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Anti-inflammatory effects of rhein and crude extracts from *Cassia alata* L. in HaCaT cells

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ABSTRACT

Cassia alata L. is a medicinal plant and is in the Thai traditional household herbal drug list. Furthermore, *C. alata* leaves extract has been reported to have anti-inflammatory activities. The rhein, an active component, can inhibit inflammation via suppressing reactive oxygen species (ROS) production, leading to decreased cytokines (TNF-α, IL-8). However, the role of anti-inflammation on skin has been investigated only in a few research. The rhein in *C. alata* leaves extract was determined by HPLC. The anti-inflammatory effects of rhein and *C. alata* leaves extract on *tert*-butyl hydroperoxide-induced oxidative stress in HaCaT cells were evaluated. Anti-inflammatory activities of *C. alata* leaves extract were compared with rhein standard via inhibition of ROS generation and production of TNF-α and IL-8. The results showed that rhein content in *C. alata* leaves extract was 0.1225% w/w. Rhein (1-50 µM) significantly reduced ROS generation in a concentration-dependent manner, and paralleled the decrease in TNF-α and IL-8 production. *C. alata* leaves extract exhibited stronger anti-inflammatory effect than rhein at same concentration. These findings indicate that rhein and *C. alata* leaves extract may reduce inflammation by decreasing TNF-α and IL-8 as a result of ROS reduction. These results indicate that *C. alata* leaves may have the possibility of role as an anti-inflammatory agent. Therefore, further studies need to be performed.

Keywords: *Cassia alata*; Rhein; HaCaT cell; ROS; Anti-inflammatory

1. INTRODUCTION

Cassia alata L. or Chum-het-thet, family Fabaceae, is generally known as ringworm bush or candle bush. It is an herbal medicine that has been used for treatment of constipation, stomach pain, ringworm, scabies, purities, eczema, herpes and skin allergy (Idu et al., 2007; Gritsanapan and Mangmeesri, 2009). In Thailand, *C. alata* has been approved as a laxative in the Thai National List of Essential Drugs. The leaves contain both aglycone and glycoside

forms of anthraquinones including rhein, aloe-emodin, chrysophanol, glycosides of rhein, emodin, physcione and sennosides A, B, C, D while rhein is the major component (Gritsanapan and Mangmeesri, 2009). It has been used as herbal lotion for skin diseases such as tinea infections, insect bites, ringworms, scabies, herpes, blotch, eczema and mycosis (Singh et al., 2012). *C. alata* leaves are also reported to have anti-inflammatory, antimutagenic, analgesic, antidiabetic, antifungal and antimicrobial properties (Idu et al., 2007).

The skin provides the first line of defense against microbial pathogens, physical and chemical insults. Keratinocyte is the major cell type of epidermis, constituting more than 90% of epidermal cells. Keratinocytes form an effective barrier against an entry of foreign matter and infectious agents into a body. Human epidermal keratinocytes are always exposed to external stimuli which consequently induce ROS generation in cells (Bito and Nishigori, 2012). Since ROS play a crucial role in inflammation, they serve as a target for inflammation therapy (Wagener et al., 2013). Following skin exposure to stimulus intracellular sensors contained in an inflammasome complex in keratinocytes are activated, leading to production of ROS and to mediating the secretion of key pro-inflammatory cytokines. This in turn results in the activation of tissue-resident immune cells that induce inflammatory response (Nestle et al., 2009). ROS can elicit direct or indirect damage to the body and contribute to a large number of diseases (Idu et al., 2007). Cytokines are key modulators of inflammation, participating in acute and chronic inflammation. The key pro-inflammatory cytokines include IL-1, IL-6 and TNF-α and pro-inflammatory chemokine, IL-8. TNF-α is a type II transmembrane protein with diverse functions in cell differentiation, inflammation, immunity and apoptosis. TNF-α is primarily secreted from activated macrophages, but it may be secreted by other cell types including monocytes, T cells, mast cells, keratinocytes, fibroblasts and neurons. It is a potent inflammatory mediator that is central to the inflammatory action of the innate immune system, including induction of cytokine production, activation or expression of adhesion molecules, and growth stimulation (Turner et al., 2014). IL-8 is one of the most widely studied chemokines and is a critical inflammatory mediator. IL-8 is a member of the CXC primary inflammatory cytokine produced by many cells such as monocytes/macrophages, T cells, neutrophils, endothelial cells, keratinocytes, and melanocytes. In many cell types, synthesis of IL-8 expression is activated by lipopolysaccharides (LPS), IL-1, TNF-α, ionizing radiation, phytohemagglutinin, double-stranded RNA, and viruses (Twentymam and Luscombe, 1987). The main role of IL-8 in inflammation is in the recruitment of neutrophils, although it is responsible for the chemotactic migration and activation of monocytes, lymphocytes, basophils, and eosinophils at sites of inflammation (Turner et al., 2014).

At present, there are many studies regarding the biological activities of *C. alata* extract. However, a few research works have been done to investigate the anti-inflammatory activities on skin, especially in keratinocytes. In this study, we evaluated the effect of *C. alata* leaves extract on *t*-BHP induced oxidative stress in human keratinocyte cell line (HaCaT) cells to determine their anti-inflammatory activities compared with standard reference rhein.

2. MATERIALS AND METHODS

2.1 Materials and cell culture

Trypsin-EDTA, dulbecco's modified eagle medium (DMEM), phosphate buffered saline (PBS), fetal bovine serum (FBS), hank's balanced salt solution (HBSS), and penicillin-streptomycin were obtained from Gibco (USA). 2′,7′-Dichlorofluorescein diacetate (DCFDA) was from Invitrogen (USA).

Acetic acid (HPLC grade) and ethanol were purchased from Merck (USA). Amphotericin B solution (tissue culture grade) was obtained from Amresco (USA). Bovine serum albumin fraction V (BSA) was from GE healthcare (Austria). Dimethyl sulfoxide (DMSO) and tween 20 (Biotech grade) were purchased from Vivantis (Malaysia). Human TNF-α screening set and human IL-8 screening set were purchased from Thermo Scientific (USA). Hydrochloric acid (1.0 N), sodium hydrogen carbonate, and sodium hydroxide (1.0 N) were from RCI Labscan (Thailand). Methanol (HPLC grade) was from Labscan. 3-(4,5-Dimethythiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) was purchased from Bio basic (Canada). Rhein and *tert*-butyl hydroperoxide were obtained from Sigma (USA). Sucrose (analytical grade) was from Ajax Finechem (Australia). HaCaT cells were obtained from Dr. Tamaki Okabayashi from MOCID, FTM, Mahidol University, and RIMD, Osaka University.

The HaCaT cells was maintained in DMEM with 10% FBS, 10% penicillin-streptomycin, and 0.02% amphotericin B. Passage number of cell used in the experiments was less than 30, due to phenotypic changes. Culture medium was removed from HaCaT cell 75 cm^2 culture flask and washed twice with PBS, then trypsinized with 1 mL of 0.05% trypsin-EDTA. After incubated at 37°C in 5% CO_2 incubator for 10 min, the reaction was stopped by adding culture medium. Cells were incubated at 37°C in 5% CO_2 incubator. The medium was replaced every 48 h. Typically, cells were sub-cultured when they reached sub-confluence of plate surface.

2.1.1 Preparation of C. alata leaves extract

C. alata leaves were collected from Nakhon Pathom Province, Thailand. They were dried at 45°C and ground. The ground leaves were macerated with 95% ethanol for 20 min on water bath then filtered through Whatman filter paper no.1 and evaporated by rotary evaporator (55°C). The dried extract was stored at 4°C. The dried extract was kept 1 week before used. The yield of dried extract was 21.10%.

2.1.2 Quantitative analysis of rhein in C. alata leaves extract

Methanolic standard solutions of rhein were prepared in five calibration points (from 2.5 to 35 µg/mL). *C. alata* extract (0.1 g) was accurately weighed, dissolved in methanol, and adjusted to 10 mL in volumetric flask. All solutions were then sonicated at room temperature and filtered through 0.45 µm nylon syringe filter prior to analysis. Separation was achieved isocratically on a C18 column (Zorbax Eclipse Plus C18, 4.6 × 250 mm). The mobile phase consisted of methanol and 0.5% acetic acid in the ratio of 70:30 v/v and was pumped at flow rate of 1 mL/min. The total running time was 30 min and injection volume was 20 µL. The quantitation wavelength was set at 254 nm. The experiments were run in triplicate. The analytical method was validated under AOAC guidelines for standard method performance requirements through linearity, specificity, accuracy and precision.

2.1.3 Determination of cell viability

Cell viability was determined by MTT (3-(4,5-dimethythiazol-2-yl)-2,5 diphenyl tetrazolium bromide) colorimetric assay (Twentymam and Luscombe, 1987). HaCaT cells were seeded in 96-well flat bottom plate at 25,000 cells/well and incubated in 5% CO_2 incubator at 37°C for 48 h. After that, cells were treated with 200 µL of 1, 25, 50, 75, 100 µM rhein or 0.01% w/v (0.43 µM rhein), 0.05% w/v (2.15 µM rhein), 0.1% w/v (4.31 µM rhein), 0.3% w/v (12.93 µM rhein) *C. alata* extract for 1 h. Then, 100 µL of MTT solution was added in each well. After incubation at 37°C for 4 h, supernatant was replaced with 100 µL DMSO to dissolve formazan crystals. Cell viability was determined by measuring the

absorbance at the wavelength 550 nm using a microplate reader.

2.1.4 Measurement of ROS production

2′,7′-Dichloroflluorescein diacetate (H_2DCFDA) has been used as a fluorescent probe to detect intracellular hydrogen peroxide (H_2O_2) (Kalyanaraman et al., 2012). HaCaT cells were seeded in 96-well flat bottom plate at 25,000 cells/well and incubated in 5% CO_2 incubator at 37°C for 48 h. After that, cells were treated with 200 µL of 1, 25, 50 µM rhein or 0.01% w/v (0.43 µM rhein), 0.05% w/v (2.15 µM rhein) *C. alata* extracts for 1 h. Then, 200 µL of 1 mM *t*-BHP (*tert*-butyl hydroperoxide) was added in each well to induce oxidative stress. After incubation at 37°C for 1 h, supernatant was replaced with 100 µL of 20 mM H_2DCFDA. Reactions were then further incubated at 37°C for 30 min. ROS production was detected by fluorescence microscopy at an excitation wavelength of 485 nm and an emission wavelength of 535 nm.

2.1.5 Measurement of inflammatory cytokines production by ELISA

TNF-α and IL-8 produced from activated keratinocytes was quantified using enzyme-linked immunosorbent assay (ELISA) kit. Briefly, HaCaT cells were seeded in 24-well flat bottom plate at 10^5 cells/well and incubated in 5% CO_2 incubator at 37°C for 96 h. After that, cells were treated with 500 µL of 1, 25, 50 µM rhein or 0.01% w/v (0.43 µM rhein), 0.05% w/v (2.15 µM rhein) *C. alata* extracts for 1 h and followed by 500 µL of 1 mM *t*-BHP for 1 h. The supernatants were collected and stored at -80°C. TNF-α and IL-8 levels were measured in the supernatant samples by ELISA kits.

2.2 Data analysis

Experiments were performed in triplicate manner. The results were shown as mean ± standard deviation. Differences between groups were analyzed by one-way ANOVA. Significance was accepted at p-value <0.05, 0.01, 0.001.

3. RESULTS AND DISCUSSION

3.1 Quantitative analysis of rhein in *C. alata* leaves extract

Quantitative analysis of rhein in *C. alata* leaves extract was analyzed using HPLC technique. Data of analysis are presented in Table 1. The chromatograms of *C. alata* leaves extract and authentic rhein standard are compared in Figure 1. Rhein was eluted at retention time of 10.7 min in the chromatograms as shown in Figure 1A. The obtained chromatograms of the extract showed no interference to rhein peak. UV spectra of rhein in *C. alata* leaves extract and of rhein standard were compared (Figure 1B and 1C). The result showed good specificity. Calibration equation with the coefficient of determination (R^2) of 0.9981, was constructed using rhein standard sample over five points (from 2.5-35 µg/mL). The relative standard deviation values for inter-day and intra-day of rhein and *C. alata* leaves extract were less than 2.0%. This indicated that the method was sufficiently precise. Method accuracy was determined by analyzing *C. alata* leaves extract fortified with known quantities of each standard analyte. The recovery results were within the acceptance criteria (80-110%). Rhein content in the extract of *C. alata* leaves was 0.1225±0.0001% w/w. It was conformed with the previous studies (Fernand et al., 2008; Gritsanapan and Mangmeesri, 2009; Panichayupakaranant et al., 2009; Wuthi-udomlert et al., 2010) that showed rhein content in the range of 0.02-0.15% w/w.

Table 1 Rhein content in *C. alata* leaves extract and method validation data

Calibration equation of rhein standard		Y = 64.509x + 34.997	
Coefficient of determination, R^2		0.9981	
Accuracy	Concentration of rhein added (µg/mL)	% Recovery	
	12	93.69 ± 2.49	
	15	92.60 ± 0.16	
	18	93.35 ± 2.32	
Precision		% RSD	
		Intra-day (n=6)	Inter-day (n=6)
	Standard solution of rhein (15 µg/mL)	1.87	1.95
	C. alata leaves extract	1.94	1.68
Rhein content in the extract		0.1225 ± 0.0001% w/w	

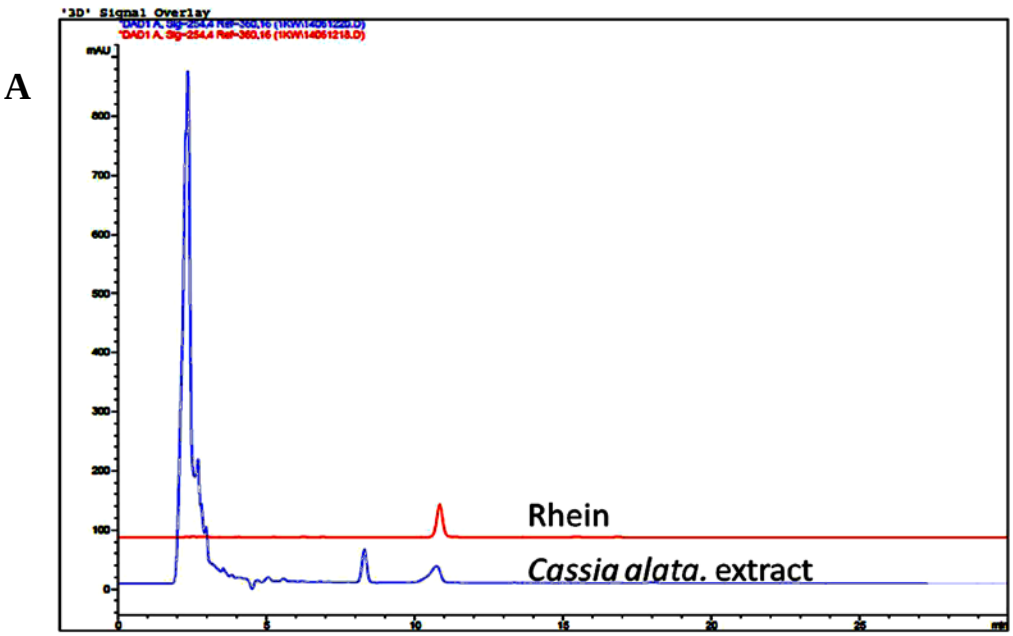

Figure 1 HPLC chromatograms of *C. alata* leaves extract and rhein standard at 254 nm (A), UV spectrum of *C. alata* leaves extract (B) and rhein standard (C), at retention time 10.7 min

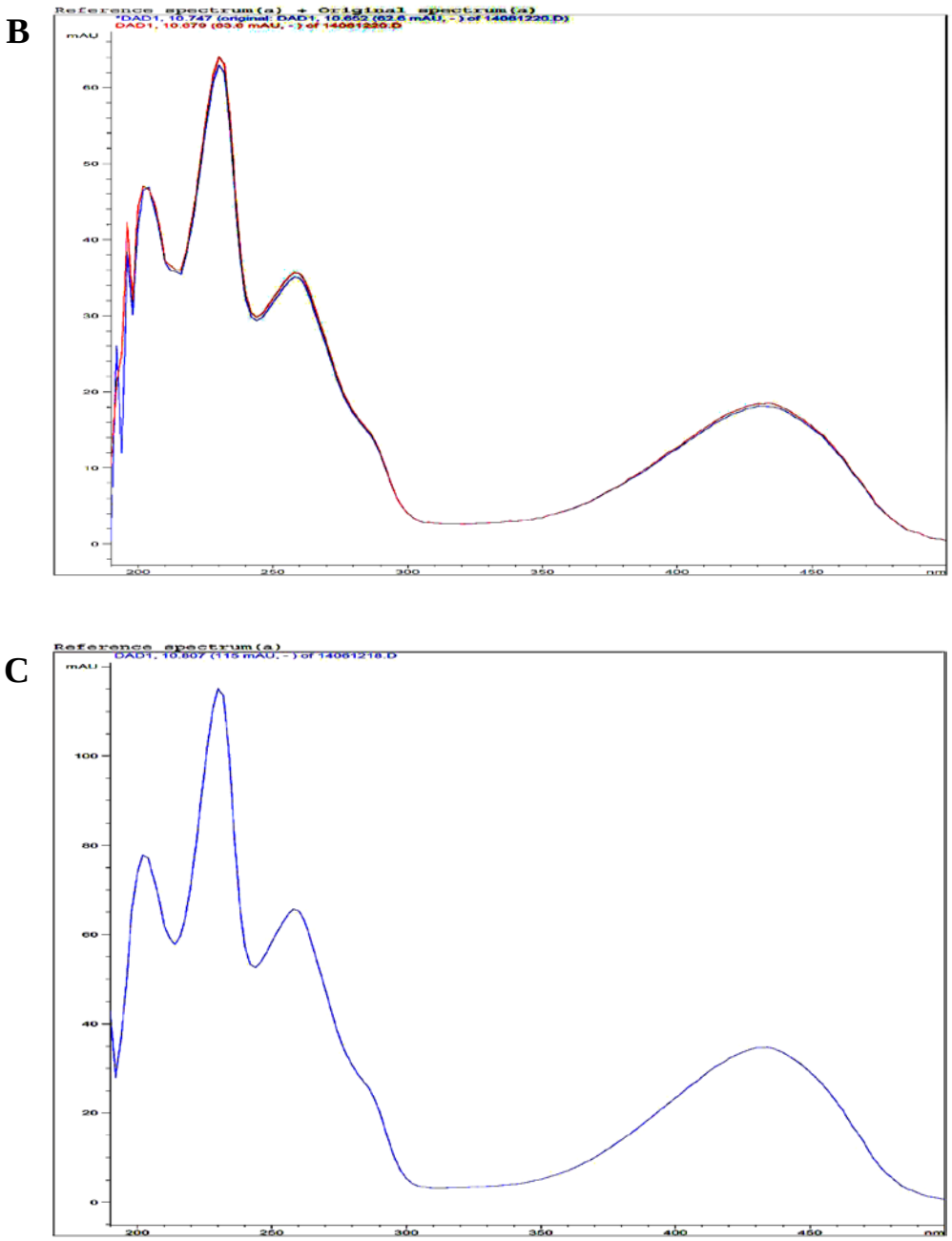

Figure 1 Continued.

3.2 Determination of cell viability

Cell viability was determined by MTT colorimetric assay. To exclude the possibility of the reduction in cell viability and the production of ROS and inflammatory cytokines by the cells occurred due to direct toxicity of rhein and *C. alata* leaves extract, we evaluated cell toxicity using various concentrations of rhein standard (1-100 µM) and *C. alata* leaves extract (0.01-0.3% w/v). Treatment with rhein standard at concentrations up to 50 µM and *C. alata*

leaves extract at 0.01% w/v (Figure 2, 3) had no effect on HaCaT cell viability. Following rhein standard and *C. alata* leaves extract incubation, cell viability was determined with MTT. Cells were also photographed before dissolving formazan crystals with DMSO under the microscope (Figure 2). As shown in Figure 3, cell viability of control was expressed as 100%. Cell viability after incubation with 1, 25, 50, 75 and 100 µM rhein standards was 97.78±9.33, 93.58±7.67, 92.57±6.87, 75.92±6.11 and 62.79±6.70%, respectively (Figure 3). Cell viability after incubation with 0.01, 0.05, 0.1 and 0.3% w/v *C. alata* leaves extract was 92.86±8.29, 76.17±8.86, 65.60±9.02 and 42.88±9.39%, respectively (Figure 3). The result showed that rhein standard at 1-50 µM and *C. alata* leaves extract at 0.01% w/v had no effect on HaCaT cell viability. Therefore, rhein standard 1-50 µM and 0.01% w/v *C. alata* leaves extract (0.43 µM rhein) were used in the subsequent experiments.

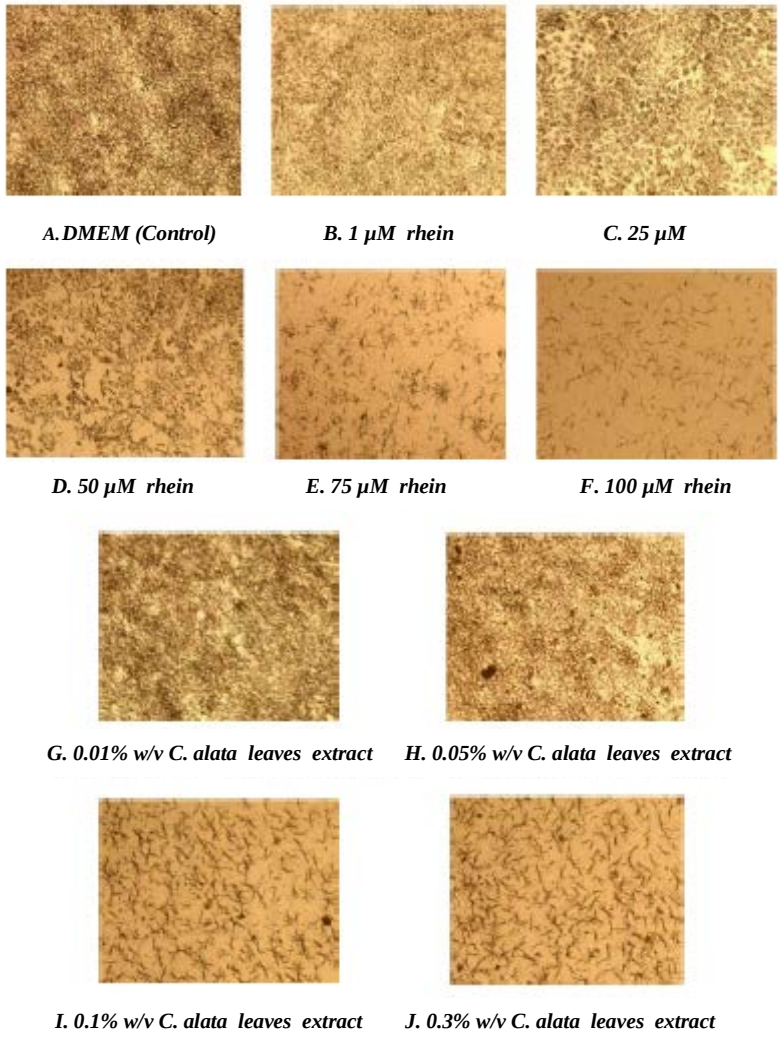

Figure 2 Formazan crystals in HaCaT cells after exposure to rhein standards (A-F) and *C. alata* leaves extracts (G- J)

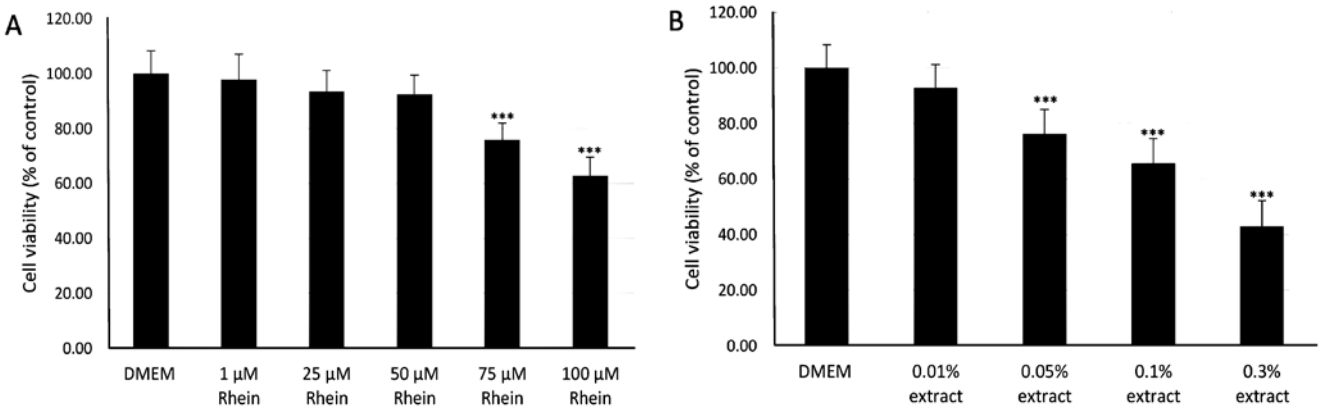

Figure 3 Effect of rhein standard and *C. alata* leaves extract on cell viability in HaCaT cells using MTT assay. HaCaT cells were treated with various concentrations of rhein standard (A) and *C. alata* leaves extract (B). Data are expressed as mean ± SD (n=3). ***$p \leq 0.001$ compared to the DMEM control group

3.3 Measurement of ROS production

To determine the effect of rhein standard and *C. alata* leaves extract on intracellular ROS production, HaCaT cells were treated with various concentrations of rhein standard (1-50 µM) and *C. alata* leaves extract (0.01% w/v (0.43 µM rhein)) for 1 h, after which they were stimulated with 1mM *t*-BHP to induce ROS production. ROS production were then detected by H_2DCFDA fluorescence probe.

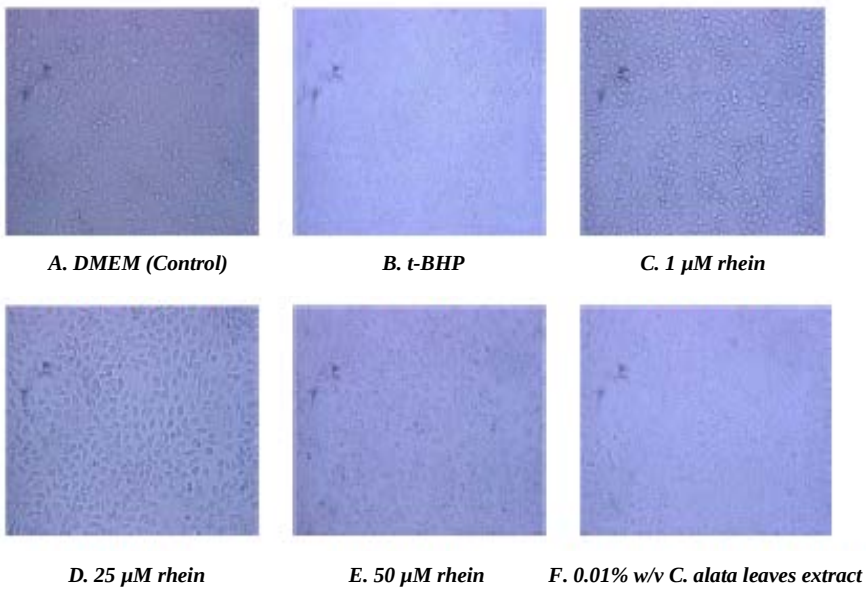

Figure 4 HaCaT cells after exposure to rhein standard and *C. alata* leaves extract then subjected to treatment with *t*-BHP

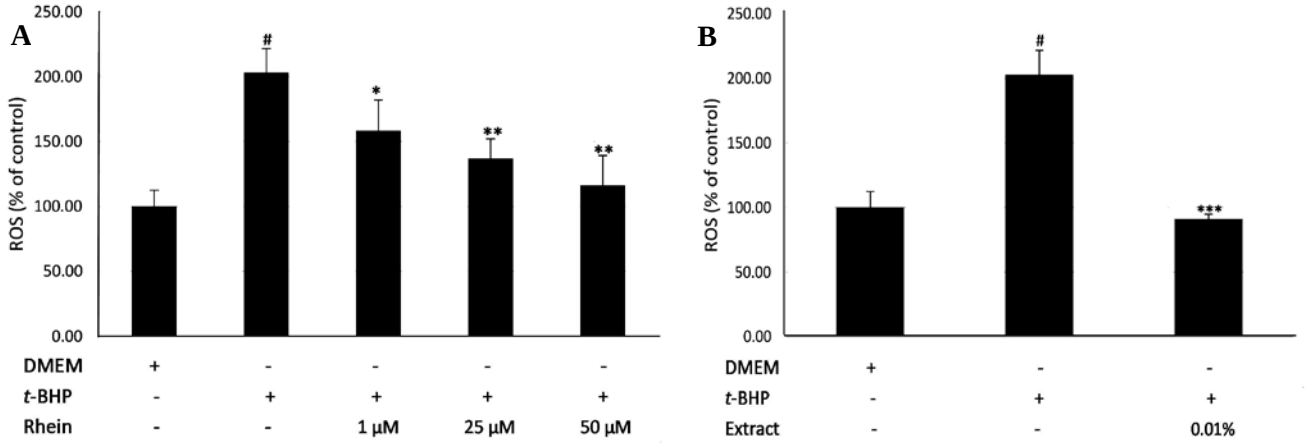

Figure 5 Effect of rhein standard and *C. alata* leaves extract on intracellular ROS production in HaCaT cells. ROS production in various concentrations of rhein standard (A) and *C. alata* leaves extract (B). Data are expressed as mean±SD (n=3). #p ≤ 0.001 compare to DMEM, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 compared to *t*-BHP treated group

As seen in Figure 4, the morphology of HaCaT cells was observed under the microscope. HaCaT cell with DMEM solution treatment showed polygonal-shaped, adherent cells growing as a confluent monolayer. Cells treated with *t*-BHP had a morphological change, shrink into spherical shape. Cells treated with rhein standard (1, 25, 50 µM) and *C. alata* leaves extract (0.01% w/v), displayed only moderate morphological changes.

Experiment data were analyzed as percentage of ROS production compared with control cells (Figure 5). The production of ROS in control group is expressed as 100%. ROS production was significantly increased after exposure to *t*-BHP (202.97±18.63%) (Figure 5A). However, treatment with rhein or *C. alata* leaves extract resulted in a significant and dose-dependent decrease in ROS production. ROS production in HaCaT cells pre-incubated with 1, 25 and 50 µM rhein standard prior to treatment with 1 mM *t*-BHP were 158.23±23.72, 136.83±15.07 and 116.00±23.08%, respectively. ROS production after pre-incubation with 0.01% w/v *C. alata* leaves extract and exposure to *t*-BHP were 91.33±3.43%. Furthermore, the effects of *C. alata* leaves extract were greater than those of rhein in *t*-BHP treated HaCaT cells.

ROS has been known to act as novel mediator for inflammation. Increased production of ROS, thereby, signifies inflammation state in the cells. *t*-BHP is a lipid hydroperoxide analog, used as a pro-oxidant substance to evaluate the mechanism that involve with oxidative stress in cells or tissue. It is changed to *t*-butoxyl and methylradicals, lead to production of lipid oxidation (Kim et al., 2012; Jayashree et al., 2015). Pre-treatment with rhein standard and *C. alata* leaves extract effectively inhibited *t*-BHP induced ROS production in a concentration-dependent manner. Similar results with rhein were reported for monocyte cell line that rhein can decreased ROS production (Heo et al., 2010). Moreover, rhein can protect the β-cells against hyperglycemia-induced cell apoptosis through suppressing ROS production (Heo et al., 2010) and against acetaminophen-induced hepatic and renal toxicity (Liu et al., 2013).

As mentioned above, percentage of rhein content in the extract of *C. alata* leaves determined by HPLC was 0.1225±0.0001% w/w. In 0.01% w/v *C. alata* leaves extract, rhein content was equivalent to 0.43 µM.

A decrease in ROS production of 55% was observed when cells were treated with 0.01% w/v *C. alata* leaves extract, while pre-treatment with 1, 25 and 50 µM rhein standard resulted in decreases in ROS production of 25%, 36%, 41%, respectively. *C. alata* leaves extract exhibited stronger anti-inflammatory effects than the rhein standard. This may be due to other compounds, such as kaempferol, aloe-emodin and emodin in the extract, which had also been reported to have anti-inflammatory effects (Liu et al., 2009; Heo et al., 2010; Zhao et al., 2011; Promgool et al., 2014).

3.4 Measurement of inflammatory cytokines production by ELISA

Inflammatory cytokines/chemokines including TNF-α, IL-8 play an important role in mediating inflammatory responses (Heo et al., 2010). To determine if rhein and *C. alata* leaves extract exerted an effect on *t*-BHP-induced cytokines/chemokines production, we pretreated HaCaT cells with 1, 25 and 50 µM rhein and 0.01% w/v (0.43 µM rhein) and 0.05% w/v (2.15 µM rhein) *C. alata* leaves extract for 1 h, after which the cells were stimulated with 1 mM *t*-BHP. The cell-free supernatants were then collected, and the cytokines/chemokines were assayed using ELISA kits for TNF-α and IL-8.

As shown in Figure 6A, the amount of TNF-α secretion of control cell was 160.33±16.95 pg/mL and was significantly increased after exposure to *t*-BHP (731.44±95.95 pg/mL). The amount of TNF-α secretion of cells treated with 1, 25 and 50 µM rhein standard prior to treatment with 1 mM *t*-BHP were 123.50±15.61, 118.39±15.22 and 108.10±10.13 pg/mL, respectively. The amount of TNF-α secretion of cells treated with 0.01% w/v *C. alata* leaves extract and exposure to *t*-BHP were 91.25±12.56 pg/mL.

As shown in Figure 6B, the amount of IL-8 secretion of control cells was 436.36±48.65 pg/mL and IL-8 secretion was significantly increased after exposure to *t*-BHP (1,313.39±219.61 pg/mL). HaCaT cells were pre-incubated with 1, 25 and 50 µM rhein standard prior to treatment with 1 mM *t*-BHP. The amount of IL-8 after expose to *t*-BHP were 777.60±61.04, 706.73±80.10 and 673.11±61.46 pg/mL, respectively. The amount of IL-8 secretion of cells treated with 0.01% w/v *C. alata* leaves extract and exposure to *t*-BHP were 504.99±148.54 pg/mL.

As shown in Figure 6, the production of TNF-α and IL-8 increased significantly following exposure to *t*-BHP, reaching values that were greater than the control values of HaCaT cells. Moreover, rhein and *C. alata* leaves extract treatment led to a marked decrease in *t*-BHP-induced TNF-α and IL-8 production, and this change occurred in a dose-dependent manner of rhein. Furthermore, the effects of *C. alata* leaves extract were greater than those of rhein.

TNF-α and IL-8 production in *t*-BHP induced HaCaT cell were elevated significantly from control, which could represent the inflammation state in the cells. Pre-treatment with rhein could diminish TNF-α and IL-8 production of *t*-BHP treated cells. We reported that *t*-BHP induced ROS production were reduced by rhein standard and *C. alata* leaves extract. Taken together, these results suggest that ROS plays a significant role in *t*-BHP induced TNF-α and IL-8 production. Thus, blocking ROS production inhibits *t*-BHP induced TNF-α and IL-8 production.

Moreover, *C. alata* leaves extract exhibited stronger anti-inflammatory effects than rhein standard. *t*-BHP induced TNF-α production was inhibited by 87% when cells were treated with 0.01% w/v *C. alata* leaves extract (0.43 µM rhein content), while 83%, 84%, 85% inhibition were seen with 1, 25 and 50 µM rhein standard, respectively. The results obtained from IL-8 production were also similar. 0.01%w/v (0.43 µM rhein) *C. alata* leaves extract inhibited *t*-BHP-induced IL-8 production by 62% while 1, 25 and 50 µM rhein standard resulted in 41%, 46%, 49% inhibition, respectively. This may be due to

other compounds, such as flavonoids and other anthraquinones in the extract, that also have anti-inflammatory effects (Liu et al., 2009; Heo et al., 2010; Promgool et al., 2014; Ramsay and Mueller-Harvey, 2016).

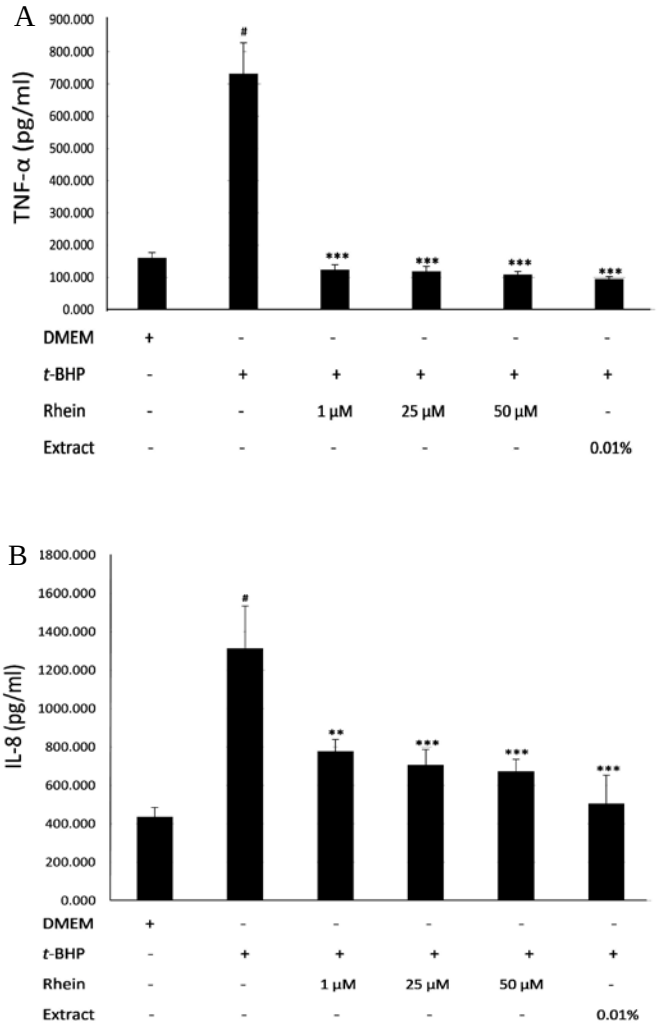

Figure 6 Inhibitory effect of rhein standard and *C. alata* leaves extract on cytokines production in HaCaT cells. TNF-α (A) and IL-8 (B). Data are expressed as mean±SD (n=3). #p ≤ 0.05 compare to control, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 compared to *t*-BHP treated group

ROS induces inflammation by stimulating the production of pro-inflammatory cytokines. TNF-α and IL-8 are pro-inflammatory cytokines which appear to be major mediators in skin inflammation (Muller and Meineke, 2007; Turner et al., 2014). Previous studies have revealed that *C. alata* leaves extract effectively inhibited ROS and TNF-α production in monocyte cells (Ramsay and Mueller-Harvey, 2016). Rhein is a major compound in *C. alata* and has been shown to have anti-inflammatory activity (Yaron et al., 1999; Borderie et al., 2001; Sanchez et al., 2003; Gritsanapan and Mangmeesri, 2009; Heo et al., 2010; Sheng et al., 2011; Cong et al., 2012; Hu et al., 2013). Rhein treatment inhibited in LIGHT-induced IL-8, MCP-1, TNF-α and IL-6 production in monocyte cell (Heo et al., 2010).

4. CONCLUSION

This research determined rhein anthraquinone content in *C. alata* leaves extract and investigated anti-inflammatory activities of rhein and *C. alata* leaves extract on *t*-BHP induced inflammation in the human keratinocytes, HaCaT cell. We found that ROS production induced by *t*-BHP was significantly increased compared to control cell. Pre-treatment with rhein standard or *C. alata* leaves extract could diminish ROS production. Pre-treatment with rhein and *C. alata* leaves extract could diminish TNF-α and IL-8 production of *t*-BHP-treated cells. *C. alata* leaves extract exhibited stronger inhibition of ROS production than rhein standard at the same concentration of rhein. Similar result was obtained concerning inhibition of *t*-BHP-induced TNF-α and IL-8 production. This may be due to other compounds in the extract that also have anti-inflammatory effects.

In summary, rhein and *C. alata* leaves extract inhibited *t*-BHP-induced inflammatory responses such as production of TNF-α and IL-8, and these effects occurred via the suppression of ROS production. Taken together, these results indicated that *C. alata*

leaves extract have the potential for use as an anti-inflammatory agent and may be particularly useful for the prevention of skin inflammation. For further study, rhein and *C. alata* leaves extract should be investigated for other anti-inflammatory mechanisms and its toxicity before further development.

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