Aquilaria crassna leaf extract selectively upregulated calreticulin surface expression, a pro-phagocytotic signal, in triple-negative breast cancer cells

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Abstract

During the last decade, studies of anti-cancer properties of extracts from several parts of *Aquilaria* species including *Aquilaria* crassna (*A. crassna*) Pierre ex Lecomte have been more attractive. Leaf extract of *A. crassna* Pierre ex Lecomte (AE) has been reported for medicinal activities except for anti-cancer activity in particular the enhancement of cancer-immune responses. Strategies that alter cancer phenotypes to be more sensitive to immune cell killing and/or to activate immune responses have become promising approaches for cancer therapy. We are interested in a strategy that sensitizes surviving cancer cells to express surface molecules for the enhancement of cancer cell killing by immune cells called immunogenic modulation. Thereby, this study aims to investigate the induction effect of AE on the expression of molecules reported for immunogenic modulation which are the surface expressions of calreticulin (ectoCRT), a major histocompatibility complex class I (MHC class I), and CD95/Fas death receptor. M.D. Anderson-Metastatic Breast 231 (MDA-MB 231) cell line, a triple-negative breast cancer; TNBC, was treated with various concentrations (0-640 μg/ml) of AE for 6 hr and 12 hr and examined for the cytotoxic effect by MTT assay. The results showed that concentrations of 20-640 μg/ml of AE treatment significantly decreased the percentages of cell viability at both time points. Sublethal doses (20-320 μg/ml) caused the cell viability > 70% were further examined for the surface expressions of ectoCRT, MHC class I, and CD95 using surface immunostaining and analyzed by flow cytometer. At 12 hr after treatment, AE selectively induced ectoCRT expression but did not show any effect on MHC class I and CD95 expressions in all AE-treated populations. To further clarify the involvement of AE-induced ectoCRT in an immunogenic modulation, AE-treated living cells were chosen to investigate the expression of ectoCRT. Our results found that AE-treated living cells showed a significant increase in the relative mean fluorescence intensity (relative MFI) of ectoCRT expression and a trend to increase in ectoCRT-positive cells compared to the untreated group (0 μg/ml). Together, this is the preliminary result reporting the upregulation of ectoCRT expression by AE. In addition, AE-induced ectoCRT has been mainly found in surviving/living cells which is accorded to the criteria of immunogenic modulation. However, the role of AE-induced ectoCRT in the enhancement of cancer cell killing especially in immune cell phagocytosis needs to be further investigated.

Keywords: *A. crassna* leaf extract, immunogenic modulation, sublethal dose, ectoCRT, triple-negative breast cancer

Introduction

Aquilaria crassna (*A. crassna*) Pierre ex Lecomte is a highly valuable plant in *Aquilaria* species of *Thymelaeaceae* family found throughout Southeast Asia (Hashim, Kerr, Abbas, & Mohd Salleh, 2016). In Thailand, it is commonly known as "Krisana" and is cultivated in several provinces including Phitsanulok province (Wongwad et al., 2019). Apart from its heartwood and the essential oil which are highly valuable in both economic and pharmaceutical benefits, leaves of *A. crassna* have been used as herbal tea to improve heart and circulatory systems (Kamonwannasit et al., 2013; Suvitayavat, Tunglert, Thirawarapan, & Bunyapraphatsara,

2005). Leaf extract of *Aquilaria* species including *A. crassna* Pierre ex Lecomte has been documented for its diverse medicinal properties, such as anti-glycemia, anti-oxidant, laxative, neuroprotection, anti-inflammation, anti-aging as well as anti-cancer activities (Adam, Lee, & Mohamed, 2017; Kakino et al., 2010; Manoka, Sungthong, Sato, Sugiyama, & Sato, 2016; Wongwad et al., 2019). Abbas et al. (2018) published the first report that leaf extract of *A. subintegra* exhibited cytotoxic and anti-attachment effects on MCF-7 breast cancer (BC) cells (Abbas, Hashim, & Salleh, 2019). The extract of *A. crassna* Pierre ex Lecomte (not mentioned for the extracted part) induced apoptosis and inhibited cancer angiogenesis in BC models, MDA-MB-231 and MCF-7 cells, as well (Jang, Lee, Kim, Park, & Jeon, 2020). Although *A. crassna* Pierre ex Lecomte has been documented for some anti-cancer activities. However, the role of *A. crassna* Pierre ex Lecomte leaf extract (AE) in the enhancement of immune-mediated cancer cell killing has not been studied.

Immunogenic modulation is an exposure of cancer cells to nonlethal or sublethal doses of agents, such as chemotherapeutic drugs, that results in the alteration of cancer phenotype or sensitizing surviving cancer cells to be more sensitive to immune cell killing (Hodge et al., 2013a). Strategies categorized in the criteria's immunogenic modulation include both downregulation and upregulation of molecules encouraging immune-mediated cancer cell killing. The examples of the upregulated molecules in which we are interested include Fas receptor or cluster of differentiation 95 (CD95), MHC class I, and ectoCRT. These inductions promote cancer antigenicity and thus elicit cancer cell killing by immune cells (Fabian, Wolfson, & Hodge, 2021). CD95 or Fas receptor (Fas/FasR/Apo-1) is a cell surface receptor that transduces apoptotic signals into cells. The binding between CD95 expressed on cancer cells and its cognate ligand called CD95 ligand on the surface of immune cells such as cytotoxic CD8+ T cells (CTL) and natural killer (NK) cells results in cancer cell apoptosis (Peter et al., 2015). MHC class I is a surface protein expressed by all nucleated cells. The best well-known function of MHC class I is to present the protein fragments of antigens to cytotoxic CD8+ T cells (Neefjes, Jongsma, Paul, & Bakke, 2011). Immunogenic peptides expressed by MHC class I on cancer cells are recognized by cytotoxic CD8+ T cells and lead to cytotoxic CD8+ T-mediated cancer cell elimination (Neefjes et al., 2011). Calreticulin (CRT or CALR) is a multifunctional protein located in the endoplasmic reticulum (ER). In healthy cells, CRT operates two main functions within the ER which are chaperoning and Ca^{2+} homeostasis. CRT also participates in antigen presentation processes of MHC class I by being a part of the peptide-loading complex (PLC) (Fucikova, Spisek, Kroemer, & Galluzzi, 2021). Besides playing those in the ER, CRT is also expressed in cytoplasm and surface membrane. The expression of CRT at the cell surface or ectoCRT does not originate from physiological pools within the ER. The translocation to the membrane of CRT occurred in stressed and dying cells which delivers potent co-stimulatory (rather than co-inhibitory) signals or pro-phagocytotic signals to antigen-presenting cells (APCs) expressing its corresponding receptor named LDL receptor-related protein 1 (LRP1, best known as CD91). The binding between ectoCRT and CD91 results in cancer phagocytosis by APCs including dendritic cells (DCs) and macrophages (Ma et al., 2013). The expression of ectoCRT is associated with the activation of both innate and adaptive immune responses against cancer (Lu, Weng, & Lee, 2015).

In this study, we aim to study the potential role of AE in immunogenic modulation by focusing on the effect AE on the expression of ectoCRT, MHC class I, and CD95/Fas death receptor on AE-treated cells. In our studies, we chose a triple-negative breast cancer (TNBC) as a cancer model. TNBC is the most aggressive type of breast cancer (BC) characterized by the poorest prognosis (earlier recurrence and metastasis), causing a short

overall survival rate, and no specific effective treatments. (Bauer, Brown, Cress, Parise, & Caggiano, 2007; Ghoncheh, Pournamdar, & Salehiniya, 2016; Tan et al., 2020). Furthermore, downregulation of MHC class I and CD95 have been reported in TNBC and correlated with disease progression and evading from T cell-mediated apoptosis, respectively (Agrawal & Kishore, 2000; Campoli & Ferrone, 2008; Muschen et al., 1999; Torigoe et al., 2012). Another data supporting our studied design is that the upregulation of ectoCRT in the TNBC model enhances DC function and CD8+ T cell proliferation (Quan et al., 2020).

Methods and Materials

Ethical approval

All protocols were approved by Naresuan University Institutional Review Board (Approval number 388/2021)

Cells and cell culture

M.D. Anderson-Metastatic Breast 231 (MDA-MB 231) is a human triple-negative breast cancer (TNBC) cell line obtained from ATCC-LGC (#HTB-26, Middlesex, UK). MDA-MB-231 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA), 100 units/ml penicillin, and 100 ng/ml streptomycin at 37°C with humidify 5% CO_2. To maintain these cells in culture for experiments, cells were subcultured twice a week by trypsinization with 0.1% trypsin in 2.5 mM EDTA and then continuously cultured in each treatment condition.

Leaf extract of *A. crassna* Pierre ex Lecompte (AE)

The AE was kindly provided and quantified by Dr. Eakkaluk Wongwad as previously described (Wongwad, Ingkaninan, Wisuitiprot, Sritularak, & Waranuch, 2020; Wongwad et al., 2019). Briefly, young leaves of *A. crassna* Pierre ex Lecompte (1-3 leaves from the top of the branch) cultivated in Phitsanulok Province, Thailand, were collected from healthy trees with an age of fewer than 10 years during February-March. The leaf sample with the collection number Wongwad001 was kept at the PNU herbarium, Faculty of Science, Naresuan University. The collected leaves were dried at 100°C for 3 hr before grounding into powder. Two grams of dried powder were dissolved in 20 ml of hot water (95°C to 100°C) for 30 min. The supernatant was filtered, lyophilized, and stored at -20°C for future use. To prepare stock for experiments at 1 mg/ml of AE, 1 mg of aqueous extract was dissolved in 1 ml of sterile distilled water and then filtered through 0.45 μM syringe nylon membrane filter. The stock solution was diluted into 200 μg/ml and injected into high-performance liquid chromatography (HPLC) with column C18 (150 mm x 4.6 mm, 5 μm particle size) (Shimadzu Corporation, Kyoto, JP) with a reference standard mixture containing 64 μg/ml of compound 1-3 (3,5-C-β-D-diglucoside = compound 1, 3,5-C-β-D-glucoside = compound 2, mangiferin = compound 3) dissolved in methanol and 8 μg/ml of compound 4 (genkwanin 5-O-β-primevoside) dissolved in ethanol.

Cell viability assay

MDA-MB-231 cells were seeded into a 96-well plate at a density of 10,000 cells/well for 24 hr before AE treatment. AE was added to the cells at various concentrations including 0, 10, 20, 40, 80, 160, 320, and 640 μg/ml for 6 hr. and 12 hr. The conditioned media were discarded and cells were added with MTT reagent [(3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium 24 bromide)] at a final concentration of 0.5 mg/ml for 2 hr. Living cells have the ability to create formazan crystals which were then dissolved into a purple solution

by adding dimethyl sulfoxide (DMSO). The percentages of cell viability were calculated from optical density value measured by microplate spectrophotometer at wavelength 570 nm and 690 nm for reference wavelength.

Immunostaining for surface expression analysis of CD95, MHC class I, and ectoCRT

MDA-MB-231 cells were treated with AE at various concentrations including 0, 10, 20, 40, 80, 160, and 320 μg/ml for 6 hr. and 12 hr. Cells were collected, washed twice with filtered 2% FBS-1X PBS, and then blocked the non-specific protein binding with 0.1% Human Ab serum for 30 min on ice. To determine the surface expression level of MHC class I, CD95, and ectoCRT, blocked cells were further separately incubated with specific primary antibodies. The expression of CD95 was detected by using the direct staining method in which blocked cells were incubated with APC-conjugated anti-CD95 (anti-APO-1/FAS, clone DX2, eBioscience™, California, USA). Meanwhile, the indirect staining method was used to observe the expression of MHC class I and ectoCRT by separately adding mouse anti-MHC class I (clone W6/32, Santa Cruz Biotechnology, California, USA) and mouse anti-calreticulin (clone FMC75, Abcam, Cambridge, UK), respectively, for 1 hr. Then, Alexa Fluoro 555-conjugated goat anti-mouse IgG2a and Alexa Fluoro 488-conjugated goat anti-mouse IgG1 secondary antibodies were added for MHC class I and ectoCRT detections. The percentages of fluorescent-positive cells and mean fluorescent intensity (MFI) were analyzed from AE-treated cells by flow cytometer (BD Accuri™ C6 plus, New Jersey, USA).

Determination of ectoCRT expression in AE-living cells

MDA-MB-231 cells were treated with AE at the same concentrations as performed in the immunostaining for surface expression analysis. At 12 hr after AE treatment, cells were firstly stained with anti-calreticulin antibody using the indirect staining method as described previously. To examine the expression of ectoCRT in living cells, cells pre-stained for ectoCRT detection were further stained with APC-conjugated AnnexinV dye and PI for 15 min. according to the manufacturer's instruction (ImmunoTools, Friesoythe, Germany). The ectoCRT-positive living cells were analyzed by observing of FITC-positive signals from gated AE-treated living cells (left lower quadrant; negative signal for both AnnexinV and PI staining) as shown in figure 4A. Data were analyzed and represented as the percentages of positive cells and relative MFI of ectoCRT.

Statistical analysis

Mean and standard error of the mean (Mean ± SEM) from at least three independent experiments were calculated and analyzed for the significant difference between two groups by one-way ANOVA and post-test with Dunnett's test using GraphPad PRISM version 8.4.3. $p<0.05$ was considered as a significant difference. (* = $p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.001$)

Results

Determination and quantification of active compounds in AE

AE contains four major active compounds which are well-known for their pharmacological activities including iriflophenone 3,5-C-β-D-diglucoside (compound 1), iriflophenone 3-C-β-D-glucoside (compound 2), mangiferin (compound 3), and genkwanin 5-O-β-primevoside (compound 4) as described by Wongwad et al. (2019). This AE was newly prepared and re-identified for their profiling by using HPLC. The result of chromatographic profiling of AE revealed that AE used in this study contains all four active compounds (Figure 1). The amounts of compounds 1-4 were calculated and presented as mg/g of dry weight

after extraction which is 47.95±0.9 mg/g, 10.36±0.1 mg/g, 5.41±3.3 mg/g, and 1.83±0.2 mg/g, respectively. The chemical structures of those four compounds are shown in Figure 2.

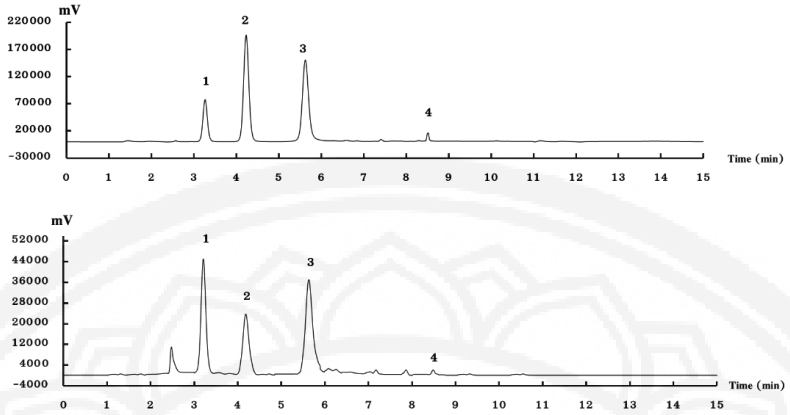

Figure 1 HPLC chromatogram profiling of *A. crassna* Pierre ex Lecompte leaf extract of (AE). Upper panel shows a profiling of a reference standard mixture which contains 64 µg/ml of iriflophenone 3,5-C-β-D-diglucoside (compound 1), iriflophenone 3-C-β-D-glucoside (compound 2), mangiferin (compound 3), and 8 µg/ml of genkwanin 5-O-β-primevoside (compound 4). Lower panel shows a profiling of AE prepared at concentration of 200 µg/ml.

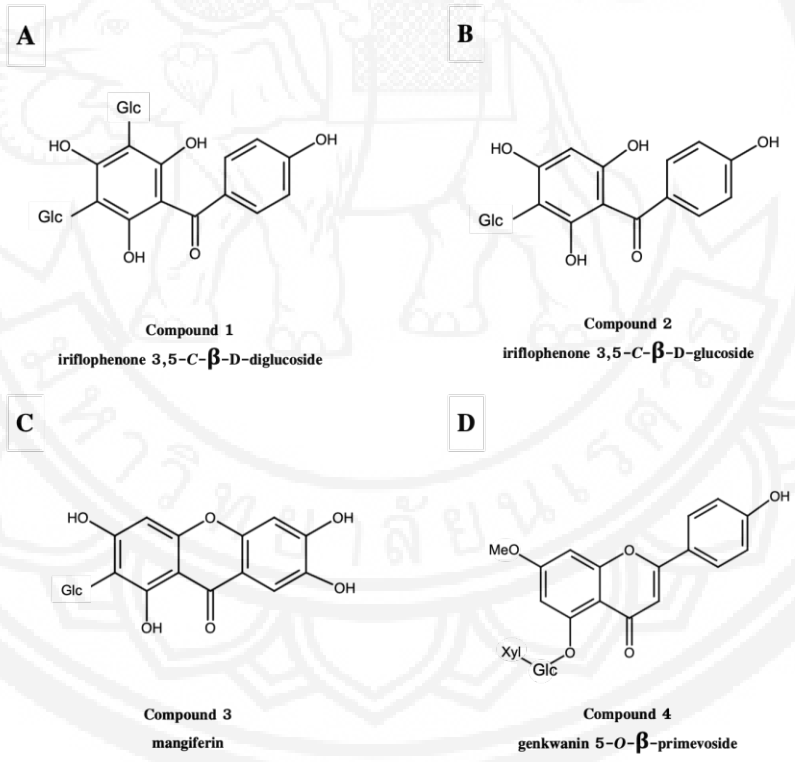

Figure 2 Chemical structures of four major active compounds in AE including compound 1 (iriflophenone 3,5-C-β-D-diglucoside), compound 2 (iriflophenone 3-C-β-D-glucoside), compound 3 (mangiferin), and compound 4 (genkwanin 5-O-β-primevoside).

Cytotoxic effect of AE on TNBC

The cytotoxic effect of AE on MDA-MB-231 cells were determined and presented as the percentages of viability cells. The results showed that AE induced the obvious cytotoxic effect on TNBC only at the highest dose of our study, 640 μg/ml, after treatment for 6 hr and 12 hr (% of cell viability were 27.56 and 21.05, respectively). Although a concentration of 20 to 320 μg/ml of AE treatment caused a significant decrease in cell viability, they still showed more than 70% of cell viability (Figure 3). Thereby, concentrations at 20-320 μg/ml were considered sublethal doses and used to further determine the expression of CD95, MHC class I, and ectoCRT.

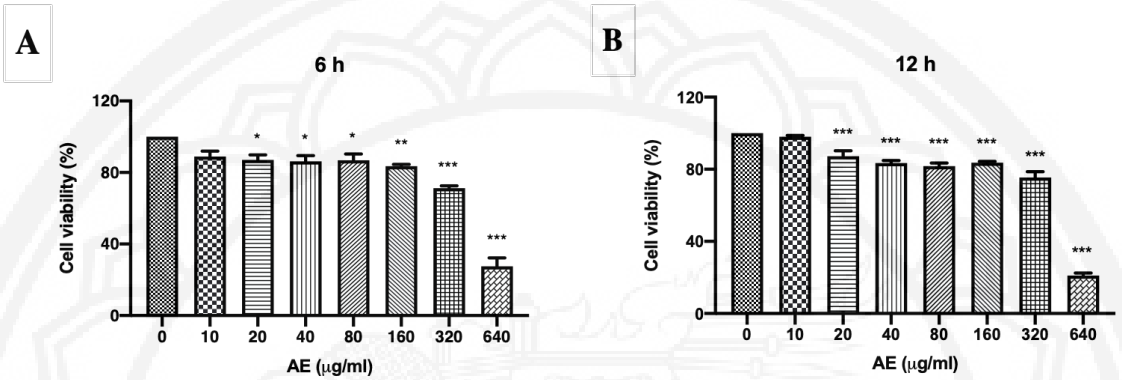

Figure 3 The percentages of MDA-MB-231 cell viability treated with AE for 6 hr (A) and 12 hr (B). MDA-MB-231 cells were treated with AE at concentrations as indicated for 6 hr and 12 hr. Cells treated and untreated (0 μg/ml) were subjected to MTT assay. The percentages of cell viability were calculated. Mean ± SEM of at least three independent experiments were represented (*p<0.05 and **p<0.001, ***p<0.001)

AE selectively upregulated the expression of ectoCRT on TNBC

To examine the surface expression of CD95, MHC class I, and ectoCRT, AE-treated cells were subjected for surface immunostaining using specific antibodies to those proteins. The results showed that AE at concentrations of 40 to 320 μg/ml selectively induced the significant increasing of ectoCRT-positive cells at 12 hr compared with untreated condition (0 μg/ml) but did not show any effect on CD95 and MHC class I expressions at both 6 hr and 12 hr (Figure 4). This result showed that AE specifically or selectively activates the upregulation of ectoCRT at the surface membrane of TNBC.

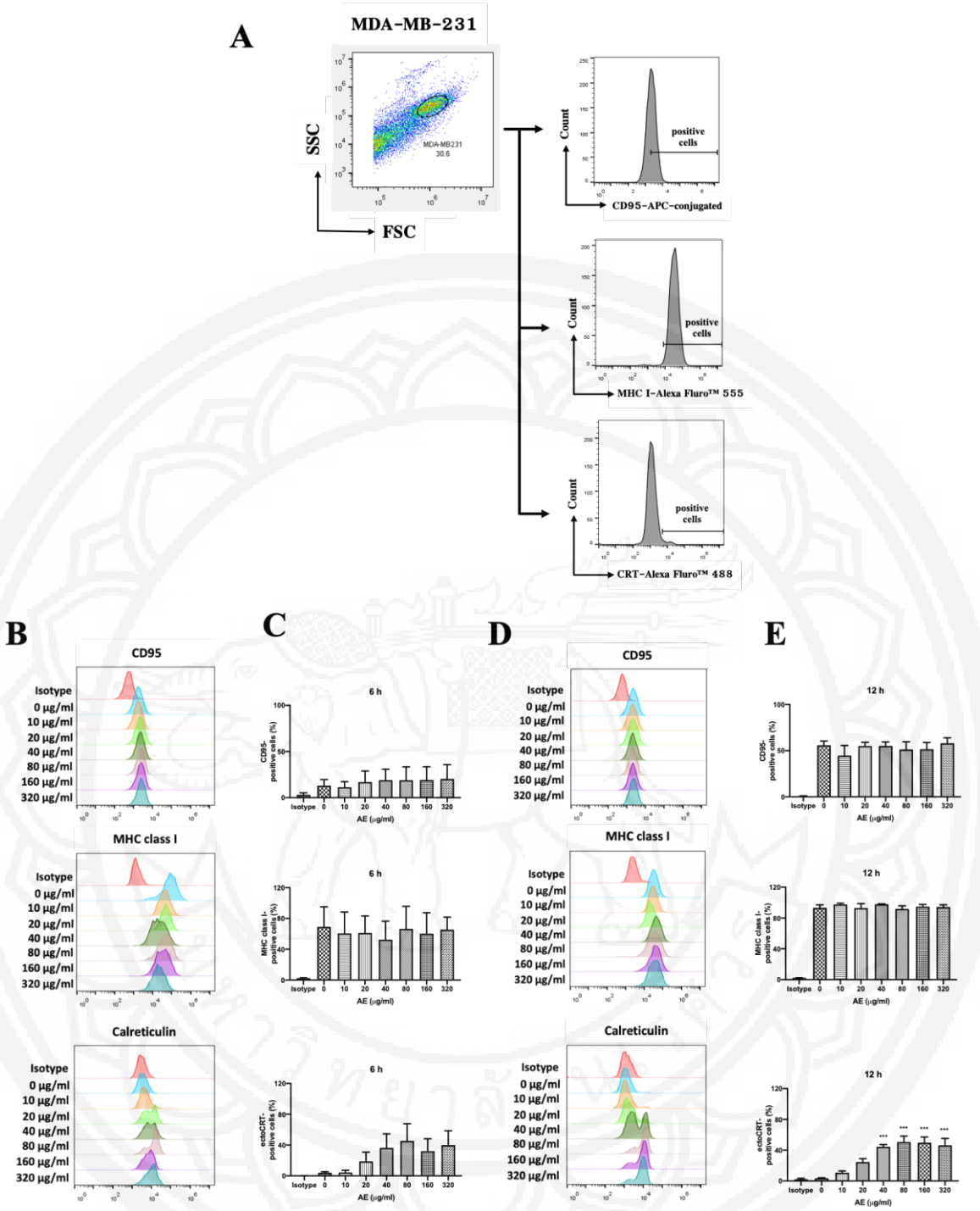

Figure 4 Surface expression of CD95, MHC class I, and ectoCRT. MDA-MB-231 cells were treated with AE at concentrations as indicated for 6 hr and 12 hr. Cells were subjected for surface immunostaining by using antibodies specific to CD95, MHC class I, and ectoCRT. Fluorescence-positive signals for each protein include APC, Alexa Fluro™555, and Alexa Fluro™488, respectively. (A) Gating strategy for choosing all AE-treated cell populations for further analysis of each protein. (B) and (D) The representative histograms of CD95, MHC class I, and ectoCRT expression detection. (D) and (E) The percentages of positive cells were calculated and presented as mean ± SEM from at least three independent experiments were represented (***p<0.001)

AE-induced ectoCRT expression in AE-treated living cells

As mentioned earlier that the translocation of CRT to surface membrane (ectoCRT) in surviving/living cells is associated with immunogenic modulation. To determine whether the upregulation of ectoCRT observed in figure 4. occurs in living cells, AE-treated living cells were gated from double-negative staining cells of both AnnexinV and PI staining (lower left in Figure 5A) and investigated for FITC-positive signals indicating the ectoCRT expression. The results revealed that AE caused a significant increase of relative MFI of ectoCRT expression at concentrations of 20-160 µg/ml (approximately 2 folds at 40 and 80 µg/ml) compared with untreated condition (0 µg/ml) (Figure 5B; right panel) and showed a slight increase of ectoCRT-positive cells (figure 5B; middle panel). These results conclude that at 12 hr of the treatment, AE caused the upregulation or increasing a number of ectoCRT molecules on the surface of AE-treated living cells.

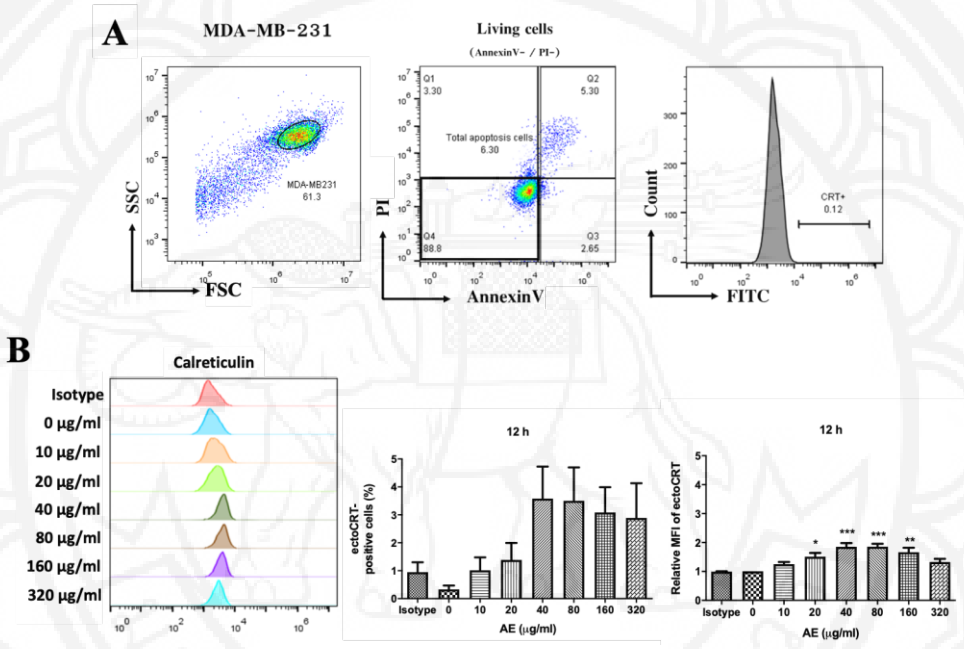

Figure 5 The expression of ectoCRT investigated from AE-treated living cells. MDA-MB-231 cells were treated with AE at concentrations as indicated for 12 hr. Cells were firstly subjected to surface staining using anti-CRT antibody followed by AnnexinV and PI staining to discriminate between dead cells and living cells. (A) Gating strategy for choosing AE-treated living cells (lower left quadrant; double-negative signal of AnnexinV and PI staining. (B; left panel) A representative histogram of ectoCRT expression analysis. (B; middle and right panels) The percentages of ectoCRT-positive cells and relative MFI were calculated and presented as mean ± SEM from at least three independent experiments (*p<0.05 and **p<0.001, ***p<0.001).

Discussion

There is a growing interest in studying of anti-cancer properties of natural products and their active components for cancer therapy developments including cancer immunotherapy (Deng et al., 2020). Due to the unique characteristics of natural products and their active components such as diversity of structure, diversity of biological activities, a wide range of sources, and especially less toxicity and side effects thereby they have become more attractive (Webb & Kukard, 2020). *Aquilaria crassna* (*A. crassna*) Pierre ex Lecomte or "Krisana" is a local plant cultivated in several provinces in Thailand including Phitsanulok province. In the past,

leaves of *A. crassna* were not a major part of the plant that was well-known in economic or marketed products. However, recently, they have been documented for pharmaceutical activities and their safety both *in vivo* and *in vitro* (Ghan, Chin, Thoo, Yim, & Ho, 2016; Wisutthathum et al., 2019). Our result in another study also showed that leaf extract of *A. crassna* Pierre ex Lecompte (AE) did not exert any toxicity on human peripheral blood mononuclear cells (PBMC) isolated from healthy donors (unpublished data). In this study, AE samples were kindly provided by Dr. Eakkaluk Wongwad and his research group. The major four active compounds (compound 1-4) found in AE have been previously characterized for their HPLC chromatogram profiling. In addition, compounds 1-3 have been determined and confirmed for their stability in an aqueous solution at 25°C and with a shelf life of 189 days for compound 1, 13 days for compound 2, and 75 days for compound 3 (Wongwad et al., 2020; Wongwad et al., 2019). The AE used in our study was re-identified for its HPLC chromatogram profiling and calculated for the amount of each compound, as illustrated in Figure 1 and in the results. The highest amount of active compound in this AE is compound 1 (iriflophenone 3,5-C-β-D-diglucoside) followed by compound 3 (mangiferin), compound 2 (iriflophenone 3-C-β-D-glucoside), and compound 4 (genkwanin 5-O-β-primevoside). Our first study showed that AE caused a significant decrease in MDA-MB-231 cell viability at 20-640 μg/ml but at 640 μg/ml exerted obvious cytotoxicity with less than 50% of cell viability. Thereby, we chose concentrations at 20-320 μg/ml for our further studies and called them as the sublethal doses. Sublethal doses of AE could specifically induce the upregulation of the surface molecules that are implicated in the activation of both innate and adaptive immune responses to cancer, ectoCRT, at 12 hr of treatment. Meanwhile the other molecules involved in cancer recognition, MHC class I, and death receptor-mediated cancer killing, CD95 or Fas, have not been changed. The possible reason to explain this specific induction is that AE, through one or some of its active components, may directly or directly activate ER-stress response by governing PERK-mediated phosphorylation of the translation initiation factor eIF2a. This event causes the translocation of CRT from the cytoplasm to the cell surface (Panaretakis et al., 2009). Among the four active compounds of AE, manigerin (compound 3) is best known for its anti-cancer properties in several cancers including TNBC. However, there is no evidence of mangiferin in the role of ER stress inducer in all types of BC and other cancers. Controversially, it has been documented as an inhibitor of ER stress in endothelial cells and liver injury model (Li et al., 2020; Song, Li, Hou, Wang, & Liu, 2015). However, the other 3 active compounds have been not reported for their anti-cancer properties or ER stress inducer (Feng, Yang, & Wang, 2011; Supasuteekul, 2017). The expression of CRT on the surface membrane (ectoCRT) is a major determinant of cellular adjuvanticity in which ectoCRT has the ability to send or deliver co-stimulatory signals including "eat me signal" to immune cells rather than deliver co-inhibitory signals. The most well-known function of ectoCRT is to deliver a pro-phagocytotic signal to APCs likes DCs and macrophages leading to the uptake of ectoCRT-expressed cells and then CTL activation (Galluzzi et al., 2020; Ma et al., 2013). Importantly, ectoCRT is implicated in two immunostimulatory events termed immunogenic modulation and immunogenic cell death (ICD). Previously, these two events have been defined as distinct processes but recently they have been reclassified as similar effects termed "immunogenic cell stress". The key point to discriminate between these two events is that immunogenic modulation is defined as a sensitizing surviving/living cancer cells to express danger signals/co-stimulatory molecules to activate immune cell killing meanwhile ICD is defined as an induction of danger signals expression occurred in cell dying (Fabian et al., 2021). In this study, we focused on the surface expression of CD95 or Fas receptor, MHC class I, and CRT in AE-treated living

cells because of their vital roles in immune-mediated cancer elimination as described previously. We found that AE exerted its effect predominantly on the upregulation of a number of ectoCRT molecules on surface of AE-treated MDA-MB-231 living cells. This implied that AE may enhance BC immune responses by increasing an immunogenicity of treated cells to be more susceptible for being killing by immune cells compared to untreated cells which is accorded to a criterion of immunogenic modulation. Our results found that AE could induce only 4 % of ectoCRT-positive cells meanwhile it clearly showed the increasing of relative MFI of ectoCRT expression instead. It implies that at least this induction by AE will make cancer cells being more susceptible to be phagocytosed than untreated cells. A study reported by Hodge et al (2013) found that MDA-MB-231 cells have to be treated with docetaxel up to 72 hr to show a significantly modulating CRT surface expression (60%) and this induction did not involve with classical ICD (Hodge et al., 2013b). Moreover, only 10% of ectoCRT expression has been found in mouse breast cancer cells (4T1-neu-HA) treated with the natural compound, *Cordyceps militarisin*, for 6 hr and 12 hr even though they did not mention which population was examined (Quan et al., 2020). Regarding this, experiments for optimization to increase percentages of ectoCRT-positive cells induced by AE needs to be further performed as well as studies to confirm that BC cells expressing AE-induced ectoCRT can activate immune responses, such as phagocytotic assay, investigations of DC/macrophage and T cell activations, and immune cell killing assay are also needed to be further examined. Interestingly, apart from our finding that AE could induce ectoCRT in living cells which maximally occurred in 4% of cases, we found 40-50% of ectoCRT-positive cells in total population of AE-treated MDA-MB-231 cells. This means that ectoCRT may be expressed in other AE-treated populations including early or late apoptotic/dead cells which coincides with the concept of classical ICD, as mentioned earlier. The expression of ectoCRT in other populations has been simultaneously investigated separately in both early and total apoptotic cell populations. Interestingly, the results showed that ectoCRT expression was mostly found in the total apoptotic population with approximately 40% at a concentration of 40-320 μg/ml at 12 hr (unpublished data). It means that AE might induce ectoCRT in dying cells which is congruent with the ICD scenario. Thereby, our group is working on this novel finding to identify the role AE in an ICD, starting from AE-induced ectoCRT dying cells. Lastly, our studies have some other limitations which need to be clarified. They include 1) which mechanism does AE use to upregulate ectoCRT expression 2) which active compound in AE exerts this action 3) Can AE induce the upregulation of ectoCRT in other types of BC and other types of cancer 4) Can induced ectoCRT induce the activation of immune cells and results in the increasing of BC cell killing. If we are able to complete an investigation of all these limitations, it would be a new finding to reveal the medicinal benefits of AE as alternative cancer therapies.

Conclusion and Suggestions

This is the first preliminary study to report the possible role of *Aquilaria crassna* (*A. crassna*) Pierre ex Lecomte leaf extract (AE) as an anti-cancer treatment relevant to the enhancement of breast cancer immune responses. Our results were that AE selectively induces ectoCRT expression in AE-treated living cells. The finding of AE-induced ectoCRT in living cells is according to a criterion of immunogenic modulation. However, to clarify the role of AE-induced ectoCRT in an immunogenic modulation, more experimentation is necessary. However, the results of this study strongly suggest that AE has a potential role in the enhancement of cancer

immune responses and may be a potential source of natural products that can be used in further cancer immunotherapy studies.

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