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Some Phytochemicals and Anti-inflammation Effect of Juice from *Tiliacora triandra* Leaves

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Abstract *Tiliacora triandra* (TT), native to Southeast Asia, has been widely used as folk medicine and in cuisines in many areas. Although little scientific evidence supports the health benefits of TT leaves, the juice extracted from TT leaves has become popular for consumers among all socioeconomic classes. Thus, this study aims to evaluate the phytochemical profile and anti-inflammatory properties of juice extracted from TT leaves. The result revealed that, the highest total phenolic contents (199.92 mg GAE/g), total flavonoid contents (29.76 mg RUE/g), and extraction yield (61.2%) of the lyophilized TT leaves juice powder were found in the solvent mixture of ethanol, water, and acetone extracts. The highest DPPH radical scavenging value (90.95%) of the lyophilized TT leaves juice powder was found in hot water extract. By HPLC analysis, total phenolic compounds of the lyophilized TT leaves juice powder was 3,938.1 mg/kg. Tannic acid, gallic acid, and rutin are the major phenolic compound and the juice is a rich source of chlorophyll compound (3,551.6 mg/kg). The lyophilized TT leaves juice down-regulated the induction of inflammatory iNOS and COX-2 proteins in LPS-stimulated macrophages. The results suggest that intake of TT leaves juice, providing various phenolic and chlorophyll compounds, has great potential for reducing the inflammatory process.

Keywords: *bioactive compounds, inflammation, cyclooxygenase-2, juice, inducible NO synthase*

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1. Introduction

Several studies have provided strong evidence that phytochemicals in vegetables and fruits protect against some cancers [1,2]. The use of these natural dietary compounds to inhibit or suppress the process of carcinogenesis is a crucial strategy for cancer prevention. The chemo-preventive effects of these natural dietary compounds in vegetables and fruits have involved various mechanisms including being an antioxidant, exhibiting anti-inflammatory activity, inducing phase II enzymes, and apoptosis [3]. Therefore, the regular intake of plant containing antioxidative and anti-inflammatory agents reduces the risk of cancer development [4]. It has now accepted that inflammation plays a crucial role in chronic inflammatory diseases and the cancer development [5,6]. Inflammatory leukocytes, such as macrophages when activated by many stimuli, result in the induction of pro-inflammatory protein and enzymes including inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) [7]. Consequently, suppressing the induction of COX-2 and iNOS is a key strategy for cancer chemoprevention in various human organs [8].

Tiliacora triandra (Colebr.) Diels (Menispermaceae) is native to Southeast Asia, called under different names from region to region, and is widespread in the northeast of Thailand, where this vegetable, generally known as Yanang [9]. Besides being used in many regional cuisines, it has been used as a traditional medicine according to folklore, as anti-pyretic, antibacterial, detoxification and an immune modulator agent [10]. A market for juices and herbal drinks has substantially developed in recent times. With the folklore and health claims of the benefits of the leaves of *Tiliacora triandra* (TT), ready to drink beverages made from TT leaves have become popular as functional beverages consumed among people of all socioeconomic classes. One report identified bisbenzylisoquinoline alkaloids from the root of TT, including tiliacorinine, nortiliacorinine, and tiliacorine, as having anti-microbacterial activity against multidrug-resistant isolates of *M. tuberculosis* [11].

There is limited information available on the phytochemicals and bioactivity effects of the TT leaves on human health. Rattana et al. (2010) reported that TT leaves possess antioxidant activity [12]. A toxicity study on water extract of TT leaves showed no toxicity up to 5,000 mg/kg in a single administration, and no adverse effects following the subchronic administration at doses of 300, 600, and 1,200 mg/kg [13]. The purpose of this current study, therefore, was to evaluate the phytochemical compounds, including phenolic acid, and the anti-inflammation activity of juice from TT leaves using RAW264.7 cells induced by lipopolysaccharide (LPS).

2. Materials and Methods

2.1. Chemicals

Lipopolysaccharide (LPS) from Escherichia coli 0127: E8, sulfanilamide, N-(1-Naphthyl) ethylenediamine dihydrochloride, and phenolic standards (gallic acid, tannic acid, kaempferol, quercetin, isoquercetin, rutin, apiginin, eriodictyol, and catechin), were purchased from Sigma Chemical Co (St. Louis, Mo., U.S.A.). All chemicals used in the experiments were of analytical grade.

2.2. Preparation of Plant Material and Juice from TT Leaves

Purchased from a farmer in the north of Thailand (Phitsanulok province), leaves of *Tiliacora triandra* (TT) were cleaned with DI water to remove soil and contaminants, and defective leaves were separated. After drying at room temperature, the leaves were processed with 6:100 w/v of water in a blender (Tefal SM, Type BL31, Thailand) at medium speed for 3 min and the blended mixture was then filtered. The filtered leaves juices were lyophilized to obtain the lyophilized TT leaves powder and kept at -20°C until the chemical analysis.

2.3. Solvent Extraction

Hot water (90-95°C), ethanol, acetone, and the mixtures of ethanol, water (room temperature) and acetone in the ration 60:20:20 v/v/v, were used to compare the effects of solvent extraction. The lyophilized leaves juices powder (5 g) was dissolved in different solvent (50 mL) following sonication (GT-1730QTS, 40 kHz, 150 W, Thailand) for 30 min. After being filtered through filter paper, the solvent was dried by rotary vacuum evaporator (Buchi, Switzerland), and the dried extract was dissolved in methanol to a final volume of 25 mL for the determination of total phenol contents, total flavonoid contents, and DPPH radical inhibition activity.

2.4. Determination of Total Phenolic Content

Phenolic concentration of samples were determined using Folin-Ciocalteu assay [14]. An aliquot of extracted sample mixed with Folin-Ciocalteu reagent (5 mL) allowed to stand for 6 min after which 7.5% Na_2CO_3 solution (4 ml) were added. The obtained mixture solution was incubated at 45°C for 15 min. The absorbance was measured at 760 nm by a UV-VIS spectrophotometer (UV 1601, Shimadzu, Japan) and the results were reported as mg of gallic acid equivalent (GAE) per g sample.

2.5. Determination of Total Flavonoid Content

Total flavonoid content was measured following an aluminum chloride colorimetric assay [15]. The extracted sample (1 mL) was mixed with 5% sodium nitrite (0.2 mL) following the addition of 0.2 mL of 10% aluminum chloride and 2 mL of 1 M sodium hydroxide. The solution was thoroughly mixed and the absorbance was evaluated at 510 nm. Total flavonoid contents were expressed as mg rutin equivalent (RUE) per g sample.

2.6. Determination of Antioxidant Activity

The DPPH radical scavenging activity was conducted according to Medini et al. (2014) with some modifications [16]. The extracted sample solution (1 mL) was added to 2 ml of DPPH–methanol solution (0.2 mM). After incubation in the dark at room temperature for 60 min, the absorbance was determined at 517 nm. The percentage of DPPH radical inhibition was calculated from $(A_{blank} - A_{sample}/A_{blank})\times 100$, where A_{blank} is the absorbance of the control reaction (DPPH solution) and A_{sample} is the absorbance of the samples.

2.7. Determination of Chlorophyll Content

The concentration of chlorophyll a, b, and the total chlorophylls, were determined by the spectrophotometric method according to AOAC (1984) and Bahloula (2014) [17]. Briefly, the lyophilized TT leaves powder (0.1 g) was added to 85% acetone solution (20 mL) and the mixture was homogenized for 3 min. After filtration with filter paper (Whatman No.1), the filtrate solution was brought to a final volume of 25 mL. The absorbance was recorded at 660 and 642.5 nm using a UV/Vis spectrophotometer (Shimadzu, Japan). The concentrations of total chlorophyll, chlorophyll a and b (ppm) were calculated by the below equations;

$$\text{Chlorophyll a} = 9.93A660 - 0.777A642.5$$

$$\text{Chlorophyll b} = 17.6\ A642.5 - 2.81A660$$

$$\text{Total chlorophyll} = 7.12\ A660 + 16.8\ A642.5.$$

2.8. Analysis of Polyphenol Content by HPLC/DAD/MS

2.8.1. Sample Extraction

The lyophilized TT leaves powder (0.1 g) was dissolved in 70% ethanol (10 mL) and then vigorously shaken for 10 min. The mixed sample solution was filtered and then centrifuged at 4,000 rpm for 10 min to get the ethanolic extract. The extractions were performed 4 times and the ethanol was removed by rotary vacuum evaporator. The obtained extract was made up to final volume of 2 mL with ethanol. Phenolic standard solutions were prepared in ethanol [18].

2.8.2. HPLC/DAD/MS Condition

The analysis of the phenolic compounds was performed on an Agilent 1100 series HPLC system (Agilent Technologies, Waldbronn, Germany) equipped with a diode array detector and an MSD SL ion trap mass spectrometer with an API-ES interface. The quantities of phenolic compounds were determined by reversed-phase (a 150 mm x 4.6 mm x 5 um, LiChroCART RP-18e) column (Purospher STAR Merk, USA) and the temperature of column was kept at 40°C. The mobile phase was a binary solvent system using water acidified with 10 mM ammonium formate buffer pH4 (solvent A) and 100% acetonitrile (solvent B), kept at a flow rate of 1.0 mL/min. The gradient elution program was 0-5 min

for 100% A; 5-10 min for 0-20% B; 10-20 min for 20% B and 20-60 min for 20-40% B. The chromatograms were obtained using 270, 330, 350 and 370 nm for analysis of phenolic compounds. The phenolic compounds including phenolic acid and flavonoids were analyzed in positive and negative ion modes with the following settings: capillary voltage -3500 and 4000 V. The N_2 nebulizer gas pressure was set at 60 psi and dry temperature of 320°C. Analysis was carried out using scans from m/z 100 to 700 in profile mode with a step size of 0.2 [19].

2.9. Determination of Anti-inflammation Activity of TT Leaves Juice

2.9.1. Sample Preparation for Inflammation Inhibition Activity

A stock solution of lyophilized TT leaves juice powder was prepared at 150 mg/mL in the dimethylsulfoxide (DMSO) solution, and the solution was centrifuged to obtain the supernatant for analysis.

2.9.2. Cell Culture

Murine macrophages RAW 264.7 cells obtained from the American type culture collection (Rockville, MD, USA) were cultured in Dulbecco's Modified Eagle's Medium with 10% endotoxin-free, heat-inactivated fetal calf serum (GIBCO, Grand Island, NY, USA), penicillin (100 units/mL), and streptomycin (100 µg/mL). The medium were removed and replaced with medium without phenol red when the cells had a density of 2–3 x 106 cells/mL. The cells were incubated in medium composing of LPS (100 ng/mL) and the sample in DMSO at various concentration was added. Cells treated with 0.1% DMSO were set as vehicle control [20].

2.9.3. Cell Viability Assay

The effect of the TT juice on cell viability of RAW 264.7 cells was analyzed by 3-(4, 5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. RAW 264.7 cells overnight seeded in a 96-well plate (1 × 106/mL) were treated with sample at various concentrations. MTT (200 µg/mL) was added following incubation at 37°C for 3 hr. The cell viability was measured by scanning with an ELISA reader with a 570 nm filter [21].

2.9.4. Nitrite Assay

According to the Griess reaction, the nitrite concentration as indicator of nitric oxide (NO) production in the culture medium was measured. The supernatant (100 µL) were mixed with Griess reagent (1% of sulfanilamide in 5% of phosphoric acid and 0.1% of naphthylethylenediamine dihydrochloride in water) and the absorbance of the mixture was measured at 550 nm in an enzyme-linked immunosorbent assay (ELISA) plate reader (Dynatech MR-7000; Dynatech Labs, Chantilly, VA, USA) [20].

2.9.5. Western Blot Analysis

The stimulated murine macrophage RAW 264.7 cells were washed with PBS and lysed in an ice-cold lysis buffer (20 mM Tris–HCl (pH 7.4)), 10 mM NaF, 137 mM NaCl, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 100 µM β-glycerophosphate, 1 mM $Na_4P_2O_7$; 5 mM EDTA; 1 mM Na_3VO_4 and protease inhibitor cocktail tablet (Roche, Indianapolis, IN) to cell pellets for 30 min, followed by centrifugation (10,000 rpm) at 4°C for 30 min. The 50 µg of protein were then combined with 5x sample buffer containing 0.3 M Tris–HCl (pH 6.8), 25% 2-mercaptoethanol, 12% sodium dodecyl sulfate (SDS), 25 mM EDTA, 20% glycerol, and 0.1% bromophenol blue following boiling for 5 min. The boiled mixture was loaded to 10% SDS–polyacrylamide mini gels at a 20 mA constant current. Proteins on the gel were transferred onto an immobile PVDF membrane (Millipore Corp., Bedford, MA, USA). Blocking solution were applied to block the membranes which later were immunoblotted with primary antibodies including iNOS, COX-2 and β-actin (Transduction Laboratories, Lexington, KY, USA) at room temperature. The blots were washed three times for 10 min each by rinsing with PBS-T buffer (0.2% Tween 20 in 1x PBS buffer) and then were incubated with the horseradish peroxidase (HRP)-conjugated secondary antibody at dilution of 1:5,000 (Zymed Laboratories, San Francisco, CA) following washing three times with PBS-T buffer. The transferred proteins were visualized with an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech, Buckinghamshire, UK) [20,22].

2.10. Statistical Analysis

The data were expressed as means ± standard deviations (SDs) of three replicate determinations and then analyzed by SPSS V.13. One way analysis of variance (ANOVA) with Duncan's new multiple-range test were used to test the differences of the mean value. P-values <0.05 were used as significantly difference. One way student's t-test was used to assess the statistical significance between the LPS-treated cells.

3. Results and Discussion

3.1. Phenolic Compounds and Antioxidant Activity of *Tiliacora triandra* Leaves Juice by Different Solvents

Most studies reported that the protective properties against oxidative damage by fruits and vegetables were attributable to phenolic compounds [23]. Consequently, TPC and TFC of plant are considered as indicators of the antioxidant capacity since the redox properties of the phenolic compounds allow them to exhibit as reducing agents via hydrogen donors and radical scavengers [24].

There are several extraction techniques to recover antioxidants. However, extraction yield and antioxidant activity not only depend on the extraction method but also on the solvents used for the extraction. Due to the different chemical characteristics of various antioxidant compounds, they may be soluble or insoluble in a particular solvent [25]. Ethanol, methanol, acetone, ethyl acetate, and the mixture of them are widely used solvents for recovering phytochemicals from plant matrices. Among them, ethanol is accepted solvent for polyphenol extraction in terms of safe solvent for human consumption while acetone is suitable solvent for extraction of higher molecular weight flavonoids such as flavanols [26].

Consequently, we investigated the effect of solvent extraction on the recovery of polyphenol contents of the lyophilized TT leaves juice powder. Ethanol and acetone were used to see the effect of extraction with these particular solvents compared with hot water. The content of the phytochemicals of the lyophilized TT leaves juices, which include total phenolic contents (TPC), total flavonoid contents (TFC), and DPPH radical inhibitory activity are shown in Table 1. The highest content of TPC of the juice, was found in the solvent mixture of ethanol, water, and acetone extracts (199.92 mg GAE/g lyophilized leaves juice powder) and the lowest in the acetone extract (20.33 mg GAE/g lyophilized leaves juice powder). This result, for the TPC, is consistent with the extraction yield which was found to be the highest in the mixture solvent and lowest in acetone. The phenomenon of the highest yield in this situation may be caused by the combination of organic solvent and water that together facilitate the extraction of all compounds soluble both in both water and organic solvents [27]. The highest TFC was found in the solvent mixture extract (29.76 mg RUE/g) followed by the ethanol extract (28.85 mg RUE/g), and the lowest in hot water extract (5.64 mg RUE/g). Wang and Helli (2001) also reported that the ethanol gave better result than aqueous methanol and acetone for extracting flavonoids from tea leaves [28].

Table 1. Extraction yield, TPC, TFC, DPPH scavenging capacity of lyophilized TT leaves juice extract

Solvent	Extraction yield[a] (%)	TPC (GAE/g)	TFC (RUE/g)	DPPH (%)
Ethanol	15.8±4.2^{c}	83.08±0.79^{c}	28.85±0.07^{b}	77.61±2.34^{b}
Hot water	55.7±11.2^{b}	126.34±0.79^{b}	5.64±0.12^{d}	90.95±0.84^{a}
Acetone	6.4±2.5^{d}	20.33±0.10^{d}	10.15±0.37^{c}	35.72±1.09^{d}
Mixture	61.2±12.5^{a}	199.92±0.38^{a}	29.76±0.25^{a}	70.07±0.22^{c}

GAE= gallic acid equivalent; RUE= rutin equivalent; TFC= total flavonoid content; TPC= total phenol content, DPPH = DPPH scavenging value (%), Mixture= Ethanol:water:acetone (60:20:20 v/v/v)
[a] Expressed as g of dry extract per g of lyophilized TT leaves powder.

For, the DPPH radical quenching activity, hot water extract exhibited the highest DPPH scavenging value (90.95%) followed by the ethanol extract (77.61%). Moreover, hot water extract contained high level of TPC (126.34 mg GAE/g) and the extraction yield (55.7%). This suggests that the TT leaves juice contains soluble phenolic compounds, resulting in a high level of DPPH radical scavenging activity, as indicated in Table 1. The result is evidence that acetone is not a suitable solvent for polyphenol extraction from TT leaves; it gave the lowest extraction yield, lowest total phenolic content and least effective DPPH scavenging activity.

3.2. Characterizing Phenolic Compounds of Lyophilized TT Leaves Juice Powder by HPLC/DAD/MS

We further investigated the individual phenolic compounds, including the flavonoid contents and chlorophyll contents, of lyophilized TT leaves juice powder using HPLC. The results are shown in Table 2. The retention times of the phenolic compounds tested are also presented in Table 1S (supplementary data). The chromatograms of the identified phenolic compounds showed that lyophilized TT leaves juice powder contains a total of phenolic compounds approximately 3,938 mg/kg. Phenolic acid, including tannic acid and gallic acid, is the major phenolic compound in the leaves juice powder which is totally 1,255.4 mg/kg of lyophilized sample. Of all the 7 flavonoid standard agents tested (see Materials and Methods), we found only 4 flavonoids that included rutin, isoquercetin, catechin and quercetin, with the rutin being the major one (1,762.1 mg/kg), followed by isoquercetin (488.1 mg/kg) and quercetin-3-O-glucoside. Numerous epidemiological studies have reported the antioxidant properties of phenolic compound [29,30,31] although their antioxidant capacities are based on their bioavailability and absorption. Most studies on the antioxidant properties of phenolic compound focused on flavonoids. Flavonoids are classified into the 7 major subgroups by their chemical structure including flavanols, flavanones, flavonols, flavones, flavanonols, isoflavones, and anthocyanins [32,33]. Apart from their antioxidant properties, they have got considerable attention due to other health promoting benefits for antiviral, antiallergic, antiplatelet, antiinflammatory, and antitumor activities [34].

By spectrophotometric method, the result revealed that lyophilized TT leaves juice powder is a rich source of chlorophyll compounds with a total chlorophyll content of 3,551.6 mg/kg. Chlorophyll a, (blue–green), (2,421.4 mg/kg lyophilized sample) is the major form of chlorophyll in the sample with chlorophyll b (yellow–green) being present in lesser amounts. Beside of antioxidant activity, natural chlorophyll and its derivatives have exhibited numerous biological activities including antimutagenic activity, modulation of xenobiotic metabolism, and initiation of apoptosis [35].

Table 2. Phenolic profile and Chlorophyll levels of juice from *Tiliacora triandra* leaves

Compounds	Contents	The retention times (min) of phenolic compounds by HPLC/DAD
Chlorophyll (mg/ kg lyophilized juice powder)		
Chlorophyll a	2,421.4 ± 212.4	
Chlorophyll b	1,132.6 ± 111.7	
Total Chlorophyll	3,551.6 ± 323.6	
Phenolic compounds (mg/ kg lyophilized juice powder)		
Tannic acid	1,213.0± 144.1	12.90
Gallic acid	42.4 ± 9.0	6.56
Rutin	1,762.1 ± 126.9	15.36
Isoquercetin	488.1± 14.3	16.72
Catechin	369.9±26.3	12.59
Quercetin	62.6±3.5	34.51

HPLC with detection limit of 5 ppm.

3.3. The Inhibitory Inflammation Effect of Lyophilized TT Leaves Juice Powder

3.3.1. Inhibition of LPS-induced Nitrite Production by TT Leaves Juice in Macrophages RAW 264.7 Cell

To investigate the inhibitory inflammation property of juice from TT leaves, we tested the effect of the lyophilized juice powder on amount of nitrite production in LPS-activated macrophages (Figure 1). The nitrite concentration of RAW 264.7 cells in the culture medium were examined after the co-treatment with LPS (100 ng/mL) and numerous concentrations of lyophilized TT juice as shown in Figure 1. The sample at the concentration of 500 µg/mL did not affect the reaction between nitrite and Griess reagents (data not shown) and the juice, at a concentration of 25–500 µg/mL, significantly suppressed LPS induced nitrite production in a dose-dependent manner. The lyophilized TT leaves juice inhibited nitrite production by maximum of approximately 56% at 500 µg/mL. MTT assay also indicated that the inhibitory property on nitrite production was not toxic (Figure 2).

3.3.2. Suppression of Lipopolysaccharide-induced iNOS and COX-2 Expression

iNOS and COX-2 are key enzymes for the production of nitrite, and therefore we further investigated, by Western blotting analysis, whether or not the lyophilized TT leaves juice powder affect the level of LPS-induced iNOS and COX-2 proteins. Figure 3 demonstrated that iNOS and COX-2 proteins were undetectable in the unstimulated RAW 264.7 cells. The protein expression of iNOS and COX-2 markedly increased after treatment with LPS for 24 hr, while the co-treatment with sample strongly suppressed both iNOS and COX-2 protein levels: this was shown to be concentration-dependent. These data indicate that translational events are involved in the down regulation of iNOS and COX-2 expression by TT leaves juice. This result was consistent with the reduction of nitrite in culture media shown in Figure 1. These data suggest that the phytochemicals including phenolic acid, flavonoids and chlorophyll in the TT leaves juice exerts anti-inflammatory activity.

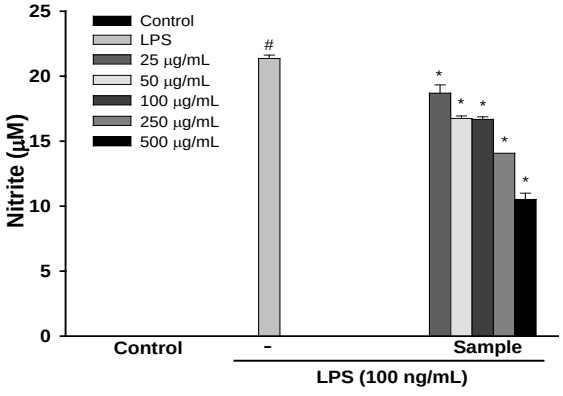

Figure 1. Effects of lyophilized TT leaves juice powder on LPS-induced nitrite production. RAW 264.7 cells were exposed to various concentration of sample with LPS (100 ng/mL) for 24 h. At the end of incubation time, 100 µL of the culture medium was collected for the nitrite assay using the Griess reagent. (*, P < 0.05 compared with the LPS treatment only; #, P < 0.05 compared with the control)

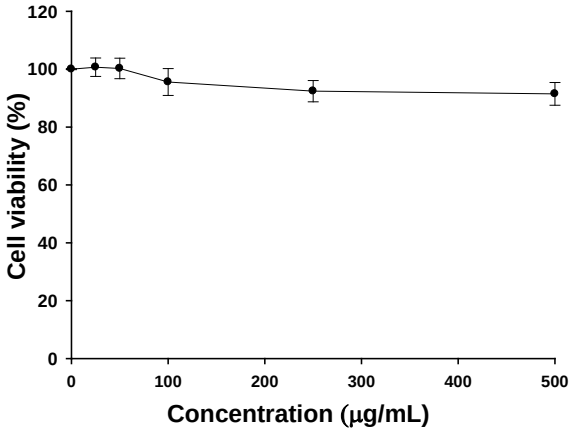

Figure 2. Effects of lyophilized TT leaves juice powder on the cell survival of RAW 264.7 cells. RAW 264.7 cells were treated with different concentrations of sample for 24 h. Viability of the cells was determined by MTT assay. RAW 264.7 cells were treated with 0.1% DMSO as vehicle control. Data were represented as means ± SE for three determinations

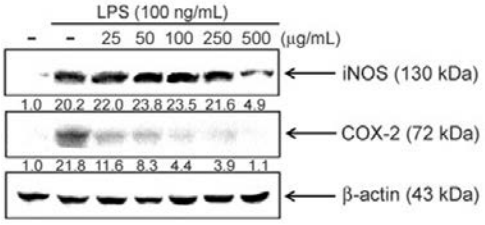

Figure 3. Effects of lyophilized TT leaves juice powder on LPS-induced iNOS and COX-2 protein expression in macrophage RAW 264.7 cells. Cells were treated with 100 ng/mL LPS only or with sample for 24 h. The total cell lysate were prepared and the levels of iNOS and COX-2 in cell lysates were analyzed by Western blotting. β-Actin was used as a loading control

4. Conclusions

T. triandra leaves juice contain different kinds of antioxidants such as phenolic compound and chlorophyll. The leaves juice as the lyophilized sample possesses high antioxidant activity. As well as being an antioxidant, the fruit juice exhibits inhibitory effects on inflammatory mediators, inducible nitric oxide synthase, and cyclooxygenase-2, suggesting anti-inflammation activity. As phenolic compounds and chlorophyll are responsible for the antioxidant, anti-inflammation, and antimutagenic activity, total phenolic and flavonoid contents may be used to predict the ability of TT extracts to scavenge free radicals and to decrease the inflammation process. TT leaves juice has high potential to be further developed as functional drink. The result suggests that increased consumption of TT juice extracted from leaves would seem to be of great health benefit to most consumers since it contains phenolic compounds and chlorophyll and *T. triandra* leaves is cheap and plentiful.

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Statement of Competing Interests

The authors have no competing interests.

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Supplementary Data

Table 1S. The retention times of phenolic compounds by HPLC/DAD

Phenolic compounds	Retention time
Gallic acid	6.56 min.
Catechin	12.59 min.
Tannic acid	12.90 min.
Rutin	15.36 min.
iso-Quercetin	16.72 min.
Hydroquinin	22.93 min.
Eriodictiol	31.37 min.
Quercetin	34.51 min.
Apiginin	42.24 min.
Kampherol	43.53 min.