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In vitro anticancer effect of *Aquilaria crassna* extract on human mammary gland cancer cells

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Abstract

Agarwood has long been known for its therapeutic effects. In the current study, anticancer effects of *Aquilaria crassna* extracts (*ACE*) on human breast cancer *in vitro* with MDA-MB-231 and MCF-7 cancer cells were studied. Cell survival and cell death were investigated mainly by Immunofluorescence (IF) assay. The gene expressions of PCNA and BCL-2 for cancer cell proliferation, Caspase-3 for apoptosis and VEGF for angiogenesis were detected. The Hoechst analysis was also made to confirm the degradation of nucleus membrane. The cancer cells treated with *ACE* showed effects of cytotoxicity with significant increase in Caspase-3 expression in the treated group compared to the control. The potential blood vessel formation induced by VEGF was significantly lower in the cancer cells treated with *ACE*. Also, MMP2 were highly expressed. We concluded that the *Aquilaria crassna* extract showed anticancerous effects in human mammay gland cells.

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Introduction

There are 22 species of Agarwood (*Aquilaria genus*) out of 47 species officially listed in *the Plant List* version 1.1 (2013). The rest of *Aquilaria* species are still unresolved by the *Plant list*. Many are confused with some synonyms of *Aquilaria* species. Different names are said in different countries such as Gaharu in south-east Asia, Oud in Middle East, Jin-koh in Japan and Chimhyang in Korea. Agarwood has long been known for its medicinal effects on various diseases and therapeutic effects through aromatic compounds. It belongs to *Thymelaeaceae* family. And also, agarwood is being commercially used as fragrance products. Dahham *et al.* (2016) studied the effect of Agarwood essential oils on prevention of blood vessel formation in vitro and reported that it prevents the formation of capillary tubules. The anti-angiogenic effect is highly crucial to block the supply of nutrients to tumor cells. Hashim *et al.* (2014) reported the Agarwood essential oil has a significant effect on anti-breast cancer using MCF-7 cell lines in vitro. Anticancer effect against pancreatic cancer cells by Agarwood essential oil in which β-caryophyllene was most abundant by GC mass analysis was studied (Dahham *et al.*, 2016). Aromatic compounds of α-gurjunene and (+)-calarene in Agarwood essential oil were found for sedative effects (Okugawa *et al.*, 1993; Takemoto *et al.*, 2008; Miyoshi *et al.*, 2013). Not only for anticancer effects, other therapeutic effects of Agarwood were also found. Analgesic effect of Agarwood extracts was studied by Okugawa *et al.* (1993) and its laxative effect of Agarwood leaf extracts was reported by Hara *et al.* (2007).

The methanol extract from agarwood leaves was abundant in alkaloids which were used for rheumatism and pains (Khalil *et al.*, 2013). Very interestingly, Agarwood trees without resin deposited, the uninfected Agarwood trees, was studied for their anticancer effect against breast cancer cells of MCF-7 (Abbas *et al.*, 2018) which indicates Agarwood barks, stems and leaves without the resinous parts also have an anticancer effect. Adam *et al.* (2017) well reviewed the various pharmacological effects of Agarwood leaves in that they have many therapeutic effects as potentially useful drugs. The objective of this study was to see the potentially therapeutic effects of Agarwood extracts from *Aquilaria crassna* Pierre ex Lecomte on anticancer effects of human breast cancer.

Materials and methods

Cell culture

Human breast cancer cells of MDA-MB-231 and MCF-7 were purchased from Korea cell line Bank, Seoul, Korea. MDA-MB-231 and MCF-7 cancer cells were cultured *in vitro* in media of RPMI 1640 (Gibco, USA) mixed with 10% fetal bovine serum (FBS; Gibco, USA). The 10㎕/1.5㎖ of Agarwood extracts were added into the culture media. Then, the cells were cultured in 5% CO_2 incubation at 38.5℃ for 48 h.

Immunofluorescence assay

Breast cancer cells of MDA-MB-231, MCF-7 were cultured on sterilized culture slide (SPL, Gyeonggi-do, Korea). After slides being fixed with 4% paraformaldehyde and blocked with 3% BSA (bovine serum albumin) in 1PBS, dehydration and permeabilization were performed by freezing the slides at -20 C in 5 mM 0.1% Triton X-100 in PBS. After blocked with 3% BSA in 1PBS, sample slides were incubated with the primary antibodies PCNA (Santa cruz, SC-7907 rabbit), VEGF (ThermoFisher, PA5-16754), BCL-2(Santa Cruz, SC-7907 rabbit), Caspase-3 (abcam, ab4051) at 1:200 dilutions. After washing, the slides were incubated with florescent secondary antibodies of anti-rabbit IgG conjugated to Alexa Fluor 488 (life technologies A-11094) or Alexa Fluor 594 ((life technologies A-11012). After counterstained with 1g/mL Hoechst 33258 solution (Sigma Aldrich, USA), and slides were mounted using fluorescent mounting medium (Dako, Carpinteria, CA, USA). Detected protein images were analyzed using Olympus AX70 fluorescence microscope fitted.

Enzyme linked immunosorbent assay (ELISA)

The 500x diluted primary antibody of MMP-2(abcam, ab97779) was inserted onto 96-well plates to detect the expression of MMP-2 by ELISA. The antigen-

antibody reaction was conducted at 4 ℃ for 24 h. Then, they were washed off twice with the washing buffer (1 × PBS containing 2.5% Triton X-100).

Thereafter, they were treated with 1% skim milk blocking solution at 4℃ for 24 h and washed again with washing buffer. Afterwards, secondary antibodies with florescence marking were treated at room temp for 3 h and the antibody-antigen reaction was detected. Finally, they were measured by intensity of transmitted light of 450 nm.

Results and discussion

Immunofluorescence analysis

After culture media were treated with *Aquilaria crassna* extract, IF analysis was made for 2 different breast cancer cell lines of MDA-MB-231 and MCF-7. The gene expressions for the cell survival, cell death and VEGF for angiogenesis were detected by IF analysis. As shown in Fig. 1, the cell proliferation protein of PCNA was well expressed in control group while the treated group (Agarwood extract; AE) showed almost non-expression of PCNA.

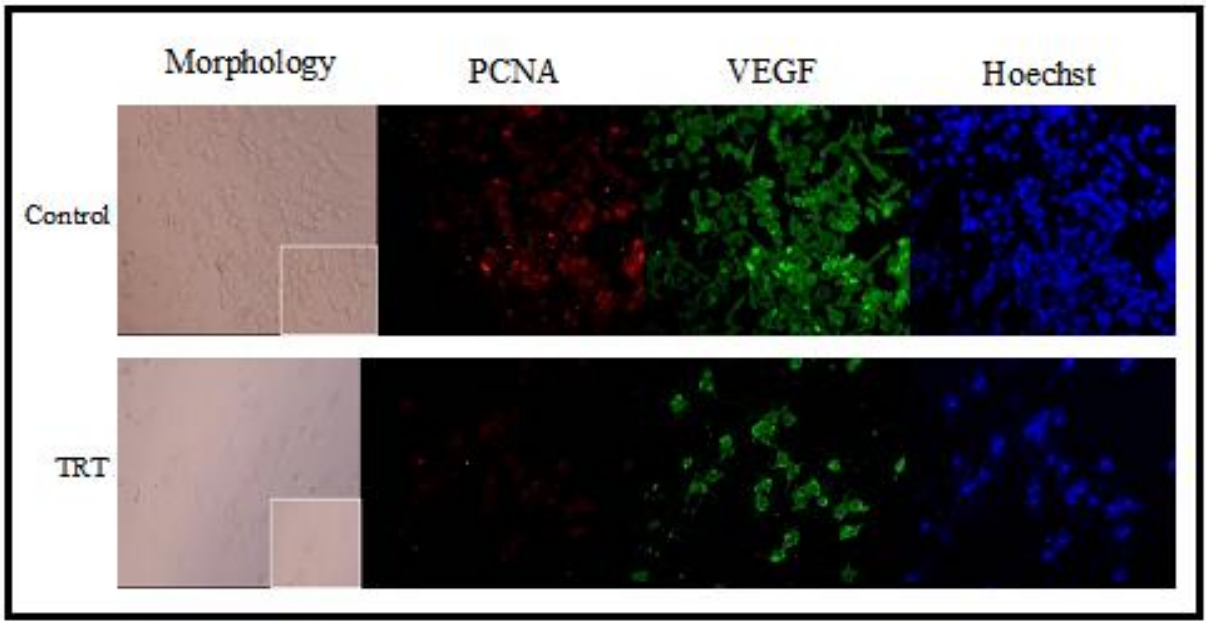

Fig. 1. Immunofluorescence analysis of MDA-MB-231 cancer cells.

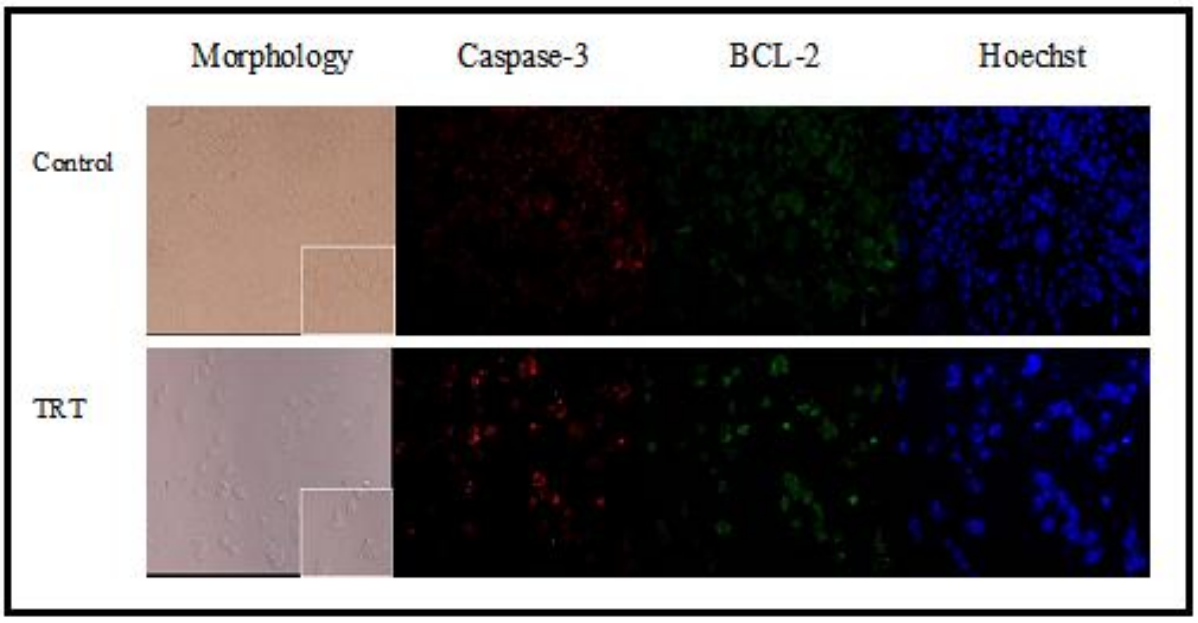

Fig. 2. Immunofluorescence analysis of MDA-MB-231 cancer cells.

The formation of blood vessel induced by VEGF was also much less expressed in AE group than control group. In cancer formation, prevention of blood vessel formation is highly important to block the supply of nutrients to the cells. In Fig. 2, for apoptotic events, not only with PCNA, BCL-2 and Caspase-3 expressions, Hoechst analyses were made for the nucleus membrane degradation and well indicated the treatment of *Aquilaria crassna* extracts highly degraded the nucleus membranes in both MDA-MB-231 and MCF-7 in Figs 1 to 4.

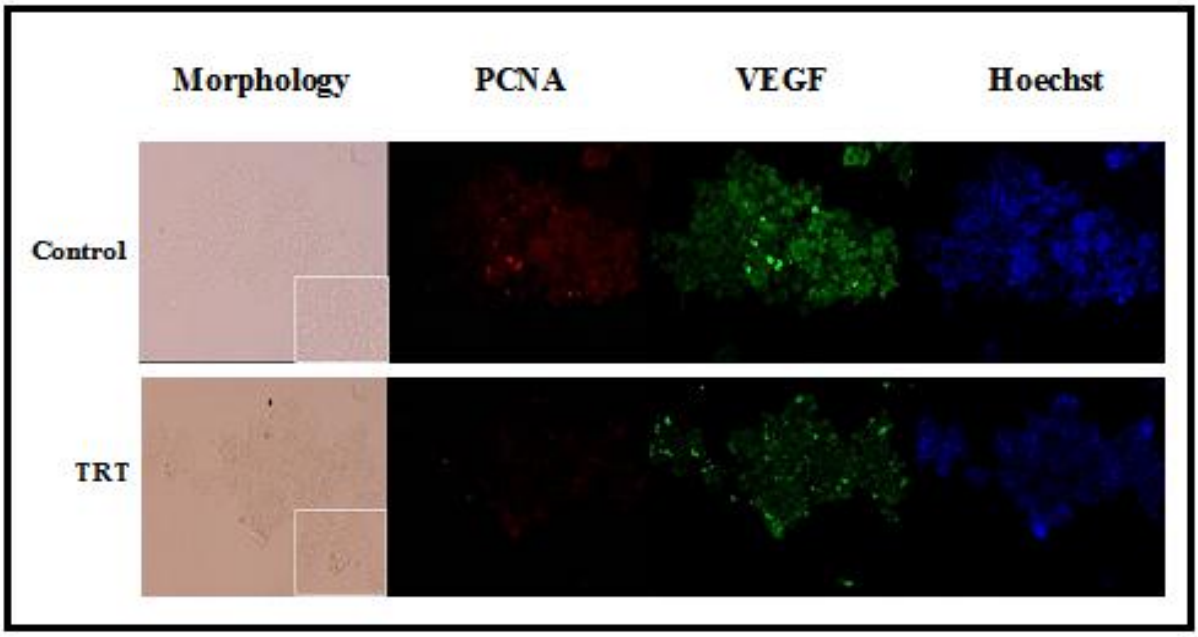

Fig. 3. Immunofluorescence analysis of MCF-7 cancer cells.

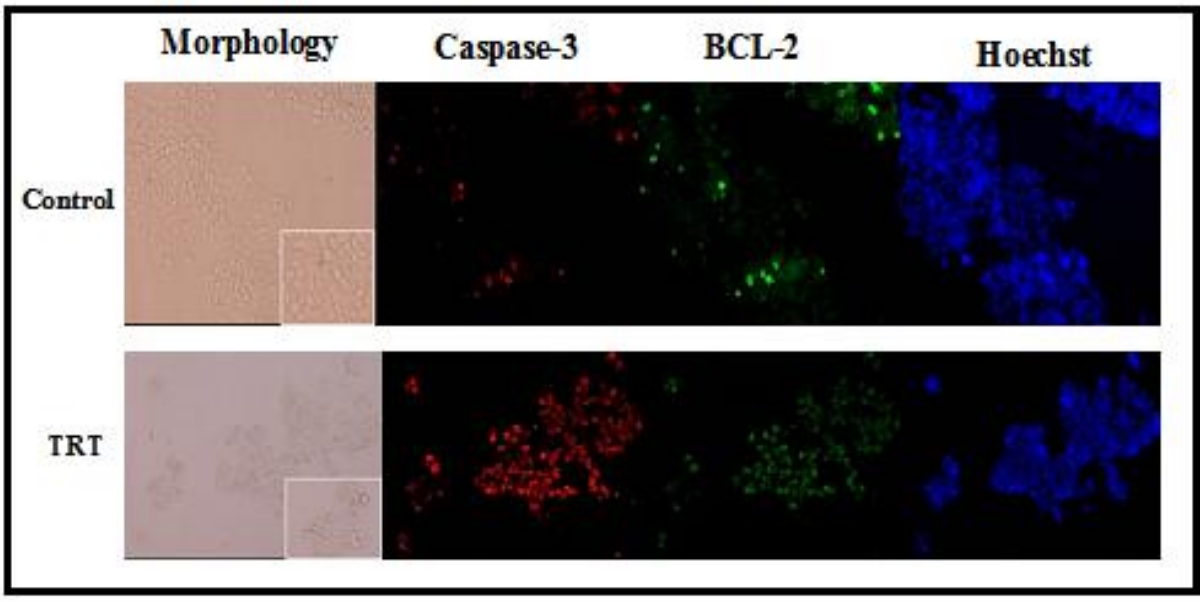

Fig. 4. Immunofluorescence analysis of MCF-7 cancer cells.

ELISA analysis

ELISA analysis was made for MMP-2, which revealed a gene expression associated with cell survival, cell death and angiogenesis. MMP-2 is highly associated with the cell death because it holds the intact of cancer cells by degrading the extracellular matrix, for which the detachment of cancer cells from the bottom of the cell culture plate reveals the death signal of the

cancer cells. As seen in Fig. 5, OD values by ELISA for MMP-2 expression were significantly higher for the treated groups in both MDA-MB-231 and MCF-7 cancer cells. These results coincide with other studies (Abbas *et al.*, 2018).

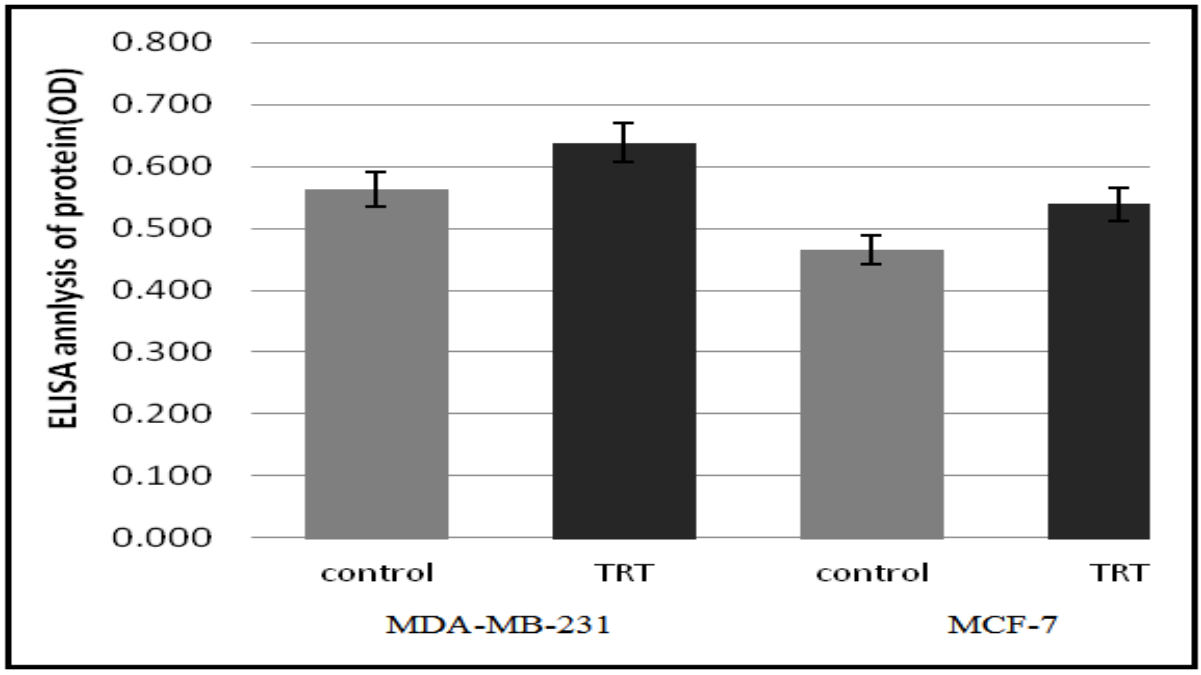

Fig. 5. ELISA analysis of MMP2 for MDA-MB-21 and MCF-7 cancer cells.

Conclusion

Aquilaria crassna is a potentially therapeutic drug effect on human breast cancer. The cell survival and cell death signals related to the corresponding gene expression by treatment of *Aquilaria crassna* extract were well detected. *Aquilaria crassna* leaves were not tested for their anticancer effects but will also be a potential drug usuage, which was studied with uninfected (no resinous parts inside) Agarwood trees.

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