

Anti-*Helicobacter pylori*, anti-inflammatory and antioxidant evaluation of crude extracts from *Amomum krervanh* fruits

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ABSTRACT: The aim of the present study was to determine the antibacterial properties against *Helicobacter pylori* and the anti-inflammatory and antioxidant activities of crude *A. krervanh* extracts. The minimum inhibitory concentrations (MICs) of ethanolic and aqueous extracts were measured against standard strain *H. pylori* standard (ATCC 43504) and six clinical isolates by disc diffusion and agar dilution. Anti-inflammatory activities were evaluated by measuring the inhibition of nitric oxide (NO) production in LPS-activated RAW 264.7 macrophages using the Griess assay and the ELISA measurement of proinflammatory cytokines, tumour necrosis factor α (TNF- α), and interleukin 6 (IL-6). Their cytotoxic activities against macrophages were determined by the MTT assay. ABTS and DPPH scavenging assays measured the antioxidant activities. Only the ethanolic extract showed antibacterial activity with mean inhibition zones of 9.0–19.3 mm for a corresponding MIC of 250 $\mu\text{g/ml}$; the clarithromycin MICs ranged from 0.5–1 $\mu\text{g/ml}$. This extract also inhibited production of NO (mean IC_{50} 98.99 \pm 0.39 $\mu\text{g/ml}$) and IL-6 (mean IC_{50} 18.68 \pm 2.16 $\mu\text{g/ml}$) and exhibited moderate antioxidant activity using DPPH and ABTS scavenging assays: mean IC_{50} s 95.92 \pm 1.26 and 90.35 \pm 2.25 $\mu\text{g/ml}$, respectively. Thus ethanolic extract showed a greater spectrum of activity that could be exploited further as adjunct treatments of *H. pylori* related gastrointestinal disease.

KEYWORDS: anti-*Helicobacter pylori*, *Amomum krervanh*, nitric oxide, tumor necrosis factor α , interleukin 6

INTRODUCTION

Functional dyspepsia (FD) which is the most common of dyspepsia is a disorder of the upper gastrointestinal tract of the stomach and duodenum and has several underlying pathophysiological mechanisms¹. A syndrome consists of epigastric pain, burning, fullness, discomfort, early satiety, nausea, vomiting, and belching that affects quality of life². Furthermore, from previous report in Thailand, 60% of patients diagnosed with FD were found to be infected with *Helicobacter pylori*³. The infection of *H. pylori* may have reduction in ghrelin secretion that may reduce gastric mobility of patients with *H. pylori* associated diseases². Furthermore, *H. pylori* is the most important cause of gastric inflammation which develop to gastritis, peptic ulcers and gastric cancer⁴. Success rates for treating dyspepsia due to an identified pathology is to reduce

or eliminate the symptoms and thus to improve the conditions of the patient. There are at least four pathophysiologic mechanisms, for example the delayed gastric emptying, impaired post-prandial fundus relaxation, hypersensitivity to gastric distension, and acid-related or acid hypersensitivity, recommended therapy includes acid suppressive therapy³. However, many patients with FD often seek out complementary and alternative therapies, such as herbal medicines, because of the limited effects of the conventional therapies.

In Thailand, patients often seek traditional remedies for common symptoms and hold beliefs related to wind and fire elements in their body. One commonly used remedy for dyspepsia is cardamom⁵.

Cardamom, *Amomum krervanh* Pierre (Zingiberaceae), is a herbaceous plant whose fruit and

seeds are fragrant⁶. The fruit is used commonly in tea in India and has been used historically to relieve indigestion and flatulence. Furthermore, cardamom has been found to possess antioxidant⁷ and antimalarial properties⁸. Cardamom fruit contains several compounds such as 1,8-cineol (eucalyptol), terpinine, borneol, camphor and limonene. The major compound of the essential oil is 1,8-cineole (eucalyptol)^{9,10} which shows a potential anti-inflammatory activity¹¹.

The body of literature on these potentially useful properties is very limited. We, therefore, conducted a study to assess the antibacterial, anti-inflammatory and antioxidant properties of *A. krervanh* fruit.

MATERIALS AND METHODS

Plant materials

A. krervanh plants were purchased from a herbal shop in January 2017 in Nakhon Pathom province, Thailand.

Chemicals

All the chemicals and reagents used were of analytical grade. 2,2-diphenyl-1-picrylhydrazyl, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), AlCl₃, potassium acetate, potassium persulphate, phosphoric acid, dimethyl sulphoxide, lipopolysaccharide from *E. coli* O55:B5 (LPS), N-(1-Naphthyl) ethylenediamine dihydrochloride, sulfanilamide, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide or thiazolyl blue tetrazolium bromide (MTT) and trypan blue dye were purchased from Sigma-Aldrich, USA. Folin-Ciocalteu's reagent and Na₂CO₃ were purchased from Fluka, USA, and Merck, Germany, respectively. Mouse TNF- α and mouse IL-6 ELISA kits were purchased from Immuno Tools, Germany. HCl and isopropanol were purchased from RCI Labscan, Thailand. Dulbecco's modified Eagle medium (DMEM), butylated hydroxytoluene (BHT), phosphate-buffered saline, brain heart infusion and anaeropack microaero were purchased from Gibco BRL, USA, Fluka, Biochrom, Germany, Difco, USA, and Mitsubishi Gas Chemical America, USA, respectively.

Ethanolic extract

A total of 50 g of dried fruit of *A. krervanh* was exhaustively extracted with aqueous 95% ethanol

(v/v) by maceration at room temperature for 3 days. The extract was obtained after filtration and concentration of the material under reduced pressure using a rotary evaporator. Then the extract was dried using vacuum dryers.

Water extract

A total of 50 g of dried fruits was boiled with deionized water for 15 min, then the aqueous extract was filtered using Whatman paper no 1. Finally, lyophilizer was used to dry extract.

Stock solution

A stock solution of the water extract was made with sterile distilled water to a concentration of 100 mg/ml; the ethanolic extract also was made with dimethylsulphoxide (DMSO) to a concentration of 500 mg/ml. Both were used in the disc diffusion and agar dilution tests. Stock solutions of 10 mg/ml (water extract) and 50 mg/ml (ethanolic extract) were also made up to perform the anti-inflammatory assays.

Bacterial strains used and culture conditions

H. pylori reference strain (ATCC 43504) was obtained from the American Type Culture Collection and six clinical isolates were obtained from human stomach biopsy specimens (kindly provided by Dr Ratha-korn Vilaichone, Faculty of Medicine, Thammasat University, Thailand). Strains were cultured on Columbia agar supplemented with 5% sheep's blood (RPD, Thailand) and incubated at 37°C under microaerobic conditions (N₂, 85%; O₂, 5%; CO₂, 10%) using a gas generating kit. *H. pylori* was subcultured every 3 days in an anaerobic jar.

Testing for anti-*H. pylori* activity by disc diffusion method

H. pylori was inoculated and incubated at 37°C under microaerobic conditions for 3 days, then adjusted equivalently to approximately 0.5 McFarland standard. Filter paper discs (6 mm in diameter) were impregnated of the extract (1 mg/disc). Air-dried discs were placed onto the Columbia 5% sheep blood agar. Clarithromycin (10 μ g/disc) was used as the positive control. The plates were incubated as previously described for 3 days. The zone of inhibition was calculated by measuring the diameter of the inhibition zone three times and taking the mean value^{12,13}.

Testing for anti-*H. pylori* activity by agar dilution

The minimum inhibitory concentration (MIC)^{14,15} was determined by the agar dilution method following the Clinical and Laboratory Standards Institute (CLSI) guidelines. The extracts were serially diluted 2-fold in Columbia agar containing 5% sheep's blood, then transferred separately into Petri dishes. The final concentrations of the extracts in the culture medium ranged from 2000 to 31.25 µg/ml. A 72-h bacterial colony of *H. pylori* was harvested and suspended in brain heart infusion. Bacterial suspensions were prepared to approximately 2.0 of the McFarland standard. Bacterial suspension (3 µl per spot) was replicated on each plate followed by incubating at 37°C for 72 h under microaerobic conditions. The MIC was defined as the lowest concentration of extract at which no visible growth was observed. All tests were conducted in triplicate, in addition to growth control with and without DMSO.

Anti-inflammatory activity

To evaluate anti-inflammatory activity, inhibition of proinflammatory cytokine (NO, TNF-α, and IL-6) was studied in RAW 264.7 macrophage cells obtained from Faculty of Medicine, Thammasat University, Thailand.

Measurement of NO production in macrophage

RAW 264.7 cells were seeded in 96-well plates (1×10^5 cells per well) and incubated at 37°C in a 5% CO₂ incubator for overnight. Then the cells were replaced and replenished with 5 ng/ml of lipopolysaccharide (LPS) and the extracts at various concentrations were incubated at 37°C in a humidified atmosphere containing 5% CO₂. At the end of 48 h, 100 µl of supernatant was mixed with 100 µl of the Griess reaction, incubated for 10 min at room temperature, and then measured absorbance at 570 nm¹⁶.

LPS-induced cytokine production assay

Production of mouse TNF-α and IL-6 was measured using an enzyme-linked immunosorbent assay (ELISA)^{17,18} according to manufacturer's instructions. Briefly, RAW 264.7 cells were seeded in 96-well plates (1×10^5 cells per well) and incubated at 37°C in a 5% CO₂ incubator for 24 h. 5 ng/ml of LPS and various concentrations of the extracts were added to the wells. After 48 h, the culture

supernatants were analysed. Briefly, a 96-well microtitre plate was pre-coated overnight with capture antibody. After blocking and several washes, samples were incubated with working standards for 1 h. After further washing, detection-antibody was added to each well and incubated for 2 h. After washing, poly-HRP-streptavidin was added and incubated for 30 min; then the substrate solution was added and incubated for 1 h with 3,5,3',5'-tetramethylbenzidine (TMB) solution. Finally, 50 µl of stop solution was added to each well and the absorbance was read within 30 min using a microplate reader at 450 nm and 630 nm.

Cytotoxicity MTT assay

Briefly, RAW264.7 cells were incubated with MTT¹⁶ (5 mg/ml in phosphate-buffered saline, pH 7.4) for 4 h. Formed MTT formazan was solubilized with 100 µl of isopropanol containing 0.04 M HCl. Cell growth was determined by reading the absorbance at 570 nm. The extract concentrations which produce more than 70% of cell survival were calculated.

DPPH radical scavenging activity

Antioxidant activity was determined using the DPPH assay, according to the modified method of Yamasaki et al^{19,20}. The extracts were prepared with different concentrations (1–100 µg/ml). Then DPPH solution was freshly prepared in absolute ethanol and adjusted to a final DPPH concentration of 100 µM. DPPH was kept in a light protected bottle. The mixture was left to stand for 30 min in the dark at room temperature. The decrease in absorbance due to DPPH was measured at 520 nm using a microplate reader and the antioxidant activity measured in µg/ml; the IC₅₀ was defined as the concentration of inhibitor necessary to reduce 50% of the DPPH radical.

ABTS radical scavenging assay

ABTS^{•+} solution was produced by mixing 7 mM ABTS stock solution in distilled water with 2.45 mM potassium persulphate. The mixture was allowed to stand in the dark at room temperature for 12–16 h and the ABTS^{•+} solution was diluted with distilled water to an absorbance of 0.68–0.72 at 734 nm before use. The reaction was carried out for 6 min, and then the absorbance was measured at 734 nm using a microplate reader. The scavenging activity of the extracts against ABTS^{•+} was measured in µg/ml; the IC₅₀ was defined as the concentration of inhibitor necessary to reduce 50% of the ABTS^{•+} radical²¹.

Determination of total phenolic content

The extract and gallic acid standard were transferred to 96-well microplates. Then Folin-Ciocalteu's reagent and 7.5% (v/w) Na_2CO_3 solution were added and mixed. The plate was incubated for 30 min at room temperature 25 °C. Absorbance at 765 nm was measured using a microplate reader. A calibration curve of gallic acid was constructed to determine the amount of total phenolic content, which was expressed as mg of gallic acid equivalent (GAE) per 1 g of dry extract²².

Determination of total flavonoid content

The extract or quercetin standard was mixed in methanol, 10% (w/v) AlCl_3 , 1 M potassium acetate and distilled water. The mixture was incubated at room temperature for 30 min. Absorbance was determined at 415 nm. A calibration curve of quercetin was constructed to determine the amount of total flavonoid content, which was expressed as mg of quercetin equivalent per 1 g of dry extract²².

Statistical analysis

All the experiments were conducted in triplicate. Results are expressed as a mean \pm standard error of the mean.

RESULTS

The extract yields of *A. krervanh* prepared by maceration and boiling methods are presented in Table 1. The higher yield was obtained from the water extract (6.98%).

The results of the anti-*H. pylori* effect of *A. krervanh* extracts are shown in Table 2. The ethanolic extract exhibited an inhibition zone of 12 mm against the standard strain with an MIC of 250 $\mu\text{g}/\text{ml}$. It also showed strong anti-*H. pylori* against the six clinical isolates with inhibition zones of 9.0–19.3 mm and minimum inhibitory concentrations (MICs) of 250 $\mu\text{g}/\text{ml}$. All of these zones of inhibition were less than those of clarithromycin and had higher MICs. There was no growth inhibition with the water based extract with corresponding MICs of 2000–4000 $\mu\text{g}/\text{ml}$.

As seen in Table 3, the ethanolic extract displayed dose-dependent and higher NO and IL-6 inhibition compared to the water extract and had IC_{50} s of 98.99 ± 0.39 and 18.68 ± 2.16 $\mu\text{g}/\text{ml}$, respectively. These results are supported by the cell viability of more than 80% by MTT assay.

The DPPH and ABTS determined antioxidant activities of the *A. krervanh* fruit extracts are summa-

rized in Table 1. The water extract scavenged DPPH radicals with an IC_{50} value of 51.08 ± 0.50 $\mu\text{g}/\text{ml}$ that was lower compared to the ethanolic extract. In contrast, the ethanolic extract possessed the highest antioxidant activity by the ABTS assay with a mean IC_{50} of 90.35 ± 2.25 $\mu\text{g}/\text{ml}$.

The phenolic content was greater in ethanolic extract than in water extract which was found to be 36.95 ± 0.65 , 31.52 ± 1.85 mg GAE/g, respectively. Additionally, the amount of flavonoids was related to total phenolic content.

DISCUSSION

Extraction is an important step in the phytochemical process for discovering bioactive constituents of plant material. The study has shown that the water and ethanolic components of the cardamom plant possessed antioxidant activity but that the ethanolic component had appreciable antibacterial and anti-inflammatory properties.

In this study, the anti-*H. pylori* activities of the water and ethanolic extracts were evaluated by disc diffusion method and by agar dilution. Although the CLSI approves only the agar dilution method¹⁵, disc diffusion is useful for screening many samples²³. Compared to the water based extract, the ethanolic extract had much greater inhibitory activity against *H. pylori* but it was considerably lower compared to clarithromycin, a standard antibiotic used in combinations to kill *H. pylori* in patients. To our knowledge, this result is the first report for the effects of *A. krervanh* extracts on *H. pylori*. However, these extracts have shown inhibitory properties against some Gram-positive (e.g., *Staphylococcus aureus*) and Gram-negative (e.g., *E. coli*) bacteria. The mechanism of action in these bacteria is thought to be distortion of cell wall and cytoplasmic membranes by the essential oils, which result in leakage of several vital intracellular constituents²⁴, as well as the disruption of DNA, RNA, protein, and polysaccharide synthesis, leading to cell death²⁵.

It is well known that proinflammatory cytokines induced by LPS, such as NO, TNF- α and IL-6, play a key role in the acute inflammatory response²⁶. We found that the ethanolic extract significantly inhibited IL-6 in a dose-dependent manner, had weak inhibitory effects on NO production and only inhibited TNF- α at the highest dose (100 $\mu\text{g}/\text{ml}$). Previous work has shown that the 1,8-cineole (Eucalytol) from *A. krervanh* suppressed the production of proinflammatory cytokines, including NF- κB , TNF- α , IL-1 β , and IL-6²⁷. In addition, 1,8-cineole also reduced significant anti-inflammatory activity

Table 1 Extraction yields (% w/w), total phenolic, total flavonoid contents and antioxidant activity (concentration of 100 µg/ml and IC₅₀ µg/ml) of *A. krervanh* extracts[†]

<i>A. krervanh</i>	Extraction (%)	Phenolic (mg GAE/g)	Flavonoid (mg GAE/g)	DPPH assay		ABTS assay	
				% inhibition [‡]	IC ₅₀ (µg/ml)	% inhibition	IC ₅₀ (µg/ml)
Water	6.98	31.52 ± 1.85	3.13 ± 0.50	72.04 ± 3.99	51.08 ± 0.50	40.80 ± 0.39	127.64 ± 0.30
95% Ethanol	1.71	36.95 ± 0.65	4.87 ± 0.50	51.94 ± 1.07	95.92 ± 1.26	53.24 ± 1.04	90.35 ± 2.25
BHT				85.61 ± 1.38	16.02 ± 0.59	102.50 ± 3.83	5.05 ± 0.69

[†] Each reported value represents the mean ± standard error of the mean of triplicates. ABTS = 2,2-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid; BHT = butylated hydroxytoluene; DPPH = 2,2-diphenyl picrylhydrazyl.

[‡] % inhibition at concentration of 100 µg/ml.

Table 2 In vitro anti-*H. pylori* activity of *A. krervanh* extracts against 6 clinical isolates and standard strain, expressed as the disc diffusion method and minimum inhibitory concentration (µg/ml). Clarithromycin was used as positive control[†]

<i>A. krervanh</i>	ATCC43504		CS24		9820		SM100	
	Inhibition zone (mm)	MIC (µg/ml)						
Water	0	2000	0	2000	0	4000	0	4000
95% Ethanol	12.0 ± 0.0	250	9.0 ± 1.0	250	14.0 ± 0.0	250	14.0 ± 1.0	250
Clarithromycin	60.0 ± 0.0	0.5	57.3 ± 0.5	0.5	60.0 ± 0.0	0.5	62.0 ± 0.0	0.5

<i>A. krervanh</i>	CS46		2693		2691	
	Inhibition zone (mm)	MIC (µg/ml)	Inhibition zone (mm)	MIC (µg/ml)	Inhibition zone (mm)	MIC (µg/ml)
Water	0	2000	0	2000	0	2000
95% Ethanol	15.0 ± 0.0	250	14.0 ± 1.0	250	19.3 ± 0.5	250
Clarithromycin	51.0 ± 0.0	1	49.3 ± 1.0	1	67.0 ± 0.0	1

[†] Final amount of crude plant extract = 1 mg/disc. Each experiment was repeated in triplicate.

in asthma patients by inhibition of mediator production²⁸. The report recently shown that 1,8-cineol revealed a steroid-like suppression of arachidonic acid metabolism and cytokine production in vitro²⁹.

We found that the radical scavenging activity of the water extract was less than the ethanolic extract when assessed by the ABTS⁺ assay; the reverse was true by the DPPH assay, consistent with earlier

reports. The crude water and ethyl acetate extracts showed radical scavenging effects of 60% and 30% at 2 mg/ml using DPPH method, respectively³⁰, and Jeong et al obtained similar results³¹, while 1,8-cineol had almost no antioxidant activity³².

The results indicated the influence of the extraction solvent on the total phenolic content. This could probably be due to their higher polarity and

Table 3 Effect of *A. krervanh* extracts on NO, TNF-α and IL-6 production by macrophage RAW 264.7 cells stimulated with 5 µg/ml of lipopolysaccharide. Prednisolone, paracetamol and andrographolide were used as positive control[†]

<i>A. krervanh</i>	NO		TNF-α		IL-6	
	% inhibition [‡]	IC ₅₀ (µg/ml)	% inhibition	IC ₅₀ (µg/ml)	% inhibition	IC ₅₀ (µg/ml)
Water	10.23 ± 3.12	>100	30.25 ± 6.97	>100	2.65 ± 1.65	>100
95% Ethanol	52.12 ± 2.54	98.99 ± 0.39	1.24 ± 0.24	>100	66.56 ± 7.61	18.68 ± 2.16
Prednisolone	–	–	95.95 ± 0.88	0.11 ± 0.00	–	–
Paracetamol	1.53 ± 0.42	>100	0.32 ± 0.05	>10	73.87 ± 0.59	32.14 ± 2.69
Andrographolide	99.13 ± 0.14	1.80 ± 0.42	92.85 ± 0.89	5.08 ± 3.48	96.12 ± 0.29	9.10 ± 0.61

[†] Each value represents the mean ± S.E.M. of triplicates.

[‡] % inhibition at concentration of 100 µg/ml.

better solubility for phenolic compounds present in this plant³³. Also, the data revealed the correlations of total phenolic content with antioxidant, anti-*H. pylori* activity and anti-inflammatory activities. A similar finding was reported for the relative extraction time, flavonoid contents and antioxidant activity in *Amomum* species³⁴.

In conclusion, our study revealed that the extracts of *A. krervanh* inhibited *H. pylori* and displayed promising anti-inflammatory activity against IL-6 and NO as well as antioxidant activity. Although the MICs against *H. pylori* were higher compared to clarithromycin, *A. krervanh* extracts could be used in the future as an adjunct treatment for treating *H. pylori* related disease, notably peptic ulceration. More preclinical work is needed before *A. krervanh* extracts can be medically examined for use in humans.

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