

Polyphenol fractions of *Pithecellobium jiringa* cause cell mortality via attenuating matrix metalloproteinase expression in lung and breast cancer cells *in vitro*

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Abstract

Phytochemical compounds from plants have been used as promising therapeutics to prevent and treat various diseases including cancer. *Pithecellobium jiringa* seed, known as jengkol, has possessed pharmacological effects, such as antioxidant, antimicrobial, and anti-inflammatory activities. In this study, anticancer activity of polyphenol fractions derived from *P. jiringa* parts (peel, seed coat, and seed) was measured by modulating cell mortality and matrix metalloproteinases (MMPs) expression in A549 lung and MCF7 breast cancer cells using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), gelatin zymography, and Real Time-PCR assays. Polyphenol fraction from each part of *P. jiringa* was extracted in 70% ethanol and identified using pyrolysis-Gas Chromatography/Mass Spectrometry (Py-GC/MS). MTT profiles showed that polyphenol fractions from *P. jiringa* parts dose dependently increased cell mortality of A549 and MCF7 cancer cells. Gelatin zymographic profiles revealed that *P. jiringa* polyphenol fractions at lowest dose (5 µg mL⁻¹) significantly decreased MMP-2 and MMP-9 activities secreted from A549 cells. At gene level, polyphenol fractions exhibited dosage effect on the decreased expression of MMP-2 mRNA in both A549 and MCF7 cancer cells. In summary, polyphenol fractions from *P. jiringa* parts may exert anticancer effect via attenuating MMP-2 expression *in vitro*.

Keywords

Pithecellobium jiringa

Anticancer activity

A549 cells

MCF7 cells

Matrix metalloproteinase-2

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Introduction

Cancer is a disease that causes by abnormal growth of cells that causing severe damage to other cells or tissues. Cancer can be caused by many factors, such as chemicals, radiation, alcohol, or smoking. There are several treatments for cancer, including chemotherapy, radiation, surgery, and combination treatment (Rudon, 2007). However, those treatments have negative effects for health and organ functions. In fact, the mortality rate caused by cancer has reached 8.2 million people and keep increasing (Cancer Research UK, 2013).

Nowadays, phytochemical compounds from plants have been used as therapeutic agents to prevent or heal many diseases, including cancer. The use of therapeutic agents is considered to be a safe and effective (Hayes *et al.*, 2008). Taxol, vinblastine, and vincristine are several natural anticancer agents that already used against cancer (Shoeb, 2006). However, these compounds are still expensive for low level economic people.

Pithecellobium jiringa, known as jengkol in Indonesia, has been traditionally used for food

or traditional medicinal for treatment of various ailments such as enuresis, hypertension, and diabetes (Muslim *et al.*, 2012). Bioactive fractions from plants have good effects for human body, such as antioxidant effects, anti-inflammatory effects, and inhibition various cancer cell lines (Motohashi, 2008). However, publication about bioactive fraction *P. jiringa* is very limited. Therefore, further study is needed to identify bioactive compound of djengkol as anticancer agent that could be applied for another alternative therapeutic treatment that more effective and affordable. This research was focused to explore the potency of polyphenol fractions from peel (PFK), seed coat (PFKA), and seed (PFB) of *P. jiringa* as anticancer agents using *in vitro* A549 lung and MCF7 breast cancer cell models.

Materials and Methods

Collection and preparation of samples

P. jiringa samples (code: DJ01 for peel, DJ02 for seed coat, and DJ03 for seed) were collected from traditional market at Balige, North Sumatera (Indonesia). It was dried under the sun and separated

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into peel, seed coat, and seed. Each dried part of *P. jiringa* peel, seed coat, and seed were crushed separately until it became powder.

Extraction of polyphenol fractions

Extraction of polyphenol fractions from each part of *P. jiringa* (peel, seed coat, and seed) was described according to the methods of Yanti *et al.* (2011). The powder of *P. jiringa* from each samples (100 g) was extracted with ethanol 70% with ratio of 1:4 (sample:solvent) ratio, then stirred with magnetic stirrer overnight. The solution was separated from the *P. jiringa* waste using Whatman filter paper. Solvent residue was evaporated to separate the polyphenol fractions using a Rotary Evaporator (BÜCHI Labortechnik AG, Flawil, Switzerland), and freeze-dried using freeze-drying system for obtaining polyphenol fractions from peel (PFK), seed coat (PFKA), and seed (PFB).

Pyrolysis-Gas Chromatography/Mass Spectrometry (py-GC/MS)

Py-GC/MS was carried out to analyse the phenolic compounds found in each polyphenol fraction (Nandagopal *et al.*, 2014). Py-GC/MS analysis was done using QP2010 (Shimadzu, Duisburg). Samples (PFK, PFKA, and PFB) were injected to the capillary column (Phase Rtx-5MS) with a film thickness of 60 m × 0.25 mm ID. Pyrolysis temperature was set to 280°C. Helium was used as the carrier gas.

Preparation of cell culture and sample

Human A549 lung cancer (ATCC®CCL-185TM) and human MCF7 breast cancer (ATCC®HTB-22TM) cells were cultured in Dulbecco's modified Eagle's Medium (DMEM) (GIBCO, Auckland, NZ). Cells were incubated at 37°C in 5% CO₂ for 24 hours. Polyphenol fractions (PFK, PFKA, and PFB) at stock concentration (103 -105 µg mL⁻¹) and working concentration (1-250 µg mL⁻¹) were dissolved in dimethyl sulfoxide (DMSO). Standard drugs of quercetin and rutin (1-25 µg mL⁻¹) were dissolved in DMSO. Ascorbic acid (10-250 µg mL⁻¹) was diluted in distilled water.

Cytotoxicity assay

Cells (5000 cells/well) were seeded into 96-well plate then incubated at 37°C in 5% CO₂ for 24 hours. Cells were treated with 100 µL for each sample of PFK, PFKA, and PFB at various concentrations (1-25 µg mL⁻¹) and then incubated for 48 hours. Cell morphology was analyzed with inverted microscope (Nikon Instruments, NY, US) with 10×100 magnification. After that, 100 µl of 3-(4,5-dimethyl-

2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were added into each well then incubated for 4 hours at 37°C in 5% CO₂. The purple formazan separated from solution and dissolved with 70% ethanol then its absorbance was measured at 565 nm using microplate reader. The mortality of cancer cells was counted by 100 - (OD_{treatment}/OD_{control} × 100%).

Sample treatment

Cells were seeded at a concentration of 105 cells mL⁻¹ in 12-well plates and cultured for 24 hours in DMEM. After that, cells were treated with 100 µL for each fraction of samples at various concentrations (5-25 µg mL⁻¹). Cells were incubated for 48 hours at 37°C. Then, media and cellular lysates were collected for measure the expression of cancer biomarker on protein and gene levels.

Gelatin zymography

Activity of MMP-2 and MMP-9 was performed using 7.5% polyacrylamide gels that copolymerized with gelatin (0.5%) (Yanti *et al.*, 2011). For each sample, 4 µL was loaded. Electrophoresis was performed at constant voltage of 80 V for 75 minutes. After that, gels were washed in aquadest and renaturation buffer (1% Triton X-100) for 30 minutes on an orbital shaker. Then, gels were incubated in ionic digestion buffer (0.1 M Tris-HCl (pH 8), 1 mM CaCl₂, 50 mM NaCl) at 37°C overnight. Zymogram gels were stained with Coomassie Brilliant Blue R-250 for 15 minutes and destained with methanol acetic acid. Enzymatic activity (MMP-2 and MMP-9) was detected as clear zones against the dark background. Relative band densities were analyzed by Gel-Doc Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA).

Real-Time polymerase chain reaction (RT-PCR)

RT-PCR was conducted to quantify the gene expression of MMP-2 and MMP-9 that regulate cancer. Total RNA from lysates were extracted with TRIzol® reagent (Life Technologies, California, US). The amount of isolated total RNA was assessed spectrophotometrically at 260/280 nm and kept at -70°C. RNA was converted to cDNA using iScript One-Step RT-PCR Kit with SYBR®Green and KAPA SYBR® FAST qPCR Kits for analysis by RT-PCR and then amplified with specific primer. MMP-2 were designed as 5'-GGCTGGTCAGTGGCTTGGGGTA-3' for forward and 5'-AGATCTTCTTCAAGGACCGGTT-3' for reverse. MMP-9 were 5'-GCGGAGATTGGAACCAGCTGTA-3' for forward and 5'-GACGCGCTGTGTACACCCACA-3' for reverse. House keeping gene of glyceraldehyde-3-

phosphate dehydrogenase (GAPDH) as control were 5'-CCATCTTCCAGGAGCGAGAT- 3' for forward and 5'-CACAGTCCATGCCATCACTG- 3' for reverse.

Statistical analysis

All data were done triplicate from samples and standards. The data were presented as average \pm deviation standard.

Results

Identification of *P. jiringa* polyphenol fractions

Polyphenol fractions from peel (PFK), seed coat (PFKA), and seed (PFB) of *P. jiringa* were analyzed using Py-GC/MS to determine the chemical compositions in each fraction. The highest peak in chromatogram of PFK fraction showed a polyphenol component known as benzenetriol trihydroxy benzene or pyrogallol (Figure 1a). It was the one major phenol content (35.52%) among 12 compounds that found in PFK fraction (data not shown). For PFKA fraction, the 3rd peak in chromatogram was identified as 1,2-benzenediol or pyrocatechol (Figure 1b). It is the only polyphenol compound (21.93%) from total of 8 compounds found in PFKA (data not shown). Meanwhile, Figure 1c showed that PFB fraction had no polyphenol content. Among 15 compounds in PFB fraction, the major peak was identified as levoglucosan (polysaccharide) with total amount of >50% (data not shown).

Effects of polyphenol fractions on cell mortality

MTT profiles showed that *P. jiringa* polyphenol fractions (PFK, PFKA, and PFB) at concentration of 10 $\mu\text{g mL}^{-1}$ caused >50% cell mortality in both A549 lung and MCF7 breast cancer cells (Figure 2). At highest concentration (25 $\mu\text{g mL}^{-1}$), all fractions inhibited cell proliferation >80% compared to that of untreated cells. Meanwhile, quercetin and rutin at lowest concentration (5 $\mu\text{g mL}^{-1}$) effectively blocked ~40% of both cell growth.

Effects of polyphenol fractions on cells morphology

Cell morphologies of A549 cancer cells were changed after treated with *P. jiringa* polyphenol fractions (PFK, PFKA, and PFB) and standard drugs (rutin and quercetin). The most severe damage of treated cells was reached at concentration of 25 $\mu\text{g mL}^{-1}$ from each fraction. Treated cells showed degradation of cell membrane, cell shrinkage, and loss contact to adjacent cells, compared to untreated cells (control) (Figure 3).

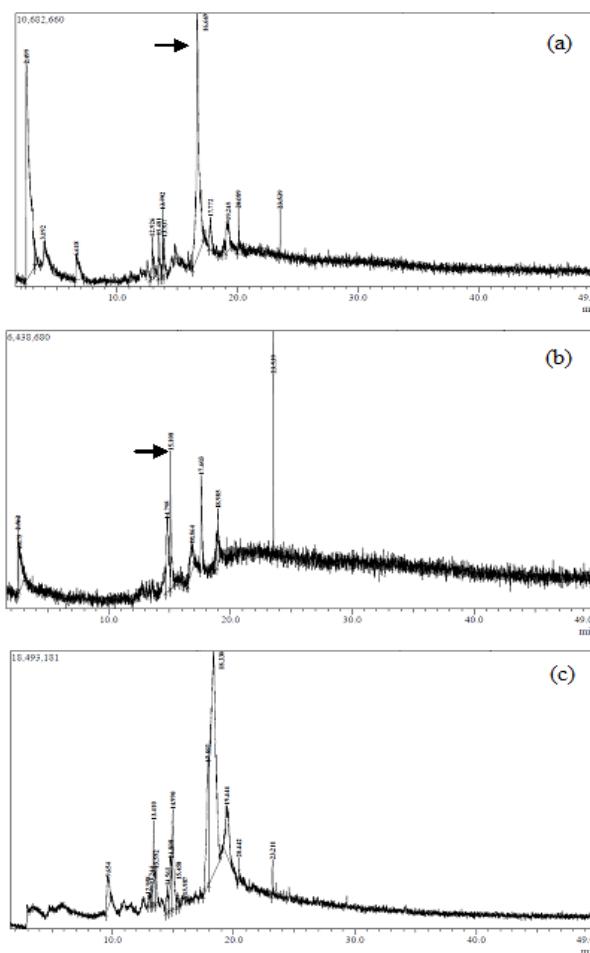


Figure 1 Chromatograms of polyphenol fractions from *P. jiringa* peel (PFK; a), coat (PFKA; b), and seed (PFB; c)

Effects of polyphenol fractions on MMP-2 and MMP-9 secretion

Gelatin zymographic profiles showed MMP-2 (72 kDa) and MMP-9 (92 kDa) secretion in A549 cancer cell media after treated with polyphenol fractions (PFK, PFKA, and PFB) and standard drugs (quercetin and rutin) (Figure 4). The increase of sample concentration caused the reduction of MMP-2 and MMP-9 band thickness compared to that of untreated cells (control). All *P. jiringa* fractions at lowest concentration (5 $\mu\text{g mL}^{-1}$) significantly decreased MMP-9 secretion (~60%) instead of MMP-2 secretion (~40%) in A549 cell media in comparison with the untreated cells (Figures 4f and 4g). Similar effects were also found in cells treated with quercetin and rutin. MMP-9 secretion was strongly attenuated by standards compared to that of MMP-2 secretion in cell system. However, our results showed there was no MMPs secretion from MCF7 cell media untreated- and treated-polyphenol fractions.

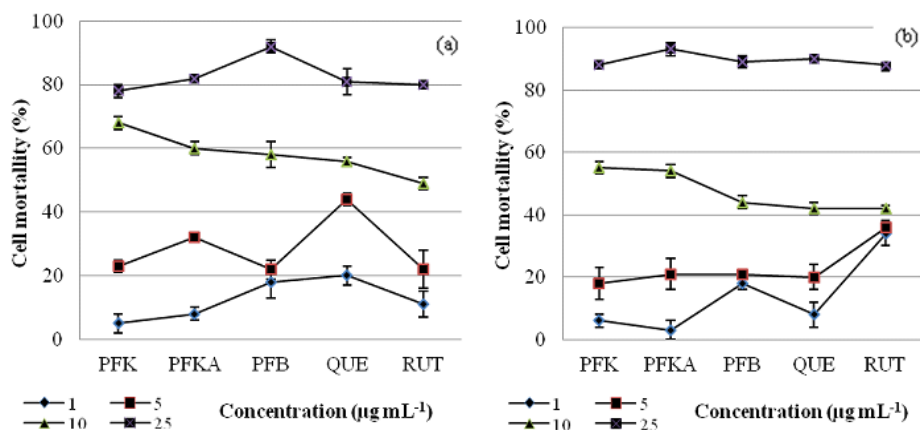


Figure 2 Mortality of A549 (a) and MCF7 (b) cancer cells using MTT assay after treated with *P. jiringa* polyphenol fractions: PFK, PFKA, PFB, quercetin, and rutin at various concentrations (1-25 µg mL⁻¹)

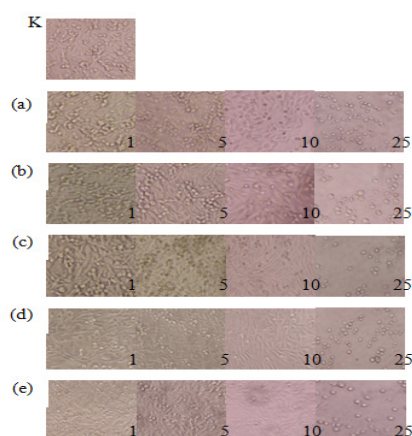


Figure 3 Morphology of A549 cancer cells after treated with *P. jiringa* polyphenol fractions: (a) PFK, (b) PFKA, (c) PFB, (d) quercetin, and (e) rutin at various concentrations (1-25 µg mL⁻¹) compared with untreated control (K)

Effects of polyphenol fractions on MMP-2 and MMP-9 mRNAs

RT-PCR profiles demonstrated that the gene expression of MMP-2 were significantly reduced up to 80% (in A549 cells) and 60% (in MCF7 cells) after treated with *P. jiringa* polyphenol fractions (PFK, PFKA, and PFB) at lowest concentration (5 µg mL⁻¹) (Figure 5). All fractions exerted the dose-dependent manner on MMP-2 gene inhibition. In linier, quercetin and rutin also performed similar inhibitory effects with *P. jiringa* polyphenol fractions. Unfortunately, polyphenol fractions had no inhibitory effect on the expression of MMP-9 mRNAs in both A549 and MCF7 cancer cells (data not shown). Moreover, treatment of polyphenol fractions caused the overexpression of MMP-9 mRNAs in comparison to those of untreated cells.

Discussion

Human mortality rate caused by chronic diseases including cancer is increasing annually. Lung cancer is type of cancer that caused highest mortality in men and breast cancer in women (WHO, 2012). In the development of cancer cells, angiogenesis and metastasis are the processes that associated with the overexpression of MMPs, such as MMP-2 and MMP-9 (Roomi *et al.*, 2009). The overexpression of MMPs is often associated with the activation of numerous pro-inflammatory cytokines in multiple cell types, including macrophages, T cells and epithelial cells (Wu and Zhou, 2009). In this study, we investigated the inhibition of MMP-2 and MMP-9 expression by *P. jiringa* polyphenol fractions in lung and breast cancer cells that initiated and caused the cancer progress *in vitro*.

Results of py-GC/MS showed that there was one phenol content in PFK and PFKA fractions (Figures 1a and 1b). Pyrogallol that found in PFK fraction is one of flavonoid compound that has three hydroxide with benzene rings (Burrows and Tyrl, 2012). Myricetin is one of polyphenolic compounds that has pyrogallol as main structure (Mahmoud and Moghazy, 2013). Similar with pyrogallol, pyrocatechol found in PFKA fraction also belongs to flavonoid compounds. In PFB fraction, there was not found any phenol compound. However, we assume that phenolic compounds in PFB fraction was degraded by heat from pyrolysis process in Py-GC/MS method. It is noted that polyphenols have heat-sensitivity characteristic (Islam, 2008).

Cytotoxicity results showed that dose of *P. jiringa* polyphenol fractions affected percentage cell mortality of A549 lung and MCF7 cancer cells (Figure 2). At highest concentration (25 µg mL⁻¹), all fractions

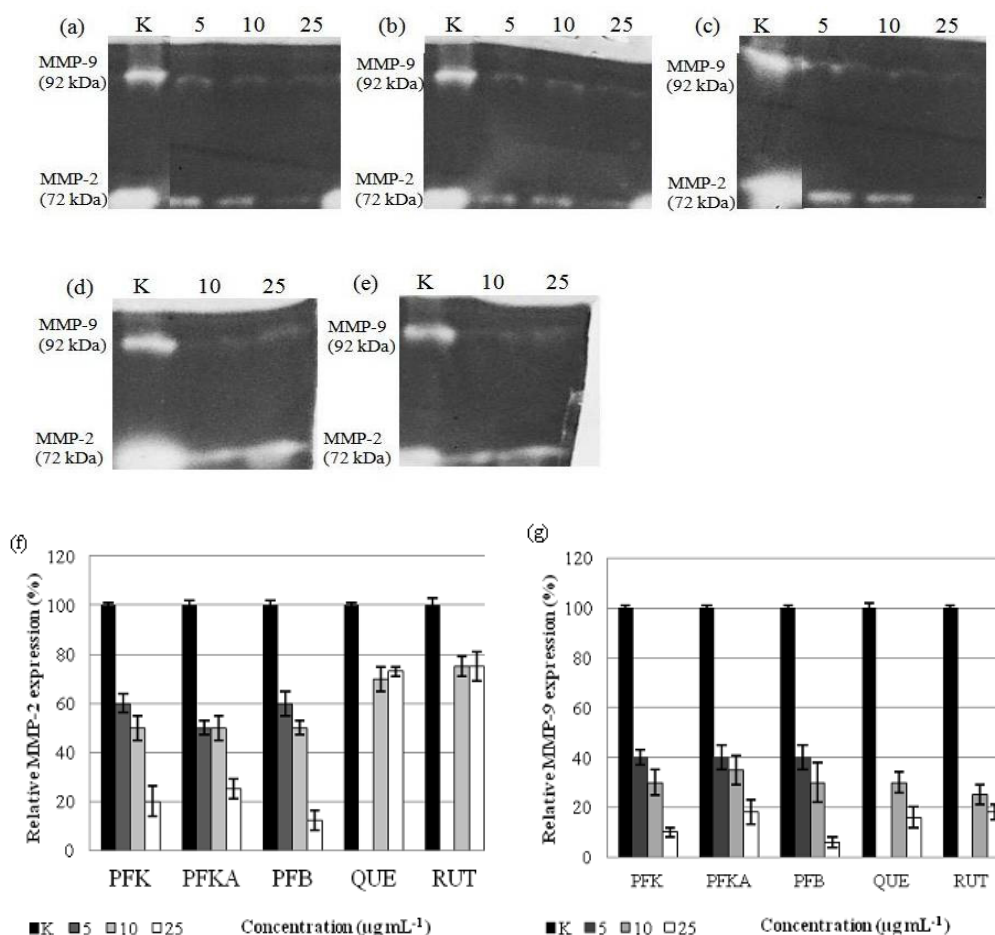


Figure 4 Gelatin zymographic profiles of A549 cancer cells media after treated with: (a) PFK, (b) PFKA, (c) PFB, (d) quercetin, and (e) rutin at various concentrations (5-25 $\mu\text{g mL}^{-1}$) compared with untreated control (K). Relative levels of MMP-2 (f) and MMP-9 secretion (g) in media after sample treatment

kill >80% cell growth in both cells, indicating that *P. jiringa* fractions may have anticancer potentials. Damage characteristics of A549 cancer cells occurred after treatment with *P. jiringa* fractions (Figure 3). Cell morphology exhibited apoptosis characteristics, such as cell shrinkage, degradation of cell membrane, loss contact with adjacent cells and blebbing of plasma membrane, chromatin condensation and nuclear fragmentation (Kettleworth, 2007; Seong *et al.*, 2013). These characteristics are similar with most cancer therapy that induce apoptosis and regression of cancer cells (Lin and Lowe, 2000).

Apoptosis is an active form of programmed cell death in development and survival of cells by eliminating damaged or otherwise unwanted cells (Kettleworth, 2007). The morphological criteria of apoptosis cells can be examined using using light microscopy. This approach has several advantages, including examination for large number of cells, low cost, and low technology. Furthermore, other assay to assess apoptotic cells, such as staining with a DNA binding dye, and examine cells under a fluorescent

microscope, or measured by biochemical criteria (Badley, 2007).

Gelatin zymographic profiles showed that MMP-2 and MMP-9 activities secreted from *P. jiringa* polyphenol fractions treated-A549 cell media were significantly decreased compared to untreated cells (Figure 4). These results suggest that polyphenol fractions have the ability to block A549 cancer cells via attenuation of MMP-2 and MMP-9 secretion at protein level. *P. jiringa* is known to have high content of polyphenols (Razab and Abdul-Aziz, 2010). Our results are in linear with Lamy *et al.* (2012) and Scoditti *et al.* (2012) that reported polyphenols have ability to inhibit angiogenesis process that related with MMP-2 and MMP-9 inhibition.

Interestingly, zymographic results of MCF7 cancer cells treated with *P. jiringa* polyphenol fractions indicated no MMP activity (data not shown). Wu and Zhou (2009) stated that *in vitro* MCF7 cancer cell is a non-metastatic cancer cell model without induction of several gene expressions. We assume that it caused the non-existence of MMP-2 and

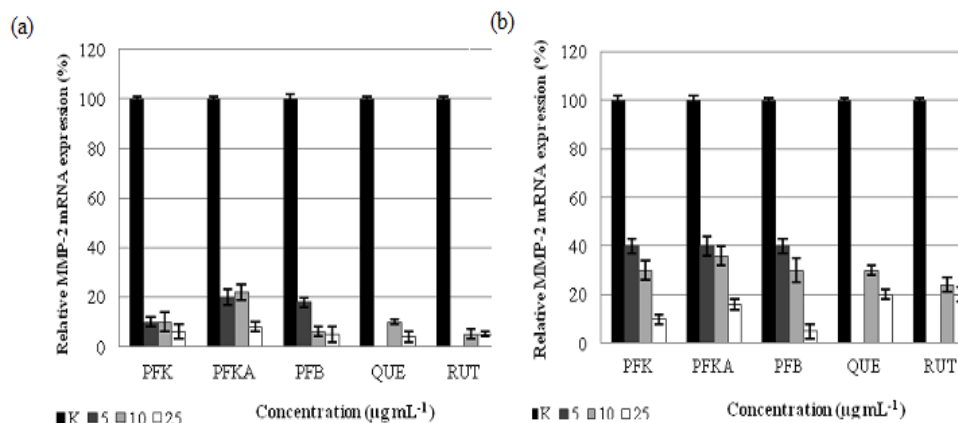


Figure 5 Gene expression of MMP-2 in A549 (a) and MCF7 (b) cancer cells after treated with *P. jiringa* polyphenol fractions (PFK, PFKA, and PFB) and reference drugs (quercetin and rutin) at various concentrations (5-25 µg mL⁻¹) compared with untreated control (K)

MMP-9 secretion in MCF7 cell media. Carcinogens are known to affect various signaling pathways, such as modulation of transcription factors (e.g. NF-κB, AP-1, STAT3), apoptotic proteins (e.g. Akt, Bcl-2, Bcl-xL, caspases, PARP), and growth factor signaling pathways (Aggarwal and Shishodia, 2006). Therefore, further study is needed to determine appropriate cancer biomarkers that work in MCF7 cancer cell model.

At gene level, we found that the expression of MMP-2 mRNAs in A549 and MCF7 cancer cells was significantly reduced after treated with *P. jiringa* polyphenol fractions in the dose-dependent manner (Figure 5). In contrast, all polyphenol fractions caused the over expression pattern of MMP-9 mRNAs in both cancer cells (data not shown). Cytokines have main functions as mediators and regulators of inflammation process that secreted by immune system. Both MMP-2 and MMP-9 have different cytokines that activate and regulate their expressions (Chakrabarti and Patel, 2005). We expect that *P. jiringa* polyphenol fractions may enhance cytokines levels in both lung and breast cancer cells that lead to the overexpression of MMP-9 mRNAs and an unbalanced production of MMP-9 mRNA and its specific inhibitor, tissue inhibitor of metalloproteinase-1 (TIMP-1). In linier with this study, Simi *et al.* (2004) also reported that MMP-9 mRNA was found to be overexpressed mainly by tumor cells in human non-small cell lung cancer. This condition, even not necessarily equate its enzymatic activity, seems to parallel a major cancer aggressiveness.

In contrast with RT-PCR results, zymographic profiles indicate that the increase of samples concentrations is in linear with the reduction of MMP-9 activity compared to untreated cells (Figure 4). MMPs are mostly secreted in pro-enzymes (zymogens) that require proteolytic processing to

release the active enzymes. Serin proteinases, such as plasmin and furin can activate MMPs, whereas some of the MMPs can activate other members of their family (Verma and Hansch, 2007). It is noted that MMP-2 is an activator of pro-MMP-9 (Toth *et al.*, 2003). Thus, our results suggest that *P. jiringa* polyphenol fractions may reduce MMP-9 activity in A549 cells due to the the lack expression of MMP-2 mRNA that lead to the decreased production of MMP-2 protein for activation of pro-MMP-9.

Conclusion

Polyphenol fractions of *P. jiringa* increased cell mortality, blocked MMP-2 and MMP-9 secretion, and inhibited MMP-2 gene expression in cancer cell models *in vitro*, indicating their potentials as alternative natural anticancer agents. Further study is needed to isolate and identified potential nutraceuticals from *P. jiringa* polyphenol fractions that have the exact efficacy in cancer prevention and treatment.

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