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Trifolin induces apoptosis *via* extrinsic and intrinsic pathways in the NCI-H460 human non-small cell lung-cancer cell line

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ABSTRACT

Background: Trifolin (kaempferol-3-O-galactoside), which is a galactose-conjugated flavonol, exhibits antifungal and anticancer effects. However, the mechanisms underlying its anticancer activities have not yet been examined.
Purpose: In this study, the anticancer effects of trifolin were examined in human lung cancer cells.
Methods: Cytotoxicity was determined by evaluating cell viability. Apoptosis was analyzed through flow cytometry and western blotting analysis. Death receptors and inhibitors of apoptosis were evaluated through RT-PCR.
Results: Trifolin induced apoptosis in NCI-H460 human non-small cell lung cancer (NSCLC) cells by inhibiting the survival pathway and inducing the intrinsic and extrinsic apoptosis pathways. Trifolin decreased levels of Akt/p-Akt, whereas levels of expression of phosphatidylinositide 3-kinase (PI3K), cyclin D1, cyclin E, and cyclin A were not altered. Trifolin initiated cytochrome c release by inducing mitochondrial outer membrane permeabilization (MOMP). Trifolin increased Bcl-2-associated X protein (Bax) levels and decreased b-cell lymphoma 2 (Bcl-2) levels, while the levels of Bcl-xL were not altered. In addition, trifolin increased the levels of the death receptor involving the Fas/Fas ligand (FasL) and Fas-associated protein with the death domain (FADD), which consequently activated caspase-8, caspase-9, caspase-3, and the proteolytic cleavage of poly (ADP-ribose) polymerase (PARP).
Conclusion: These results suggested that trifolin induced apoptosis *via* death receptor-dependent and mitochondria-dependent pathways and that trifolin can be used as a therapeutic agent in human lung cancer.

Introduction

Lung cancer is the leading cause of cancer deaths worldwide. Because little is known about how to treat and prevent lung cancer, many studies of lung cancer are still required. Commonly, lung cancer cells have chromosomal abnormalities, such as defects of the p53 and pRb cancer suppressor genes, which result in the failure of the regulation of apoptotic signals and cell proliferation (Carbone and Robinson, 2003; Thafeni et al., 2012). Lung cancer is classified as a non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC) (Carbone and Robinson, 2003). SCLC commonly occurs in bronchial regions and results in malignant cancer. The 5-year survival rate of a patient with SCLC is under 21%, which is very low. NSCLC, which accounts for approximately 85% of all lung cancer cases (Herbst et al., 2008), includes adenocarcinoma, squamous cell carcinoma, and large cell carcinoma. Adenocarcinoma commonly occurs in pulmonary alveoli, while squamous cell carcinoma, which is caused by smoking, is mainly found in the large airway and bronchial regions of male patients (Cross, 2012). Large cell carcinoma occurs in any part of the lung, and its growth and metastasis is aggressive (Schuller, 1989; Yamamoto et al., 1987). In this study, large cell carcinoma NCI-H460 cells were used.

The development of alternative lung cancer agents is required. Plant extracts have begun to receive attention as therapeutic agents in cancer treatment due to their capacity to inhibit tumor growth, angiogenesis, and metastasis with minimal side effects. Plants contain several alkaloids, glycosides, and polyphenols, which are secondary metabolites that often found in

Abbreviations: NSCLC, non-small cell lung cancer; MOMP, mitochondrial outer membrane permeabilization; PARP, poly (ADP-ribose) polymerase; Bax, Bcl-2-associated X protein; FasL, Fas ligand; FADD, Fas-associated protein with the death domain.

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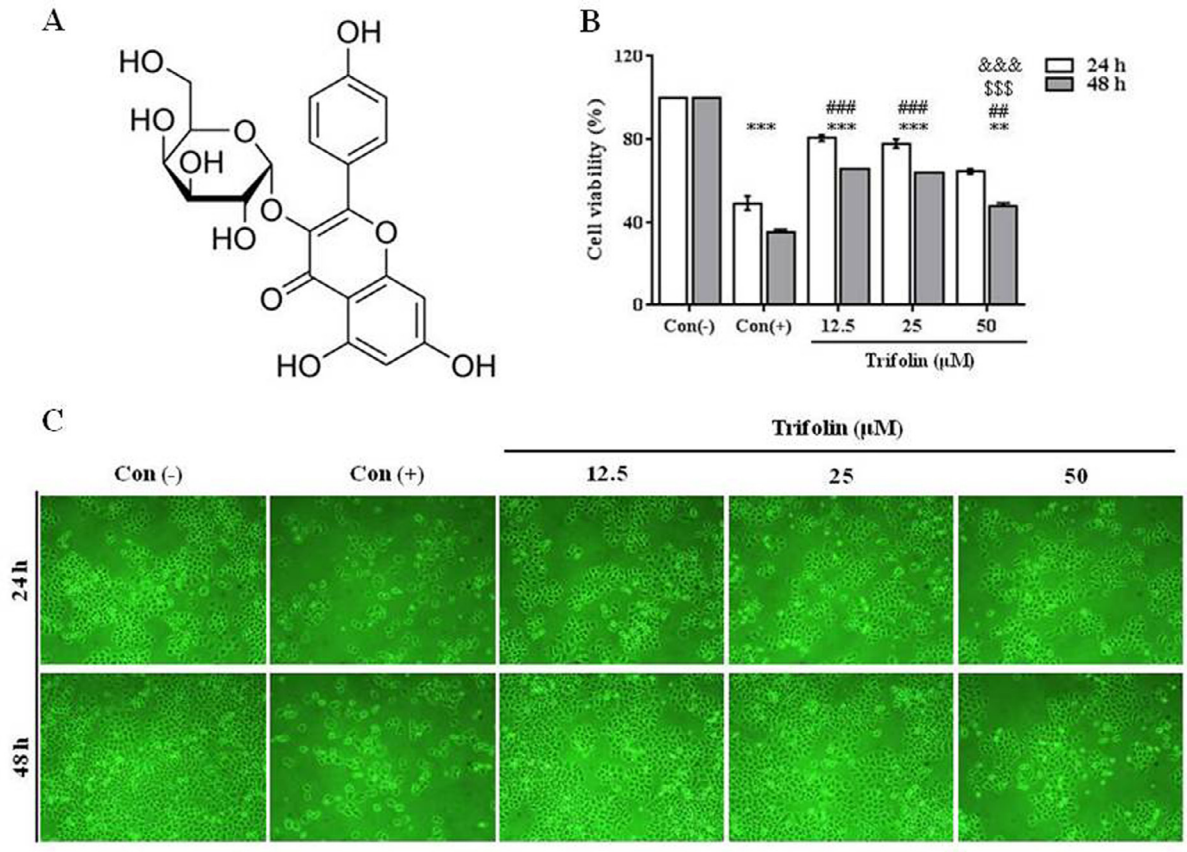

Fig. 1. Cytotoxic effects of trifolin on NCI-H460 cells. Kaempferol was used as a positive control at the concentration of 50 μM. (A) The chemical structure of trifolin, (B) Effects of trifolin on the viability of NCI-H460 cells. The NCI-H460 cells were incubated with various concentration of trifolin (12.5–50 μM) for 24 h and 48 h, and (C) Micrographs of NCI-H460 cells treated with the indicated concentrations of trifolin. The micrographs were taken with phase-contrast microscopy at a magnification of 100X. The data are presented as mean ± standard deviation (n = 3). *, #$p < 0.05$ and **, ##$p < 0.005$ versus control and positive control cells. &, \$$p < 0.05$ and &&, \$\$$p < 0.005$ versus 12.5 and 25 μM cells.

fruits, vegetables, and even phytogenic beverages (Galluzzo and Marino, 2006). Trifolin, which is also known as kaempferol-3-O-galactoside (Fig. 1A), is a flavonol, which is a type of flavonoid (Vierstra et al., 1982). Flavonoids are relatively abundant in the human diet and in promising anticancer agents (Havsteen, 2002; Middleton et al., 2000; Ren et al., 2003). Trifolin is a galactoside-conjugated kaempferol that results from conjugation by kaempferol 3-O-galactosyltransferase according to the following formula: UDP-galactose + kaempferol → UDP + kaempferol 3-O-beta-D-galactoside (Miller et al., 1999). Trifolin is found in many plants, such as *Diospyros blancoi* (Candolle, 1844), *Camptotheca acuminata* (Perdue, 1966), *Euphorbia condylocarpa* (Roshchin, 1977), and *Consolida oliveriana* (Arboretum, 1967). The bark and leaves of *Diospyros blancoi* are used for the treatment of itchy skin, while the bark is used for the treatment of coughs, fevers, dysentery and diarrhea (Ragasa et al., 2009). It has been reported the antifungal activity of *Camptotheca acuminata* (Li et al., 2005). In addition, the anticancer property of *Consolida oliveriana* against some human cell lines HL-60, U-937 and SK-MEL-1 also has been reported (Diaz et al., 2008). However, there is no report regarding on medicinal property of *Euphorbia condylocarpa.* Kaempferol, which is the mother compound, has been studied in various fields (Devi et al., 2015), and many studies have suggested that kaempferol reduces the risk of various cancers and that it has antibacterial, antifungal, and antioxidant abilities (Ackland et al., 2005; Calderon-Montano et al., 2011; Devi et al., 2015; Kataoka et al., 2001). In lung cancer cells, kaempferol upregulates the levels of the proapoptotic proteins b-cell lymphoma 2 (Bcl-2)-associated X protein (Bax) and Bcl-2-associated death protein (Bad), while it down-regulates the levels of the antiapoptotic proteins Bcl-2 and b-cell lymphoma-extra large (Bcl-xL). These changes result in an increase in apoptosis in the cancer cells (Kim and Choi, 2013). However, the effects of trifolin on cancer cells have not yet been studied. Only a few studies have reported that trifolin exhibits antifungal and antioxidant activities and that trifolin acetate can induce cell death in human leukemia cells (Torres et al., 2008). Therefore, in this study, we investigated the anticancer effects of trifolin in the NCI-H460 human NSCLC cell line.

Results and discussion

Growth inhibitory effects of trifolin in human NSCLC NCI-H460 cells

Plant extracts have the potential to become anticancer therapeutic agents because of their abilities to inhibit tumor growth, angiogenesis, and metastasis with few side effects (Cragg et al., 1997). Importantly, these effects are demonstrated by flavonoids, which are components of plant extracts. This study examined the effects of trifolin, which is a type of flavonoid that is conjugated to galactoside (Fig. 1A).

First, the viability of the trifolin-treated NCI-H460 cells was evaluated. NCI-H460 cells were treated with various concentrations of trifolin up to 50 μM to investigate the time- and dose-dependent effects of trifolin. The cell viability of the trifolin-treated NCI-H460 cells was analyzed with a MTS assay and compared to untreated control cells. The results revealed that trifolin decreased