนเปอร์ออกไซด์ โดยคุณสมบัติดังกล่าว จะสอดคล้องกับระดับความเข้มข้นของสารสกัดที่ใช้ และเมื่อใช้สารสกัดความเข้มข้น 1,000 มิลลิกรัม/มิลลิลิตร เม็ดเลือดแดงของผู้ที่พร่องเอนไซม์ G6PD จะมีการยับยั้งการสร้าง Heinz body ได้สูงสุดที่ 12.8% และสามารถป้องกันการแตกของเม็ดเลือดแดงสูงสุดที่ 4.3% สำหรับการทดสอบความเป็นพิษของสารสกัดที่มีต่อเซลล์มะเร็งเม็ดเลือดขาวเพาะเลี้ยง พบว่า สารสกัดจะมีความเป็นพิษต่อเซลล์ KG-1a มากกว่าเซลล์ Molt4 โดยมีค่า IC20 ของสารสกัดต่อเซลล์ KG-1a ที่ 79.2 ± 0.5 ไมโครกรัม/มิลลิลิตร อย่างไรก็ตาม สารสกัดจากใบอ่อนของกระถินนี้ไม่มีคุณสมบัติในการยับยั้งเชื้อแบคทีเรียที่ได้จากสิ่งส่งตรวจของผู้ป่วย ดังนั้น จากผลการศึกษาทั้งหมดนี้ จึงควรมีการศึกษาวิจัยในมนุษย์ต่อไป ถึงผลของสารสกัดธรรมชาติจากใบอ่อนของกระถินเพื่อหาระดับของการใช้สารสกัดในรูปแบบของผลิตภัณฑ์ยาที่ไม่เป็นพิษต่อร่างกายเพื่อนำมาใช้ในการป้องกันการเกิดปฏิกิริยาจากสารอนุมูลอิสระและป้องกันการแตกของเม็ดเลือดแดงในผู้ป่วยที่มีภาวะพร่องเอนไซม์ G6PD ต่อไป

คำสำคัญ: กระถิน, ภาวะพร่องเอนไซม์ G6PD, การต้านอนุมูลอิสระ, การเกิด Heinz body, มะเร็งเม็ดเลือดขาว

Introduction and Objectives

The leaves of *Leucaena leucocephala*, which belong to the Fabaceae family have been widely used in foods and traditional herbal medicine. Extracts of *L. leucocephala* are traditionally used as control stomach diseases and it is often used as a complementary treatment for diabetes[1]. Additionally, its young shoots are generally consumed as side dish in Thailand. Previous studies have

demonstrated several biological activities, including antioxidant, antibacterial, and antidiabetic properties of *L. leucocephala* plant materials[2-4]. Furthermore, it exhibits anticancer on human oral cancer cell lines[5].

Phenolic compounds, in addition to the flavonoids, found in different parts of Leucaena were condensed tannins, quercetin and myricetin glycosides, gallocatechin, epigallocatechin, and epicatechin[6]. Flavonoids are known to prevent cell damage caused by oxidative stress. They act as antioxidants, anti-inflammatory[7-8] and cytotoxic activities (toward human breast adenocarcinoma (MCF-7), human colon carcinoma (HT29), human cervical carcinoma (HeLa), and human liver carcinoma (HepG2) cell lines in cancer cells through in vitro experiments[9]. Excessively high levels of reactive oxygen species (ROS) are detected in almost all types of cancer, suggesting that ROS play a major role in cancer development[10]. The ROS level were decreased by administering an antioxidant.[11]

Glucose-6-phosphate dehydrogenase (G6PD) deficiency is a genetic abnormality that results in an inadequate amount of G6PD in the blood. G6PD catalyzes the first step in the pentose phosphate pathway to generate NADPH which is subsequently utilized in processes that reduce oxidative stress in erythrocytes. The cells with reduced levels of G6PD are especially sensitive to oxidative stress, and the hemolytic damage in this cell is considerably higher when they are exposed to oxidizing agents. Moreover, in the absence of the enzyme, Heinz bodies result in the production, which can cause intravascular hemolysis associated with unstable hemoglobin[12]. Several studies have described the antioxidant activities of flavonoids, as free-radical scavengers. Antioxidant agents have been shown to prevent oxidative alterations in G6PD-deficient erythrocytes[13].

Leukemia is hematological disease caused by an overproduction of abnormal white blood cells. Treatments for leukemia include chemotherapy, radiation therapy, targeted therapy, antibiotics, blood transfusion, and stem cell transplant[14]. Many chemical substances derived from herbs are known to be effective anticancer agents in experimental models[14-15]. Oxidative stress is known to induce cancer. The regulation of oxidative stress is an important factor in tumor development and responses to anticancer therapies.

No study has evaluated the anti-leukemic effect and erythrocyte protective effects of *L. leucocephala* young leave extract. The present study was therefore designed to screen *L. leucocephala* young leave extract for its phytochemical constituents, evaluate antimicrobial activity, cytotoxicity on leukemic cell lines, inhibition of Heinz body formation, its hemolytic effect, and protection of normal and

G6PD enzyme-deficient human erythrocytes against oxidative hemolysis. Based on our knowledge, this is the first report about the assessment of antileukemic activity, inhibition of Heinz body formation, hemolytic effect, and protective activity of this extract.

Methodology

1. Materials

1.1 plant material

L. leucocephala young leave (3-4 layers from shoot) were collected from Western University, Kanchanaburi, Thailand in December and authenticated by Manop Poopath (Senior Botanists, Forest and Plant Conservation Research Office, Department of National Parks, Wildlife and Plant Conservation, Thailand).

2. Methods

2.1 preparation of extracts

The fresh young leaves were dried in a hot air oven at 60°C. The dried leaves were grounded and extracted by the maceration method. The powder was soaked using 80% ethanol at 1:10 w/v. The mixture was macerated for four days. The extract was filtered. The filtrate was evaporated using a rotary evaporator at 70°C, thus leaf condensed extract was obtained. The condensed extract was heated in a water bath to obtain an extract with a solid. Finally, the crude extract was kept in the refrigerator at -20°C until used and suspended in DMSO to prepare the stock solution.

2.2 total phenolic content

The total phenolic content was determined by using the Folin-Ciocalteu assay[16]. The crude extract was prepared to yield a concentration of 1 mg/mL. About 100 μL of the extract (1 mg/mL) was combined and mixed with 0.75 mL of the Folin-Ciocalteu reagent in the test tube. The liquid mixture was allowed to stand for 5 minutes at room temperature. The mixture was then added about 0.75 mL of sodium carbonate (Na_2CO_3), and the test tube was shaken gently to mix them. After 90 minutes, the absorbance against the reagent blank was measured at 725 nm with the UV-Vis spectrophotometer.

Gallic acid (range of concentration from 0.1 to 10 mg/mL) was used to calibrate the standard curve. Total phenolic content was revealed as milligrams of gallic acid equivalents per grams of the extract sample (mg GAE/g extract).

2.3 blood samples collection

The human test procedure was approved by the Western University Ethics Committee on Human Research (WTU 2564-007).

Human peripheral blood (3 mL) from 17 G6PD deficient (6 male and 11 female) and 20 healthy donors (10 male and 10 female) was collected after their consent. Erythrocytes were separated by centrifugation and then suspended in sterile PBS to obtain the 10%

erythrocyte suspension.

2.4 inhibition of Heinz body induction

Two milliliters of extract (100, 500, and 1,000 μg/mL) was mixed with 0.1 mL blood and incubated for 2 hours, and then 2 mL acetylphenylhydrazine (APH) was added. The mixture was incubated at 37°C for another 2 hours, and then Heinz bodies were counted. Positive control was prepared by adding 2 mL of APH into 0.1 mL of packed red blood cells. Negative control was prepared by adding 2 mL of buffer solution into 0.1 mL of packed red blood cells. The positive and negative control were incubated at 37°C for 2 hours. We performed the counterstain of Heinz body by transferring solutions from each test and mixed it with crystal violet solution at equal volume. The mixture was left undisturbed at room temperature for 5 minutes. A thin smear was performed, and the Heinz body was observed in at least 1,000 red blood cells under a microscope (1,000×).

2.5 hemolysis test

The properties of *L. leucocephala* extract to prevent oxidative hemolysis was evaluated by spectrophotometer method. A volume of 0.5 mL of erythrocyte suspension was added to 1 mL of the plant extract (100, 500, and 1,000 μg/mL). Then 0.5 mL of hydrogen peroxide solution was added. The reaction mixtures were incubated (4 hours, 37°C) and then centrifuged. The free hemoglobin found in the supernatant was used for measurement at 540 nm. Hydrogen peroxide solution and PBS were used as positive and negative hemolytic controls, respectively. The percentage hemolysis and protection were calculated as follows[17].

$$\% \text{ Hemolysis} = \frac{(\text{Absorbance}_{\text{sample}} - \text{Absorbance}_{\text{neg control}}}{\text{Absorbance}_{\text{pos control}} - \text{Absorbance}_{\text{neg control}}} \times 100$$

% Protection=100 % Hemolysis

2.6 microorganisms preparation

Five pathogens in total, (*Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Proteus mirabilis,* and *Klebsiella pneumoniae*), were kindly provided and identified by Maharaj Nakorn Chiang Mai Hospital, Thailand. These bacteria were isolated from clinical samples. An overnight culture of each bacterial organism was prepared. Each organism in the stock was sub-cultured into freshly prepared nutrient broth aseptically using a flamed wire loop and then incubated at 37°C for 24 hours in the incubator. This culture was used for the antimicrobial assay.

2.7 antimicrobial assay

Disk diffusion assay was performed for antimicrobial screening. The inoculums were adjusted to contain approximately 108 cells/

mL of microorganisms. Microorganisms were seeded onto Muller-Hinton agar (MHA) by using a sterile cotton swab. Sterilized filter paper disks (Whatman no. 1; 6 mm) were individually impregnated with 10 μL of 200 mg/mL extracts (resuspended in DMSO) to obtain 2 mg/disk. The filter paper discs impregnated with the extracts were placed onto the seeded plates. Ceftazidime 30 μg/disk was used as the positive control. DMSO was used as negative control. The plates were incubated at 37°C for 24 hours. All tests were performed in triplicate and the antibacterial activity was expressed as mean of inhibition diameters (mm) produced.

2.8 cell line culturing and maintenance

Molt4 and KG-1a were kindly provided by Dr. Songyot Anuchapreeda (Associate Professor of Medical Technology within Faculty of Associated Medical Sciences at Chiang Mai University). Molt4 and KG-1a were used for cytotoxicity assay. The cells were cultured in modified RPMI-1640 complete medium with 2.05 mM L-glutamine and 25 mM HEPES, supplemented with 10% heat-inactivated FBS at 37°C in a 95% humidified atmosphere with 5% CO_2. Cell counts and viability estimation by trypan blue dye exclusion test were performed regularly. Percentages of dead cells were in the range of 0-3%.

2.9 cytotoxicity assay

The cytotoxicity of crude extract was evaluated using the MTT assay. Molt4 and KG-1a were seeded at a density of 1.0×10^4 cells/well in 96-well plates and incubated overnight at 37°C with 5% of CO_2. Then, cells were treated with extract (0-100 μg/mL) for 48 hours and the complete medium with DMSO was used as vehicle control (VC). After that 15 μL of MTT dye (Sigma-Aldrich, St Louis, MO, USA) solution (5 μg/mL) was added to each well and the plate was incubated at 37°C for 4 hours. Formed formazan crystals were dissolved with 200 μL of DMSO, and the absorbance was measured at 578 nm by a AccuReader™ microplate reader (Metertech-Inc, Taipei, Taiwan) and the reference blank was 630 nm. The % cell viability was calculated by the formula as follows.

$$\% \text{ Cell viability} = \frac{\text{Absorbance of test} \times 100}{\text{Absorbance of control}}$$

2.10 statistical analysis

All data were expressed as mean ± SE. Analyses were performed in triplicates. The data were statistically evaluated using analysis of variance (ANOVA) with IBM SPSS Statistics 28.0.0.0 version. Significance levels were defined using $p < 0.05$.

Results

1. Total phenolic contents

The yield of *L. leucocephala* ethanolic extract was about 25.85%. Estimation using

Folin-Ciocalteu reagent revealed that the total phenolic contents of *L. leucocephala* young leave extract was 325.6 ± 0.7 mg gallic acid equivalent/g of dry extract (mg GAE/g extract).

2. Inhibition of Heinz body induction

Levels of Heinz body formation in G6PD-deficient erythrocytes were significantly higher than those in normal controls. The percentages of Heinz body formation by APH induction are shown in Figure 1. The *L. leucocephala* extracts could significantly inhibit the Heinz body formation of G6PD-deficient human erythrocytes in a concentration-dependent manner. The highest inhibition of Heinz bodies formation was about 12.8% at the concentration 1,000 μg/mL for G6PD deficient erythrocytes. Heinz body formation is often not recognized in normal erythrocytes (Normal control).

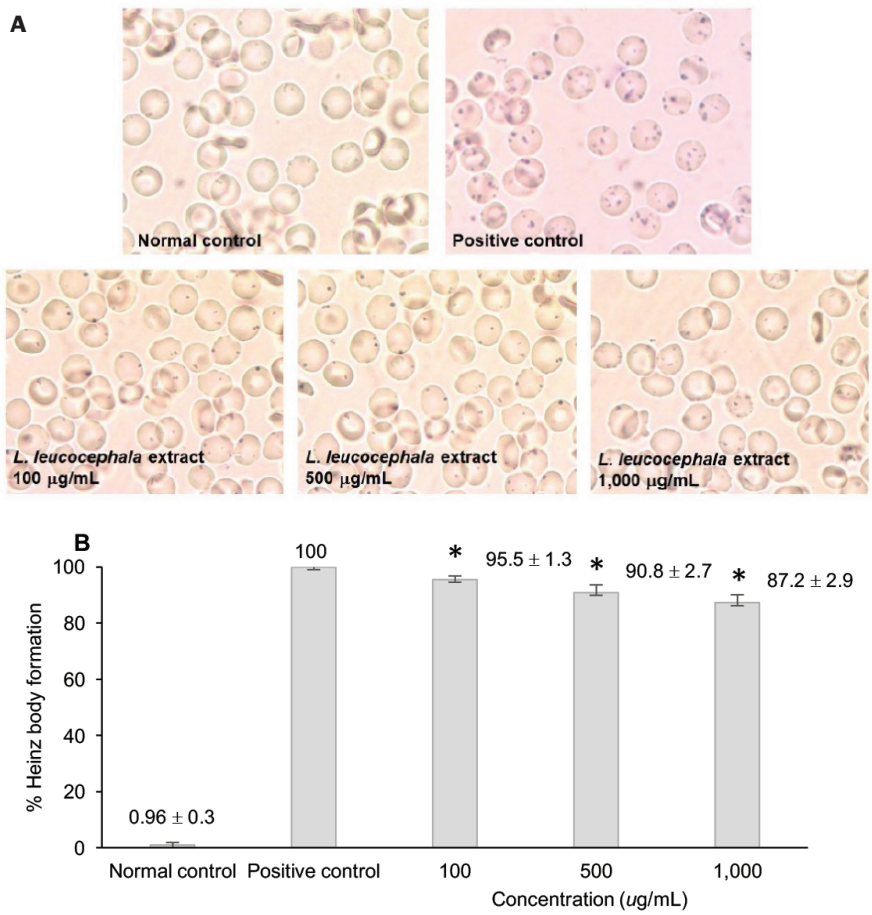

Figure 1 Effect of young leaf extract from *L. leucocephala* on Heinz body formation by APH induction, showing: (A) Heinz body formation was induced in erythrocytes and (B) Heinz body formation are represented as mean ± SE, (*) significant differences at $p < 0.05$.

3. Anti-hemolytic activity

The hemolysis induced by hydrogen peroxide is reduced in a concentration-dependent manner in the presence of the *L. leucocephala* extract. However, the protection values were significantly increased in the case of G6PD deficient erythrocytes. The activity of *L. leucocephala* extract in the protection of G6PD-deficient erythrocytes were revealed at the concentration of 500 and 1,000 ug/mL. The highest protection values were about 4.3% and 1.8% for G6PD deficient and normal erythrocytes respectively. Significant differences between extract and control are represented in Table 1.

Table 1 The percentages of hemolysis and protection by *L. leucocephala* extract against oxidative hemolysis

***L. leucocephala* concentration**	**G6PD-deficient erythrocytes**		**Normal erythrocytes**	
(μg/mL)	**%Hemolysis**	**%Protection**	**%Hemolysis**	**%Protection**
0	100%	0%	100%	0%
100	98.6 ± 1.4	1.4 ± 1.4	99.7± 0.2	0.3 ± 0.2
500	97.3* ± 1.6	2.7* ± 1.6	99.1 ± 0.2	0.9 ± 0.2
1,000	95.7* ± 1.9	4.3* ± 1.9	98.2 ± 0.1	1.8 ± 0.1

All values are represented as mean ± SE, (*) significant differences at $p < 0.05$

4. Antimicrobial screening

The growth inhibition zones measured by using agar disc diffusion assay are presented in Table 2. Extract of *L. leucocephala* young leave had no effect against *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Proteus mirabilis,* and *Klebsiella pneumoniae.*

Table 2 Diameter of zones of inhibition (mm) of bacterial isolates by different concentrations of extract

Microorganism	***L. leukocephala* extract (2 mg/disk)**	**Positive control (Ceftazidime 30 μg/disk)**	**Negative control (DMSO)**
S. aureus	0	12 ± 0.9	0
E. coli	0	16 ± 0.8	0
P. aeruginosa	0	14 ± 0.5	0
P. mirabilis	0	20 ± 0.9	0
K. pneumoniae	0	6 ± 0.5	0

5. Effects of the extracts on viability of leukemic cell line

The cytotoxic effect of *L. leucocephala* young leave extract on leukemic cell lines was investigated by MTT assay. The result revealed that KG-1a cell was more sensitive to extract than Molt4 cell. The IC_{20} of extract on KG-1a cell was 79.2 ± 0.5 μg/mL. No cytotoxic effect was obtained with Molt4 leukemic cells compared to vehicle control. The cell inhibition activity showed in Figure 2.

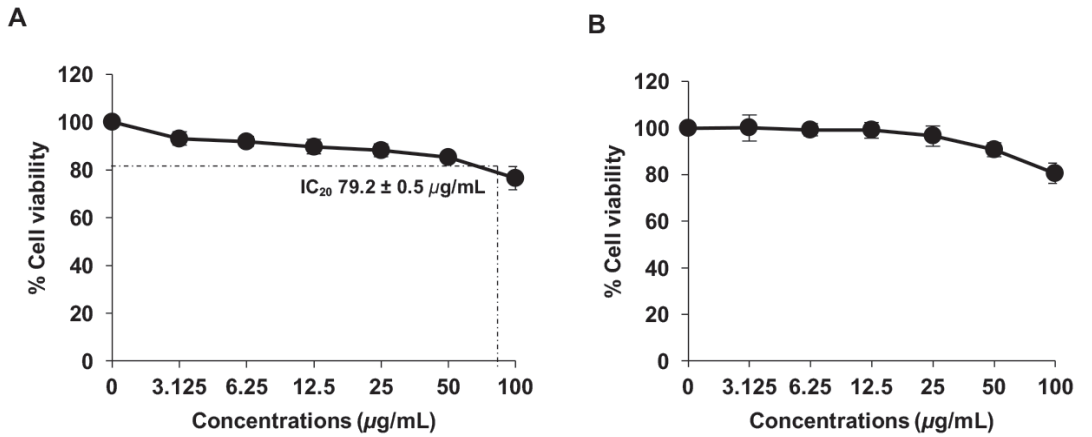

Figure 2 Cytotoxic effect of *L. leucocephala* young leave extracts on (A) KG-1a and (B) Molt4 cell line. The average of cell viability was obtained from three independent experiments.

Discussion

L. leucocephala leaves extract contains high condensed tannins, terpenes, sterols, coumarins, and flavonoids[6]. The result of the total phenolic content determination test showed that the extract was 325.6 ± 0.7 mg gallic acid equivalent/g of dry extract (mg GAE/g extract). The antioxidant properties of extract from *L. leucocephala* found in this study are mainly due to the phenolic content present in the extract.

Reactive oxygen species (ROS) including hydrogen peroxide, superoxides, hydroxyl radicals cause oxidative stress and damage cell membrane[18-19]. In general, the activity of the extract in the protection of erythrocyte membrane is due to its ability to act as antioxidant. Recently, phenolic compounds including tannins, phenolic acids and flavonoids have been well recognized as antioxidants[20].

G6PD enzyme is rate-limiting enzyme in pentose phosphate pathway (PPP) that is responsible for nicotinamide adenine dinucleotide phosphate (NADPH) production. NADPH is necessary for maintaining glutathione in reduced form to remove oxidative stress

from red blood cells. Enhanced susceptibility against oxidative stress in G6PD deficient erythrocytes is caused by ingestion of fava beans, infection, or exposure to an oxidative drug[8,21-22]. Faba bean (*Vicia faba* L.) is a leguminous plant belonging to the Fabaceae family. Faba beans are rich in two glucosidic aminopyrimidine derivatives, vicine, and convicine, and their respective aglycones, divicine and isouramil[23]. Divicine and isouramil have been identified as the main factors of G6PD deficient[24], which was not found in *L. leucocephala* leaves extract. The phytochemical investigation of *L. leucocephala* leaves revealed the presence of terpenes, sterols, coumarins and 8 flavonoids including quercetin-3-O-(2′′-trans-p-coumaryl)-α-rhamnopyranosyl-(1′′′→6′′)-β-glucopyranoside (1), quercetin-3-O-α-rhamnopyranosyl-(1′′′→2′′)-β-glucopyranoside (2), quercetin-7-O-α-rhamnopyranosyl-(1′′′→2′′)-β-glucopyranoside (3), quercetin-3-O-α-rhamnopyranoside (4), quercetin-3-O-β-glucopyranoside (5), isovitexin (6), vitexin (7) and quercetin (8)[6,25]. Other studies on the leave extracts had shown antioxidant activity[25-26]. In the present study, when G6PD-deficient erythrocytes were incubated with the extract in the presence of APH, the extract showed activity in significantly reducing the Heinz body formation was noticed at the concentrations 100, 500, and 1,000 μg/mL.

The treatment of erythrocytes with hydrogen peroxide resulted in the rupture of cell membranes and caused visual hemolysis. This study demonstrates that concomitant administration *L. leucocephala* young leave extract and oxidating agent can significantly decrease the oxidative hemolysis in G6PD deficiency erythrocytes in comparison with negative control. The obtained results confirmed *L. leucocephala* extract possesses a dose-dependent protective capacity in peroxidation conditions and decreases the Heinz body formation in G6PD deficiency erythrocytes.

It was reported that medicinal plants containing phenolic and flavonoid compounds possess antimicrobial activity[23-28]. In disk diffusion test, clinical strains of bacteria are not sensitive to *L. leucocephala* young leave extract. Despite the previous study showed significant antimicrobial activity against *E. coli, Pseudomonas,* and *Salmonella typhimurium,* moderate activity against gram positive bacteria[29]. Because of efficacy of extract varies depending on the microbial species and strains. The bacterial properties of American Type Culture Collection (ATCC) have different from clinical strains. The relation between bacterial strain and composition of *S. aureus* has been reported[30]. Our finding also implies poor activity of *L. leucocephala* young leave extract against clinical isolation stain.

It is very well known that all parts of

L. leucocephala young tree especially leaves and seeds contain mimosine which is proven to have inhibitory activities of many cancers, cell divisions, cell proliferation, and differentiation[31]. In this study, *L. leucocephala* young leave extract effect is more selective to KG-1a leukemic cell line than Molt4 leukemic cell line. Furthermore, *L. leucocephala* young leave extract was previously shown to inhibit cell proliferation in human breast adenocarcinoma (MCF-7), human colon carcinoma (HT29), human cervical carcinoma (HeLa), and human liver carcinoma (HepG2) cell lines in cancer cells through in vitro experiments[9].

Conclusions

Through this study can be concluded that *L. leucocephala* young leave extract showed inhibition of Heinz body formation and inhibition of leukemic cell line proliferation. The results also revealed that the extract had phenolic contents and was able to protect G6PD deficient erythrocytes from oxidative hemolysis. These results suggest that *L. leucocephala* is a useful natural products and can be foreseen to *in vivo* studies aimed to design suitable drugs for preventing oxidative stress induced peroxidation and hemolysis in G6PD deficient erythrocytes.

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Conflict of Interests

No conflicts of interest.

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