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Research Article

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Suppressive effect of ethanolic extract of *Annona squamosa* L. leaves on the expression of Id1 biomarker: Phytochemical investigation and antioxidant activity study

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ABSTRACT

Recognized as an incurable disease, psoriasis is a chronic inflammatory disease, which is characterized by hyperproliferation and abnormal differentiation of keratinocytes. Although psoriasis has become a public health concern, its precise causes remain to be elucidated. The current study aimed at evaluating the suppressive effect of each of these following Thai plant extracts with anti-psoriatic activity, Alpinia galanga L. (rhizome), Curcuma longa L. (rhizome) and Annona squamosa L. (leaf), on the expression of the helix-loop-helix transcription factor Id1 (inhibitor of differentiation or inhibitor of DNA binding), which is known as a biomarker for psoriasis. In addition, we would like to assess their antioxidant contents and activities in order to provide insights into the mechanism underlying the inhibitory effect. Using confocal immunofluorescence technique, we found that the expression of Id1 protein was clearly reduced upon treating the HaCaT cells with 6.3, 3.15 or 1.575 µg/ml (IC_{50}= 6.3µg/ml) of Annonas quamosa leaf extract. Western blot analysis confirmed that the Id1 protein was significantly attenuated after treating HaCaT cells with all tested concentrations of ethanolic extract of Annona squamosa leaves ($P<0.05$). No inhibitory effect of Alpinia galanga and Curcuma longa extracts on the Id1 expression was observed. Regarding antioxidant contents and antioxidant activities, although it exhibited the inhibitory effect on the Id1 expression, the extract of Annona squamosa was not the richest. This suggests that the suppressive effect of Annona squamosa extract may not be primarily responsible by antioxidant contents and activities.

Keywords: Id1, herbal medicine, total phenolic content, total flavonoid content, antioxidant activity

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INTRODUCTION

Psoriasis is a chronic inflammatory skin disorder, and the psoriatic lesions are uniquely filled with hyperproliferation and abnormal differentiation of keratinocytes. It is also classified as an autoimmune disease since dysregulation of the immune system is recognized as a critical occurrence in this ailment [1-3]. This particular disease seriously affects the patients' quality of life. Furthermore, the psoriatic patients have been shown to be at high risk with other complications, thus worsening their health [4-10]. Psoriasis is incurable, and purpose of current treatment is to alleviate the disease condition. In Thailand, this disease has become a public health concern [11]. Herbal medicine may help reducing not only side effects, but also saving expenses from imported drugs. Based on

the fact that Thailand is rich of herbs, we previously demonstrated that ethanolic extracts of *Alpinia galanga* L. (rhizome), *Curcuma longa* L. (rhizome) and *Annona squamosa* L. (leaf), exhibited antipsoriatic activity [12]. We then have attempted to elucidate the underlying mechanisms responsible by the mentioned plant extracts [13, 14].
According to the study of cholesteatoma, the helix-loop-helix transcription factor Id1 (inhibitor of differentiation or inhibitor of DNA binding) was demonstrated to contribute to hyperproliferation of keratinocytes via augmentation of cell cycle progression, elimination of cell cycle inhibition, and concurrently promoted keratin production [15]. A hallmark of cholesteatomais is hyperproliferation of keratinocytes, which is comparable to the cellular episode found in psoriasis, thus suggesting Id1 as a biomarker for both diseases. In fact, this particular transcription factor was already well recognized to function in suppressing cell differentiation and promoting cell proliferation. HaCaT keratinocyte cell line, an *in vitro* cellular model for psoriasis, was shown to express Id1 mRNA in a high level [16]. Of note, a raised Id1 gene expression was uncovered in keratinocytes of psoriatic patients' skin [17], and an upregulated Id1 pathway was further reported [18]. We showed that the levels of Id1 transcripts in peripheral blood mononuclear cells of psoriatic patients were about 2-fold greater than those in controls, thus emphasizing the molecular role of Id1 in psoriasis [19]. Furthermore, we recently demonstrated the inhibitory effect of the antipsoriatic drug dithranol on mRNA and protein expression levels of Id1 in the HaCaT keratinocyte cell line [20].
The antipsoriatic activity of tested Thai herbs may actually mediate by suppressing the expression of Id1 protein. Therefore, we would like to investigate their effect on the expression of Id1 in this study. In addition, we aimed at evaluating their antioxidant contents and activities in order to provide insights into the mechanism underlying the inhibitory effect.

EXPERIMENTAL SECTION

Plant materials

Annona squamosa (leaf) was collected from Ratchaburi province, Thailand. *Alpinia galanga* (rhizome) and *Curcuma longa* (rhizome) were collected from the Princess Maha Chakri Sirindhorn Herbal Garden, Rayong province, Thailand. At Department of Botany, Faculty of Science, Chulalongkorn University, Bangkok, Thailand, the herbs were authenticated by Professor Dr. Thaweesakdi Boonkerd and deposited at the Professor Kasin Suvatabhandhu Herbarium with assigned voucher specimen numbers, 013509 (BCU), 013397 (BCU) and 013396 (BCU), respectively.

Preparation of Thai medicinal plant extracts

With respect to the determination of Id1 expression, *Annona squamosa* leaf extract used was prepared by maceration at room temperature with ethanol using a 1:5 (w/v) ratio in a shaking incubator at 120 rpm for 48 h as formerly described [12-13]. For other subsequent experiments, all Thai medicinal herbs were successively extracted with three different solvents, petroleum ether, dichloromethane and ethanol (Merck, Hohenbrunn, Germany) with a 1:10 (w/v) ratio by a Soxhlet extractor. Crude extracts were concentrated using the MiVac Quattro concentrator at 30°C (for petroleum ether and dichloromethane) and 45°C (for ethanol). Eventually, the crude extracts were dissolved in dimethyl sulphoxide (DMSO) (Merck) as stock solutions at a concentration of 100 mg/ml, stored at -20°C and protected from light. Crude extracts were filtered through a 0.2-µm-pore-size filter (Corning Inc., Corning, NY, USA).

Determination of Id1 protein expression

The expression of Id1 protein was evaluated quantitatively and qualitatively using confocal immunofluorescence microscopy and Western blot, respectively as previously described [14].

Determination of total phenolic and flavonoid contents and antioxidant activities

Total phenolic content in the plant extracts was determined using Folin-Ciocalteu assay as formerly described [21]. Gallic acid was used a standard at varying concentration (0.78 - 50 µg/ml). Briefly, 50 µl of each extract (1 mg/ml) or gallic acid was mixed with 50 µl of working Folin and 50 µl of 0.1 M Na_2CO_3. After incubation at room temperature for 1 hr in the dark, the absorbance of the reaction mixture was measured at 750 nm. The concentrations of phenolic compounds are expressed as mg of gallic acid equivalent (GAE) per gram of dry sample (mg GAE/g of sample).

Total flavonoid content in the plant extracts was determined using aluminum chloride ($AlCl_3$) colorimetric assay as previously described [22]. Quercetin at varying concentrations (0.78 - 50 µg/ml) was used in this assay in order to construct a standard curve. In brief, 50 µl of each extract (1 mg/ml) or quercetin was mixed with 10 µl of 1 M

sodium acetate, 150 µl of 95% ethanol and 10 µl of 10% $AlCl_3$. The absorbance of the reaction mixture was measured immediately at a wavelength of 415 nm after incubation for 40 min in the dark. The concentrations of flavonoid compounds are expressed as mg of quercetin equivalent (QE) per gram of dry sample (mg quercetin/g of sample). For antioxidant activities, DPPH and ABTS radical scavenging assays were performed as described elsewhere [23].

Statistical analysis

All values are presented as the mean ± S.E.M (standard error of mean). Each experiment consisted of at least three independent experiments.

RESULTS AND DISCUSSION

Effect of ethanolic extract of *Annona squamosa* leaves on the expression of Id1 protein

In this part, *Annona squamosa* leaves were extracted by maceration at room temperature with ethanol using a 1:5 (w/v) ratio in a shaking incubator at 120 rpm for 48 h as described elsewhere [12-13]. Using confocal immunofluorescence technique, we found that the expression of Id1 protein was clearly reduced upon treating the HaCaT cells with 6.3, 3.15 or 1.575 µg/ml (IC_{50}= 6.3 µg/ml) of *Annonas quamosa* leaf extract as demonstrated in Figure 1. Then, a qualitative protein assay using Western blot was performed. In Figure 2, Id1 protein was significantly attenuated after treating HaCaT cells with all tested concentrations of ethanolic extract of *Annona squamosa* leaves ($P<0.05$).

In this work, we did not find any inhibitory effect of the extracts of *Alpinia galanga* and *Curcuma longa* on the Id1 expression (data not shown). Our result with a significant impact of *Annona squamosa* encouraged us to further investigate the potential mechanism underlying this effect. Like the recent study of Tada and colleagues [24] revealing the inhibitory effect of the Japanese apricot namely Ume on the Id1 expression, we hypothesized that this particular suppressive activity was primarily driven by phytochemicals and antioxidants in *Annona squamosa* leaves. However, the first part of our investigation, we extracted *Annona squamosa* leaves using maceration with ethanol. To narrow them down, we then extracted them using a successive technique with a Soxhlet extractor as detailed in the experimental section. The resulting extracts were employed in all subsequent experiments of this study. Therefore, we then performed the phytochemical and antioxidant evaluation since this particular natural product may exert its antipsoriatic effect via an inhibition of Id1 expression.

Phytochemical evaluation

In this study, we determined total phenolic and total flavonoid contents of Thai medicinal plant species with antipsoriatic activity including *Annona squamosa, Alpinia galanga* and *Curcuma longa* successively extracted using three different solvents (petroleum ether, dichloromethane and ethanol). As in Figure 3, *Annona squamosa* extracts were found to contain phenolic compounds only in extracts derived from dichloromethane and ethanol in a quantity of 4.70±0.49 mg GAE/g of sample and 23.01±1.01 mg GAE/g of sample, respectively. However, the phenolic content of *Annona squamosa* extracts was obviously less than that of *Curcuma longa* extracts with a quantity of 71.84±2.29, 64.71±3.19 and 12.54±1.14 mg GAE/g of sample in extracts derived from dichloromethane, ethanol and petroleum ether, respectively. As we expected, it was true that ethanolic extract of *Annona squamosa* was substantially high, and this might be supporting evidence with respect to its antipsoriatic effect via inhibition of Id1 expression. Nevertheless, the ethanolic extract of *Curcuma longa*, without a suppressive effect on Id1 expression, was around 2.8 fold greater than that of *Annona squamosa*. This might suggest that the inhibitory effect on Id1 expression was not primarily originated from the phenolic compounds, and this finding actually encouraged us to do another experiment, the total flavonoid content assay.

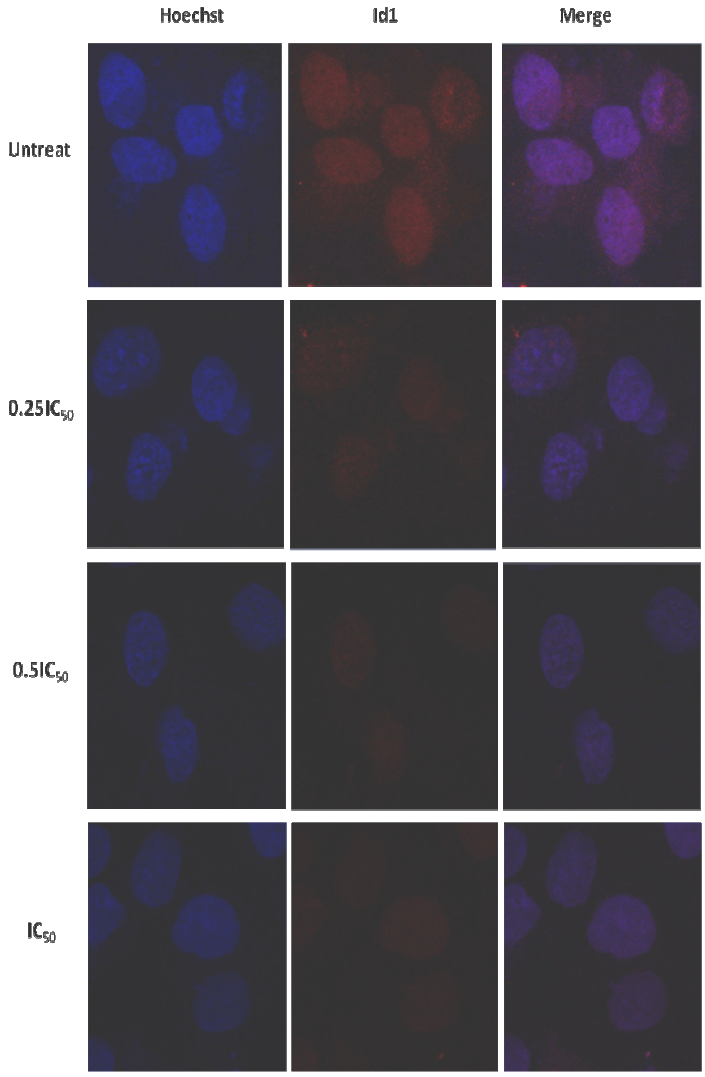
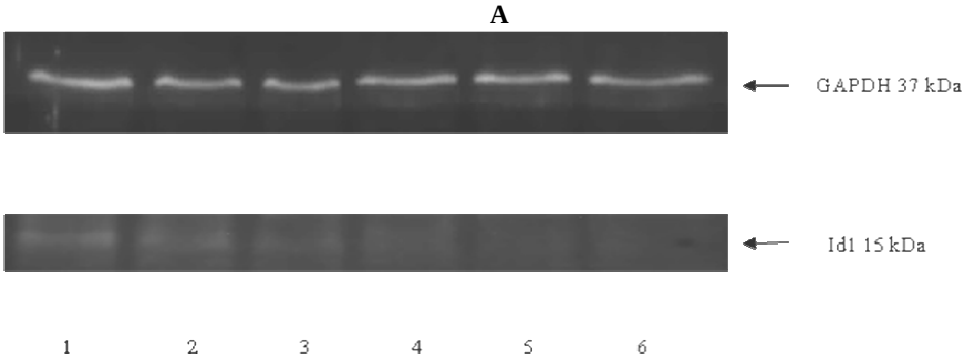


Figure 1. Expression of Id1 protein as detected by immunocytochemistry and confocal microscopy in HaCaT cells when treating cells with 6.3, 3.15 or 1.575 µg/ml (IC_{50}= 6.3) of *Annona squamosa* extract for 48 h. Untreat: HaCaT cells treated with IFN-γ and TNF-α, $0.25IC_{50}$: HaCaT cells with IFN-γ, TNF-α and 1.575 µg/ml ($0.25IC_{50}$) of *Annona squamosa*, $0.5IC_{50}$: HaCaT cells with IFN-γ, TNF-α and 3.15 µg/ml ($0.5IC_{50}$) of *Annona squamosa*, IC_{50}: HaCaT cells with IFN-γ, TNF-α and 6.3 µg/ml (IC_{50}) of *Annona squamosa*.



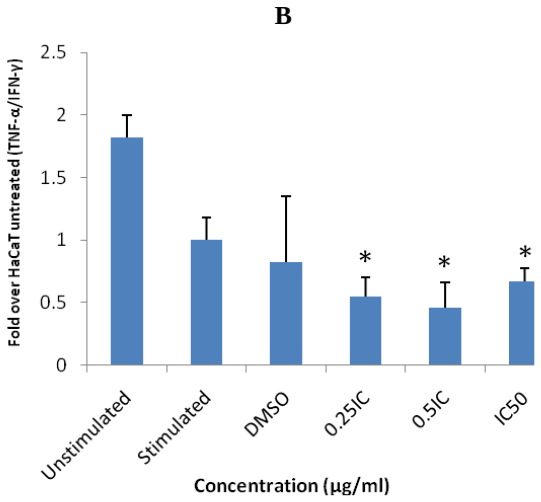


Figure 2. Expression of Id1 and GAPDH proteins as detected by Western blotting analysis in HaCaT cells when treating cells with 6.3, 3.15 or 1.575 µg/ml (IC_{50}= 6.3) of *Annona squamosa* extract for 48 h. A. The expression levels of Id1 and GAPDH were shown as a result of Western blotting analysis. Lanes 1: HaCaT cells with medium alone (unstimulated condition), 2: HaCaT cells with IFN-γ and TNF-α (control or stimulated condition), 3: HaCaT cells with IFN-γ, TNF-α and 0.0067% DMSO, 4: HaCaT cells with IFN-γ, TNF-α and 1.575 µg/ml (0.25IC_{50}) of *Annona squamosa*, 5: HaCaT cells with IFN-γ, TNF-α and 3.15 µg/ml (0. 5IC_{50}) of *Annona squamosa*, 6: HaCaT cells with IFN-γ, TNF-α and 6.3 µg/ml (IC_{50}) of *Annona squamosa*. B. After Id1 protein is normalized against that of GAPDH, data represent the fold expression over that of control. A symbol, *, indicating a statistically significant difference (P< 0.05)

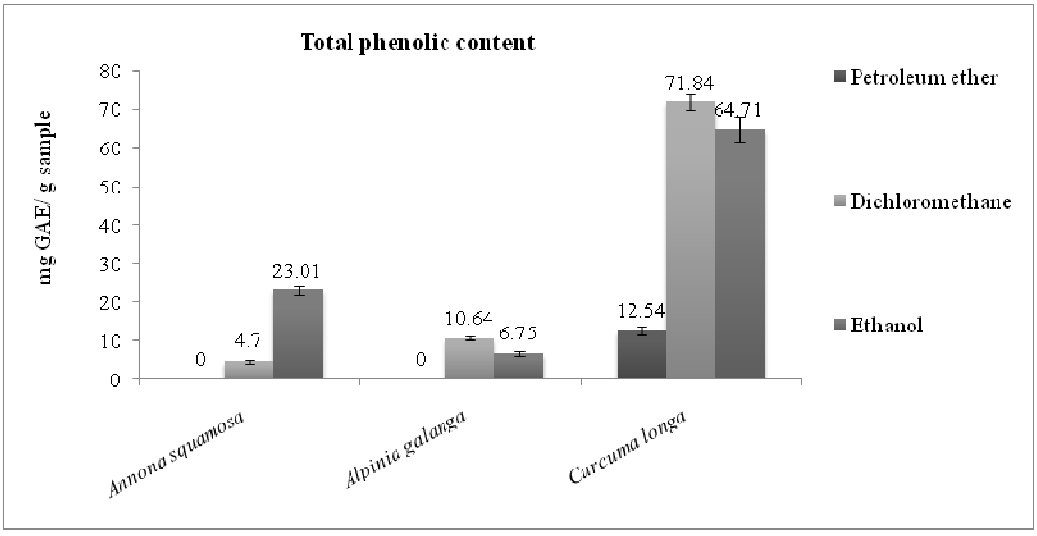


Figure 3. Total phenolic content in various extracts of *Annona squamosa, Alpinia galanga* and *Curcuma longa* as determined using Folin-Ciocalteu assay

Total flavonoid content in all plant extracts was determined using aluminum chloride colorimetric assay. As shown in Figure 4, *Annona squamosa* extracts were found to possess total flavonoid content in a quantity of 63.76±2.58, 40.07±1.49 and 19.59±1.98 mg quercetin/g of sample in dichloromethane, ethanol and petroleum ether, respectively. In line with the total phenolic content, the highest content of total flavonoid with regard to each extracting solvent was found in those of *Curcuma longa* when comparing among all three plant species. While the lowest was found in extracts of *Alpinia galanga* and *Curcuma longa* extracts had a huge quantity of flavonoids, 775.90±10.30, 596.00±7.14 and 20.27±3.07 mg quercetin/g of sample, in dichloromethane, ethanol and petroleum ether, respectively.

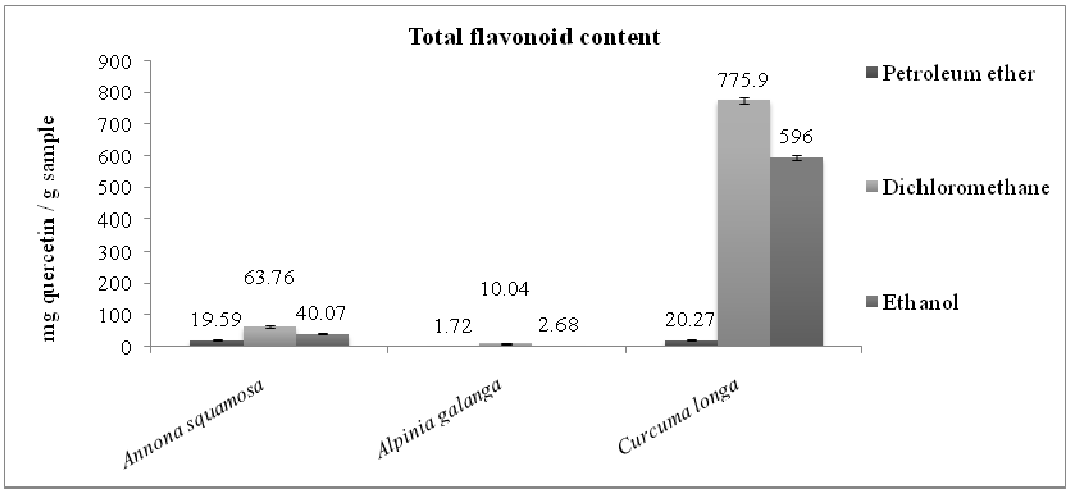


Figure 4. Total flavonoid content in various extracts of *Annona squamosa, Alpinia galanga* and *Curcuma longa* as determined using aluminum chloride colorimetric assay

Determination of antioxidant activities

Two different experimental approaches were utilized for determination of antioxidant activities. DPPH method is based on hydrogen donor property of antioxidants and is commonly applied in natural antioxidant studies because of its sensitivity and simplicity. Another assay, ABTS has also been broadly employed to assess antioxidant activities due to its applicability in both aqueous and lipid phases [25-26]. In this present work, results of the DPPH and ABTS assays are listed in Tables 1 and 2, respectively. In all antioxidant activity assays, *Curcuma longa* extract from dichloromethane fraction had the richest antioxidant activity (87.85±1.50 % SC by the DPPH assay and 98.80±0.32 % SC by the ABTS assay), followed by *Curcuma longa* extract from ethanolic fraction (84.47±1.71 % SC by the DPPH assay and 97.11±0.94 % SC by the ABTS assay). For *Annona squamosa* extracts, the ethanolic fraction was found to compose of the highest antioxidant activity (33.88±0.83% SC by the DPPH assay and 56.30±1.35% SC by the ABTS assay) as compared to other two fractions. Concerning the solvent types, the extracts gained from solvents with lower polarities (petroleum ether) showed lower antioxidant activities than those of solvents with higher polarities (dichloromethane and ethanol).

Table 1. Antioxidant activities of three species of Thai plant extracts derived from different solvents by DPPH assay

Types of plant	% scavenging activity (% SC)			mg ascorbic acid/g weight of sample		
	Petroleum ether	Dichloromethane	Ethanol	Petroleum ether	Dichloromethane	Ethanol
Annona squamosa	8.45 ± 0.42	14.96 ± 2.29	33.88 ± 0.83	1.68 ± 0.33	8.40 ± 3.21	25.18 ± 0.69
Alpinia galanga	N.A.	32.05 ± 0.46	22.49 ± 1.62	N.A.	24.67 ± 1.63	15.35 ± 0.32
Curcuma longa	16.19 ± 0.32	87.85 ± 1.50	84.47 ± 1.71	10.19 ± 0.57	63.00 ± 2.72	58.56 ± 0.50

Values are means ± standard error of mean of triplicate independent analyses; N.A.: not available.

Table 2. Antioxidant activities of three species of Thai plant extracts derived from different solvents by ABTS assay

Types of plant	% scavenging activity (% SC)			mg ascorbic acid/g weight of sample		
	Petroleum ether	Dichloromethane	Ethanol	Petroleum ether	Dichloromethane	Ethanol
Annona squamosa	10.93 ± 1.83	27.63 ± 2.36	56.30 ± 1.35	6.22 ± 0.26	16.43 ± 0.96	30.28 ± 4.83
Alpinia galanga	14.67 ± 3.24	57.40 ± 1.75	39.23 ± 4.18	7.97 ± 0.75	31.29 ± 3.93	23.80 ± 1.02
Curcuma longa	41.03 ± 2.23	98.80 ± 0.32	97.11 ± 0.94	24.16 ± 0.77	57.68 ± 8.31	50.32 ± 2.60

Values are means ± standard error of mean of triplicate independent analyses.

According to our previous report, we found the high correlations when regression analyses were used to evaluate the correlations between the two assays for antioxidant activities and between the antioxidant contents and antioxidant activities [23]. In this study, we observed the high correlation as well between the antioxidant contents and antioxidant activities, thus validating the experimental methods employed in our study. For instance, the dichloromethane extract of *Curcuma longa* had the highest antioxidant contents with the highest antioxidant

activities, and the second best in this regard was the ethanolic extract of *Curcuma longa*. This suggests that antioxidant activities are primarily contributed by phenolic and flavonoid compounds in these three Thai plants. The high antioxidant activities and high levels of phenolics and flavoniods in *Curcuma longa* found in this study support its beneficial health effects. This current finding was also in agreement with our previous report, and of particular interest, the maximum UVA absorption was found in *Curcuma longa* extracts from the ethanol and dichloromethane fractions [23]. Although it exhibited the inhibitory effect on Id1 expression, the extract of *Annona squamosa* was not the richest with respect to antioxidant contents and antioxidant activities. This suggests that the suppressive effect of *Annona squamosa* extract may not be primarily responsible by antioxidant contents and activities. It is worth addressing that phenylated flavone derived from *Annona squamosa* may be a major contributor to inhibit the expression of epidermal growth factor receptor (EGFR), thus being an interesting herb with antitumor effect [27]. Cancer and psoriasis are similar because of the hyperproliferative feature of cells. This highlights the feasible applications of *Annona squamosa* leaf extract and should be additionally studied for drug discovery. Of note, phytochemical and pharmacological studies on *Annona squamosa* leaf extracts were widely carried in India. In particular, there were only a few reports recently conducted by Thai researchers while at least five reports were published in 2011 alone by Indian researchers [28-32].

With regard to our present investigation, the ethanolic extracts of *Annona squamosa* leaves and *Curcuma longa* rhizomes were found to be truly rich of antioxidant contents and activities, thus making the two herbs potential candidates for further studies and applications. Creating a combination of the two plants is a concept for preventive and therapeutic uses. Previously, the significant anti-inflammatory activity of polyherbal formulation of the ethanolic extracts of *Annona squamosa* leaves and *Curcuma longa* rhizomes was reported using a rat model [33].

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