Original Article

Anti-Proliferation and Apoptosis Induction in Epidermoid Carcinoma A431 Cells by *Terminalia bellirica* Extract

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Background: Squamous cell carcinoma (SCC) is a member of non-melanoma skin cancer (NMSC), which is slow-growing and high metastasis. UV exposed from the sun or tanning beds is one of the risk factors of SCC developing. Nowadays, SCC treatments include surgery, photodynamic therapy, radiation therapy and chemotherapy, but it is unsatisfactory. Therefore the finding of new therapeutic target is necessary.

Objective: To investigate the effect of *Terminalia bellirica* extract on anti-proliferation and apoptosis induction in highly metastasis epidermoid carcinoma A431 cells.

Materials and Methods: Cytotoxicity, cell viability and cell proliferation were determined by MTT assay. Nuclear morphological changes and protein expression were determined by Hoechst staining and Western blot analysis, respectively.

Results: *T. bellirica* extract showed anti-proliferative effect on A431 cells. Hoechst staining revealed chromatin condensation and apoptotic bodies induction in A431 treated cells by *T. bellirica* extract. In addition, apoptosis induction via up-regulartion of Bax, Bik, and Bok as well as down-regulation of Mcl-1 was obtained. Moreover, *T. bellirica* extract showed apoptosis induction through caspase-7 protein activation.

Conclusion: The present study showed that *T. bellirica* extract inhibited cell proliferation and induced apoptosis induction in A431 treated cells. The results indicated that *T. bellirica* extract may be further developed as skin cancer protection product. However the underlying mechanisms of apoptosis induction in A431 cells should be further studied.

Keywords: Skin cancer, *T. bellirica* extract, Apoptosis, Anti-proliferation

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Skin cancer is the uncontrolled growth of abnormal skin cells occurs when unrepaired DNA damage to skin cells from ultraviolet radiation from sunshine or tanning beds. Skin cancers are classified into two main groups, non-melanoma skin cancer (NMSC) and malignant melanoma (MM)[1]. The NMSC can be divided into two groups, basal cell carcinoma (BCC) and squamous cell carcinoma (SCC). A431 is an epidermoid carcinoma cell line that belongs in SCC group. This type of cancer was used as a SCC model for skin cancer study. It shows overexpression of epidermal growth factor receptor (EGFR), which possess high growth and survival rate.

T. bellirica, known as "Bahera" or Beleric or bastard myrobalan. Previous studies showed that *T. bellirica* exhibits many pharmacological properties comprising anti-diabetic[2], anti-oxidant[3], anti-bacterial[4,5], anti-HIV, anti-malarial, anti-fungal[6], and hepatoprotective property[7]. *T. bellirica* extract is a natural compound composes of various phytochemicals identified as tannins, phenols, belleric acid, bellericoside, thermilignan and ellagic acid[7-9]. In addition, 70% methanolic extract of fruits of *T. bellirica* induced apoptosis in lung and breast cancer through regulation of Bax/Bcl-2[10]. Previous study showed that *T. bellirica* fruit extract possessed anti-oxidant and anti-proliferative activity by different solvent fractions in various cancer cells[11]. Therefore, this study aims to investigate the effect of *T. bellirica* extract on apoptosis induction in SCC, A431 cells.

Apoptosis is a major type of cell death that regulated cell homeostasis in multicellular organism. It is an essential physiological process including development of embryo, tissue homeostasis and elimination of damaged cells[12]. Mediator proteins that involve in apoptosis induction are including Bcl-2 family (divide to two group, pro-apoptotic proteins and anti-apoptotic proteins) and caspases protein. The morphological changes in apoptotic cell are including the chromatin condensation and apoptotic bodies. These characteristic reveal that cell enter to final step of apoptosis

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process[13]. Aberrant of apoptosis leads to various diseases including cancer. Many studies showed that apoptosis induction is important process to eliminate cancer cells. Herein, the effect of *T. bellirica* extract on anti-proliferation and apoptosis induction in highly metastasis epidermoid carcinoma A431 cell was explored. The finding will be used as therapeutic target for NMSC.

Materials and Methods

Cell culture

Epidermoid carcinoma cell line A431 was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were maintained as a monolayer in DMEM medium (Invitrogen Life Science, USA) supplemented with 10% FBS (GE Healthcare, UK), 100 U/ml penicillin and 100 µg/ml streptomycin (PAA Laboratories, Pasching, Austria).

Plant extract and chemicals

The fruit extract of *T. bellirica* included 50% hydroglycol was obtained from Dr. Malin Chulasiri, S&J international enterprises public company limited (Thailand). The fresh fruit of *T. bellirica* were dried at 50°C and then ground. The powdered of *T. bellirica* was macerated in 50% hydroglycol for 3 day. The *T. bellirica* extract was filtered through Whatman filter paper No.1 and kept refrigerated until use. 3-(4,5-dimethylthaiazol-2-yl)-2,5-dipheny ltetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) was purchased from Merck Calbiochem (San Diego, CA, USA). Hoechst 33342 dye was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell proliferation and cell viability assays

Cytotoxicity of *T. bellirica* extract was measured by MTT assay. A431 cells were seeded in a 96-well plate at $1x10^4$ cells/well for 24 h. The cells were treated with *T. bellirica* extract at various concentrations, whereas the control group was treated with 50% hydroglycol for 24 h. After incubation, MTT solution was added to each well and incubated for 2 h at 37°C. DMSO was added to each well to solubilize purple formazan crystals. Then, the absorbance was measured by using a microplate reader at 570 nm (Epoch Microplate Spectrophotometer, BioTek Instruments, Inc., USA). The IC_{50} value was calculated using the GraphPad Prism 3.03 (GraphPad Software, Inc., San Diego, CA, USA).

Nuclear morphological staining

A431 cells were seeded in 6-well plate at $20x10^4$ cells/well for 24 h and treated with 2.4 and 4.8 µL/mL of *T. bellirica* extract for 24 h, 50% hydroglycol was used as a control. After incubation, 10 µM Hoechst 33342 was added to stain nucleic acid for 30 min at 37°C and examined under a fluorescence microscope (IX73; Olympus, Tokyo, Japan).

Western blot analysis

To confirm apoptosis induction via pro-apoptotic proteins and caspase activation, Western blot analysis was carried out. A431 cells were seeded in 6-well plate at $20x10^4$ cells/well for 24 h and treated with 2.4 µL/mL of *T. bellirica* extract for 24 h, 50% hydroglycol was used as a control. After incubation, cells were harvested and lysed with RIPA lysis buffer containing complete mini protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). Then, proteins were separated by 10% SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF) membranes (Merck Millipore Corp., Merck KGaA) for 1 h at 100 V with a Mini Trans-Blot Cell® (Bio-Rad). The membrane was blocked with 5% nonfat milk in TBST buffer (10 mM Tris, pH 7.5, 150 mM NaCl and 0.1% Tween 20), then incubated overnight with caspase-7, Bax, Bok, Bik, Mcl-1, and β-actin antibody (dilution 1: 1,000) at 4°C (Cell Signaling Technology, Beverly, MA.). After incubation, membranes were washed in TBST and incubated with appropriate secondary antibody conjugated with horseradish peroxidase (Cell Signaling Technology, Beverly, MA) for 1 h at room temperature. The specific protein bands were detected by chemiluminescent HRP substrate (Merck Millipore Corp., Merck KGaA).

Statistical analysis

All data presented were obtained from at least three independent experiments and were presented as mean $\pm$ standard deviation (SD). Statistical analysis was performed using SPSS statistical software package (version 20).

Results

T. bellirica extract inhibits cell viability in A431 skin cancer cells

The anti-proliferation activity of *T. bellirica* extract in A431 cells was determined by MTT assay. The IC_{50} value of *T. bellirica* extract was 0.93 ± 0.745 µL/mL, which inhibited cell viability in a dose-dependent manner. The concentration of 2, 4, 6, 8 µL/mL *T. bellirica* extract significantly reduced cell viability to less than 40% as compared to the control group (Figure 1).

T. bellirica extract induce nuclear morphological changes in A431 cells

The Hoechst 33342 staining showed that 2.4 and 4.8 µL/mL *T. bellirica* extract induced chromatin condensation and apoptotic bodies in A431 treated cells compared with the control group (Figure 2). Therefore, our results suggested that *T. bellirica* extract showed apoptosis induction through chromatin condensation and apoptotic bodies induction in A431 cells, characteristics of apoptotic cells.

T. bellirica extract induce apoptosis through Bcl-2 family proteins and caspase-7 activation

The Western blot analysis revealed that 2.4 µL/mL *T. bellirica* extract induced apoptosis through caspase activation in A431 treated cells. *T. bellirica* extract showed up-regulation of pro-apoptotic proteins including Bax, Bik and Bok that involved in promotion of apoptosis induction in the intrinsic apoptosis pathway. *T. bellirica* extract down-

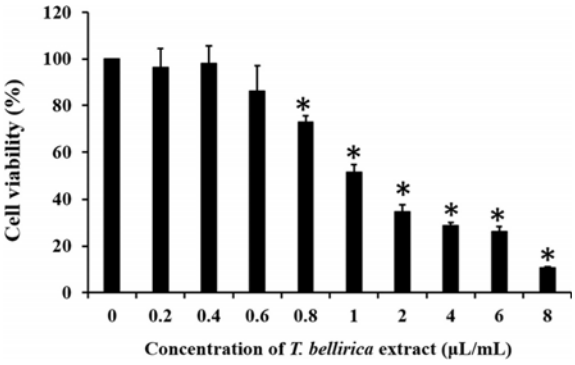

Figure 1. Effect of *T. bellirica* extract on cell viability. A431 cells were treated with different concentrations of *T. bellirica* extract at 24 h, IC_{50} 0.93±0.754 µL/mL. The results were expressed as mean ± SD, n = 3. *p<0.05 significantly compared with the control group.

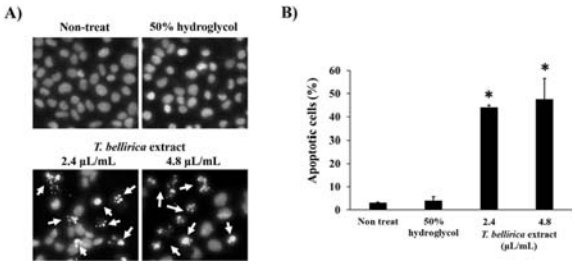

Figure 2. Effects of *T. bellirica* extract on apoptosis induction in A431 cells. Cells were treated with 2.4 and 4.8 µL/mL *T. bellirica* extract for 24 h. (A) Cells were stained with Hoechst 33342 and examined under a fluorescent microscope (magnification, x40) (White arrow = apoptotic cell). (B) Percentage of apoptotic cells of A431 treated with *T. bellirica* extract as compared with untreated cells. *p<0.05 significantly compared with the 50% hydroglycol group.

regulated anti-apoptotic protein Mcl-1, whereas it induced caspase-7 activation in A431 treated cells. Therefore, our study suggested that *T. bellirica* extract induced apoptosis induction through intrinsic apoptosis pathway by pro-apoptotic and caspase proteins activation.

Discussion

In this study, A431 cell line was used as a model for epidermoid carcinoma cell line. Recent research has demonstrated that *T. bellirica* extract showed cytotoxicity, anti-tumor and anti-oxidant properties against various human tumor cell lines. Ghate et al reported that *T. bellirica* extract exhibited strong cytotoxicity against human lung and breast carcinoma, A549 and MCF-7 cell, respectively[10]. These results showed that *T. bellirica* extract induce apoptosis via Bax/Bcl-2 ratio up-regulation, caspase-9 and caspase-3 activation as well as PARP inactivation but remained nontoxic to normal cells, human lung fibroblast cell line, WI-38[14]. Moreover, Basu et al found that *T. bellirica* extract showed anti-oxidant and anti-proliferative activity on various cancer cells including MCF-7, HeLa (cervical cancer), U87 (glioblastoma cell), A549 and HepG2 (hepatocellular cells)[11]. Moreover, octyl gallate and gallic acid isolated from methanolic fruit extract of *T. bellirica* extract showed inhibition of the survival of breast cancer cells, MCF-7 and MDA-MB-231 without any toxic effect on normal breast cells, MCF-10A. Both octyl gallate and gallic acid showed inhibition of cell cycle progression by regulation of cell cycle regulators including cyclin D1, D3, CDK4/6, p18, p21 and p27[14]. Since the mechanisms of *T. bellirica* extract induce apoptosis in skin cancer A431 cells have not yet been reported. Therefore, our study showed that *T. bellirica* extract inhibited cell growth and cell proliferation in a dose-dependent manner with an IC_{50} value of 0.93±0.745 µL/mL (Figure 1). After that we confirm apoptosis induction by investigation characteristic nuclear morphological changes in A431 treated cells. Hoechst staining revealed that *T. bellirica* extract induced condensed chromatin and apoptotic bodies in A431 cells as shown in Figure 2. Furthermore, we confirm that *T. bellirica* extract induce apoptosis in A431 cells by using Western blot analysis. The results showed that *T. bellirica* extract induced the increase of Bax, Bik and Bok proteins at 12 and 24 h after treatment with 2.4 µL/mL *T. bellirica* extract in A431 cell (Figure 3). *T. bellirica* extract also decreased Mcl-1 at 12 and 24 h (Figure 3). In addition, *T. bellirica* extract induced the expression of cleaved-caspase-7 (active form) expression in A431 treated cells (Figure 3). These data correlated with Ghate et al reported that *T. bellirica* extract induced nuclear fragmentation and apoptosis via caspase activation in MCF-7 and A549 cells[10]. In summary, the data suggested that *T. bellirica* extract induced apoptosis through Bcl-2 family proteins and caspase-7 activation in A431 cells. However, further studies on mechanism of apoptosis induction and signaling pathway were under investigation.

Conclusion

The present study demonstrated that *T. bellirica* extract possessed anti-proliferative and apoptosis induction in A431 cells determined by nuclear morphological changes including chromatin condensation and apoptotic bodies in A431 treated cells. In addition, *T. bellirica* extract induced apoptosis through pro-apoptotic proteins and caspase activation. The data suggested that *T. bellirica* extract induce apoptosis induction via intrinsic apoptosis pathway. Therefore, *T. bellirica* extract may be used as an anti-cancer agent to induce apoptosis in cancer cells. However, further studies on mechanism of apoptosis induction in another cell type of SCC and animal model need to be further explored.

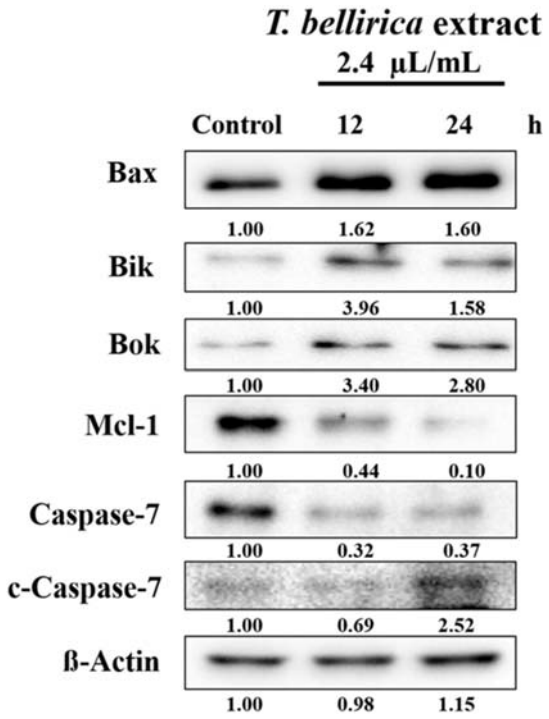

Figure 3. Effect of *T. bellirica* extract on Bcl-2 family proteins and caspase-7 expression. Cells were treated with 2.4 µL/mL *T. bellirica* extract for 12 and 24 h. *T. bellirica* extract induced the up-regulation of pro-apoptotic proteins, Bax, Bik, Bok. In contrast, *T. bellirica* extract showed down-regulation of anti-apoptotic protein, Mcl-1 in A431. In addition, *T. bellirica* extract-treated cells showed the increase cleaved caspase-7 expression. β-actin was used as an internal control. The intensity was calculated by Image J program.

What is already known on this topic?

T. bellirica extract contains effective bioactive compounds that have been reported to possess several activities including anti-diabetic, anti-oxidant, anti-bacterial, anti-HIV, anti-malarial and anti-fungal, anti-proliferative and apoptosis induction in several cancer cell lines.

What this study adds?

T. bellirica extract exhibited inhibition of cell proliferation and apoptosis induction in epidermoid carcinoma A431 cells through intrinsic apoptosis pathway.

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Potential conflicts of interest

The authors declare no conflicts of interest.

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