Kaemperia parviflora EXTRACT INHIBITS CASPASE-DEPENDENT APOPTOSIS STIMULATED BY *H. pylori* INFECTION

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ABSTRACT:

Background: Apoptosis process is one of the major pathogenesis in gastric cancer causing by *Helicobacter pylori* infection. *Kaempferia parviflora* (KP) has been traditionally used for treating gastrointestinal tract disorders, which exhibited anti-*Helicobacter pylori*, anti-inflammatory and anti-gastric ulcer. Our study aimed to evaluate the anti-apoptosis effects of KP extract in *H. pylori*-infected gastric epithelial cells.

Methods: The cytotoxic effect of KP extract was assessed by MTT assay in AGS cells at various concentrations and incubation times. The level of caspase-8 and caspase-3/7 activities and DNA fragmentation were determined by luminescence assays and ELISA, respectively.

Results: KP ethyl acetate extract significantly decreased AGS cells viability in dose and time-dependent manners, no cytotoxic effect was observed at 8 and 16 µg/ml of KP extract even 24 hours. *H. pylori* activated apoptosis mainly via caspase-3/7 more effectively than caspase-8. Interestingly, KP extract significantly inhibited both caspase-3/7 and caspase-8 in *H. pylori* infected and uninfected cells, although DNA fragmentation was not suppressed.

Conclusions: Thus, KP extract may be a prominent alternative therapy for gastric cancer induced by *H. pylori* infection through the inhibition of initiator caspase-8 and effector caspase-3/7 which play important roles in apoptosis pathway.

Keywords: *Helicobacter pylori*; *Kaemperia parviflora*; Apoptosis; Caspase-3/7; Caspase-8; DNA fragmentation

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INTRODUCTION

Helicobacter pylori is a causative agent of gastritis, gastric ulcer, mucosa-associated lymphoid tissue lymphoma and gastric cancer. The WHO classification indicated that *H. pylori* is a class I carcinogen. The majority of people in the world are infected by *H. pylori* which the highest prevalence area is in the southeast Asia [1]. In this region, there is difference prevalent rate in each country, Vietnam is about 75% [2] which is the highest rate and Thailand is about 57% [3]. Because *H. pylori* infection is a major cause of gastritis and gastric cancer, serious concerns aim at killing the organism with antibiotics and prevention of gastric cancer. Antibiotic is the primary eradication therapy which clarithromycin and metronidazole combination is still used in first-line therapy [4]. Unfortunately, many *H. pylori* antimicrobial drugs resistance strains dramatically increase which results in persistent infection and subsequently develops to gastric cancer [5]. The first step of *H. pylori* infection begins with the agent colonization to gastric mucosa; its virulent factors such as cytotoxin-associated protein A (CagA) massively activate the immune responsiveness in host gastric cells by secretion of certain cytokines and chemokines. The immune modulatory in gastric cells trigger aberrant proliferation and apoptosis [6].

Apoptosis pathway composes of extrinsic (death

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receptor-mediated) and intrinsic (mitochondria-mediated) pathway. The products of inflammatory response could stimulate apoptosis pathway through death ligand receptor or p53 activation such as TNF-alpha from macrophages [7] and reactive oxygen species produced by polymorphonuclear white blood cells [8], respectively. Moreover, vacuolating toxin A (VacA) from *H. pylori* could stimulate Bax and cytochrome c release in mitochondria-mediated pathway [9, 10]. Each caspase enzyme family plays important role in apoptosis process in different pathways, which subsequently activate endonuclease enzyme to produce DNA fragmentation, a key feature of apoptosis. The caspase-8 and caspase-10 implicate with apoptosis by death receptor-mediated pathway. The caspase-9 activation is a signaling of mitochondria-mediated pathway. The caspase-3/7 involves in both extrinsic and intrinsic pathways. A higher apoptosis index was found in gastric carcinoma than non-ulcer dyspepsia in patients infected with *H. pylori* [11]. Inflammation mediated by *H. pylori* infection also participates in cell apoptosis via leukocyte activities, especially ROS production [12-14]. The apoptotic and proliferation indices were increased and correlated to the severity of gastric inflammation in *H. pylori* induced gastritis [15]. Finally, cell apoptosis develops chronic gastritis to gastric atrophy stage and intestinal metaplasia [16, 17] and DNA might lose its stability leading to cell transformation and over proliferation which finally causes gastric cancer [18]. Thus, apoptosis is an important cell response *H. pylori* infection which participates in the gastric pathogenesis and early stage of carcinogenesis.

According to the ineffective regimens for treating *H. pylori*, natural substances have been used as alternative therapy in many countries, which have effective activities and low cytotoxicity. Several medicinal plants have been traditionally used for gastric ailments for many centuries in many countries such as Greek [19], India [20] and Thailand [21]. The prominent anti-*H. pylori* activity was founded in several herbal medicines including curcumin, garlic extract, ginger, and ginseng [22-25]. The Thai ginseng or *Kaempferia parviflora* (KP) also known as Krachaidum (in Thai) is a medicinal plant belong to *Zingiberaceae* family. The superior of pharmacological activities of KP has been demonstrated including antiallergenic, anti-inflammatory, antimutagenic, antimicrobial, anticancer and anti-peptic ulcer [26]. In previous study, KP could reduce TNF-alpha, nitric oxide and prostaglandin production of inflammatory cells [27]. KP extract has anti-gastric ulcer and anti-*H. pylori* activities including the inhibitory effects against *H. pylori* growth, anti-adhesion and anti-internalization into HEp-2 cell [28, 29]. However, the anti-carcinogenesis effects of KP are obscure in *H. pylori*-infected gastric cells. Hence, in the present study, we determined the anti-apoptosis effects of KP extract on *H. pylori* infected AGS cells including DNA fragmentation, caspase-3/7 and caspase-8 activities. The benefit of *H. pylori* eradication using traditional therapy of KP extract will be explored in the aspect of apoptotic responses which may improve eradication rate and reduce gastric cancer.

MATERIALS AND METHODS

Plant materials and extraction

A voucher specimen of *Kaempferia parviflora* Wall. Ex Baker was authenticated under ES280306, which deposited at the herbarium of the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand. The extraction process was described previously [29]. Briefly, roughly ground air-dried KP was extracted twice with 200 ml ethyl acetate solution for 48 hours at room temperature. The filtrate was evaporated at 40°C to dryness and dissolved at 10 mg/ml or 100 mg/ml with dimethyl sulfoxide (DMSO). The stock solutions were sterile by 0.2 µm pore size filter and stored at -20 ºC in the dark.

Bacterial stain, culture condition and inoculum preparation

H. pylori ATCC 43504 (vacA s1a/m1, cagA positive) strain was cultured on 7% (v/v) sheep blood enriched with brain heart infusion agar (Oxoid, United Kingdom) and incubated at 37ºC for 3 days in anaerobic jar with Gas Pack (Anaero Pack-MicroAero, Japan) to create microaerobic condition (6-12% O_2 and 5-8% CO_2). The inoculum was prepared in cell culture medium and adjusted to $1x10^8$ CFU/ml by Densitometer (Biomérieux Biotechnology, France).

AGS cells culture

AGS cells or human gastric adenocarcinoma epithelial cells (ATCC CRL1739) was kindly provided by Assist. Prof. Dr. Panan Ratthawongjirakul, Faculty of Allied Health Sciences, Chulalongkorn University, Thailand. The cells were cultured in RPMI 1640 medium (Caisson Laboratories, USA) containing 10% fetal bovine serum (Caisson Laboratories,

USA) and 1% antibiotic/antimycotic (Capricorn Scientific, Germany) at 37ºC in 5% CO_2 and 80% humidity. AGS cells with 80-90% confluency were harvested by 0.5% trypsin (Caisson Laboratories, USA).

Cell viability assay

AGS cells were seeded in 96-well plate at a density of $1x10^4$ cells/well and incubated overnight. After the cells attached, the medium was removed and the cells were incubated in RPMI 1640 containing two-fold serial dilution of the KP extract (8-128 µg/ml). For vehicle control, DMSO was tested instead of the extracts. The cytotoxic effects were examined at various times of 6, 12 and 24 hours. Five milligrams per milliliter of MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphynyl tetrazolium bromide (Bio Basic INC., Canada) was added and incubated in the darkness at 37ºC for 4 hours. Then, formazan crystals were solubilized in DMSO for 5 minutes and read absorbance at 570 nm and 630 nm for reference wavelength with a microplate reader (Synergy HT, BioTek). The % cell viability was calculated comparing to untreated AGS cells (Negative control) by the formula below.

$$\%Viability = \left[\frac{Mean\ absorbance\ test}{Mean\ absorbance\ control}\right]_{570} - \left[\frac{Mean\ absorbance\ test}{Mean\ absorbance\ control}\right]_{630} \times 100$$

Determination of caspase-3/7 and caspase-8

AGS cells were seeded in 96-well plate at a density of $1x10^4$ cells/well and incubated overnight to reach 70 - 80% confluence. Three culture conditions were undertaken in the assay. Co-culture condition, AGS cells were infected with 3 days old *H. pylori* (Multiplicity of Infection, MOI = 1:100) and treated with KP extract at various times of 6, 12 and 24 hours. Pre-treated condition, AGS cells were incubated with the KP extract for 3 and 6 hours and then washed with 1X PBS before infected with *H. pylori* for more 3 and 6 hours. Pre-infected condition, AGS cells were infected with *H. pylori* for 3 and 6 hours and then washed with 1X PBS before incubated with the KP extract for more 3 and 6 hours. Vehicle control was treated with DMSO. The level of caspase-3/7 and caspase-8 activities were determined by Caspase-Glo® 3/7 and Caspase-Glo® 8 luminescence assay kit (Promega Corporation, USA). The subsequent steps were performed according to the manufacturer's instructions. Briefly, 100 µl of Caspase-Glo® 3/7 or Caspase-Glo® 8 reagents was added to each well, incubated at room temperature for 30 minutes and measured with luminometer microplate reader (Synergy HT, BioTek).

Determination of DNA fragmentation

AGS cells were seeded in 96-well plate at a density of $1x10^4$ cells/

Awell and incubated overnight. Cells were infected with 3 days old *H. pylori* (MOI = 1:100) and co-cultured with the extract for 6, 12 and 24 hours. After washing twice with 1X PBS, cells were lysed and centrifuged at 20,000× g for 10 minutes. The 100 µl of supernatant was added to each well of anti-histone coated microtiter plate supply in Cell Death Detection ELISAPLUS (Roche Ltd, Switzerland) kit. The plate was incubated for 90 minutes in the darkness and washed three times with washing buffer. The peroxidase conjugated mouse monoclonal antibody against DNA was added, incubated for 90 minutes and washed three times. One hundred microliters of substrate solution was added, incubated for 15 minutes on rotary shaker and measured the absorbance at 405 nm with a microplate reader (Synergy HT, BioTek). The released of enrichment factor of mono- and oligonucleosomes was calculated by the formula below.

$$\text{Enrichment factor} = \frac{\text{(Mean absorbance sample)}}{\text{(Mean absorbance negative control)}}$$

Statistical analysis

The data are presented as mean ± standard deviation (SD). Statistical analyses were performed using SPSS version 22.0 (IBM Corp, 2013). All data was analyzed by using one-way ANOVA statistical analysis and followed by Dunnett's post hoc test. The symbol * indicates statistical significant (*p*-value < 0.05).

RESULTS

Effect of KP extract on AGS cell viability

The growth inhibitory activity against *H. pylori* was confirmed in our KP ethyl acetate extract with MIC at 32 µg/ml (data not shown). Thus, the AGS cell viability was tested with KP extract ranging from 8-128 µg/ml using MTT assay. The KP extract at 8 and 16 µg/ml were not affected the cell viability after 6, 12 and 24 hours of incubation. At 6 hours, the cell viability was significantly reduced to 19.38% in KP extract at 128 µg/ml (Figure 1A). While, KP extract ranging from 32-128 µg/ml significantly decreased cell viability to 64.8%-1.7% at 12 hours and 54.2%-0.97% at 24 hours, respectively (Figure 1B and 1C). The highest concentration of 0.128% DMSO containing in

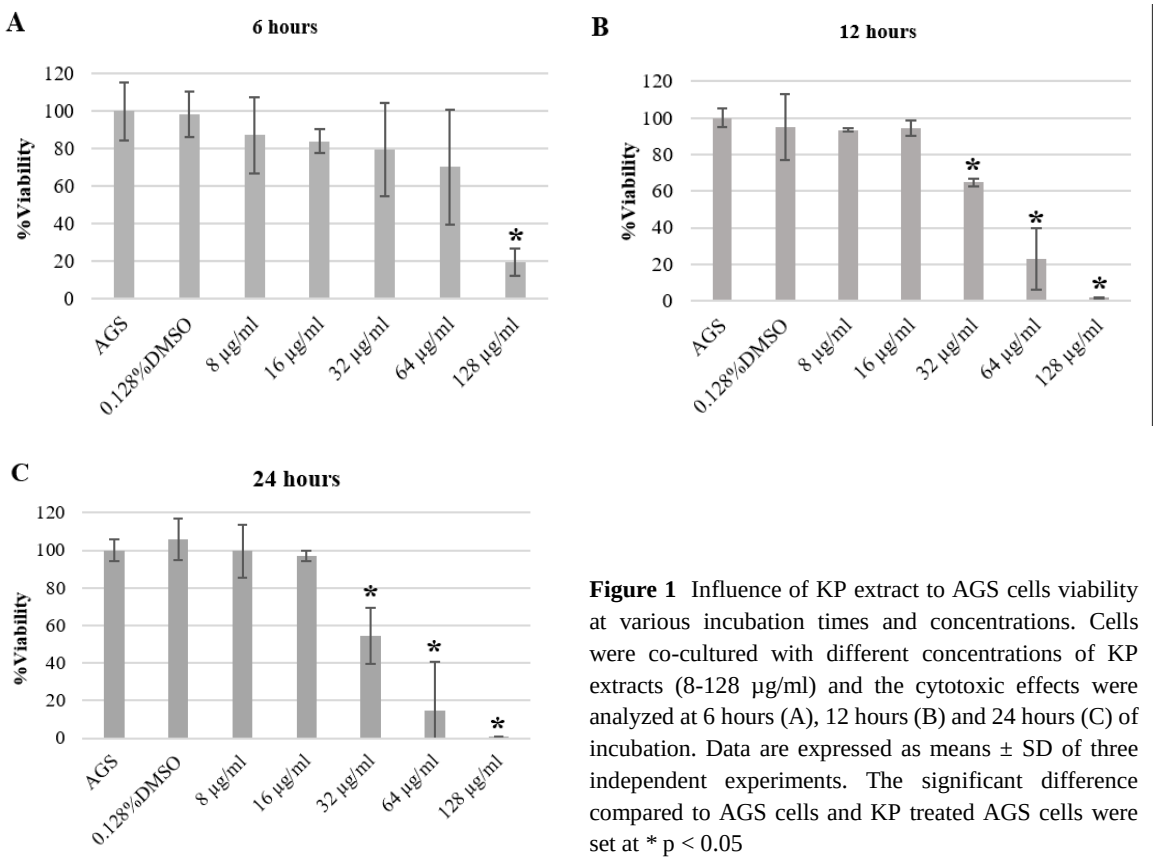

Figure 1 Influence of KP extract to AGS cells viability at various incubation times and concentrations. Cells were co-cultured with different concentrations of KP extracts (8-128 µg/ml) and the cytotoxic effects were analyzed at 6 hours (A), 12 hours (B) and 24 hours (C) of incubation. Data are expressed as means ± SD of three independent experiments. The significant difference compared to AGS cells and KP treated AGS cells were set at * $p < 0.05$

KP extract was not affected to cell viability at all incubation period, as demonstrated in the vehicle control group. Thus, KP extract affected to AGS cells viability in dose and time-dependent manners.

Anti-apoptosis property of the KP extract on *H. pylori*-infected AGS cells

1. Effect of KP extract against caspase-3/7 activity at various times and conditions

Due to KP extract at 8 and 16 µg/ml had no cytotoxic effects against AGS cells, the subsequent determination of caspase-3/7 level was performed at various times among co-culture, pre-treatment with KP extract and pre-infection with *H. pylori*. Both concentrations of KP extract significantly inhibited caspase-3/7 activity at all incubation period of co-culture. The inhibitory activity of KP extracts at 8 and 16 µg/ml were 53.9% and 75.13%, 64.2% and 81.3%, 45.8% and 85.5% at 6, 12 and 24 hours, respectively (Figure 2A). The preventive and therapeutic effects of KP extract were initially determined at 6 and 12 hours. In pre-treated groups, both 8 and 16 µg/ml of KP extract lacked preventive action against caspase-3/7 activity. After washout of KP extract, *H. pylori* were still capable to activated caspase-3/7 activity in AGS cells at 12 hours (Figure 2B). On the contrary, pre-infected condition, KP extract at 8 and 16 µg/ml significantly inhibited 80.6% and 90.5% of caspase-3/7 activation, respectively, at 12 hours (Figure 2C). The upmost %DMSO showed no effect to caspase-3/7 activity at all times.

2. Effects of KP extract against caspase-8 activity

According to caspase-3/7 activity was markedly inhibited at 16 µg/ml of KP extract, the inhibitory effect against caspase-8 synthesis was further carried out at 16 and 32 µg/ml. As result in Figure 3, the level of caspase-8 enzyme does not difference between *H. pylori* infected and uninfected AGS cells. At 32 µg/ml of KP extract significantly inhibited the production of caspase-8 enzyme only in infected AGS cells with 47.3% inhibitory activity. However, KP extract tend to reduce caspase-8 synthesis. At 16 µg/ml of KP extract showed 24.2% inhibitory activity in infected AGS cells and 32 µg/ml of KP extract revealed 25.8% inhibitory activity in uninfected AGS cells. The highest concentration of 0.032% DMSO solubilized in KP extract was not affected to caspase-8 activity.

3. KP extract irresponsible to *H. pylori* induced DNA fragmentation

As in Figure 4, *H. pylori* infection extremely activated DNA fragmentation at all incubation

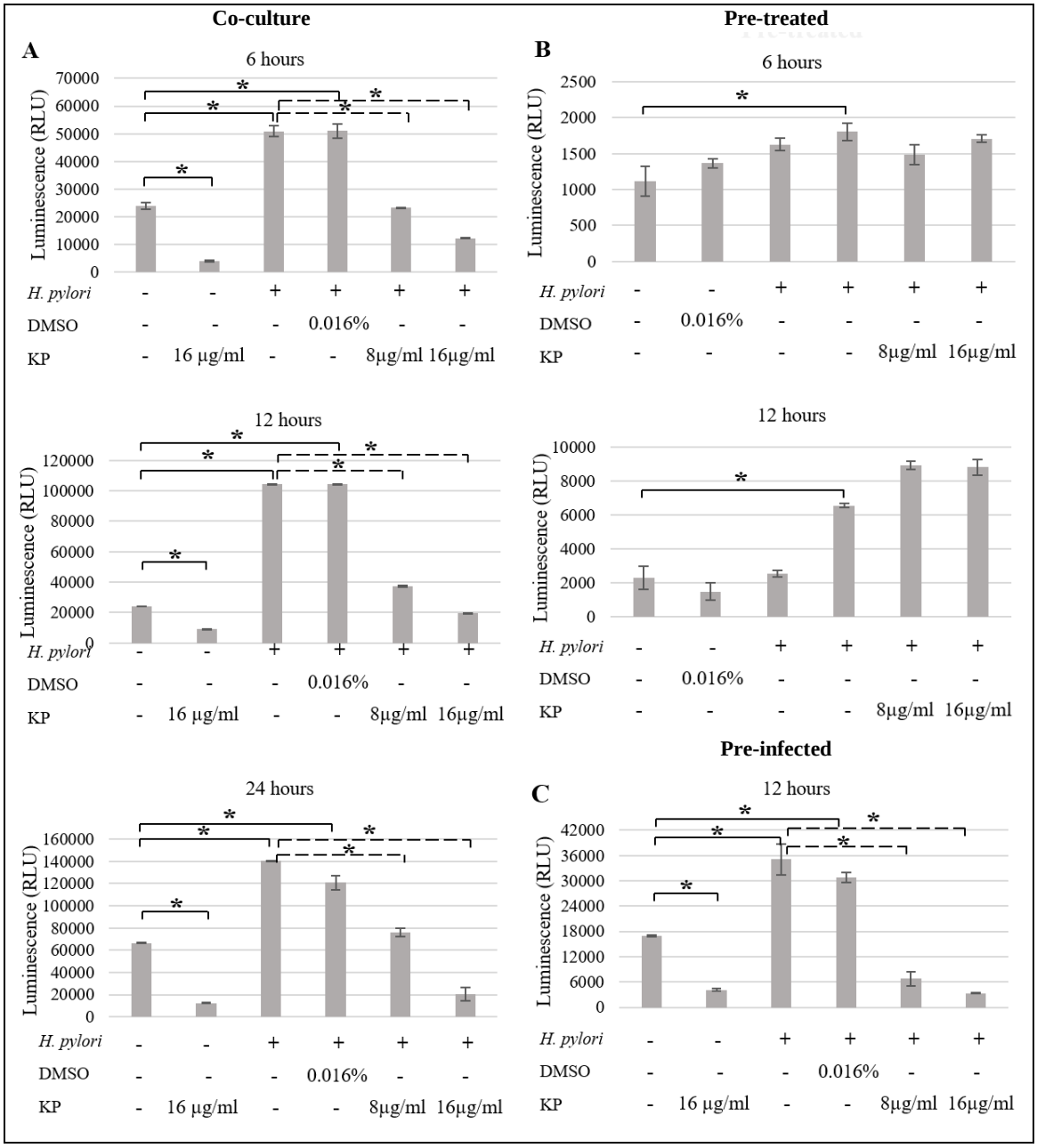

Figure 2 Effect of KP extract at 8 and 16 µg/ml against caspase-3/7 activity on uninfected and *H. pylori* infected AGS cells in various culture conditions and time points. Cells were co-cultured (A) at 6, 12, and 24 hours; pre-treated (B) at 6 and 12 hours; pre-infected (C) at 12 hours. The mixture of supernatant and lysate was determined for caspase-3/7 activity. Data are expressed as means ± SD of two independent experiments. The significant difference compared to AGS cells and *H. pylori* infected AGS cells were set at * $p < 0.05$

times. The level of DNA fragmentation was gradually increased by *H. pylori* induction from 6 to 24 hours. However, KP extract at 8 and 16 µg/ml failed to inhibit DNA fragmentation in *H. pylori* induced AGS cells at every time points.

DISCUSSION

H. pylori induced cell apoptosis is one of the most important mechanism of gastric cancer development. Two majors independent apoptotic signaling pathways are death receptor and mitochondrial pathways which activate various caspase enzymes including caspase-3, caspase-8 and caspase-9 [30]. In our study found that *H. pylori* enhanced apoptosis in AGS cells greatly through caspase-3/7 not caspase-8. The level of caspase-3/7 activity was more obviously boost up than caspase-8 in *H. pylori*-infected compared to uninfected AGS cells. Our data strongly supported that the major apoptotic process in *H. pylori* mediated through the

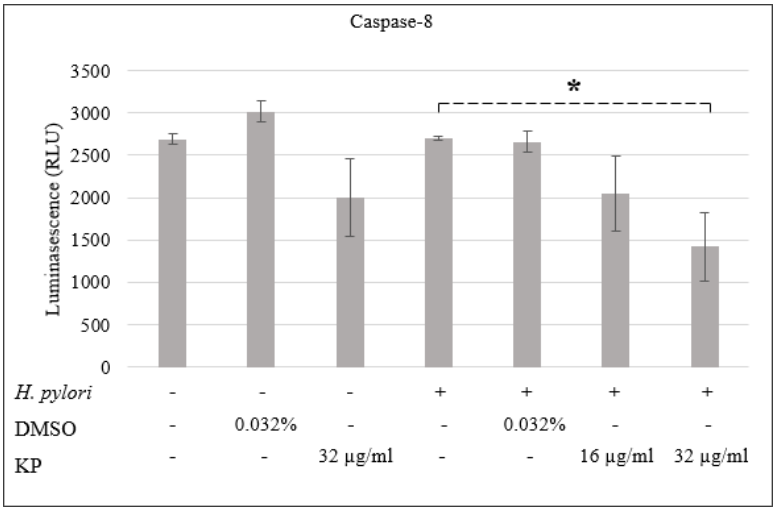

Figure 3 Effect of KP extract against caspase-8 activity on AGS cells and *H. pylori* infected AGS cells. Cells were co-cultured with *H. pylori* and KP extract at 16 and 32 µg/ml for 24 hours. The mixture of supernatant and lysate was determined for caspase-8 activity. Data are expressed as means ± SD of two independent experiments. The significant difference from control of each group was set at * $p < 0.05$

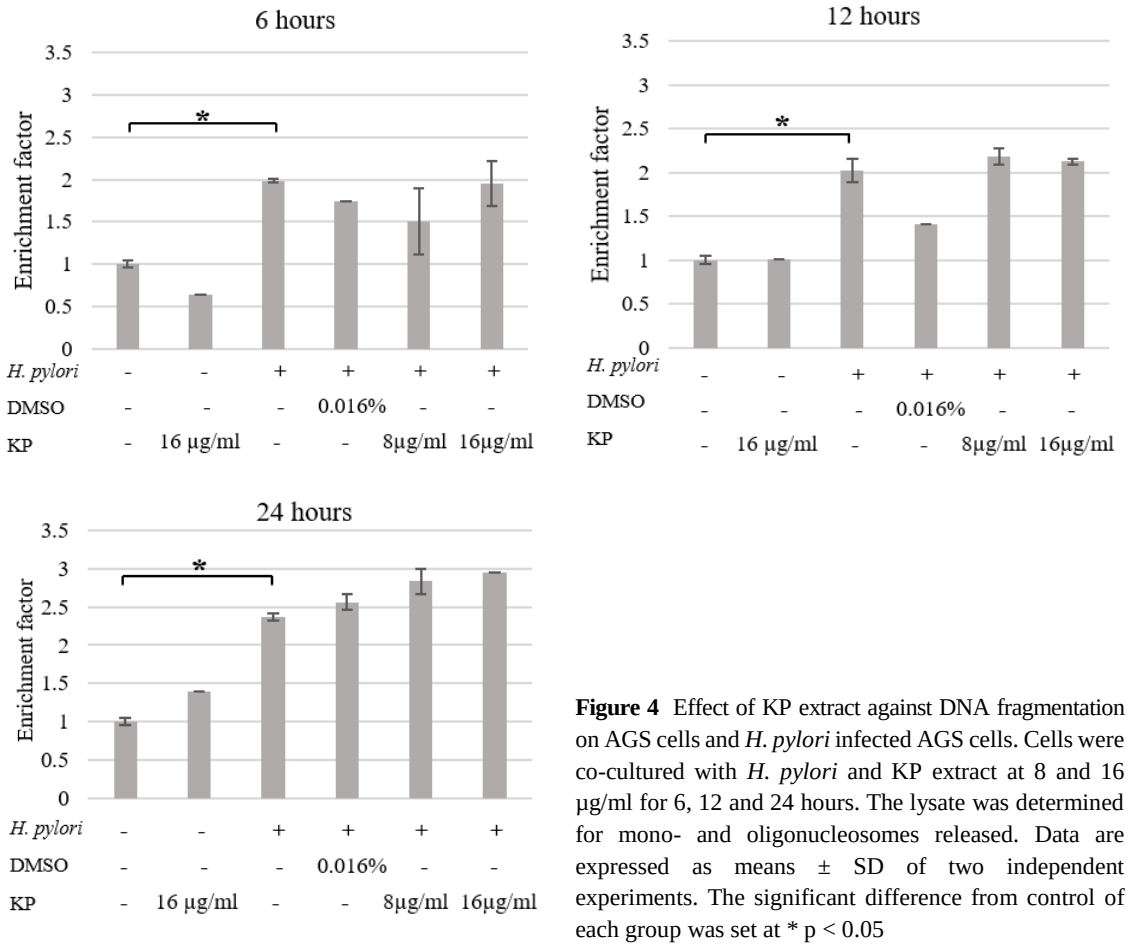

Figure 4 Effect of KP extract against DNA fragmentation on AGS cells and *H. pylori* infected AGS cells. Cells were co-cultured with *H. pylori* and KP extract at 8 and 16 µg/ml for 6, 12 and 24 hours. The lysate was determined for mono- and oligonucleosomes released. Data are expressed as means ± SD of two independent experiments. The significant difference from control of each group was set at * $p < 0.05$

mitochondrial pathway and independent of caspase-8 activation. Similarly, caspase-8 did not involve in *H. pylori* associated-apoptosis in SGC-7901 cells. Only 20% of the apoptosis rate was reduced by caspase-8 inhibitor while caspase-3 and 9 inhibitors significantly suppressed apoptosis rate at 72% and 45%, respectively [31]. Recently, *H. pylori* was found to inhibit the cleavage of tumor necrosis

factor receptor-associated factor 1 (TRAF1) and caspase-8 activation via cagA dependent mechanism or via other signaling pathways mediated by activation of NF-kB [32]. In addition, vacA N-terminal peptide could activate cytochrome c release and procaspase-3 as determined by poly (ADP-ribose) polymerase (PARP) cleavage [33].

Since the antibiotic resistance is problematic for recent *H. pylori* treatment [34], herbal medicines have become the alternative treatment for *H. pylori* infection by their several pharmacological activities [35]. *Kaempferia parviflora* (KP) is commonly found in Asia including Thailand and used as a traditional remedy to treat gastrointestinal disorders for a long time. In this study, the ethyl acetate extract of KP showed time and concentration effect on survival of AGS cells. However, 8 and 16 µg/ml of KP extract does not displayed significant cytotoxic action even at 24 hours. Similarly, KP maceration extract at 20 µg/ml affected AGS cell viability only 10% at 48 hours [36]. Interestingly, our KP extract significantly inhibited both caspase-3/7 and caspase-8 synthesis in *H. pylori* infected and uninfected AGS cells. KP extract showed superior suppressive action against caspase-3/7 in co-culture and pre-infected conditions which benefit for its therapeutic purpose in *H. pylori* infected patient. While, the preventive action was not noticed with KP extract, the caspase-3/7 activation was still induced by *H. pylori* in pre-treated KP extract to AGS cells. The low concentration of KP ethanolic extract (10 and 20 µg/ml) expressed anti-apoptosis function in U937 cell, but high concentration (80 and 100 µg/ml) induced apoptosis through caspase-3 activation [37]. KP.8.10 or 5,7,4-trimethoxyflavone**,** a bioactive compound of KP extract, significantly increased caspase-3 activity at 18 hours in cholangiocarcinoma cell lines [38]. However, DNA fragmentation which is a hallmark of apoptosis could not suppressed by our KP extract in *H. pylori* induced AGS cells even 24 hours of co-culture. This result supported that the caspase-independent cell apoptosis plays a major role in DNA fragmentation in *H. pylori* infected tissues especially the intrinsic activation of reactive oxygen radicals through mitochondria pathway [39]. Several investigators have investigated the regulation of *H. pylori* induced apoptosis leading to releasing of cytochrome c, apoptosis-inducing factor (AIF) and endonuclease-G (ENDO-G) from mitochondria [40]. Moreover, the vacA toxin of *H. pylori* can efficiently trigger apoptosis by dissipation of mitochondrial membrane potential, recruitment of Bax and Bak, and release of cytochrome c into the cytosol [9]. Although, KP extract could inhibit the activity of caspase-3/7 and caspase-8, the effect of KP extract has to be further elucidated against other mitochondrial pathways in order to effectively inhibit downstream apoptotic events.

CONCLUSION

Our results demonstrate that *H. pylori* infection activates apoptosis using caspases-independent as the main pathway. KP ethyl acetate extract exhibited less cytotoxicity with time and dose-dependent and significantly suppressed caspase-8 and caspase-3/7 activities in both of *H. pylori* infected AGS cells and uninfected cell. Further investigation should be carried out for a better understanding of *H. pylori* induced-apoptosis pathway and anti-apoptotic response of KP ethyl acetate extract.

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