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Full Length Research Paper

Modulatory effects of Thai medicinal plant extract on proinflammatory cytokines-induced apoptosis in human keratinocyte HaCaT cells

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It has been experimentally proven that proinflammatory cytokines, interferon (IFN)-γ and tumor necrosis factor (TNF)-α are able to synergistically induce apoptosis in HaCaT keratinocyte cells. The present study aimed to elucidate modulatory effects of ethanolic extracts derived from Thai traditional medicinal plants on IFN-γ/TNF-α-caused HaCaT apoptosis and correlate with their natural phenolic content. Using 3-(4-5 dimethylthiozol-2-yl) 2-5 diphenyl-tetrazolium bromide (MTT) assay, we found that herbal extracts derived from members of the Acanthaceae family, *Rhinacanthus nasutus* (L.) Kurz (0.1, 1 and 10 µg/ml) and *Clinacanthus nutans* (Burm.f.) Lindau (1 and 100 µg/ml), significantly inhibited the IFN-γ/TNF-α-induced HaCaT apoptosis, while members of the Zingiberaceae family, *Curcuma longa* L. and *Alpinia galanga* (L.) Willd, significantly enhanced apoptosis when a concentration of 100 µg/ml was used. Furthermore, the ethanolic plant extracts were found to possess different amounts of total phenolics ranging from 1.64 to 10.04 mg GAE/g as determined using Folin-Ciocalteu assay. The richest phenolic sources were *R. nasutus* (10.04 ± 1.12 mg GAE/g) and *C. longa* (7.49 ± 0.50 mg GAE/g), whereas the least phenolic source was *Centella asiatica* (1.64 ± 0.84 mg GAE/g). Taken together, we found certain modulatory effects of Thai medicinal extracts on IFN-γ/TNF-α-induced apoptosis in HaCaT cells, but these findings might not be directly correlated with their natural phenolic content. Therefore, further investigations on different types of natural phenolic contents in these Thai medicinal extracts and their relevant molecular mechanisms in keratinocytes, should be carried out in the near future.

Key words: Interferon, tumor necrosis factor, Thai medicinal herbs, apoptosis, phenolics, human keratinocytes.

INTRODUCTION

Skin, the largest human body organ, provides a major interface between the body and the environment and is vulnerable to pathological conditions caused by various factors including chemical and microbial agents, thermal and electromagnetic radiation, and mechanical trauma (Caroline and Thomas, 1999). In addition, the skin is constantly exposed to oxidative stress induced by reactive oxygen species (ROS), which are derived from both endogenous sources, such as enzyme activities, and exogenous sources, such as ultraviolet (UV) (Bickers and Athar, 2006). There are several potential targets in the skin layers, and ROS-mediated oxidative damage involves many biological molecules such as DNA modification, lipid peroxidation and secretion of inflammatory cytokines (Briganti and Picardo, 2003; Pillai et al., 2005). Thus,

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Abbreviations: DDW, Distilled deionized water; **DMEM,** Dulbecco's modified eagle's medium; **DMSO,** dimethyl sulphoxide; **FBS,** fetal bovine serum; **GAE,** gallic acid equivalents; **IFN-γ,** interferon-γ; **PBS,** phosphate buffered saline; **ROS,** reactive oxygen species; **TNF-α,** tumor necrosis factor; **UV,** ultraviolet; **MTT,** 3-(4-5 dimethylthiozol-2-yl) 2-5 diphenyl-tetrazolium bromide; **DMEM,** Dulbecco's modified eagle's medium.

oxidative stress has received much attention with regard to its pivotal roles in alterations of aging, proliferation and apoptosis of keratinocytes.

Apoptosis is a crucial event necessary to maintain tissue homeostasis for all organ systems, including the epidermis (Paus et al., 1993; Polakowska and Haake, 1994; Norris, 1995; Lippens et al., 2009). Nevertheless, apoptosis of keratinocytes in the basal and spinous layers, combined with the impaired cell proliferation of the basal layer, may result in the decreased overall thickness of the aged epidermis (Haake et al., 1998). A list of skin diseases associated with apoptosis or with alterations in the regulation of apoptosis in keratinocytes of the epidermis has been previously addressed (Teraki and Shiohara, 1999). It has been correlated with aging, decreased epidermal proliferation and increased keratinocyte apoptosis (Gilhar et al., 2004).

In inflammatory conditions, imbalanced levels of circulatory cytokines can lead to a number of pathophysiologic processes of keratinocytes, including apoptosis. For instance, it has been particularly demonstrated that a synergistic action of interferon (IFN)-γ with tumor necrosis factor, (TNF)-α strongly induces apoptosis of HaCaT keratinocyte cells (Konur et al., 2005). The IFN-γ/TNF-α- simulated apoptosis has also been shown in other cells, including human intestinal epithelial cells (Francoeur et al., 2004). Since intensive apoptotic death of keratinocytes in such inflammatory status is likely to cause adverse outcomes, it is of great particular interest to restore the keratinocyte cell numbers. There have been certain Thai herbs known for their biological activeties. These have been utilized for the treatment of various skin diseases according to Thai traditional medicine. Nevertheless, to the best of our knowledge, their modulatory effects on such proinflammatory cytokines-induced apoptosis of keratinocytes have not been elucidated to date. Herein, we aimed at investigating the modulatory effects of Thai medicinal plant extracts, on IFN-γ/TNF-α-induced apoptosis in the HaCaT keratinocyte cell line. Also, total phenolic content in the extracts from studied herbs was determined in order to correlate with the observed modulatory effects.

MATERIALS AND METHODS

Plant materials

Thai traditional medicinal plants, *Curcuma longa* L., *Alpinia galanga* (L.) Willd., *Punica granatum* L. var. *granatum*, *Annona squamosa* L., *Centella asiatica* (L.) Urb., *Houttuynia cordata* Thunb., *Rhinacanthus nasutus* (L.) Kurz, *Clinacanthus nutans* (Burm.f.) Lindau and *Zingiber officinale* Roscoe, were collected from the Princess Maha Chakri Sirindhorn Herbal Garden (Rayong Province, Thailand). They were authenticated by Professor Dr. Thaweesakdi Boonkerd (Department of Botany, Faculty of Science, Chulalongkorn University, Thailand). The voucher specimens [013396 (BCU) – 013400 (BCU), 013402 (BCU), 013404 (BCU), 013405 (BCU) and 013407 (BCU)] were deposited at the Professor Kasin Suvatabhandhu Herbarium, Department of Botany, Faculty of Science, Chulalongkorn University, Thailand.

Preparation of Thai medicinal plant extracts

All Thai medicinal plants were extracted with ethanol (Merck, Hohenbrunn, Germany) using ratio 1:5 (w/v) by maceration in shaking incubator at 120 rpm at room temperature for 48 h. Thai medicinal plant extracts were filtered, and the residues were subsequently extracted twice. After the two filtrates were combined, the crude extracts were concentrated using the MiVac Quattro concentrator at 45°C. Eventually, the resulting crude extracts were dissolved in dimethyl sulphoxide (DMSO) (Merck, Hohenbrunn, Germany) as stock solutions (100 mg/ml), stored at -20°C and protected from light. In order to sterilize before using for bioassays, the crude extracts were filtered through a 0.2 μm pore size filter (Corning Inc., Corning, NY).

Cell culture

The human keratinocyte cell line HaCaT (kindly provided by Professor Dr. N. E. Fusenig, German Cancer Research Centre, Heidelberg, Germany) was cultured in Dulbecco's modified Eagle's medium with high glucose (DMEM/high glucose), supplemented with 10% heat-inactivated fetal bovine serum (FBS) containing 100 U/ml of penicillin and 100 μg/ml of streptomycin. The cells were maintained at 37°C in a humidified atmosphere at 5% CO_2. All media, supplements and drugs for cell culture were purchased from Hyclone (Logan, UT).

Cell treatment

To actively induce apoptosis of HaCaT keratinocytes, we performed the respective experiments by following the previously described protocol (Konur et al., 2005) with minor modifications. Briefly, the HaCaT cells, seeded in a 96-well plate (1×10^4 cells/well), were incubated at 37°C in 5% CO_2 incubator overnight. Subsequently, the cells were pretreated for 24 h with both IFN-γ and TNF-α (PeproTech, Rocky Hill, NJ) with a final concentration of 10 ng/ml for each. Finally, the cells were treated with different concentrations (0.1, 1, 10 and 100 μg/ml) of each crude ethanolic extract of Thai medicinal plants, and were incubated at 37°C in a humidified atmosphere at 5% CO_2 for 48 h. Control cells were only pretreated with IFN-γ/TNF-α without any crude extract.

MTT assay

HaCaT cell viability was assessed using MTT assay as previously described (Mosmann, 1983) with minor modifications. MTT reagent was purchased from Merck (Hohenbrunn, Germany). Briefly, following the cell treatment, HaCaT cells in each well of the 96-well plate, were subjected to MTT assay by mixing with 20 μl of MTT [5 mg/ml in phosphate buffered saline (PBS)], and incubated at 37°C in 5% CO_2 incubator for 4 h. Subsequently, the medium was removed, and 200 μl of DMSO was added into each well to dissolve the resulting formazan. The absorbance was measured at 550 nm. The percentage cell survival was calculated according to following formula:

$$\% \text{ cell survival} = [(\text{absorbance of treatment group} - \text{blank}) / (\text{absorbance of control group} - \text{blank})] \times 100.$$

Total phenolic assay

Total natural phenolic content in the Thai medicinal plant extracts was determined using Folin-Ciocalteu assay (Singleton and Rossi,

1965) with minor modifications. Briefly, after a test tube containing either 500 µl of either standard solutions of gallic acid (50, 25, 12.5, 6.25, 3.125, 1.5625 and 0.78125 µg/ml) or crude extracts [diluted 400 - fold with distilled deionized water (DDW)] was prepared, 500 µl of 10% Folin-Ciocalteu's phenol reagent (in DDW) was added into each test tube and mixed. After 20 min, 350 µl of 1 M Na_2CO_3 solution was added into the mixture. After incubation for 20 min at room temperature, the absorbance was determined at 750 nm against the parallely prepared blank (500 µl of DDW + 500 µl of 10% Folin-Ciocalteu's phenol reagent + 350 µl of 1 M Na_2CO_3 solution). Total phenolic content in each Thai herbal extract was expressed as mg gallic acid equivalents (GAE)/1 g wet weight. All samples were analyzed in triplicate.

Statistical analysis

The data represented the mean ± S.E.M. of three independent experiments. Differences between control and treatment groups were analyzed by one way analysis of variance, (One-way ANOVA) followed by Games-Howell post hoc test for multiple comparisons. Differences at $P < 0.05$ were considered to be significant.

RESULTS AND DISCUSSION

To prove the synergistic effect of IFN-γ and TNF-α on apoptosis induction in HaCaT cells, we performed experiments in the present study according to the method described by Konur et al. (2005) with minor modifications. In accordance we found that the two proinflammatory cytokines (in a final concentration of 10 ng/ml each) significantly reduced the HaCaT cell numbers ($P < 0.05$), about 5-fold decrease when compared to those left untreated, as demonstrated using the MTT assay. Although, it was not considered significant to determine if the HaCaT cell numbers in our experiments were really diminished due to apoptosis, since IFN-γ/TNF-α-caused HaCaT apoptosis has already been reported, mainly due to an activation of caspases (Konur et al., 2005). Interestingly, they have also reported induction of autocrine TNF-α expression, therefore increasing the possibility of further sensitizing keratinocytes for apoptosis. Notably, the release of large amount of ROS occurs during the inflammatory process for destroying and invading microorganisms and/or degrading damaged tissue structures. Nevertheless, ROS not only induce a number of transcription factors, but also activate apoptosis in keratinocytes by altering mitochondrial membrane permeability (Dhar et al., 2002; David et al., 2006). It is worth addressing that IFN-γ has been recognized as one of the key mediators in inflammatory skin diseases, which mediates Th1 type inflammatory and growth inhibition of keratinocytes (Hattori et al., 2002), and TNF-α has been known to play a crucial role in inflammatory and immunelogical responses in human skin (Chen et al., 2008).

After the HaCaT cells were pretreated with both IFN-γ and TNF-α, the cells were subsequently treated with various concentrations of nine different Thai medicinal plant extracts to observe their potential modulatory effects. As shown in Figure 1, although, most herbal extracts did not exert any biological activity, we found that *R. nasutus* (L.) Kurz (0.1, 1 and 10 µg/ml) and *C. nutans* (Burm.f.) Lindau (1 and 100 µg/ml) significantly suppressed apoptosis of the keratinocytes ($P < 0.05$). Both medicinal plants were members of the Acanthaceae family. It should be noted that, although not significant, there was a trend for *C. nutans* (Burm.f.) Lindau at a concentration of 10 µg/ml to be critical in inhibiting apoptosis as similar to its concentrations of 1 and 100 µg/ml. In contrast, members of the Zingiberaceae family, *C. longa* L. and *A. galanga* (L.) Willd., significantly promoted apoptosis ($P < 0.05$) when a concentration of 100 µg/ml was applied to HaCaT cells. Furthermore, two herbal extracts, derived from *P. granatum* L. var. *granatum* and *A. squamosa* L., almost significantly aggravated apoptosis at a concentration of 100 µg/ml.

The anti-apoptotic effect exerted by these two plant extracts in the present study might be due to their natural phenolics. In particular, phenolics are usually present in plants, vegetables and fruits, and several lines of evidence have shown that they are antioxidants. For instance, cytoprotective effects of phenolics against apoptosis have been reported in neural cells (Jung et al., 2007; Huang et al., 2008) and peripheral blood mononuclear cells (Khanduja et al., 2006). In addition, phenollics have been proved beneficial to prevent UV-induced skin damage (Svobodová et al., 2003). Therefore, we determined total phenolic content in the studied Thai herbal extracts using the Folin-Ciocalteu assay (Table 1). The ethanolic extracts derived from various Thai traditional plants were found to possess different amounts of total phenolics ranging from 1.64 to 10.04 mg GAE/g. The richest phenolic sources were *R. nasutus* and *C. longa*, whereas the least phenolic source was *C. asiatica*. Therefore, these findings might not directly reflect the modulatory effects of these plant extracts. As far as the technique is concerned, the Folin-Ciocalteu method was employed for measuring total phenolic content in the present study due to its simplicity and reproducibility (De Beer et al., 2004). Nonetheless, this particular technique might not be able to measure all different types of phenolics. It would be better if we could determine total phenolic contents in these samples, using other methods in parallel. In addition, there might be only specific types of natural phenolics capable of suppressing apoptosis of keratinocytes during inflammatory status. Those specific phonolic compositions might not be detected using the Folin-Ciocalteu approach.

In summary, certain modulatory effects were found in Thai traditional medicinal plant extracts. In inflammatory state, ethanolic herbal extracts derived from members of the Acanthaceae family, *R. nasutus* (L.) Kurz and *C. nutans* (Burm.f.) Lindau, significantly, inhibited the IFN-γ/TNF-α-induced HaCaT apoptosis, while members of the Zingiberaceae family, *C. longa* L. and *A. galanga* (L.) Willd, significantly, enhanced apoptosis. Nevertheless, the concentrations of these medicinal plants with respect to their biological activities were taken into account. All Thai

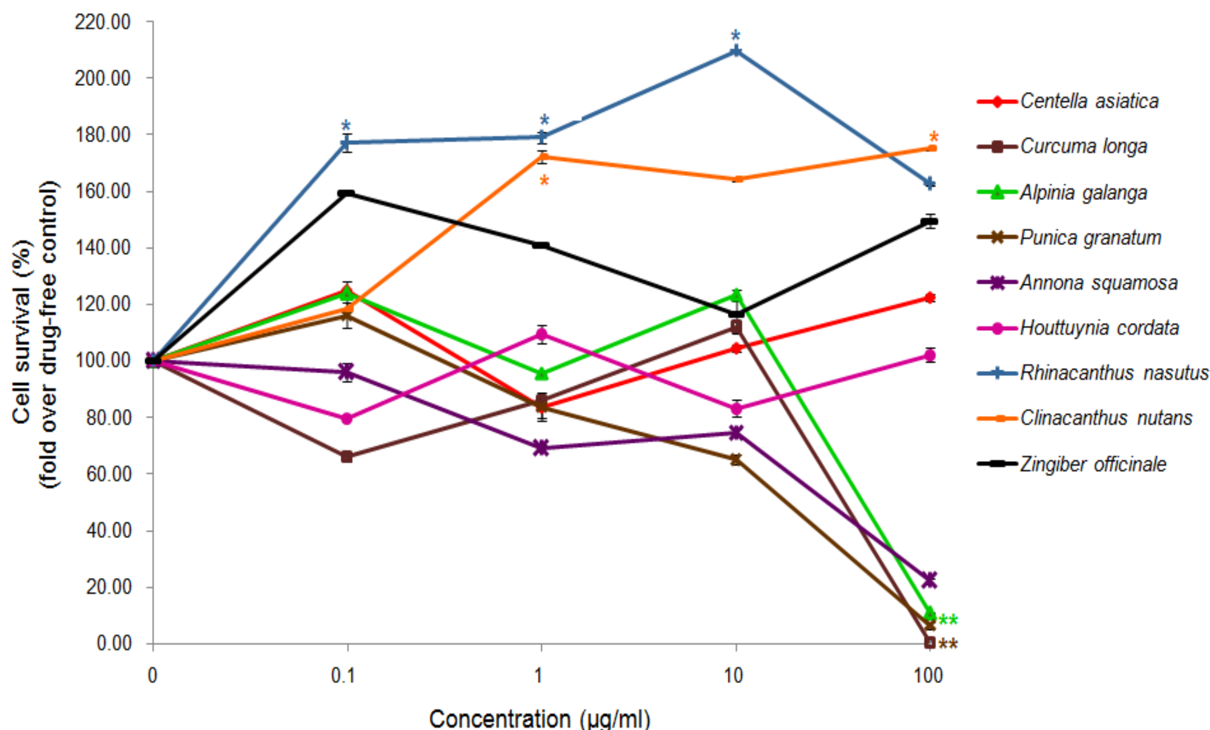

Figure 1. Modulatory effects of ethanolic extracts derived from Thai medicinal plants on IFN-γ/TNF-α-induced apoptosis of HaCaT cells. Three independent experiments were performed in triplicate; * and **, indicate a statistically significant difference ($P < 0.05$) with regard to inhibited apoptosis and promoted apoptosis, respectively, when compared to the IFN-γ and TNF-α-induced HaCaT apoptosis in the absence of any Thai medicinal extracts.

Table 1. Details of nine ethanol extracts derived from Thai medicinal herbs and their total phenolics evaluated using the Folin-Cioculteau method.

Name of medicinal materials	Family	Part used	Herbarium number	Extract yield (%)	Total phenolic content (mg GAE/ g sample)
Curcuma longa L.	Zingiberaceae	Rhizome	013396 (BCU)	9.6	7.49 ± 0.50
Alpinia galanga (L.) Willd.	Zingiberaceae	Rhizome	013397 (BCU)	7.7	2.72 ± 1.37
Punica granatum L. var. *granatum*	Punicaceae	Fruit	013398 (BCU)	30.3	6.16 ± 2.39
Annona squamosa L.	Annonaceae	Leave	013399 (BCU)	11.7	4.02 ± 1.97
Centella asiatica (L.) Urb.	Umbelliferae	Whole body	013400 (BCU)	9.2	1.64 ± 0.84
Houttuynia cordata Thunb.	Saururaceae	Leave	013402 (BCU)	7.0	5.08 ± 0.40
Rhinacanthus nasutus (L.) Kurz	Acanthaceae	Leave	013404 (BCU)	5.0	10.04 ±1.12
Clinacanthus nutans (Burm.f.) Lindau	Acanthaceae	Leave	013405 (BCU)	10.0	4.67 ± 3.60
Zingiber officinale Roscoe	Zingiberaceae	Rhizome	013407 (BCU)	15.0	4.44 ± 2.97

medicinal extracts in the present investigation were found to possess different amounts of natural phenolics, which might not be firmly correlated with the apoptosis modulatory effects. However, our results indicated the beneficial effects of Thai traditional herbs with respect to their potential antioxidant properties on restoring skin homeostasis and therapeutic skin disorders during inflammatory status. A further investigation on different types of natural phenolic contents is studied. Thai medicinal extracts and their relevant molecular mechanisms in keratinocytes remain to be carried out.

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