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Ethyl Alcohol Extract of *Hizikia Fusiforme* Synergistically Enhances Apoptosis In TRAIL-Administrated Hep3B Cells

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Abstract: Edible brown algae, Hizikia fusiforme has been used as traditional medicinal herbs for a long time in East Asian countries. Ethyl alcohol extract of H. fusiforme (EAHF) possesses cancer invasion and metastasis; however, whether EAHF enhances tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis is not still understood. Therefore, we investigated the molecular mechanism by which EAHF triggers TRAIL-induced apoptosis. High dosages (40 μg/ml and 50 μg/ml) of EAHF significantly downregulated cell proliferation, and increased DNA fragmentation, sub-G_1 phase, and annexin V^+-cell populations in response to TRAIL. The combined treatment with EAHF and TRAIL also increased apoptotic protein expression such as caspases and poly (ADP-ribose) polymerase, and suppressed anti-apoptotic proteins such as X-linked inhibitor protein, the inhibitor of apoptosis proteins, and Bcl-2. Intriguingly, combined treatment-induced apoptosis was completely inhibited by a pan-caspase inhibitor and chimeric antibody against death receptor 5 (DR5) which is a specific receptor of TRAIL, suggesting that combined treatment enhances the DR5-induced death signal pathway by activing caspases and consequently promotes apoptosis. Additionally, EAHF increased stabilization of DR5 protein, but not DR5 mRNA, and also stimulated ROS generation; ROS inhibitors partially inhibited combined treatment-induced apoptosis. Taken together, EAHF enhances TRAIL-induced apoptosis by stabilizing of DR5 protein and increasing ROS generation, and caspase activation.

Keyword: Hizikia fusiforme, tumor necrosis factor-related apoptosis-inducing ligand, reactive oxygen species, death receptor 5

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I. INTRODUCTION

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a member of the TNF superfamily and is known to be an effective cancer therapeutic owing to its cancer cell specificity [1]. TRAIL induces apoptosis of cancer cells and abnormal cells, whereas avoiding the destruction of normal cells, indicating that TRAIL is more effective target for cancer therapeutics than the other members of the TNF superfamily such as TNF-α and CD95 [2]. TRAIL selectively induces apoptosis of specific cancer cells by binding specific death receptors (DRs), which subsequently upregulated Fas-associated protein with death domain (FADD) and caspase-8 to form death-inducing signal complex (DISC) [3]. However, previous study has shown that cancer cells can develop resistance to TRAIL-induced apoptosis through disturbance of the expression and stability of DR and adaptor molecules that are responsible for downstream caspase activation and/or inducing expression of apoptotic proteins [4]. Therefore, recent studies have focused on specific components that stimulate TRAIL-induced apoptosis by enhancing DR expression and stability, as well as the apoptotic signaling pathway [5,6].

Marine algae are excellent source of biological compounds and are used in the development of medicines [7]. Certain seaweeds are a crucial source of vitamins, minerals, and essential proteins. Several species of seaweeds contain secondary metabolites, glycoproteins, and polysaccharides possessing biological functions such as anti-viral, anti-tumor, and immune-stimulatory activity [8]. *Hizikia fusiforme* is one of the most abundant edible brown algae species of the Sagassaceae family. It is typically found in the coastal areas of Northeastern Asia and has been widely used in herbal remedies and health foods for centuries. *H. fusiforme*

contains a number of compounds with anti-oxidant, anti-cancer, and anti-inflammatory effects [9,10]. Although many studies have been carried out on the ability of *H. fusiforme*, its cancer-killing pharmaceutical properties have not been well documented.

In the current study, the novel activity of ethyl alcohol extract of *H. fusiforme* (EAHF) in TRAIL-administered cancer cell was investigated. It was found that EAHF synergistically sensitizes Hep3B human carcinoma cells to TRAIL-induced apoptosis through reactive oxygen species (ROS)-mediated caspase activation and DR5 stabilization.

II. MATERIALS AND METHODS

2.1. Reagents and antibodies

EAHF was obtained from Institute of Oriental Medicine (Dongeui University, Busan, Republic of Korea). Briefly, fresh *H. fusiforme* was washed three times with tap water to remove salt, epiphyte, and sand on the surface of the samples before storage -20ºC. The frozen samples were lyophilized and homogenized using a grinder before extraction. The dried powered was extracted with ethyl alcohol and evaporated in vacuo. Recombinant human TRAIL/Apo2 ligand (the non-tagged 19 kDa protein, amino acids 114-281) and peroxidase-labeled donkey anti-rabbit immunoglobulin were purchased from KOMA Biotechnology (Seoul, Republic of Korea). The chimeric antibody against DR5 was purchased from R&D Systems (Minneapolis, MN, USA). z-VAD-fmk was purchased from Calbiochem (San Diego, CA, USA). 6-Carboxy-2',7'-dichlorofluorescein diacetate (DCFDA) was purchased from Molecular Probes (Eugene, OR, USA). Glutathione (GSH) and *N*-acetyl-L-cysteine (NAC) were purchased from Sigma (St. Louis, MO, USA). Antibodies against caspase-3, caspase-8, caspase-9, XIAP, IAP-1, IAP-2, Bcl-2, DR5, and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. Cell line and cell viability assay

Human hepatocarcinoma Hep3B cells were cultured in RPMI 1640 (WelGENE, Daegu, Republic of Korea) supplemented with 10% fetal bovine serum and antibiotics (WelGENE). The cells were seeded at 1×10^5 cells/ml, incubated for 12 h, and then treated with the indicated concentrations of EAHF (0-50 μg/ml) 1 h before addition of TRAIL (100 ng/ml) for 24 h. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays were performed to determine relative cell viability.

2.3. DNA fragmentation assay

After treatment with EAHF, TRAIL, and combined treatment with EAHF and TRAIL for 24 h, Hep3B cells were lysed in DNA fragmentation lysis buffer (10 mM Tris, 150 mM NaCl, 5 mM EDTA, and 0.5% SDS) for 1 h on ice. Lysates were vortexes and cleared through centrifugation at 13,000 *g* for10 min. Fragmented DNA in the supernatant was extracted with equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) mixture and analyzed electrophoretically on 1.5 % agarose gels.

2.4. Analyses of apoptotic cells by PI and annexin V-FITC staining

Hep3B cells were pretreated with EAHF (50 μg/ml) for 1 h and then administered with 100 ng/ml of TRAIL for 24 h. After harvesting, the cells were washed twice with PBS. The cells were fixed with 1 U/ml RNase A (DNase free) and 10 μg/ml propidium iodide (PI, Sigma) for 1 h at room temperature in the dark. For annexin V staining, live cells were incubated with annexin V (R&D systems) according manufacturer´s instructions. A FACSCalibur flow cytometer (Becton Dickenson, San Jose, CA, USA) was used to analyze the level of apoptotic cells containing sub-G_1 DNA content and annexin V^+ population.

2.5. Western blot analysis

Hep3B cells were treated with 100 ng/ml TRAIL 24 h after administration of EAHF for 1 h. After harvesting, the cells were washed two times with PBS. Whole-cell lysates were prepared by PRO-PREP protein extraction solution (iNtRON Biotechnology, Sungnam, Republic of Korea). The cell lysates were harvested from the supernatant after centrifugation at 13,000 *g* for 20 min. Total cell proteins were separated on polyacrylamide gels and standard procedures were used the transfer them the nitrocellulose membranes. The membranes were developed using an ECL reagent (Amersham, Arlington Heights, IL, USA).

2.6. RT-PCR analysis

Hep3B cells were cultured in 6 wells overnight, pretreated with EAHF (0-50 μg/ml) for 1 h, and then treated with TRAIL (100 ng/ml) for 24 h. Butein (Sigma) was used as a positive control. Total RNA was extracted from Hep3B cells using Easy-Blue reagent (iNtRON Biotechnologya). For *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*), the sense primer 5´-CGT CTT CAC CAT GGA GA-3´ and the anti-sense primer 5´-CGG CCA TCA CGC CCA CAG TTT-3´ were used. The sense primer 5´-GTC TGC TCT GAT CAC

CCA AC-3´ and the anti-sense primer 5´-CTG CAA CTG TGA CTC CTA TG-3´ were used to amplify human *DR5* mRNA. The PCR initiated at 94°C for 5min followed by cycles (25 cycles for *GAPDH* and 31 cycles for *DR5*) of 94°C for 30 sec, annealing temperature (at 63°C for *GAPDH* and at 60°C for *DR5*) for 30 sec, 72°C for 30 sec followed through final extension at 72°C for 5min.

2.7. Measurement of intracellular ROS

ROS were detected using a FACSCalibur flow cytometer after staining with DCFDA for 30 min at 37°C. Briefly, Hep3B cells were treated with EAHF (0-50 μg/ml) for 1 h. Sample harvested and stained with DCFDA for 30 min. Fluorescence intensity of DCFDA was analyzed using a FACSCalibur flow cytometer.

2.8. Statistical analysis

All data were derived from at least three independent experiments. Statistical analyses were conducted using SigmaPlot software (version 12.0) Values were presented as mean ± SE. Significant differences between the groups were determined using the unpaired one-way ANOVA test. Statistical significance was regarded at *, $p < 0.05$.

III. RESULTS

3.1. EAHF augments TRAIL-induced apoptosis in Hep3B cells

We first addressed whether EAHF possesses cytotoxicity in the presence or absence of TRAIL. MTT assay was performed to evaluate the effects of combined treatment with EAHF and TRAIL on the viability of Hep3B cells. The cells were treated with different concentrations of EAHF (0-50 μg/ml) in the presence or absence of TRAIL (100 ng/ml) for 24 h. Fig. 1A demonstrated that EAHF and TRAIL alone had no anti-proliferative and in Hep3B cells (Figure 1A). However, combined treatment with high doses of EAHF (40 μg/ml and 50 μg/ml) and TRAIL (100 ng/ml) resulted in significant induction of cell death, suggesting that EAHF is a promising cell death-stimulating agent of TRAIL. Additionally, DNA fragmentation analysis displayed representative ladder forms of DNAs in Hep3B cells treated with EAHF and TRAIL; however, no DNA ladder forms were observed in treating EAFH and TRAIL alone (Figure 1B). Combined treatment with EAHF and TRAIL significantly increased the percentage of sub-G_1 phase from 2.9% to 35.0% (Figure 1C, top) and annexin V^+-cell populations from 4.8% to 29.1% (Figure 1C, bottom). EAHF and TRAIL alone induced a slight increase in the number of sub-G_1 phase and annexin V^+-cells. These results indicate that combined treatment with EAHF and TRAIL significantly induces apoptosis in Hep3B cells.

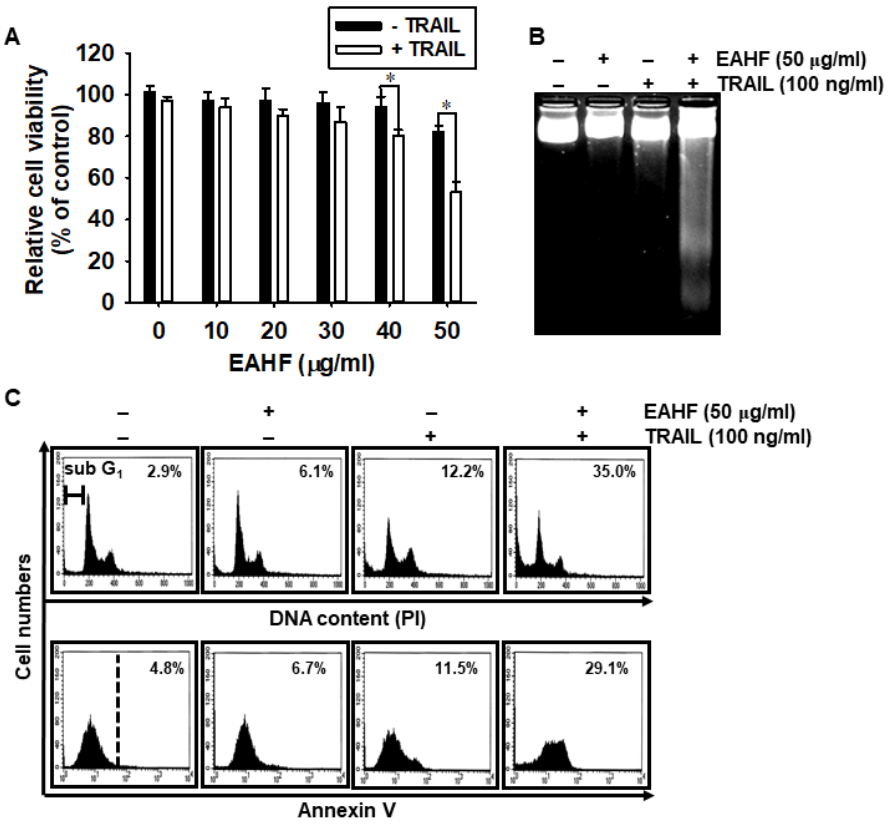

Figure 1. Effect of ethyl alcohol extract of *H. fusiforme* (EAHF) in TRAIL-induced apoptosis. Hep3B cells were pretreated with EAHF (0-50 μg/ml) for 1 h, followed by treatment with TRAIL (100 ng/ml) for 24 h. (A) Cell viability was measured by MTT assay. (B) Fragmented DNA was analyzed on 1.5 % agarose gel. (C) The percentage of sub-G_1 DNA content and annexin V^+-cell population are indicated in each panel. Statistical significance was determined by a one-way ANOVA test (*, $p < 0.05$ *vs*. TRAIL-untreated group).

3.2. EAHF reinforces TRAIL-induced apoptosis by inducing stabilization of DR5 protein

We, next, examined whether EAHF increases functional DR5 expression in TRAIL-induced apoptosis because DR5 was known as a specific receptor of TRAIL. After 24-h treatment with EAHF, DR5 expression was measured by western blot analysis and RT-PCR. Western blot analysis showed that over 40 μg/ml EAHF dramatically increased the expression of DR5 protein levels (Figure 2A). Contrary to assumptions, compared to treatment with butein used as a positive control, treatment with EAHF did not affect the mRNA levels of DR5 (Figure 2B). This indicates that EAHF significantly enhances stabilization of DR5 protein, without affecting *DR5* gene expression. Treatment of the cells with DR5 chimeric antibodies significantly restored the sub-G_1 and annexin-V^+ population, compared to that observed after combined treatment (Figure 2C). Taken together, these results indicated that EAHF increased DR5 stability, causing to sensitization of TRAIL-mediated apoptosis.

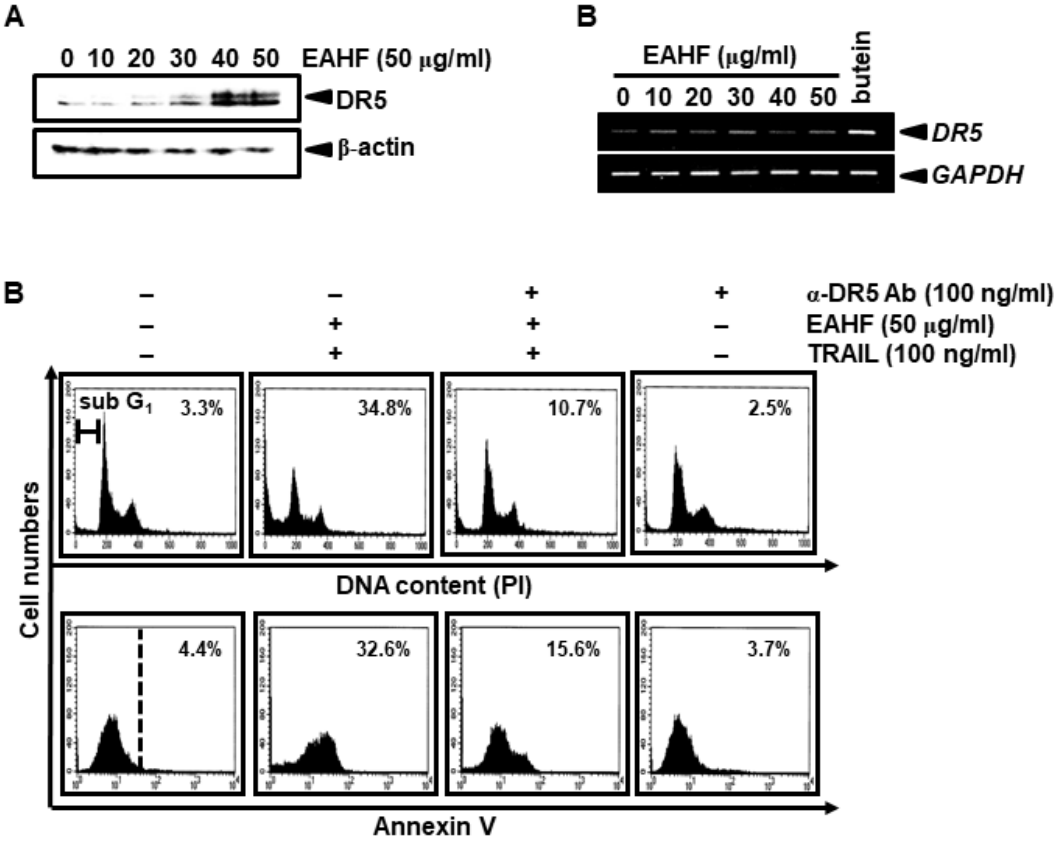

Figure 2. Expression level of the DR5 protein and mRNA by ethyl alcohol extract of *H. fusiforme* (EAHF). Hep3B cells were pretreated with EAHF (0-50 μg/ml) for 1 h, followed by treatment with TRAIL (100 ng/ml) for 24 h. (A) Expression levels of DR5 protein were determined by western blot analysis. (B) Expression levels of DR5 mRNA were determined by RT-PCR analysis. (C) Hep3B cells were treated with EAHF and TRAIL in the presence or absence of DR5 blocking antibody (100 ng/ml). The percentage of sub-G_1 DNA content and annexin V^+ cell population are indicated in each panel.

3.3. EAHF enhances TRAIL-induced apoptosis by upregulating caspase activation and downregulating anti-apoptotic protein expression

Previous report demonstrated that TRAIL-induced apoptosis is principally triggered by the extrinsic and the intrinsic apoptotic pathway, involving caspase cascade and apoptosis-regulating proteins [11]. Therefore, expression levels of pro-apoptotic and anti-apoptotic proteins were measured. Western blot analysis showed that EAHF and TRAIL alone slightly inhibited expression of caspase-8, -9, and -3 proforms; however, combined treatment with EAHF and TRAIL significantly decreased the expression of caspase proforms and increased PARP cleavage (Figure 3A). Additionally, combined treatment remarkably decreased the expression of anti-

apoptotic proteins such as XIAP, IAP-1, IAP-2, and Bcl-2 (Figure 3B). Furthermore, to assess whether caspases are crucial effectors in the combined treatment-induced apoptosis, apoptotic cell death was examined in the presence of a pan-caspase inhibitor, z-VAD-fmk. PI and annexin V^+ analyses demonstrated that z-VAD-fmk completely suppressed combined treatment-induced apoptosis (Figure 3C). The result suggests that EAHF and TRAIL-induced apoptosis inevitably requires the activation of the caspase cascade.

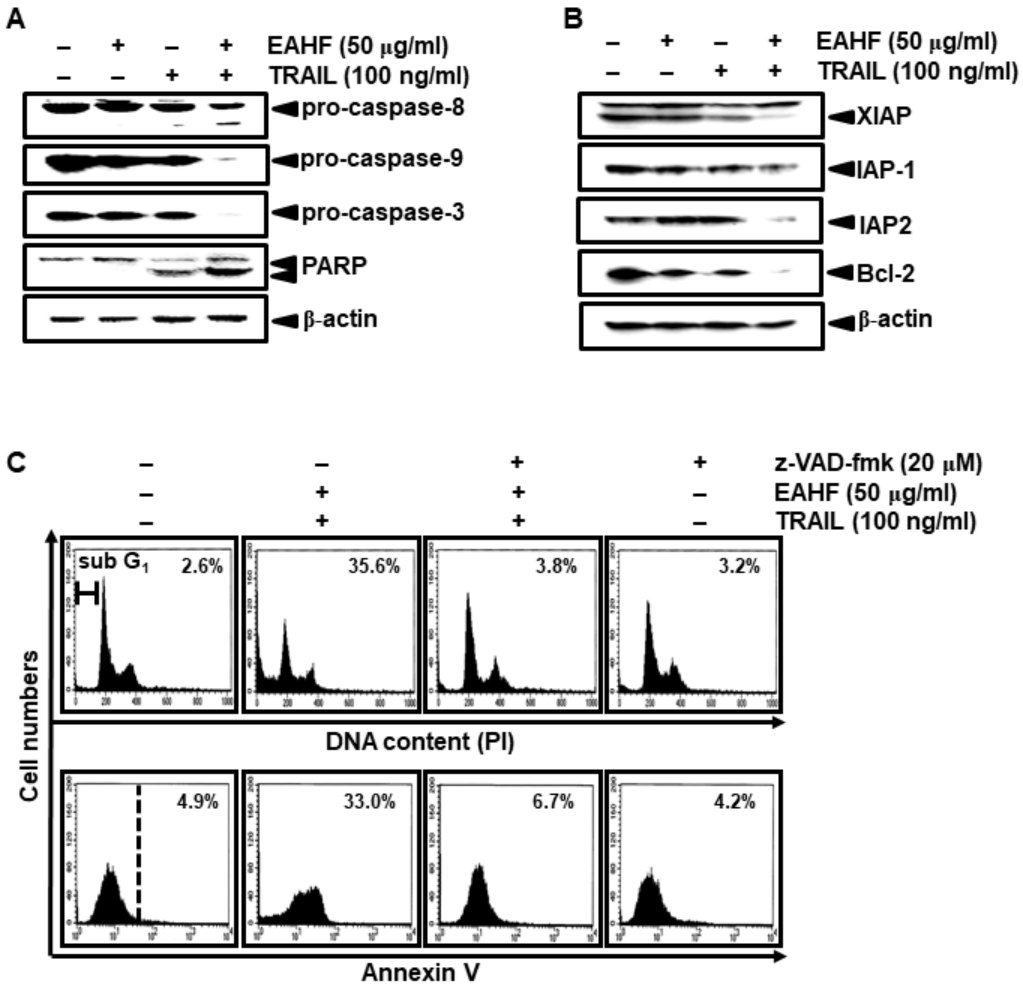

Figure 3. Synergistic induction of apoptosis by ethyl alcohol extract of *H. fusiforme* (EAHF) and TRAIL. Hep3B cells were pretreated with EAHF (0-50 μg/ml) for 1 h, followed by treatment with TRAIL (100 ng/ml) for 24 h. (A) Expression levels of procaspase forms and PARP cleavage were determined by western blot analysis. (B) The expression of anti-apoptotic proteins was detected by western blot analysis. (C) Hep3B cells were treated with EAHF and TRAIL in the presence or absence of z-VAD-fmk (20 μM). The percentage of sub-G_1 DNA content and annexin V^+-cell population are indicated in each panel.

3.4. EAHF enhances TRAIL-induced apoptosis by increasing ROS generation

Previous studies proved that EAHF induces ROS-dependent apoptosis in human leukemia cells [12], which confirmed using DCFDA staining in the current study. After treatment with EAHF, we found that levels of ROS generation significantly increased in a dose-dependent manner (Figure 4A). To examine whether ROS generation is directly associated with the combined treatment-induced apoptosis, Hep3B cells were treated with ROS inhibitors, NAC and GSH. Combined treatment significantly decreased cell viability; however, pretreatment with NAC and GSH inhibited the apoptotic activity and restored cell viability (Figure 4B). Furthermore, pretreatment with NAC and GSH significantly inhibited the combined treatment-induced increase in sub-G_1 phase population (Figure 4C). These results suggest that combined treatment with EAHF and TRAIL stimulates ROS generation, which consequently induces apoptosis.

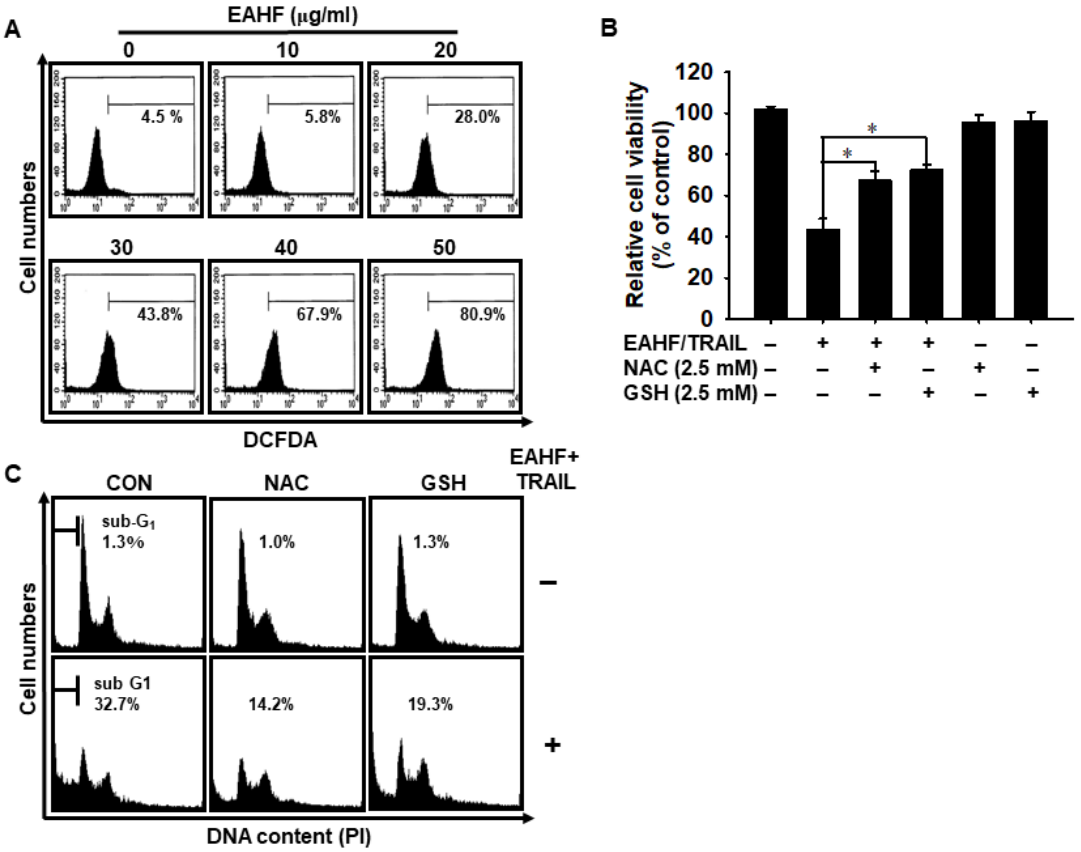

Figure 4. Effect of ROS on ethyl alcohol extract of *H. fusiforme* (EAHF) and TRAIL-induced apoptosis. Hep3B cells were pretreated with EAHF (0-50 μg/ml) for 1 h, followed by treatment with TRAIL (100 ng/ml) for 24 h. (A) The cells were stained with 20 μM DCFDA for 30 min and analyzed using a FACS. Hep3B cells were treated with EAHF and TRAIL in the presence or absence of NAC (2.5 mM) and GSH (2.5 mM). Cell viability was analyzed by MTT assay (B) and sub-G_1 population by PI staining (C). Statistical significance was determined by a one-way ANOVA test (*, $p < 0.05$ *vs.* combined treatment).

IV. DISCUSSION

The effectiveness of chemotherapeutic agents is limited by adverse effects, systemic toxicity, and drug resistance [1,2]. TRAIL is being investigated as therapeutic strategy against cancers since it selectively targets cancer cells to undergo apoptosis [11,12]. In particular, previous report demonstrated that TRAIL-induced apoptosis is principally triggered by the extrinsic and the intrinsic apoptotic pathway involving caspase cascade and apoptosis-regulating proteins [13]. However, recent research indicates that many cancers develop resistance against TRAIL by inhibiting caspase activity and upregulating the expression of anti-apoptotic proteins [14]. Therefore, new strategies are required to overcome TRAIL resistance in cancer cells. In the current study, EAHF sensitizes apoptosis TRAIL-resistant Hep3B cells by inducing caspase activity and ROS generation, as well as by stabilizing DR5 expression.

The increase and stabilization of DR5 expression prove to be a useful alternative to combat resistance against TRAIL-induced apoptosis in cancer cells [4]. Previous reports showed that ectopic overexpression of DR5 enhanced TRAIL sensitivity in TRAIL-resistant cancer cells [15,16]. Results from the current study demonstrated that EAHF stabilizes the expression of DR5 protein, but not that of its gene. It was hypothesized that EAHF-mediated DR5 stabilization enhances binding activity of TRAIL to DR5 in cancer cells. Finally, TRAIL binding to DR5 promotes DISC formation in the cytoplasmic domains of DR5 and triggers caspase-8, an initiator of the death signal [3]. Consequently, downstream molecule of caspase-8 induces depolarization of mitochondrial membrane potential and subsequently permit the release of ROS to the cytosolic compartments [17]. Alternatively, drug-induced oxidative stress overlapped with the intrinsic oxidative stress, resulting in a potent ROS-induced cell death response that primarily kills cancer cells or suppresses their proliferation [18]. EAHF appeared to induce apoptosis of human leukemia cell lines by inducing oxidative stress that leads to the production of ROS [19]. In the present study, caspase activation and ROS generation significantly increased after combined treatment with EAHF and TRAIL owing to binding between DR5 and TRAIL (Figure 5). ROS is perceived to be associated with carcinogenesis and metastasis, even though the correlation is often complex and

controversial [20]. Although ROS generation tends to be involved in the upregulation or stabilization of DR5 expression [21,22], the current study shows that EAHF only induces stabilization of DR5 protein, not its gene expression, by inducing ROS generation. Additionally, the ROS inhibitors (NAC and GSH) significantly suppressed the combined treatment-induced apoptosis, suggesting that ROS generation leads to the stabilization of DR5 and caspase cascade, and eventually enhances cell death. Taken together, the present study showed that EAHF enhances TRAIL-induced apoptosis, at least in part, via the caspase and ROS signaling pathways as well as by enhancing stability of DR5 protein.

V. CONCLUSION

In summary, EAHF triggers TRAIL-induced apoptosis by caspase activation and ROS generation as well as by DR5 stabilization. EAHF may enhance TRAIL efficacy in cancer treatment.

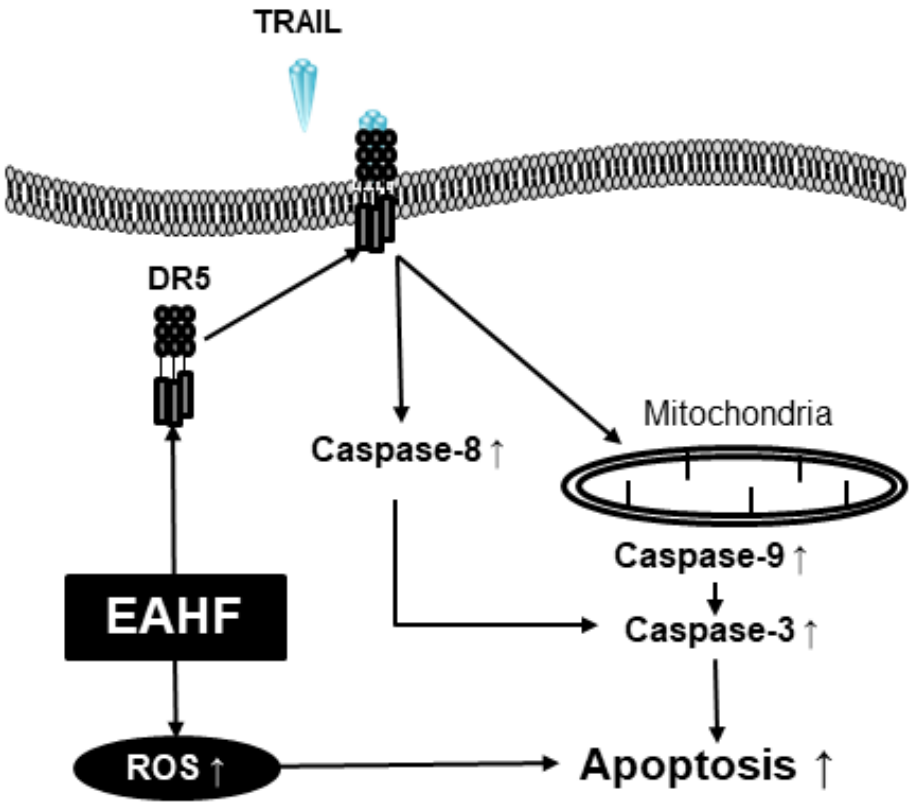

Figure 5. Scheme of TRAIL sensitization by EAHF. EAHF activates caspases, which induces mitochondrial depolarization and ROS generation, resulting to TRAIL-mediated apoptosis. In addition, EAFH promotes stabilization of DR5, leading to an increase of TRAIL-binding activity.

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Conflict of interests

The authors declare to have no conflict of interest at all.

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