Anti-*Helicobacter pylori* Agents from the Cashew Apple

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Anacardic acids and (*E*)-2-hexenal characterized from the cashew *Anacardium occidentale* L. (Anacardiaceae) apple have been found to exhibit antibacterial activity against the Gram-negative bacterium *Helicobacter pylori*, which is now considered to cause acute gastritis. The same antibacterial compounds have also been found to inhibit urease (EC 3.5.1.5).

Keywords: *Helicobacter pylori; anacardic acids; (E)-2-hexenal; antibacterial activity; urease inhibitory activity*

INTRODUCTION

Peptic ulcer is one of the most common human diseases, affecting close to 50% of the population in industrialized countries. For many years, this ulcer was believed to be caused by acid hypersecretion in the stomach until sufficient evidence showed a causal link with *Helicobacter pylori* isolated from the gastric mucosa of patients with duodenal-ulcer disease (Warren and Marshall, 1983). The current medical consensus shows that it is the primary causative agent of acute gastritis (Marshall et al., 1985). *H. pylori* is characterized by very high urease activity that may be associated with virulence (Mégraud et al., 1992). In the absence of urea, *H. pylori* is sensitive to acidic pH (Goodwin et al., 1986). Urease activity may be an important colonization and survival factor by generating ammonia in the immediate bacterial microenvironment, thus protecting *H. pylori* from the deleterious effects of gastric acid. The concept that peptic ulcer is an infectious disease changed therapy from treating symptoms with antiacid drugs to antibiotic eradication of infection. A number of antibiotics have been evaluated for *H. pylori* eradication, including combinations of metronidazole, tetracycline, amoxicillin, and clarithromycin as well as bismuth compounds. Chemotherapy with antibiotics, however, sometimes poses serious side effects such as diarrhea, nausea, abnormal taste, dyspepsia, abdominal pain/discomfort, headache, and angioedema. Therefore, there is a strong demand for compositions having all of the beneficial properties of antibiotics but with reduced side effects. Since the drug will directly come in contact with the lining of the stomach, edible plants may be a good source of oral antiulcer agents. With this concern in mind, we have screened for new antibacterial and urease inhibitory agents from various fruits and vegetables that have been continuously consumed by many people for many years. In our preliminarily screening, the ethanol extract of the fresh cashew *Anacardium occidentale* apple was found to inhibit the growth of *H. pylori* and was subjected to further fractionation.

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MATERIALS AND METHODS

General. General procedures are the same as previous work (Kubo et al., 1986; Himejima and Kubo, 1991; Muroi and Kubo, 1993, 1996).

Plant Material. Fresh cashew apples (1 kg) were purchased at market places in Salvador, Brazil, and were immediately immersed into ethanol at the sites. These were kept at ambient temperatures until their use.

Isolation and Characterization. After concentration of the solvent, the water-based suspension was partitioned with *n*-hexane and ethyl acetate. Subsequent bioassay indicated the ethyl acetate portion (2.85 g) to be active but not the *n*-hexane fraction (6.28 g). Similar to our previous experiment, the active principles were isolated by recycle-HPLC (R-HPLC) using an ODS C_{18} column and identified as 6-(8(*Z*),11(*Z*),14-pentadecatrienyl)salicylic acid (**1**) ($C_{15:3}$) (28 mg), 6-(8(*Z*),11(*Z*)-pentadecadienyl)salicylic acid (**2**) ($C_{15:2}$) (20 mg), and 6-(8(*Z*)-pentadecenyl)salicylic acid (**3**) ($C_{15:1}$) (12 mg) by spectroscopic methods (Kubo et al., 1986, 1993a). The yield of anacardic acids from the fresh cashew apple was about 0.00006%. This result is consistent with our previous data of 500 μg/mL since 1 kg of the fresh cashew apple provides approximately 100 mL of the juice. It should be noted, however, that the amount of the juice greatly varies when it was squeezed out at the purchasing sites.

Chemicals. Anacardic acids (**1**-**3**) and cardanol used for the assay were available from our previous work (Kubo et al., 1986), but the repurification was achieved by the R-HPLC method. The saturated alkyl side chain analogues, 6-pentadecylsalicylic acid (**4**) ($C_{15:0}$) and 6-dodecylsalicylic acid (**5**) ($C_{12:0}$), were previously synthesized (Yamagiwa et al., 1987). Salicylic acid, (*E*)-2-hexenal, (*E*)-2-octenal, and (*E*)-2-decenal were purchased from Aldrich Chemical Co. (Milwaukee, WI). *N*,*N*-Dimethylformamide (DMF) was obtained from EM Science (Gibbstown, NJ). Tetracycline was purchased from Sigma Chemical Co. (St. Louis, MO). Clarithromycin was obtained from Abbott Laboratories, Ltd. (Queensborough, U.K.).

Test Strain. The test strain *H. pylori* ATCC 43504 used for the bioassay was purchased from American Type Culture Collection (Rockville, MD). The other three strains (*Pseudomonas aeruginosa* ATCC 10145, *Enterobacter aerogenes* ATCC 13048, and *Escherichia coli* ATCC 9637) were also obtained from the same source.

Medium. Columbia agar base (pH 7.3) and horse blood were obtained from Oxoid Ltd. (Basingstoke, England). Selective supplements (500 mL of Columbia blood agar supplemented with 5.0 mg of vancomycin, 2.5 mg of cefsulodin, 2.5 mg of trimethoprim, and 2.5 mg of amphotericin B) and bovine serum were purchased from Sigma Chemical Co. (St. Louis, MO).

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Columbia broth was obtained from BBL Microbiology System (Cockeysville, MD).

Antibacterial Assay. The minimum inhibitory concentrations (MICs) were determined by the agar dilution method. Test compounds were first dissolved in DMF and used for the assay at 2-fold dilution. The highest concentration tested was 1600 μg/mL unless otherwise specified. An aliquot of 0.01 mL of test solution was added to a multiwell plate, containing 0.99 mL of agar media in each well. The multiwell plate was placed on a heat block and maintained at 45 °C. The initial concentration of test agar solution was obtained. Serial 2-fold dilutions were made by mixing 0.5 mL of test agar solution with 0.5 mL of blank media. And 0.5 mL of agar solution of diluted inoculum containing 5×10^6 cfu/mL *H. pylori* was then added into each well containing 0.5 mL serial dilution of the test compound. After being incubated for 5 days at 37 °C, the read-out for visible growth colony and MIC was determined as the lowest concentration of compounds that prevent visible bacterium growth. A culture growth control without compound (solvent only) and several culture-sensitive reference agents were used as positive controls.

After determining the MIC, 0.2 mg of agar media was extracted from each well exhibiting no colony growth using a sterile straw with a diameter of 5 mm. The sample agar media was added to 1.8 mL of PBS (pH 7.4) and homogenized with glass beads to make the final suspension. A total of 2 μL of the suspension was used to do a subculture in agar plating. After 96 h of incubation, the growth colony was visibly evaluated. No growth of colony in the agar plate was regarded as the minimum bactericidal concentration (MBC). The MBC was the lowest concentration of antibacterial compound that decreased 99.9% of initial inoculum. The assays were performed in duplicate on separate occasions. The assay against the three Gram-negative bacteria (*P. aeruginosa*, *E. aerogenes*, and *E. coli*) was performed as previously described (Muroi et al., 1993). The antibacterial assay including *Salmonella typhimurium* was also carried out by Panlabs (Taipei, Taiwan), and the results were consistent with ours.

Enzyme Assay. The jack bean urease (EC 3.5.1.5) used for the study was purchased from Sigma Chemical Co. (St. Louis, MO). Although the jack bean urease differs somewhat from *H. pylori* (Dunn et al., 1990; Cesareo and Langton, 1992), this plant source was used for the experiment because of its ready availability. The assay was performed as previously described (Gorin and Chin, 1966). Test samples were first dissolved in ethanol and used for the experiment at 30 times dilution.

RESULTS

The ethanol extract was suspended in water, and the suspension was successively partitioned with *n*-hexane and ethyl acetate. Subsequent bioassay showed that the ethyl acetate fraction retained the antibacterial activity against *H. pylori*. In addition, the same ethanol extract was found to exhibit urease inhibitory activity. The antibacterial activity-guided fractionation using *H. pylori* led to the isolation of three active principles that were identified as anacardic acids (**1**−**3**) (see Figure 1 for structures) by means of spectroscopic methods. These three were previously isolated from various parts of *A. occidentale* (Tyman, 1979), and subsequently their diverse biological activities were reported. For example, their molluscicidal (Sullivan et al., 1982; Kubo et al., 1986), antibacterial (Gellerman et al., 1969; Himejima and Kubo, 1991), and cytotoxic (Itokawa et al., 1989; Kubo et al., 1993b) activities have been described. Anacardic acids are also known to inhibit various enzymes such as tyrosinase (Kubo et al., 1994), glycerol-3-phosphate dehydrogenase (Irie et al., 1996), prostaglandin synthase (Bhattacharya et al., 1987; Kubo et al., 1987), lipoxygenases (Shobha et al., 1994), cyclooxygenases (Grazzini et al., 1991), aldose reductase (Toyomizu et al., 1993), and β-lactamase (Coates et al., 1994).

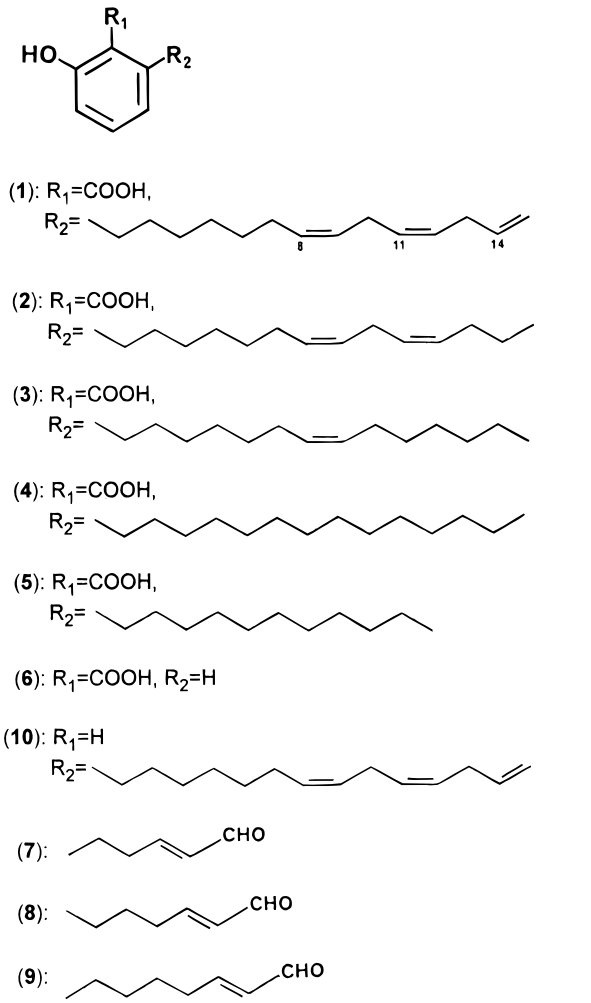

Figure 1. Chemical structures of anacardic acids (**1**−**5**), salicylic acid (**6**), (*Z*)-2-alkenals (**7**−**9**), and cardanol (**10**).

Table 1. Antibacterial Activity of Anacardic Acids, (*Z*)-2-Alkenals, and Antibiotics against *H. pylori*

compounds tested	MIC (μg/mL)	MBC (μg/mL)
anacardic acids		
($C_{15:3}$)	200	800
($C_{15:2}$)	200	–[a]
($C_{15:1}$)	400	–
($C_{15:0}$)	>800	–
($C_{12:0}$)	200	800
salicylic acid	>1600	–
(*E*)-2-hexenal	400	800
(*E*)-2-octenal	200	400
(*E*)-2-decenal	800	–
tetracycline	6.25	–
clarithromycin	0.1	0.78

[a] –, not tested.

In addition to the three anacardic acids (**1**−**3**) isolated from the cashew apple, their synthetic analogues, **4** ($C_{15:0}$) and **5** ($C_{12:0}$) (Yamagiwa et al., 1987; Kubo et al., 1993b), were also tested for comparison. Moreover, since they are the derivatives of salicylic acid (**6**) with a nonisoprenoid alkyl side chain at the C-6 position, their activity was compared with that of salicylic acid. Their MICs and MBCs against those of *H. pylori* are listed in Table 1. Among the compounds tested, **1** ($C_{15:3}$), **2** ($C_{15:2}$), and **5** ($C_{12:0}$) were the most potent, each having an MIC of 200 μg/mL, while **4** ($C_{15:0}$) did not exhibit any activity up to 800 μg/mL. It should be noted, however,

that the two saturated side chain anacardic acids, **4** ($C_{15:0}$) and **5** ($C_{12:0}$), are hardly soluble in the water-based medium. This caused variations with O.D. readings, which were essential in determining the MIC. Therefore, their exact data may not be established unequivocally. Interestingly, their parent compound (salicylic acid) did not show any activity up to 1600 μg/mL. It appears that the addition of an alkyl side chain plays an important role in eliciting the activity. In previous studies of the antibacterial activity of C_{15}-anacardic acids (Gellerman et al., 1969; Himejima and Kubo, 1991), a decrease in the number of double bonds in the side chain was reported to decrease the activity against Gram-positive bacteria. The current study against *H. pylori* also observed the similar result. The MIC of anacardic acids **1**, **2**, and **5** is 2000-fold less effective than clarithromycin, which is one of the most commonly used antibiotics for treatment of peptic ulcer. The MIC of this semi-synthetic antibiotic obtained was 0.1 μg/mL. Tetracycline, another antibiotic used for the same purpose, showed less effective as compared to clarithromycin but still was 32-fold more effective than the anacardic acids. The MIC of the latter antibiotic was found to be 6.25 μg/mL.

In a previous paper, we reported that anacardic acids **1**−**4** did not exhibit any activity against the three Gram-negative bacteria tested up to 100 μg/mL, although they showed potent antibacterial activity against Gram-positive bacteria and weak antifungal activity against molds (Himejima and Kubo, 1991). After finding their antibacterial activity against *H. pylori*, a Gram-negative bacterium, the three anacardic acids **1**−**3** were retested at higher concentrations against the same three Gram-negative bacteria (*P. aeruginosa*, *E. aerogenes*, and *E. coli*) but were found to show no activity up to 800 μg/mL. It appears now that the activity of anacardic acids **1**−**3** against Gram-negative bacteria seems to be specific against *H. pylori*.

In addition, although the *n*-hexane fraction did not exhibit any activity against *H. pylori*, one of the characteristic flavor compounds of the cashew apple, (*E*)-2-hexenal (**7**), was previously reported to have broad antimicrobial activity including the three Gram-negative bacteria (*P. aeruginosa*, *E. aerogenes*, and *E. coli*) with MICs ranging between 400 and 800 μg/mL (Muroi et al., 1993). We now found this alkenal to be antibacterial against *H. pylori* with an MIC and MBC of 400 and 800 μg/mL, respectively. After finding this activity, the two additional alkenals, (*E*)-2-octenal (**8**) and (*E*)-decenal (**9**), characterized from the fresh fruits of *Olea europaea* L. (Oleaceae) known as olive (Kubo, A., et al., 1995) were also tested. The former alkenal exhibited slightly more potent activity than **7** with an MIC of 200 μg/mL while the latter did not show any activity up to 800 μg/mL. Subsequently, (*E*)-2-hexenal was found to inhibit the growth of *S. typhimurium* with an MIC of 400 μg/mL.

Colonization and survival of *H. pylori* in the hostile environment of the stomach are aided by four to six strong flagella, which allow the bacterium to swim in the viscous mucus layer of the stomach, and a potent urease, which may neutralize the acid environment by hydrolyzing urea to ammonia and carbon dioxide (Blaser, 1993). Therefore, the two representative antibacterial agents characterized in the cashew apple, anacardic acid (**1**) ($C_{15:3}$) and (*E*)-2-hexenal (**7**), were tested to see if they inhibit urease. Both were found to inhibit the enzyme. The former showed a dose-dependent inhibitory effect on the ammonia liberated by jack bean urease, and its IC_{50} was established as 125 μg/mL, which is close to its MIC. The latter volatile compound also showed a dose-dependent inhibitory effect with an IC_{50} of 50 μg/mL. Although **5** ($C_{12:0}$) and (*E*)-2-octenal (**8**) were not characterized in the cashew apple, both were also tested. The result is listed in Table 2. Interestingly, salicylic acid did not exhibit any inhibitory activity up to 1600 μg/mL.

Table 2. Urease Inhibitory Activity and Mode of Inhibition of Anacardic Acids and (*Z*)-2-Alkenals

compounds tested	ID_{50} (μg/mL)	mode of inhibition
anacardic acids		
($C_{15:3}$)	125	competitive
($C_{12:0}$)	125	−
salicylic acid	>1600	−
(*E*)-2-hexenal	50	noncompetitive
(*E*)-2-octenal	50	−

[a] −, not tested.

DISCUSSION

It should be noted that 6-pentadecylsalicylic acid (**4**) ($C_{15:0}$) was not previously isolated from the cashew apple but from the cashew nut shell oil in minute amounts (Kubo et al., 1986). In general, anacardic acids with a fully saturated alkyl side chain are relatively rare and occur only in traces admixed with unsaturated analogues (Spencer et al., 1980). Nevertheless, the result that this $C_{15:0}$ anacardic acid did not show any activity against *H. pylori* while $C_{12:0}$ anacardic acid (**5**) exhibited the activity indicates that the double bond in the side chain is not essential to elicit the activity. This is not in agreement with the previous conclusion (Gellerman et al., 1969; Himejima and Kubo, 1991) but suggests that the alkyl side chain length is important instead, similar to the case against Gram-positive bacteria (Kubo et al., 1993b). The activity gradually increased with their side chain length: C_{12} exhibited the maximum, and C_{15} was inactive. In anacardic acids **4** and **5**, the saturated $C_{15:0}$ and $C_{12:0}$ alkyl side chains that can exist usually assume the extended form, which requires the least amount of energy to maintain. The unsaturated $C_{15:3}$, $C_{15:2}$, and $C_{15:1}$ alkyl side chains in anacardic acids **1**−**3**, on the other hand, have a bend of about 30° in the hydrocarbon side chain, imposed on the molecule by the cis configuration of the double bond. Two double bonds in the cis configuration in the $C_{15:3}$ and $C_{15:2}$ side chain create more bends and significantly shorten the side chain length. It is apparent that the cis double bond in the C_{15} alkyl side chain seems to play an important role. The data so far obtained indicate that ancardic acids act as a detergent (Kubo, I., et al., 1995). However, the role of the hydrophilic salicylic acid moiety remains unclear. It should be remembered here that cardanol (**10**) did not exhibit any antimicrobial activity (Himejima and Kubo, 1991).

Antibacterial treatment of *H. pylori* is difficult because of the habitat occupied by the organism below the layer of mucus adherent to gastric mucosa. Access of antibacterial agents to this site is limited from the lumen of the stomach and also from the gastric blood supply. The ideal therapy for *H. pylori* eradication should be simple, safe, and free from side effects with 100% efficacy. The total amount of lipophilic anacardic

acids **1**−**3** in the fresh cashew apple was found to be around 500 μg/mL (Kubo et al., 1986), which is slightly more than MIC but less than MBC as compared to those of **1** ($C_{15:3}$) and **2** ($C_{15:2}$). Antibacterial agents from a regularly consumed fruit like the cashew apple may be superior as *H. pylori* control agents as compared to many non-natural products. It seems that continuing consumption of the fresh cashew apple as well as its processed products such as juice, soft candy, condiments, and jam may be effective to control *H. pylori*. In recent years, the cashew apple has increased in value, especially in the countries where it is grown such as Brazil. Although (*E*)-2-hexenal was characterized as one of the key flavor compounds in the cashew apple (Maciel et al., 1986), it will not significantly contribute in controlling *H. pylori* because of its existence in minute amounts. However, this alkenal is known as a green leaf aldehyde in many plants and easily available in quantities since it has been widely used as a food flavor (Bauer et al., 1990).

Urease is a nickel-containing enzyme, and anacardic acid **1** ($C_{15:3}$) was reported to show somehow selectivity toward Ni^{2+} (Nagabhushana et al., 1995). Therefore, its urease inhibition mechanism may come from its ability to chelate nickel in the enzyme. In addition, since salicylic acid did not inhibit urease up to 1600 μg/mL, the protein pocket likely contributes to the stability of binding of the conjugated ligands by interacting with the planar salicylic acid portion (Duckworth and Coleman, 1970). It is not clear however if the antibacterial activity of anacardic acids against *H. pylori* comes from their urease inhibitory activity, although this explains their specific activity against this bacterium. It should be noted, however, that a urease inhibitor is now considered only to function during initial colonization during bacterial transit across the gastric lumen in vivo.

It is known that single agents are generally ineffective or poorly effective in eradicating *H. pylori*. In addition, the problem of resistance is very important since it readily occurs with monotherapy, which therefore should never be used for *Helicobacter*. The combination of two or more antibacterial agents is therefore recommended (Harris and Misiewicz, 1996). It may be advisable to use anacardic acids or (*E*)-2-hexenal in combination with antibacterial agents in order to make the development of resistance mechanisms in microorganisms less likely as well as enhancing the total antibacterial activity. Their structure−activity relationship and combination studies with antibiotics used for treatment of peptic ulcer such as clarithromycin and erythromycin will be reported separately.

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