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Inhibition of *Helicobacter pylori in vitro* by various berry extracts, with enhanced susceptibility to clarithromycin

Archana Chatterjee,[1,2] Taharat Yasmin,[2] Debasis Bagchi[2] and Sidney J. Stohs[2]

[1]*Department of Pediatrics, Creighton University Health Sciences Center, Omaha, NE, USA;* [2]*Department of Pharmacy Sciences, Creighton University Health Sciences Center, Omaha, NE, USA*

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Abstract

The objective of this study was to evaluate the effects of various berry extracts, with and without clarithromycin on *Helicobacter pylori*. Resistance to clarithromycin by *H. pylori* has been reported, leading to interest in alternatives/adjuncts to therapy with clarithromycin. *H. pylori* American type culture collection (ATCC) strain 49503 was grown, cell suspensions were made in PBS and diluted 10-fold. One hundred μl of the suspension was then incubated for 18 h with extracts of raspberry, strawberry, cranberry, elderberry, blueberry, bilberry, and OptiBerry®, a blend of the six berries, at 0.25–1% concentrations. Serially diluted cell suspensions were exposed for 1 h to clarithromycin at 15 μg/ml. Ten μl of bacterial samples from the 10^{-7} dilution tube were plated and incubated for 18 h and the number of colonies were counted. Growth of *H. pylori* was confirmed by the CLO® test. All berry extracts significantly ($p < 0.05$) inhibited *H. pylori*, compared with controls, and also increased susceptibility of *H. pylori* to clarithromycin, with OptiBerry® demonstrating maximal effects. (Mol Cell Biochem **265**: 19–26, 2004)

Key words: berry extracts, clarithromycin, *Helicobacter pylori*

Introduction

Approximately 50% of the earth's population is infected with *Helicobacter pylori* [1], which has been implicated in the etiology of chronic gastritis and peptic ulcer, both in adults and children [2]. The World Health Organization has declared *H. pylori* a carcinogen, predisposing infected persons to gastric cancer and lymphoma [3]. Several oral antimicrobial agents have efficacy against *H. pylori*. Clarithromycin is a key component of many therapeutic regimens recommended for eradication of gastric *H. pylori*. Antibiotic treatment for *H. pylori* infection is often accompanied by side effects including development of resistance to antimicrobial agents, including clarithromycin [2]. Considerable interest has focused recently on alternative/adjunct approaches such as biologically active compounds including antioxidants from plants and other natural sources [4–7].

Previous publications from our laboratory have focused on the role of *H. pylori* in production of oxygen-free radicals such as superoxide anion and hydroxyl radicals, which lead to increased oxidative damage (a major cause of gastric injury), as demonstrated by enhanced lipid peroxidation and increased DNA damage in the gastric tissues [8, 9]. Natural antioxidants may thus serve as novel therapeutic tools in alleviating *H. pylori*-induced oxidative damage. Several recent studies have demonstrated the inhibitory effect of cranberry juice and its constituents on *H. pylori* adhesion to human gastrointestinal cells [5–7]. The exact mechanism of this inhibition is unclear at this time, but a plausible explanation may be the antioxidant property of cranberry juicewhich is due

Address for offprints: Archana Chatterjee, Department of Pediatrics, Creighton University Health Sciences Center, 601 N. 30th St, Suite 2300 A, Omaha, NE 68131, USA (E-mail: achatter@creighton.edu)

to the presence of anthocyanins [10]. For example, Howell *et al.* [11], have demonstrated the inhibitory effects of proanthocyanidin extracts from cranberries on the adherence of P-fimbriated *Escherichia coli* to uroepithelial-cell surfaces. Other edible berries including bilberries, blueberries, strawberries, elderberries and raspberries have also been shown to have antioxidant properties [12–16].

In this study, we evaluated the inhibitory effects of various berry extracts with and without clarithromycin, against a pathogenic strain of *H. pylori* (ATCC strain 49503) which is known to produce an 87 kDa cytotoxin responsible for gastric injury [17]. This strain was chosen based on a previous study that demonstrated that in a tissue culture model, the greatest lactate dehydrogenase (LDH) leakage and superoxide anion production were caused by it compared with other strains of *H. pylori* [8]. We also evaluated the inhibitory effects of several antioxidants against this strain of *H. pylori* [8]. In a comparative antioxidant study, we have demonstrated the superior performance of a grape seed proanthocyanidin (GSPE) compared with vitamins C, E and β-carotene both *in vitro* and *in vivo* [18]. Recently, it has been shown that berry mix is a superior antioxidant compared with GSPE [19]. On the basis of these observations of the anitoxidant properties of berry extracts, we chose to study the inhibitory effects of various berry extracts on *H. pylori*.

Materials and methods

Chemicals

Clarithromycin (Biaxin® was obtained from Abbott laboratories, North Chicago, IL . Extracts of raspberry, cranberry, elderberry, strawberry, bilberry, blueberry and OptiBerry® (a blend of the six berries) were obtained from InterHealth Nutraceuticals Incorporated, Benicia, CA . All other chemicals unless otherwise stated, were obtained from Sigma Chemical Co. (St. Louis, MO, USA), and were of analytical grade.

Stock solutions of 5 mg/ml for clarithromycin were prepared in DMSO and stored at 4 °C. Further dilutions of the drug were made in phosphate buffered saline for use in the bactericidal tests. Fresh solutions were prepared for each experiment.

Bacterial strain

Freeze dried cytotoxin producing *H. pylori* strain ATCC 49503 was obtained from American Type Culture Collection (Rockville, MD). Freeze dried bacterial samples were redissolved in sterile *Brucella* broth and incubated at 37 °C for 30 min. before being cultured on fresh blood agar plates.

Growth media and growth conditions

Lennox broth (Fisher Chemicals) was used for growth of *H. pylori*. Trypticase soy agar (TSA) plates with 5% defibrinated sheep blood (BBL, Becton Dickinson, MD) were used for determining viable bacterial counts. Bacterial plates and culture tubes were incubated at 37 °C, under microaerophilic conditions (oxygen 5%, carbon dioxide 10% and nitrogen 85%) in an incubator [8].

Determination of bacterial counts with berry extracts

H. pylori was initially grown on 5% blood agar plates overnight. Cell suspensions were then prepared in 2 ml of PBS and diluted 10-fold. The various berry extracts mentioned above were incorporated into Lennox broth in concentrations of 0.25, 0.5 and 1%, with control tubes having only the broth. One hundred μl of the bacterial cell suspension was then added to each tube and incubated under microaerophilic conditions for 18 h. Samples from each culture tube were then serially diluted, and 10 μl aliquots from the 10^{-7} dilution tube were plated on fresh 5% blood agar plates which were incubated under microaerophilic conditions for 18 h and the number of colonies counted. Growth of *H. pylori* was confirmed by the CLO® test . All experiments were conducted in triplicate.

CLO test®

The CLO® test is a rapid urease test initially developed to detect the urease enzyme of *H. pylori* in gastric mucosal biopsies [20]. It has also been used to detect urease production from *H. pylori* infection in tissue culture [8]. Test slides were obtained from (Ballard Medical Products, Draper, UT). After 16 h of incubation, bacterial cultures were tested for urease activity to confirm growth of *H. pylori*.

Determination of bactericidal effects of clarithromycin and berry extracts on H. pylori

Each of the serially diluted experimental and control samples were incubated with 15 μg/ml of clarithromycin for 1 h. Three replicates of 10 μl from each tube were then plated on 5% blood agar plates, incubated under microaerophilic conditions for 18 h, and the number of colonies counted. Once again, growth of *H. pylori* was confirmed by the CLO® test.

Statistical analysis

Study results were entered into a database and analyzed using the ABSTAT® software. Chi-square test was used to compare

results at different concentrations. Level of significance was set at $p < 0.05$.

Results

The *in vitro* bactericidal activities of the various berry extracts with and without clarithromycin, against *H. pylori* are shown in Figs. 1–6. All of the extracts at all concentrations tested, inhibited the growth of *H. pylori*, compared with controls, with maximal effects noted with OptiBerry®. Even at the lowest concentration of 0.25%, significant inhibition of *H. pylori* was noted with elderberry (30%), bilberry (50%), blueberry (50.5%) and OptiBerry® (62%) (Fig. 1). A concentration-dependent increase in inhibition of *H. pylori* with the higher concentrations of 0.5 and 1% of all the berry extracts was observed (Figs. 2 and 3, respectively). Modest increases in bactericidal effect were seen with the 0.5% concentration of strawberry, raspberry and cranberry extracts, compared with the increases noted for elderberry, bilberry, blueberry and OptiBerry® (Fig. 2). At the 1% concentration, all extracts showed >70% inhibition, with cranberry, elderberry, bilberry and blueberry extracts showing >90% inhibition, and OptiBerry® exhibiting 100% inhibition (Fig. 3). However, the differences were not significant for the latter four berry extracts and OptiBerry®.

The addition of clarithromycin to the 0.25% berry concentrations, led to a significant increase in the bactericidal effects of the elderberry, bilberry, blueberry and OptiBerry®extracts against *H. pylori* compared with the berry extracts alone (Fig. 4). When clarithromycin was added to the 0.5% berry concentrations, a significant increase in the inhibition of *H. pylori* was observed with all the extracts tested (Fig. 5). Finally, when clarithromycin was added to the 1% berry concentrations, >90% inhibition was noted for all extracts, with elderberry, bilberry, blueberry and OptiBerry® exhibiting 100% inhibition (Fig. 6).

Discussion

The objective of this study was to evaluate the inhibitory effects of various berry extracts with and without clarithromycin on *Helicobacter pylori in vitro*. *H. pylori* is now recognized as an important human pathogen/carcinogen [21–24]. It is estimated to infect 50% of the world and US populations [22, 25]. Various gastrointestinal disorders including chronic gastritis, gastric inflammatory diseases, peptic ulcer disease and gastric cancer have been associated with *H. pylori* infection [21–24, 26–30].

Oxygen free radicals and oxidative stress have been implicated in several gastrointestinal diseases, such as small-intestinal ischemia, ulcerative colitis, pancreatitis and gastric ulcer [31]. They appear to be important in the development of gastrointestinal injury after intestinal ischemia and reperfusion [32], and after hemorrhagic shock [31]. They have also been implicated in ischemia-reperfusion injury to the liver [33]. In gastrointestinal inflammatory diseases,such as acute

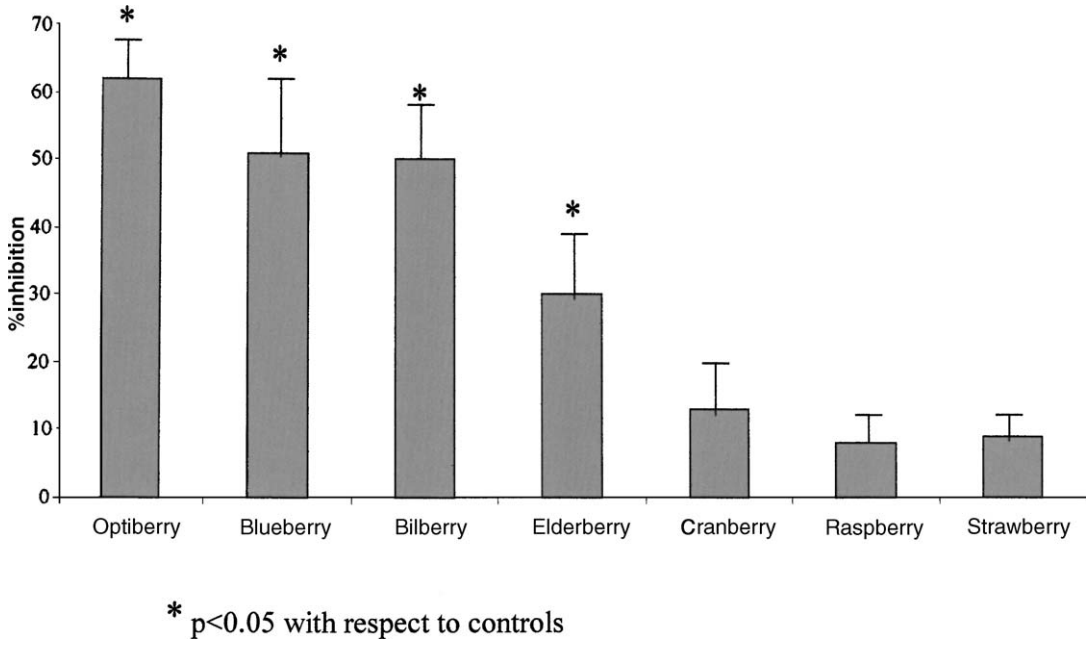

Fig. 1. Effects of berry extracts on *H. pylori* growth at 0.25% concentration.

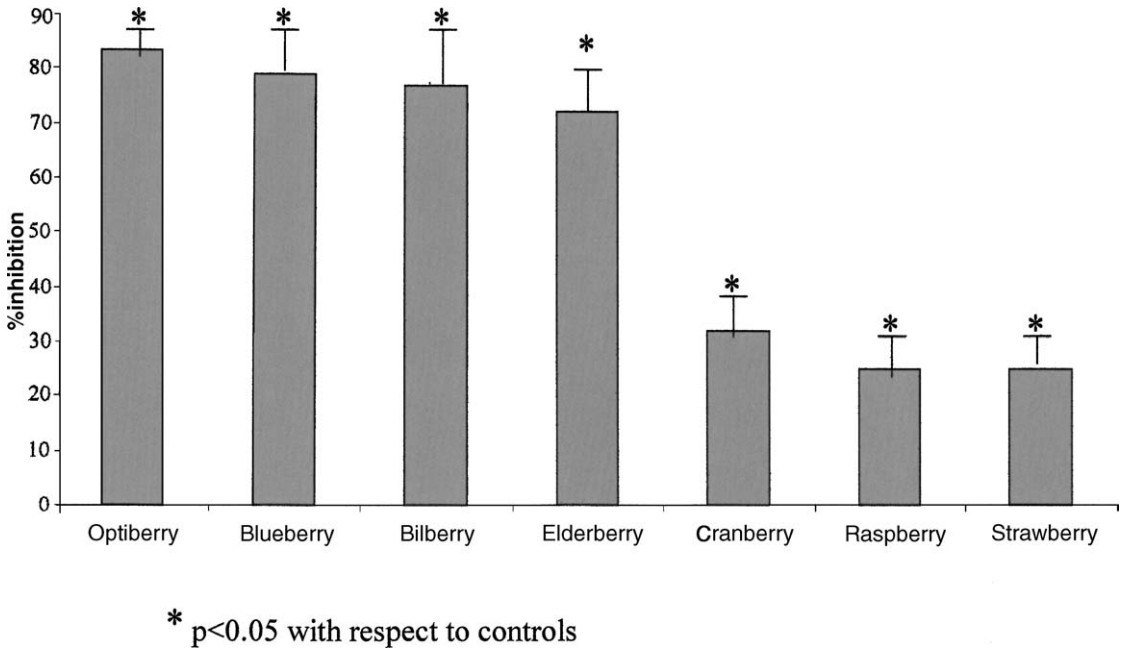

Fig. 2. Effects of berry extracts on *H. pylori* growth at 0.5% concentration.

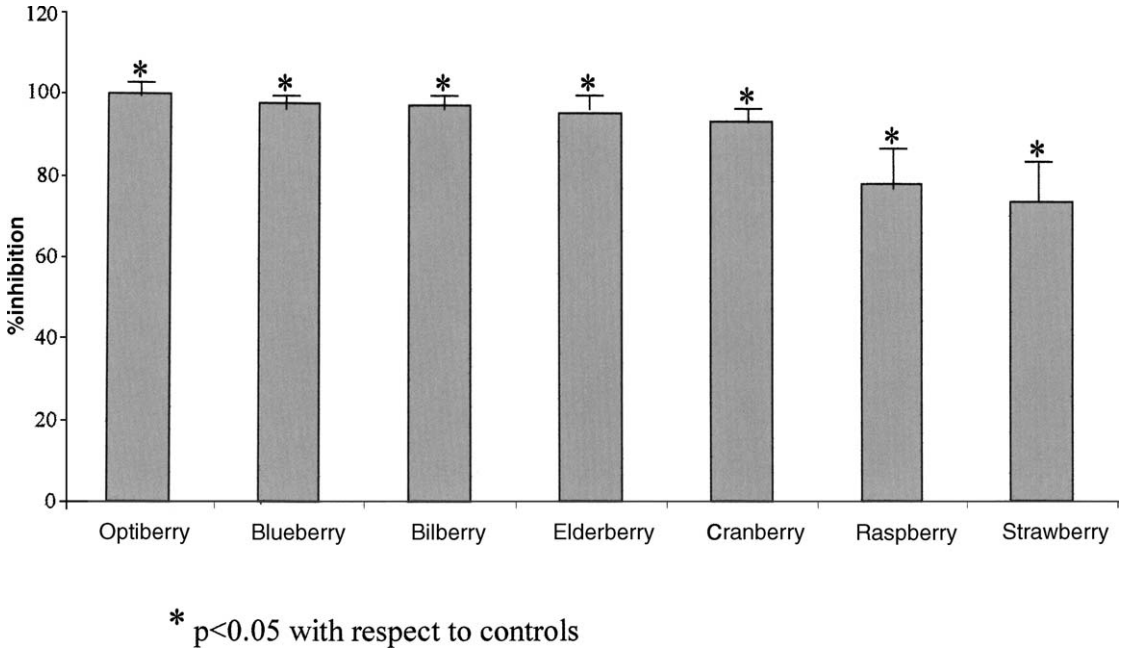

Fig. 3. Effects of berry extracts on *H. pylori* growth at 1% concentration.

pancreatitis [34] and inflammatory bowel diseases [35], oxidative stress and oxygen free radicals have been shown to have a role. Finally, increased production of free radicals have also been demonstrated to occur during the gastrointestinal metabolism of xenobiotics, which may lead to intestinal disorders [36].

Elevated levels of free radicals in duodenal biopsies from patients with active duodenal ulcers have been reported [26]. Greater chemiluminescence in *H. pylori* positive compared with negative tissues was observed when samples were grouped by equivalent macroscopic or microscopic damage, indicating free radical production. In part, this difference

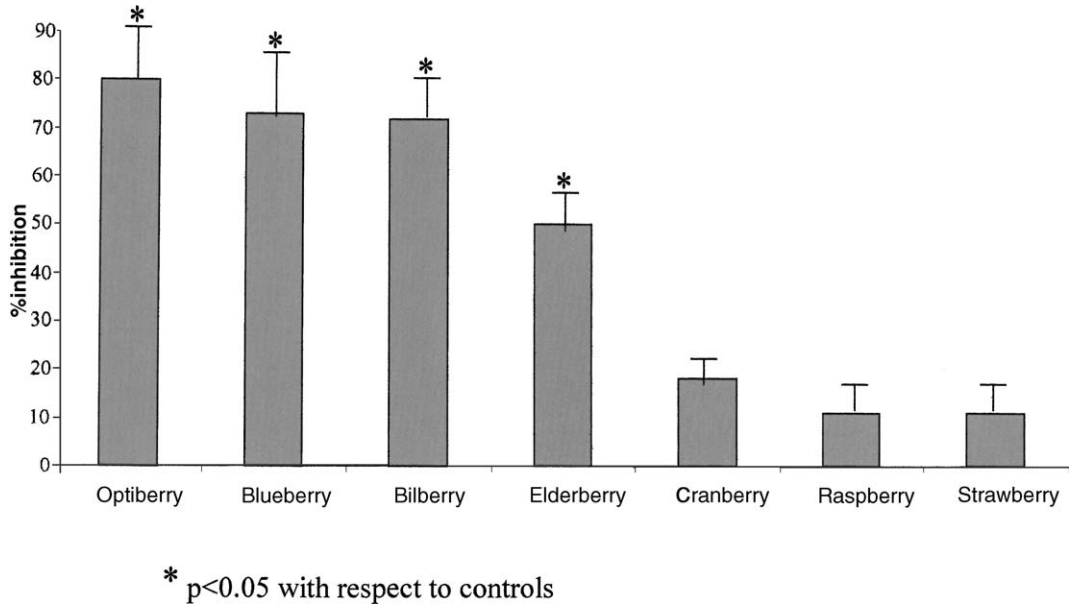

Fig. 4. Effects of clarithromycin on *H. pylori* growth after exposure to berry extracts at 0.25% concentration.

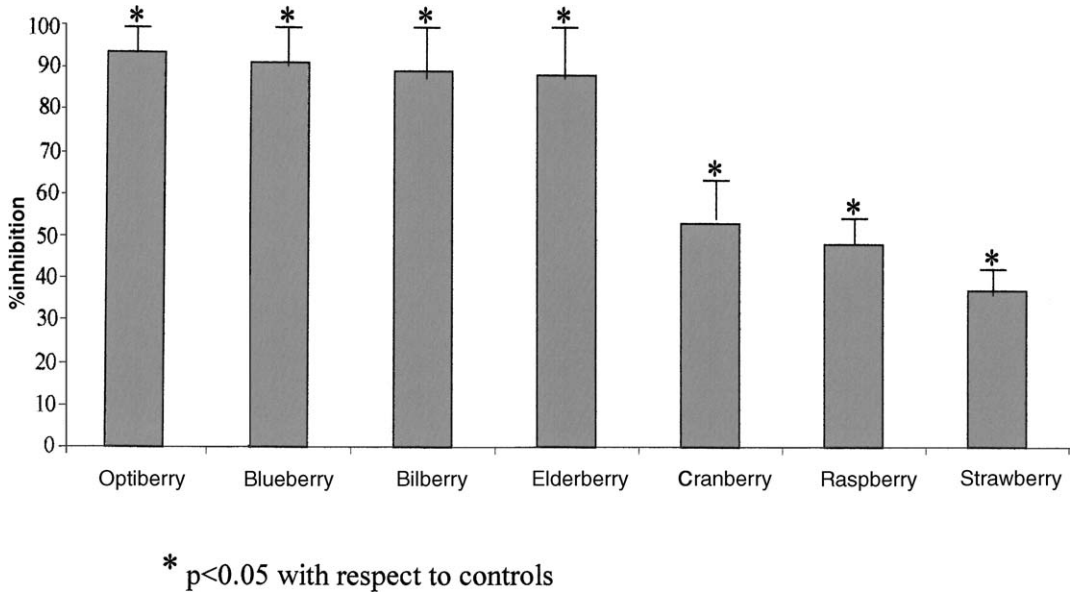

Fig. 5. Effects of clarithromycin on *H. pylori* growth after exposure to berry extracts at 0.5% concentration.

was accounted for by a greater neutrophil infiltration in the *H. pylori* positive mucosa, but when biopsy specimens with equivalent neutrophil infiltration were compared directly, *H. pylori* positive specimens gave greater chemiluminescence than negative tissue specimens. No evidence was found for a role of free radicals in the pathogenesis of gastric mucosal injury in cases unrelated to *H. pylori* infection [26]. These results indicate that production of free radicals is associated with *H. pylori* positive antral infection and may be an important pathogenic mechanism. In addition, enhanced production of free radicals in *H. pylori*-induced duodenal ulceration has been demonstrated [37]. Interestingly, cimetidine, an H_2-receptor antagonist and a widely used gastroprotective medication, is a potent hydroxyl radical scavenger [38].

In recent studies, we have demonstrated increased production of free radicals in human gastric mucosal cells following incubation with various strains of *H. pylori*, as evidenced by

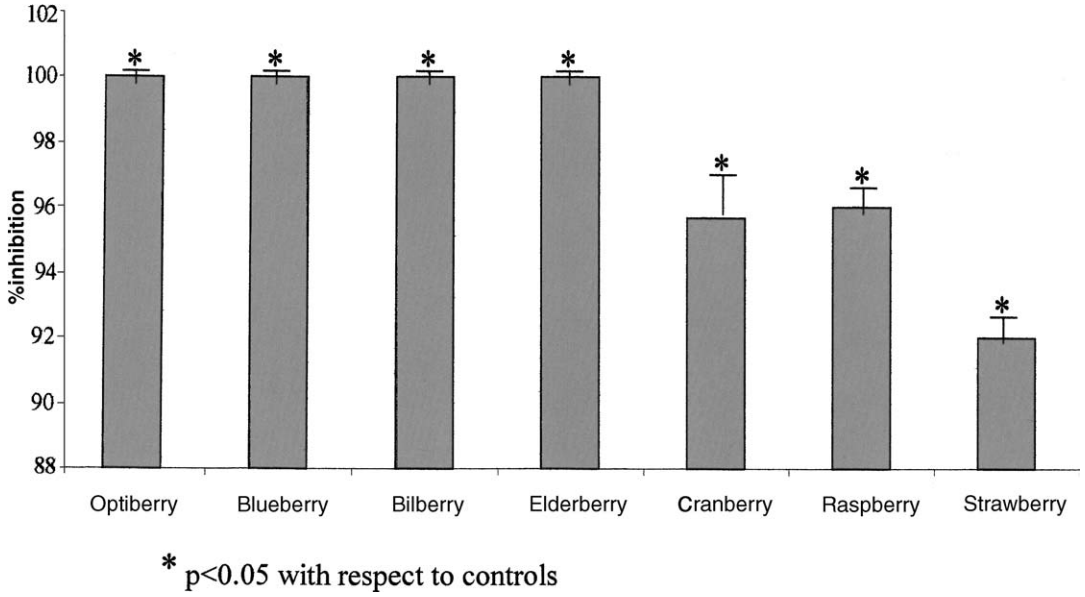

Fig. 6. Effects of clarithromycin on *H. pylori* growth after exposure to berry extracts at 1% concentration.

enhanced production of superoxide anion and hydroxyl radicals, and increased lipid peroxidation and DNA damage in gastric tissues [8, 9]. We have also shown the bactericidal effects of the potent antioxidant garcinol (a polyisoprenylated benzophenone derived from *Garcinia indica* fruit rind) against a pathogenic strain of *H. pylori* [4].

A number of antimicrobial agents, including amoxicillin, tetracycline, metronidazole, clarithromycin and bismuth salts have activity against *H. pylori*, but none have proven therapeutic effectiveness as single agents [39]. In general, therapeutic regimens for *H. pylori* infection consist of 1–2 weeks of one or two effective antimicrobial agents plus bismuth subsalicylate, or a proton pump inhibitor (lansoprazole, omeprazole, esometrazan or rabeprazole sodium). Depending on the regimen used, such therapies result in eradication rates ranging from 61 to 94% in adults [39]. Triple-drug therapy regimens are more effective for eradication of *H. pylori* than two-drug therapy regimens. These regimens are expensive and may result in adverse effects such as diarrhea and drug allergies.

Increasing antibiotic resistance is a major threat to treatment of many infectious diseases including *H. pylori*. Recent studies have shown increasing resistance of *H. pylori* strains to clarithromycin, which is widely used to treat infections caused by this pathogen [40, 41]. Rates of clarithromycin resistance in *H. pylori* isolates from children are higher than in adults [41], probably due to increased exposure of children to macrolide antibiotics for treatment of respiratory tract infections. Alternatives to antibiotic therapy have therefore been sought for treatment of *H. pylori* infections. For example, garcinol [42] and resveratrol [43, 44], two plant products with antioxidant activity, have recently been shown to have antiulcer properties. In a previous study, we have demonstrated that garcinol inhibited *H. pylori in vitro*, while resveratrol did not [4]. In the current study, we evaluated the effects of various berry extracts that have been shown to have antioxidant properties [12–16] on *H. pylori*. The experiments were repeated with clarithromycin, because these extracts could potentially be used for therapeutic purposes in combination with antibiotics such as clarithromycin in the future. It was thus important to demonstrate that there was no interference with the effects of clarithromycin on *H. pylori*. Indeed, we observed improved inhibition of *H. pylori* by the combination of various berry extracts and clarithromycin.

In the present study, we chose to evaluate the antibacterial properties of various berry extracts against the same pathogenic strain of *H. pylori*. To the best of our knowledge, only one of these (cranberry) has previously been evaluated for this purpose [5–7]. This is therefore, the first report of inhibition of *H. pylori in vitro* by extracts of strawberries, raspberries, elderberries, bilberries, blueberries, and a blend of the six berries (OptiBerry®. As our results indicate, there was a concentration-dependent inhibition of *H. pylori*, with the highest antibacterial activity noted at the 1% concentration of all extracts. It is also important to note that not all extracts had equivalent activity. OptiBerry® demonstrated the greatest effect at all concentrations tested, although the differences with several of the individual berry extracts were not significant. Blueberry and bilberry had significantly better activity against *H. pylori* compared with raspberry, cranberry and strawberry, particularly at lower concentrations.

Finally, an additive inhibitory effect was noted when clarithromycin was used in combination with all concentrations of the various berry extracts tested. Our study thus shows the potential of using the antibacterial activity of berry extracts alone or in combination with clarithromycin against *H. pylori*. Since this is the first report of this activity for most of the extracts tested, our findings need to be confirmed by other investigators and further studies initiated to determine the mechanism of inhibition, as well as potential clinical applications of our results.

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