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Research Article

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Effect of Thai fruit mesocarp extracts on growth of *Helicobacter pylori* and their anti-adhesion activities to HEp-2 cells

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ABSTRACT

*The increasing of antibiotic resistance is the serious problem of most pathogenic bacteria including Helicobacter pylori, which is the etiologic agent of chronic gastritis, peptic ulceration, gastric carcinoma and gastric MALT-lymphoma. An alternative treatment using traditional plants is advantageous in less or nontoxic, cheap and availability in rural areas. In this study, we examined the activities of 19 Thai fruit mesocarp extracts on growth of H. pylori by agar dilution method and anti-adhesion activities of H. pylori ATCC*43504 *against HEp-2 cells by spectrofluorometry. The ethanolic extract of sugar apple (Annona squamosa L.) showed the most potent with* MIC_{50} *of 20 mg/ml and superior anti-adhesion activity of 76.3% against HEp-2 cells. The potential anti-H. pylori activities were observed from ethanolic extracts of mangosteen, durian, jujube, papaya, rambutan juice, mango juice. While, potential anti-adhesion activities were derived from ethanolic extracts of mangosteen, mango, sapodilla, rose apple and both ethanolic and aqueous extracts of guava. Although, pharmacological activities of mesocarp of fruits were lower than exocarp (peel), endocarp (seed), leaf and other parts, it is the consumable part that people could gain pharmacological substances directly apart from great flavor.*

Keywords: *Helicobacter pylori*, Thai fruits, mesocarp, MIC, anti-adhesion

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INTRODUCTION

The incidence of gastric cancer is the second leading cause of cancer death worldwide. For primary prevention, it is important to combine *Helicobacter pylori* eradication with other medical and laboratory screening in order to reduce the mortality in risk population [1]. *H. pylori* associated diseases include peptic ulcer, gastritis, gastric adenocarcinoma and MALT lymphoma. Approximately half of the world population becomes infected. The prevalence of *H. pylori* infection varied between geographical area, ethnic, social and age groups, which associated with socioeconomic status, educational level and hygiene. Transmission is via person-to-person contact by oral-oral, gastro-oral and fecal-oral. The major source of spreading is from contaminated water and food [2]. The pathogenesis of *H. pylori* infection starts from the adhesion of bacteria to stomach microvilli and the translocation of CagA into the cells, which triggers the inflammation and apoptosis process [3]. The first line of treatment is standard triple therapy with a proton pump inhibitor (PPI) and two antibiotics of clarithromycin, amoxicillin, or metronidazole for 7 days [4]. However, the problems of stains resistant to these antibiotics are rising in many countries rendering the failure of eradication. The alternative approaches of natural products have been extensively studied worldwide to explore novel anti-*H. pylori* agents [5, 6]. Anti-adhesion agents are also the attractive therapy due to adhesion is the critical step of infection. Several herbal medicines, spice and food plants had been screened for their anti-*Helicobacter pylori* activities including garlic, wasabi and honey [7-9]. While, cranberry juice and *Pelargonium sidoides* (EPs7630) exhibited superior anti-adhesion activities [10, 11].

Fruits are healthy foods with the great flavor and a lot of nutritional benefits. Consumption of fruits and vegetables was associated with lower risk of duodenal ulcer, stomach and colon cancer [12]. The phytochemicals of fruits including polyphenols, carotenoids, flavonoids, and anthocyanins possess anti-oxidants, anti-neurodegenerative, anti-cancer, anti-inflammatory and anti-microbial properties, which are different among fruits and their parts. The anti-oxidants effect was observed in apple peel, cherry and grape seeds [13-15]. The anti-cancer activity was demonstrated in berry fruits (black raspberry and strawberry), apples, and pears [16, 17]. The most potent anti-microbial effect was observed in seed, peel and leave such as pomegranate fruit peel and apple seed [18, 19]. Thailand is one of the tropical countries that have many kinds of fruits. Some of famous Thai's fruits are durian, mango, rambutan, pomelo and mangosteen. Most of tropical fruits must peel off the skin and consume only the pulp, while cold-climate fruits usually eat whole. Although, the pharmacological and biological activities are abundant in the skin of fruits, which protect the pulp from environmental attack such as sunlight, insects and microorganism, the mesocarp or pulp is also the important consumable part with the great flavor and treasured part of fiber, nutritional and therapeutic substances. Thus, this study aimed to screen the anti-*H. pylori* and anti-adhesion of the mesocarp of nineteen Thai fruits, which will be advantageous for *H. pylori* prevention and treatment by consumption of fruit in daily diet.

EXPERIMENTAL SECTION

Bacterial strains and cultivation

Two *H. pylori* standard strains (ATCC 43504 and ATCC 43526) were used in this study. Seventeen clinical isolates were obtained from Division of Gastroenterology, Department of Medicine, Thammasat University Hospital, Pathumthani, Thailand. All bacterial strains were cultured on brain heart infusion agar containing 7% (v/v) sheep blood and were incubated at 37°C for 3-5 days in a microaerophilic jar system with gas generating kit.

Preparation of fruits extracts

Nineteen Thai fruits were purchased from local markets including sugar apple, mangosteen, rambutan, durian, longkong, jujube, salak pulm, pomelo (variety Thong Dee), pomelo (variety Kao Pan), guava (variety Pan-Sri-Thong), guava (variety Klom Sali), pineapple, watermelon, tangerine, sapodilla, mango, rose apple, papaya and coconut. Only the mesocarp parts were subjected to aqueous and ethanolic extraction. Briefly, 1.5 kilograms of fresh fruits were washed and processed the uneaten parts away (skin and seeds). The mesocarp parts were cut into small pieces and dried in an oven at 50 ºC for 24-48 h except watermelon and tangerine were freshly extracted.

For aqueous extraction, one half of the dehydrated fruits was mechanically crushed, squeeze through gauze cloth, centrifuged at 5,000 rpm for 10 min, and filtered through Whatman Grade No. 1 filter paper. The supernatant was lyophilized until dryness.

For ethanolic extraction, another half of the dehydrated fruits were mechanically crushed, extracted twice with 95% ethanol and dried by rotary evaporator at 60 ºC followed by lyophilization until dryness. All extracts were dissolved in dimethyl sulfoxide (DMSO) and stored at -20°C until used.

Minimum inhibitory concentration (MIC) determinations

An agar dilution method was used according to the Clinical and Laboratory Standards Institute guideline. A stock solution of each extract was serially diluted twofold in Mueller-Hinton broth and 1 ml of each dilution was mixed with 24 ml Mueller-Hinton agar supplemented with 5% sheep blood and poured in a petri dish to give a final concentration of the extracts in the medium ranged from 0.625-20 mg/ml. Each *H. pylori* strain was suspended in 0.85 NaCl to a turbidity of McFarland standard No. 2 to yield approximately 10^7-10^8 CFU/ml. Three microlitres of each bacterial suspension was spotted on each diluted plate and incubated for 3 days as previously described. Growth control plates consisting of medium alone and medium with the upmost concentration of DMSO for each extraction diluents were performed in each experiment. The MIC was determined from the lowest concentration of each extract with no bacterial growth.

Cell Culture

The HEp-2 cell line from a patient with human larynx carcinoma was kindly provided by Dr. Pornthep Tiensiwakul, Faculty of Allied Health Sciences, Chulalongkorn University, Thailand. The cells were cultured in 75 cm^2 flasks containing RPMI 1640 supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) antibiotic-antimycotic solution at 37°C in 5% CO_2 with 80% humidity. The cells were passaged every 3-5 days at a 1:2-1:4 split ratio using Dulbecco's phosphate buffered saline (DPBS) without calcium and magnesium as washing buffer and trypsin-EDTA solution to detach the adherent cells.

Labeling of bacteria

The 3 day culture *H. pylori* ATCC43504 was resuspended in 1 ml RPMI 1640 and bacterial numbers were adjusted by optical density at 600 nm to 2.0. The bacterial suspension was incubated with 10 μ0.1% FITC in DMSO for 1 h at room temperature in the dark. The FITC-labeled bacteria were recovered by centrifugation

Adhesion assay of *H. pylori* to Hep-2 cells

The HEp-2 cells were seeded at a density of 10^5 cells on 24-well plates and left overnight to obtain 80% confluence. The cell monolayers in each well was washed twice with RPMI 1640 and replaced with RPMI 1640 containing no antibiotics. The cells were infected with 100 μ1 FITC-labeled *H. pylori* ($5x10^6$ CFU/ml) in the absence and presence of the extracts. After incubation at 37°C for 2 h, nonadherent bacteria were washed three times in RPMI 1640. The cell monolayers were lysed with sterile distilled water at room temperature for 10 min. The bacterial adherence was quantified by fluorescence microplate reader (excited at 485 nm and detected at 528 nm).

Statistical analysis

All experiments were carried out independently in at least duplicate experiments. The levels of adhesion were expressed as mean±SE by compared to the untreated control (100% adhesion). One way analysis of variance (ANOVA) was used to evaluate the significance at the probability values < 0.05.

RESULTS AND DISCUSSION

The anti-*H. pylori* activities of fruits

The emerging problem of *H. pylori* resistance has a serious impact for successful treatment regimens. An alternative treatment using traditional herbal medicines is advantageous in less or nontoxic, cheap and availability in rural areas. Fruits are favorite foods for people all over the world. Both fiber and vitamins are important for good health. In addition, fruits contain many pharmacological values, including anti-oxidant [20] anti-inflammation [21], anti-aging [22], anti-cancer [23], anti-bacterial activities [24]. In this study the effect of 19 Thai fruit extracts on growth of *H. pylori* were screened using standard agar dilution method as CLSI recommendation. The MICs of both aqueous and ethanolic extracts from mesocarp of fruits against *H. pylori* was shown in Table 1. The ethanolic extract of sugar apple exhibited the highest anti-*H. pylori* activity with MIC_{50} = 20 mg/ml. The potential growth inhibition were demonstrated in ethanolic extracts of mangosteen, durian, jujube and papaya and aqueous extracts of rambutan and mango with MIC_{40} = 20 mg/ml. The upmost concentration of DMSO diluents of each extract ranging from 4-25% v/v were not effected to *H. pylori* growth. Although the anti-bacterial activity of mesocarp part of fruits were not as good as exocarp (peel) and endocarp (seed), their potential bacteriostatic effects could directly gain by daily consumption besides other health benefits. The low antibacterial activities of fruits had been demonstrated in some previous reports. The MIC of pericarps of mangosteen against *Candida albicans* was 1 mg/ml [25]. The MIC of mango against methicillin-resistant *Staphylococcus aureus* (MRSA) and extended spectrum beta-lactamase (ESBL) were 0.31-6.25 mg/ml and 0.32-7.5 mg/ml, respectively [26]. The MIC of guava leave against multidrug-resistant *Vibrio cholera*e O1 and multidrug resistant *S. aureus* was 1.25 mg/ml [27] and 7.5 mg/ml [28], respectively.

Table 1. Minimum inhibitory concentrations (MICs) of Thai fruit extracts against 19 *H. pylori* strains

NO	Scientific Name	Common Name	Aqueous extracts		Ethanolic extracts	
			MIC 50	MIC 40	MIC 50	MIC 40
1	*Garcinia mangostana* L.	Mangosteen	> 20 mg/ml	> 20 mg/ml	> 20 mg/ml	**20 mg/ml**
2	*Nephelium lappaceum* L.	Rambutan	> 20 mg/ml	**20 mg/ml**	> 20 mg/ml	> 20 mg/ml
3	*Annona squamosa* L.	Sugar Apple	> 20 mg/ml	> 20 mg/ml	**20 mg/ml**	**20 mg/ml**
4	*Durio zibethinus* L.	Durian	> 20 mg/ml	> 20 mg/ml	> 20 mg/ml	**20 mg/ml**
5	*Lansium domesticum*	Longkong	> 20 mg/ml	> 20 mg/ml	> 20 mg/ml	> 20 mg/ml
6	*Zizyphus mauritiana* Lamk.	Pudsa, Jujube	> 20 mg/ml	> 20 mg/ml	> 20 mg/ml	**20 mg/ml**
7	*Salacca edulis* Reinw.	salak pulm	> 20 mg/ml	> 20 mg/ml	> 20 mg/ml	> 20 mg/ml
8	*Citrus maxima* (Burm.) Merr. (variety Thong Dee)	Pomelo	> 20 mg/ml	> 20 mg/ml	> 20 mg/ml	> 20 mg/ml
9	*Citrus maxima* (Burm.) Merr. (variety Kao Pan)	Pomelo,	> 20 mg/ml	> 20 mg/ml	> 20 mg/ml	> 20 mg/ml
10	*Psidium guajava* L. (variety Pan-Sri-Thong)	Guava	> 20 mg/ml	> 20 mg/ml	> 20 mg/ml	> 20 mg/ml
11	*Psidium guajava* L. (variety Klom Sali)	Guava	> 20 mg/ml	> 20 mg/ml	> 20 mg/ml	> 20 mg/ml
12	*Ananas comosus* (Linn.) Merr.	Pineapple	> 20 mg/ml	> 20 mg/ml	> 20 mg/ml	> 20 mg/ml
13	*Citrullus lanatus* Mats & Nakai	Watermelon	> 20 mg/ml	> 20 mg/ml	> 20 mg/ml	> 20 mg/ml
14	*Citrus reticulata* Blanco	Tangerine	> 20 mg/ml	> 20 mg/ml	> 20 mg/ml	> 20 mg/ml
15	*Manilkara achras* Fosberg	Spodilla	> 20 mg/ml	> 20 mg/ml	> 20 mg/ml	> 20 mg/ml
16	*Syzygium samarangense* (Blume) Merr. & Perry	Rose apple	> 20 mg/ml	> 20 mg/ml	> 20 mg/ml	> 20 mg/ml

17	*Cocos nucifera* Linn.	Coconut	> 20 mg/ml	> 20 mg/ml	> 20 mg/ml	> 20 mg/ml
18	*Carica papaya* Linn	Papaya	> 20 mg/ml	> 20 mg/ml	> 20 mg/ml	**20 mg/ml**
19	*Mangifera indica* L.	Mango	> 20 mg/ml	**20 mg/ml**	> 20 mg/ml	> 20 mg/ml

The anti-adhesion properties of fruits against *H. pylori*

According to adhesion of *H. pylori* to host epithelial cells or host tissue is a prerequisite for initial virulent process. The strategy of blocking adhesion at mucosal surfaces is advantageous not only to prevent infection but also reduce bacterial resistance [29]. In this study the extracts from mesocarp of all 18 fruits were compared for their anti-adhesion activity against HEp-2 cells using the concentration at 20 mg/ml of each. The result was shown in Figure 1. The ethanolic extract of sugar apple and aqueous extract of mango exhibited the most potent anti-adhesion activity at 76.2 % and 70%, respectively ($p < 0.05$). The traditional use of sugar apple is mostly for anti-malarial and insecticidal activities. Recently, the extract of sugar apple leaves shown to prevent and heal peptic ulcer by reduce the excretion of pepsin and plasma gastrin and increase mucin [30] and its MICs against gram-positive and gram-negative bacteria were 53-380 μand i[24]. While, mango leaves extract was reported to contain anti-typhoid activity of multidrug resistant strains with MICs of 10-50 mg/ml [31]. The potential anti-adhesion activities were observed between 38.1-74.3% from ethanolic extracts of mangosteen, mango, sapodilla, rose apple, guava and guava juice. The inhibitory activity of ethanolic extract of mangosteen could not determine directly due to the HEp-2 cells detachment by the extract. The preliminary study by pre-incubation of the mangosteen extract with *H. pylori* and washing off before adding to the HEp-2 cells demonstrated significant anti-adhesion activity (data not shown). The anti-adhesive effect against *H. pylori* was reported in some natural products such as cranberry juice [32], *Pelargonium sidoides* root extract [11] and green tea leaf [33]. The compound in the extracts that shown to influence *H. pylori* binding on gastric tissue were glucuronic acid-enriched polysaccharides from okra fruit [34] and acidic polysaccharide from green tea [35]. The polysaccharide fractions from *Artemisia capillaris* and *Panax ginseng* reduced the *H. pylori* binding to AGS cells by 11-44% and 35-67%, respectively [36]. The fresh extract from okra fruits exhibited 68.8% reduction in *H. pylori* adhesion to AGS cells [37].

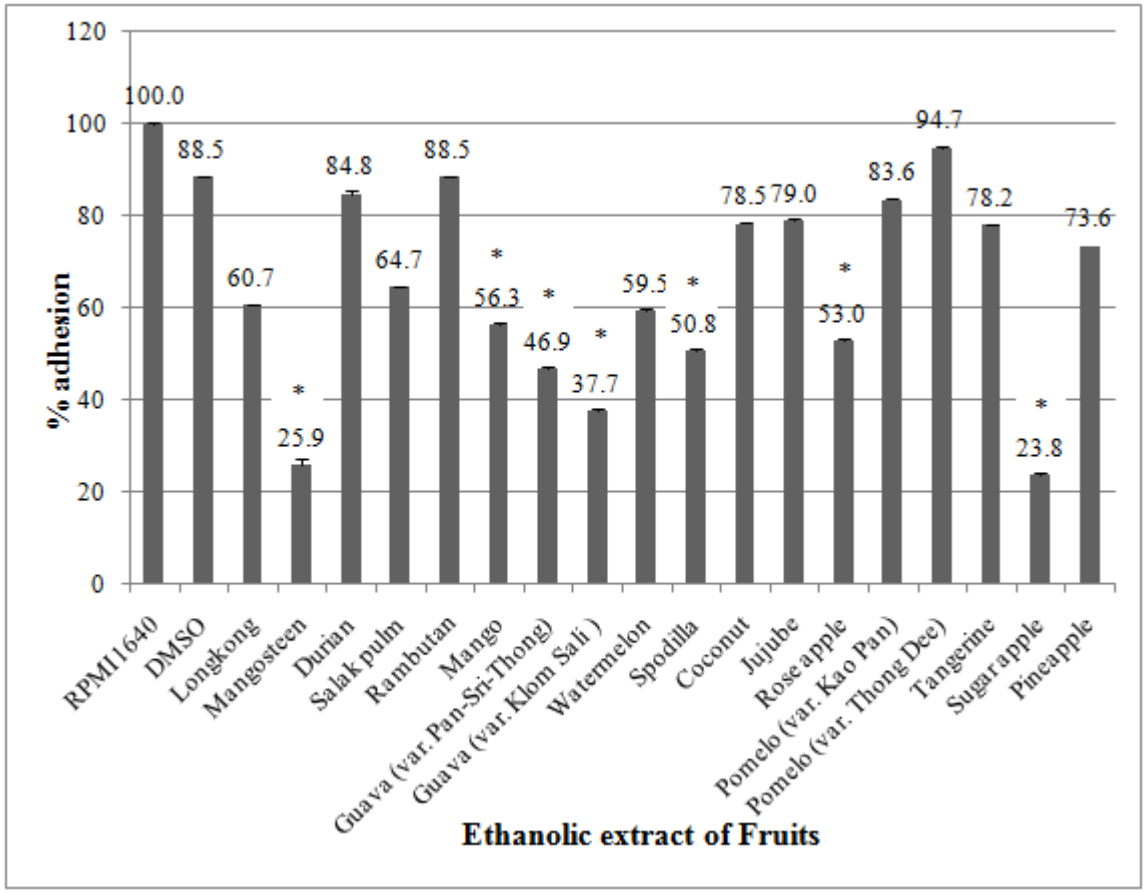

Figure 1. Adhesion assay of *H. pylori*-infected HEp-2 cells in the absence or presence of 18 ethanolic fruit extracts at 2 h of incubation.
The extracts were added at the concentration of the MIC. Data are presented as mean ±SD. The significa nt difference from control without the extract was set at p<0.05.

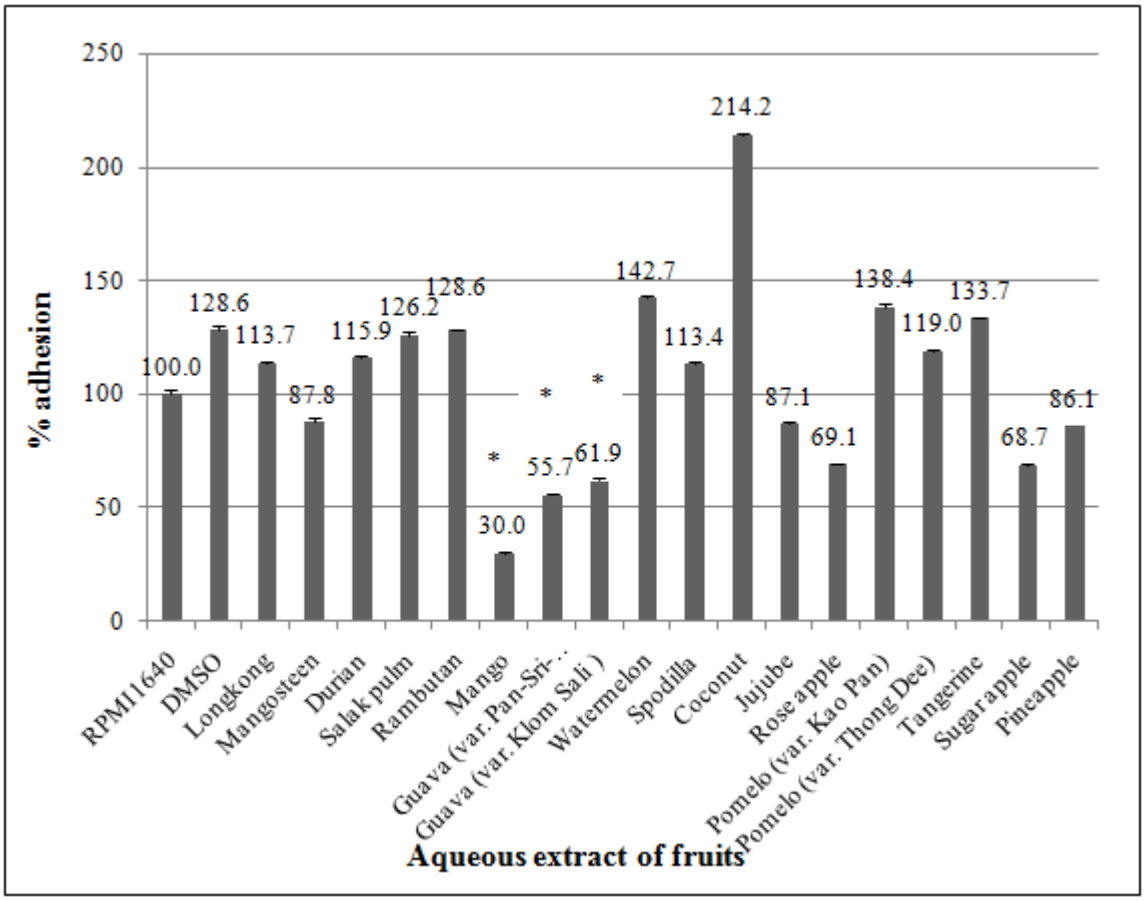

Figure 2. Adhesion assay of *H. pylori*-infected HEp-2 cells in the absence or presence of 18 aqueous fruit extracts at 2 h of incubation.
The extracts were added at the concentration of the MIC. Data are presented as mean ±SD. The significa nt difference from control without the extract was set at $p<0.05$.

CONCLUSION

In this study, the ethanolic extract from mesocarp part of sugar apple exhibited the strongest inhibitory activity against *H. pylori* growth and showed highest anti-adhesion activity compared to other fruits. The extracts of mangosteen and mango demonstrated the potential anti-*H.pylori* and anti-adhesive effects. Although, the bactericidal activities from mesocarp of fruits were inferior to other parts of fruit and fruit tree, it is the consumable part that contains various nutritional substances and fiber. Thus, daily intake of fruits is beneficial for health and could also gain the pharmacological agents for treatment and prevention of *H. pylori* infection.

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