

The Effect of *Punica granatum* L. Peel Extract against *Plasmodium yoelii* 17XL Infected Mice ผลของสารสกัดจากเปลือกทับทิมในการต่อต้านการติดเชื้อปรสิตชนิดพลาสโมเดียม โยอีไลด์ เซเวนทีน เอ็กซ์แอล ในหนูทดลอง

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ABSTRACT

Malaria is a global health problem that requires knowledge and understanding of the pathogenesis of the disease for appropriate management. Drug-resistant malaria parasites are constantly being discovered. One of the safest and cheapest alternative therapies is the use of medicinal plants. In this study, pomegranate (PG) was of interest to the researcher. It has been used as antioxidant and antiparasite. Therefore, its effects were examined effects were on murine malaria-induced liver injury and oxidative stress. *P. yoelii* 17XL mice was treated with PG peel extract administered in 150 mg/kg (low dose) and 300 mg/kg (high dose), respectively per body weight of mouse via intraperitoneally. Pomegranate peel was investigated by ORAC at 28,367 μM TE/100g. The results showed that it significantly reduced parasitemia at 3.40% in the high dose treatment. In contrast, the low dose treatment group showed suppression on the ring stage at 34.78%. Furthermore, PG extracts could able to be reduce liver histological examination score which showed better assistance in the PG-treated group and showed a significant difference in high dose treatment group. This study showed that PG extract seem to have parasite inhibit ability against malaria infection and histopathological recovering. However, it is necessary to investigate in the clinical phase so that it could be developed as an alternative therapy to treating malaria.

บทคัดย่อ

มาลาเรียเป็นปัญหาที่แพร่หลายทั่วโลกเกี่ยวกับภาวะด้านสุขภาพที่ต้องใช้ความรู้และการจัดการเพื่อให้เข้าใจถึงสาเหตุของโรค การศึกษาครั้งนี้ผู้วิจัยเลือกใช้เปลือกทับทิมไทยแดงเจ้าพระยา มีวัตถุประสงค์เพื่อศึกษาผลกระทบของมาลาเรียโดยการเหนี่ยวนำให้เกิดการบาดเจ็บในเซลล์ตับรวมถึงผลของความเครียดออกซิเดชัน สารสกัดเปลือกทับทิมถูกนำมาใช้ในหนูที่การติดเชื้อ พลาสโมเดียม โยอีไลด์ 17 XL หนูที่ติดเชื้อได้รับสารสกัดจากเปลือกทับทิม ปริมาณ 150 มิลลิกรัม / กิโลกรัม (ขนาดต่ำ) และ 300 มิลลิกรัม / กิโลกรัม (ขนาดสูง) ตามลำดับต่อน้ำหนักตัวของ ผ่านทางช่องท้อง เปลือกทับทิมได้นำไปวิเคราะห์ปริมาณของสารต้านอนุมูลอิสระได้เท่ากับ 28,367 μM TE / 100 กรัม ผลการศึกษาพบว่าเปอร์เซ็นต์ปรสิตลดลงอย่างมีนัยสำคัญที่ 3.40% ในกลุ่มติดเชื้อได้รับสารสกัดในปริมาณสูง ในทางตรงกันข้ามกลุ่มติดเชื้อได้รับสารสกัดขนาดต่ำ มีการยับยั้งปรสิตในระยะ ring stage ที่ร้อยละ 34.78 แสดงให้เห็นว่าสารสกัดมีฤทธิ์ในการต้านมาลาเรียและต้านอนุมูลอิสระ นอกจากนี้ยังช่วยลดจำนวนปรสิตและลดความเสียหายของตับได้อย่างมีนัยสำคัญทางสถิติ การศึกษานี้แสดงให้เห็นว่าสารสกัดจากเปลือกทับทิมมีความสามารถในการยับยั้งเชื้อมาลาเรียและฟื้นฟูสภาพเนื้อเยื่อ อย่างไรก็ตามจำเป็นต้องมีการศึกษาเพิ่มเติมในขั้นตอนทางคลินิกเพื่อที่จะสามารถพัฒนาในการรักษาโรคมมาลาเรียต่อไป

Keywords: Malaria, *P.yoelii*, *Punica granatum* L.

คำสำคัญ: มาลาเรีย พลาสโมเดียม โยอีไลด์ ทับทิม

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Introduction

Malaria remains an important cause of illness and death in children and adults in countries in which it is endemic. According to the World Health Organization 2017 reported, 446,000 deaths from malaria globally African Region accounted for 91% of all malaria deaths in 2016, The WHO African Region accounted for 91% of all malaria deaths in 2016, followed by the WHO Southeast, normally from *Plasmodium falciparum* (World Health Organization, 2017). The causative agents of malaria disease are Plasmodium parasite. There are five different species of parasites that infect in human life; *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae*, and *P. knowlesi*. *P. falciparum* and *P. Vivax* are the most prevalent, Particularly, *P. falciparum* remains the most devastating form of malaria species (Cox FE, 2010) *P. falciparum* is the most dangerous, can cause cerebral malaria with the highest rates of complications and dead (White, 2008). Effect malaria leads to clinical symptoms such as moderate to severe shaking chills, high fever, sweating. Other signs and symptoms may include headache, vomiting, diarrhea. In most cases, malaria deaths are related to one or more serious complications, including: breathing problems, organ failure, anemia, Low blood sugar, cerebral malaria and death (Information PCH. Diseases & Conditions, 2018).

P. falciparum infected have resulting to cyto-adherence property come from subsequent develop of oxidative stress (Gomes et al., 2015). In previous, studies suggest that the generation of reactive oxygen and nitrogen species (ROS and RNS) associated with oxidative stress, plays a crucial role in the development of systemic complications caused by malaria. Malaria infection induces the generation of hydroxyl radicals (OH^\bullet) in the liver, which most probably is the main reason for the induction of oxidative stress and apoptosis (Guha et al., 2006). Additionally, (Atamna et al., 1993) observed that erythrocytes were infected with *P. falciparum* produced OH^\bullet radicals and H_2O_2 about twice as much compared to normal erythrocytes. A potential source of free radical production in this disease is the host's hemoglobin molecule, since the parasite uses this molecule as a source of amino acids for its own nutrition during the erythrocytic stage of the disease, resulting in the liberation of large amounts of circulating heme (Kumar, Bandyopadhyay, 2005). By having Fe^{2+} -associated groups, these heme groups can induce intravascular oxidative stress, causing changes in erythrocytes and endothelial cells and facilitating the internalization of the parasite in tissues such as the liver and brain. Especially liver, it is the first target organ for malaria infection (Bertolino, Bowen, 2015).

Antioxidants act as radical scavenger, hydrogen donor, electron donor, peroxide decomposer, singlet oxygen quencher, enzyme inhibitor, synergist, and metal-chelating agents. Both enzymatic and nonenzymatic antioxidants exist in the intracellular and extracellular environment to detoxify ROS (Frei et al., 1998). It is well known that plants are a source of antioxidants. There are several reports showing antioxidant potential of fruits (Wang et al., 1996). And there were found that *Punica granatum* L. (Punicaceae) (PG), commonly called pomegranate or “Tub-Tim” in Thai, recently described as nature’s power fruit, is a plant used in folkloric medicine for the treatment of various diseases (Moneim, 2012). It is widely cultivated in the Mediterranean region and has been used extensively in the folk medicine of many countries. In India, PG used as such or are made into juice. Alternatively, PG can be used for preparation of different value-added products such as concentrate, canned beverages, wine, jam, and jelly (Negi, Jayaprakasha, 2013). This fruit has been used for centuries to treat common ailments such as microbial and parasitic infections, stomach ache, ulcers, diarrhea and dysentery. In addition, the health benefits of PG consumption in preventing cardiovascular diseases and cancers have been widely investigated in both laboratory and clinical studies (Braidly et al., 2013; Spilmont et al., 2014; Wang et al., 2015). In previous study, it was found that a major bioactive

ingredient in pomegranate juice, has been to exert protective effects on acute lung injuries induced by lipopolysaccharides (Shao, 2016). The fruit is composed of two parts (Gouda et al., 2015): PG, that is the edible part, constitutes 52% of the total fruit (w/w), contains 78% juice and 22% seeds, and (Czerska et al., 2015) the non-edible part or the peel, have been traditionally used in folk medicine. A large number of phytochemicals have been identified in the two parts of pomegranate, including polyphenolics like hydrolysable tannins (ellagic and gallic acids) and anthocyanin in the peel. The main benefit of PG has been attributed to its unique polyphenols composition, which has been shown to exhibit high anti-oxidant and anti-inflammatory capacities (Hafiz et al., 2016). As mentioned occur, our will study is focus on the antimalarial effect of the peel extract of *Punica granatum* L. (Punicaceae) extract in *P.yoelii* 17XL infected mice that whether will decrease percentage parasitemia, decrease a free radical from parasite, suppress the parasite development, kill the parasite and promising in the hepatoprotective effect of the liver.

Objective of the study

To investigate the effect of *Punica granatum* L. (Punicaceae) (PG) extracts on anti-plasmodial activities under malaria condition after treatment with *Punica granatum* L. (Punicaceae) (PG) peel extract in *Plasmodium yoelii* 17XL infected mice.

Materials and methods

Plant material

Peels of *Punica granatum* L. (Punicaceae) were authenticated by Assoc. Prof. Dr. Aikkarach Kettawan, Institute of Nutrition, Mahidol University, Nakorn Pathom, Thailand.

Preparation of methanol extract

PG peel will be washed wash through clear water to remove fraction. According to (Abdel, 2012) with slight modifications. Then, air dried under shade, and grinded into powder PG was prepared. Using percolation at room temperature, air-dried powder (100 g) of pomegranate peels was extracted with 70% methanol and retained at 4 °C for 24 h. The extract was concentrated under reduced pressure (bath temperature 50 °C) before being dried in a vacuum evaporator. Afterwards, normal saline will be used to dissolve the residue to be used in the experiment.

Malaria parasite

Frozen *P. yoelii* 17XL strain infected blood stock obtained from Assist. Prot. Dr. Thanaporn Runguang (Generate from Prof. Dr. Motomi Torii from Ehime University School of Medicine and Prof. Dr. Osamu Kaneko from Institute of Tropical Medicine, Nagasaki University, Japan). *P. yoelii* 17XL blood stock was preserved as serial blood passage and kept in liquid nitrogen until used.

Infection of mice

Blood stages of *P.yoelii* 17XL were passaged in Swiss albino mice. Experimental animals were challenged with 10⁷*P.yoelii* 17XL-parasitized erythrocytes. Giemsa-stained blood smear were evaluated parasitaemia.

Animals

Female ICR mice from SIAM NOMURA CO., LTD. Thailand, were used in this experiment. Thirty female ICR mice age around 6-7 weeks and weighting between 25-30 g. Animals were kept in plastic cage at room temperature and illuminated environment with a 12:12 hour dark/light cycle. They got standard diet and tap water ad libitum. The procedures were approved by Ethical Committee on Animals experiments from Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand (Ethical certificate no. SI-ACUP 011/2561).

Experimental design

Thirty ICR mice divided into 6 groups of five animals (n=5) randomly and received different treatment as follow. Vehicle control group (NC), a group infected with 10^7 *P.yoelii* 17XL-parasitized erythrocytes (PC). Next, two groups that low (LC) and high dose (HC) were administered 150 mg/kg (low dose) and 300 mg/kg (high dose) per body weight of mouse dosage of pomegranate peel extract (PG) and two groups that treated group infected with 10^7 *P.yoelii* 17XL-parasitized erythrocytes with low (P_LC) and high dose (P_HC) follow, were administered low and high doses per bodyweight of mouse dosage of PG via intraperitoneally. Mice were sacrificed after finish. For groups of NC, HC, and LC, mice were sacrificed on day that the last mice in P_LC and P_HC groups were died. All of them were sacrificed by anesthesia with NEMBUTAL® (pentobarbital sodium), took their blood by cardiac puncture, and liver were collected. The determination of the PG extract activity was done by calculated the percentage reduction of parasitemia in treated groups compared to untreated infected mice. After that blood were collected in centrifuge tube at 4 °C and liver of mice were maintained in 10% formalin.

Histopathological study

Six-micrometer sections were prepared from liver after being formalin fixed at room temperature and embedded in paraffin. Liver sections were stained with hematoxylin-eosin (H&E) method. At least Histopathological recovering of liver was studied under Light microscope by taking a photograph in five different fields of every mouse. In one field had to measure the diameter of five hepatocytes, five Kupffer cells, five areas of hepatic sinusoid randomly. Hemozoin or malaria pigment also was determined.

Determination antioxidant capacity determination of *Punica granatum* L. (Punicaceae) peel extract by ORAC assay

Oxygen radical absorbance capacity (ORAC) use to evaluate oxidant inhibition and antioxidant properties in plants. It is a kinetic reaction of antioxidant compounds and peroxy radicals. The ability of antioxidants against free radicals was measured through the change in fluorescence intensity. This assay was used to evaluate antioxidant activity from PG extract. The ORAC assay use free radical damage to fluorescent probe and result of fluorescent intensity in downward change. The degree of change is amount of radical damage. The antioxidant results presence inhibition in free radical damage to fluorescent compound. The antioxidants and blanks are run in parallel, so protection reaction calculated by area under curve (AUC). Comparison to set of standard solution to calculate equivalents and compare result from sample. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) is a vitamin E derivative used as calibration standard. ORAC results are expressed as Trolox equivalent (μM).

Determination ORAC assay antioxidant capacity determination of *Punica granatum* L. (Punicaceae) peel extract in mice serum

Antioxidant capacity in mice serum was determined using oxygen radical absorbance capacity (ORAC) assay to measured antioxidant inhibition against peroxy radical generated by thermal decomposition of AAPH. For determination, the blank solution, fluorescein solution, standard Trolox solution and serum solution were prepared. Phosphate buffer (pH 7.2) was used as blank solution. 0.5 ml of blank solution.

Sample extraction

1. Weigh 2-5 g of sample into 125 ml Erlenmeyer flask.
2. Add 20 ml of 50% acetone.
3. Shake with 400 rpm speed by Shaking machine at room temperature, 1 hour.
4. Transfer to 15 ml centrifuge tube.
5. Centrifuge at 2,500 rpm for 15 min.
6. Transfer the supernatant of extracted sample into a test tube.

Spectrofluorometer measurement

1. Set temperature of water bath at 37°C.
2. Set the spectrofluorometer with an excitation wavelength at 493 nm, emission wavelength at 515 nm.

Warm the instrument for 30 min.

3. Add 3 ml of fluorescein working solution into the cuvette.
4. Add 0.5 ml of Trolox standard or blank or sample supernatant.
5. Add 0.5 ml of AAPH
6. Measured the intensity of fluorescence, every 1 min until the value nearly constant or equal zero.
7. Put curve between time and relative fluorescein.

Determination percent reduction of parasitaemia

Collected mice blood from lateral tail vein after that thin blood smear was prepared. Slides were fixed with methanol, Giemsa stained and determined under light microscope at 100X magnification. The parasitemia would counted on the photograph which took from microscope. Then calculated daily from day 1 of infected mice to the end of experimental.

Determination percent reduction of parasitemia

PG peel extract effects against malaria infection on blood stage was evaluate by calculate percent reduction of parasitemia. Percent reduction of parasitemia is percentage related between number of infected red blood cells and total number of red blood cells. Collecting mice blood from tail snip bleed after that thin blood smear was prepare. Slides were fixed with methanol, Giemsa stained and determined under light microscope at 100X magnification. The parasitemia was counted on the photograph which took from microscope. Then calculated daily from day 1 of infected mice to the end of experimental. Day 1 is the day that parasites were supposed to be found in red blood cells.

Statistical analysis

This study expressed data result as mean \pm standard error of the mean (Mean \pm SEM) and statistical analysis had to performed by using SPSS version 18. One Way ANOVA was used to examine statistical difference. The differences at the $p < 0.05$ considered as the significantly difference.

Results

1. Anti-oxidant capacity of *Punica granatum* L. (Punicaceae) (PG) peels methanol extract

The anti-oxidant capacity of *Punica granatum* L. (Punicaceae) (PG) peels methanol extract was examined by ORAC assay. For ORAC assay use to analyze free radical damage to fluorescent probe and result of fluorescent intensity in downward change. The anti-oxidant results presence inhibition in free radical damage to fluorescent compound. Therefore, *Punica granatum* L. extract sample was calculated equivalents compared to set of *Punica granatum* L. (Punicaceae) (PG) peels methanol extract standard Trolox solution results expressed as Trolox equivalent (μ M). The value of compared with standard Trolox was presented 28,367 μ M TE/100g.

2. Anti-oxidant capacity in plasma of *Punica granatum* L. (Punicaceae) peel methanol extract

The result of antioxidant capacity in mice plasma from LC and HC groups present significantly increasing antioxidant property when compare to NC group similar to compared with PC group. After statistical analysis compare means, the data of LC group compared NC group showed $p < 0.021$. In contrast, when compared LC group and PC group founded that not significantly difference at $p = 0.543$. Next, the data of HC group compared NC group showed $p = 0.000$ and as same as when compared HC group and PC group showed at $p < 0.001$. In addition to P_LC and P_HC groups antioxidant capacity showed significantly high more than PC group. From this result presented $p < 0.000$ between P_LC and PC groups and $p < 0.000$ between P_HC and PC groups. Therefore, comparison of P_LC group compared to NC group showed significantly decreasing antioxidant capacity at $p < 0.000$ and when compared P_HC to NC showed at $p = 0.121$ level.

Table 1 Determination of antioxidant capacity of mice plasma in each group (Mean \pm SEM).

Groups (n=5)	Antioxidant capacity (μ M TE/ml)
Normal control	14.32 \pm 0.29
Parasite control	8.72 \pm 0.40 *
Low dose PG	16.25 \pm 0.23 *
High dose PG	18.11 \pm 0.53 **,
Parasite low dose PG (n=4)	9.71 \pm 0.23 **,
Parasite high dose PG (n=3)	12.62 \pm 0.75**

* The mean difference is significant at p -value < 0.05 compared to NC group (N)

** The mean difference is significant at p -value < 0.05 compared to PC group (P)

3. Anti-malarial activity on erythrocytic stages of mice infected *P. yoelii* 17XL after treatment with *Punica granatum* L. (Punicaceae) (PG) peel methanol extract

Percentage parasitemia of infected *P. yoelii* 17XL erythrocytic stage in NC, PC, P_{LC}, and P_{HC} groups from Day 1 until day that the last mouse died. The data showed effect of PG extract on antiplasmodial (P_{LC} and P_{HC} group) when compared PC group. At percentage parasitemia 57.48±6.22 of PC group was increasing maximum but less than P_{LC} and P_{HC} groups (59.24±3.61, 59.76±8.37). At percentage parasitemia 57.70±6.10 in PC group the mouse was died but the mouse in P_{LC} and P_{HC} groups were alived and percentage parasitemia in both groups were continuously reducing at 41.41±6.26 ,31.50±8.50 respectively. After the mouse in PC group was died but the mouse were P_{LC} and P_{HC} groups were alived until day 11. Between percentage parasitemia of both group (P_{LC} and P_{HC}) were continuous reducing. Day 11, the last mouse in P_{LC} and P_{HC} groups were died at percentage parasitemia 5.4% and 3.4% followed. For suppressive percentage parasitemia in P_{LC} and P_{HC} group were -2.72±11.27 and -3.98±14.56 respectively. There was no significantly difference in suppressive percentage parasitemia of both groups when compared to PC group.

Ring stage, The data showed effect of PG extract on antiplasmodial (P_{LC} and P_{HC} group) when compared PC group. At percentage parasitemia 57.48±6.22 of PC group was increasing maximum but less than P_{LC} and P_{HC} groups (59.24±3.61, 59.76±8.37). At percentage parasitemia 57.70±6.10 in PC group the mouse was died but the mouse in P_{LC} and P_{HC} groups were alived and percentage parasitemia in both groups were continuously reducing at 41.41±6.26 ,31.50±8.50 respectively. After the mouse in PC group was died but the mouse were P_{LC} and P_{HC} groups were alived until day 11. Between percentage parasitemia of both groups (P_{LC} and P_{HC}) were continuous reducing. Day 11, the last mouse in P_{LC} and P_{HC} groups were died at percentage parasitemia 5.4% and 3.4% followed. For suppressive percentage ring stage 34.78±14.08 in P_{LC} group and there was significantly difference ($p=0.041$) compared to PC group. For suppressive percentage ring stage in P_{HC} group was 32.48±8.81 that presented reported no significantly difference ($p=0.060$) compared to PC group.

Trophozoite stage, The result of percentage trophozoite stage in PC group was 0.00±0.00 increased continuously to maximal percentage at 32.00±4.44 but less than in P_{LC} (39.20±2.48) and P_{HC} (39.92±5.90) groups. After that, percentage trophozoite stage in PC group decreased to 24.9±8.70. While P_{LC} and P_{HC} groups percentage parasitaemia were reducing more than PC group. P_{LC} group was 16.69±6.24.P_{HC} group was 18.80±7.60. At percentage parasitemia 32.00±4.44 in PC group the mouse was died and the mouse in P_{LC} and P_{HC} groups were alived and percentage parasitemia in both groups were continuously increasing at 39.20±2.48, 39.92±5.90 respectively. After the mouse in PC group was died but the mouse were P_{LC} and P_{HC} groups were alived until day 11. Between percentage parasitemia of both groups (P_{LC} and P_{HC}) were begin reducing. Until, the last mouse in P_{LC} and P_{HC} groups were died at trophozoite stage percentage parasitemia 0.6% and 1.2% followed. For suppressive percent trophozoite stage were -118.58±17.14 in P_{LC} group, and -114.33±22.31 in P_{HC} group. After statistical analysis compared PC group to P_{LC} group showed $p=0.653$ and PC group to P_{HC} group was $p=0.867$. From this result mean significantly difference between PC group to P_{LC} and P_{HC} groups in percent trophozoite suppression at $p=0.000$ level.

The percentage schizont stage data of PC group were not found in Day 1. At ,percentage schizont stage 0.08 ± 0.04 presented in this day and increased continuously to 11.24 ± 4.53 . After that PC group was 13.30 ± 4.90 percent schizont stage then almost of mice in this group died with percentage schizont stage of parasitemia in 13.30%. Then, the percentage schizont stage data of P_LC group there was 3.20 ± 0.97 . After that, there was reducing at 2.84 ± 0.78 percent schizont stage and increased continuously to maximal schizont at 11.24 ± 4.53 . Later percent schizont stage begin decreased but almost of mice in P_LC group died with 11.24 ± 4.53 . There was one mouse of P_LC group still lived to Day 11 with 2.60% schizont stage and died in Day 11. Next, the result of percentage schizont stage was found in P_HC group in Day 1 showed 0.08 ± 0.48 . Percent schizont stage of P_HC group increased continuously 7.20 ± 2.63 . After that, percentage schizont stage reduced continuously but almost of mice in P_HC group died with 2.20%. Then, percentage schizont stage increased to maximum 11%. The last mouse in P_HC group was died on Day 11 with 2.20% percentage schizont stage. For suppressive percent trophozoite stage were -45.00 ± 17.87 in P_LC group and -61.28 ± 32.95 in P_HC group. From statistical analysis compared PC group to P_LC group showed $p=0.339$ and $p=0.137$ to P_HC group. There was no significantly difference in suppressive percentage parasitemia of both groups when compared to PC group.

4. Histology and morphology of liver

Table 2 Diameter of each cell (μm) (Mean \pm SEM)

Groups (n=5)	Diameter of each cells (μm)			
	Hepatocytes	Hepatic sinusoid	Kupffer cells	Hemozoin(%)
Normal control	13.61 ± 0.56	3.91 ± 0.11	6.10 ± 0.16	-
Parasite control	$15.99 \pm 0.39^*$	10.09 ± 0.48	13.44 ± 0.18	6.34 ± 0.51
Low dose PG	14.17 ± 0.32	4.38 ± 0.19	6.38 ± 0.15	-
High dose PG	14.55 ± 0.72	4.58 ± 0.43	6.21 ± 0.39	-
Parasite low dose PG	13.84 ± 0.25	7.58 ± 0.40	12.66 ± 0.21	4.68 ± 0.46
Parasite high dose PG	14.24 ± 0.68	7.22 ± 0.46	10.49 ± 0.22	4.14 ± 0.39

Table 3 The data of morphological and histopathological liver tissue (Mean \pm SEM)

Groups (n=5)	Relative Weight (%)	Liver Width (cm)	Liver Length (cm)
Normal control	3.80 ± 0.14	1.78 ± 0.10	2.46 ± 0.10
Parasite control	6.26 ± 0.18	2.50 ± 0.00	3.14 ± 0.29
Low dose PG	4.66 ± 0.16	2.58 ± 0.17	2.48 ± 0.02
High dose PG	4.90 ± 0.11	2.40 ± 0.10	2.30 ± 0.12
Parasite low dose PG	6.36 ± 0.17	2.56 ± 0.06	2.66 ± 0.10
Parasite high dose PG	6.50 ± 0.13	2.58 ± 0.21	2.80 ± 0.20

Color of liver Figure 1 presented liver in each group; Normal control group (Figure 1A) showed reddish brown color, low dose PG (Figure 1C) and high dose PG (Figure 1D) group showed reddish brown color not different from normal control group. In contrast to parasite control group (Figure 1B) showed brown color, parasite low dose PG (Figure 1E), and parasite high dose PG (Figure 1F) groups black color appeared.

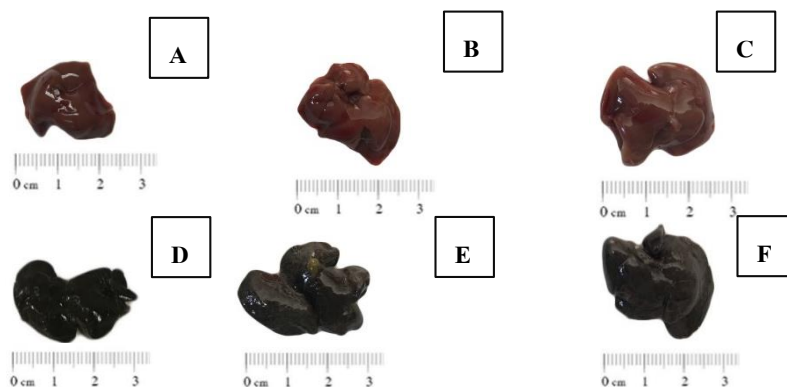


Figure 1 Morphology of mice livers; A: Normal control mice, B: Low dose PG extract mice, C: High dose PG extract mice, D: Parasite control mice, E: Parasite low dose PG extract mice, F: Parasite high dose PG extract mice

Histology of liver

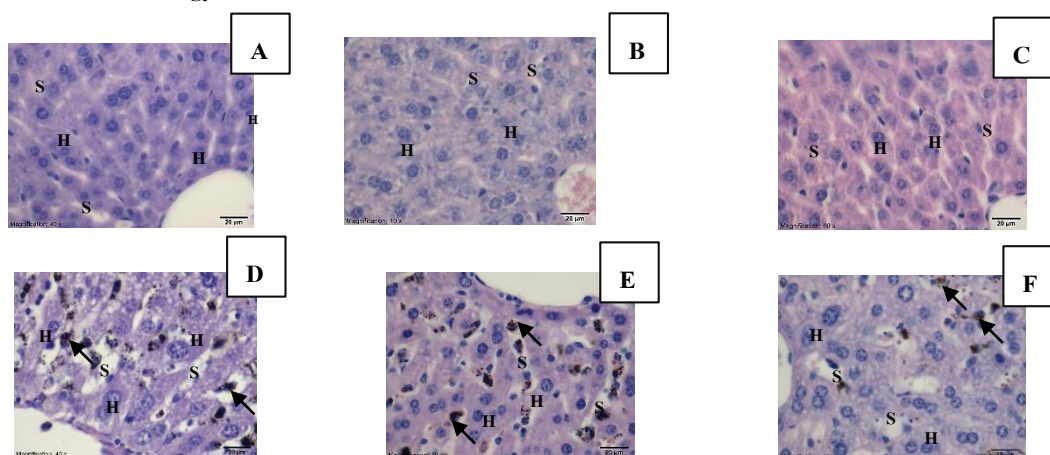


Figure 2 Histological changed of mice livers in magnification 40X; A: Normal control mice (NC), B: Low dose PG extract mice (LC), C: High dose PG mice (HC), D: Parasite control mice (PC), E: Parasite low dose PG extract mice (P_LC), F: Parasite high dose PG extract mice (P_HC), H: Hepatocyte, S: Hepatic sinusoid, and arrow: Hemozoin in Kupffer cell.

Discussion

Punica granatum L. (Punicaceae) peels extract or pomegranate have antioxidant property. Previous report n phenolic acids and ellagitannins showed that they are effective antioxidants (Osawa et al., 1990; Aviram et al., 2002). The observed antioxidant property in the peel extract in this study could be attributed to polyphenol compounds such

as ellagic tannins, ellagic acid and gallic acid (Lansky, Newman, 2007; Gil et al., 2000). The strong antioxidant potency of tannins has been explained in terms of the number of phenolic hydroxy groups, and formation of stable reaction products. Polyphenols act as scavengers of ROS, peroxide decomposers, quenchers of singlet oxygen, electron donor, labile hydrogen donor, and inhibitors of lipoxygenase (Porter, 1980). In addition, the FRAP assay measures in pomegranate peels the ability of an antioxidant to reduce ferric (III) to ferrous (II) in a redox-linked colorimetric reaction that involves single electron transfer (Li et al., 2006). The reducing power of a compound serves as a significant indicator of its potential antioxidant activity. Antioxidant capacity based on both the free radical scavenging and the oxidation–reduction mechanisms may be determined by several methods. Previous studies have shown strong antioxidant activity in pomegranate fruit peel extracts (Hajimahmoodi et al., 2008). When measured antioxidant activity of pomegranate peel by the bleaching of β -carotene, measurement of lipid peroxidation by Thiobarbituric Acid (TBA) Assay, hydroxyl radical scavenging activity, antioxidant activity on human Low-Density Lipoprotein (LDL) Oxidation, radical scavenging activity using DPPH method and HPLC analysis were found pomegranate peel as an enriched source of the antioxidants exhibiting higher activity (Singh et al., 2002). The results show that pomegranate peel may have great relevance in the prevention and therapies of diseases in which oxidants or free radicals are implicated (Singh et al., 2002). From this study, the value of PG peel methanol extract presented 28,367 TE/100g compared with standard Trolox.

During malaria infection, the liver is the first target organ which *Plasmodium* attack. The *Plasmodium*-infected mosquito injects sporozoite forms into the human host, and these migrate to the liver, where they can pass through Kupffer cells and invade hepatocytes within which they develop into liver merozoites. These merozoites are released into the bloodstream, where they invade erythrocytes. They develop through ring, trophozoite, and schizont stages, replicating to produce from 16 to 32 daughter merozoites that are released during egress. The free merozoite is then able to invade other erythrocytes to continue the asexual blood-stage life cycle (Cowman, Crabb, 2006). *Plasmodium* parasites release hemozoin or malaria pigments after digested hemoglobin of host cell for detoxified and they collect ferrous (Fe^{2+}), ferric (Fe^{3+}) and superoxide anions inside their food vacuoles (Schwarzer et al., 2003). In liver, sporozoites develop to schizonts and multiply by mitosis up to merozoites before release millions of merozoites into the blood stream.

In this study, NC group showed normal morphology. In this group, showed reddish brown color similar to LC and HC groups. In contrast to P_LC and P_HC groups appeared black color similar to PC group. Nevertheless, liver histology investigation for the amount of hemozoin presented in all mice infected with *P.yoelii* 17XL. The result of P_LC and P_HC groups showed significantly reducing accumulated of hemozoin in Kupffer cells compared to PC group. This could be determined that PG peel extract 150 and 300 mg/kg doses haven't hemozoin clearance activity. The result in relative weight, the data showed that mice in infected groups have heavier weight more than uninfected group because when malaria infection cause hepatomegaly. Hepatomegaly is reported to be a common finding in acute primary malaria infection, especially in children, and hyperplasia of the reticuloendothelial cells of the liver occur as part of the response to malaria (Viriyavejakul et al., 2014). They were studied also showed an association between hepatomegaly and parasite load in the liver. There was a significant correlation between the size of a palpable liver and

parasite sequestration in the liver (Krugliak et al., 2002). Therefore, results of P_LC and P_HC groups were showed increasing relative weight significantly difference when compared to NC group. Liver size of mice result, the data in P_LC and P_HC groups presented no significantly difference in liver size when compared to PC group. For LC and HC groups showed significantly difference in relative weight and size of liver but color of liver is similar when compared to NC group. However, this result of PG peel extract examined able to accumulation inside liver during malaria infection. Pathological changes in liver with severe malaria presented hepatomegaly because accumulation and congestion which cellular lead to increasing weight of liver (Banerjee et al., 2002). This data examined that PG peel extract 150 and 300 mg/kg doses have no hepatotoxicity effect to liver.

Liver histological study, hepatocytes of NC group show polyhedral hepatocytes contain spherical nucleus and well arrangement of hepatic cords similar liver cells characteristic of LC and HC groups. The cells are polygonal, round nucleus with one or two nucleoli. Their sides can be in contact either with sinusoids (sinusoidal face) or neighboring hepatocytes (lateral faces). In P_LC and P_HC groups found diameter of hepatocyte were not significant difference when compared NC group. Hepatic sinusoid are low pressure vascular channels that receive blood from terminal branches of the hepatic artery and portal vein at the periphery of lobules and deliver it into central veins. Hepatic sinusoid area lined by Kupffer cells without any pigment inside in NC group similar to LC and HC groups. At the same time, in PC, P_LC and P_HC groups which infected groups showed enlargement of hepatic sinusoids but less than PC group According, the studied of Prommano et al., (Prommano et al., 2005). They studied ultrastructural features of *Plasmodium* infected liver. They performed a retrospective study of 25 patients who died of severe *falciparum* malaria in Thailand and Vietnam using electron microscopy. The result showed hepatic sinusoids were filled with NRBC (nucleated red blood cells), PRBC (parasitized red blood cells), schizonts and phagocytes (Prommano et al., 2005). The histological parameters (hepatic sinusoids) were significant difference reduction diameter of hepatic sinusoid. This supports the role of pomegranate peel extract from previous study was found pomegranate peel extract could be reduce diameter of exhibited hepatoprotective activities upon histopathological examination (Hafiz et al., 2016). As well as, kupffer cells are important macrophage liver inside liver by attract to hepatic sinusoids, they phagocytize to pathogens entering from blood circulation. The result of diameter kupffer cells, P_LC and P_HC groups, presented kupffer cells distention by accumulation of hemozoin or malaria pigments less than PC group. PG peel extract 150 and 300 mg/kg could be reduced hepatic sinusoid distention. Malaria parasite resides inside the erythrocytes of the infected host during the asexual blood stage of its life cycle. *Plasmodium* degrade hemoglobin and use the amino acids for their biosynthetic requirements (Francis et al., 1997). A large amount of toxic free heme is released as a byproduct of hemoglobin degradation. Malaria parasite utilizes a unique pathway of hemozoin formation to avoid heme toxicity (Rudzinska et al., 1965). Inside the food vacuole of malaria parasite, toxic heme is converted into hemozoin, or “malaria pigment,” an almost inert form of heme (Slater et al., 1991). This hemozoin pigment is a complex of heme units linked through an iron–carboxylate bond, formed by linking of central ferric iron of one heme unit with the propionate side chain of another heme (Pandey et al., 1995). Hemozoin pigment is released into the host blood supply after the infected erythrocytes burst open at the end of malaria life cycle and the released hemozoin pigment gets deposited inside the tissues of the infected host and increases with the increase in parasitemia (Pandey, TekwaniL, 1996; Pagola et al., 2000)

In this study, the accumulation of hemozoin in P_{LC} and P_{HC} groups showed reducing of hemozoin in Kupffer cells when compared to PC group. From the result of this study, P_{LC} (Parasite low dose 150 mg/kg) and P_{HC} (Parasite high dose 300 mg/kg) of PG peels methanolic extract could be reduced cell accumulation of hemozoin in liver during malaria infection. Similar to previous study of Dell' Agli, et al., (Dell' Agli, et al., 2003) previously investigated the activity of recombinant plasmepsin II, one of the hemoglobin proteases, and the in vitro detoxification of hemozoin into β -hemozoin. They showed that ellagic acid inhibited the formation of hemozoin like some quinoline antimalarial drugs because of its ability to form a complex and had an IC₅₀ three times higher than that of chloroquine. Thus, this study found PG peel extract could be able to reduce percentage parasitemia that was the result which followed by reduction of hemozoin.

During its blood stage, the malaria parasite *Plasmodium* is known to digest between 60% and 80% of the haemoglobin present in the red blood cell (Banerjee et al., 2002). This process occurs in an acidic organelle within the parasite called a digestive vacuole (or food vacuole) (Eggleston et al., 1999; Banerjee et al., 2002). The short peptides produced are then further degraded to amino acids in the parasite cytoplasm probably by aminopeptidases (Rosenthal et al., 2002). It has long been assumed that the amino acids are a food source for the parasite and recent evidence confirms this, although a surprisingly small fraction is actually utilised in protein synthesis (averaging about 15% over the whole trophozoite stage of the lifecycle) (Schwarzer et al., 2003). It has been suggested that haemoglobin degradation is also required for maintenance of osmotic balance as well as to make space available in the red blood cell for parasite growth. During the process of degradation all of the haem present in the haemoglobin is released into the digestive vacuole. The iron is oxidized from the Fe (II) to Fe (III) state by a mechanism that call "Fenton reaction" that is not yet fully explored but is presumed to involve O₂ as the oxidant. Indeed, it has been shown that oxyhaemoglobin is rapidly oxidized to methaemoglobin at low pH. In the process vast quantities of superoxide (O₂⁻), are believed to be generated. Under the acidic conditions of the digestive vacuole O₂⁻ dismutates to O₂ and H₂O₂ spontaneously, leaving the parasite with the problem of disposing of the even more dangerous H₂O₂. It has been further suggested that catalase activity eliminates H₂O₂, thus disproportionation it to H₂O and O₂. The resulting Fe (III)PPIX produced also presents the organism with a major potentially toxic insult. Even although Fe (III)PPIX (presumably present as H₂O-Fe (III)PPIX) is relatively insoluble in aqueous solution at digestive vacuole pH, it can still potentially dissolve in a non-polar environment such as a lipid membrane (Eggleston et al., 1999; Rosenthal et al., 2002; Gavigan et al., 2001). Atamna et al. (Atamna et al., 1993) observed that erythrocytes infected with *P. falciparum* produced OH⁻ radicals and H₂O₂ about twice as much compared to normal erythrocytes. A potential source of free radical production in this disease is the host's hemoglobin molecule, since the parasite uses this molecule as a source of amino acids for its own nutrition during the erythrocytic stage of the disease, resulting in the liberation of large amounts of circulating heme. By having Fe²⁺-associated groups, these heme groups are able to induce intravascular oxidative stress, causing changes in erythrocytes and endothelial cells and facilitating the internalization of the parasite in tissues such as the liver and brain (Kumar, Bandyopadhyay, 2005). In this study found the PG peels methanolic extract have anti-plasmodial activity in infected mice.

The percentage parasitaemia in PC, P_{LC} and P_{HC} groups were increasing maximum at 57.48 ± 6.22 , 59.24 ± 3.61 and 59.76 ± 8.37 respectively but followed percentage parasite of mice in P_{LC} and P_{HC} were decreasing until mice were died. While mice in PC group were died with higher percentage of parasite than P_{LC} and P_{HC} groups. Furthermore, Percentage suppression used four days suppressive test of percentage ring stage of P_{LC} and P_{HC} groups compared to PC group showed PG peels extract 150 and 300 mg/kg in P_{LC} and P_{HC} groups could be suppressed parasite in ring stage at 34.78 and 32.48 percent respectively. In addition to, in P_{HC} group found PG peels extract 300 mg/kg could be able to reduce percentage parasitemia at presented 3.40 percent. In the present study, were found to be major constituents of PG peel extract that produce antiparasitic effects. Similar to the previous study in 2007, a study on isolation, identification, and assessment of antioxidant activity in a cell-based assay, and antimicrobial and antiplasmodial activities. This report represents the first comparative evaluation of antioxidant activity in a cell-based assay, and antimicrobial and antiplasmodial activities in POMx. In these studied, found purified gallic acid and punicalins were antiplasmodial activities against the *P. falciparum* D6 and W2 clones. Both compounds could be inhibited both *P. falciparum* D6 and W2 strains, with IC₅₀ values of 10.9 and 10.6, and 7.5 and 8.8 mM, respectively (Reddy et al., 2007) According to same previous study of Njomnang et al., that found antimalarial properties of ellagic acid were explored. The results obtained have shown high activity in vitro against all *Plasmodium falciparum* strains whatever their levels of chloroquine and mefloquine resistance (50% inhibitory concentrations ranging from 105 to 330 nM). Ellagic acid was also active in vivo against *Plasmodium vinckei petteri* in suppressive, curative, and prophylactic murine tests, without any toxicity (50% effective dose by the intraperitoneal route inferior to 1 mg/kg/day). The study of the point of action of its antimalarial activity in the erythrocytic cycle of *Plasmodium falciparum* demonstrated that it occurred at the mature trophozoite and young schizont stages. Moreover, ellagic acid has been shown to potentiate the activity of current antimalarial drugs such as chloroquine, mefloquine, artesunate, and atovaquone (Soh et al., 2009). The point of action of ellagic acid in the parasite life cycle corresponded with protein and nucleic acid synthesis. The pharmacological targets of ellagic acid and its mechanism of action on *P. falciparum* are not well known. According to the report in 2016, the potential role of *Punica granatum* treatment on murine malaria-induced hepatic injury and oxidative stress were found flavonoids and hydrolyzable tannins (ellagitannins) were found to be major constituents of PG that produce antiparasitic effects (Hafiz et al., 2016). However, in present study, it is still necessary to more study what constituents of the pomegranate peel have anti-malarial activity and investigate biological marker, chemical marker followed so that it can be used as an antimalarial agent in the future.

Conclusion

This study showed that PG extract seem to have parasite inhibit ability against malaria infection and histopathological recovering. However, further studies are needed in order to determine of action of pomegranate and necessary to investigate in the clinical phase so that it may be developed as an alternative therapy to treating malaria.

Acknowledgement

This study was granted by Faculty of Medicine, Siriraj Hospital, Mahidol University, Thailand.

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