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Original Article

In vitro biological activities of *Thunbergia laurifolia* stem and leaf with reference to rosmarinic acid

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Abstract

Thunbergia laurifolia Lindl. (Thunbergiaceae family) was shown to be a natural antioxidant source to cope with oxidative stress. By DPPH and FRAP antioxidant assays, this study found that rosmarinic acid exhibited the highest antioxidant activity followed by leaf and stem ethanolic extracts of *T. laurifolia*. By intracellular anti-oxidation assay against H_2O_2-induced ROS in EA.hy926 endothelial cells using 2′,7′-dichlorodihydrofluorescein diacetate as a probe, all tested samples exhibited marginal protection, i.e. *T. laurifolia* leaf extract (77.31±0.66%), rosmarinic acid (88.10±6.105%), and *T. laurifolia* stem extract (91.30±0.86%), compared to the control (100%). Cytotoxicity against five cancer and two normal cell lines were evaluated by MTT cell viability assay. Rosmarinic acid and the leaf extract inhibited cell viability selectively in each cell line, whereas the stem extract revealed no cytotoxicity (IC_{50}≥100 µg/mL) in any of the cell lines

Keywords: *Thunbergia laurifolia*, rosmarinic acid, reactive oxygen species, antioxidant activity

1. Introduction

Reactive oxygen species (ROS), or simply free radicals, are molecules that contain reactive oxygen which can be found in all aerobic organisms. In a normal physiological condition, these molecules are endogenously generated at low concentrations to function in cellular signaling (Zhang *et al.*, 2016). In contrast, a high concentration of ROS, as a result of an imbalance between ROS generation and degradation, is defined as oxidative stress. Oxidative stress is a serious pathological causative factor for numerous diseases due to the high reactivity to proteins, nucleic acids, and lipids. Therefore, a prolonged period of cell exposure to high concentrations of ROS leads to diseases becoming more severe and disorders are generated. Oxidative stress with high levels of ROS can commonly present as vascular inflammation which can lead to complications of vascular disorders and diseases such as atherosclerosis, ischemic heart disease, cerebrovascular disease, and diabetes mellitus (Siti, Kamisah, & Kamsiah, 2015).

Plants containing polyphenols are considered to be natural sources of antioxidants to prevent oxidative stress (Ghasemzadeh & Ghasemzadeh, 2011). *Thunbergia laurifolia* Lindl. (Family Thunbergiaceae) is a woody climber plant which is commonly found in Asia. The plant is called “Rang Jued” in Thai and has been used in traditional Thai medicine for hundreds of years. The fresh or dried crude drug of the leaves and bark can be individually prescribed to treat inflammation, fever, and poisoning (Thongsaard & Marsden, 2002). There are numerous scientific reports on the leaf of the *Thunbergia laurifolia* that focused on anti-oxidation (Suwan chaikasem, Chaichantipyuth, & Sukrong, 2014), antiproliferation (Jetawattana, Boonsirichai, Charoen, & Martin, 2015), antidiabetes (Kosai, Jiraungkoorskul, & Jiraungkoor skul, 2015), detoxifying effect (Chattaviriya, Morkmek, Lertprasertsuke, & Ruangyuttikarn, 2010), anti-nociceptive,

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and anti-inflammatory effects (Boonyarikpun chai, Sukrong, & Towiwat, 2014).

Despite the medicinal properties of *Thunbergia laurifolia* stem, there is still a lack of scientific reports on its biological activity. However, a phytochemical study of the aerial part of *Thunbergia laurifolia* indicated that some flavonoids and many antioxidant compounds were found as the constituents in the plant (Kanchanapoom, Kasai, & Yama saki, 2002). Rosmarinic acid, a phenolic ester that consists of caffeic acid and 3,4-dihydroxyphenylacetic acid, is one of the main phytochemical compounds in *Thunbergia laurifolia* (Suwanchaikasem *et al.*, 2014). This natural compound was reported to have remarkable biological and pharmacological properties in many scientific studies (Amoah, Sandjo, Kratz, & Biavatti, 2016).

In this work, the leaf and stem of *Thunbergia laurifolia* and its phytochemical constituent, rosmarinic acid, were investigated for the free radical scavenging activity, reducing power, and intracellular anti-oxidation. Additionally, the cytotoxic effects against five cancer and two normal cell lines were also examined.

2. Materials and Methods

2.1 Chemicals and reagents

Rosmarinic acid, 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), RpMI-1640 medium, and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (MO, USA). Dulbecco's Modified Eagle's medium (DMEM), fetal bovine serum (FBS) and penicillin-streptomycin solution were purchased from GIBCO (New Zealand). All chemicals and reagents were of analytical grade.

2.2 Plant collection and preparation of extracts

T. laurifolia stems and leaves were collected in Thailand and authenticated by Associate Professor Dr. Nijsiri Ruangrungsi. Plant voucher specimens were deposited at the College of Public Health Sciences, Chulalongkorn University, Thailand. Dried plant materials were pulverized and then extracted with ethanol in a Soxhlet apparatus to obtain exhausted ethanolic extracts. The obtained residues were evaporated *in vacuo* and stored at 4 °C until further use.

2.3 DPPH radical scavenging assay

Fifty microliters of different concentrations of extracts, rosmarinic acid, and positive controls (ascorbic acid and butylated hydroxytoluene [BHT]) in ethanol were added to 150 µL of 120 µM diphenylpicrylhydrazyl (DPPH) ethanolic solution. A 96-well plate containing the mixtures was incubated in the dark at room temperature for 30 min and the absorbance was measured at 517 nm. The test was done in triplicate. The inhibition percentage was evaluated following this formula:

$$\% \text{ Inhibition} = \frac{(\text{Absorbance of the control} - \text{Absorbance of the sample})}{\text{Absorbance of the control}} \times 100$$

2.4 Ferric reducing antioxidant power (FRAP) assay

The FRAP reagent solution was prepared in the ratio of 10:1:1 including 300 mM acetate buffer (pH 3.6), 10 mM 2,4,6-tripyridyl-s-trlazine solution, and 20 mM ferric chloride, respectively. Twenty-five microliters of the extracts, rosmarinic acid, and positive controls (ascorbic acid and BHT) in ethanol were added to 175 µL of FRAP reagent solution. The 96-well plate containing the mixtures was incubated at room temperature for 30 min and the absorbance was measured at 593 nm. The test was done in triplicate. A ferrous sulfate calibration curve was established to evaluate the reducing antioxidant power expressed in mM of ferrous iron per milligram of the samples.

2.5 Intracellular antioxidative assay

EA.hy926 human umbilical vein endothelial cells from American Type Culture Collection (ATCC) were grown in DMEM media supplemented with 10% FBS and 1% penicillin-streptomycin and grown in an incubator with a humidified atmosphere containing 5% CO_2 at 37 °C. A subculture of confluent cells was done using 0.25% trypsin in sterile phosphate buffered saline (PBS) solution. At first, cell viability under various concentrations of rosmarinic acid, hydrogen peroxide (H_2O_2) as well as *T. laurifolia* stem and leaf extracts were assayed.

MTT cell viability assay was slightly modified from a previous study to evaluate the cytotoxic effect according to the previous study with a slight modification (Carmichael, DeGraff, Gazdar, Minna, & Mitchell, 1987). Briefly, the cells were seeded into a 96-well plate ($1x10^5$ cells/mL) at 37 °C for 24 h, thereupon, the cells were incubated with different concentrations of *T. laurifolia* (stem, leaf) extracts, rosmarinic acid, and H_2O_2 for 24 h. After removing the medium, MTT solution (0.4 mg/mL) was added and incubated for 4 h. Thereafter, the MTT solution was removed and replaced by 100% DMSO. The resulting formazan was measured at 570 nm for half maximal inhibition concentration (IC_{50}). The next step was an anti-oxidation assay against H_2O_2-induced intracellular ROS.

The assay was slightly modified from a previous study to quantitate the amount of ROS using DCFH-DA which could diffuse into the cells and then enzymatically hydrolyzed by cytoplasmic esterase to produce dichlorodi-hydrofluorescein as a non-fluorescent and it could rapidly oxidize to a fluorescent dichlorofluorescein (DCF) by intra-cellular ROS (Mutsuko, Rintaro, Jae-Hak, Kaeko, & Junji, 2002). Briefly, following the cell treatments, the cells were washed twice with cold-PBS solution and incubated with 5 µM of DCFH-DA for 30 min. The solution of DCFH-DA was removed and the cells were then washed twice with cold-PBS solution and incubated with 0.05 mg/mL of H_2O_2 for 30 min. The absorbance was measured with excitation at 485 nm and emission at 535 nm. The results were expressed as percentage of ROS which is in direct proportion to DCF fluorescence.

2.6 Cytotoxic assay against cancer cell lines

WI-38 (human lung fibroblast), BT-474 (human breast ductal carcinoma), ChoGo-K-1 (human bronchogenic carcinoma), HepG2 (human hepatocellular carcinoma),

KATO III (human gastric carcinoma), and SW620 (human colorectal adenocarcinoma) were purchased from ATCC. All cell lines were grown in RpMI-1640 media, supplemented with serum growth factor, and antibiotic as mentioned in the previous assay. All cell lines were grown to obtain a density of $1x10^5$ cells/mL in a 5% CO_2 incubator at 37 °C. The ethanolic extracts of *T. laurifolia* (leaf and stem), rosmarinic acid, and doxorubicin (positive control) were subjected to cytotoxic determination using the MTT cell viability assay as previously described.

3. Results and Discussion

Oxidative stress is a deleterious key that contributes vascular dysfunction, which has been found to be the causative factor for several fatal diseases (Gracia, Llanas-Cornejo, & Husi, 2017). It was found that antioxidants obtained from natural sources are potential compounds to cope with oxidative stress (Lobo, Patil, Phatak, & Chandra, 2010). *T. laurifolia* is a herb that is used to treat inflammation, fever, and poisoning. In a previous study, rosmarinic acid, a polyphenolic phytoconstituent in *T. laurifolia*, was proven to be a potential natural antioxidant (Suwanchaikasem *et al.*, 2014). Furthermore, rosmarinic acid which was isolated from the ethanolic extract of *T. laurifolia* leaf showed anti-inflammatory and antinociceptive properties in mice (Boonya rikpunchai, Sukrong, & Towiwat, 2014). The findings of this study demonstrated the bioactive potentials of the ethanolic extracts of *T. laurifolia* leaf and stem in regard to rosmarinic acid.

3.1 Effects of *T. laurifolia* extracts and rosmarinic acid on the *in vitro* antioxidant activities

The yields of ethanolic extracts of *T. laurifolia* leaf and stem were found to be 15.2 and 9.0 g/100 g, respectively. The results of all test samples for *in vitro* antioxidant activities are shown in Table 1. The DPPH scavenging activity of all samples exhibited a dose-response relationship (Figure 1). *T. laurifolia* leaf and stem extracts showed IC_{50} values of 151 and 195 µg/mL, respectively. However, the IC_{50} value of rosmarinic acid showed better activity than ascorbic acid (52 µg/mL vs. 118.29 µg/mL). Reducing power of the antioxidant was expressed as the amount of ferrous sulfate ion obtained by FRAP assay. *T. laurifolia* leaf and stem extracts showed reducing power ability with FRAP values of 0.26 and 0.18 mM Fe(II)/mg extract, respectively. Rosmarinic acid exhibited a similar value as ascorbic acid and BHT (positive controls) with FRAP values of 0.46, 0.42, and 0.55 mM Fe(II)/mg extract, respectively. A previous report indicated that an aqueous extract of microwave-dried leaves of *T. laurifolia* exhibited the highest scavenging property comparing to fresh leaves and commercial *T. laurifolia* leaves tea with IC_{50} values of 0.50, 0.99, and 0.64 mg/mL, respectively (Chan & Lim, 2006). The use of different solvents (water, ethanol, and acetone) to extract *T. laurifolia* leaves also indicated that all of the leaf extracts possessed antioxidant activity. (Ratchadaporn Oonsivilai, M. G. Ferruzzi, & S. Ningsanond, 2008). These studies concluded that the plant had antioxidant potency.

Table 1. *In vitro* antioxidant activities of test samples.

In Vitro models	*T. laurifolia* leaf extract	*T. laurifolia* stem extract	Rosmarinic acid	Ascorbic acid	BHT
DPPH IC_{50} (µg/mL)	151.47	195.12	52.11	118.29	-
FRAP mM Fe(II)/mg extract	0.26	0.18	0.46	0.42	0.55

BHT=butylated hydroxytoluene, DPPH=diphenylpicrylhydrazyl, FRAP=ferric reducing antioxidant power.

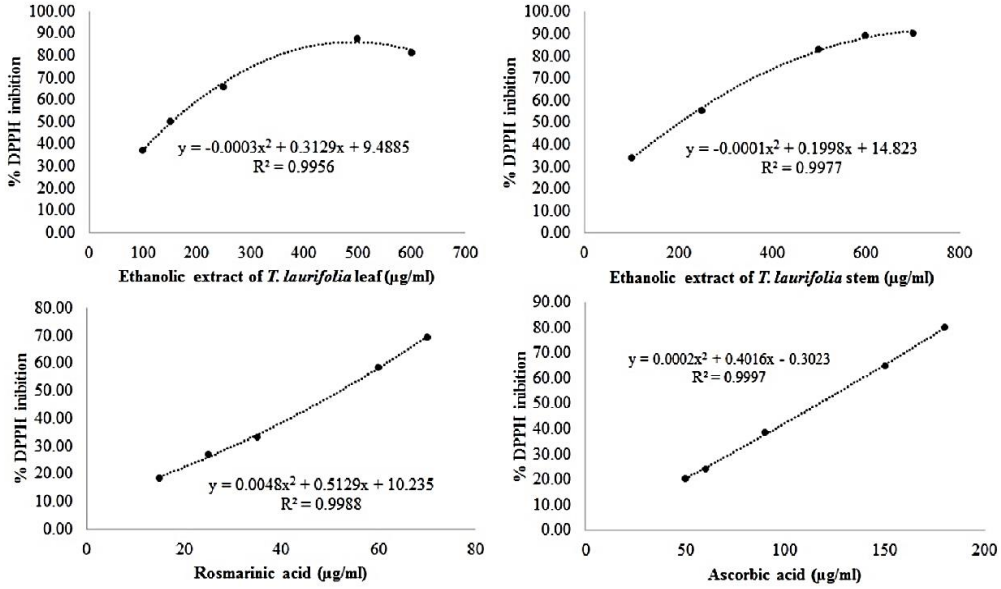

Figure 1. DPPH scavenging activity of test compounds.

3.2 Effects of *T. laurifolia* extracts and rosmarinic acid on intracellular ROS anti-oxidation

EA.hy926 cells were used as the study model to investigate intracellular ROS inhibition. This immortalized cell line is one of the most commonly used endothelial cell lines because it best characterizes the human vascular system, especially the large vessel endothelium (Bouis, Hospers, Meijer, Molema, & Mulder, 2001). Cell viability was tested prior to investigating ROS inhibition. It was shown that *T. laurifolia* leaf and stem extracts, rosmarinic acid, and H_2O_2 inhibited cell viability with IC_{50} values of 0.48, >1, 0.23, and 0.05 mg/mL for EA.hy926 cells, respectively, after 24-h treatment (Figure 2). Then the IC_{50} of H_2O_2 (0.05 mg/mL) was used to induce ROS production in EA.hy926 cells. The results are expressed in percentage of ROS. At 1 mg/mL, all test samples exhibited marginal intracellular ROS anti-oxidation. *T. laurifolia* leaf exhibited 77.31±0.66% of intracellular ROS production in H_2O_2-induced oxidative stress in the cells, followed by rosmarinic acid (88.10±6.10%) and the stem extract (91.30±0.86%) (Figure 3). Another study on intracellular ROS inhibition in HepG2 cells using the DCFH-DA assay indicated that cells preincubated with 80% ethanol extract of *T. laurifolia* leaf at 800 µg/mL for 29 h significantly decreased oxidative stress by approximately 60% of cellular oxidative stress compared to the control (Rocejanasaroj, Tencomnao, & Sangkitikomol, 2014). Similar work in different cell lines using human umbilical vein endothelial cells pretreated with rosmarinic acid for 12 h at 25–200 µM reduced intracellular ROS in a dose-dependent manner by 31–59% (Huang & Zheng, 2006). EA.hy926 cells might not be suitable cell lines to test rosmarinic acid and the extracts containing rosmarinic acid according to its cytotoxicity on this cell line.

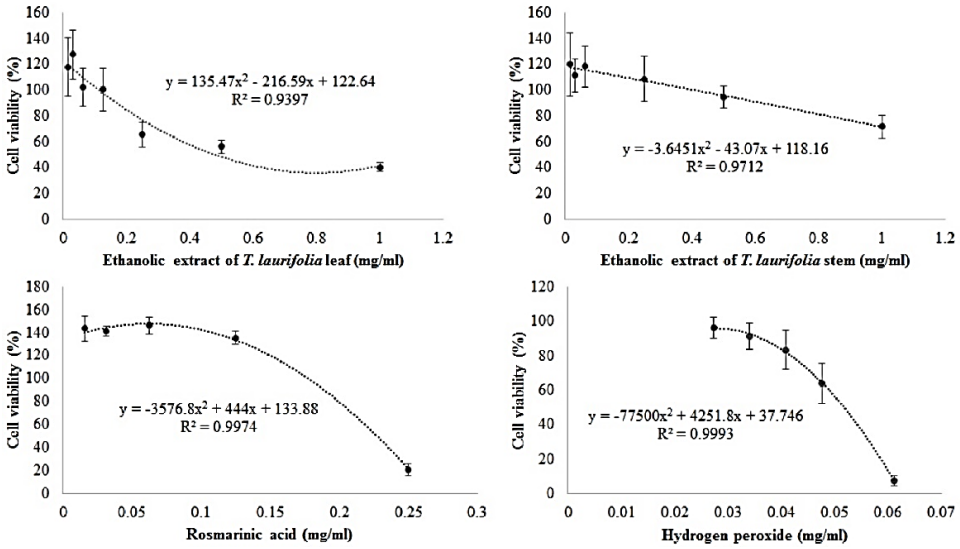

Figure 2. Effects of *T. laurifolia* extracts, rosmarinic acid, and H_2O_2 on cell viability of EA.hy926 cells determined by the MTT assay.

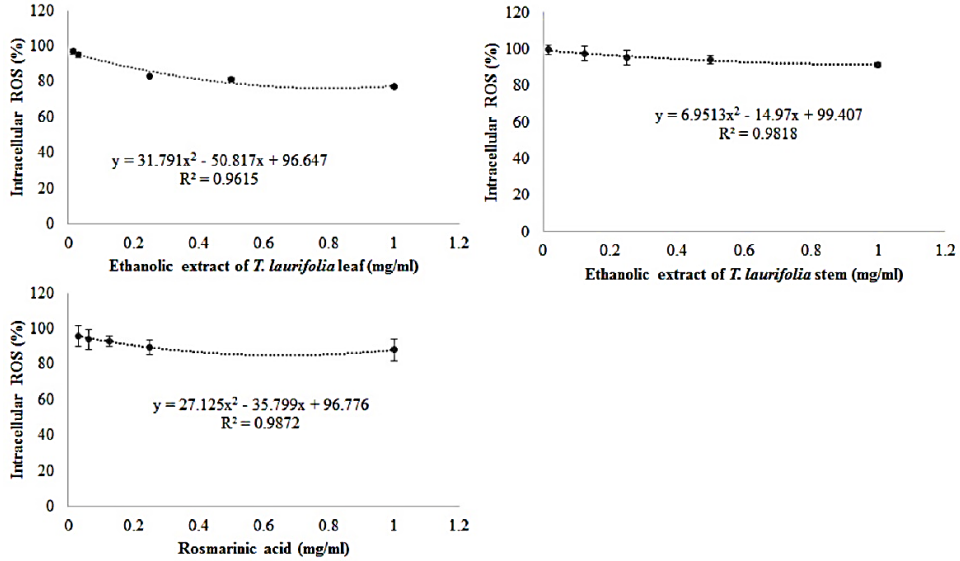

Figure 3. Effects of *T. laurifolia* extracts and rosmarinic acid on the level of intracellular ROS in EA.hy926 cells using H_2O_2 as the ROS inducer.

3.3 Cytotoxic effects of *T. laurifolia* extracts and rosmarinic acid on cancer cell lines

Cytotoxicity was determined using the MTT cell viability assay. The results are expressed as the IC_{50} on cell viability after the cells were exposed to the test samples. Cytotoxic potential against all five cancer cell lines as well as one normal cell line were shown mostly in the *T. laurifolia* leaf extract. According to the National Cancer Institute, the criteria for cytotoxicity determination in plant extract and pure compounds are that the plant extract must have a IC_{50} value lower than 20 µg/mL and the pure compound must have a IC_{50} value lower than 4 µg/mL (Geran, Greenberg, MacDonald, Schumacher, & Abbott, 1972). Therefore, all test samples in this study were not effective against cancer cell lines (Table 2). Another report of four cancer cell lines tested with water, ethanolic, and petroleum ether extracts of *T. laurifolia* leaf exhibited IC_{50} values >100 µg/m_L against all of the test cell lines indicating low cytotoxicity (Ratchadaporn Oonsivilai, Mario G. Ferruzzi, & Suwayd Ningsanond, 2008). A similar study revealed that rosmarinic acid decreased cell viability of HepG2 cell lines as its concentration increased in a time-dependent manner with a IC_{50} value of 33±0.74 µg/mL after 48 h of incubation (Ma *et al.*, 2018). In this study, it was found that *T. laurifolia* ethanolic leaf extract was more toxic against ChoGo-K-1 than other cell lines (IC_{50} 29.92 µg/mL). From this study, the cytotoxic potential of *T. laurifolia* leaf against cancer cell lines as well as normal cell lines was exhibited. The toxicity of *T. laurifolia* leaf should be investigated further.

4. Conclusions

T. laurifolia leaf and stem extracts as well as its phytoconstituent, rosmarinic acid, revealed marginal potentials on *in vitro* cytotoxicity against cancer cell lines. Rosmarinic acid exhibited strong antioxidant activity *in vitro* via the DPPH and FRAP assays compared to the positive control in each test.

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Table 2. Cytotoxic activity (IC_{50}) against various cell lines.

Test samples	IC_{50} (µg/mL)					
	WI-38	BT-474	ChoGo-K-1	Hep G2	KATO III	SW620
T. laurifolia leaf extract	45.30	38.54	29.92	31.28	36.46	63.71
T. laurifolia stem extract	>100	>100	>100	>100	>100	>100
Rosmarinic acid	>100	93.01	79.86	>100	>100	81.69
Doxorubicin	0.22	0.80	0.65	0.12	0.71	2.57

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