

Full Length Research Paper

The effects of *Kaempferia parviflora* on anti-internalization activity of *Helicobacter pylori* to HEp-2 cells

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***Helicobacter pylori*, an etiological agent of active chronic gastritis and peptic ulcer disease, is now considered to be an invasive enteropathogen. Anti-adhesion and anti-internalization are new strategies for prevention and treatment of bacterial infection including the alternative of medicinal plants. In this study, four parts of *Kaempferia parviflora*'s extracts composing of volatile oil, hexane, ethyl acetate and methanol were examined for their antibacterial and anti-internalization activities of *H. pylori* against HEp-2 cells. All extracts except volatile oil showed significant antibacterial activity and had a minimum inhibitory concentration (MIC) ranging from 32 - 64 µg/ml. The most active extract of ethyl acetate exhibited significant anti-internalization activity which corresponded to dose and time of treatment. Moreover, *K. parviflora*'s ethyl acetate extract could significantly inhibit the invasion of both *H. pylori* virulent strains (*cagA*⁺) and non-virulent strains (*cagA*⁻) in HEp-2 cells. Thus, *K. parviflora* is one of the effective herbs for potential prevention and treatment of *H. pylori* infection.**

Key words: *Helicobacter pylori*, *Kaempferia parviflora*, HEp-2 cells, anti-internalization activity.

INTRODUCTION

Helicobacter pylori is a causative agent in gastroduodenal diseases such as chronic gastritis, peptic ulceration, gastric adenocarcinoma and mucosa-associated lymphoid tissue lymphoma (MALT) (Graham, 1997; Raus et al., 1987; Wotherspoon et al., 1993). Its colonization most likely occurs during childhood and affects about half of the world's population by majority of fecal-oral transmission (Blaser, 1999; Covacci et al., 1999). The *H. pylori* infection is successfully treated by a combination of a proton pump inhibitor (PPI) and two antibiotics of

clarithromycin, amoxicillin, or metronidazole. However, the incidence of antibiotic resistance has increased, causing the disease eradication to fail. According to previous reports, *H. pylori* has been considered an intracellular microorganism which may be responsible for the chronic infection and the development of gastric cancer (Anderson and Holck, 1990; Evans et al., 1992; Wilkinson et al., 1998). The histomicrobiological study from transmission electron microscope revealed spiral *H. pylori* in the gastric lumen and between the gastric epithelium cells and coccoid *H. pylori* within the endocytotic vesicles in the apical cytoplasmic part of the epithelial cells, which confirmed the internalization capability of this organism (Ozbek et al., 2009). The adherence and invasion of *H. pylori* in gastric epithelial cells triggers the process of immune response and virulence properties including secretion of pro-inflammatory cytokines, cell proliferation, and apoptosis (Day et al., 2004; Ledig et al., 2004; Karabay

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Abbreviations: MIC, Minimum inhibitory concentration; CFU, colony forming unit; *cagA*⁺, *Helicobacter pylori* virulent strains; *cagA*⁻, *Helicobacter pylori* non-virulent strains.

et al., 2006). Therefore, a non-antibiotic agent, capable of inhibiting the growth of *H. pylori* and the invasion to the target cells may be an effective way for preventing or treating the infections. Medicinal plants are useful alternative source of novel drugs containing various pharmacological activities such as antimicrobial activities, anti-inflammatory and anti-ulcerogenic effects. *Kaempferia parviflora* Wall. Ex Baker or Kra-chai-dam belonging to the Zingiberaceae family is one of the attractive plants for the reasons of its anti-gastric ulcer activity (Rujjanawate et al., 2005). It has long been used in Thai traditional medicine for rectifying male impotence, body pains, relief symptoms of colic and gastrointestinal upset. The extract could reduce the time in the first 10 min of rat courtship behavior with no toxicity (Sudwan et al., 2006). Furthermore, the extracts and flavone derivatives from the rhizome of *K. parviflora* could suppress function of multidrug resistance associated-proteins (MRP), in addition to P-glycoprotein, in cancer cells, (Patanasetthanont et al., 2006; Patanasetthanont et al., 2007).

In this study, the anti-*Helicobacter pylori* activity of extracts from *K. parviflora* were investigated. In addition, internalization ability of *H. pylori* against HEp-2 cells was compared between the presence and absence of extracts using gentamicin internalization assay.

MATERIALS AND METHODS

Plant materials and extraction

K. parviflora Wall. Ex Baker were bought from a Thai traditional drug store at Pak Klong market in Bangkok, Thailand. The plant material was identified by one of the authors (E. Saifah, Chulalongkorn University). A voucher specimen (No. ES280306) was deposited at the herbarium of the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand. One kilogram of roughly ground air-dried plant material was extracted for 3 h by hydrodistillation method and volatile oil was collected. The residue was further extracted twice for 48 h with 200 ml of hexane at room temperature. The combined extracts were filtered and concentrated by evaporation at 40°C until dryness. The residue was dissolved in 200 ml of ethyl acetate and extracted twice for 48 h at room temperature. The filtrate was evaporated to dryness as described. The remaining residue was finally extracted twice for 48 h at room temperature with 200 ml of methanol. After filtration, the extract was evaporated similarly to dryness. The yield obtained under each extraction type was 0.129% for volatile oil, 0.046% for hexane, 0.721% for ethyl acetate and 1.27% for methanol. Each extract was dissolved at 0.25 g/ml with dimethyl sulfoxide (DMSO) except for hexane extract at 0.125 g/ml. These stock solutions were filtrated through 0.2 µm Millipore and stored at -20°C before use.

Bacterial strains and culture condition

Three reference strains of *H. pylori* (ATCC 43504, ATCC 43526 and ATCC 51932) were purchased from the American Type Culture Collection (Rockville, MD). Nine clinical isolated were a kind gift from Dr. Ratha-korn Vilaichone, Division of Gastroenterology, Department of Medicine, Thammasat University Hospital (C7, C53, 742, 818, 820, 849, 867, 912 and 949) and 2 clinical isolates were

purchased from Department of Medicinal Sciences, Ministry of Public Health, Thailand (DMST 20165 and DMST 20885). The bacteria were grown on brain heart infusion agar containing 5% (v/v) sheep blood and were incubated at 37°C under microaerophilic conditions (N₂, 85%; O₂, 5%; CO₂, 10%) using gas generating kit (Oxoid, UK) for 3-5 days.

Minimum inhibitory concentrations

Minimum inhibitory concentrations (MIC) were determined, in duplicate, by an agar dilution method following the National Committee for Clinical Laboratory Standards guidelines using Mueller-Hinton agar with 5% (v/v) sheep blood. The concentrations of the extracts were ranging from 16 - 2,048 µg/ml using two-fold serial dilutions. A 72-h bacterial colony of *H. pylori* strains were suspended in 0.85% NaCl to a turbidity of McFarland standard 2 (10⁷ - 10⁸ CFU/ml). Subsequently, three microlitres of each inoculum was spotted on each plate and incubated as described for 3 days. The MIC was defined as the lowest concentration of each extract at which no visible growth was observed.

Culture of HEp-2 cells

The HEp-2 cells (kindly provided by Dr. Pornthep Tiensiwakul, Faculty of Allied Health Sciences, Chulalongkorn University, Thailand) were grown on tissue culture plasticware in Dulbecco's modified Eagle's medium (DMEM; Life Technologies Inc., Rockville, Md) supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) antibiotic-antimycotic solution (Gibco BRL Laboratories, Grand Island, N.Y.) at 37°C in 5% CO₂ with 80% humidity.

Gentamicin internalization assay

HEp-2 cells were washed two times with Hank's balanced salt solution (HBSS) treated with trypsin, and counted. Approximately 1X10⁶ cells were seeded on six wells tissue culture plates and incubated overnight to obtain 80% confluency. Each well was washed twice with HBSS and added with DMEM containing no antibiotics. *H. pylori* was harvested and resuspended into HBSS (about 10⁸ CFU/ml). 100 µl of bacterial suspension was added to each well in the absence or presence of the extracts and incubated for 3 h. After incubation, the cells were gently washed two times with HBSS, and treated with HBSS containing 100 µg/ml gentamicin and incubated for one hour to kill extracellular bacteria. HEp-2 monolayers were then washed three times with HBSS and treated with distilled water for 10 min to release intracellular bacteria. Serial 10-fold dilutions of the lysed monolayer were placed on brain heart infusion agar supplemented with 5% (v/v) sheep blood, incubated as previously for 3 - 5 days to determine the number of viable intracellular bacteria.

Statistical analysis

All experiments were carried out independently in duplicate experiments. The inhibition of internalization by various *K. parviflora*'s extracts and various concentrations of *K. parviflora* ethyl acetate extract were evaluated by one way analysis of variance (ANOVA). While, the effect of *K. parviflora*'s extracts on internalization activities of each *H. pylori* strains and the effect of treatment time were compared under treated and untreated condition using the student's *t* test. Statistical significance was accepted at the P = 0.05 level.

Table 1. Minimum inhibitory concentrations (MICs: $\mu\text{g/ml}$) of *Kaempferia parviflora*'s extracts against 13 *Helicobacter pylori* strains.

Extracts	MIC ($\mu\text{g/ml}$)
Volatile oil	1,024
Hexane extract	64
Ethylacetate extract	32
Methanol extract	64

RESULTS

Minimum inhibitory concentrations

Four parts of *K. parviflora*'s extracts, volatile oil, hexane, ethyl acetate and methanol, were screened in vitro for the anti-*Helicobacter pylori* actions. As summarized in Table 1, the strongest growth inhibition against all thirteen test strains of *H. pylori* was ethyl acetate extract with MIC of 32 $\mu\text{g/ml}$. The MICs of the extracts from hexane and methanol were 64 $\mu\text{g/ml}$. While, the MICs of volatile oil was 1,024 $\mu\text{g/ml}$.

Internalization assay

All *K. parviflora*'s extracts exhibited anti-internalization activities against HEP-2 cells at the concentration of MIC except volatile oil (Figure 1). It was found that volatile oil affected the cellular structure and adherence ability of HEP-2 cells. HEP-2 cells were deformity and detached from the culture well. Moreover, volatile oil inhibited the growth of *H. pylori*, no viable bacteria was detected when incubated the volatile oil with *H. pylori* for 3 h (data not shown). However, the anti-internalization activities were not significantly different between hexane, ethyl acetate and methanol extracts (Figure 1; $P > 0.05$). In order to determine the optimum treatment condition, the ethyl acetate extract with the highest MIC was tested for the inhibitory effect on *H. pylori* internalization at various times and concentrations. It was found that the bacterial conferred higher capability to enter into the cells at the extended incubation times (Figure 2). However, ethyl acetate extract of *K. parviflora* still maintained effective anti-internalization activity upon *H. pylori* during the longer treatment period. The number of treated intracellular *H. pylori* was significantly lower than the untreated at 3, 6, 12 and 24 h (Figure 2; $P < 0.05$). Due to the number of bacterial viability was decreased during the longer treatment time (data not shown), the effect of dose was examined at 3 h according to low effect of growth inhibition. The invasion efficiency had been shown to respond to the concentrations of *K. parviflora*'s ethyl acetate extract in the range from 8 to 128 $\mu\text{g/ml}$ (Figure 3). The proportions of intracellular bacteria were significantly different from the control without the extract ($P <$

0.05). The percent of internalized bacteria was 59.28, 42.96, 32.34, 11.63 and 8.47% at 8, 16, 32, 64 and 128 $\mu\text{g/ml}$, respectively. However, *K. parviflora*'s ethyl acetate extract of 128 $\mu\text{g/ml}$ markedly effected the growth of *H. pylori*, the number of viable bacteria dramatically reduced about 100 fold from the initial inoculum (data not shown). Thus, the optimum treatment condition of *K. parviflora*'s ethyl acetate extract against *H. pylori* internalization into HEP-2 cells was selected at 64 $\mu\text{g/ml}$ at 3 h of incubation. Comparison of the effect of *K. parviflora*'s ethyl acetate extract between five *H. pylori* strains (3 *cagA*⁺ strains of ATCC 43504, ATCC 43526 and clinical isolated no. 820 and 2 *cagA*⁻ strains of ATCC 51932 and DMST 20165) for the anti-internalization activity against HEP-2 cells was demonstrated in Figure 4. The extract was significantly inhibited the invasion of all tested *H. pylori* strains ($P < 0.05$). The bacterial invasion activities in overall treated strains were reduced more than 75% compared to the untreated control.

DISCUSSION

H. pylori colonization of the human stomach leads to a chronic infection associated with duodenal and gastric ulcers (Logan, 1994; Forman, 1996). In view of the rapid increase of bacterial resistance to antibiotic, medicinal plants are now more explored for their bioactivity. Here, we demonstrated the anti-*Helicobacter pylori* activity of *K. parviflora*, a Thai traditional herb for treatment of colic disorder and gastric ulcer (Rujjanawate et al., 2005). The ethyl acetate extract of *K. parviflora* exhibited the highest MIC of 32 $\mu\text{g/ml}$ against *H. pylori*. *K. parviflora* had been reported to possess antibacterial, anti-allergic activities (Tewtrakul and Subhadhirasakul, 2007; Tewtrakul et al., 2008), anti-peptic ulcer (Rujjanawate et al., 2005), antiviral proteases (Sookkongwaree et al., 2006) and anti-inflammatory activity (Sae-wong et al., 2009). Recently, the ethanolic extract of *K. parviflora* was found to exhibited the anti-cancer action against human cholangiocarcinoma (Leardkamolkarn et al., 2009). It was suggested that some bioactive flavonoids containing in the rhizome of *K. parviflora* was the major active compound comprising of 5-hydroxy-3,7-dimethoxyflavone, 5-hydroxy-7-methoxyflavone, 5-3,7,4'-trimethoxyflavone, 5-hydroxy-7,4'-dimethoxyflavone, 5-hydroxy-3,7,3'4'-tetramethoxyflavone, 3,5,7-trimethoxyflavone, 3,5,7,4'-tetramethoxyflavone, 5,7,4'-trimethoxyflavone and 5,7,3'4'-tetramethoxyflavone (Yenjai et al., 2004). Some flavonoids derivatives and their metabolite such as genistein, hesperitin, ponciretin, irisolidone and cabreuvin displayed anti-*Helicobacter pylori* activity (Bae et al., 1999).

H. pylori has been proven an intracellular organism in both cell lines and human gastric biopsies (Bjorkholm et al., 2000). Moreover, it was found that the antibiotic resistance in *H. pylori* was associated with higher internalization activity due to the ability to escape from antibiotic

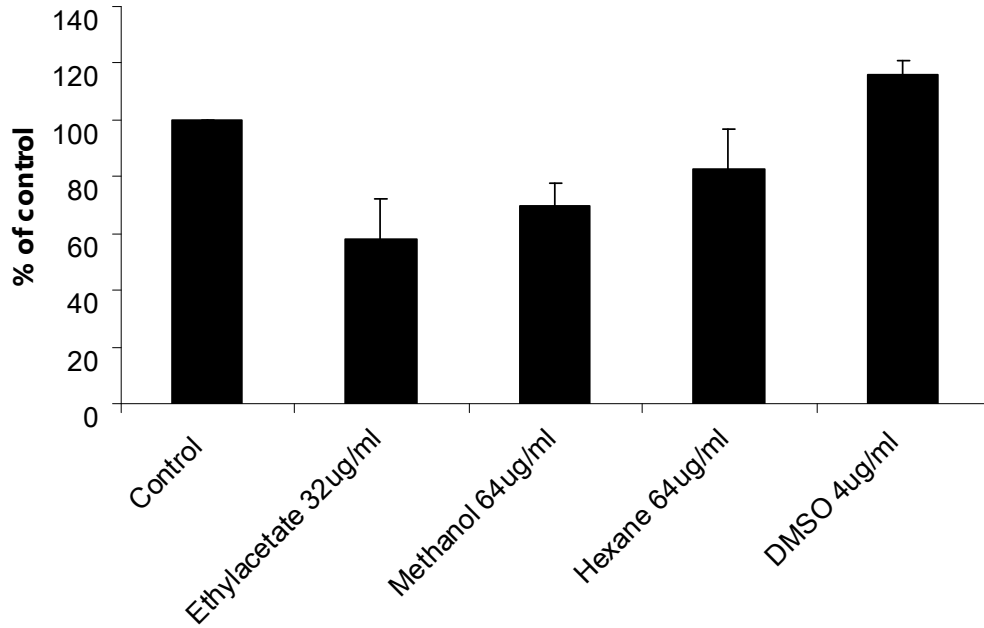


Figure 1. Internalization assay of *H. pylori*-infected HEp-2 cells in the absence or presence of *K. parviflora*'s extracts at 3 h of incubation. The extracts were added at the concentration of the MIC. Results are presented as the proportion of internalized bacteria versus control. The proportion of internalized bacteria was calculated as the rate of the number of internalized bacteria divided by the bacterial inoculum. Data are presented as mean±SD of separate duplicate experiments with two wells per experiment and duplicate plate counts from each well. The significant difference from control without the extract was set at * p<0.05.

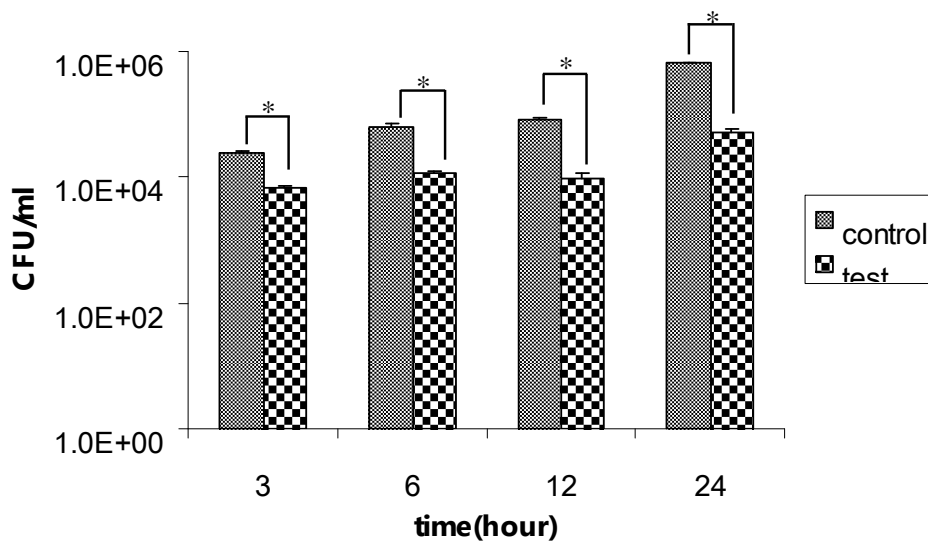


Figure 2. Quantitative internalization assay in HEp-2 cells incubated with *H. pylori* ATCC 43504 with and without ethyl acetate extract of *K. parviflora* at 3, 6, 12 and 24 h. The concentration of *K. parviflora*'s ethyl acetate extract was used at 32 µg/ml. The number of internalized *H. pylori* was assessed by log CFU/ml. Data are presented as mean±SD of separate duplicate experiments with two wells per experiment and duplicate plate counts from each well. The significant difference from each control was set at * p<0.05.

treatment. The close relationship between the elevated invading efficiency and clarithromycin resistance was

investigated (Lai et al., 2006). Therefore, anti-invasion agents may open a new avenue for effective prevention

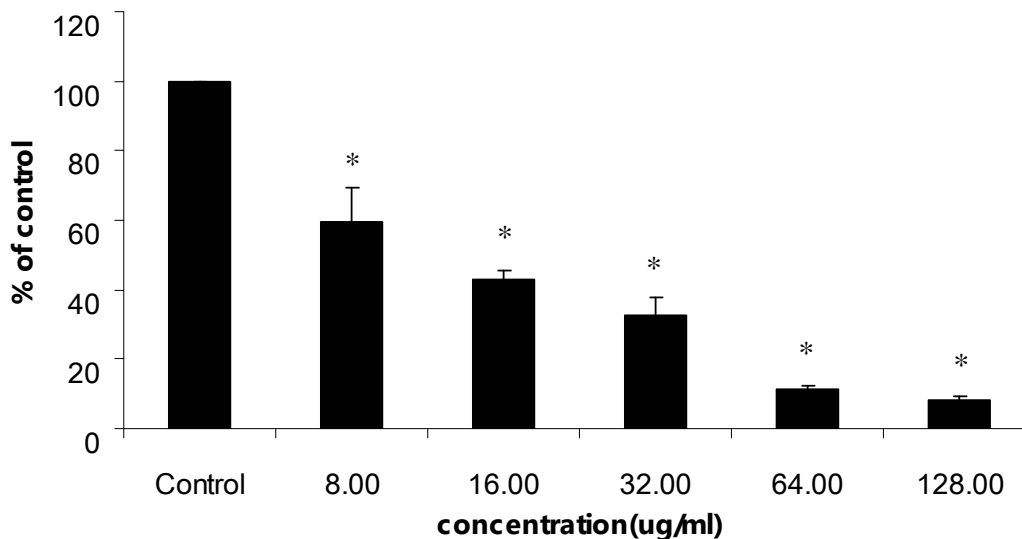


Figure 3. The effect of various concentrations of *K. parviflora*'s ethyl acetate extracts on *H. pylori* ATCC 43504 internalization of HEp-2 cells at 3 h of incubation. The data are presented as mean±SD of independent duplicate experiments with two wells per experiment and duplicate plate counts from each well. The significant difference from control without the extract was set at * p<0.05.

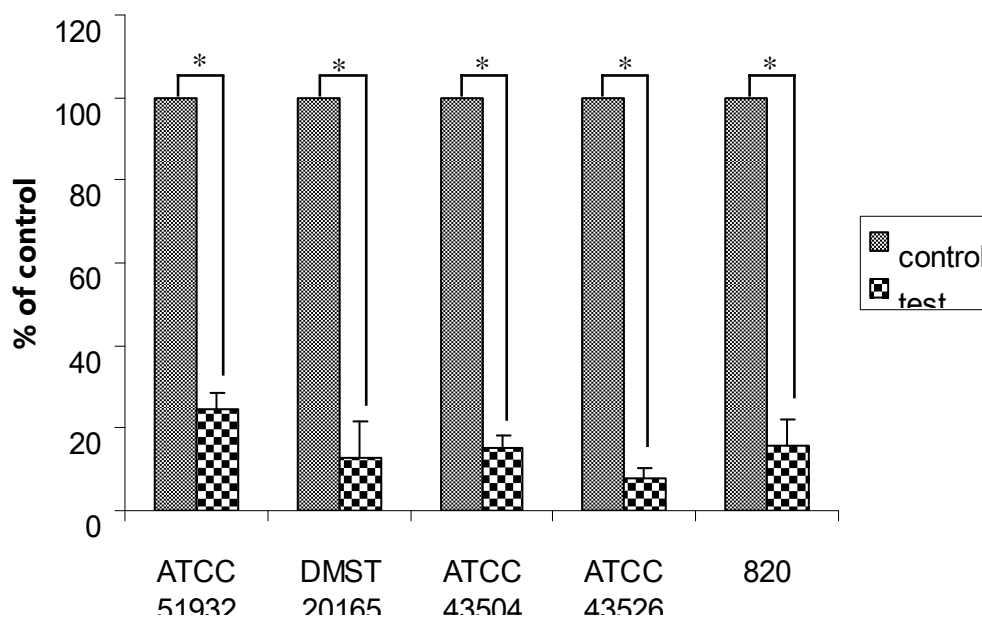


Figure 4. The effect of *K. parviflora*'s ethyl acetate extract on internalization of different strains of *H. pylori* into HEp-2 cells. HEp-2 cells were co-cultured with *H. pylori* in the absence and presence of 64 µg/ml of the extract for 3 h. Data are presented as mean±SD of independent duplicate experiments with two wells per experiment and duplicate plate counts from each well. The significant difference from each control without the extract was set at * p<0.05.

and treating infections. In the present study, the anti-internalization activities of *H. pylori* into HEp-2 cells were observed in *K. parviflora* extracted with hexane, ethyl acetate and methanol. The invasion frequency of *H. pylori* against HEp-2 cells was accelerated with time. This correlated with previous study which found that HEp-2

cells penetration of *H. pylori* were inoculum and time dependent (Wilkinson et al., 1998). Although, higher number of internalized *H. pylori* was observed after the extended period, *K. parviflora*'s ethyl acetate extract still significantly inhibited the invasion ability. Moreover, the extract inhibited the internalization activity in a dose

dependent pattern. The inhibitory activity was independent of the strain virulence. Both *cagA*⁺ and *cagA*⁻ strains were inhibited by *K. parviflora*'s ethyl acetate extract in the same manner. The anti-internalization activities of *K. parviflora* implicated one possible mechanism for anti-peptic ulcer of this plant. Oral administration of *K. parviflora* at 30, 60 and 120 mg/kg significantly inhibited gastric ulcer formation in rats. Its gastroprotective potential was suggested to be related partly to preservation of gastric mucus secretion (Rujjanawate et al., 2005). So, blocking the *H. pylori* invasion by *K. parviflora* was suggested to enhance the curing rate of *H. pylori* and could synergize the conventional antibiotic treatment. However, the anti-invasion mechanism of the *K. parviflora*'s extract has to be deeply elucidated by both biochemical and genetically based studies.

In conclusion, to achieve the therapy against *H. pylori*, both extracellular and intracellular organism have to be eliminated. In this regard, *K. parviflora* may be considered in addition to current antimicrobial therapy for *H. pylori* eradication due to the potent anti-*H. pylori* activity and anti-internalization activity.

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