Original Article

ความเป็นพิษต่อเซลล์เม็ดเลือดขาวชนิดโมโนนิวเคลียร์ของรงทองสะตุและรงทองที่ไม่ผ่านการสะตุ

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บทคัดย่อ

ความเป็นพิษต่อเซลล์เม็ดเลือดขาวชนิดโมโนนิวเคลียร์ของรงทองสะตุและรงทองที่ไม่ผ่านการสะตุ

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รงทองเป็นเครื่องยาที่ต้องนำมาผ่านกรรมวิธีทางการแพทย์แผนไทย (สะตุ) เพื่อลดความเป็นพิษก่อนนำไปใช้ปรุงยา ซึ่งวิธีดังกล่าวยังไม่มีการพิสูจน์ทางวิทยาศาสตร์มาก่อน งานวิจัยนี้จึงมีวัตถุประสงค์เพื่อพิสูจน์การลดพิษของรงทองด้วยวิธีการสะตุ โดยการเปรียบเทียบความเป็นพิษต่อเซลล์เม็ดเลือดขาวชนิดโมโนนิวเคลียร์ (PBMC) ของรงทองสะตุและรงทองที่ไม่ได้สะตุ **วัสดุและวิธีวิจัย:** ทำการสะตุรงทองโดยใช้ความร้อน 3 วิธี แล้วนำไปทดสอบความเป็นพิษต่อเซลล์ PBMC เปรียบเทียบระหว่างรงทองสะตุ รงทองที่ไม่ได้สะตุ และกรดแกมโบจิก **ผลการศึกษา:** รงทองที่ไม่ได้สะตุแสดงความเป็นพิษต่อ PBMC มากกว่ารงทองที่ผ่านสะตุทั้ง 3 วิธี ประมาณ 3-6 เท่า รงทองที่ผ่านการสะตุที่ความเข้มข้นต่ำซึ่งมีปริมาณกรดแกมโบจิก 0.0028 – 0.0159 ไมโครโมลาร์ มีผลกระตุ้นการแบ่งตัวของ PBMC ในขณะที่รงทองสะตุที่มีปริมาณกรดแกมโบจิก 0.0159 – 0.2902 ไมโครโมลาร์ ไม่มีความเป็นพิษต่อเซลล์ และรงทองสะตุที่มีปริมาณกรดแกมโบจิกมากกว่า 0.2902 ไมโครโมลาร์ แสดงความเป็นพิษต่อ PBMC และรงทองสะตุที่มีปริมาณกรดแกมโบจิกมากกว่า 1.14 ไมโครโมลาร์ ทำให้เซลล์ PBMC ตายทั้งหมด **สรุปผลการวิจัย:** รงทองที่ผ่านการสะตุทั้ง 3 วิธี มีความเป็นพิษต่อ PBMC ลดลง เมื่อเปรียบเทียบกับก่อนสะตุ ซึ่งสอดคล้องกับภูมิปัญญาการแพทย์แผนไทยที่กำหนดให้สะตุรงทองเพื่อลดความเป็นพิษ ในโอกาสต่อไปควรศึกษากลไกการเป็นพิษและระบุสารเคมีที่เป็นพิษชนิดอื่นในรงทองก่อนและหลังการสะตุ

คำสำคัญ: รงทอง, กรดแกมโบจิก, การแพทย์แผนไทย, การเตรียมเครื่องยาก่อนใช้, สะตุ

Cytotoxicity Against Peripheral Blood Mononuclear Cells of Processed and Unprocessed Gamboge

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Abstract

Cytotoxicity Against Peripheral Blood Mononuclear Cells of Processed and Unprocessed Gamboge

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Garcinia hanburuyi Hook. f. resin or gamboge is a Thai crude drug which has to be processed in Thai traditional medicine, so called “Sa-Tu”, in order to detoxify prior to use for drug preparation. No scientific testing has been done for the toxic reduction of gamboge by Sa-Tu. This research aimed to prove the toxic reduction by comparing the toxicity of processed and unprocessed gamboge against human peripheral blood mononuclear cells (PBMC). **Methods:** Gamboge was Sa-tu by heating by 3 different methods. PBMC were assessed for cell viability following treatment with Sa-tu gamboge, unprocessed gamboge and gambogic acid (GA). **Results:** Unprocessed gamboge was 3-6 fold more toxic than all processed gamboges against PBMC. The processed gamboge containing GA 0.0028 – 0.0159 μM showed PBMC proliferation, while 0.0159-0.2902 μM had non-cytotoxic effect and more than 0.2902 μM was cytotoxic. The processed gamboge with GA concentration higher than 1.14 μM caused all cell death. **Conclusion:** Processed gamboge by 3 Sa-Tu methods showed reduced toxicity to PBMC. The decreasing of cytotoxicity of processed gamboge related to Thai traditional medicine wisdom of Sa-Tu process for gamboge detoxification. Further study should be performed to explain mechanisms of cytotoxicity and to identify the other toxic compounds in the unprocessed and processed gamboge.

Keywords: *Garcinia hanburyi*, Gambogic acid, Thai traditional medicine, Crude drug processing, Sa-Tu

Introduction

Gamboge or Rong Thong (in Thai), is orange-yellowish resin which is the exudate of *G. hanburuyi* Hook. f. stem (Clusiaceae). Gamboge is composed of more than 100 xanthones, mainly gambogic acid, together with isomorellic acid, morellic acid, desoxymorellin, and isomorellinol (Chantarasriwong *et al.*, 2010) and including other components such as triterpenoids, volatile oil and potassium bitartrate (Wang *et al.*, 2008). The biological activities of gamboge are anticancer, antituberculosis, antimalaria (He *et al.*, 2009; Tan *et al.*, 2011; Qin *et al.*, 2007), anti-inflammation, antinociceptive and antipyretic (Norkaew, 2003). Gamboge has been used in Chinese traditional medicine for intoxication, stop bleeding and antiparasitic (Qin *et al.*, 2007). In Thai traditional medicine (TTM), gamboge was used for purgative, wound healing and anthelminthic (Tao *et al.*, 2009) and also used as an active ingredient in several TTM recipes (Poomchusri, 1973).

In TTM, gamboge has to be processed before use, so called “Sa-Tu”, by wrapping it in 7 layers of galanga (*Alpinia galanga* (L.) Willd.) or lotus (*Nelumbo nucifera* Gaertn.) leaves, and roast until gamboge become orange color and crispy. The TTM theory described Sa-Tu as process for reduction of strength, side effects and toxic of crude drugs, however the exact effects of Sa-Tu to gamboge is still unclear. From our previous study, it was found that gambogic acid content decreased approximately 29% after being processed by the Sa-Tu (Poowanna *et al.*, 2014). However, the reduction of toxicity of the processed gamboge was not proved yet.

PBMC (peripheral blood mononuclear cells) are blood cells with single round nucleus. It includes cells related to human immunity such as lymphocytes, monocytes, and macrophages or dendritic cells. The immune cells i.e. natural killer cells (NK cell), and $CD4^+$ T cell and $CD8^+$ T cell are also PBMC members (Corkum *et al.*, 2015). PBMC have been used for cell toxicity effect, anti-inflammation assay, and used for immunological assessment of drugs and natural products such as anticancer, anti HIV, and infectious diseases (immunostimulator or immunosuppressive) (Chuchawankul *et al.*, 2012; Okonogi *et al.*, 2007). Separation of PBMC could be performed by using Ficoll which is polysaccharide in the density of 1.077 g/mL. PBMC will form a buffy layer on the upper interface of the Ficoll layer after gradient centrifugation.

This study aimed to prove the detoxification of gamboge by Sa-Tu process through cell viability assay of human PBMC. The obtained results could be used to explain the reason of Sa-Tu of gamboge in term of crude drug detoxification.

Materials and Methods

Ficoll-Hypaque (density 1.077±0.001 g/mL) was purchased from GE Healthcare (Sweden), blood collection tube 6 mL (LH Lithium heparin 13x100, Vacuette) was obtained from Greiner Bio-one (Thailand), RPMI-1640, Trypan blue, Fetal bovine serum, and penicillin/ streptomycin were purchased from Gibco (USA), Prestoblue^R (resazurin cell viability reagent) was obtained from Invitrogen (USA). The chemical standard (Gambogic acid) was purchased from Chengdu Biopurify Phytochemicals (China). Acetonitrile (HPLC grade) and trifluoroacetic acid (TFA) were purchased from Merck (USA).

1. Gamboge

Gamboge was purchased from a Thai traditional herbal drug store in Bangkok, Thailand. It was kept in a tight container at room temperature (28 ^{O}C) and protected from light. It was ground to be fine powder before use.

2. Gamboge processing (Sa-Tu)

Gamboge was performed Sa-Tu by 3 methods. The first and second methods were done by wrapping 20 g of gamboges powder in 7-layered galanga leaves (*A. galanga*) or 7-layered lotus leaves (*N. nuclfera*), respectively, and grilled with charcoal brazier until the 6th layer of galanga or lotus leaves were burned and the processed gamboge became orange color and crispy. The

third Sa-Tu method was developed by Poowanna *et al.* (2014), briefly, 20 g of gamboge powder was wrapped in aluminum foil and burned in a furnace for 30 min at 120 ^{O}C. All processed gamboges were kept in a tight container at room temperature (28 ^{O}C) and protected from light until use.

3. Analysis of gambogic acid

Gambogic acid content in the processed and unprocessed gamboges was analyzed by high performance liquid chromatography (HPLC) (LC-10AD vp, Shimadzu), photo diode array detector (SPD-M20A), prominance system controller (SCL-10A Shimadzu), guard column (GL Sciences), silica gel RP18 column (GL Sciences 5 µm 4.6 X 10 mm). The HPLC mobile phase was according to Poowanna (2014) which acetonitrile was used as solvent A and 0.5% trifluoroacetic acid in water as solvent B in the gradient manner of A:B as followed: 80:20 to 90:10 at 0-8 min; 90:10 to 95:5 at 8-10 min, 95:5 to 95:5 at 10-23 min and 95:5 to 100:0 at 23-25 min, with the flow rate of 0.8 mL/min. The amount of gambogic acid was calculated from the standard graph of gambogic acid which were plotted between area under curve and concentrations (0.50, 0.38, 0.25, 0.19 and 0.12 mg/mL). Each sample was done in triplicate.

4. Cell viability assay on human peripheral blood mononuclear cell (PBMC)

4.1 Preparation of PBMC

The heparinized blood from a healthy volunteer was separated by density gradient centrifugation with Ficoll-Hypaque to obtain the buffy layer of PBMC. The heparinized blood was mixed with phosphate buffer saline (PBS) in a ratio 1:1. Diluted blood 6 ml was then carefully layered onto the top of 3 mL Ficoll-Hypaque. The mixture was centrifuged at 1500 rpm for 30 min at 25°C. The PBMC layer was collected, washed 2 times with PBS, centrifuged at 1000 rpm for 10 min and resuspended PBMC pellet in RPMI-1640 complete medium which was the medium supplemented with 10% FBS and 1% penicillin/streptomycin. Cells were mixed with trypan blue and counted for the viable cells by using haematocytometer under inverted microscope.

4.2 Optimization of cell amount and incubation time

Determination of optimal cell amount was performed in 96-well plate. PBMC $3.8x10^{4}$ – $5.1x10^{5}$ cells/well were incubated with Prestoblue and measured by microplate reader (Sunrise^{TM}, Tecan, Switzerland) for the absorbance at 570 nm and 600 nm (reference wavelength) after incubation at 37 ^{O}C in 5%CO_2 incubator for 30 min to 3 hr.

4.3 Cell viability assay

Cell viability assay was carried out in 96-well plate. PBMC 2.5 x 10^{5} cells/well were cultured in the RPMI-1640 complete medium with adding of unprocessed gamboge, processed gamboge, or gambogic acid at concentrations 0.01 – 4.0 µg/mL, and was incubated in 5% CO_2 incubator at 37 ^{O}C for 48 hr. All samples were dissolved in the RPMI-1640 complete medium in the presence of DMSO at final concentration of 0.1%. Cell viability was determined by adding Prestoblue in a ratio of Prestoblue: cell volume (1:9) and incubated for 2 hr, then the plate was measured at wavelengths 570 and 600 nm (reference wavelength) by microplate reader. Cell viability (%) was calculated by the following formula: % cell viability = $(A_{sample}/A_{control})$ x 100, where A_{sample} = normalized absorbance of the sample and $A_{control}$= normalized absorbance of the untreated control. Three independent experiments were performed in quadruplicate (n = 4). The negative control was the untreated PBMC and the positive control was PBMC which was heated at 65 ^{O}C for 25 min. Cell viability less than 80% was considered to be toxic to PBMC.

5. Statistical analysis

The results are presented as % cell viability ± SD. The value of LD_{50} was calculated from a graph between log concentrations (x-axis) versus percentage of viable cells (y-axis). The statistical analysis was done by using One-way ANOVA for comparison between the treated group and the untreated control. *P* value < 0.05 was regarded as significant.

6. Ethic consideration

This research was approved by the ethics committee, Mahasarakham University, No. 0098/2556

Results

1. Determination of gambogic acid content in processed and unprocessed gamboges

The HPLC profile of the unprocessed and processed gamboges indicated the similar pattern of chemical components and showed the major peak of gambogic acid (Figure 1). The calibration curve of gambogic acid provided the linear equation: $Y = 4x10^7X + 894009$ ($R^2 = 0.999$). The percent average amount of gambogic acid in unprocessed gamboge, processed gamboge-L (lotus leaves), processed gamboge-G (galanga leaves), and processed gamboge-F (aluminum foil) were 25.77± 0.26, 21.20 ± 1.47, 21.58 ± 0.35 and 20.47 ± 1.44 % respectively. The amount of gambogic acid in all processed gamboges was significantly decreased (*P* value < 0.05) when compared to unprocessed gamboges.

2. Optimization of cell viability assay

2.1 Cell amount

Resazurin in Prestoblue was converted to resorufin in direct proportion to the amount of viable PBMC. The negative values of the normalized absorbance could be obtained by low conversion of Prestoblue. It was found that 2.55 x 10^5 cells PBMC gave the average normalized absorbance above zero within 2 hr (Table 1), therefore this cell amount was used for further experiments.

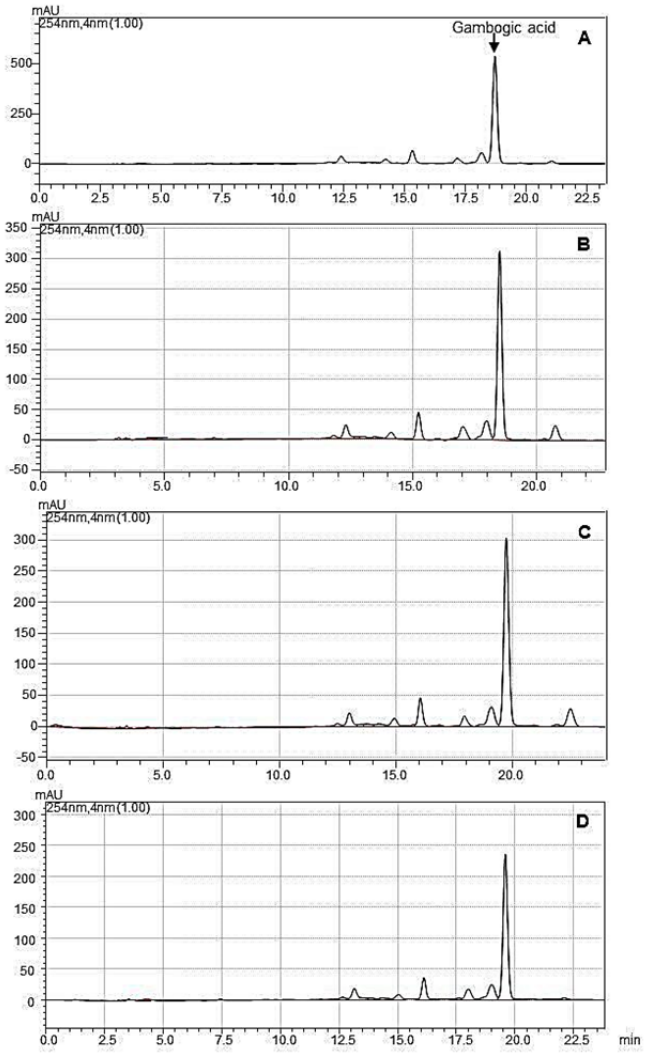

Figure 1 HPLC profile of unprocessed and processed gamboges

(A: unprocessed gamboge; B: processed gamboge in lotus leaves; C: processed gamboge in galangal leaves; D: processed gamboge in aluminum foil)

Table 1 Optimization of the PBMC amount in the presence of Prestoblue for 2 hr

PBMC (x10^4 cells/well)	Average normalized absorbance*
0	-0.1163±0.0112
3.18	-0.0760±0.0341
6.35	-0.0273±0.0061
12.7	-0.0173±0.0028
25.5	0.1267±0.0041
51.0	0.2613±0.0264

*Negative values could be obtained by low conversion of Prestoblue (resazurin is reduced to resorufin).

2.2 Optimal concentrations of gamboge for cell viability assay

Since cell viability assay of PBMC against gambogic acid was reported in the concentration range of LD_{50} 5.2–11.0 μM (Batova *et al.*, 2010; Hahnvajanawong *et al.*, 2010) which was equal to 3.27–7.10 μg/mL. Therefore, the concentration of gamboge 1, 5, 10, 50, 100 μg/mL was preliminary screen for cytotoxicity. It was found that after 48 hr incubation, gamboge up to 5 μg/mL resulted 0% cell viability. Therefore, the concentration range of gamboge 0.01-4 μg/mL was chosen for further experiment.

3. Cell viability assay

The viability of PBMC in the presence of processed gamboges, and gambogic acid were significantly different from the unprocessed gamboge after 48 hr incubation. From Figure 2, all samples at 0.01 μg/mL resulted in PBMC viability more than 80%, and showed cell proliferation with gambogic acid, processed gamboge-L and processed gamboge-F ($p<0.01$). The LD_{50} of gamboges in Table 2 indicated that unprocessed gamboge was the most potent cytotoxic to PBMC. The cytotoxicity (% cell viability < 80%) of the unprocessed gamboge to PBMC began from the concentration 0.1 μg/mL, while all processed gamboges were toxic to PBMC from the concentration 1 μg/mL. Gambogic acid at 1 μg/mL led to cell death (0% cell viability), while unprocessed and processed gamboges showed 0% cell viability at higher concentration (4 μg/mL).

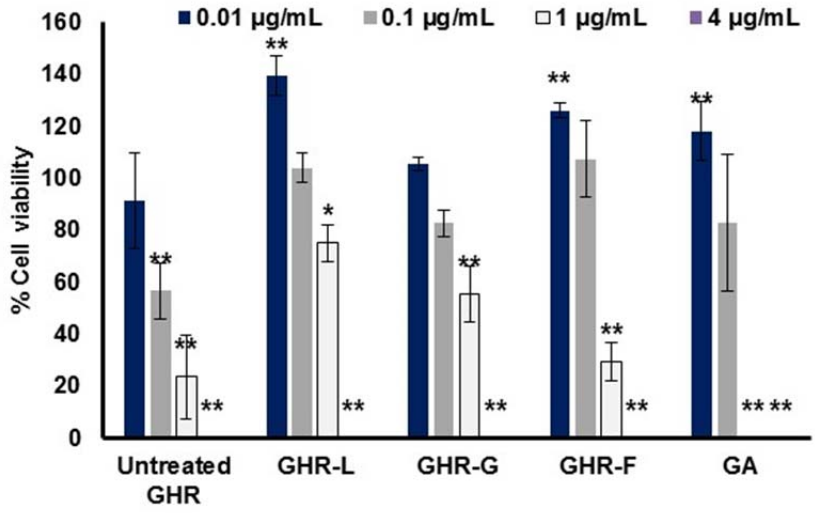

Figure 2 Cell viability (%) of PBMC in the presence of unprocessed gamboge (Untreated GHR), processed gamboges (GHR-L = processed gamboge in lotus leaves; GHR-G = processed gamboge in galangal leaves; GHR-F = processed gamboge in aluminum foil), and gambogic acid (GA). (* = $P < 0.05$; ** = $P < 0.01$ when compared with untreated PBMC)

Table 2 LD_{50} of gamboges and its gambogic acid content

Sample	LD_{50} of gamboge (μg/mL)	Gambogic acid content at LD_{50} (μM)
Unprocessed gamboge	0.1566	0.0642
Processed gamboge-L	1.0137	0.2904
Processed gamboge-G	0.4905	0.1430
Processed gamboge-F	0.5104	0.1481
Gambogic acid	-	0.3084

Discussion and Conclusion

Gambogic acid content of all processed gamboges (gamboge-G, gamboge-L and gamboge-F) was significantly decreased when compared to unprocessed gamboge (P value < 0.05) but there were not significant differences among the processed gamboges. The percent viability of PBMC in all processed gamboges was significantly higher than unprocessed gamboge but not different among them. These results showed the inverse relationship between amount of GA and PBMC viability in processed and unprocessed gamboges. The toxicities of all processed gamboges (gamboge-G, gamboge-L and gamboge-F) at the concentration of 0.1 µg/mL were significantly less than that of the unprocessed gamboge (P value < 0.05) but the toxicities within the group of all processed gamboges were not significantly different. Both processed and unprocessed gamboges caused cell death at the concentration of 1 and 4 µg/mL, therefore 0.1 µg/mL gamboges, which gave various viability effect to PBMC, was a suitable concentration for comparison of cytotoxicity in this study. It was interesting that gambogic acid, processed gamboge-L and processed gamboge-F at low concentration (0.01 µg/mL) could stimulate proliferation of PBMC, indicating potential immunomodulatory properties (Alnajar *et al.*, 2012; Yeap *et al.*, 2010). The increasing cell proliferation effect of the low concentration of extract and toxic effect of increased concentration was also shown in *Ganoderma lucidum* and *Rhaphidophora korthalsii* (Gill and Rieder, 2008; Yeap *et al.*, 2010).

The toxicity of gamboge was related to gambogic acid content. By using HPLC technique, the amount of gambogic acid in gamboges was calculated into µM. From the results, gambogic acid content in gamboges could be divided into 3 categories on the basis of their effects against PBMC: as cell proliferation (0.0028 to 0.0159 µM gambogic acid), non-cytotoxic (0.0159-0.2902 µM gambogic acid), and cytotoxic (≥ 0.2902 µM gambogic acid). Gamboges that contained gambogic acid ≥ 1.14 µM led to all PBMC death (Figure 3). Interestingly, unprocessed gamboge with gambogic acid content 0.0410 µM fell into non-cytotoxic range, but exhibited cytotoxic effect (Figure 3, arrow). This result indicated that the unprocessed gamboge may contain other unknown substances which are highly toxic to PBMC and could be destroyed by the process of Sa-Tu.

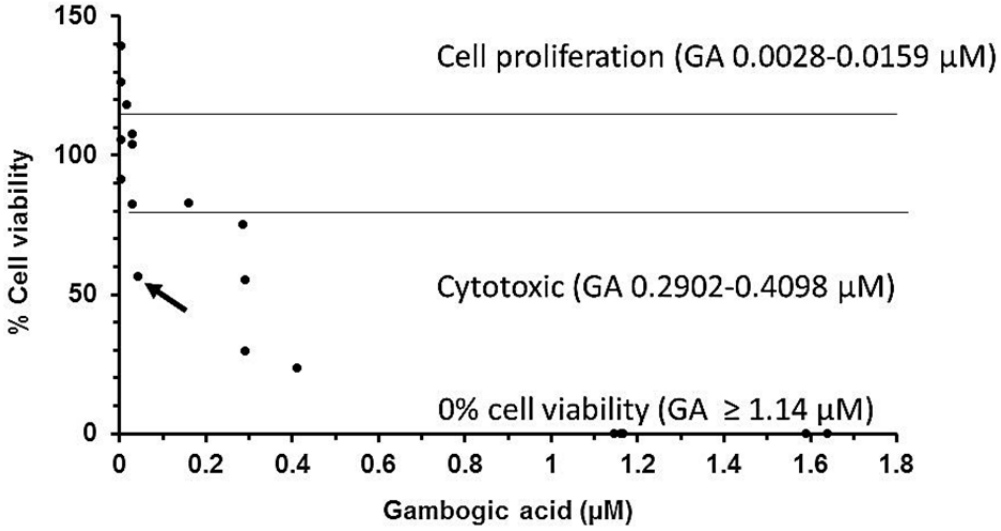

Figure 3 Relationship between gambogic acid (µM) and % cell viability (Arrow = unprocessed gamboge showed high toxicity to PBMC even its GA content was in non-toxic range, this indicated the presence of other toxic compositions in unprocessed gamboge which could be destroyed by Sa-Tu.

Gamboge is known to be composed of gum 15-25% which are mostly arabinose and galactose, and resin 70-80% (mainly xanthones). The other substances are also found such as triterpenoids, volatile oil, and potassium bitartrate (Wang *et al.*, 2008). The components which are labile to heat should be gum and volatile oil. The latter is cytotoxic to PBMC as the description from Tilaoui *et al.* (2015) that the essential oil of aerial parts of *Artemisia herba-alba* from 1.6 µg/mL caused 50% cytotoxicity to PBMC. Since gamboge is a complex mixture of several chemical components, the cytotoxic effect might be the result of synergism among those components which are heat labile and gambogic acid, and lead to more potent cytotoxicity to PBMC in the unprocessed gamboge. This was clearly supported by the high LD_{50} of gambogic acid alone (0.3804 µM gambogic acid) whereas the unprocessed gamboge that contained the lowest amount of gambogic acid (0.0642 µM) showed the highest toxicity to PBMC (Table 2). Further studies should be designed to clarify this hypothesis.

It could be proved that the gamboges processing (or “Sa-Tu”) led to reduce cytotoxicity against PBMC. The relationship of gamboge concentration and its pharmacological activities should be studied in detail for the dosage optimization. In conclusion, this study clearly indicated that Sa-Tu of gamboge by either old methods or developed method could reduce cytotoxicity against PBMC of gamboges. This finding corresponds with the Thai traditional medicine wisdom of processing the highly toxic crude drug for safe use.

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