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Screening Thai plants for DNA protection, anti-collagenase and suppression of MMP-3 expression properties

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ABSTRACT

Objective: To explore the suppression effect of six Thai plants on matrix-degrading enzymes such as collagenase and matrix metalloproteinases (MMPs).
Methods: Six Thai plant extracts, *Phyllanthus niruri* (*P. niruri*), *Vernonia cinerea*, *Pluchea indica*, *Rhinocanthus nasutus* (*R. nasutus*), *Zingiber cassumunar* (*Z. cassumunar*) and *Cissus quadrangularis* (*C. quadrangularis*) were tested for total phenolic content, antioxidant, DNA protection, anti-collagenase properties and inhibitory effects on IL-1β-acitvated MMP-3 expression. Additionally, the ethanolic extracts of *P. niruri* and *Z. cassumunar* were assessed for MMP-2 and -9 production using gelatin zymography.
Results: An evaluation of antioxidant activity and total phenolic content revealed that the ethanolic extract of *P. niruri* had the highest activity (72.17 and 93.05 mg gallic/g extract, respectively). The ethanolic extracts of *P. niruri*, *Vernonia cinerea*, *R. nasutus* and *C. quadrangularis* performed a strong activity of DNA protection against hydroxyl radicals. The extracts of *C. quadrangularis*, *R. nasutus* and *P. niruri* (IC_{50} = 0.3, 0.82 and 0.91 mg/mL, respectively) possessed good activity for the inhibition of bacterial collagenase activity. Using the promoter activity assay, the ethanolic extract of *P. niruri* and *Z. cassumunar* (IC_{50} = 26.94 and 27.82 μg/mL, respectively) decreased IL-1β-stimulated MMP-3 expression in human chondrosarcoma cells (SW1353) cells. Besides, both the ethanolic extracts of *P. niruri* and *Z. cassumunar* could alleviate the production of MMP-2 and -9 in IL-1β-activated SW1353.
Conclusions: Taken together, the ethanolic extract of *P. niruri* had several beneficial effects.

1. Introduction

Tissue remodelling is a common process occurring in a variety of tissues for the balance of the extracellular matrix (ECM). The key enzymes responsible for tissue remodelling are matrix degrading enzymes such as matrix metalloproteinases (MMPs)[1,2]. Although these enzymes are typically produced under physiological conditions, the overproduction of these enzymes results in the pathogenesis of various diseases, such as osteoarthritis, tumor invasion and atherosclerosis[3,4]. The treatment of any ailments caused by an irregularity of ECM remodeling is, therefore, an inhibition of their production or/and activity. MMP-3 is potentially one of the most effective Zn^{2+}-endopeptidases that could regulate ECM remodelling. Most substrates of MMP-3 involve the composites of ECM, such as collagen IV, aggrecan, gelatin, versican and proteoglycan-linked protein, *etc*. Several studies have reported that MMP-3 is associated with osteoarthritis, rheumatoid and cancer invasion[1,5,6]. Hence, MMP-3 is an interesting alternative target for the therapeutic treatment of various diseases like metastatic cancer, as well as osteoarthritis. Thus, in this study, we have constructed the reporter plasmid to determine the level of MMP-3 expression. Reporter gene technology is another effective tool in better understanding the relevant cellular events, especially the signal transduction pathways in controlling the gene expressions through both intracellular and extracellular stimulations. An

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example of a reporter gene would be the secreted embryonic alkaline phosphatase (SEAP) gene, which has been used as the reporter gene in RAW 264.7 murine macrophages that express the SEAP gene stimulated by transcriptional activation of the noncanonical nuclear factor (NF)-κB and activator protein 1 (AP-1) to develop a screen for immunopharmacological activity of cell wall-active antifungal agents[7].

In recent years, the attention of natural products as supplementary food and treatment purposes has been increasing. Despite the fact that they are relatively low cost, their prolonged consumption has fewer adverse side effects than the traditional/synthetic drugs, especially, nonsterodial anti-inflammatory drugs (NSAIDs)[2,8]. Plants contain a wide variety of compounds including polyphenols, such as flavanoids, tocopherals, phenolic acid, tannins and lignins. These compounds have been found to provide many health benefits, such as those of anti-inflammation, anti-oxidation, anti-peroxidation, anti-collagenase and anti-UV irradiation[2,8-11]. These compounds may be a platform from which the design of active molecules can be based to increase their efficacy in treatments.

Therefore, the purpose of this study was to determine the activities of six Thai plants extracted by ethanol and water, including *Phyllanthus niruri* L (*P. niruri*), *Vernonia cinerea* Less. (*V. cinerea*), *Pluchea indica* Less. (*P. indica*), *Rhinocanthus nasutus* Karz (*R. nasutus*), *Zingiber cassumunar* Roxb. (*Z. cassumunar*) and *Cissus quadrangularis* Linn. (*C. quadrangularis*). The inhibitory effect of these plants on MMP-3 expression was observed, and their anti-collagenase activity and DNA protection ability were assessed.

2. Materials and methods

2.1. Chemicals and reagents

Human chondrosarcoma cells (SW1353) were obtained from American Type Culture Collection (Manassas, VA, USA). Dulbecco's modified eagle's medium (DMEM), trpysin-ethylene diamine tetraacetic acid, penicillin/streptomycin, fetal bovine serum (FBS), type II bacterial collagenase, *Clostridium histolyticum* and other cell culture materials were purchased from Gibco BRL, Life Technologies (USA). The 3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent, 1,1-diphenyl-2-picrylhydrazyl (DPPH), gallic acid, Folin-Ciocalteau solution, para-nitrophenyl phosphate and dimetyl sulfoxide (DMSO) were purchased from Sigma Chemical Co. All other chemicals and solvents were of analytical grade.

2.2. Preparation of plant extracts

Medicinal plants used in this study as *P. niruri* (whole plant), *V. cinerea* (whole plant), *P. indica* (leaves), *R. nasutus* (leaves), *Z. cassumunar* (tumeric) and *C. quadrangularis* (stem) were purchased from Lampang Herb Conservation, Thailand. Plants and plant-materials were dried in a ventilated oven at 60 ˚C for 72 h, then ground and extracted using two solvents including distilled water, and 95% ethanol with the proportion of 1:10 (w/v). In brief, 250 g of each plant were extracted with distilled water at 45 ˚C for 3 h or macerated with 95% ethanol for 72 h with frequent agitation, at room temperature[12]. The plant extracts were filtered through Whatman's No.1. The solvent was removed from the filtrate of the plant extracts by evaporation at 45 ˚C under reduced pressure at 50 mbar in a rotary evaporator (Buchi TM). The plant extracts were then dissolved in DMSO to give a concentration of 50 mg/mL.

2.3. Determination of total antioxidant activity using DPPH radical scavenging

The DPPH radical scavenging assay was conducted according to the modified method of Brand-Williams *et al*[13]. The extracts were dissolved with methanol in order to prepare various concentrations, then 0.5 mL of each concentration was incubated with 1.5 mL of 0.1 mmol/L methanolic solution of DPPH in the dark at room temperature for 20 min. The samples were subsequently determined at 517 nm. Methanol was used as a blank solution and DPPH without extract was used as a control. The percentage of free radical inhibition by the extract was calculated by the following equation: % Inhibition = $[(A_0 - A_1) / A_0] \times 100$, where A_0 is the absorbance of control and A_1 is the absorbance of the tested sample after treatment with extracts. Antioxidant activity of the extracts was expressed as gallic acid equivalent antioxidant capacity (GAE).

2.4. Determination of total phenolic content

The total phenolic compound contents were determined by Folin-Ciocalteau method[14]. Briefly, the plant extract was dissolved with methanol to obtain a concentration of 1 mg/mL. Afterwards, 250 μL of the extract was mixed with 1.25 mL of water and then 250 μL of 95% ethanol and 125 μL of 50% Folin-Ciocalteau were added and mixed thoroughly. The mixture was incubated for 5 min. Subsequently, 250 μL of 5% Na_2CO_3 was added and incubated for 1 h. The absorbance of the samples was recorded at 725 nm. The standard curve was prepared using a concentration range of 10-100 μg/mL solution of gallic acid. The equivalents were read off the straight line generated by linear regression. The value of the total phenolic content was expressed as gallic acid equivalent antioxidant capacity (GAE).

2.5. DNA protective assay

The plasmid pSEAP2-control (clontech) DNA (200 ng) was incubated in H_2O_2 with and without the extracts to evaluate the effect of the extracts on the protection of plasmid before being exposed to UV-C irradiation (254 nm, 100 μJ/cm^2) for 5 min[15]. The reaction mixtures (10 μL) were comprised of 2.5 mmol/L H_2O_2, 200 ng of super coiled pSEAP2-control, 10 or 100 μg/mL of the plant extracts (dissolved in DMSO) and deionized water. The vehicle control contained only 2% DMSO and 2.5 mmol/L H_2O_2. At the end of the reactions, the plasmid DNA from all samples was subjected in 1% agarose gel in 1 × Tris-acetate-Ethylene Diamine Tetraacetic Acid at 120 V for 40 min. The gel was stained by EtBr and visualized under a UV transilluminator to detect the conformations [super coiled (SC), open circular (OC) and linear (LIN)] of plasmid DNA.

2.6. Examination of plant extracts on the activity of bacterial collagenase type II

To evaluate the effect of six Thai plants extracts on bacterial collagenase, a colorimetric method for gelatinolytic assay was performed[16]. The reaction mixture was prepared in 20 μL in each well of a 96-well plate. Each experiment contained 1 × collagenase buffer (50 mmol/L Tris-HCl, 10 mmol/L $CaCl_2$ and 0.15 mol/L NaCl),

100 ng bacterial collagenase and the plant extracts giving the final concentrations of 0.01-5.00 mg/mL after being incubated at 37 ˚C for 30 min. Each well was then added with 10 μg of gelatin and incubated at 37 ˚C for 4 h. The plant extracts were not included in the control group. Subsequently, an amount of gelatin remaining was quantified by the addition of the heated commassie brilliant blue, 20 μL, followed by shaking, either by hand or by a shaker for 5 minutes to mix the reagents. By pipetting, the supernatant was discarded and 50 μL of DMSO was added to dissolve the pellet. The plate was finally read for the absorbance at 600 nm. All experiments were done in triplicate. The inhibitory effect of these agents was expressed in percentage of the control and calculated with the following formula; [(A_{600} of sample - A_{600} of blank) - (A_{600} of control - A_{600} of blank)] / (A_{600} of gelatin - A_{600} of blank) × 100, where A_{600} of the sample is the absorbance value of the sample tested with the agents at different concentrations, A_{600} of the control is the absorbance value of the sample without the agents, A_{600} of gelatin is the absorbance value of total gelatin (10 μg) and A_{600} of the blank is the absorbance value of the well containing only commassie brilliant blue and DMSO.

2.7. Cell culture and viability measurement of plant extracts

SW1353 were cultured in DMEM supplemented with 10% FBS in the humidified atmosphere of 5% CO_2 at 37 ˚C. Cells were seeded in 96-well plates at 1 × 10^4 cells/well. The plate was incubated overnight and was then exposed with the plant extracts at various concentrations ranging from 0 to 1 000 μg/mL in a total volume of 100 μL, which were diluted in a two-fold series with serum-free DMEM. Cells in the control group were left untreated. All experiments were done in triplicate. After 3 days, cell viability was determined using tetrazolium compound that was MTT based on it's the mitochondrial-dependent reduction to formazone. A total of 20 μL of 2.5 mg/mL MTT was added to each well and the plate was incubated for another 4 h under 5% CO_2 at 37 ˚C. Next, the formation of formazan was determined using 50 μL of DMSO to dissolve the pellets and then read at 540 nm. All data were calculated as the percentage of viable cells vs. the control, and were then considered as the IC_{50} of each extract.

2.8. Construction and validation of MMP-3 reporter gene

Genomic DNA was extracted from human lung fibroblasts obtained from the Human and Animal Cell Culture Technology Research Unit by the phenol-chloroform method. The upstream regulatory element (URE) of MMP-3 was cloned with the PCR procedure using specific primers, MMP3-forward: 5' GCC ACC ACT CTG TTC TCC TTG TCC T 3' and MMP3-reverse: 5' TTA AGC CCC AGT GCT GCT ACT GCT A 3'. The PCR product of MMP-3 URE was 2 128 bp (interval of MMP-3 promoter used in this study form -1 761 bp to +438 bp). The MMP-3 URE was attached to the SEAP reporter plasmid (P_{SV40} deleted pSEAP2-control; Clontech). The plasmid was called pMMP3-SEAP (Figure 1). The constructed plasmid was transiently transfected into SW1353 obtained from the American Type Culture Collection. SW1353 were transiently transfected using 3 μL of X-treamGENE HP DNA transfection reagent (Roche Applied Science, USA) mixed with two micrograms of pMMP3-SEAP in 100 μL serum free DMEM. Then, the solution was transferred at 10 μL per well. The transfected cells were incubated in a 37 ˚C, 5% CO_2 incubator for 24 h. Then the cells were treated with serum free DMEM containing either recombinant human interleukin (IL)-1β (1-50 ng/mL), or bacterial lipopolysaccharides (LPS; 0.1-10.0 μg/mL), for 48 h. The control groups were treated with only serum-free DMEM. All experiments were tested in triplicate per treatment. The cultured medium was collected to measure the SEAP activity as described by Berger *et al*[17].

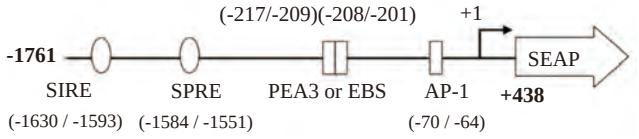

Figure 1. Construction of pMMP3-SEAP plasmid.
The plasmid contains upstream regulatory element (URE) of MMP3 (-1691 to +437) for reporter gene assay and DNA binding sites at MMP-3, stromelysin IL-1 responsive element (SIRE), stromelysin PDGF responsive element (SPRE) and ETS (E26 transformation-specific or E-twenty six) binding site (EBS), PEA3 and AP-1.

2.9. Real-time PCR

To establish whether pMMP3-SEAP could be used in examining the expression level of MMP-3, the correlation between SEAP reporter gene assay and real-time PCR was evaluated at mRNA level. SW1353 cells were seeded (1 × 10^6 cells/dish) into 60 mm dish for 24 h in DMEM supplemented 10% FBS. Then, the cells were incubated in serum-free DMEM containing IL-1β at various concentrations (1, 10 and 50 ng/mL) for 48 h, or left untreated as a control. The cells were harvested by trypsinization and the RNA was extracted by Trizol® reagent with DNaseI treatment. cDNA obtaining from RNA by Moloney murine leukemia virus reverse transcriptase (Fermentas, USA) was used as a template for real-time PCR by using Maxima® SBYR green qPCR master mix (Fermentas, USA) normalized with glyceraldehydes-3-phosphate dehydrogynase (GAPDH). The relative expression of MMP-3 normalized by GAPDH were calculated through cycle threshold with $2^{-\Delta\Delta CT}$ method. Oligonucleotides of primers used for real-time PCR were shown in Table 1.

Table 1
Primer used for real-time PCR.

Genes (Accession number)	Sequence	Ta (°C)	Size (bp)
MMP3 (NM_002422)	Forward: 5'-CTTTTGGCGAAAATCTCTCAG-3' Reverse: 5'-AAAGAAACCCAAATGCTTCAA-3'	57	404
GAPDH (NM_002046)	Forward: 5'-TGGTATCGTGGAAGGACTCAT-3 Reverse: 5'-GTGGGTGTCGCTGTTGAAGTC-3'	57	370

Ta: annealing temperature

2.10. The effect of doxycycline on IL-1β induced MMP-3 expression using promoter activity assay

Preliminary validation of the MMP-3 reporter plasmid system for the inhibitory effect of chemicals on MMP-3 expression was evaluated. Thereby, the transfectant cells were exposed with the different concentrations of deoxycycline, giving final concentration of 1, 10, 50 and 100 μg/mL in serum free DMEM, containing 10 ng/mL of IL-1β. The control group was treated without doxycycline and the plate was incubated for 48 h. After incubation, the conditioned media was determined for SEAP activity.

2.11. Determination of plant extracts on MMP-3 expression by promoter activity assay

SW1353 (3×10^4 cells/well) were seeded into the 96-well plate and maintained in monolayer culture at 37 ˚C for 24 h, in DMEM supplemented 10% FBS. SW1353 were transiently transfected, using 3 μL of X-treamGENE HP DNA transfection reagent (Roche Applied Science, USA) with 2 μg pMMP3-SEAP, and then maintained at 37 ˚C in 5% CO_2 incubator for 24 h. The transfected cells were exposed, or not exposed, to plant extracts at various concentrations (1, 10, 50 and 100 μg/mL) in serum-free DMEM and co-stimulated with 10 ng/mL IL-1β for 48 h. Following that, the conditioned media were collected to measure SEAP activity. The control groups were treated only with serum-free DMEM containing 0.4% DMSO. All experiments were tested in triplicate. The results were expressed in IC_{50}.

2.12. SEAP activity assay

To assess SEAP activity, the collected condition medium in a 96–well plate was treated with chemical agents and incubated at 65 ˚C for 30 min to eliminate the endogenous alkaline phosphatase. Then, 100 μL of 4 mg/mL para-nitrophenyl phosphate in buffer (1 mol/L diethanolamine, 0.5 mmol/L $MgCl_2$, pH 9.8) was added and the mixture was incubated at 37 ˚C for 3-4 h, or until the color changed to yellow. The absorbance of the plate was then read at 405 nm. This protocol was modified from Berger *et al*[17]. The MMP-3 expression was calculated in terms of fold induction by the following formula: fold induction = (A_{405} of treatment - mean A_{405} of blank) / (A_{405} of control-mean A_{405} of blank), where A_{405} of treatment is the absorbance value of the sample tested with the agents (IL-1β and/or extracts) at different concentrations, A_{405} of the control is the absorbance value of the sample without agents and A_{405} of the blank is the absorbance value of medium alone.

2.13. Gelatin zymography assay

Gelatin zymography was performed for MMP-2 and -9 assay[18]. Conditioned media obtained from the cells, exposed with different concentrations of ethanolic extracts of *P. niruri* and *Z. cassumunar*, were precipitated with 2 volume of acetone and then incubated at -80 ˚C for at least 1 h. Subsequently, the samples were centrifuged at 12 000 × g for 15 min 4 ˚C and were then resuspended in 20 μL of phosphate buffered saline. After that, they were mixed in a sample buffer containing 62.5 mmol/L Tris-HCl (pH 6.8), 10% glycerol, 2% sodium dodecyl sulphate, and 0.00625% (w/v) bromophenol blue and loaded, without being boiled, in 7.5% acrylamide: bisacrylamide (29:1) separating gel containing 0.1% (w/v) gelatin. Electrophoresis was carried out at a constant voltage of 120 V. After electrophoresis, the gels were soaked in 0.25% Triton X-100 (2 × 30 min) at room temperature and rinsed in deionized water. The gel containing gelatin was incubated at 37 ˚C for 20 h in the incubation buffer containing 50 mmol/L Tris-HCl (pH 7.6), 20 mmol/L NaCl, 5 mmol/L $CaCl_2$ and 0.02% NaN_2. The gel was then stained for 15–30 min in 0.25% (w/v) Coomassie blue R-250 (30% methanol and 10% acetic acid), and de-stained in the same solution without the Coomassie brilliant blue dye. The clear zone in the dark field confirmed the gelatinolytic activity.

3. Results

3.1. Total antioxidant activity and total phenolic content of six Thai plant extracts

As a rapid and simple assay of antioxidant activity, DPPH, a free radical compound, has been widely used to determine the ability of plant extracts for free radical scavenging. The total antioxidant activity of both ethanolic and aqueous extracts of six plants was expressed as gallic acid equilibrium (GAE) ranging from (72.17 ± 1.09) to (0.76 ± 0.03) mg gallic/g extract, as shown in Table 2. The highest antioxidant activity was found in the ethanolic extract of *P. niruri*, with (72.17 ± 1.09) mg gallic/g extract, followed by the ethanlic extract of *R. nasutus* [(16.53 ± 0.70) mg gallic/g extract] and *V. cinerea* [(16.48 ± 0.14) mg gallic/g extract], whereas the aqueous extract of *Z. cassumunar* had the weakest activity in terms of free radical scavenging with (0.76 ± 0.03) mg gallic/g extract. *C. quadrangularis* exhibited a moderate effect for the ethanolic extract and the relatively low effect for the aqueous extract. Overall, the ethanolic extracts seemed to have a greater tendency of antioxidant activity than the aqueous extracts.

The consequences of total phenolic content assay revealed that the ethanolic extract of *P. niruri* had the highest total phenolic content, with (93.05 ± 0.55) mg gallic/g extract. Both ethanolic and aqueous extracts of *C. quadrangularis* had relatively low total phenolic content, with (27.94 ± 0.52) and (12.95 ± 0.21) mg gallic/g extract, respectively.

3.2. Protection effect of the plant extracts on the plasmid DNA

The electrophoretic pattern of plasmid DNA (Figure 2) revealed the multimeric forms of plasmid DNA (super coiled, open circular and linear) after UV-photolysis of H_2O_2 to generate hydroxyl radicals ($^{\bullet}OH$), a reactive oxygen species (ROS). The plasmid pSEAP-control DNA provided two bands: super coiled and open circular in the agarose gel. After the plasmid DNA was combined with H_2O_2 induced by UV-C, there were not any bands of DNA in the gel. This indicated that $^{\bullet}OH$ radicals could thoroughly damage DNA

Table 2

The antioxidant activity and total phenolic content of six Thai plant extracts in ethanol and water solvents (mean ± SD).

Scientific name	Antioxidant (DPPH) (mg gallic/g extract)		Total phenolic content (mg gallic/g extract)	
	Ethanol	Water	Ethanol	Water
P. niruri L	72.17 ± 1.09	10.05 ± 0.18	93.05 ± 0.55	31.37 ± 1.09
V. cinerea Less.*	16.48 ± 0.14	7.45 ± 0.10	29.06 ± 0.59	21.94 ± 1.07
P. indica Less.*	9.30 ± 0.26	1.77 ± 0.01	27.50 ± 0.66	13.83 ± 0.37
R. nasutus Karz.*	16.53 ± 0.70	9.09 ± 0.24	33.13 ± 1.45	22.38 ± 0.49
Z. cassumunar Roxb.*	11.24 ± 0.35	0.76 ± 0.03	43.17 ± 0.67	11.35 ± 0.14
C. quadrangularis Linn.	6.01 ± 0.03	1.82 ± 0.02	27.94 ± 0.52	12.95 ± 0.21

*: Data obtained from Pukumpuang et al., 2012[11].

molecules. However, UV-C irradiation contributed to DNA strand breaks resulting in the relaxation of the super coiled form to the linear form. The assay showed that all plant extracts had no activity on the protection of the plasmid DNA from UV-C irradiation, because the linear form could be found in all samples. However, these plant extracts could protect against the destruction of plasmid DNA from the •OH radical, particularly in the ethanolic extracts. Both ethanolic and aqueous extracts of *P. niruri*, at 100 μg/mL, possessed the greatest actions on the DNA defense against •OH radicals, whereas the plasmid DNA was damaged entirely at 10 μg/mL. The ethanolic extracts of *V. cinerea*, *R. nasutus* and *C. quadrangularis* seemed to possess relatively stronger activity than the aqueous extracts of them, while the results of the *P. indica* extracts were reversed. Additionally, it was found that the extract of *Z. cassumunar* in both ethanol and water had the weakest activity on DNA protection from the •OH radical.

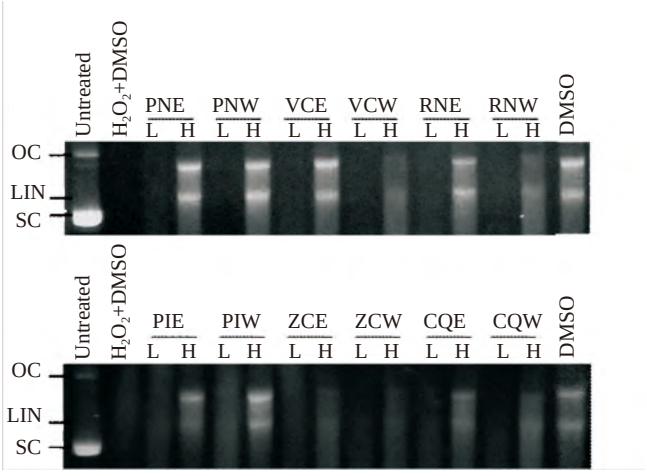

Figure 2. The effect of plant extracts on the inhibition of DNA fragmentation against hydroxyl radicals.

Plasmid DNA (pSEAP2-control) were incubated with H_2O_2 and then irradiated by UV-C in the presence of 10 μg/mL (L) or 100 μg/mL (H) plant extracts. All stocks of the plant extracts were dissolved with DMSO. The vehicle control thus contained only 2% DMSO. PN: *P. niruri* L; VC: *V. cinerea* Less.; PI: *P. indica* Less.; RN: *R. nasutus* Karz; ZC: *Z. cassumunar* Roxb.; CQ: *C. quadrangularis* Linn. These plants were extracted using two solvents, ethanol (E) and water (W).

3.3. The effect of plant extracts on the activity of bacterial collagenase, type II

Bacterial collagenase was incubated with gelatin as a substrate in the presence of the different concentrations of plant extracts ranging from 0-5 mg/mL. The results of the tests on the inhibitory effect of the extracts on the enzyme are shown in Figure 3. The results showed that the ethanolic extracts of *C. quadrangularis*, *R. nasutus* and *P. niruri* (IC_{50} = 0.3, 0.82 and 0.91 mg/mL, respectively) possessed a strong anti-collagense activity. Moderate anti-collagenase activities were exhibited by the aqueous extracts of *C. quadrangularis*, *P. niruri* and *Z. cassumunar*, as well as the ethanolic extract of *Z. cassumunar*, with IC_{50} ranging from 1.02-1.81 mg/mL. Some plant extracts had no or less inhibition of collagenase activity (IC_{50} > 5 mg/mL), such as *P. indica* and *V. cinerea* in water and ethanol extracts, as well as the aqueous extract of *R. nasutus*.

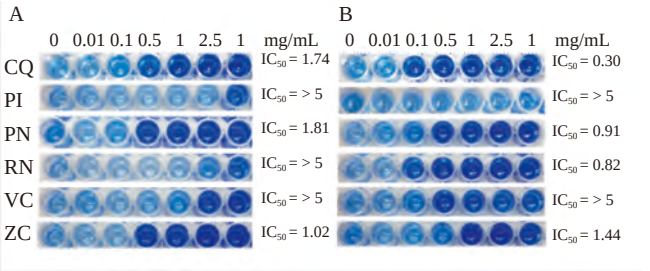

Figure 3. Effect of the six plant extracts in water and ethanol solvents.

A: Aqueous plant extracts; B: Ethanol plant extracts. CQ: *C. quadrangularis* Linn.; PI: *P. indica* Less.; PN: *P. niruri* L; RN: *R. nasutus* Karz; VC: *V. cinerea* Less.; ZC: *Z. cassumunar* Roxb.. Their activities were expressed in terms of IC_{50} (mg/mL).

3.4. Validation of MMP-3 reporter plasmid to analyze the level of MMP-3 expression

The SEAP activity was found to have increased in a dose-dependent manner after treatment with MMP-3-induced factors, such as recombinant human IL-1β or LPS, indicating an up-regulation of MMP-3 expression (Figure 4A and 4B). These results showed that a significant increase over the control was found when either was incubated with recombinant human IL-1β or LPS, from the concentration ranges of 1-50 ng/mL or 0.1-10.0 μg/mL, respectively.

The results of the level of MMP-3 expression activated by IL-1β showed that the MMP-3 transcript was up-regulated in a dose-dependent manner, using real-time PCR. Furthermore, the MMP-3 expression determined by the MMP-3 promoter activity assay was correlated to the MMP-3 transcript, in terms of the log (normalized MMP-3 expression) by real-time PCR (Figure 4C). Furthermore, by the MMP-3 reporter plasmid system, it was found that doxycycline decreased the SEAP activity in a dose-dependent manner compared to the IL-1β treated group (Figure 4D), indicating that doxycycline tended to inhibit MMP-3 expression from IL-1β induction in SW1353.

Table 3

IC_{50} of plant extracts on the suppression of IL-1β-induced MMP-3 expression and cytotoxicity on SW1353 cells.

Scientific name	IC_{50} of IL-1β-induced MMP-3 expression inhibition by promoter activity assay (μg/mL)		IC_{50} of cytotoxicity on SW1353 cells (μg/mL)	
	Ethanol	Water	Ethanol	Water
P. niruri L	26.94	>100	199.0	759.4
V. cinerea Less.	>100	74.26	626.0	>1000
P. indica Less.	60.54	36.78	357.2	>1000
R. nasutus Karz.	36.49	>100	321.5	>1000
Z. cassumunar Roxb.	27.82	ND	124.1	>1000
C. quadrangularis Linn.	ND	>100	754.4	>1000

ND= not determined.

3.5. Inhibitory effect of six Thai plant extracts on the viability cell and IL-1β induced MMP3 expression and the production of MMP-2 and -9

The cytotoxicity of plant extracts on SW1353 cells was assessed using MTT procedure, as expressed in IC_{50}. The range of cytotoxicity of six plant extracts was 124.1 to >1 000 μg/mL (Table 3). The results demonstrated that all ethanolic extracts had a higher level of cytotoxicity than the aqueous extracts. Among the aqueous extracts, *P. niruri* displayed the highest cytotoxicity, at 759.4 μg/mL. In most aqueous extracts, the values of IC_{50} were more than 1 000 μg/mL. Moreover, the ethanolic extract of *Z. cassumunar* had more cytotoxicity than any of the others with 124.1 μg/mL. Therefore, we selected the range of concentrations for treatment on IL-1β-induced MMP-3 expression as 1, 10, 50 and 100 μg/mL.

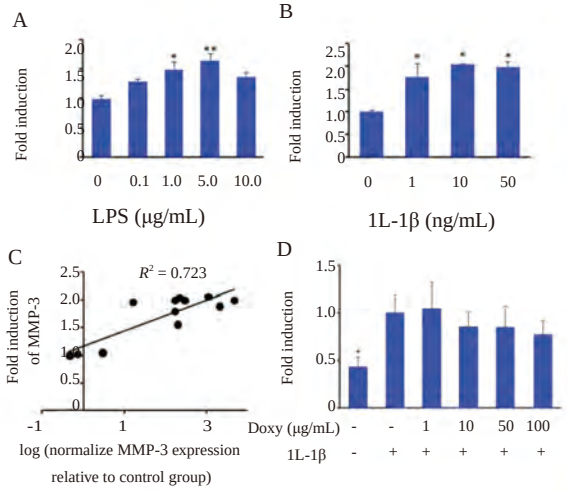

Figure 4. The MMP-3 expression obtained by examining the SEAP activity in medium conditions of pMMP3-SEAP transfected SW1353 cells. A: Transfected SW1353 cells treated with LPS; B: Transfected SW1353 cells treated with IL-1β. Bar graphs show the varied inductions of MMP-3 when compared to the control group treated with serum free DMEM alone (mean ± SD, n = 3), *: $P < 0.05$ and **: $P < 0.01$. C: Correlation of IL-1β induced MMP-3 expression at mRNA level that was determined by two methods: real-time PCR (X axis) and SEAP reporter gene assay (Y axis). The points show the values of each group of both methods (0, 1, 10 and 50 ng/mL IL-1β in serum free DMEM). D: The inhibitory activity of doxycycline (Doxy) on IL-induced MMP-3 expression by SEAP reporter gene assay in medium conditions from pMMP3-SEAP transfected SW1353 with 10 ng/mL IL-1β stimulation. Bar graphs show the induction of MMP-3. +: Present; -: Absent. The control groups were treated with serum free DMEM at 10 ng/mL IL-1β (mean ± SD, n = 3), *: $P < 0.05$.

To determine whether the plant extracts have the inhibitory effect on IL-1β activated MMP-3 expression, the transiently transfected SW1353 cells were exposed with the plant extracts in the presence of 10 ng/mL of IL-1β (Table 3). The outcome of the treatment exhibited that the activity of these plant extracts was relatively greater in the ethanolic extracts than aqueous extracts. The greatest activity was found in the ethanolic extract of *P. niruri*, with 26.94 μg/mL. In addition, the ethanolic extract of *Z. cassumunar* (27.82 μg/mL) displayed slightly less activity than that of *P. niruri*. The ethanolic extract of *R. nasutus* (36.49 μg/mL) and the aqueous extract of *P. indica* (36.78 μg/mL) revealed quite high levels of activity, while the ethanolic extract of *V. cinerea* and the aqueous extracts of *P. niruri* and *R. nasutus* had values of IC_{50} of more than 100 μg/mL, indicating that these possessed weak inhibitory effects on IL-1β-induced MMP-3 expression. The moderate effect of suppression on IL-1β-induced MMP-3 was revealed by the aqueous extract of *V. cinerea* (74.26 μg/mL) and the ethanolic extract of *P. indica* (60.54 μg/mL). There were two extracts (the aqueous extract of *Z. cassumunar* and the ethanolic extract of *C. quadrangularis*), for which the IC_{50} could not be calculated, due to the fact that these extracts increased or remained at the level of MMP-3 expression.

Gelatin zymography were preformed to assess the inhibitory activity of the ethanolic extracts of *P. niruri* and *Z. cassumunar* on the production of the MMP-2, and -9 in SW1353 cells activated by IL-1β. Their concentrations were used under the same conditions as those of MMP-3 expression. Consequently, the MMP-2 results were found to be greater than those of MMP-9, owing to the fact that MMP-2 expresses constitutively in various tissues. Moreover, both extracts significantly decreased the levels of MMP-2 and -9 productions that are stimulated by IL-1β, following the dose-dependent manner (Figure 5). This indicated that both extracts possessed a level of potency on the reduction of both MMP-2 and -9 productions.

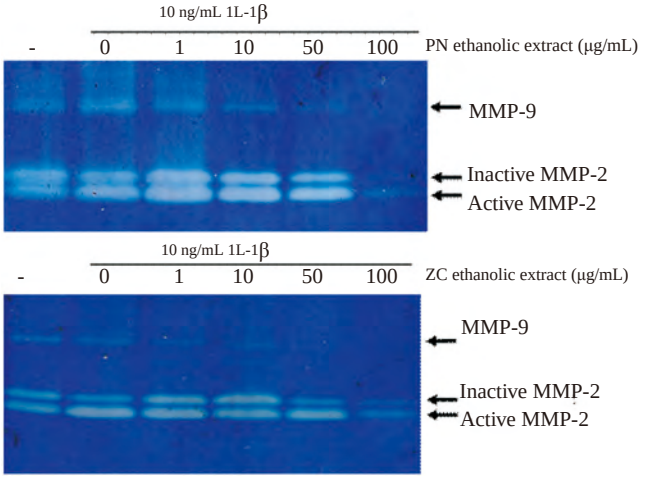

Figure 5. Gelatin zymography shows the effect of the ethanolic extract of *P. niruri* and *Z. cassumunar* on the production of MMP-2 and MMP-9 in IL-1β-activated SW1353 cells. PN: *P. niruri*; ZC: *Z. cassumunar*.

4. Discussion

The plant kingdom is one of the most important sources for bioactive compound discovery called naturally chemical library. The supplement foods, cosmetics and medicine derived from the extract of natural products, especially in plant kingdom, have been widely focused to be developed as marketing products because they are relatively safer than some synthetic drugs. Moreover, they are available and low cost for the screening of some Thai plants for antioxidant and total phenolic compounds. From the results, the ethanolic extract of *P. niruri* exhibited better antioxidant property than others. Fang *et al*.[19] reported that there were many bioactive ingredients in *Phyllanthus urinaria*, which has a very close relationship with *P. niruri*. The bioactive compounds methybrevifoncarboylate, trimethyl-3,4-dehydrochebulate,

methylgallate and quercitrin have the great capacity for free radical scavenging using DPPH, while phyllanthin and phyltetralin displayed a relatively mild level of antioxidant activity. Furthermore, the ethanolic extract of *P. niruri* has the highest total phenolic content which are considered the major secondary metabolite in the plant kingdom. They possess a wealth of useful biological functions such as antioxidant, anti-inflammation and antibacterial activity. Generally, the antioxidant activity of the plant extracts was related with the total phenolic content[10,11].

Oxidative DNA damage can be resulted from ROS produced during normal metabolic processes inside the cell. DNA damage can cause and contribute to pathogenesis such as inflammatory response, aging, brain/nervous system and cancers[20]. The *P. niruri* extract could inhibit DNA fragmentation from $^{\bullet}OH$ radical generated UV-induced H_2O_2. ROS are implicated in cytotoxins, such as cell proliferation, apoptosis, and cell senescence[21]. H_2O_2 in low concentrations is one of the second messengers triggering a genetic program associated with cell growth[22]. Thus, it is indicated that, in normal conditions of the cell, the production of H_2O_2 naturally occur. $^{\bullet}OH$ radical is derived from $O_2^{\bullet}$ and H_2O_2 can contribute to the oxidative damage in the biological system[20], in which the $^{\bullet}OH$ radical can react with a number of target molecules including proteins, membrane lipids, as well as DNA molecules. Bioflavanoids (rutin, catechin and narigin), generally found in the plant kingdom, had effects on the inhibition of DNA fragmentation[15].

Collagenase plays critical roles in physiological conditions. On the other hand, in the case of excessive collagenases, this leads to the pathogenesis of many diseases. Inhibition of the activity of collageanase is considered another way to ameliorate these ailments. It is notable that three out of the six plants exhibited anti-collagenase activity in both water and ethanol extracts, such as *C. quadrangularis*, *Z. cassumunar* and *P. niruri*. The plant kingdom contains numerous bioactive compounds, especially polyphenolic compounds. These compounds exhibit various activities, such as antioxidant, anti-inflammatory, anti-microbial, anti-mutagenic and anti-proteolytic effects (anti-collagenase and anti-elastase). Catechin, epigallocatechingallate and tannic acid have been proven to possess beneficial activities, such as anti-collagenase activity[9]. Curcumin (diferuloylmethane), a polyphenolic compound, is the chief component of the spice turmeric including *Z. cassumunar*, which could suppress collagnase activity. Isolated green tea polyphenols could change the conformation of collagenase by the hydroxyl group and benzene ring, which would block the high activity of collagenase[9]. Furthermore, it is clear that this method is a promising assay for pharmaceutical applications.

To enable quickly screening of any agents on MMP-3 expression in cell culture, a reporter gene for MMP-3 expression, pMMP3-SEAP, was constructed and validated for its potential in MMP-3 expression analysis. The pMMP3-SEAP transfected SW1353 could respond to the inflammatory agents, IL-1β and LPS by the increase of secreting SEAP protein in conditioned media. IL-1β and LPS have been known to be stimulating agents that cause ECM degradation though MMPs activity. Furthermore, the signal transductions of LPS induced the expression of other cytokines, such as IL-1β and tumor necrosis factors (TNF-) via a toll like receptor[23]. This evidence provides the amplification signal of inflammation that leads to ECM destruction, since IL-1β and TNF- can stimulate proteases production, as well as MMP-3[24]. Besides, The doxycycline could inhibit the increase of IL-1β induced MMP-3 expression in SW1353 using reporter gene assay, which was similar to the previous reports[25,26]. These results exhibited that pMMP3-SEAP has the potential to analyze the MMP-3 expression since the plasmid could respond to environmental stimulation in transfected SW1353 cells. Thus, this would suggest that a pMMP3-SEAP reporter gene plasmid was an ideal system for determining the MMP-3 expression, which could be useful for identifying active pharmacological agents and their relative potency.

Although there are now several chemicals that have been experimentally and clinically proven to be capable of attenuating both the activity and/or production on various MMPs and some have been clinically used as drugs in treatment, some have side effects depending on the individual and his/her race. Thus, the discovery of drugs from new sources is important in terms of the novel bioactive molecules, resulting in more efficient treatment. *P. niruri* extract has been used for hepatoprotection, anticancer, anti-inflammation and anti-diabetic action as well as for the reduction of the quantity of hepatitis B virus in the blood. The standardized extract of *P. niruri* was reported to inhibit the induction of inducible nitric oxide synthase, cyclooxygenase-2 and TNF- production by inhibiting NF-κB activation. Besides, the stimulation of IL-1β, IL-10 and interferon-γ were attenuated in human whole blood by *P. niruri* extracts, which showed the anti-inflammatory action of *P. niruri* extract[27]. Furthermore, geraniin, one of the chemical constituents of the *P. niruri* extract, had an inhibitory effect on NF-kB by blocking the phosphorylation of IkB and could reduce the increase in nitric oxide synthase levels in activated macrophages[28]. *Z. cassumunar* has been widely used as a fork medicine for the treatment of various disorders, such as diseases accompanied by tissue inflammation, sprains, rheumatism, muscular pain, wounds and asthma. *Z. cassumunar* is enriched with phenylbutanoids and cassumunols and is capable of anti-invasion of huma fibrosarcoma HT 1080 cells, especially (E)-1-(2,4,5-trimethoxyphenyl)buta-1,3-diene [inhibition = (46.8 ± 7.2)% ($P < 0.05$) at 30 μmol/L], which is one of the phenylbutanoids[29]. Besides, *Z. cassumunar* contains curcuminoid as cassumuin A and B. Curcumin inhibits the phorbol 12-myristate 13-acetate-induced mRNA expression of MMP-1, -3, -9, and -14. Curcumin repressed the DNA binding and transcriptional activities of AP-1, which is a common upstream modulator of the MMP-1, -3, and -9 gene expressions[30]. These results indicated that *P. niruri* and *Z. cassumunar* contain bioactive molecules regulating the transcriptional factors and signal transduction pathways, resulting in attenuating the expression of MMP-3 and the production of MMP-2 and -9. Further investigations involving the elucidation on the mechanism of the plant extracts on suppression of MMP-3 expression are needed.

The ethanolic extract of *P. niruri* seemed to exhibit good effects on the suppression of DNA damage against hydroxyl radicals, anti-collagenase and the inhibitory effect on IL-1β-induced MMP-3 expression. Besides, it could reduce the production of MMP-2 and -9. It was indicated that *P. niruri* had various effects. Hence, it could be a promising herbal agent to be used for pharmaceutical applications.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

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