Original Article

Proven Inhibitory Effect on Lipopolysaccharides-Induced Nitric Oxide Production of *Zanthoxylum rhetsa* under Accelerated Condition and Stability Affecting Factors

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Abstract

Introduction: *Zanthoxylum rhetsa* preparations were used in ethnomedicine for the treatments of muscle and tendon spasms. The essential oil from its fruit has been reported for its anti-inflammatory activity and previous study has shown that the hexane extract of *Z.rhetsa* pericarp showed highest *in vitro* anti-inflammatory effect. Nitric oxide (NO) is an inflammatory mediator associated with osteoarthritis. Therefore, *in vitro* production inhibition of NO in RAW264.7 macrophages was performed. The accelerated stability test and the forced degradation test were studied to estimate storage life and to ensure appropriate conditions for its activity.

Methods: The hexane extracts obtained from the pericarp (PHE), fruit (FHE) and seed (SHE) were tested for production inhibition of LPS-induced NO in RAW264.7 macrophages. The active extract was treated with accelerated storage conditions and stress conditions and assessed for the LPS-induced NO production inhibition.

Results: PHE showed the most potent activities on NO (IC_{50} 11.99 ± 1.66 µg/ml) production inhibition but less active than prednisolone. After accelerated stability test, the activity of PHE was less stable than 30 days. Forced degradation on PHE revealed that oxidation condition did not affect NO production inhibition while moisture, heat and alkaline conditions weakened its activity. The acidic condition rendered PHE to be inactive.

Conclusion: PHE was the most active fraction for NO production inhibition associated with osteoarthritis. Its estimated shelf-life was between 9 to 12 months at room temperature. However, moisture, heat, alkaline, and acid conditions should be avoided in order to maintain its activity.

Keywords: *Zanthoxylum rhetsa,* Nitric oxide, Osteoarthritis, Stability, Forced degradation

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Introduction

Osteoarthritis occurs most often in the elderly. It is a degenerative joint disease with an important characteristic of the deterioration of cartilages.[1] Thailand has become an aging society since 2007. The percentage of older adults has been rising continuously at 16.70% in 2017.[2, 3] The inflammatory mediator nitric oxide (NO) are associated with pain, swelling and limited movement of knee joint causing pain in older people with osteoarthritis.[4] Therefore, high levels of NO production in the synovium and cartilages are found in osteoarthritis.[5] NO produces peroxynitrite which is highly toxic to tissues and normal neighboring cells.[6] The high concentration of nitrite causes degradation of hyaluronan and collagen type II in chondrocytes,[7] causing pain.[8]

Zanthoxylum rhetsa is the medicament with a spicy flavor and intense aroma. It is a member plant in the Rutaceae family and distributes in northern Thailand, called *Ma-khwaen*.[9] Whole fruit consists of pericarp and seed. Fruits have been used as a food spice and as a medicament in Pa-Ra-Ti-Tri oil and Bee-Pra-Sen ointment recorded in Pra-O-Sod-Pra-Narai scripture for reducing pain and swelling in musculoskeletal diseases.[10] Pericarp and fruit were also used to relax muscle and tendon according to Thai traditional household remedies.[11] Previous studies reported that fruit essential oil could inhibit COX-1 (70.40%) and COX-2 (88.90%),[12] inhibited swelling, redness and inflamed cells in the rat (P-value < 0.05)[13] and decreased pain score at calf muscle in volunteers (*P-value* < 0.05).[14] However, previous study revealed lower NO production inhibition activity of pericarp essential oil than pericarp hexane extract (PHE).[15] Moreover, PHE gave the strongest NO production inhibition[15], thus PHE appeared to be a promising active ingredient in osteoarthritis preparation. In pharmaceutical product development, the accelerated stability test is essential for shelf-life estimation and suggests appropriate drug product storage conditions to maintain its biological activity.[16] Forced degradation test is a necessary process in product development to determine suitable conditions for activity preservation of herb.[17] Forced degradation condition such as moisture hydrolysis, acid hydrolysis, alkaline hydrolysis, temperature degradation, and oxidation are important conditions in formulating herbal product and avoiding degrading conditions on biological activity.[17] There has been no study on the biological activity of the hexane extract from *Z.rhetsa* under accelerated storage conditions and forced degradation. Therefore, the inhibitory effect of *Z.rhetsa* hexane extracts on lipopolysaccharides (LPS)-induced NO production in RAW264.7 macrophages was performed. After accelerated stability and forced degradation tests to gain information on its shelf life and appropriate conditions.

Methods

Plant material

Z.rhetsa was collected from Chiang Rai province, Thailand and identified by the Department of National Park, Wildlife and Plant Conservation. The voucher specimen BKF193835 was stored at the office of the Forest Herbarium, Bangkok, Thailand.

Chemicals and reagents

Analytical grade dimethyl sulfoxide (DMSO), hexane, hydrochloric acid (HCl) and isopropanol were purchased from RCI Labscan (Thailand). Distilled water was produced by the Milli-Q water purification system from Millipore (USA). Sodium hydroxide (NaOH) was purchased from Univar (Australia). Hydrogen peroxide 30% (H_2O_2) was purchased from Merck (Germany). Griess reagent (1% sulfanilamide and 0.1% *N*-(1-naphthyl) ethylenediamine dihydrochloride in 2.5% phosphoric acid), thiazolyl blue tetrazolium bromide (MTT), lipopolysaccharide (LPS) from *E.coli* (O55:B5) and prednisolone were purchased from Sigma-Aldrich (U.S.A.). Fetal bovine serum (FBS), penicillin-streptomycin (P/S), RPMI 1640 medium were purchased from Gibco (U.S.A.).

Preparation of extracts

Z.rhetsa whole fruits were sun-dried and separated into three parts: pericarp, fruit and seed then ground to coarse powder. The powder of each part was extracted by maceration. For maceration: crude powder of each part (1 kg) was macerated with hexane (crude powder : solvent ratio as 1:2) for three days then filtered and repeated maceration twice with residue. All filtrates were dried by rotary evaporation to give hexane extracts from pericarp (PHE), fruit (FHE) and seed (SHE).

Cell culture and culture medium

RAW264.7 macrophages from mouse (*Mus musculus*) (ATCC®TIB-71) (USA) were cultured in RPMI 1640 medium supplemented with 10% FBS and 1% P/S (100 unit/ml). The cells were incubated in an incubator at 37 °C, 5% CO_2 and 95% humidity.

Biological activity testing

Determination of viable cells

The determination of viable cells was done in triplicate following the established protocol of MTT assay.[18] Briefly, the cells (1 x 10^5 cells/well) were seeded in 96 well-plate (100 µl/well) and incubated for 24 h. Subsequently, the medium was replaced with a fresh medium (100 µl/well) and treated with various concentrations of sample solution (100 µl/well) without LPS, incubated for 24 h. Subsequently, the supernatants (100 µl/well) were removed and added 10 µl/well of MTT solution (5 mg/ml), then incubated for another 2 h. After medium removal, isopropanol containing 0.04 M HCl (100 µl/well) was added to dissolve formazan in the cells. The absorbance was measured by microplate reader at 570 nm and viable cells higher than 70% compared with negative control (0.2% DMSO in final concentration). This demonstrated that the inhibitory activity of the tested samples without LPS was not due to cytotoxicity.[18] The percentage of viable cells were calculated by using the equation:

$$\text{The \% viable cells} = \left[\frac{\text{OD sample without LPS}}{\text{OD negative control without LPS}}\right] \times 100$$

where, OD = optical density; OD sample without LPS = mean of sample without LPS ODs; OD negative control without LPS = mean of control solvent (0.2% DMSO in final concentration) without LPS ODs

Inhibitory effect on LPS-induced NO production

Inhibitory effect on LPS-induced NO production was done in triplicate according to the established method.[18] Briefly, the cells (1x10^5 cells/well) were seeded in 96 well-plate (100 µl/well), then incubated for 24 h. Subsequently, the medium was replaced with a fresh medium (100 µl/well) without LPS and another was replaced with a fresh medium (100 µl/well) containing LPS (2 ng/ml final concentration), after that all medium containing LPS and without LPS were treated with various concentrations of tested samples (100 µl/well), then incubated for 24 h. An aliquot of the supernatant (100 µl/well) was transferred into another 96 well-plate and added Griess reagent (100 µl/well). The absorbance was measured by a microplate reader at 570 nm. The percentage of inhibition on LPS-induced NO production was calculated using the equation below and IC_{50} was calculated using the Prism program (CA, USA). Prednisolone was used as a positive control.

$$\text{The \% inhibition} = \left[\frac{\text{OD negative control - OD sample}}{\text{OD negative contro}}\right] \times 100$$

where, OD = optical density; OD negative control = mean of control solvent (0.2% DMSO in final concentration) containing LPS ODs – mean of control solvent (0.2% DMSO in final concentration without LPS ODs; OD sample = mean of sample containing LPS ODs – mean of sample without LPS ODs

Accelerated stability test

The stability test of the active extract was conducted according to the International Conference on Harmonization (ICH) Q1A (R2) for stability testing of drug substances[19] and an established protocol.[20] Active extract was exposed to accelerated storage condition of 40 ± 2°C with 75 ± 5% RH for six months. After a specified time, from day 0th to days 15th, 30th, 60th, 90th, 120th, 150th, and 180th, the treated samples were evaluated for the inhibitory effect on LPS-induced NO production in RAW264.7 macrophages, compared with active extract at day 0th, along with viable cells test by MTT assay. All tests were conducted in triplicate.

Forced degradation test

The forced degradation test on active extract was conducted in triplicate and according to the guidelines of the International Conference on Harmonization (ICH) Q1A (R2) on stability testing of drug substances[18] and an established protocol.[20] Active extract (5 x 50 mg) was subjected to five forced degradation conditions: 1) temperature degradation: heating at 80°C for 3 h, 2) moisture hydrolysis: adding 3 drops of DI water and heated

at 80°C for 3 h, 3) acid hydrolysis: adding 3 drops of 3N HCl and heated at 80°C for 3 h then neutralized by adding 3N NaOH, 4) alkaline hydrolysis: adding 3 drops of 3N NaOH and heated at 80°C for 3 h then neutralized by adding 3N HCl and 5) oxidation: adding 3 drops of 30% H_2O_2 and heated at 80°C for 3 h. All treated samples were tested for the inhibitory effect on LPS-induced NO production compared with the untreated sample. The MTT assay was used to determine viability of the cells.

Statistical analysis

Viable cells, percentage of inhibitory effect on LPS-induced NO production and IC_{50} were presented as mean ± standard error of means (SEM). The means of control and treatment groups were compared using one-way analysis of variance followed by Dunnett's Multiple Comparison Test. The means in treatment groups were compared by using an unpaired t-test. The level of significance was $P < 0.05$.

Results

Plant material extractions

The percentage yield of hexane extracts obtained from fruit (FHE = 6.51%) and seed (SHE = 8.09%) was higher than the hexane extract obtained from pericarp (PHE = 5.89%) (Table 1).

Biological activity

Determination of viable cells

The percentage of viable cells after exposure to the tested samples and prednisolone as a positive control was greater than 70% for the inhibitory effect on LPS-induced NO production (Figure 1).

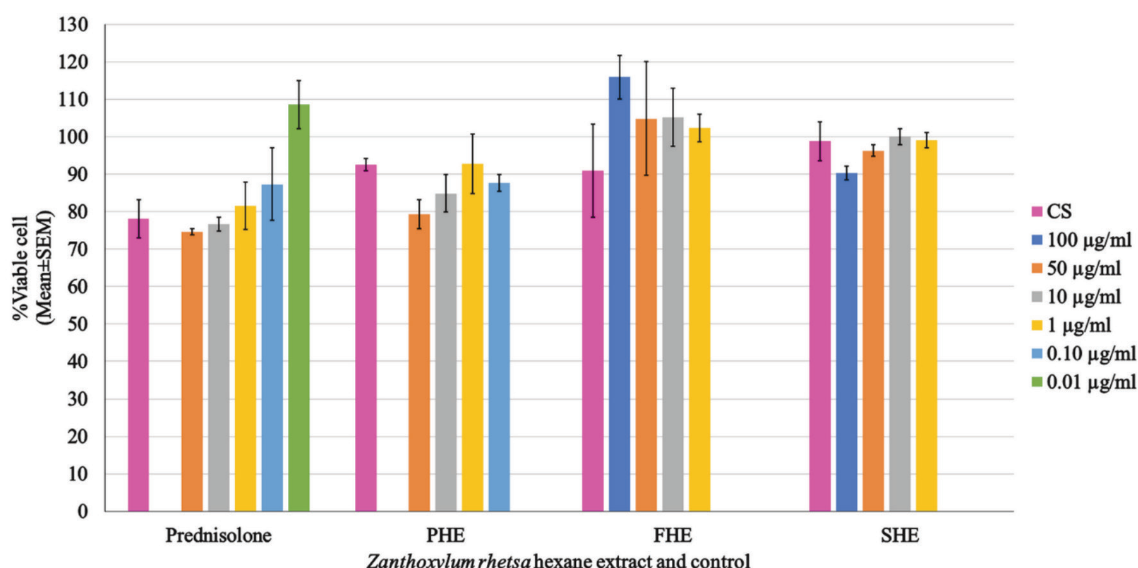

Figure 1 Percentage viable cells without LPS (n = 3). PHE, FHE, SHE: hexane extract from pericarp, fruit and seed *Zanthoxylum rhetsa*. Prednisolone as a positive control, CS: 0.2% DMSO final concentration as a solvent and negative control.

Inhibitory effect on LPS-induced NO production

PHE at 50 µg/ml and FHE at 100 µg/ml showed the highest percentage inhibition of NO production as 97.15% ± 0.37 and 91.55% ± 3.04, respectively which was not significantly different from prednisolone (96.82% ± 0.34) at 50 µg/ml. However, the IC_{50} of PHE (11.99 ± 1.66 µg/ml) and FHE (39.81 ± 0.53 µg/ml) were significantly different (*P*-value < 0.01 and *P*-value < 0.001, respectively) from prednisolone (0.07 ± 0.001 µg/ml or 0.19 ± 0.001 µM) while PHE was more active than FHE (*P*-value < 0.001). SHE was not active (IC_{50} > 100 µg/ml) (Table 1).

Table 1 Percentage yield, inhibitory effect and IC_{50} values of LPS-induced nitric oxide (NO) production in RAW264.7 macrophages of various *Zanthoxylum rhetsa* hexane extracts

Sample		Percentage yield	Percentage inhibition of negative control	Percentage of inhibition at various concentrations						IC_{50} (µg/ml)
Solvent	**Extract and positive control**			**100 µg/ml**	**50 µg/ml**	**10 µg/ml**	**1 µg/ml**	**0.10 µg/ml**	**0.01 µg/ml**	
0.2% DMSO	-	-	21.66 ± 2.92	-	-	-	-	-	-	>100
	PHE	5.89	-	-	97.15 ± 0.37	38.47 ± 8.69	-11.70 ± 3.94	-12.38 ± 3.61	-	11.99 ± 1.66**
0.2% DMSO	-	-	18.38 ± 0.95	-	-	-	-	-	-	>100
	FHE	6.51	-	91.55 ± 3.04	60.95 ± 0.84	8.69 ± 3.68	-5.20 ± 4.00	-	-	39.81 ± 0.53***, [a]
0.2% DMSO	-	-	17.88 ± 0.85	-	-	-	-	-	-	>100
	SHE	8.09	-	45.92 ± 1.91***	22.75 ± 1.03	-1.17 ± 5.23	-11.93 ± 8.02	-	-	>100***
0.2% DMSO	-	-	33.87 ± 1.35	-	-	-	-	-	-	>100
	Prednisolone	-	-	-	96.82 ± 0.34	89.32 ± 0.31	81.49 ± 6.98	72.90 ± 2.26	5.16 ± 1.25	0.07 ± 0.001 (0.19 ± 0.001 µM)

The results were shown as mean ± standard error of mean (SEM) (n = 3). IC_{50}: the half maximal inhibitory concentration; LPS: lipopolysaccharide; 0.2% DMSO mean 0.2% DMSO final concentration as a solvent and negative control; PHE, FHE, SHE: hexane extract from pericarp, fruit and seed *Zanthoxylum rhetsa*. **P-value < 0.01, ***P-value < 0.001 compared with prednisolone as a positive control; a mean different significantly statistic (P-value < 0.001) between PHE and FHE; (–) not tested.

Accelerated stability test

Due to PHE showed the highest inhibitory activity on LPS-induced NO production. Therefore, PHE was kept for six months under accelerated storage conditions. The extract was tested for stability and inhibitory effect on LPS-induced NO production in RAW264.7 macrophages at day 0 compared with those at days 15, 30, 60, 90, 120, 150 and 180. Results were shown in Figure 2, indicating that IC_{50} values of PHE on day 15 (14.63 ± 0.90 µg/ml) was not significantly different from day 0 (11.99 ± 1.66 µg/ml). However, the IC_{50} values on day 30 (19.94 ± 0.70 µg/ml) and day 60 (21.74 ± 0.85 µg/ml) were significantly different (P-value < 0.05 and P-value < 0.01, respectively) from day 0 while IC_{50} values at day 90 (24.86 ± 1.20 µg/ml), day 120 (30.86 ± 1.18 µg/ml), day 150 (36.76 ± 1.22 µg/ml), and day 180 (43.96 ± 3.05 µg/ml) were highly significantly different (P-value < 0.001) from day 0. Viable cells in all tests were greater than 70% (Figure 2).

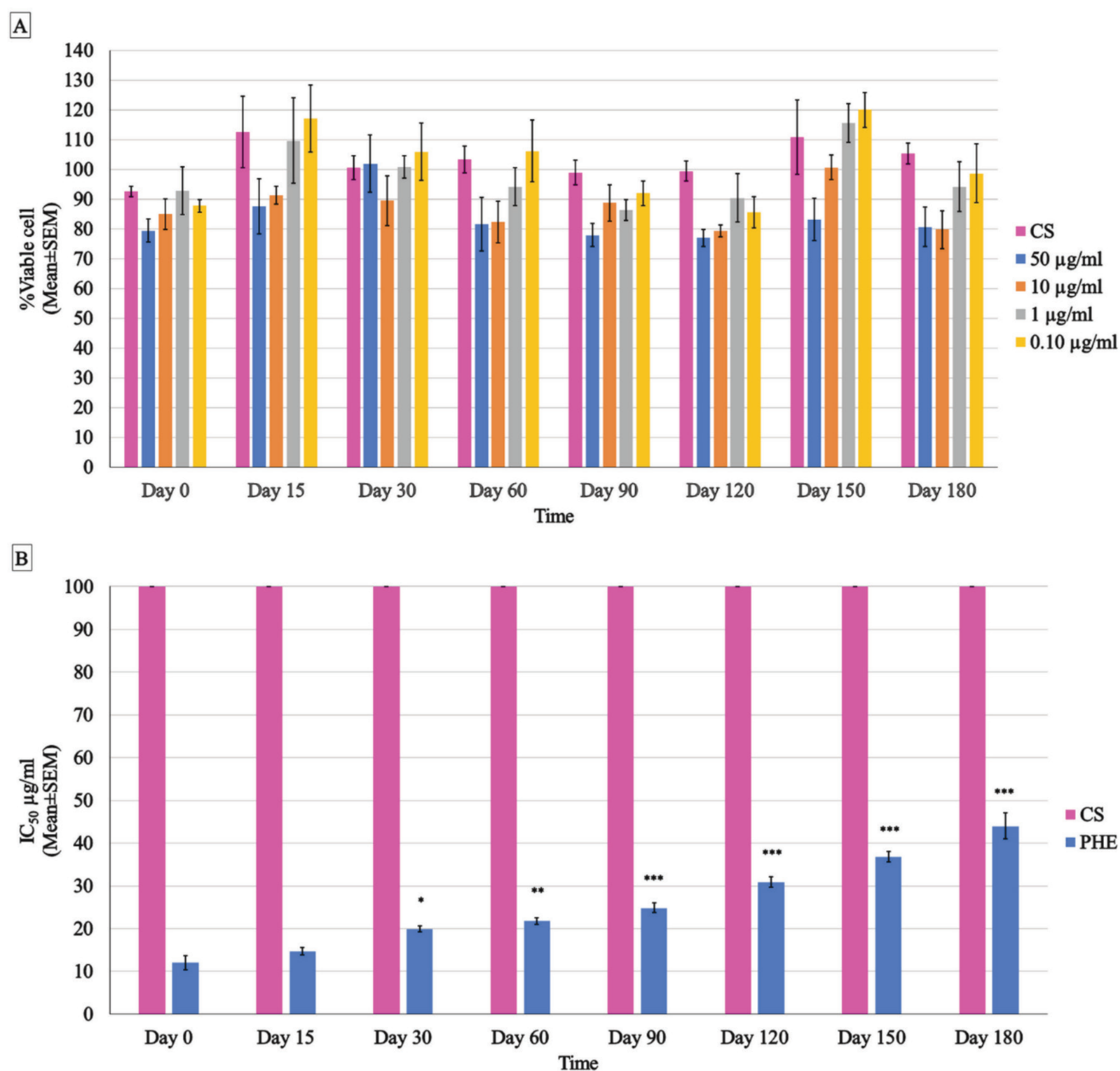

Figure 2 Accelerated stability test of hexane extract of *Zanthoxylum rhetsa* pericarp (PHE) (n = 3). Percentage viable cells without LPS (A) and inhibitory effect of lipopolysaccharides (LPS)-induced nitric oxide (NO) production (B). IC_{50}: the half maximal inhibitory concentration; CS: 0.2% DMSO final concentration as a solvent and negative control (IC_{50} yielded > 100 µg/ml). *P-value < 0.05, **P-value < 0.01, ***P-value < 0.001 compared with day 0 (PHE in normal condition).

Forced degradation test

Due to PHE showed inhibitory activity on LPS-induced NO production. Therefore, PHE was kept under forced degradation conditions, then tested for LPS-induced NO production inhibition. It was found that IC_{50} of PHE under oxidation condition (17.87 ± 0.61 µg/ml) was not significantly different from PHE kept under normal condition (11.99 ± 1.66 µg/ml). The values of IC_{50} of PHE under moisture hydrolysis (22.63 ± 3.97 µg/ml) were significantly different (P-value < 0.05) from PHE in the normal condition. Temperature degradation and alkaline hydrolysis yielded lesser activity (IC_{50} 27.64 ± 1.50 µg/ml and 38.44 ± 2.30 µg/ml, respectively) which was significantly different from PHE in normal condition (P-value < 0.001). Whereas PHE treated with acid hydrolysis lost its activity (IC_{50} > 100 µg/ml) (Figure 3). Viable cells in all tests were greater than 70% (Figure 3).

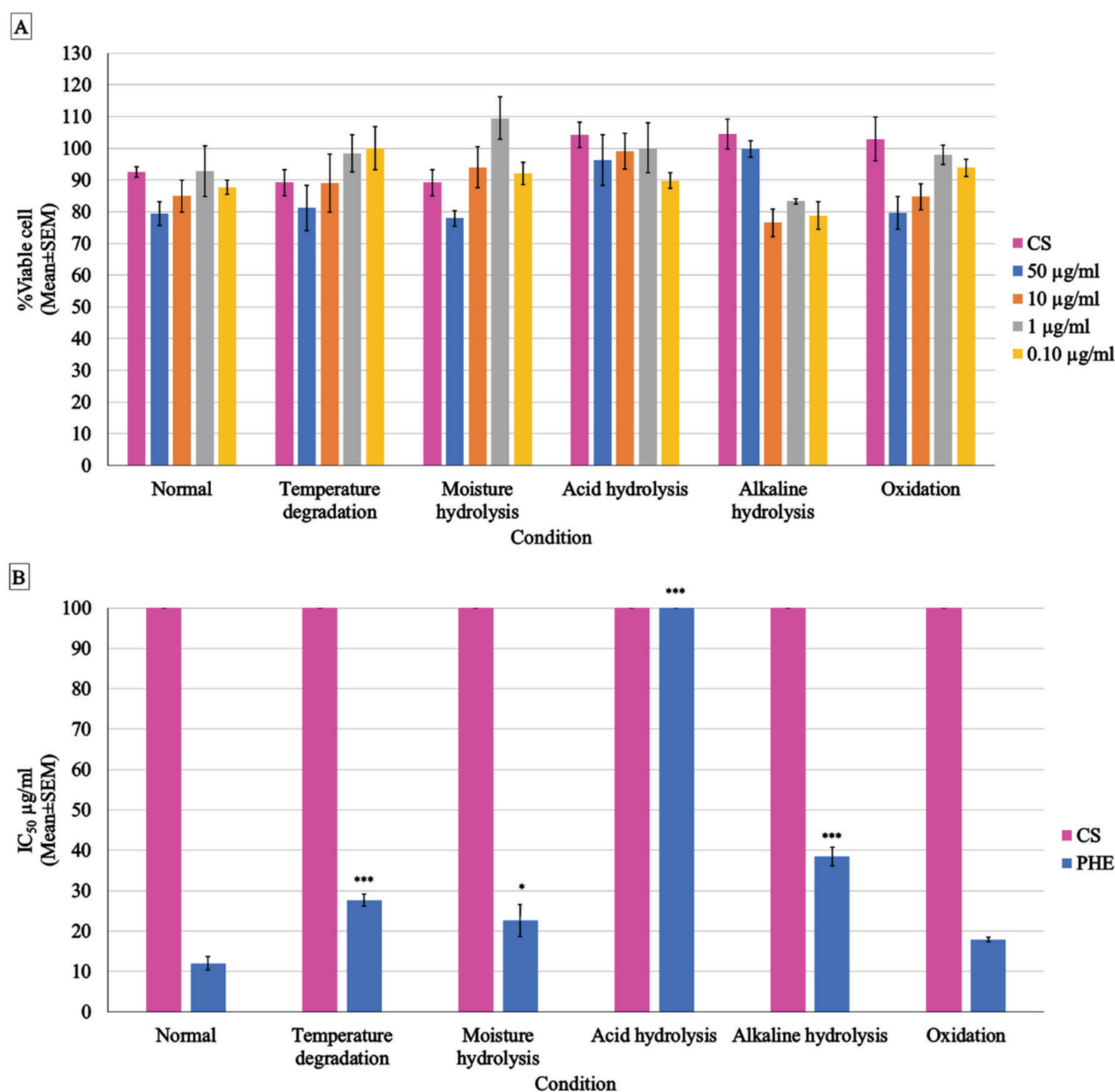

Figure 3 Forced degradation test of hexane extract of *Zanthoxylum rhetsa* pericarp (PHE) (n = 3). Percentage viable cells without LPS (A) and inhibitory effect of lipopolysaccharides (LPS)-induced nitric oxide (NO) production (B). IC_{50}: the half maximal inhibitory concentration; CS: 0.2% DMSO final concentration as a solvent and negative control (IC_{50} yielded > 100 µg/ml). *P-value < 0.05, ***P-value < 0.001 compared with PHE in normal condition. Acid hydrolysis condition IC_{50} yielded > 100 µg/ml.

Discussion

Osteoarthritis is a degenerative disease with deterioration of cartilages. Excessive production of NO induction of oxidative stress results in apoptosis of chondrocytes and progressive loss of cartilages.[7, 21] NO is associated with osteoarthritis. The hexane extract of the pericarp (PHE) of *Z.rhetsa* showed the highest potency in the production inhibition of NO (Table 1). Therefore, PHE showed the highest potency in reducing progressive osteoarthritis and pain due to inhibition of tissue damage induced by NO.[6, 7, 8] NO is an inflammatory mediator that causes edema in chronic inflammation,[22] PHE may be able to decrease swelling in osteoarthritis. This finding supported the use of fruit (pericarp and seed) in ethnomedicine (namely, Pa-Ra-Ti-Tri oil and Bee-Pra-Sen ointment in Pra-O-Sod-Pra-Narai scripture) which could relieve pain and muscle spasm.[10] Pericarp was used as a medicament for relaxing muscle and tendon in Thai traditional household remedies.[11] In our study, we separately prepared extracts from the pericarp, seed and fruit yielding different activity. However, a previous study reported only fruit essential oil contained potent production inhibition of NO in RAW264.7 macrophages (IC_{50} was 16.42 ng/ml)[23] while PHE was not tested.

According to Thai Traditional preparation the herbal oil was prepared by hot oil extraction, therefore in this study the herbal materials were macerated with hexane without heat intervention to avoid any deterioration to their activity.[24] The results demonstrated different potency in NO production inhibition from PHE at 50 μg/ml and FHE at 100 μg/ml as 97.15% ± 0.37 and 91.55%± 3.04, respectively, which was not significantly different from prednisolone (96.82% ± 0.34) at 50 μg/ml. However, the IC_{50} of PHE (11.99 ± 1.66 μg/ml) and FHE (39.81 ± 0.53 μg/ml) were significantly different (P-value < 0.01 and P-value < 0.001, respectively) from prednisolone (0.07 ± 0.001 μg/ml or 0.19 ± 0.001 μM) while PHE was more active than FHE (P-value < 0.001). SHE was not active (IC_{50} > 100 μg/ml) (Table 1).

PHE was the most active extract for reducing pain and swelling, which were symptoms and signs causing uncomfortable of daily activities in the elderly with osteoarthritis.[4] Therefore, our results suggested that separating the pericarp from fruit would give higher analgesic action.

All tested samples showed greater than 70% viable cells at all concentrations (Figure 1) indicating that their activities were not due to cytotoxicity.[18]

Although medications such as non-steroidal anti-inflammatory drugs (NSAIDs) and steroids effectively relieve pain and inflammation, they often cause side effects in the gastrointestinal tract.[25] PHE has been considered to be an alternative therapy for analgesic drugs. According to herbal medicine preparation, Thai traditional pharmacy theory, Hung-Nam-Mun or hot oil extract is one of the twenty-eight methods.[26] This traditional method uses oil for frying plant materials to give a spicy flavor and pungent aroma, the oils from plai, ginger, capsicum and pepper were obtained by this method.[26] The obtained herbal oils were applied to the muscles and joints to relieve pain. The proven activity of PHE was supportive to the topical oil preparation by the traditional Thai method. In this study the herbal materials were macerated by hexane and were not exposed to heat to avoid any deterioration to their activity.[24]

Both stability testing and forced degradation tests were performed according to International Conference on Harmonization (ICH) Q1A (R2) for stability testing of drug substances[19] and an established protocol.[20] Moreover, Thailand is a country in a humid tropical zone with general climate conditions are similar to those described in the accelerated test. Therefore, the conditions of 40ºC and 75% humidity were appropriate. The inhibitory activity on NO production was tested at intervals which revealed gradual reduction of this activity since day 30th. This result implied that storage condition for PHE should be below these temperature and humidity.

The composition of PHE has been earlier identified by GC-MS, they were bicyclo (3.1.1) hept-2-ene, 2,6,6-trimethyl-, dimer or alpha-pinene dimer ($C_{20}H_{32}$, 11.28%), neryl acetate ($C_{12}H_{10}O_2$, 7.65%), caryophyllene oxide ($C_{15}H_{24}O$, 7.50%), spathulenol ($C_{15}H_{24}O$, 6.65%) and cetanol or palmityl alcohol ($C_{16}H_{34}$, 3.78%). It also contained common constituents with seed oil i.e. terpinen-4-ol ($C_{10}H_{18}O$, 1.07%), terpinenyl acetate ($C_{12}H_{20}O_2$, 1.57%), and γ-terpinene ($C_{10}H_{16}$, 0.68%).[15] These compounds were monoterpene and derivatives

which were thermolabile, therefore degradation of these compounds could occur at high temperature used in the stability study and forced degradation study. However, hexane is a lipophilic solvent therefore other active lipophilic compounds would also be present in PHE.

A phytochemical study on the 95% ethanolic extract of *Z.rhetsa* pericarp revealed the presence of β-sitosterol, a mixture of β-sitosteryl glucoside and stigmasteryl glucoside (58:42), and lupeol (0.195% w/w).[27] β-sitosterol is only slightly soluble in hexane[28] but lupeol would be extractable by hexane since it is freely soluble in non-polar solvents such as ether, benzene and petroleum ether.[29] Therefore, lupeol could be considered as the compound of interest in PHE.

Lupeol is a naturally occurring lupane triterpene found in various plants and has been shown to reduce the inflammatory response of adjuvant arthritis in rats. In this study the rats were treated orally with lupeol (50 mg/kg body weight daily for 8 days), a significant decrease of the inflammatory symptoms was observed, while the activity of the antioxidative enzymes SOD and CAT were elevated.[30] Lupeol was also tested for acute anti-inflammatory effects *in-vivo* by topical application of lupeol (0.5 mg or 1 mg/ear) which showed significantly reduction of 12-O-tetradecanoylphorbol acetate (TPA)-induced oedema by 36.2% and 52.0%, respectively.[31] In the same study it was demonstrated that lupeol at 100 µM significantly reduced nitrite production in lipopolysaccharide-stimulated peritoneal macrophages.[31] The effects were stronger with PGE2, TNF-α and IL-1β production inhibition in the same model.[31]

Another study on 80% ethanolic extract from separate parts (fruit, pericarp and seed) of *Z.limonella* (a synonym of *Z.rhetsa*) revealed highest content of total phenolic compounds (as gallic acid equivalent) and flavonoids (as quercetin equivalent) in the pericarp. The antioxidant property by DPPH scavenging was not affected by elevated temperature (30°C and 50°C).[32] These compounds could also contribute to the anti-inflammatory activity of PHE, apparently, they were susceptible to heat, humidity and stressed conditions as well. Further studies should be focused on these non-volatile components of PHE.

Results of the stability study could be used for shelf-life estimation of herbal drugs. As PHE inhibitory effect on LPS-induced NO production significantly changed under accelerated storage conditions within six months, its normal shelf-life could be estimated to be up to one and half times period (1.5X) of its accelerated storage life but not exceeding X + 6 months (where X = accelerated storage life).[33] Therefore, the estimated shelf-life of PHE was nine months (1.5 x 6) but not exceeding 12 months (6 + 6) at room temperature.

A forced degradation test is typically used to determine appropriate conditions for maintaining herbal activity.[17] It was found that exposure to moisture, heat, alkaline and especially acidic condition should be avoided in the product development due to diminishing or losing activity (Figure 3).

However, the oxidation condition did not affect PHE activity. This could be due to the anti-oxidant property of *Z.Rhetsa*[34, 35] as well as lupeol and other antioxidants which were not affected by high temperature.[32] Therefore, this work was the first report on the inhibitory effect of PHE on LPS-induced NO production after accelerated stability test and forced degradation test.

Our results suggested that the hexane extract of *Z.rhetsa* (PHE) was suitable for relieving pain and swelling in chronic inflammation. PHE could be used in the preparation of analgesic drugs in osteoarthritis, which is a chronic disease. However, in the form of extract, it was stable for 9-12 months and unstable in acid, alkaline, heat and humid conditions. On the other hand, it was stable under oxidation condition with improved heat tolerance, therefore further study on composition of PHE after oxidation should be investigated. The appropriate dosage form of PHE should be ointment, spray, coated tablet or capsule to avoid hydrolysis and other degradation conditions.

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Conflicts of interest

The authors declare no conflict of interest regarding the contents and publication of this article.

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