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In Vitro Effect of Thai herbal extracts with anti-psoriatic activity on the expression of caspase 9

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ABSTRACT

Psoriasis, known as the incurable disease, is a chronic inflammatory disease characterize by hyperproliferative keratinocytes. The causes of this disease are unclear, and many side effects are evident as a result of certain treatments of psoriasis. In this work, we aimed at studying the in vitro effect of each of these following Thai herbal extracts, Alpinia galanga L. (rhizome), Curcuma longa L. (rhizome) and Annona squamosa L.(leaf), containing anti-psoriatic activity on the expression of caspase 9, the protein known to play a critical role in the intrinsic apoptotic pathway. The HaCaT keratinocyte cell line was chosen as an in vitro model. Based on Western blot analysis, the extract derived from Annona squamosa L. leaf at all three concentrations tested significantly reduced the expression of procaspase 9 ($P<0.05$), and two higher concentrations, $0.5IC_{50}$ (3.15 µg/mL) and IC_{50} (6.3 µg/mL), of this herbal extract significantly increased the expression of caspase 9 protien ($P<0.05$). Confocal immunofluorescence microscopy subsequently confirmed this finding. Taken together, this might suggest that the ethanolic extract of Annona squamosa leaf with anti-psoriatic activity could exert its biological effect by activating the cleavage of procaspase 9 into caspase 9, thus inducing the intrinsic apoptotic pathway.

Keywords: Psoriasis, Thai herbal extracts, Apoptosis, Caspase 9, HaCaT.

INTRODUCTION

Psoriasis is a chronic inflammatory skin disease that affects 1%-3% of the population worldwide. It is characterized by hyperproliferation and abnormal differentiation of keratinocytes. Psoriasis can be found in all ages and found in males more than female, especially

in ages 25-45 years [1]. The precise cause of disease remains unknown, but numerous factors including genetics, immune system and environmental stimuli can induce the disease [2]. During treatment, psoriatic patients experienced difficulties in their health, work and quality of life. In other words, these difficulties were similarly found in patients suffered from other chronic diseases such as depression, hypertension, heart disease and type 2 diabetes mellitus[3]. In the present time, psoriasis is incurable. Although there are current anti-psoriatic drugs, they are not only expensive and ineffective, but also lead to certain side effects.

Caspase 9, a critical enzyme playing a role in the intrinsic pathway of apoptosis, was found to be decreased in epidermis of psoriatic patients [4]. Without appropriate cell death, this might lead to an increase of skin cells, thus contributing to psoriasis. It should be reasonable to search for certain naturally therapeutic compounds containing an enhanced expression of caspase 9, particularly in keratinocytes. According to our previous study, we found three Thai herbal extracts with *in vitro* anti-psoriatic activity, *Alpinia galanga* L. (rhizome), *Curcum longa* L. (rhizome) and *Annona squamosa* L. (leaf) [5]. Recently, we demonstrated that these mentioned plant ethanolic extracts might function in a molecular level to reduce psoriatic cells by regulating the expression of NF-κB signaling biomarkers in a HaCaT keratinocyte cell line [6]. These Thai medicinal herb extracts may also mediate other effects with regard to the signaling network. Therefore, the present study was aimed at investigating the *in vitro* effect of all three Thai herbal extracts with anti-psoriatic property on the expression of caspase 9 in the HaCaT cells. Western blot was performed to assess the expression level of caspase 9 after cells were treated with various concentrations of each studied herbal extract. We also confirm the significant difference using immunocytochemistry in conjunction with confocal microscopy.

EXPERIMENTAL SECTION

Plant materials and preparation of Thai herbal extracts

Plant materials and preparation of Thai herbal extracts were described elsewhere [5, 6].

Cell culture and treatment of HaCaT cells using Thai herbal extracts

HaCaT cells, purchased from Cell Lines Service (CLS, Heidelberg, Germany), were seeded $3x10^6$ cell/mL in 15x60 mm of Petri dish and grown in Dulbecco's Modified Eagle Medium/high glucose supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin (Hyclone, Logan, UT) at 37 ºC in 5% CO_2. The cells were pretreated with pro-inflammatory cytokines, IFN-γ (10 ng/mL) and TNF-α (10 ng/mL) (Peprotech, Rocky Hill, NJ) for 24 h. Subsequently, HaCaT cells were treated with various concentrations of each plant ethanolic extract: 6.3, 3.15, 1.575 µg/mL (IC_{50}= 6.3) of *Alpinia galanga* L. rhizome extract, *Annona squamosa* L. leaf extract or 6.7, 3.35, 1.675 µg/mL (IC_{50}= 6.7) of *Curcuma longa* L. rhizome extract for the period of 48 h at 37ºC in 5% CO_2. Controls were performed with DMSO and medium alone. Each sample concentration was tested in triplicates.

Protein extraction and Western blotting analysis

Total protein isolation from the HaCaT cells was carried out using Nonidet P40 lysis buffer. Protein concentrations were determined using the Bradford method [7]. The protein samples were frozen at -80°C for later analyses.

For Western blotting, thirty µg of protein extracts were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a polyvinyldine fluoride (PVDF) membrane. The membrane was then blocked with 5% non-fat dry milk in Tris-buffered saline Tween20 and incubated with either polyclonal rabbit anti-caspase 9 (1: 10,000, Cell Signaling Technology, Danvers, MA) or polyclonal rabbit anti-GAPDH (1: 10,000, Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h. After washing, the membrane was incubated with anti-rabbit IgG, horseradish peroxidase-linked secondary antibody (Cell Signaling Technology), diluted at an appropriate dilution in 5% BSA, for 45 min at room temperature. The proteins, full length caspase 9 (procaspase 9, 47 kDa), caspase 9 (active form, 35 kDa) and GAPDH (37 kDa), were visualized using an enhanced chemiluminescence system with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo scientific, Rockford, IL). The results were normalized to GAPDH values.

Confocal immunofluorescence microscopy

The HaCaT cells were seeded $1x10^5$ cell/mL on coverslip in a 6-well plate at 37 ºC in 5% CO_2. The cells were pretreated with 10 ng/mL IFN-γ and 10 ng/mL TNF-α for 24 h. Subsequently, the cells were treated with various concentrations, 6.3, 3.15, 1.575 µg/mL (IC_{50}= 6.3), of *Annona squamosa* L. leaf extract for 48 h at 37ºC in 5% CO_2. Cells were washed with cold PBS three times and fixed with absolute methanol at -20°C for 3-5 min. Fixed cells were washed with PBS and then permeabilized by adding 0.5% Triton X-100 with 0.05% Tween20 in PBS for 1 h at room temperature. Cells were exposed to primary antibody (caspase 9 antibody, Cell signaling Technology), and detection using anti-rabbit Alexa 555 (Cell signaling Technology) was carried out. After staining, cells were washed for 5 min in PBS three times and stained with Hoechst 33258 for 15 min at room temperature. After washing for 5 min in PBS three times, cells were observed using a confocal laser scanning microscope (LSM 700, Carl Zeiss, Germany).

Statistical analysis

All values are expressed as means ± SEM. The data were analyzed by the Student *t*-test. $P < 0.05$ was considered to be significant. Each experiment consisted of at least three replicates per condition.

RESULTS AND DISCUSSION

Effect of Thai herbal extracts with anti-psoriatic activity on the expression of caspase 9

The biological effect of Thai herb ethanolic extracts with anti-psoriatic property on the expression of caspase 9 was determined in the HaCaT cells. In all concentrations of ethanolic extracts used for treating the HaCaT cells, no significant changes in the caspase 9 levels were found with regard to either *Alpinia galanga* L. rhizome extract (Figure 1) or *Curcuma longa* L. rhizome extract (Figure 2). Interestingly, the extract derived from *Annona squamosa* L. leaf at all three concentrations tested significantly reduced the expression of procaspase 9 *($P<0.05$)*, and two higher concentrations, $0.5IC_{50}$ (3.15 µg/mL) and IC_{50} (6.3 µg/mL), of this herbal extract significantly increased the expression of caspase 9 protien *($P<0.05$)* (Figure 3). Although an increased caspase 9 protein was not significant after HaCaT cells treated with the *Annona squamosa* L. leaf extract at the lowest concentration tested, 1.575 µg/mL ($0.25IC_{50}$), the tendency toward increasing the expression of caspase 9 was obvious. Particularly, this

concentration of *Annona squamosa* L. leaf extract resulted in about 2-fold increase in the protein level of caspase 9 as compared to the control (HaCaT cells stimulated by IFN-γ and TNF-α).

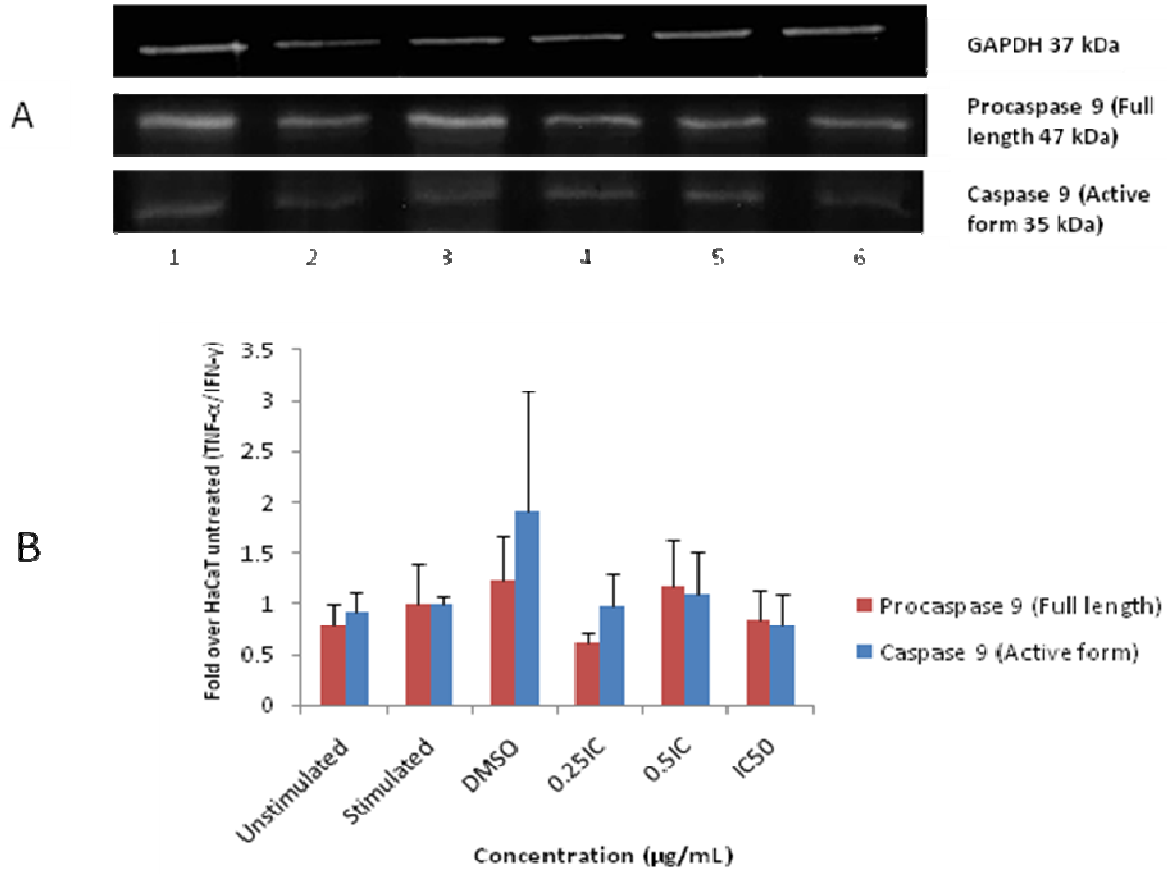
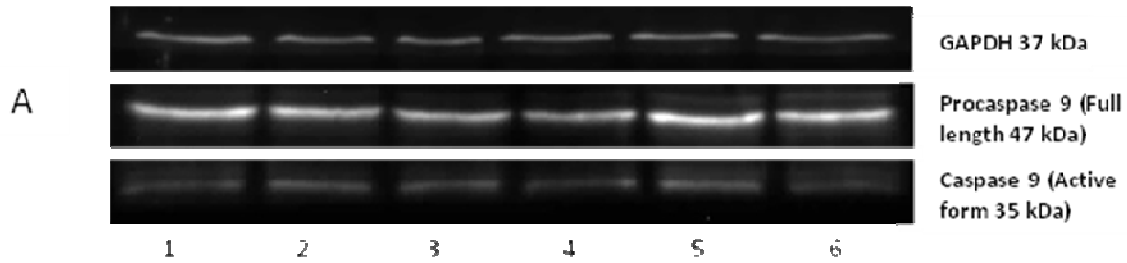


Figure1 Expression of caspase 9 protein as detected by Western blotting analysis in HaCaT cells when treating cells with 6.3, 3.15 or 1.575 µg/mL (IC_{50}= 6.3 µg/mL) of *Alpinia galanga* L. for 48 h. A. The expression levels of GAPDH, procaspase 9 and caspase 9 were detected by Western blotting analysis. B. The graph showing normalized procaspase 9 and caspase 9 protein expression when cells were treated with *Alpinia galanga* L. 1: HaCaT cells with medium alone, 2: HaCaT cells with IFN-γ and TNF-α, 3: HaCaT cells with IFN-γ, TNF-α and DMSO, 4: HaCaT cells with IFN-γ, TNF-α and 1.575 µg/mL ($0.25IC_{50}$) of *Alpinia galanga* L., 5: HaCaT cells with IFN-γ, TNF-α and 3.15 µg/mL ($0.5IC_{50}$) of *Alpinia galanga* L., 6: HaCaT cells with IFN-γ, TNF-α and 6.3 µg/mL (IC_{50}) of *Alpinia galanga* L.



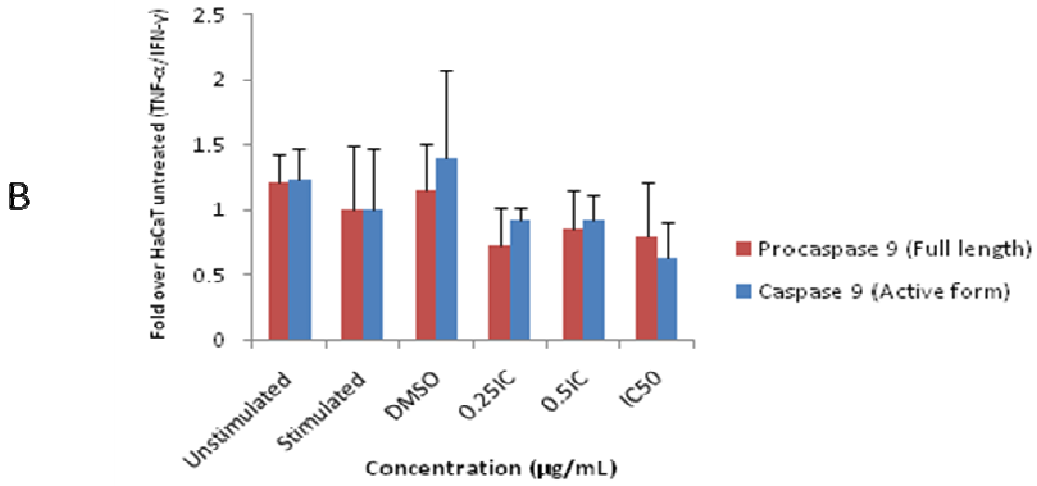
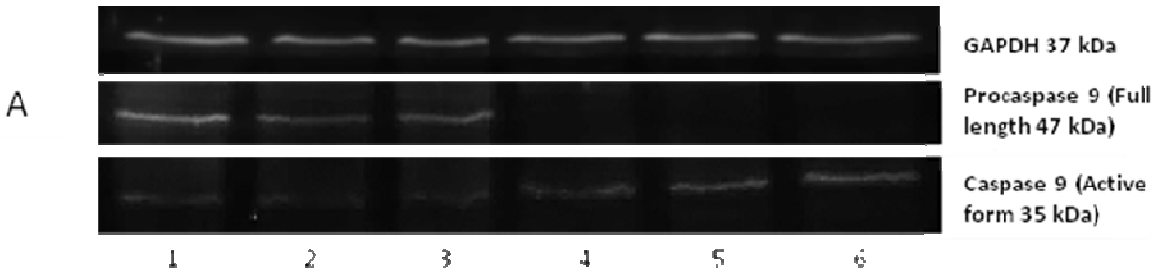


Figure 2 Expression of caspase 9 protein as detected by Western blotting analysis in HaCaT cells when treating cells with 6.7, 3.35 or 1.675 µg/mL (IC_{50}= 6.7) of *Curcuma longa* L. for 48 h. A. The expression levels of GAPDH, procaspase 9 and caspase 9 were detected by Western blotting analysis. B. The graph showing normalized procaspase 9 and caspase 9 protein expression when cells were treated with *Curcuma longa* L. 1: HaCaT cells with medium alone, 2: HaCaT cells with IFN-γ and TNF-α, 3: HaCaT cells with IFN-γ, TNF-α and DMSO, 4: HaCaT cells with IFN-γ, TNF-α and 1.575 µg/mL ($0.25IC_{50}$) of *Curcuma longa* L., 5: HaCaT cells with IFN-γ, TNF-α and 3.15 µg/mL ($0.5IC_{50}$) of *Curcuma longa* L., 6: HaCaT cells with IFN-γ, TNF-α and 6.3 µg/mL (IC_{50}) of *Curcuma longa* L.

Effect of Thai herbal extracts with anti-psoriatic activity on the expression of caspase 9 by confocal immunofluorescence microscopy

Since the ethanolic extract derived from *Annona squamosa* L. leaf significantly altered the protein expression levels of caspase 9 in relation to procaspase 9, we further confirmed this finding using confocal immunofluorescence microscopy. When treating the HaCaT cells with 6.3, 3.15 or 1.575 µg/mL (IC_{50}= 6.3µg/mL) of *Annona squamosa* L. leaf extract, we demonstrated the increase in caspase 9 (Figure 4). In addition, we found that caspase 9 immunostaining was located diffusely throughout cells, thus being in accordance with the previous report [8].



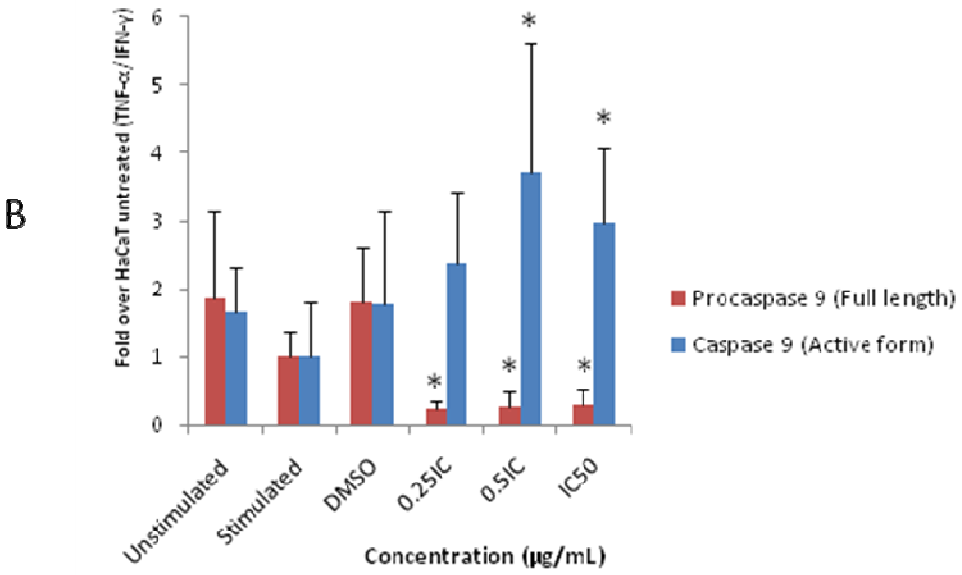


Figure 3 Expression of caspase 9 protein as detected by Western blotting analysis in HaCaT cells when treating cells with 6.3, 3.15 or 1.575 µg/mL (IC_{50}= 6.3) of *Annona squamosa* L. for 48 h. A. The expression levels of GAPDH, procaspase 9 and caspase 9 were detected by Western blotting analysis. B. The graph showing normalized procaspase 9 and caspase 9 protein expression when cells were treated with *Annona squamosa* L. 1: HaCaT cells with medium alone, 2: HaCaT cells with IFN-γ and TNF-α, 3: HaCaT cells with IFN-γ, TNF-α and DMSO, 4: HaCaT cells with IFN-γ, TNF-α and 1.575 µg/mL (0.25IC_{50}) of *Annona squamosa* L., 5: HaCaT cells with IFN-γ, TNF-α and 3.15 µg/mL (0. 5IC_{50}) of *Annona squamosa* L., 6: HaCaT cells with IFN-γ, TNF-α and 6.3 µg/mL (IC_{50}) of *Annona squamosa* L.

The potential molecular mechanism of Thai herbal extracts with anti-psoriatic activity was investigated. Since apoptosis might be one of the prime candidate mechanisms, we analyzed the expression level of caspase 9, a biomarker decreased in the psoriatic epidermis [4], under treatment conditions using three Thai ethanolic extracts. The HaCaT keratinocyte cell line was chosen for this study since it was shown to be a proper *in vitro* model as previously described [5, 6, 9]. The HaCaT cells were stimulated or pretreated with IFN-γ and TNF-α, the two cytokines significantly increased in psoriatic patients. Our result showed that *Annona squamosa* L. leaf extracts at all concentrations, 0.25IC_{50} (1.575 µg/mL), 0. 5IC_{50} (3.15 µg/mL) and IC_{50} (6.3 µg/mL), significantly decreased the expression of procaspase 9 in the HaCaT cells *(P<0.05)*, whereas only two higher concentrations of the ethanolic leaf extracts significantly enhanced the expression of caspase 9 *(P<0.05)*. Nevertheless, we observed about 2-fold increase in the caspase 9 expression when treated with *Annona squamosa* L. leaf extract at a concentration of 0.25IC_{50} (1.575 µg/mL). Furthermore, the enhanced expression of caspase 9 was substantiated using the confocal immunofluorescence microscopy. Taken together, these findings suggest that the ethanolic extract of *Annona squamosa* L. leaf may stimulate the cleavage of procaspase 9, thus transforming from its inactive form to active caspase 9. Nonetheless, we did not perform a caspase 9 activity assay in the present study.

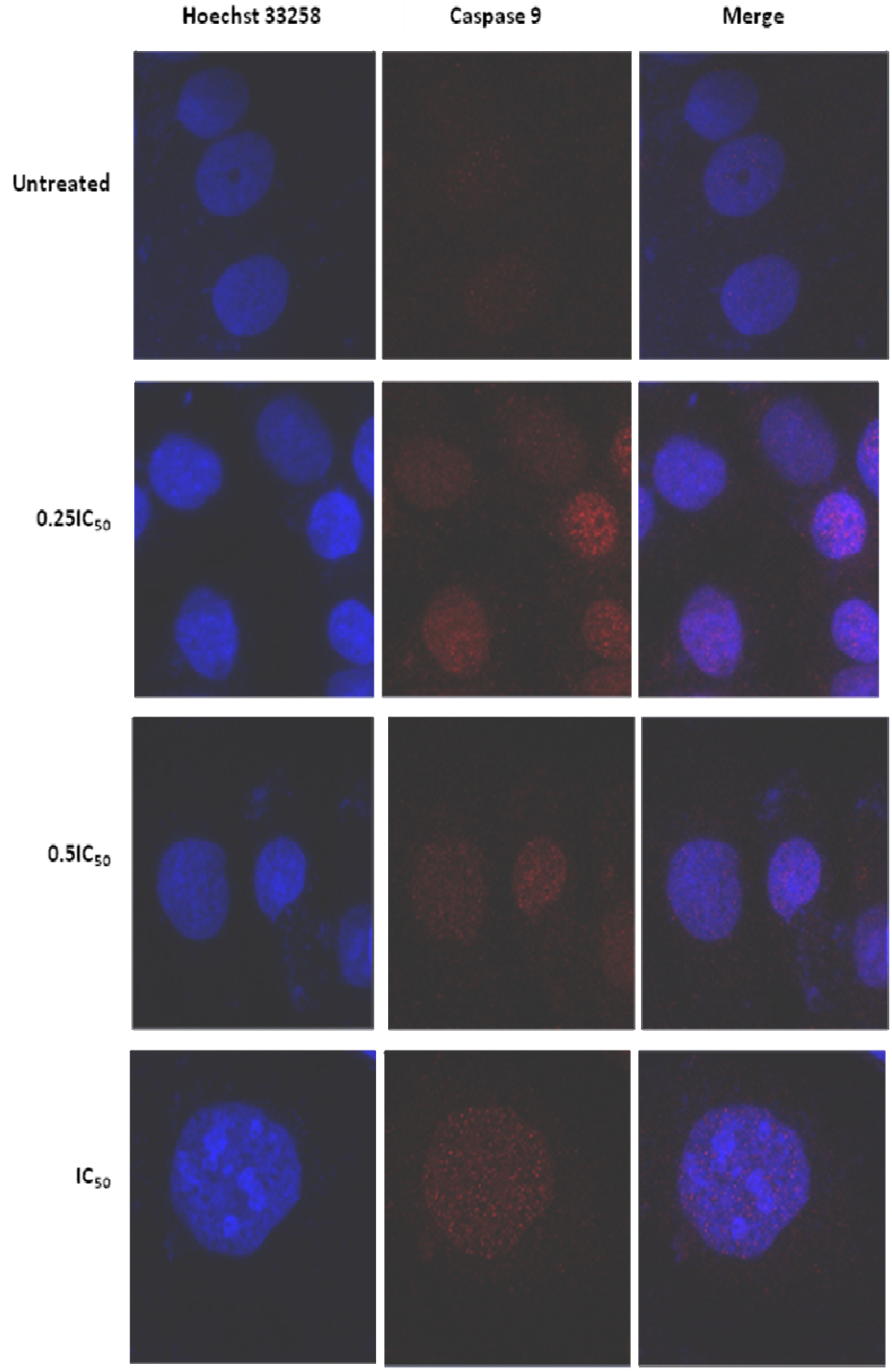


Figure 4 Expression of caspase 9 protein as detected by Immunocytochemistry and Confocal microscopy in HaCaT cells treating cells with 6.3, 3.15 or 1.575 µg/mL (IC_{50}= 6.3) of *Annona squamosa* L. for 48 h. Untreat: HaCaT cells with IFN-γ and TNF-α, 0.25IC_{50}: HaCaT cells with IFN-γ, TNF-α and 1.575 µg/mL (0.25IC_{50}) of *Annona squamosa* L., 0.5IC_{50}: HaCaT cells with IFN-γ, TNF-α and 3.15 µg/mL 0.5IC_{50}) of *Annona squamosa* L., IC_{50}: HaCaT cells with IFN-γ, TNF-α and 6.3 µg/mL (IC_{50}) of *Annona squamosa* L.

Interestingly, quercetin, known as a flavonoid with antioxidant property, was found to be an active constituent of *Annona squamosa* leaves [10]. Ouercetin was shown to induce apoptosis by the activation of caspases 3 and 9, but not caspase 8 in a human hepatoma cell line, HepG2 [11]. Consistently, another study showed that quercetin activated a caspase 3-depended mitochodrial pathway to induce apoptosis in a human leukemia cell line, HL-60 [12]. Therefore, both studies have supported the role of quercetin in apoptosis induction via the intrinsic pathway, which is in accordance with our present investigation. However, there was a study demonstrating that both intrinsic and extrinsic pathways were involved in quercetin-induced apoptosis since quercetin treatment promoted activation of caspase 3, 8 and 9 in a human breast cancer cell line, MDA-MB-231 [13]. We did not analyze the possible involvement of extrinsic apoptotic pathway in our present work. Based on our curry investigation, it should be worth noting that the anti-psoriatic effect, possibly contributed by the enhanced cleavage of procaspase 9 into caspase 9, of *Annona squamosa* leaf extract may be due to the active role of quercetin. Further studies should be performed to provide insights into an understanding of processes at molecular, biochemical and cellular levels.

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