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**In-Vitro anti-inflammatory activity evaluation of Gardenia fruit extract
using SRBC membrane stabilization**

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Introduction

Gardenia jasminoides Ellis is a Gardenia genus, Rubiaceae family, common names are cape jasmine, cape jessamine, common gardenia, garden gardenia, gardenia and jasmin. It's woody shrub, 1-2 m. high. It's grown in a humid climate. China called Zhi Zi^(8, 14, 15). *Gardenia jasminoides* Ellis is a commonly used traditional Chinese, Korean and Japanese medicine. The major constituents of the Gardenia fruit extract are geniposide and crocins. Geniposide is one of iridoid glycosides providing strong anti-inflammatory activity^(5, 7, 12). The non-steroidal anti-inflammatory drugs (NSAIDs) work by inhibiting the cyclooxygenase enzymes that responsible for conversion of arachidonic acid to prostaglandins, inflammatory mediators and inhibiting lysosomal enzymes or stabilizing the lysosomal membrane which possesses anti-inflammatory activity. Lysosomal membrane and red blood cell (RBC) membrane are resemble. RBC has been used as an in-vitro model to evaluate anti-inflammatory activity by hypotonic induced haemolysis and heat induced haemolysis. The aim of this study was to evaluate in-vitro anti-inflammatory activity using sheep red blood cells (SRBC) membrane stabilization of gardenia fruit extract and compare with the reference drugs aspirin and indomethacin (NSAIDs)^(2, 3, 4, 6, 13).

Methodology

Chemical: The dried gardenia fruit was purchased from Vejpong Pharmacy Co., Ltd. (Bangkok, Thailand). Acetonitrile and water (HPLC grade) were purchased from RCI Labscan Co., Ltd. (Bangkok, Thailand). Aspirin was purchased from Bureau of drug and narcotic (Nonthaburi, Thailand). Indomethacin was from Department of Pharmaceutics and Industrial Pharmacy, Faculty of Pharmaceutical Sciences, Chulalongkorn University (Bangkok, Thailand). Sheep blood was purchased from Department of Animal Husbandry, Faculty of Veterinary Science, Chulalongkorn University (Bangkok, Thailand).

Extraction: Gardenia fruit extraction process was from Sunatwanichkul et al.,2017. Weighted 10 g of gardenia fruit was washed and soaked in 50 ml of distilled water for 60 min and then the filtrate was collected as portion 1. The crude was soaked again in 50 ml of 50% ethanol for 60 min and then the filtrate was collected and mixed with portion 1. The decoction was concentrated with a rotary evaporator under reduced pressure. The percent yield of the crude extract was calculated using equation 1. Then content of geniposide in the dried extract was determined using HPLC method. Percent yield of geniposide in the dried fruit and percentage of geniposide in the extract were calculated using equation 2 and 3, respectively.

$$\% \text{ Yield of extract} = \frac{(\text{weight of the extract (g)} \times 100)}{\text{weight of gardenia fruit (g)}} \quad \text{equation 1}$$

$$\% \text{ Yield of geniposide in gardenia fruit} = \frac{(\text{weight of geniposide (g)} \times 100)}{\text{weight of gardenia fruit (g)}} \quad \text{equation 2}$$

$$\% \text{ of geniposide in the extract} = \frac{(\text{weight of geniposide (g)} \times 100)}{\text{weight of the extract (g)}} \quad \text{equation 3}$$

Analysis of geniposide in gardenia extract using HPLC technique: Gardenia extract solution (1 mg/ml) was prepared in ultrapure water. Separation of geniposide and crocins was performed on a Shimadzu LC20A equipped with a C18 Column (5 µm), 150 x 4 mm (Agilent®). Separation condition was from Sunatwanichkul et al., 2017. The elution profile was 10% acetonitrile at start, the linear gradient to 18% acetonitrile from 0 to 15 min, the linear gradient to 28% acetonitrile from 15 to 20 min, the linear gradient to 38% acetonitrile from 20 to 40 min, the linear gradient to 50% acetonitrile from 40 to 50 min, the final elution at 50% acetonitrile from 50 to 55 min, and then the linear gradient to 10% acetonitrile from 55 to 64 min. The flow rate was 1 ml/min and injection volume of sample was 10 µl. The geniposide was detected at 238 nm.

Measurement of concentration of geniposide: Concentration of geniposide in a sample was determined using following procedure. Five concentrations between 0.00432 to 0.0864 mg/ml were prepared from standard geniposide and injected to the HPLC system. A linear correlation of peak area at 238 nm and geniposide concentration was plotted. The diluted sample solution of gardenia extract was injected to the HPLC system and its concentration was calculated from the correlation.

SRBC membrane stabilization: SRBC induced haemolysis was modified from Anosike et al., 2012 and Sachin et al., 2009.

Preparation of SRBC suspension: 10 ml of sheep blood was transferred to centrifuge tubes, then centrifuged at 3000 rpm for 20 min and washed three times with equal volume of isosaline (0.85% NaCl). The volume of the blood was measured and reconstituted to 10% v/v suspension using isosaline.

Hypotonic induced haemolysis: In sample tubes, the mixture (3.5 ml) consisted of 2 ml hyposaline (0.36% NaCl), 1 ml of 10 mM phosphate buffer saline (0.2 g of NaH₂PO₄, 1.15 g of Na₂HPO₄ in 1 L of distilled water, pH 7.4) mixed with gardenia fruit extract at 100 µg/ml. The negative control was prepared in the same manner without gardenia extract. In the positive control, aspirin or indomethacin was added in place of gardenia extract at the concentration of 120 and 100 µg/ml respectively. All the assay mixtures were incubated at 37°C for 30 min and centrifuged at 3000 rpm for 20 min. The haemoglobin content in the supernatant solution was estimated using UV spectrophotometer measuring absorbance at 560 nm. The percentage inhibition of haemolysis or membrane stabilization was calculated. (equation 4)

Heat induced haemolysis: In sample tubes, the mixture (3.5 ml) consisted of 2 ml isosaline, 1 ml of 10 mM phosphate buffer saline mixed with gardenia fruit extract at 100 µg/ml. The mixture without gardenia extract was prepared and used as the negative control. In the positive control, gardenia extract was replaced by aspirin or indomethacin at the concentration of 120 and 100 µg/ml respectively. All the assay mixtures were incubated at 56°C for 30 min and centrifuged at 3000 rpm for 20 min. The haemoglobin content in the supernatant solution was estimated using UV spectrophotometer measuring absorbance at 560 nm. The percent inhibition of haemolysis or membrane stabilization was calculated. (equation 4)

$$\% \text{ Inhibition of haemolysis} = \frac{(\text{Absorbance}_{\text{negative control}} - \text{Absorbance}_{\text{sample}})}{\text{Absorbance}_{\text{negative control}}} \times 100 \quad \text{equation 4}$$

Statistical analysis: The data was reported as mean ± standard deviation for three replicates.

Results

Extraction: Percent yields of gardenia fruit extract and geniposide are shown in Table 1. Table 1. The percent yields of gardenia fruit extract and geniposide.

Percent yield of extract (mean±SD)	Percentage of geniposide in the extract (mean±SD)	Percent yield of geniposide in gardenia fruit (mean±SD)
65.72 ± 0.75	5.50 ± 0.13	3.61 ± 0.16

Analysis of geniposide in gardenia extract using HPLC technique: Chromatogram of geniposide in gardenia fruit extract was detected wavelength at 238 nm in retention time at 9 min and standard curve of five concentrations between 0.00432 to 0.0864 mg/ml were prepared from standard geniposide are shown in figure 1.

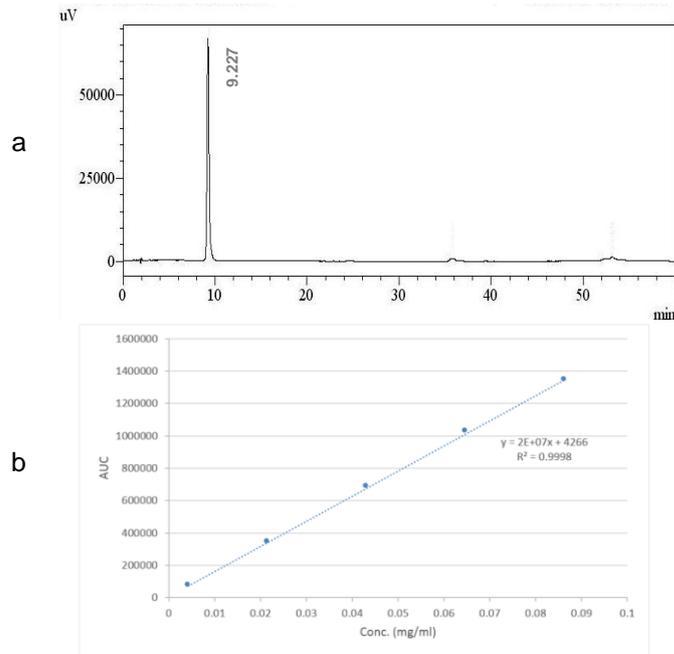


Figure 1. Chromatogram of geniposide detected at 238 nm (a) and the correlation plot of geniposide (b).

Hypotonic induce haemolysis: The result is shown in figure 2 and table 2

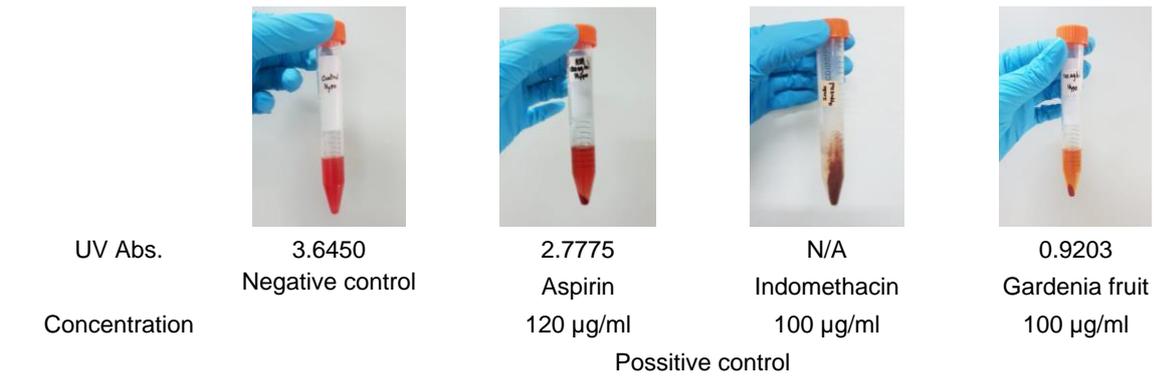


Figure 2. Haemolysis induced by hypotonic.

Heat induce haemolysis: The result is shown in figure 3 and table 2

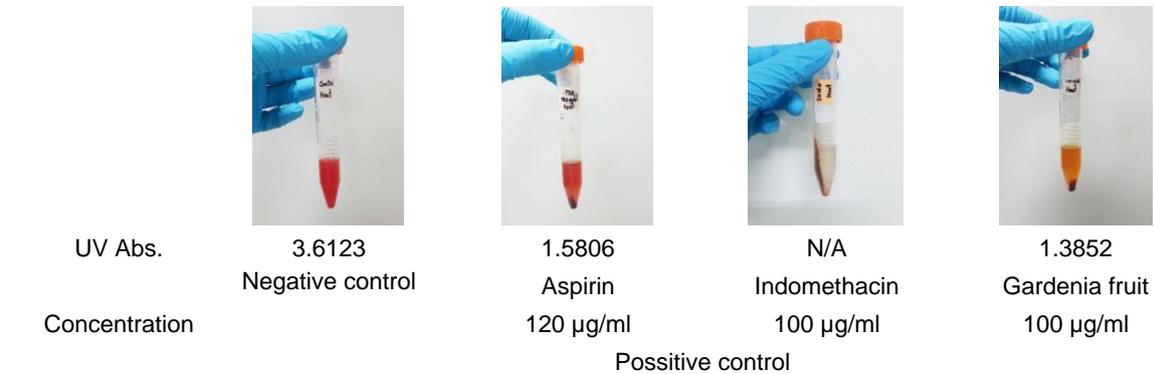


Figure 3. Haemolysis induced by heat.

Table 2. Percent inhibition of haemolysis.

	Hypotonic technique	Heat technique
Aspirin	23.80 ± 1.75%	56.24 ± 0.95%
Indomethacin	N/A	N/A
Gardenia extract	74.75 ± 1.28%	61.65 ± 1.78%

The results of hypotonic and heat induced haemolysis of the tested substances, Gardenia fruit extract at 100 µg/ml were protected the SRBC against lysis as shown by the percent inhibit haemolysis are more than 50% and were potent anti-inflammatory activity more than aspirin.

Discussion

Exposure to hypotonic condition and heat of RBC causes injury to the cell membrane leading to cell lysis and haemoglobin emerge. Hypotonic induced haemolysis is related to the difference of fluid inside and outside of the cell causing RBC swollen and broken⁽³⁾. When temperature is set at greater than 50°C in heat induced haemolysis, RBC is broken due to the loss of lipid and phospholipid in the cell membrane⁽¹⁾. Haemoglobin content is used as an indicator of haemolysis. Lysosomal membrane and RBC membrane are resemble. Lysosome and lysosomal enzymes involve in the inflammation process. Thus, substance stabilizing cell membrane are expected to inhibit inflammation. In negative control, high haemoglobin content was observed as expected which meaned complete haemolysis. Both aspirin and gardenia extract showed less haemolysis meaning that the membrane of RBC was stabilized. Moreover, gardenia extract was more potent than aspirin since less haemolysis was observed at a lower concentration. In the use of indomethacin, the mixture turned turbid due to the precipitation of indomethacin in aqueous medium. The solubility in water indomethacin is 0.937 mg/l⁽¹⁰⁾. Therefore, insoluble form of indomethacin could not provide the activity.

Conclusion

The use of 10 g gardenia fruit with water and 50% ethanol as extraction solvents of gardenia fruit provided the percent yield of extract 65.72 ± 0.75, the percentage of geniposide in the crude extract 5.50 ± 0.13 and the percent of geniposide in gardenia fruit 3.61 ± 0.16. The gardenia fruit extract effectively inhibited or stabilized RBC from the hypotonic induced and heat induced haemolyses and the extract presented higher potency than aspirin meaning stronger anti-inflammatory activity.

Acknowledgements

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