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Efficacy of *Thunbergia laurifolia* (Rang Jued) aqueous leaf extract for specific biological activities using RAW 264.7 macrophage cells as test model

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Abstract

Thunbergia laurifolia or Rang Jued (RJ) has been used in Thailand in folklore medicine since ancient times and has been claimed have anti-inflammatory, and antioxidant properties and to be an antidote for several toxins. The phytochemical screening, antioxidant and anti-inflammatory activities including anti-dote of aqueous leaf extract on RAW 264.7 macrophage cells were evaluated. Data indicated that the extract contained phenols, flavonoids, tannins, sterols and cardiac-glycoside groups. Total extractable phenolic contents (TPC) and antioxidant activities of the extract were quite high level compared with other researches' data. The high-performance liquid chromatographic (HPLC) analysis of phenolics and flavonoids indicated the presence of pyrogallol, caffeic acid, rosmarinic acid and rutin compounds. The extract could not only reduce nitric oxide production but also increased cell proliferation and was judged as cytotoxicity even in low level. In addition, the extract could be used as preventing agent not for curing treatment for RAW 264.7 macrophage cells treated with cadmium (Cd) and chlorpyrifos (CP). However, preventing efficacy of the extract to CP toxicity was higher compared with Cd.

Introduction

The increasing use of pesticide in Thailand poses a serious problem because increased exposure is related to variety of human illnesses (Sapbamrer *et al.*, 2011; Panuwet *et al*., 2012). Moreover, a toxic organophosphate pesticide has been used for many crops since last 60 years (Eleršek and Filipič, 2011) and its residues could be found in water, and aquatic ecosystems as well as in agricultural products. Another serious global problem is contamination of soils with toxic heavy metals such as mercury (Hg), lead (Pb) and cadmium (Cd). Actually, heavy metals are metallic elements which can be present in both natural and polluted environments (Palipoch *et al*., 2011). Increasing contamination with toxic agents, particularly Cd is mainlydue to human activities via both light and heavy industry and increasing urbanization. Residues of heavy metals such as arsenic (As), Pb, Cd and Hg are recognized as a serious international problem since they can spread into the food chain and endanger human health (Järup, 2003). Cd in particular has an extremely long biological half-life of almost 30 years in humans, accumulating in liver and kidneys resulting in both acute and chronic toxicities which are a burden toindividual human health and public health services (Gonick, 2008; Zhu and Chan, 2012). Therefore, it is desirable to reduce or remove both pesticides and heavy metals such as Cd.

Rang Jued, Thunbergia laurifolia belongs to family of Acanthaceae and has been used as a folklore medicine in Thailand for centuries. Fresh and dried leaves, bark and roots are utilized as antioxidants, anti-inflammatories, antidotes for pesticides, drugs and chemical toxins including, Cd (Thongsaard and Marsden, 2002; Oonsivilai, 2006; Rocejanasaroj *et al*., 2014; Maneenoon *et al*., 2015). In general, Thais consume *T. laurifolia* as tea, capsules and in powder form. To make a herbal tea, dried and ground leaves packed in the tea bag will be steeped in hot water (Chan *et al*., 2012; Singtonat and Osathanunkul, 2015). Actually, our preliminary tests, revealed that *T. laurifolia* juice seemed to exhibit some cytotoxicity even (Oonsivilai, 2006; Ioset *et al*., 2009) though other reports suggested that its toxicity was very low or that it was non-toxic. In addition, the Food and Drug Administration of Thailand (Thai FDA) has just announced a ban on using *T. laurifolia* as a food or drink product (Thai FDA, 2015a; Thai FDA, 2015b) because it may have negative effect to blood system, liver and kidney. Therefore, the objective of this work was to investigate the biological activities of the aqueous extracted of *T. laurifolia* by using RAW

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264.7 macrophage cells as a model system.

Materials and Methods

Chemical

Murine macrophage cell (RAW 264.7) was purchased from American Type Culture Collection (ATCC). Most of the chemicals used for determination of antioxidant activities, phytochemical screening and high-performance liquid chromatographic (HPLC) analysis were purchased from Sigma-Aldrich, Seelze, Germany otherwise from Merck, Darmstadt, Germany; Ajax Finechem, Auckland, New Zealand; QRAC, Selangor, Malaysia; Fisher Scientific, Leicestershire, England; and LAB-SCAN, Dublin, Ireland.

Plant material and preparation of extract

The *T. laurifolia* leaves were collected at developing or intermediate stage which was green in color, and leaves could be folded without breaking easily. Leaves were directly purchased from the contact farmer in Bangkok, Thailand and transported to laboratory within 24 h. Then the samples were washed with tap water, drained and air dried for 5-8 d following a folk medicine method, for to reduce the moisture content to 8-10 % (w/w), ground to be a fine powder, sieved with 20-40 mesh, and stored in a dark bottle at room temperature before being used within 6 mo. The powder of the leaves were soaked in hot water, 98±1ºC (1:10 w/v) for 1 h, and then filtered through three layers of gauze followed by Whatman No. 4 filter paper. The filtrate was freeze-dried and stored at 4°C for further study as crude extract (Ruangyuttikarn *et al.*, 2013).

Phytochemical screening assay

The crude extract was analyzed for phytochemical screening following the method of Sofowara (1993) and Harborne (1973). Firstly, crude extract was dissolved in distilled water to obtain the concentration at 5 mg/ml as working solution for further analysis (except for the cardiac-glycosides test)as following.

Phenol test

The extract solution, 2 ml was warmed up at 45-50ºC by water bath. Then 2 ml of 3% iron (III) chloride ($FeCl_3$) was added. Formation of green or blue color was recorded for the presence of phenols.

Flavonoid tests

For flavonoid I test; 1 ml of the extract solution was added to 1 ml of 10% lead acetate then gently shaken. A muddy brownish precipitate represented the presence of flavonoid was noted.

For flavonoid II test; 1 ml of the extract solution was added with a few drops of 10% FeCl3 before shaken. A wooly brownish precipitate was reported the presence of flavonoids.

Alkaloid tests

The extract solution, 1 ml was stirred with 5 ml of 1% hydrochloric acid (HCl) using a water bath (60ºC) for 15 min to complete the reaction before taken to filter. Thereafter, alkaloids (I) test was checked by adding 1 ml of Dragendorff reagent into 1 ml of the filtrate. The formation of cloudy orange was recorded. Test for alkaloid II was performed by adding 1 ml of Mayer reagent into 1 ml of the filtrate. A light yellow color was noted. Test for alkaloid III was evaluated by adding 1 ml of Wagner reagent into 1 ml of the filtrate. The observation of turbid brown color indicated the presence of alkaloids.

Tannin test

The extract solution, 1 ml was added in to 1 ml of 3% FeCl3. A greenish black precipitate represented the presence of tannins.

Saponin test

The extract solution, 0.2 ml was mixed with 5 ml of distilled water then shaken vigorously for 5 min. Persistence of foams was the indicator for saponins.

Terpenoid test

The extract solution, 5 ml was mixed in 2 ml chloroform. Then, 3 ml concentrated sulfuric acid (H_2SO_4) acid was carefully added to observe a reddish brown coloration between upper and lower layer.

Sterol test Sterol (Salkowski's test) test was started with adding 2 ml of concentrated H_2SO_4 acid into 2 ml of the extract solution. A red precipitate indicated steroidal ring.

Cardiac-glycoside test

The extract powder, 100 mg was dissolved in 1 ml glacial acetic acid containing 1 drop of 3% $FeCl_3$. The formation of brown ring at the interface under layer of concentrated H_2SO_4 indicated the presence of de-oxy sugar characteristic of cardenolides.

Total extractable phenolic content

The total phenolic content of the extracts were measured using a modified Folin-Ciocalteu method (Tan and Kassim, 2011). Briefly, 20 µl of 0.5 mg/ml of the extract was added to 96-well microplate. Then, an amount of 100 µl of Folin penol reagent (10% v/v) and 80 µl of sodium carbonate (7.5% w/v) were added

and mixed thoroughly. After incubation for 30 min in the dark at ambient temperature, the absorbance was measured at 765 nm using the microplate reader. The total phenolic contents were expressed as mg of gallic acid, Trolox and caffeic acid equivalents (GAE, TE and CAE/g extract) via the calibration curve.

ABTS radical scavenging activity

ABTS assay, the procedure followed the method of Arnao *et al*. (2001) with a modification by using microplate reader. The stock solutions included 7.4 mM ABTS solution and 2.6 mM potassium persulphate solution. The working solution was prepared by mixing the two stock solutions in equal quantities and allowed them to react for 12-14 h at room temperature in the dark. The solution was then diluted by mixing 1 ml of ABTS solution with 48 ml of distilled water in order to obtain an absorbance of 1.1±0.02 units at 734 nm. Fresh ABTS solution was prepared and used within 2 h. The prepared extract (15 µl) was mixed with 285 µl of ABTS solution and the mixture was kept at room temperature for 2 h in the dark. The absorbance was then measured at 734 nm using the micro-plate reader. A standard curve of gallic acid, Trolox and caffeic acid were prepared. The activity was expressed as mg equivalent of GAE, TE and CAE/g extract.

DPPH radical scavenging activity

DPPH radical scavenging activity was determined by DPPH assay using the modified method from Shimada *et al.* (1992) by using micro-plate reader. Briefly, 150 µl of the extract was added to 150 µl of 0.2 to mM DPPH in 95% ethanol. The mixture was shaken lightly and stand at ambient temperature for 30 min in the dark. The absorbance was determined at 517 nm using the microplate reader. Standard curves were prepared using gallic acid, Trolox and caffeic acid and reported as mg equivalent (GAE, TE and CAE) /g extract.

FRAP reducing antioxidant power activity

The FRAP assay was done according to Benzie and Strain (1996) with a modification by using microplate reader. The stock solutions included 300 mM acetate buffer [3.1 g sodium acetate trihydrate ($C_2H_3NaO_2.3H_2O$) and 16 ml acetic acid ($C_2H_4O_2$)], pH 3.6, 10 mM of 2, 4, 6- tripyridyl-s-triazine (TPTZ) solution in 40 mM HCl, and 20 mM Iron(III) chloride hexahydrate ($FeCl_3.6H_2O$) solution. The fresh working solution was prepared by mixing 25 ml acetate buffer, 2.5 ml TPTZ solution, and 2.5 ml $FeCl_3.6H_2O$ solution and then warmed up at 37ºC before using. The extract at 0.5 mg/ml (15 µl) as allowed to react with 285 µl of the FRAP solution for 30 min in the dark condition. Readings of the colored product (ferrous tripyridyltriazine complex) were then performed at 593 nm. The standard curves were prepared using gallic acid, Trolox and caffeic acid and reported as mg equivalent (GAE, TE and CAE) /g extract.

Total extractable flavonoid content

Total extractable flavonoid content was measured by colorimetric method (Kim *et al*., 2003) with some modifications. Briefly, 800 µl of distilled water were added into 200 µl of 0.5 mg/ml of the extract followed by 60 µl of 5% (w/v) sodium nitrite solution and 60 µl of 10% (w/v) aluminium chloride solution. The mixture was allowed to stand at ambient temperature for 5 min then 400 µl of 1 M sodium hydroxide was added. Then, the volume of reaction mixture was made up to 2 ml with distilled water and mixed thoroughly. Absorbance of solutions was measured with spectrophotometry at 510 nm. Total extractable flavonoid contents were calculated from the standard curve of catechin and expressed as mg of catechin equivalent (CE)/g extract.

Iron chelating activity

The chelating activity on ferrous (Fe^{2+}) was determined using the method of Decker and Welch (1990). One milliliter of extract solution was mixed with 3.7 ml of distilled water. The mixture was then reacted with 0.1 ml of 2 mM iron (II) chloride ($FeCl_2$) and 0.2 ml of 5 mM ferrozine for 20 min at ambient temperature. The absorbance was read at 562 nm. The control was prepared in the same manner except that distilled water was used instead of the sample. A standard curve was prepared using ethylene diamine tetra-acetic acid (EDTA). The activity was expressed as mg EDTA equivalent (EDTAE)/g extract.

High-performance liquid chromatographic (HPLC) analysis for phenolic and flavonoid compounds

The quantitative of phenolic and flavonoid compounds of *T. laurifolia* extract were measured by HPLC. Firstly, the extract was subjected to hydrolyze by 6 M HCl and shaken at 70ºC for 1 h then filtered through a 0.22 µm syringe filter into a vial and 20 µl and analyzed by a high performance liquid chromatography system (Agilent Technologies 1200 series coupled with a UV-Vis photodiode array detector, DAD) equipped with an Eclipse XDB C18 column (4.6 x 250 mm, 5 µm). The mobile phase consisted of 0.1% trifluoroacetic acid (solvent A) and acetonitrile (solvent B). Column temperature was controlled at 40ºC. The mobile phase was

programmed using a constant flow rate of 0.8 ml/min with the following gradient as 95% solvent A and 5% solvent B for 50 min thereafter the ratio of solvent B was increased to 80% in 50 min. Phenolic and flavonoid compounds consisted of pyrogallol, protocatechuic acid, syringic acid, vanillic acid, caffeic acid, ρ-coumaric acid, ferulic acid, quercetrin, rosmarinic acid, quercetin, cinnamic acid, catechin, rutin, apigenin and kaempferol were used as standard agents.

Cell culture

RAW 264.7 cells were grown and maintained in RPMI – 1640 medium supplemented with 1% penicillin-streptomycin and 10% fetal bovine serum (FBS) in culture flasks at 37°C, 5% carbon dioxide (CO_2), in a fully humidified incubator. The cells were harvested with 0.25% trypsin-EDTA and suspended in a fresh medium. Cells were counted by a standard trypan blue cell counting technique.

Cytotoxicity as determined by MTT assay

The cells were seeded in a 96-well plate with 1×10^6 cells/ml adjusted by culture medium and counted by a standard trypan blue cell counting technique and incubated for 2 h to allow cell adhered to the flask. The extracts (0.01-2.00 mg/ml) were added to the wells and incubated for 24 h. The test sample was removed from the cell cultures and then tested with 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT). Briefly, 100 µl of MTT (0.5 mg/ml) was added to each well and allowed to incubate for 2 h at 37°C. After incubation, the media was aspirated and 100 µl of DMSO was added to each well to dissolve the formazan. Cell was incubated for 10 min at 37°C and before taken to read an absorbance at 570 nm with microplate spectrophotometer. Percentage of cell viability was calculated as followed equation;

$$\% \text{ Cell viability} = [OD_{sample} / OD_{control}] \times 100$$

To determine effect of the extract on MTT assays, experiment was set up as followed.

Normal control : Media + Media.
Tested sample : Media + Sample.

Nitric oxide (NO) inhibition of T. laurifolia in RAW 264.7 cells

This method was measured the production of NO in macrophages cell line. Briefly, RAW 264.7 at the density of 1×10^6 cells/ml were cultured in RPMI-1640 medium and incubated at 37°C with 5% CO_2 for 2 h to allow cell adhered to the flask. Cell density was adjusted by culture medium and counted by a standard trypan blue cell counting technique. The medium was replaced with fresh medium containing 0.4 µg/ml of lipopolysaccharide (LPS) to stimulate NO production and followed by the addition of the extract at various concentrations (0.01 to 0.5 mg/ml) and further incubated for 24 h. Then, the supernatant (100 µl) was moved to 96-well plate for NO production assay and another plate was tested for cytotoxicity by using MTT assay. To determine NO production assay, 100 µl Griess's reagent was added into the well plate which has 100 µl of supernatant. Then, NO production was measured using a microplate reader at 570 nm. The inhibition (%) of NO production was calculated by using the following equation.

$$\% \text{ Inhibition of NO} = \frac{[(\text{Negative control-Normal control})-(\text{Tasted sample-Blank sample})]}{(\text{Negative control-Normal control})} \times 100$$

The effect of sample extract on MTT and NO inhibition assays, the experiment was set up as followed.

Normal control : Media + Media.
Negative control : Media + LPS.
Tested sample : Sample+Media+LPS.
Blank sample : Sample + Media.

Anti-dote activity determine by MTT assay

Cytotoxicity of both Cd and chlorpyrifos (CP), a representative of organophosphate pesticides were tested MTT assay with a modified method of Mosmann (1983). The concentration 50% of cytotoxicity (CC_{50}) of each agent was calculated by plotting the percentage of cells survival versus the concentrations of toxin. Therefore, the T. laurifolia extract was tested on cell line with 3 different manners. Group 1; the extract was added before added with toxin (CC_{50}), group 2; the toxin (CC_{50}) was added before then the extract was followed, and group 3; the extract and the toxin (CC_{50}) was mixed together before tested on cell line as followed.

Group 1 (added sample before toxin)

- Normal control : Media (24 h) + Media (24 h)
- Negative control: Media (24 h) + Toxin (24 h)
- Tasted sample : Sample (24 h) + Toxin (24 h)

Group 2 (added sample after toxin)
- Normal control : Media (24 h) + Media (24 h)
- Negative control: Toxin (24 h) + Media (24 h)
- Tasted sample : Toxin (24 h) + Sample (24 h)

Group 3 (added sample and toxin together)
- Normal control : Media (24 h)
- Negative control : Media and toxin (24 h)
- Tasted sample : Sample and toxin (24 h)

RAW 264.7 cells were used for cytotoxicity by using MTT assay. These cell lines were seeded into a 96-well plate with $1x10^6$ cells/ml. Cells were allowed to attach to the plate for 2 h before treated with the toxic substances or sample extract. The toxin or sample extract was added and incubated for 24 h and then removed before the sample extracts or toxin was added to the wells. The condition of mix with sample and toxin, the mix solution was added on cell and incubated for 24 h while, added sample before and after toxin conditions use incubating totally for 48 h and then discard before tested with MTT. Briefly, 100 µl of MTT (0.5 mg/ml) was added to each well and allowed to incubate for 2 h at 37°C. After incubation, the media were aspirated and 100 µl of DMSO was added to each well to dissolve the formazan. Cell was incubated for 10 min at 37°C and read the absorbance at 570 nm with microplate spectrophotometer. Percentage of cell viability was calculated as followed equation;

$$\% \text{ Cell viability} = [OD_{sample} / OD_{control}] \times 100$$

Statistical analysis

Completely randomized design (CRD) was used throughout in this experiment. Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple range tests. Significance was declared at $p<0.05$ using the statistical software.

Results and Discussion

Phytochemical screening

The results showed that phytochemicals presented in *T. laurifolia* leaves extracted by high polarity media such as water were phenols, flavonoids, tannins, sterols and cardiac-glycoside as showed in Table 1. It was hypothesized that many of the various active compounds contained in the leaves should be highly water soluble compounds. From our own observations and according to folklore medical tradition, this plant is rarely invaded by insect and animals, maybe due to its terrible taste which is related to the tannin content. Putiyanan *et al.* (2008) reported that ethanolic extracts of the plant leaves showed only sterol but not tannin, alkaloid and glucosides. While, Oonsivilai *et al.* (2007) and Chuthaputti (2010) reported that the leaves contained sterols, phenols, steroids and

Table 1. Preliminary Phytochemical screening and antioxidant activities of *T. laurifolia* aqueous leaf extract

Activities	*T. laurifolia* aqueous leaf extract
Phytochemicals screening	
- Phenols	+
- Flavonoids (Test I and II)	+
- Terpenoids	-
- Alkaloids (Test I, II and III)	-
- Tannins	+
- Saponins	-
- Sterols	+
- Cardiac-glycosides	+
TPC	
- mg GAE/g extract	123.68±2.94^b
- mg TE/g extract	603.41±21.38^a
- mg CAE/g extract	77.79±1.89^c
ABTS	
- mg GAE/g extract	41.66±1.63^c
- mg TE/g extract	277.89±9.09^a
- mg CAE/g extract	88.36±2.63^b
DPPH	
- mg GAE/g extract	156.61±1.47^c
- mg TE/g extract	238.02±1.72^a
- mg CAE/g extract	187.21±1.48^b
FRAP	
- mg GAE/g extract	39.08±0.26^c
- mg TE/g extract	192.13±1.25^a
- mg CAE/g extract	87.50±0.58^b
TFC (mg CE/g extract)	62.83±2.85
Iron chelating (mg EDTA /g extract)	2.36±0.37

+ mean present; - mean absent. $^{a-c}$ Means within a column with different letters are significantly difference ($p<0.05$). TPC mean total extractable phenolic content; TFC mean total extractable flavonoid content; GAE mean gallic acid equivalent; TE mean Trolox equivalent; CAE mean caffeic acid equivalent and CE mean catechin equivalent. Values are represent as mean ± standard deviation (n=3).

glycosides. These differing results may be due to growth location and conditions, extract preparation and leaf age (Oonsivilai, 2006; Chan *et al.*, 2013). From an intensive review of the literature, it was found that there is no document reporting tannins in the *T. laurifolia* leaves. Therefore, to our knowledge, this is the first report. In addition, previous studies mentioned only glucoside compounds but not specifically cardiac-glycosides which are related to pharmaceuticals (Winnicka *et al.*, 2006; Solihah *et al.*, 2012). Therefore, identification of tannin and cardiac-glycosides were targeted for further study. Alkaloids and terpenoids are well known as a toxic substances and are associated with bitter flavors while, saponins can be classified as steroidal saponin or triterpenoid saponin or steroidal alkaloids (Solihah *et al.*, 2012; Saxena *et al.*, 2013). However, actually terpenoids, alkaloids and saponins also related to anti-carcinogenic, anti-microbial, anti-inflammatory and antioxidant activities from these phytochemical groups (Dolan *et al.*, 2010; Solihah *et al.*, 2012; Saxena

et al., 2013; Yalavarthi and Thiruvengadarajan, 2013). Though, screening test could not identify a single of pure compound containing in the crude extract, at least it is a good guide line to narrow the chemical compound group which is easier to operate with HPLC profiling further.

Antioxidant activities

Total extractable phenolic content (TPC), total extractable flavonoid content (TFC), ABTS radical scavenging, DPPH radical scavenging, FRAP reducing antioxidant power of the extract were determined and the results were showed in Table 1. Three different standard agents i.e. Trolox, gallic acid and caffeic acid were used to determine TPC. The result showed that caffeic acid expressed highest ability to react with Folin's phenol reagent. Therefore, mg equivalent of each standard antioxidant was ranked as TE>GAE>CAE. Generally, gallic is used as standard antioxidant for TPC determination based on its high functional group, -OH group, however, in this experiment it was found that caffeic acid was better. Stratil *et al*. (2006) suggested caffeic acid can respond to Folin-Ciocalteu method better than gallic acid, however, antioxidant activities based on FRAP and DPPH assay of gallic acid were higher than caffeic acid. Similar to this experiment, it was found that activity of gallic was highest when taken to evaluate antioxidant property based on ABTS, DPPH and FRAP assay, therefore the antioxidant value of each assay was ranked as TE>CAE>GAE. It pointed out that it was difficult to make a good conclusion for TPC determination in each plant if used standard antioxidant was different. Based on TPC which also may relate to some antioxidant property, the product having TPC as ≥5,000 mg GAE/100 g, ≥1,000 mg GAE/100 g and <1,000 mg GAE/ 100 g was appointed as high, moderate and low antioxidant, respectively (Chan *et al*., 2012). It pointed out that the aqueous extract of *T. laurifolia* in this experiment was followed in a high antioxidant category with TPC as 12,368 mg GAE/100 g sample. Moreover, Oonsivilai *et al*. (2008) reported that the aqueous extract of *T. laurifolia* was highest in antioxidant activities compared with ethanol and acetone extract when using DPPH free radical scavenging and FRAP reducing power as testing models. This results confirmed that main bioactive compounds in the plant leaves responded to high polar compounds consisting of phenolic and flavonoid compounds. Result of TFC indicated that simple phenols was used to generate polyphenols including flavonoid compounds. The result also revealed that the extract exhibited iron chelating with 2.36±0.37 mg EDTA/g sample.

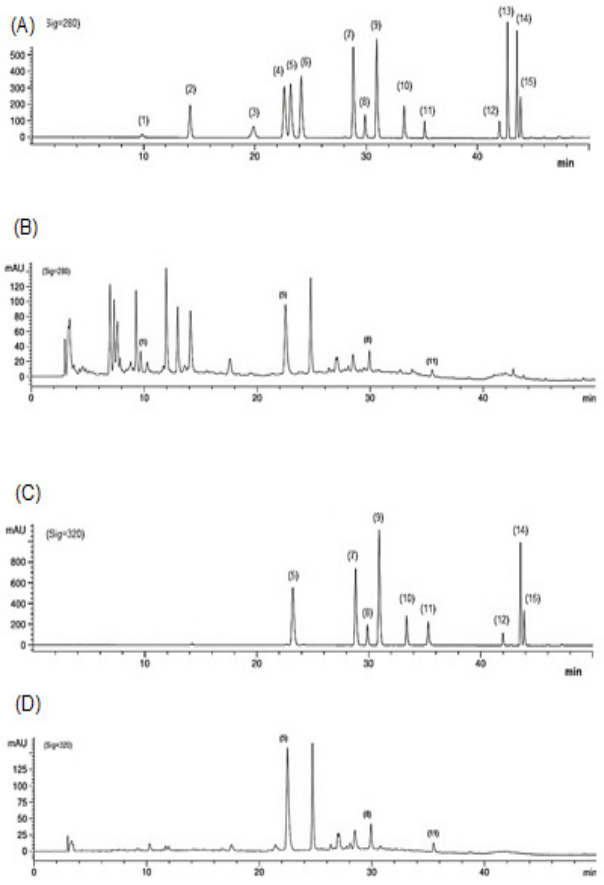

Figure 1. HPLC chromatograms; (A) standard compounds at wavelength 280 nm; (B) *T. laurifolia* aqueous leaf extract at wavelength 280 nm; (C) standard compounds at wavelength 320 nm; (D) *T. laurifolia* aqueous leaf extract at wavelength 320 nm. Peaks 1=Pyrogallol, 2=Protocatechuic acid, 3=Catechin, 4=Vanillic acid, 5= Caffeic acid, 6= Syringic acid, 7= ρ-Coumaric acid, 8=Rutin, 9=Ferulic acid, 10=Quercetrin, 11=Rosmarinic acid, 12=Quercetin, 13= Cinnamic acid, 14=Apigenin and 15= Kaempferol.

In fact, active chelating compound was typical characteristic of flavonoids. However, Oonsivilai *et al.* (2007) reported that not only apigenin, a flavonoid compound, but also caffeic acid (not flavonoids) (Psotová *et al*., 2003; Symonowicz and Kolanek, 2012) which assumed to be main compounds were reported in the aqueous leaf extract having metal chelating.

Phenolic and flavonoid profiling determined by HPLC

HPLC technique was used to determine of phenolic and flavonoid compounds presented in T. laurifolia aqueous leaf extract. In fact, the phenolic and flavonoid compounds were identified based on their retention times and quantified according to respective standard calibration curves (Figure 1A and 1C). Based on chromatogram peak, it was found that the phenolic and flavonoid compounds from the

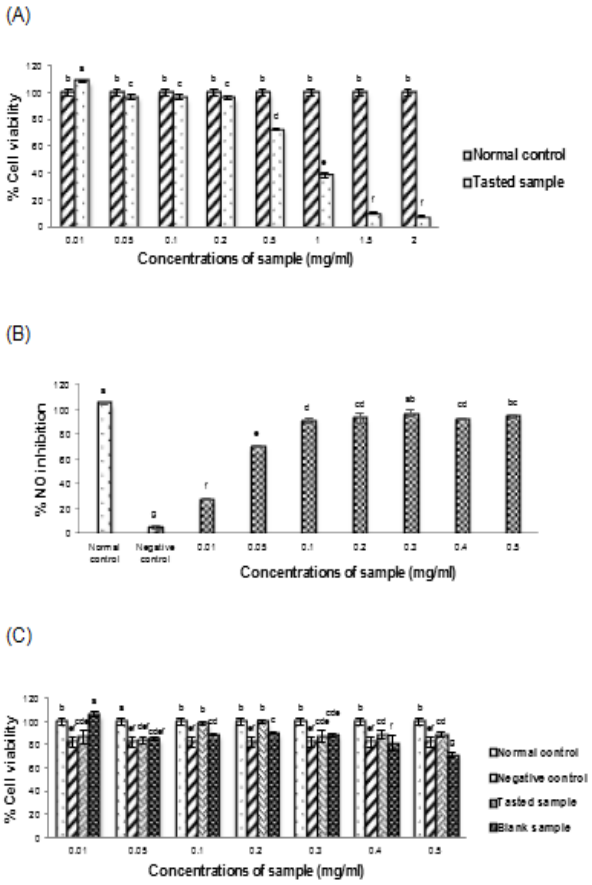

Figure 2. Effect of *T. laurifolia* leaf extract on cell viability and NO inhibition in RAW 264.7 macrophage cells; (A) percentage of cell viability of sample on cells; (B) percentage of NO inhibition induced by LPS of sample on cell; (C) percentage of cell viability induced by LPS of sample on cells. [a-g] Means within a figure with different letters are significantly different ($p<0.05$). Values are mean ± standard deviation (n=3).

extract were identified as pyrogallol, caffeic acid, rosmarinic acid and rutin (Figure 1B and 1D) with values of 57.20±1.62, 5.79±0.01, 3.56±0.05 and 1.66±0.16 mg/g dry extract, respectively (data not shown). This result revealed that caffeic acid, rutin and other phenolic and flavonoid compounds were responsible for the antioxidant activity and biological activity in this plant. Actually, there were some scientific data reported that the aqueous leaf extract of *T. laurifolia* contained gallic acid, protocatechuic acid, catechin and apigenin (Oonsivilai, 2006; Oonsivilai *et al*., 2007; Mahasarakul *et al*., 2013). The differences in phenolic and flavonoid profiles of each researcher group may due to the variation of plant cultivars, location and environmental conditions of plant growth (Oonsivilai, 2006) as well as plant preparation. As mentioned in material preparation that the plant used in this experiment was dried in ambient temperature without any leaching processed which was different from other research groups. However, the profiling of this extract showed many unknown phenolic and flavonoid compounds that should be identified further with other techniques or protocols.

Cytotoxicity of leaf extract to RAW 264.7 macrophage cells and inhibition of NO synthesis

Cytotoxicity is the quality of a substance being toxic to cells. The potential toxicity (CC_{50}) of leaf extracts was determined using the MTT test for cell viability. The results showed that using the extract at 0.5 mg/ml reduced RAW 264.7 macrophage cell viability by approximately 20% (Figure 2A). In addition, CC_{50} of the extract was calculated and reported as 870 µg/ml (data not shown) which was classified as low cytotoxicity or non-toxic, based on the threshold of 90 µg/ml (Oonsivilai, 2006; Ioset *et al*., 2009). This result was in agreement with finding of Oonsivilai *et al*. (2008) who found that the aqueous extract of *T. laurifolia* was judged as of low cytotoxicity with CC_{50}>100 µg/ml after testing with mouse connective tissue (L929), baby hamster Syrian kidney (BHK(21)C13), human liver hepatocarcinoma (HepG2) and human colon adenocarcinoma (Caco-2) cell lines. Moreover, Chivapat *et al.* (2009) suggested that using aqueous leaf extracts at doses ranging from 20 to 2,000 mg/kg/day did not affect body weight, food consumption, behavior and general health on Wistar rats. In addition, at 0.01 mg/ml of the extract in this experiment showed higher cell viability than control condition. It has been hypothesized that at lower doses, the extract may help cell proliferation or strength as a result of some nutrition or bioactive compounds as glucoside and phenolic compounds which was claimed as supplementary compound for cell proliferation and protecting cell death via its antioxidant and anti-inflammatory effect (Chattaviriya *et al*., 2010; Ruangyuttikarn *et al*., 2013). However, it is possible that at high concentration, most compounds would act as toxic agents even if it is normally essential to life at a certain amount. This finding could be supported by some flavonoids and phenolic compounds which showed pro-oxidant activity under certain conditions such as at high doses or in the presence of metal ions (Yordi *et al*., 2012) and can injure sensitive cells. Moreover, hydrophobicity of the individual phenolic compounds affects the solubility of the phenol in a cell fraction and thus possibility of interaction of the compound with specified cell and tissue structures led to affects toxic on cell (Michałowicz and Duda, 2007; Bazrafshan *et al*., 2013).

It was accepted that NO production, an important

molecule in the inflammatory response is generally used for anti-inflammatory activity determination in RAW 264.7 induced by lipopolysaccharide (LPS). However, it should keep in mind that the inflammatory effect involves in various mediators including histamine, prostaglandins (PGs), leukotrienes (LTB4), platelet activation factor (PAF), bradykinin, serotonin, lipoxins, cytokines and growth factors (Nile and Park, 2013).In addition, the inflammatory effect can be generated through COX-2 (Lee, Lee, Ha *et al*., 2015) not only pathway of iNOS. Based on NO inhibition, a highest inhibition which was comparable to normal control found when the extract was applied at 0.3 mg/ml ($p<0.05$). In fact, the extract seemed to significantly reduce NO production when the extract was applied at 0.01 mg/ml as shown in Figure 2B. The results also indicated that NO inhibition showed saturation when the extract was used in the range 0.01 to 0.5 mg/ml. In addition, the extract exhibited 50% inhibitory concentration (IC_{50}) at 23.31 µg/ml (data not shown) with cell viability higher than 80%. Surprisingly, cell viability of RAW 264.7 induced inflammation by LPS and followed with the extract (tasted sample) was significantly increased (Figure 2C) when compared with treated cells with the extract without inflammatory condition (blank sample). It pointed out that the extract would induce cell death at higher doses (>0.2 mg/ml) but if the cell was already induced with LPS to get some stress then the extract would help the cell to survive. In addition, this may support the claimed of folklore medicine doctors who normally used the T. laurifolia leaves for any patient who just approached the toxic compounds as pesticide or hangover. TPC has been accepted to play a major role for cytotoxicity and anti-inflammatory activity (Dolara *et al*., 2005; Saxena *et al*., 2013), it was found that TPC in this experiment was higher than that finding of Oonsivilai (2006). This may due to many factors such as location of plant growth, preparation of extract, used solvent and standard antioxidant, period of leaves as well as month of harvesting (Oonsivilai, 2006; Chan *et al*., 2012; Chan *et al*., 2013).

Kanchanapoom *et al.* (2002) and Oonsivilai *et al.* (2007) accordingly stated that main bioactive compounds found in *T. laurifolia* leaves were gallic acid, caffeic acid and apigenin. Flavonoids have been reported to possess anti-inflammatory activity in vitro and *in vivo* (García-Lafuente *et al*., 2009; Rathee *et al*., 2009; Funakoshi-Tago *et al.*, 2011). In addition, apigenin either aglycone or glucoside also have been reported a good anti-inflammatory effect (Rathee *et al.*, 2009; Lee, Ha, Cho *et al*., 2015) by protecting endothelial cell inflammation (Duarte *et al.*, 2013). Recently, Lee, Ha, Cho *et al.* (2015) reported that apigenin significantly suppressed production of NO in RAW 264.7 stimulated with LPS through inducible nitric oxide synthase (iNOS) inhibition. However, Zhang *et al*. (2014) stated that LPS-induced macrophage inflammation was inhibited by applying apigenin through its multi mechanisms, for example; TNF-α and IL-6 cytokines. Moreover, Lee, Lee, Ha *et al.* (2015) suggested that rutin, a low molecular weight of flavonoid glycoside, reduced inflammatory effect by suppressing the expression of COX-2 and iNOS in RAW 264.7. Not only flavonoid apigenin but many phenolic compounds such as caffeic acid, gallic acid and protocatechuic acid obtained from the aqueous extract of *T. laurifolia* leaves as well as rosmarinic acid from ethanolic extract also expressed their anti-inflammatory activity (Huang *et al*., 2009). Actually, both caffeic acid and caffeic acid ester were reported to have both antioxidant and anti-inflammatory properties (Chao *et al*., 2010; Veres, 2012; Zhang *et al.*, 2014) via multi pathways such as the inhibition of NF-κB, p38/ERK including iNOS expression (Song *et al*., 2002; Shin *et al.*, 2004; Jung *et al.*, 2008; Liu *et al*., 2014; Zhang *et al*., 2014).

Anti-Cd activity of leaf extract tested with RAW 264.7 macrophage cells

To study the effect of various extract concentrations on Cd toxicity, cells were treated with the extract and/or Cd as 3 groups including treatment with the leaf extract before Cd, after Cd and together with Cd for 24 h. The results showed that cell viability significantly increased when concentration of added sample extract increased in the group 1 (Figure 3A) compared with negative control (Control + Cd). It pointed out that the extract expressed its protective effect to the cells. However, it was found that this phenomenon did not appear in the group 2 and 3 (Figure 3B and 3C). In addition, cell viability was continuously reduced without no signal of recover. Moreover, increasing of sample extracts tended to increase cell death particularly in group 3. The result confirmed that the extract exhibited more a preventing effect rather than a curative effect. This result was in agreement with the finding of Morkmek *et al.* (2010) who reported that abnormal appearance and behavior of rats fed with the extract prior to Cd exposure was lesser than those fed with the extract after Cd exposure. Chattaviriya *et al.* (2010) stated that the aqueous extract significantly protected kidney damage caused by Cd exposure in Wistar rats. And Tangpong and Satarug (2010) suggested that the extract can reduce neuronal cell death caused by lead uptake in mice due to both antioxidant and

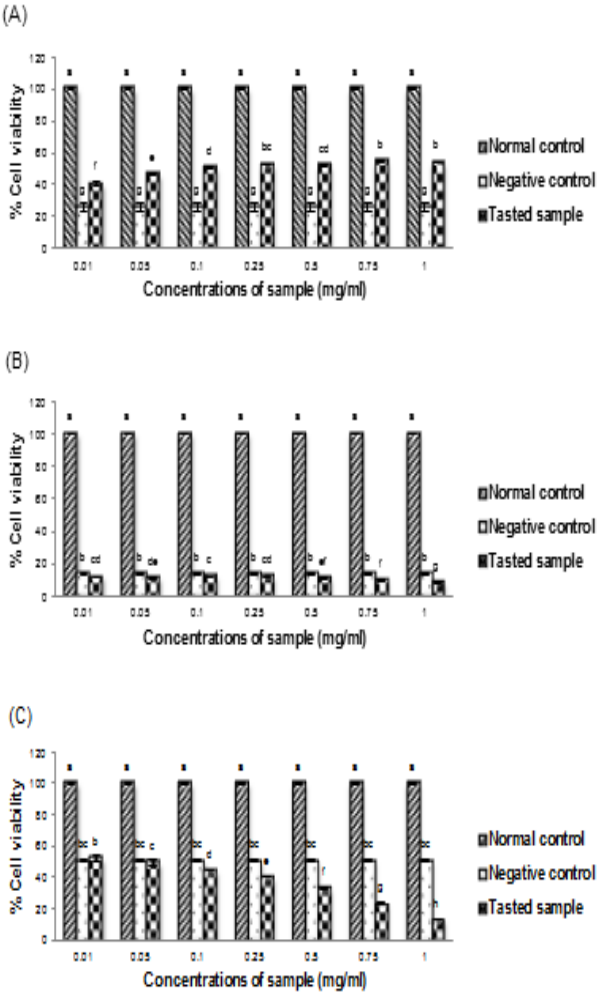

Figure 3. Percentage of cell viability of *T. laurifolia* aqueous leaf extract in RAW 264.7 macrophage cells; (A) sample added before Cd (CC_{50}); (B) sample added after Cd (CC_{50}); (C) sample and Cd (CC_{50}) added together on cells. [a-h] Means within a figure with different letters are significantly different ($p<0.05$). Values are mean ± standard deviation (n=3).

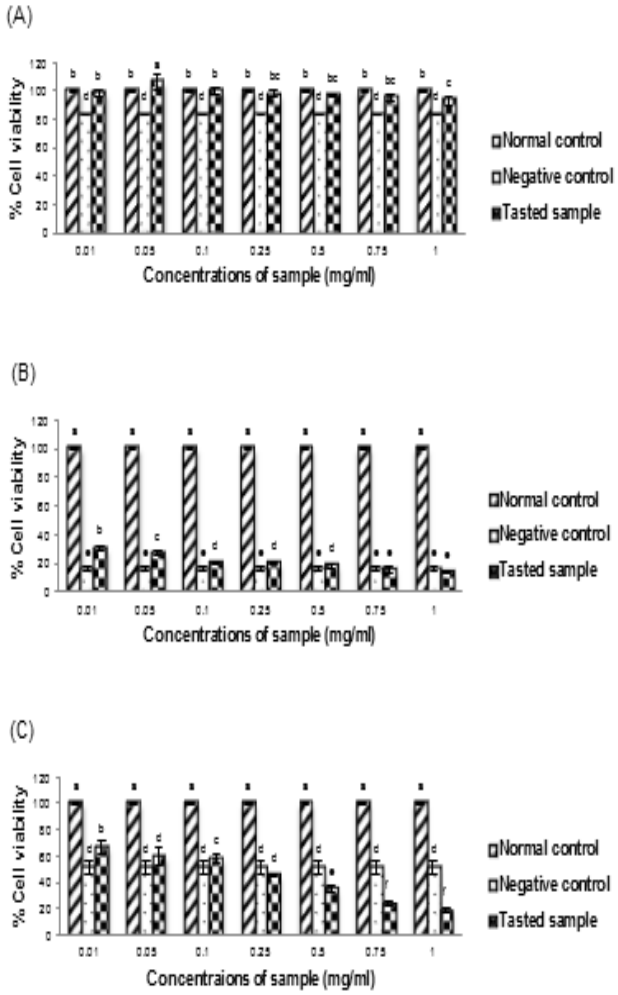

Figure 4. Percentage of cell viability of *T. laurifolia* aqueous leaf extract in RAW 264.7 macrophage cells; (A) sample added before CP (CC_{50}); (B) sample added after CP (CC_{50}); (C) sample and CP (CC_{50}) added together on cells; CP mean chlorpyrifos toxicity. a-f Means within a figure with different letters are significantly different ($p<0.05$). Values mean ± standard deviation (n=3).

anti-inflammatory effect of phenolic compounds. In addition, there was proposed that the *T. laurifolia* leaves contained aromatic and glucoside compounds (Ruangyuttikarn *et al.*, 2013) which were responsible for mitigation effects. Boonpeng *et al.* (2014) also reported that Cd was a very toxic substance then both fresh and pickled garlic extract could pronounce only cytotoxicity preventing not curing when human embryonic kidney 293 cells (HEK 293) were treated with similar way as before, after and together. The phenomenon of Cd effect on cell death may due to acute toxicity via several apoptotic pathways such as disruption of the cellular antioxidant enzyme, disruption of cell adhesion, induction of reactive oxygen species, including inhibition of DNA repair (Ercal *et al*., 2001; Fujiwara *et al.,* 2012; Zhu and Chan, 2012). Therefore, more cell death in group 2 and 3 (Figure 3B and 3C) compared with control sample were occurred as a result of combined cytotoxicity effect.

Anti-chlorpyrifos (CP) activity by using RAW 264.7 macrophage cells

Chlorpyrifos (CP), an insecticide originated from organophosphate at CC_{50}, 0.25 µl/ml (data obtaining from standard curve), was tasted similarly to the Cd toxicity assay. It was found that the extract could reduce cell death when the cells were treated with CP as showed in Figure 4A and 4B. A significant increase of cell viability was observed particularly when cells were treated with leaf extract before CP was applied (Figure 4A). This effect may explained by finding the of Usanawarong *et al.* (2000) who showed that the aqueous leaf extract of *T. laurifolia* can decrease

plasma malonaldehyde (MDA), an indicator of lipid peroxidation derived from free-radical mediated reactions of rat with paraquat. In fact, various toxins caused cell death via oxidative stress (Verma and Srivastava, 2003; McCarthy *et al.*, 2004) through cell membrane which compose of poly-unsaturated fatty acids, a primary target for reactive oxygen attack leading to cell membrane damage (Repetto *et al*., 2012). Cellular membrane damage from oxidative stress can be retained or inhibit when appropriate phenolic and flavonoid concentrations were applied (Nijveldt *et al*., 2001; Karimi *et al*., 2012). In addition, Chinacarawat *et al.* (2012) reported that using the leaf extract on reduce poisoning of organophosphate and carbamate pesticides showed without side effects in high risk volunteer. However, in this experiment the results revealed that the extract could act as prevetative better than as a curative when the cells were treated with CP. A comparison showed that was more toxic than CP and that cells recovered less well. This may be due to different toxicity mechanisms of those compounds, individual half-life and cellular targets. Mostafalou and Abdollahi (2013) stated that Cd can cause cell injury or death via disrupting oxidative balance and damaging cell components including lipids, protein and DNA.

However, more extract added, more cell death in condition of after and together with both of toxic compounds (Cd and CP). This may due to synergist toxicity effect of some phenolic and flavonoid compounds as well as toxic agents used (Kyselova, 2011). Chang *et al.* (2010) reported that caffeic acid induced apoptosis in HeLa cells, human cervical cancer cells via the mitochondrial apoptotic pathway. While, Zbidah *et al.* (2012) stated that apigenin induced suicidal erythrocyte cell death by stimulation of Ca^{2+} entry through Ca^{2+} permeable cation channels, ceramide formation and ATP depletion, eventually leading to eryptosis by cell shrinkage and cell membrane scrambling. Hence, this phenomenon needs to be carefully studied to improve the utilization and application from *T. laurifolia* extract in the future.

Conclusion

Water extract of the *Thunbergia laurifolia* leaves provided 5 groups in the phytochemical screening test; phenols, flavonoids, tannins, sterols and cardiac-glycosides with high of antioxidant activities based on TPC, TFC, ABTS, DPPH, FRAP and iron chelating properties. Phenolic and flavonoid profiled of the extract alone matched with of pyrogallol, caffeic acid, rosmarinic acid and rutin. The extract its own showed a low cytotoxicity and helped reduce nitric oxide production. The leaf extract exhibited a preventative rather than a curative effect when RAW 264.7 cells were treated with Cd and CP. Since, toxicity of Cd was higher than CP therefore, preventing effect of Cd toxicity was lower compared with CP toxicity. This experiment supported the Thai folk wisdom for using *T. laurifolia t*ea as anti-dote, however, the agent application method still needs more investigation to ensure safety.

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