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Original Research Article

Anti-inflammatory, antioxidant and quinone reductase inducing effects of Lumyai Thao (*Dimocarpus longan* var. *obtusus*) seed extract

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ABSTRACT

Dimocarpus longan var. *obtusus*, commonly called Lumyai Thao (LT) is a scandent shrub belonging to Sapindaceae family. LT is the local longan cultivar planted in Chonburi province and found only in Thailand. LT seeds have been used as traditional medicines among local Thai people to relieve swelling. However, there is still little information of their biological activities including anti-inflammation and antioxidant activities. The objective of this study was to investigate in vitro anti-inflammatory, antioxidant and cancer preventive effects of crude extract of LT seeds. Dried LT seeds were sequentially extracted with ethyl acetate, ethanol, and water. The extracts were then evaluated for their anti-inflammation using nitric oxide (NO) inhibitory effect in lipopolysaccharide (LPS)-activated RAW 264.7 cells. The results showed the IC50 of ethyl acetate, ethanol, and water extracts of LT seeds were 957.8±49.9, 638.2±15.7 and 465.9±15.7 µg/ml, respectively in a dose dependent manner and no cell toxicity was detected. The crude extracts of LT seeds were analyzed for their antioxidant activities using DPPH and FRAP assays. LT water extract showed the highest potency of antioxidant activity. Furthermore, LT water extract exhibited the induction of quinone reductase (QR) in cultured Hepa1c1c-7 (mouse hepatoma) cells with CD value of 389.8±64.5 µg/ml. HPLC analysis tentatively identified gallic acid as a major constituent of LT extract. This finding may provide the basis for the purported anti-inflammatory, antioxidant and anti-cancer effects of LT seed.

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INTRODUCTION

Cancer is a major public health issue and a cause of mortality in many countries (Ferlay et al., 2015). Carcinogenesis is a multiple-step process, typically characterized by three stages: initiation, promotion and progression (Moolgavkar, 1978).

Inflammation is an essential component of immune-mediated protection against pathogens, infection, and tissue damage. However, long-standing inflammation or chronic inflammation may also be a contributing factor for some diseases including cancer (Mantovani et al., 2008). Macrophages play an important role in inflammatory diseases through the release of cytokines and pro-inflammatory enzymes factors such as inducible NOS (iNOS) and cyclooxygenase (COX-2). In mammalian cells, iNOS and COX-2 are responsible for elevated levels of nitric oxide (NO) and prostaglandins E_2 (PGE2), respectively (Hseu et al., 2005; Vane et al., 1994). During the inflammatory process, NO accumulation increases and becomes cytotoxic to the cells by reacting with superoxide anion to produce peroxynitrite (ONOO-) which induce oxidative damage in the host tissue by modifying several cellular targets (Sautebin, 2000). Induction of iNOS has been shown in several cell lines after exposure with lipopolysaccharide (LPS) (Patel et al., 1999). Therefore, inhibition of nitric oxide production is a potential important approach to study for anti-inflammatory activities.

Quinone reductase or QR (EC 1.6.99.2) is a representative of Phase II detoxifying enzymes and is widely distributed in mammalian tissues (Deller et al., 2008). This enzyme is a broadly distributed FAD-dependent flavoprotein that catalyzes the two-electron reduction of a wide variety of these quinone compounds to hydroquinones, which can be inactivated by glucuronidation (Prochaska and Santamaria 1988). QR utilizes both NADH and NADPH as electron donors. Therefore, this enzyme is useful to protect the cells against quinone-mediated redox cycling. In addition to its antioxidant effect, QR is also involved in cancer prevention as a phase II detoxifying enzyme (Radjendirane et al., 1998). Therefore, QR is often used as a phase II enzyme biomarker for the screening of potential chemoprotective effect against the initiation stage of cancer (Song et al., 1999)

Dietary vegetables and fruits have attracted increasing attention as contributors to chemopreventive dietary strategies for reducing cancer according to their low toxicity and high diversity of their chemical structures (Crowell, 2005). Specific phytochemicals for example; resveratrol from grapes, lycopene from tomatoes, polyphenol from tea and sulforaphane from broccoli have been shown to have beneficial effects including anti-inflammatory, anticancer and antioxidant activities (Pan and Ho, 2008).

Longan (*Dimocarpus longan*) is a tropical fruit belonged to Sapindaceae family. It is widely cultivated in Thailand, Vietnam and China and popularly consumed among Asian people. The family Sapindaceae that has been found in Thailand can be divided into two species based on various characteristics such as seed, stem and fruit (Subhadrabandhy, 1990) which are *Dimocarpus longan* Lour. and *Dimocarpus longan* var. *obtusus*. *Dimocarpus longan* Lour. has been reported to have biological properties such as anticancer activities, anti-inflammation and antioxidant (Huang et al., 2012; Rangkadilok et al., 2007). Previous reports found that dried *Dimocarpus longan* Lour. seed contained high level of phenolic compounds compared to peel and pulp. The major components were gallic acid, corilagin and ellagic acid (Rangkadilok et al., 2005). No adverse effects of longan seed have been reported in the literature so far. The seed extract might be safe to use as a herbal medicine due to no significant toxicological effect of oral administration has been found in acute and repeated dose (4 and 13 weeks) (Worasuttayangkurn et al., 2012). *Dimocarpus longan* var. *obtusus*. or Lumyai Thao (LT) is the creeping plant or scandent shrub with 6 m tall found only in Thailand (Subhadrabandhy, 1990). People usually grow this tree for ornamental. The fruit tastes sweet with a small black and brown seed. In previous studies, *D. longan* var. *obtusus* seed water extract contained high total phenolic compound and exhibited a potential antioxidant activity and scavenging effect (Nitteranon et al., 2018). These LT seeds might be a usable source of natural phenolic antioxidant and anti-inflammation. The aims of the present study were to investigate the effects of LT seeds, including their bioactive compounds on in vitro anti-inflammatory, antioxidant and QR-inducing activities.

MATERIALS AND METHODS

Chemicals and reagents

Folin-Ciocalteu's phenol reagent was purchased from Loba Chemie (Mumbai, India). 2,2-Diphenyl-1-picrylhydrazyl (DPPH), methylthiazolyl diphenyltetrazolium bromide (MTT), lipopolysaccharide (LPS), 2,4,6-Tris(2-pyridyl)-s-trizaine (TPTZ), and gallic acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Sodium carbonate, dimethyl sulfoxide (DMSO) were obtained from Fisher Scientific (Pittsburgh, PA, USA). HPLC-grade ethyl acetate (EtOAc), ethanol (EtOH) and methanol (MeOH) were purchased from Merck (Darmstadt, Germany). Dulbecco's modified Eagle's (DMEM) medium, fetal bovine serum (FBS), L-glutamine, 0.5% trypsin-EDTA and penicillin/streptomycin were obtained from Gibco (Grand Island, NY, USA). All chemicals and reagents used in the study were of analytical grade.

Preparation of LT seed extracts

LT fruits were obtained from Taladlang community, Chonburi, Thailand. The fruits were washed, peeled and dissected seeds. LT seeds were washed and dried using tray dryer (Since OFM 1997, Thailand) at 50ºC for 72 hours. Then dried seeds were ground into a fine powder using a hammer mill through a 25-mm sieve (Schmersal, Germany). Dried LT seeds powder (~50 g) was first extracted with ~500 ml EtOAc. The residue was subjected to further sequential extracts with EtOH, and finally aqueous extraction at 40^{oC} for 1 hour. For each extract, solvent was removed by vacuum rotary evaporation (Büchi, Switzerland) or lyophilization (Christ, Germany) to obtain extracted dry matter.

Determination of total phenolic content (TPC)

The content of total phenolic was measured spectrophotometrically using Folin-Ciocalteu colorimetric method as followed by Kaur and Kapoor, 2002. In brief, a 0.25 ml aliquot of Folin-Ciocalteu's phenol reagent was added to a test tube containing 0.2 ml of appropriately diluted sample extract. A 2.5 ml of saturated Na_2CO_3 (7% w/v) was added to the mixture. Then, 5.8 ml of ddH2O was added and the mixture was incubated at room temperature for 25 minutes. The absorbance of the mixture was measured at 725 nm using a UV-VIS spectrophotometer (Shimadzu, Japan). All data was determined in triplicate, and the results are expressed as mg gallic acid equivalent (GAE)/g of extract.

Determination of DPPH radical scavenging activity (DPPH assay)

Antioxidant activity was measured using DPPH radical scavenging method (Braca et al., 2001). 30 µl of sample extracts were added to a 3 ml of 0.004% DPPH solution in MeOH. The mixture was incubated for 30 minutes in the dark at room temperature. The absorbance of the reaction mixtures were measured at 517 nm using Spectra MR microplate reader (Dynex Technologies, USA). The experiment was performed in triplicate. The samples were quantified as mg gallic acid equivalent (GAE)/g of extract.

Determination of ferric reducing antioxidant power activity (FRAP assay)

FRAP assay was performed as previously described (Benzie and Strain, 1996). A 0.1 mL aliquot of each extract was mixed with 3 mL of FRAP reagent (300 mM acetate buffer, pH 3.6, 10 mM TPTZ in HCl and $FeCl_3.6H_2O$). After 15 minutes incubation at room temperature in the dark, the absorbance was spectrophotometrically recorded at 593 nm. The results were expressed as mg gallic acid equivalent (GAE)/g of extract.

HPLC analysis

Water crude LT seed extract were profiled by analytical HPLC (Shimadzu, Japan) using 150 × 4.6 mm, 5 µM reversed phase column, Intersil® ODS-3 column (GL Sciences Inc, Japan) generally using a previously reported protocol (Rankgadilok et al., 2012). The mobile phase consisted of 0.1% formic acid in water (eluant A) and methanol (eluant B) at a flow rate of 1.0 ml/min. The temperature of the column was 25°C with the UV detection at 270 nm. The injection volume was 10 µl. The gradient system started with 4%B at 0 minute to 80%B in 27 minutes. The total run time was 30 minutes. Retention time of gallic acid was 6.2 minutes.

Cell culture

Mouse macrophage cells (RAW 264.7), a commonly used model for inflammatory processes, obtained from ATCC (Rockville, USA) were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, USA) supplemented with 10% fetal bovine serum (FBS) with 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco, USA). The cells were grown in a humidified incubator at 37°C under 5% CO_2. Cultured murine hepatoma cells (Hepa 1c1c-7 cells) (ATCC, USA) were grown in α-minimal essential medium (MEM; Gibco, USA) supplemented with heat-inactivated FBS, 100 U/ml of penicillin and 100 µg/ml of streptomycin at 37°C in humidified condition under 5% CO_2.

Determination of nitric oxide (NO) in RAW 264.7 cells

Mouse macrophage (RAW 264.7) cells were cultured in DMEM phenol red-free medium in 96-well plates at 5 × 10^3 cells per well for 24 hours according the method of Nitteranon et al. (2011). The medium was then replaced with fresh DMEM containing either 1 µg/ml lipopolysaccharide (LPS) alone or LPS with various concentrations of test compounds (62.5-1000.0 µg/ml) and the cells were incubated for another 24 hours. NO was measured as nitrite in the culture supernatant using the Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphtylethylene diamine dihydrochloride) with absorbance at 542 nm determined using an optical microtiter plate spectrophotometer (Dynex Technologies, USA). Results were expressed as % inhibition relative to control. The level of isolated required to inhibit NO evolution by 50% was defined as an IC50 value and was interpolated from the dose response results.

Determination of cell viability

Cell viability was evaluated using MTT assay according to the method as previously described (Levitz and Diamone, 1985). Briefly, RAW 264.7 macrophage cells were seeded in 96-well plates at 5 × 10^3 cells per well for 24 hours. The medium was then replaced with fresh DMEM containing either 1 µg/ml LPS alone or LPS with the presence of EtOAc, EtOH and Water LT seed extract (62.5-1000.0 µg/ml) for 24 hours. At the end of incubation period, 100 µl of an MTT solution (0.1 mg/ml in PBS buffer) was added to each well and the plate was further incubated at 37°C for 4 hours. DMSO (200 µl) was added to each well to dissolve tetrazolium dye and after 15 minimes of agitation at 37°C, the absorbance was determined at 570 nm using microplate reader.

Determination of quinone reductase (QR) inducting activity in Hepa1c1c-7 cells

Induction of QR specific activity was used as a biomarker for phase II enzyme as described earlier (Prochaska and Santamaria, 1988). Murine Hepa 1c1c-7 cells at 5000 cells/well were plated in duplicate 96-well plates containing α-MEM (Gibco, USA) supplemented with 10% FBS and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin) for 24 hours at 37°C in 5% CO_2 in air. The medium was then replaced with fresh α-MEM medium containing test compound, and the cells were incubated for an additional 48 hours.

After induction, one plate of cells was used for QR activity assay. The cells were lysed by adding 50 µl of 0.08% digitonin. QR activity was determined after adding 200 µl of a standard cocktail assay. Reduction of tetrazolium dye was continuously measured over 10 minutes period in absorbance at 490 nm using microplate reader. A duplicate plate was used for cell protein measurement using crystal violet assay and measuring absorbance at 610 nm. Relative QR specific activity was calculated as the following: [ΔAbs490nm for QR assay ÷ Abs610nm for protein assay], with the ratio for control cells set to 1.0. The concentration required to double specific QR activity (CD value) was used as an indicator of inducer potency. All samples were performed in triplicate.

Statistical analysis

All experiments were performed at least triplicate. The results were expressed as means ± standard deviation (SD). A one way analysis of variance (ANOVA) was used to calculate significant differences, and multiple comparisons of means were assessed by Duncan's Multiple Range Test (DMRT) using the statistic software SPSS version 11.5. $P < 0.05$ was considered significant

Results and discussion

Total phenolic content (TPC) and antioxidant activity of LT seed extracts

The purpose of this study was to evaluate TPC and *in vitro* antioxidant capacities of crude extract (EtOAc, EtOH and Water) from LT seeds using DPPH radical-scavenging and FRAP assays. As shown in Table 1, it can be clearly seen that water extract of LT seed had the highest % yield after extraction with dark brown color.

Table 1. %Yield, total polyphenols and antioxidant capacities of LT seed extracts.

LT seed extracts	% yield	Total phenolic contents (mg GAE/g of extract)	Antioxidant capacities	
			DPPH (mg GAE/g of extract)	FRAP (mg GAE/g of extract)
EtOAc	6.02±1.12[b]	1.16±0.09[a]	0.40±0.04[a]	0.33±0.01[a]
EtOH	3.42±0.89[a]	2.24±0.09[b]	1.50±0.02[b]	0.78±0.07[b]
Water	20.32±1.26[c]	17.04±0.53[c]	10.18±1.26[c]	8.56±0.46[c]

Values are expressed as means ± SD (n=3).
Different letters in the same column indicate significant difference at $p < 0.05$.

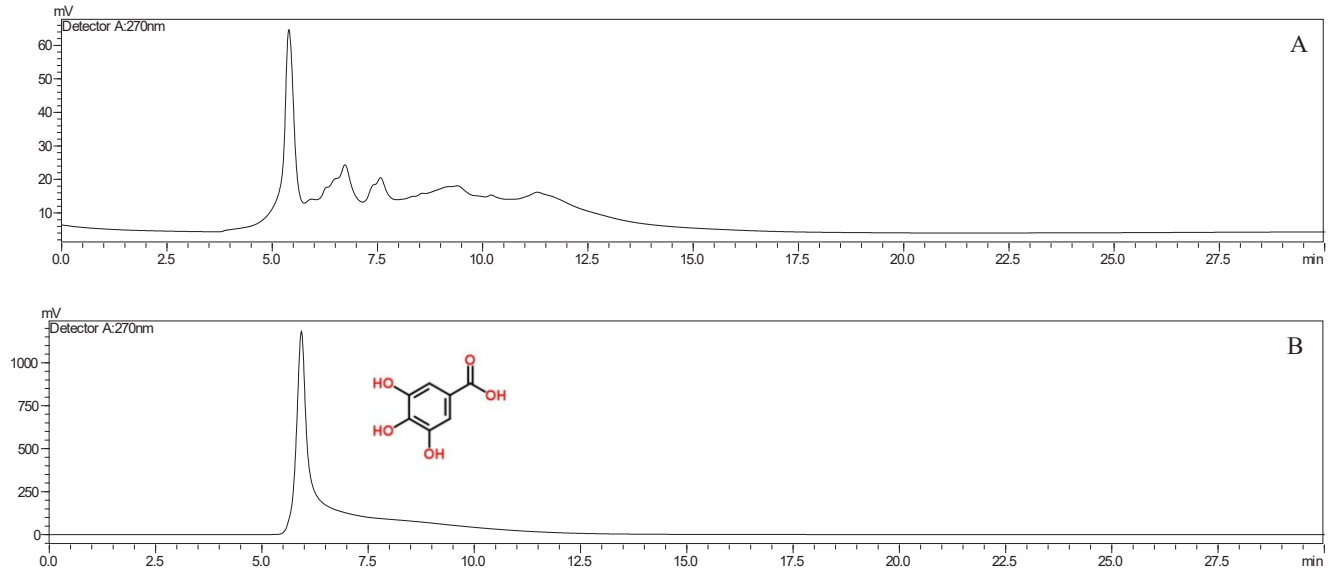

Figure 1. HPLC chromatograms of A) Water extract of LY seeds and B) gallic acid as a standard

Furthermore, the strongest of scavenging potencies was shown in water LT seed extract. The lowest scavenging capacity was observed in EtOAc extract of LT seed. These results were in agreement with the previous finding that *Dimocarpus longan* Lour. seed water extract contained the highest yield of 20.32±1.26% as well as exhibited highest total phenolic content and DPPH-radical scavenging activities (Chindaluang and Sriwattana 2014).

The FRAP assay is the method to measure total antioxidant content, based on the reduction of ferric (Fe^{3+}) to ferrous (Fe^{2+}) ions at low pH which causes a formation of a colored ferrous-tripyridyltriazine complex (Benzie and Strain, 1996). Water extract from LT seeds had the highest total antioxidant content which was similar to the result of DPPH method (Table 1). There was a strong relationship between the amount of total phenolic compounds and antioxidant activities in LT seed extract. Fruit seeds are known to contain several phenolic compounds to protect them from oxidative damage and microorganisms such as fungi, yeast and bacteria that might restrain their germination (Soong and Barlow, 2005). The simple phenolic substances including monophenols with a single benzene with hydroxyl group were found in fruits and seeds (Gülcin, 2012). A previous study demonstrated that *Dimocarpus longan* Lour. seed water extract contained several phenolic compounds such as gallic acid, corrilagin and ellagic acid (Rangkadilok et al., 2005). Especially, gallic acid, the phenolic compound that has high polarity, has been shown strong antioxidant effect (Zheng and Wang 2001). It is also a major component in plants having anti-cancer properties (Saeki et al., 2000).

HPLC analysis

Figure 1 presents the chromatograms of water LT seed extract. The findings indicated the major active compound was gallic acid by comparison with the retention time of standard gallic acid (retention time = 6.1). The contents of gallic acid of water extract of LT seed were 13.82 ± 1.82 mg/g DW. HPLC chromatogram of gallic acid in seed extract was similar to previous study of Rangkadilok et al. (2012). Therefore, gallic acid might partially contribute to the DPPH-radical scavenging and antioxidant activities of LT seeds. However, there were other peaks present in the extract that need to be clarified.

NO inhibiting activity and cell viability of LT seed extracts

Nitric oxide (NO) is the mediator of chronic inflammation in macrophage cells. This study used EtOAc, EtOH and Water extracts from LT seeds to test for NO inhibiting activity in LPS induced RAW 264.7 macrophage cells. Figure 2 shows that water LT seed extract had the most potent in inhibiting NO production in a concentration-dependent response with IC_{50} of 465.94±15.67 µg/ml, followed by EtOH LT seed extract with the IC50 of 638.20±15.73 µg/ml and the EtOAc extract was the least potent with the IC50 of 957.48±15.73 µg/ml ($P<0.05$). Kunworarath et al., 2016 studied the extraction from flower, seed and pulp of *Dimocarpus longan* Lour. It was clear that flower extract showed the highest ability of inhibiting NO production in LPS-activated RAW 264.7 cells, followed

by seed and pulp with the IC50 of 128.2, 1127.4 and 1260.2 µg/ml, respectively. The mechanisms were underlying the inhibition of iNOS enzyme activity via the suppression of NF-κB and AP-1 transcription factors (Kunworarat et al., 2016). It has been indicated that gallic acid inhibited histamine release and pro-inflammatory cytokine production in mast cells (Kim et al., 2006). Gallic acid also suppresses LPS-induced NF-κB signaling in A549 lung cancer (Choi et al., 2009). Normally, LT seeds were traditionally used for swelling treatment and stomach pain. This is the scientific evidence to support the use of LT seeds to treat inflammatory-related diseases.

RAW 264.7 macrophage cells were treated with various concentrations (62.5-1000.0 µg/ml) of test samples for 24 h. As shown in Figure 3, more than 90% of RAW 264.7 cells were viable. All crude extracts from LT seeds with the concentration of did not induce cell toxicity in comparison to control cells.

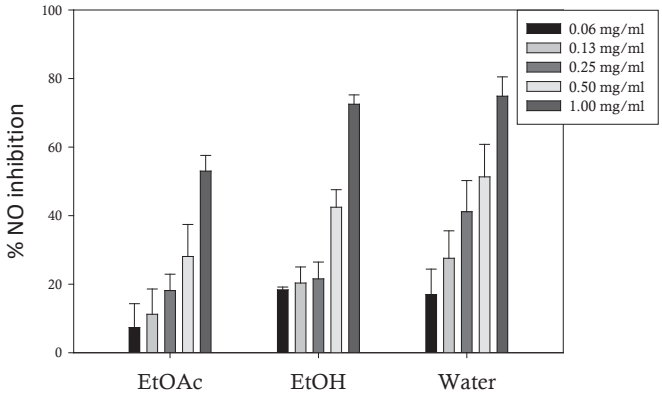

Figure 2. % NO inhibition of LPS-stimulated RAW 264.7 cells treated with 0.06-1.00 mg/ml of EtOAc, EtOH and Water extracts of LT seeds.

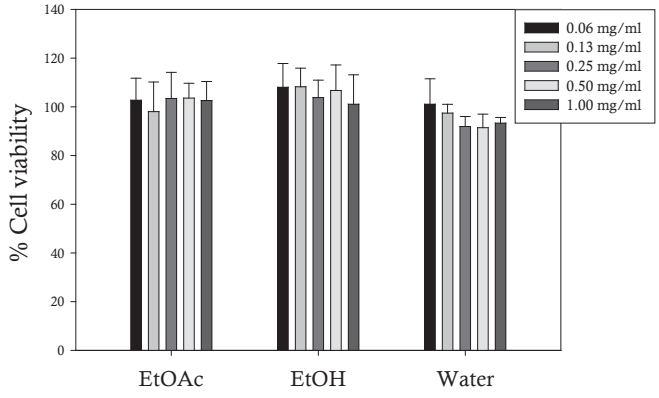

Figure 3. % Cell viability of LPS-stimulated RAW 264.7 cells treated with 0.06-1.00 mg/ml of EtOAc, EtOH and Water extracts of LT seeds.

QR inducing activities of LT seed extracts

The induction of Phase II enzymes or detoxifying enzymes has an important role to protect the cells against oxidative stress. In this study, EtOAc, EtOH and Water extracts were treated in Hepa1c1c-7 cells to test for the induction of quinone reductase (QR) enzyme activity. The results showed that Water extract of LT seeds could induce QR activity with the CD value (concentration required to double specific activity) of ~350 µg/ml whereas EtOAc and EtOH extracts could not induce QR activity (Figure 4A). Cell viability of Hepa1c1c-7 treated with all samples was maintained at > 50 % (data not shown). Standard gallic acid could induce QR activity with the CD value of ~240 µg/ml (Figure 4B). A transcription factor, Nrf2 has been shown to play an essential role in the antioxidant response element (ARE)-mediated expression of phase II detoxifying enzymes and stress-inducible genes including heme oxygenase-1 (HO-1), glutathione S-transferase (GST) and NAD(P)H:quinone oxidoreductase (QR or NQO1). Gallic acid has been shown to contain the highest potency of increasing Nrf2/ARE activity and up-regulating the expression of QR (Liu et al., 2018). Therefore, phenolic compound in water crude extract of LT seeds could be partially responsible for QR inducing activities in Hepa1c1c-7 cells. However, further efforts should be focused on isolation and identification of other bioactive compounds that present in LT seed.

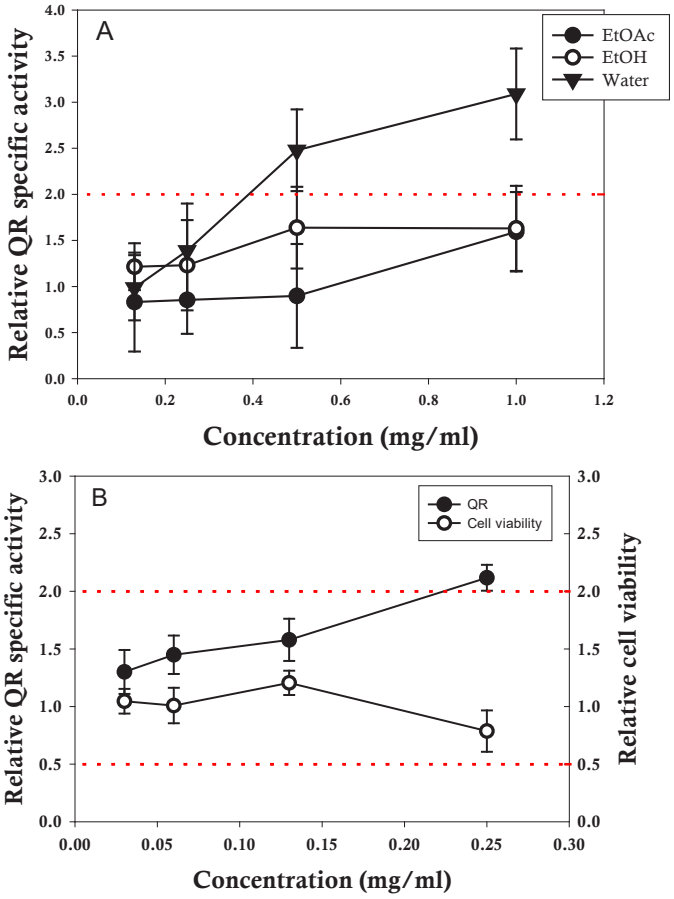

Figure 4. Induction of QR specific activity in Hepa1c1c-7 cells treated with A) EtOAc, EtOH and Water extracts of LT seeds and B) standard gallic acid

CONCLUSIONS

In this study, *Dimocarpus longan* var. *obtusus* or Lumyai Thao (LT), a relative species of *Dimocarpus longan* Lour. was examined for their biological activities. Water extract of LT seeds contained the highest level of antioxidant, anti-inflammatory and QR inducing activities. It might due to the presence of phenolic compounds in the extracts for example gallic acid. Here is the first study of LT seeds which might be used as health-beneficial compounds rather than discarded as waste. However, study of antioxidant, inflammatory inhibitor and cancer prevention should be further investigated in animal models to optimize utilization of the LT seeds in pharmaceutical and food products.

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