RESEARCH ARTICLE

Radical Intermediate Generation and Cell Cycle Arrest by an Aqueous Extract of *Thunbergia Laurifolia* Linn. in Human Breast Cancer Cells

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Abstract

***Thunbergia Laurifolia* Linn. (TL) is one of the most familiar plants in Thai traditional medicine that is used to treat various conditions, including cancer. However, the antitumor activity of TL or its constituents has never been reported at the molecular level to support the folklore claim. The present study was designed to investigate the antitumor effect of an aqueous extract of TL in human breast cancer cells and the possible mechanism(s) of action. An aqueous crude extract was prepared from dried leaves of TL. Folin-Ciocalteu colorimetric assays were used to determine the total phenolic content. Antiproliferative and cell cycle effects were evaluated in human breast adenocarcinoma MCF-7 cells by MTT reduction assay, cell growth inhibition, clonogenic cell survival, and flow cytometric analysis. Free radical generation by the extracts was detected using electron paramagnetic resonance spectroscopy. The exposure of human breast adenocarcinoma MCF-7 cells to a TL aqueous extract resulted in decreases in cell growth, clonogenic cell survival, and cell viability in a concentration-dependent manner with an IC_{50} value of 843 μg/ml. Treatments with extract for 24h at 250 μg/ml or higher induced cell cycle arrest as indicated by a significant increase of cell population in the G1 phase and a significant decrease in the S phase of the cell cycle. The capability of the aqueous extract to generate radical intermediates was observed at both high pH and near-neutral pH conditions. The findings suggest the antitumor bioactivities of TL against selected breast cancer cells may be due to induction of a G1 cell cycle arrest. Cytotoxicity and cell cycle perturbation that are associated with a high concentration of the extract could be in part explained by the total phenolic contents in the extract and the capacity to generate radical intermediates to modulate cellular proliferative signals.**

Keywords: *Thunbergia laurifolia* - tumor growth inhibition - cell cycle arrest - radical intermediates

Asian Pac J Cancer Prev, **16 (10)**, 4357-4361

Introduction

The efficacy of polyphenols for cancer treatment has emerged as alternative to pharmacological agents in recent years. *Thunbergia Laurifolia* Linn. (TL) is a medium sized climbing plant of the family *Acanthaceae* usually found in moist and gallery evergreen areas. It is commonly known as Babbler's Bill Leaf or Laurel Clock Vine or Rang Chuet in Thai. TL has long been described in Thai traditional medicine for its mitigation against various conditions such as fever, food poisoning, drug addiction, insecticide poisoning, infammation, and various chronic diseases including cancer. Dry powder capsules, herbal teas, and instant teas are available in the market for consumer convenience.

Most studies on TL have been conducted on phytochemical profiles and in animals to determine its physiological responses and pharmacological effects. Aqueous, ethanolic, and organic solvent-extracts of TL were demonstrated to exhibit powerful antioxidant activity *in vitro* and showed pharmacological effectiveness in various animals models (Hanchaipiboonkun, 2008; Palipoch et al., 2011; Wonkchalee et al., 2012; Thongsaard and Marsden, 2013; Boonyarikpunchai et al., 2014; Khobjai et al., 2014; Rojsanga et al., 2015). Methanolic extracts of TL were demonstrated to minimize the adverse effects of toxicants by regulating P-glycoprotein activity, CYP450, and lipid metabolism gene expression in HepG2 cells (Rocejanasaroj et al., 2014). Aqueous extracts of TL were shown to have antimutagenicity effects by inhibit the formation of micronuclei in rat polychromatic erythrocytes induced by *Pueraria mirifica* (Saenphet et al., 2005). However, the cytotoxic effects of TL and it's likely mechanism in modifying the phenotype of human cancer cells have never been reported.

In this study, an aqueous extract of TL was investigated for antiproliferative activity in human breast adenocarcinoma MCF-7 cells and its possible mechanisms

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of action were also studied.

Materials and Methods

Reagents and chemicals

MTT (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide), DMSO (dimethyl sulfoxide), gallic acid and Folin-Ciocalteu's phenol reagent were from Sigma (St. Louis, MO, USA). Tissue culture medium and supplements were from Invitrogen (Carlsbad, CA, USA).

Plant specimen collection and preparation of botanical extracts

Whole aerial parts of *Thunbergia Laurifolia* Linn. (TL) were collected from local area at Tambon Klong Muang, Pakchong, Nakhon Ratchasima province, northeastern region of Thailand, N 14o 40.074' E 101o 39.796' Altitude +345 m. Samples were air-dried in the shade to preserve all parts of the plants. Voucher specimens (no. 173174) were preserved at the Forest Herbarium (BKF), Department of National Parks, Wildlife and Plant, Bangkok, Thailand.

An aqueous crude extract was achieved as follows. The leaf parts were air dried at room temperature and crushed into a fine powder using a blender. The extract was prepared 1:10 (w/v) in boiling distilled water for 10 min and left at room temperature for 1h before being paper filtered. The extract was freeze-dried (ScanVac, Denmark) and kept at -20°C until use.

Total soluble phenolic contents determination

The total soluble phenolic content of the crude extract was quantified using Folin-Ciocalteu's phenol reagent and external calibration with gallic acid (Waterhause 2001). Briefly, 10 mg of crude extract was prepared in 10 ml water. The 20 μl aliquot of the mixture was thoroughly mixed with 1.58 ml of water and 100 μl of Folin-Ciocalteu reagent and allowed to incubate at room temperature for 5 minute. A sodium carbonate solution (20%) was added, mixed, and incubated for 2h at room temperature. The absorbance was spectrophotometrically determined at 756 nm (KB Biochrom ultrospec II). A standard calibration curve was prepared by measuring the absorbance of a series of standard gallic acid concentrations in water: 5, 10, 25, 50, and 100 mg/ml. The unit was expressed as g of gallic acid equivalents/g dry weight extract. The calibration equation for gallic acid was y=0.0091x+0.0077 (R^2=0.99987). HPLC analysis was performed with UV detection (Agilent, Santa Clara, CA, USA) at 315 nm.

Cells and culture conditions

Human breast adenocarcinoma MCF-7 cells (ATCC, Manassas, VA, USA) were cultured in Eagle's MEM supplemented with 10% fetal bovine serum, 1 mmol/l sodium pyruvate, and 0.1 mmol/l nonessential amino acids. Cells were routinely maintained at 37°C in a humidified atmosphere with 21% O_2 and 5% CO_2.

Cell viability and proliferative response

Antiproliferative effects of TL against cancer cells were determined *in vitro* using the MTT-based assay and clonogenic survival assay as previously described (Hematulin et al., 2012; Wang et al., 2014). Percentage of viable cells and cell survival fraction were calculated based on these two assays. The half maximal inhibitory concentration (IC_{50}) of the extract was analyzed using GraphPad Prism Software (La Jolla, CA, USA). For determination of cell growth, MCF-7 cells were seeded into 12-well plate 24 h before exposure to the aqueous TL extract. The number of cells in the presence or absence of the extract was counted at 2 day interval for 12 days. To do this, cells in each of three dishes for each concentration of extract were trypsinized with trypsin-EDTA, resuspended in isotonic solution, and then counted using Coulter Counter (Beckman, Brea, CA, USA).

Analysis of cell cycle progression

Cells were seeded 1x10^5 in 12-well plate 24h before fresh media was replaced. Different concentrations of TL extract were introduced to the cells for 24h. Culture media were collected in 15 ml tube and cells were washed once with PBS and then trypsinized to harvest. Cell pellets were collected by centrifugation at 3,000 rpm at 4°C and stained with cell cycle reagent containing propidium iodide (Kang et al., 2012). Flow cytometric analysis of cell cycle was conducted using BD FACSCalibur (Becton Dickinson, CA, USA), and results were analyzed using FlowJo software (TreeStar, Ashland, OR, USA).

EPR spectroscopy

To determine whether the extracts were able to generate radicals, EPR spectroscopy was used. This technique involved a change in pH of the samples and/or additional reagents to form an EPR detectable radical product. The intensity of the EPR signal corresponds to the amount of short-lived radicals detected. EPR spectra were recorded using a Bruker EMX spectrometer equipped with a high-sensitivity cavity. The spectra were obtained as an average of 4 scans with a modulation amplitude of 1 G; scan rate 80 G/81 s; receiver gain 2x10^5; microwave power 20 mW; and modulation frequency of 100 kHz. The EPR peak heights were presented in arbitrary units and were obtained at room temperature. The extract was prepared as 1 mg/ml in glass vial using water or 100 mM potassium phosphate buffer (KPB). All solutions above neutral pH (alkaline solutions) were prepared by adjusting with 5 M sodium hydroxide solution.

Statistical analysis

All data represent the mean±standard error of mean (SEM) from three independent experiments. The differences between means were analyzed using one-way ANOVA, followed by a post hoc Tukey test, or Student's t-test. $p<0.05$ was considered as significant.

Results and Discussion

Total soluble phenolic content of TL crude extract

Total soluble phenolic content of TL crude extracts was 28.68±1.06 g of gallic acid equivalents/g dry weight extract. The presence of high level of total phenolic content from TL extracts indicates a tendency to have

high antioxidant capacity as demonstrated in several spices and plant materials (Shan, 2005; Suwanchaikasem et al., 2013). The chromatogram of TL extract indicated seven distinct peaks at 11.58, 12.22, 12.64, 13.59, 15.14, 15.63, and 16.88 minutes after injection. The largest peak was observed at 15.63 minute (Figure 1) but the major constituents were not identified. Though, primary constituents of TL aqueous extract were reported to be caffeic acid and apigenin (Oonsivilai et al., 2007).

TL aqueous extracts affected the viability and clonogenic survival of MCF-7 cells

The viability of MCF-7 cells was demonstrated to be influenced by the aqueous crude extract in a concentration-dependent manner as shown in Figure 2A. The resulting inhibition of cell viability was found to significantly increase with increasing concentration of the aqueous extract at 500 μg/ml or higher relative to untreated control cells ($p < 0.01$). The IC_{50} value was determined to be 843 μg/ml. In the cancer cell lines, L929, BHK-21[C13], HepG2 and Caco-2, toxicity of TL aqueous crude extracts were observed at concentrations beyond 100 μg/ml but the extract preparation method was different (Oonsivilai et al., 2008). However, water extract capsule at dose 600 mg/day for 14 days on healthy volunteers were reported to be safe but elevated liver enzymes have to be cautious (Sittiprom et al., 2012).

For clonogenic survival, only cells exposed to 500 μg/ml aqueous extract showed a significant decrease ($p<0.05$) in survival fraction relative to untreated cells as shown in Figure 2B. In addition to reduced clonogenicity observed at 500 μg/ml TL extract treatment, a significant decrease in cell number, relative to untreated control cells, was observed during 12 days of culturing (Figure 2C). These results confirm the inhibitory effects in MCF-7 cells are dependent on concentration of the extract.

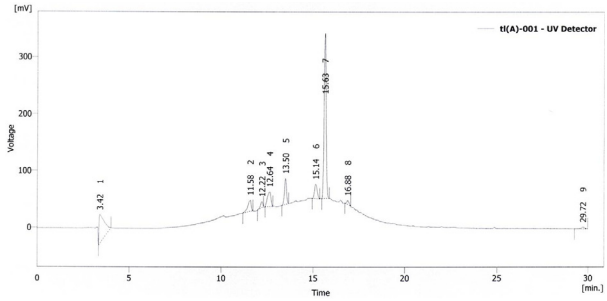

Figure 1. Chromatogram of TL Aqueous Crude Extract

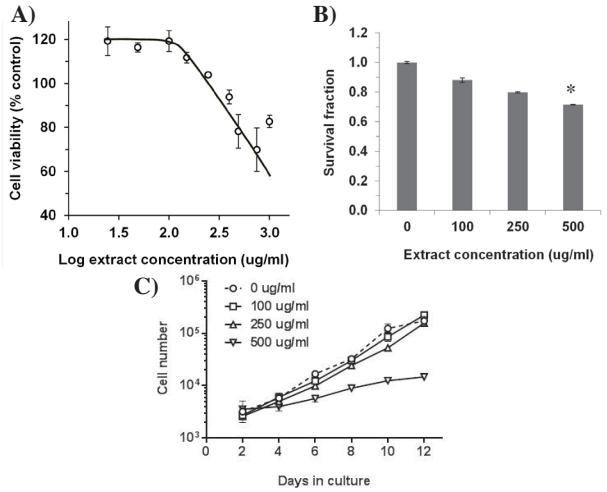

Figure 2. TL Aqueous Crude Extracts Inhibits Cells Viability, Clonogenicity, and Cell Growth of MCF-7 Cells in Concentration Dependent Manner. Cells were treated with aqueous extract at different concentrations for 24 h following by **(A)** MTT reduction assay. **(B)** The ability to form clones were examined after 14 days of extract treatment. **(C)** Growth curves of MCF-7 cells in the presence and absence of aqueous crude extract were plotted as cell number and time of treatment (day). All data represent the mean±standard error of mean (SEM) from three independent experiments. *p<0.05 relative to untreated control

Aqueous extract of TL induced G1 cell cycle arrest

To further analyze the effects of increasing TL extract concentration on MCF-7 cells, the cell cycle distribution was studied by measuring the DNA content of individual cells using flow cytometry. An increase in the G1 population was observed with increasing concentration of extract. This G1 delay was significant at TL concentrations of 250 μg/ml and higher ($p<0.05$). Concomitantly, a significant decrease of cell population in S phase was also observed with the increasing extract concentration of 250 μg/ml or higher ($p<0.05$). It should be noted that, no change in cell population of G2M phase was observed (Figure 3).

Aqueous extract of TL generated radical intermediates at near-neutral pH

An EPR spectrum from a free radical intermediate(s) was easily detected when the crude extract prepared in water was spiked to an alkali condition (pH 12) as shown in Figure 4A. We further determined the generation of this radical intermediate(s) when aqueous extracts were prepared in KPB at near-neutral pH (pH 7.4-7.8) in the presence or absence of the enzyme horse radish peroxidase (HRP) and hydrogen peroxide (H_2O_2). Initially, in the presence of HRP and H_2O_2, a one-electron oxidation product is formed. The organic components in the extracts should then be expected to be oxidized by HRP using H_2O_2 as the oxidizing agent, and then produce a characteristic change that is detectable by EPR spectroscopy. It was

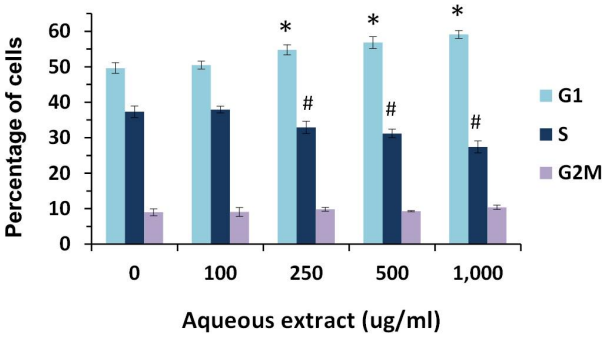

Figure 3. Aqueous Crude Extract of TL Induced G1 Cell Cycle Arrest in MCF-7 Cells after 24h Exposure. All data represent the mean±standard error of mean (SEM) from at least three independent experiments; *, #p<0.05 relative to untreated cell population in G1 and S phase, respectively

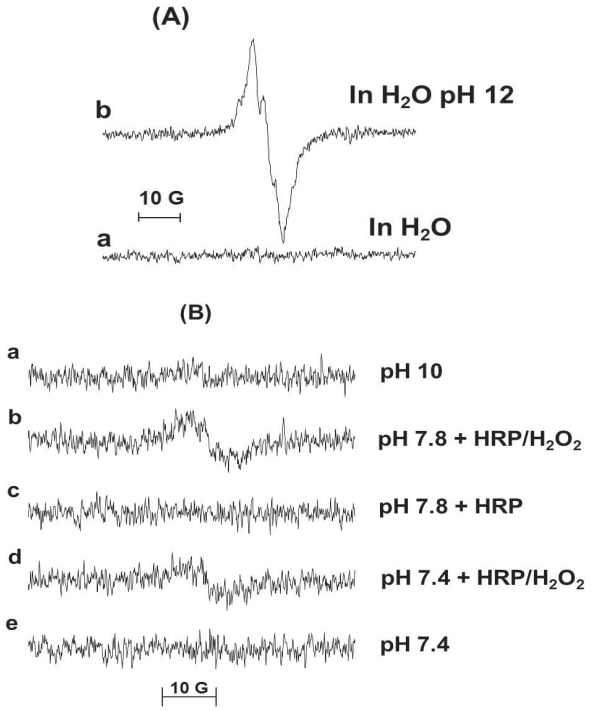

Figure 4. Radical Intermediates Generation Capability of TL. (A) Representative EPR spectra of radical intermediates generated from TL aqueous crude extracts in water (a) and in water at pH 12 (b) measured at room temperature. **(B)** TL aqueous crude extract generated radical intermediates at near-neutral pH. Representative EPR spectra of the extract were prepared in potassium phosphate buffer (a) pH 10 (b) pH 7.8+50 milliunits of HRP/ml+100 uM H_2O_2, (c) pH 7.8+50 milliunits of HRP/ml (d) pH 7.4+50 milliunits of HRP/ml+100 uM H_2O_2 and (e) pH 7.4

found that no complex spectrum was observed when aqueous extracts of TL were only prepared in KPB pH 7.4 or, KPB pH 7.8 without 50 milliunits of HRP and 100 uM H_2O_2, or in KPB pH 10 as shown in Figure 4B (a, c, e).

In contrast, radical intermediate spectra were detected when the extracts were prepared in KPB both at pH 7.4 and 7.8 in the presence of 50 milliunits of HRP/ml and 100 uM H_2O_2, Figure 4B (b and d). However, the types of organic radicals generated were not identified and the exact major component of TL extracts responsible for this radical generation is still unclear but the results suggested that the radical generation could be pH-dependent. This interesting observation needs to be further clarified.

Though, several plant-derived phenolic compounds have been reported to contain free radical-scavenging activity and show protection from intracellular reactive oxygen species. It should be noted that H_2O_2 and peroxidase enzymes such as glutathione peroxidase are always present in cells. The detection of the unidentified complex spectra that was produced from the extract in the presence of H_2O_2 and HRP at near-neutral pH *in vitro* would cause one to question reliability of any ROS measurements in the cells treated with TL extracts. Here, we have demonstrated that an aqueous crude extract of TL is able to produce a free radical intermediate(s) at near-neutral pH, therefore the experiment designed to investigate whether TL can modulate the steady-state levels of ROS, such as superoxide and H_2O_2, in breast cancer cells should be treated with caution since the MCF-7 cells were cultured and maintained in medium at pH 7.4.

Conclusions

In recent years, diverse extracts from plant derived materials were demonstrated to affect cell viability and relevant cell functions through various mechanisms in different cancer cell types (Calderon-Montano et al., 2013; Takashima et al., 2013; Jung, 2014; Wang et al., 2014). Our present study demonstrated for the first time the inhibitory and potential antitumor effects of TL on MCF-7 breast cancer cells using multiple cellular and molecular approaches. The decrease in cell viability, cell growth, and clonogenic cell survival appeared to be strongly correlated with cell cycle perturbations when high concentrations of TL crude extracts were applied to MCF-7 breast cancer cells. The antiproliferative results of TL observed in an *in vitro* system suggested a biologically relevant effect of high amounts of total phenolic contents present in the extracts. To provide additional evidence for a possible antitumor mechanism, we demonstrated that the aqueous crude extracts of TL were able to produce free radical intermediates at both high pH and near-neutral pH conditions as determined by EPR spectroscopy. These results were consistent with many reports when various kinds of polyphenolics from plant-derived materials were shown to produce phenoxyl radicals or other reactive oxygen species and induce cytotoxicity (Galati et al., 2002; Tsuji and Walle, 2008). In another study, a phenolic compound, gallic acid, was found to produce different free radicals in the pH range 7-10 as detected by EPR (Eslami et al., 2010). These finding suggest to us that the oxidation of phenolic component of TL extract at near-neutral pH might produce radical intermediates that could partially influence the cellular redox environment and subsequently affects the viability and proliferation process of the cancer cells.

Acknowledgements

This work was supported by Thailand Research Fund (TRF) and Thailand Institute of Nuclear Technology (Public Organization), Thailand, DBG5380048. The authors gratefully thank Prof. Garry R. Buettner for access to the EPR Core Facility at The University of Iowa and valuable discussions; Mr. Brett Wagner for EPR technical guidance; Department of Pathology, Carver College of Medicine, University of Iowa for flow cytometer facility access; and Mrs. Wiranee Sriwieng for HPLC technical assistance.

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