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Inhibition of Gastric H^+,K^+-ATPase and *Helicobacter pylori* Growth by Phenolic Antioxidants of *Curcuma amada*

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Gastric ulcer is the most prevalent gastrointestinal disorder, resulting from oxidative stress, *Helicobacter pylori* infection, up-regulation of proton potassium ATPase (PPA) activity, down-regulation of gastric mucosal defense, etc. In this paper it is reported that phenolic fractions of *Curcuma amada*, commonly known as mango ginger, acted as potent inhibitors of PPA and *H. pylori* growth. Mango ginger free phenolics (MGFP) and mango ginger bound phenolics (MGBP) inhibited PPA at IC_{50} values of 2.2 ± 0.21 and 0.7 ± 0.08 μg/mL, respectively, exhibiting 9−27-fold better potency over lansoprazole (IC_{50} of 19.3 ± 2.2 μg/mL). MGFP is constituted by caffeic (26%), gentisic (24%), ferulic (20%), gallic (10%), cinnamic (7%), and protocatechuic acids (7%) and MGBP by ferulic (47%), cinnamic (29%), *p*-coumaric acid (11%), and syringic (5%) acids as major phenolic acids. MGFP and MGBP further exhibited free radical scavenging (IC_{50} of 2.2 ± 0.17 and 4.2 ± 0.36 μg/mL), reducing power abilities (193−104 units/g), inhibition of lipid peroxidation (IC_{50} of 10.3 ± 0.91 and 15.6 ± 1.6 μg/mL), and DNA protection (80% at 4 μg), indicating strong antioxidative properties. MGFP and MGBP thus may be potential and inexpensive multistep blockers against ulcers.

KEYWORDS: Antioxidant activity; free and bound phenolics; H^+,K^+-ATPase; *Helicobacter pylori*; *Curcuma amada*

INTRODUCTION

Oxidative stress (OS) arising from an imbalance between reactive oxygen species (ROS) accumulation and defense mechanisms in the body contributes to OS-induced diseases such as cancer (*1*), ulcer, inflammation (*2*), and brain dysfunction. Dietary antioxidants play an important role against this OS and hence have been known to prevent many degenerative diseases (*3*). Diets rich in phenolic acids, particularly free and bound phenolics constituting different phenolic acids, have been shown to possess antiulcer activity (*4*, *5*). Phenolic acids such as caffeic, ferulic, cinnamic, and protocatechuic acids have recently been shown from our laboratory to exert antioxidant and antimicrobial activities (*6*). The antioxidant activity of phenolics appears to be an important factor contributing to antiulcer activity because free radicals and ROS are the main causative factors for ulcer (*7*).

Curcuma amada belonging to the family of Zingiberaceae, popularly known as mango ginger, has been known for its potent antioxidant activity. Mango ginger cultivated mainly in the Indo-Malaysian regions is well-known for culinary preparations such as pickles due to its exotic mango aroma. Mango flavor is mainly attributed to δ-3-carene and *cis*-ocimene among the 68 volatile aroma components present in the essential oil of mango ginger rhizome (*8*). It has been used as a stomachic and carminative (*9*). Antioxidant and antibacterial activities of mango ginger have been also reported recently (*10*). In traditional and ayurvedic medicine systems, mango ginger was found to be a classic herb for the digestive system because of its stomachic and carminative properties, which in turn help to prevent major gastric problems such as hyperacidity, gastritis, and ulcer.

Gastric hyperacidity and ulcer are major recurrent diseases of the gastrointestinal tract affecting all geographical regions (*11*). It is an imbalance between damaging factors within the lumen and protective mechanisms within the gastroduodenal system. Hyperacidity is due to excessive secretion of HCl from the mucosa, which is due to hyperactivity of proton pumping

Table 1. Total Phenolic Content and Yield of Phenolics per 100 g (Weight) of MGFP and MGBP Fractions[a]

phenolic fraction	yield (mg/g of dry wt)	% yield (g/100 g)	phenolic content (mg/g)
MGFP	2.8b ± 0.22	0.28	2.1a ± 0.20
MGBP	2.3a ± 0.18	0.23	1.9a ± 0.17

[a] Free and bound phenolic constituents were isolated from 2 g of defatted dry powder as per the protocol mentioned under Materials and Methods. Two milligrams of powder was solubilized in methanol, and phenol was estimated in soluble fraction; to calculate the yield from 2 g of mango ginger powder, total solubles were dried and weighed. MGFP, mango ginger free phenolic fraction; MGBP, mango ginger bound phenolic fraction. All data are the mean ± SD of three replicates; mean values followed by different letters in the same column indicate that they differ significantly ($p > 0.05$).

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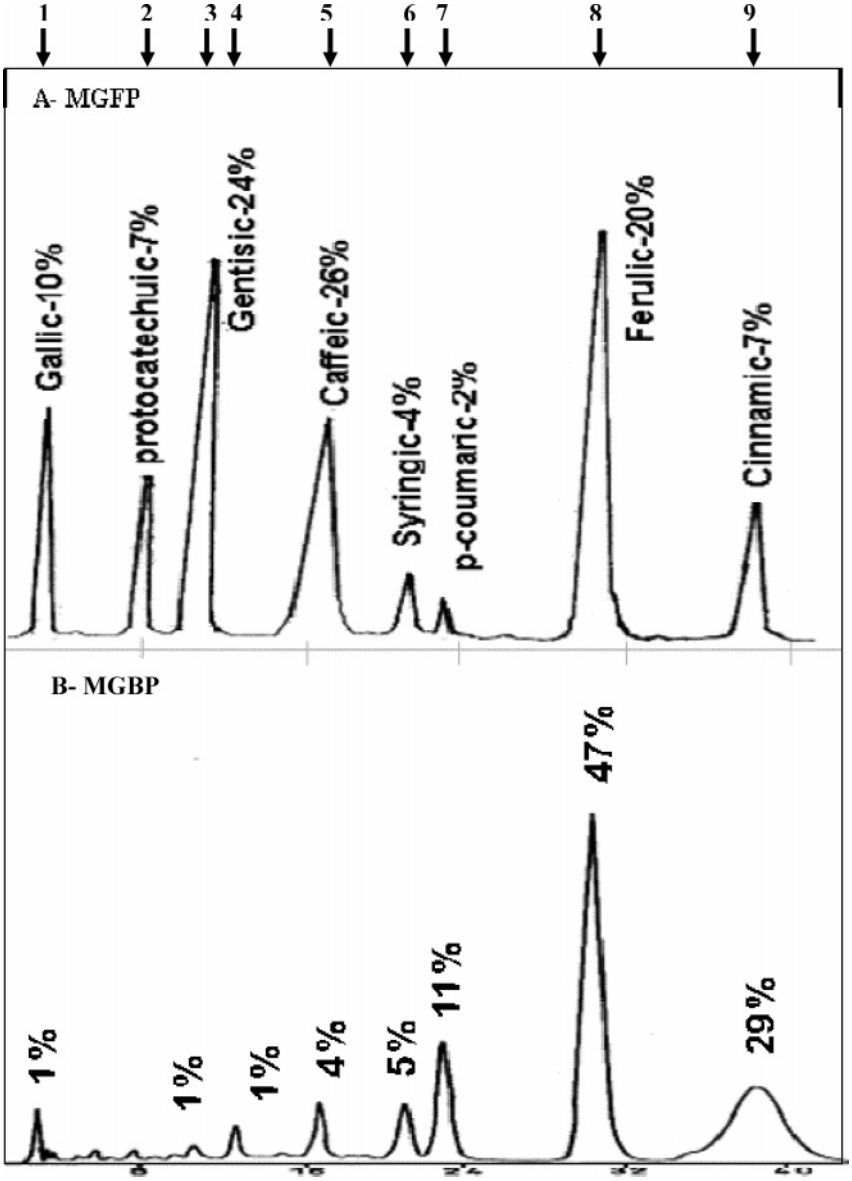


Figure 1. HPLC analysis of phenolic acid constituents in free (MGFP) and bound (MGBP) phenolic fractions. Phenolic acids in each fraction were identified by comparison of their retention time with known standards. Arrows 1–9 indicate the location as retention time in minutes of standard phenolic acids. 1, gallic; 2, protocatechuic; 3, gentisic; 4, vanillic; 5, syringic; 6, *p*-coumaric; 7, caffeic; 8, ferulic; 9, cinnamic acid.

by H^+,K^+-ATPase of parietal cells (*12*). Gastroduodenal ulcers, on the other hand, are caused by the loss of gastroprotection by various factors such as stress-related gastric mucosal damage, nonsteroidal anti-inflammatory drug induced gastric lesions (*13*), and *Helicobacter pylori* mediated ulcer lesions (*14*). Apart from the contributing role of acid to mucosal damage, ROS, especially the hydroxyl radical [OH], plays a major role in causing oxidative damage to mucosa, which in turn leads to ulcer. The damaged mucosa becomes a susceptible site for the entry of *H. pylori*. Mucosal damage together with *H. pylori* growth aggravates the gastric conditions. In the stomach there is a 1000-fold higher production of ROS compared to that in other tissues or plasma, which makes it most susceptible to damage. ROS also play a major role in the multistep process toward the development of gastric adenocarcinoma from ulcerous condition (*15*). Dietary antioxidants with the potent ability to scavenge oxygen and nitrogen free radicals, breaking lipid chain peroxidation reactions, may act as gastroprotective factors. Phenolic compounds are one of the major classes of dietary antioxidants that, apart from their action as radical scavengers, exhibit several indirect effects such as inhibition of lipoxygenase (*16*), reduction of platelet aggregation and inflammation (*17*), and potential reduction of entry of human pathogens into the gut. The modern approach to therapy for ulcer disease therefore includes proton pump blockers, free radical scavengers, and antimicrobials from dietary sources that do not cause side effects such as known available antiulcer drugs (*18*). In this regard the current study addressed the isolation of antioxidant fraction free and bound phenolics from mango ginger and evaluated their potential antiulcer effect by examining proton potassium ATPase blockade and anti *H. pylori* properties.

MATERIALS AND METHODS

Plant Material. Mango ginger (*Curcuma amada*) rhizome was purchased from the local market at Mysore, India, and used for studies. One kilogram of fresh mango ginger rhizome was cleaned, washed under running tap water, cut into small pieces, air-dried, and powdered at a particle size of 20 mesh. Free and bound phenolics were isolated, and proton potassium ATPase inhibition (PPI) as well as their antioxidant and anti *H. pylori* activities was determined.

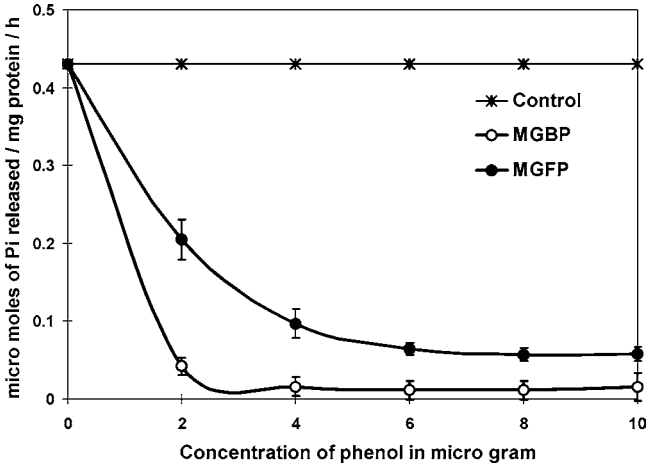


Figure 2. Inhibition of proton potassium ATPase enzyme activity by free (MGFP) and bound (MGBP) phenolic fractions of ginger. Sheep parietal cell extract was employed as gastric H^+,K^+-ATPase source, and activity was determined by employing the protocol described under Materials and Methods. Three hundred and fifty micrograms of enzyme protein per milliliter of reaction volume was incubated with 2–10 μg of GAE of MGFP (●) and MGBP (○) and with no inhibitor (∗). Enzyme activity is represented as micromoles of P_i released per milligram of enzyme protein per hour. All data are the mean ± SD of three replicates.

Chemicals. Agarose, calf thymus DNA, 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2-thiobarbituric acid (TBA), and phenolic acid standards such as gallic, tannic, caffeic, *p*-coumaric, ferulic, gentisic, protocatechuic, syringic, and vanillic acids were obtained from Sigma (St. Louis, MO) (the purity of these compounds was verified by HPLC, and they were found to be homogeneous). Folin−Ciocalteu reagent, ferric chloride, glutaraldehyde, trichloroacetic acid, sodium carbonate, ferrous sulfate, and ascorbic acid were of the highest quality purchased from Qualigens Fine Chemicals (Mumbai, India). The HPLC column (Shimpak C_{18}) was obtained from Shimadzu Corp. (Kyoto, Japan), and HPLC grade solvents employed for HPLC analyses were purchased from Spectrochem Biochemicals Pvt. Limited (Mumbai, India).

Isolation of Free and Bound Phenolic Fractions of Mango Ginger. Free phenolics were extracted according to the method of Ayumi et al. (*19*). Briefly, 2 g of mango ginger powder was extracted with 4 × 50 mL of 70% ethanol and centrifuged at 3000*g* for 10 min at room temperature. Clear supernatant was concentrated by flash evaporation (Buchi 011, Flawil, Switzerland), and the pH was adjusted to 2.0 with 4 N HCl followed by centrifugation and concentration. Phenolic acids were separated by ethyl acetate phase separation (5 × 50 mL), and the pooled fractions were treated with anhydrous disodium sulfate to remove moisture, filtered, evaporated to dryness, and taken in 2 mL of methanol (w/v); this is designated the mango ginger free phenolic fraction (MGFP).

Bound phenolics were extracted according to the method of Nordkvist et al. (*20*). Two grams of mango ginger powder was extracted with 4 × 50 mL of 70% ethanol, followed by 4 × 50 mL of hexane to remove free phenolics and fat, respectively. The dried samples were extracted with 2 × 100 mL of 1 M sodium hydroxide containing 0.5% sodium borohydride under nitrogen atmosphere for 2 h, and the clear supernatant was collected followed by centrifugation at 3000*g* for 10 min. The combined supernatants were acidified with 4 N HCl to pH 1.5, and phenolic acids were processed as mentioned in the case of free phenolics; this is designated the mango ginger bound phenolic fraction (MGBP).

Estimation of Total Phenolic Content. The Folin−Ciocalteu reagent assay was used to determine the total phenolic content (*21*). A sample aliquot of 100 μL was added to 900 μL of water, 1 mL of Folin−Ciocalteu reagent previously diluted with distilled water (1:2 v/v), and 2 mL of 10% sodium carbonate solution in distilled water, which was then mixed in a cyclo mixer. The absorbance was measured at 765 nm with a Shimadzu UV−visible spectrophotometer (Shimadzu UV-160 spectrophotometer, Kyoto, Japan) after incubation for 2 h at room temperature. Gallic acid was used as standard for the calibration curve. The total phenolic content was expressed as gallic acid equivalents (GAE) in milligrams per gram of sample.

HPLC Analysis of Phenolic Antioxidants of MGFP and MGBP. Phenolic acids of MGFP and MGBP were analyzed by HPLC (model LC-10A, Shimadzu Corp.) on a reversed phase Shimpak C_{18} column (4.6 × 250 mm, Shimadzu Corp.) using a diode array UV detector (operating at λ_{max} = 280 nm). A solvent system consisting of water/acetic acid/methanol (isocratic, 80:5:15 v/v/v) was used as mobile phase at a flow rate of 1 mL/min (*22*). Phenolic acid standards such as caffeic, coumaric, cinnamic, ferulic, gallic, gentisic, protocatechuic, syringic, and vanillic acids were employed for the identification of phenolic acids present in MGFP and MGBP by comparing the retention time under similar experimental conditions.

Determination H^+,K^+-ATPase Inhibition (PPAI) by MGFP and MGBP Fractions in Comparison with Lansoprazole (PPA Inhibitor). Fresh sheep stomach was obtained from a local slaughterhouse at Mysore, India. The mucosa of gastric fundus was cut off, and the inner layer was scraped for parietal cells (*23*), which were homogenized in 16 mM Tris buffer (pH 7.4) containing 10% Triton X-100 and centrifuged at 6000*g* for 10 min. The supernatant (enzyme extract) was used for the assay. Protein content was determined according to Bradford's method using BSA as standard.

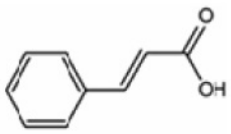
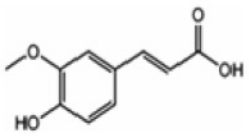
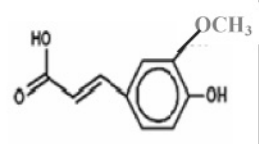
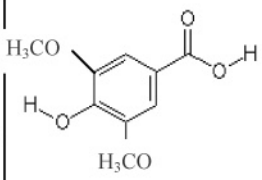
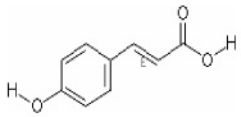
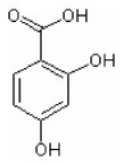
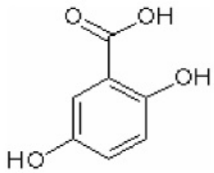
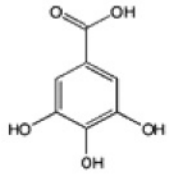
The enzyme extract (350 μg/mL) was incubated with different fractions of mango ginger phenolics, MGFP and MGBP, in a reaction mixture containing 16 mM Tris buffer (pH 6.5), and the reaction was initiated by adding substrate 2 mM ATP, in addition to 2 mM $MgCl_2$ and 10 mM KCl. After 30 min of incubation at 37 °C, the reaction was stopped by the addition of assay mixture containing 4.5% ammonium molybdate and 60% perchloric acid. Inorganic phosphate formed was measured spectrophotometrically at 400 nm. Enzyme activity was calculated as micromoles of P_i released per hour at various doses (2−10 μg) of MGFP and MGBP. Results were compared with the known antiulcer proton potassium ATPase inhibitor drug lansoprazole and with standard phenolic acids, because the active fraction of mango ginger contained phenolic acids.

Anti *H. pylori* Activity. *H. pylori* was obtained by endoscopic samples of ulcer patients from Karnataka Cardio Diagnostic Centre (KCDC, Mysore, India) and cultured on the specified medium Ham's F-12 nutrient agar medium with 5% fetal bovine serum (FBS) at 37 °C for 2−3 days in a microaerophilic condition (*24*). *H. pylori* culture was characterized by specific tests such as urease, catalase, oxidase, Gram staining, colony characteristics, and morphological appearance under scanning electron microscope and was also confirmed by the growth of culture in the presence of antibiotics for which it is resistant/susceptible.

Antimicrobial activity against *H. pylori* was tested by the standard agar diffusion method (*25*). Briefly, the Petri plates were prepared with Ham's F-12 nutrient agar medium containing 5% FBS inoculated with 100 μL of *H. pylori* culture (10^5 cells/mL). Sterile disks of high-grade cellulose of 5.5 mm diameter were impregnated with 20 μL of known extract at 5, 10, and 15 μg/disk of MGFP and MGBP placed on the inoculated petri plates. Amoxicillin was used as a positive reference standard and 0.9% saline as negative control. Anti *H. pylori* activity of pure phenolic compounds such as cinnamic, ferulic, caffeic, syringic, *p*-coumaric, gentisic, protocatechuic, and gallic acid (200 μg/disk) were also examined in order to understand the anti *H. pylori* activity of the individual phenolic acids. *H. pylori* growth inhibition was determined as the diameter of the inhibition zones around the disks. The growth inhibition diameter was an average of four measurements taken at four different directions, and all tests were performed in triplicates.

Minimal Inhibitory Concentration (MIC). MIC values were determined by conventional broth dilution method (*6*). Serial dilutions (final volume of 1 mL) of MGFP and MGBP (5−100 μg/mL) were performed with 0.9% saline. Afterward, 9 mL of specified medium was added. Broths were inoculated with 100 μL of *H. pylori* suspension [5 × 10^4 colony-forming units (CFU)] and incubated for 24 h at 37 °C. Amoxicillin and polymyxin were used, respectively, as positive and negative controls because *H. pylori* is susceptible to amoxicillin

Table 2. Antioxidant Potency and Potency of Inhibition of H^+,K^+-ATPase and *H. pylori* by Phenolics of MGFP and MGBP[a]

standard phenolic acids	structures	antioxidant activity[a] IC_{50} (μg)	PPA activity IC_{50} (μg)	*H. pylori*-inhibition zone (mm)	Phenolic acid content (mg/g)	
					MGFP	MGBP
Cinnamic acid (3-Phenyl-2-propenoic acid)		$4.6^{bc} \pm 0.3$	$15.1^{b} \pm 1.8$	34 ± 4.1	52.5	237
Caffeic acid (3,4-Dihydroxy cinnamic acid)		$1.8^{ab} \pm 0.14$	$27.1^{cde} \pm 3.1$	28 ± 3.4	195	30.7
Ferulic acid (4-hydroxy-3-methoxy cinnamic acid)		$6.60^{c} \pm 0.51$	$33.6^{de} \pm 3.8$	25 ± 2.6	150	391.5
Syringic acid (4-Hydroxy-3,5-dimethoxybenzoic acid)		$64.90^{d} \pm 5.4$	$37.4^{de} \pm 4.1$	18 ± 2.1	30	38.8
p-coumaric acid (p-Hydroxy cinnamic acid)		$1.9^{ab} \pm 0.20$	$39.7^{e} \pm 3.2$	18 ± 2.8	15	95
Protocatechuic acid (3,4-Dihydroxybenzoic acid)		$1.35^{a} \pm 0.16$	$47.1^{f} \pm 4.2$	16 ± 1.8	52.5	-
Gentisic acid (2,5-Dihydroxy benzoic acid)		$3.0^{b} \pm 0.28$	$59.1^{g} \pm 6.1$	16 ± 2.1	180	4.9
Gallic acid (3,4,5-Trihydroxybenzoic acid)		$1.1^{a} \pm 0.09$ 39	$132.0^{h} \pm 14$	14 ± 1.6	75	11.5

[a] MGFP and MGBP containing different phenolic acids given with their yield (mg/g) and structure. To understand the probable contribution of identified phenolic acids in mango ginger phenolic fractions, under similar experimental conditions PPAI and anti-*H. pylori* activities were also performed for standard pure phenolic acids. Values are expressed as IC_{50} (in μg/mL) for antioxidant activity and PPAI activity, and inhibition zone for *H. pylori* is expressed in millimeters. All data are the mean ± SD of three replicates; mean values followed by different letters in the same column differ significantly ($p > 0.05$). *Antioxidant activity measured as free radical scavenging activity.

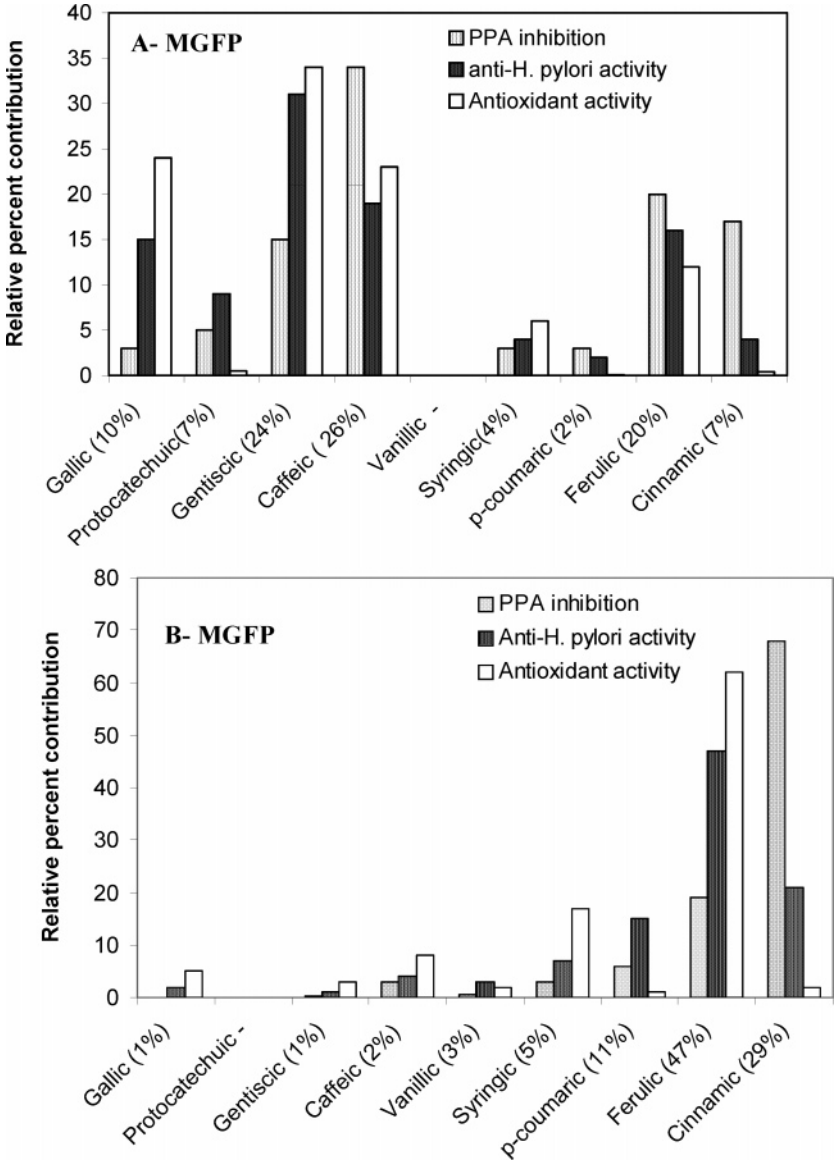


Figure 3. Relative percentage contribution of individual phenolic acids toward antioxidant, anti *H. pylori*, and PPA inhibition. This graph depicts the relative percent contribution of each phenolic acid found in MGFP and MGBP against H^+,K^+-ATPase activity (gray bars), anti *H. pylori* (black bars), and antioxidant activity (white bars). Percentage indicated in parentheses under each phenolic acid depicts the actual percent of them as revealed by HPLC (**Figure 1**).

and resistant to polymyxin. After 24 h, *H. pylori* growth was assayed by measuring the absorbance at 625 nm. MIC was defined as the lowest concentration in micrograms of GAE to restrict the growth of *H. pylori* to an absorbance value of <0.05 at 625 nm.

Scanning Electron Microscopy (SEM). The bacterium was grown overnight in broth at 37 °C, and 100 μL (8 log CFU/mL) in 5 mL of broth medium was incubated separately with (a) amoxicillin (10−30 μg/mL), (b) MGFP, (c) MGBP (10−50 μg/mL), and (d) major phenolic acids such as cinnamic, gentisic, ferulic, and gallic acids (10−50 μg/mL) for 6 h at 37 °C, and the suspension without treatment was taken as control. After incubation, a 100 μL aliquot was processed for SEM studies as described earlier (*25*). Multiple fields of visions were viewed, and results were documented by photography at different magnifications.

Measurement of Antioxidant Activity in MGFP and MGBP. *Free Radical Scavenging Activity.* The antioxidant activity of MGFP and MGBP, on the basis of the scavenging activity of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical, was determined according to the method described by Braca et al. (*26*). An aliquot of 100 μL of MGFP and MGBP at various concentrations from 2 to 10 μg/mL was added to 3 mL of 0.004% methanol solution of DPPH. The mixture was shaken vigorously and left to stand for 20 min at room temperature in the dark. The absorbance of the resulting solution was measured spectrophotometrically at 517 nm. The capability to scavenge the DPPH radical was calculated using the following equation:

$$\text{scavenging effect (\%)} = \left(\frac{\text{absorbance of control at 517 nm} - \text{absorbance of sample at 517 nm}}{\text{absorbance of control at 517 nm}}\right) \times 100$$

Reducing Power Ability. The reducing power of MGFP and MGBP was determined according to the method of Yen and Chen (*27*). The extract of MGFP and MGBP (2−10 μg/mL) was mixed with an equal volume of 0.2 M phosphate buffer (pH 6.6) and 1% potassium ferricyanide. The mixture was incubated at 50 °C for 20 min. An equal volume of 10% trichloroacetic acid was added to the mixture and centrifuged at 3000*g* for 10 min. The upper layer of the solution was mixed with distilled water and 0.1% $FeCl_3$ at a ratio of 1:1:2 (v/v/v), and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture compared to blank in a dose-dependent manner due to reduction of potassium ferricyanide by the antioxidant resulted in blue color formation that absorbed maximally at 700 nm. Hence, increased absorbance compared to blank indicates the increased antioxidant potency.

Inhibition of Lipid Peroxidation of Rat Liver Homogenate. In vitro lipid peroxidation levels in rat liver homogenate were measured as thiobarbituric acid reactive substances (TBARS). Ten percent fresh liver homogenate was prepared in 20 mM phosphate-buffered saline (PBS; pH 7.4) (*28*). Briefly, 0.25 mL of liver homogenate was incubated with 4−20 μg/mL of MGFP and MGBP in 20 mM PBS (pH 7.4). After 5 min of pretreatment, 0.5 mL of ferric chloride (400 mM) and 0.5 mL of ascorbic acid (400 mM) were added and incubated at 37 °C for 1 h. The reaction was terminated by the addition of 2.0 mL of TBA reagent (15% TCA, 0.375% TBA in 0.25 N HCl), and tubes were boiled for 15 min at 95 °C, cooled, and centrifuged; their absorbance was then measured at 532 nm. TBARS were measured by using a standard 1,1,3,3-tetramethoxypropane (TMP) calibration curve (0.1−0.5 μg) and expressed as percent inhibition of lipid peroxidation by extracts.

DNA Protection Assay. The DNA protective effect of phenolic fractions was determined electrophoretically (Submarine electrophoresis system, Bangalore Genei, Bangalore, India) using calf thymus DNA (*29*). Calf thymus DNA (1 μg in 15 μL) was subjected to oxidation by Fenton's reagent (30 mM H_2O_2, 50 mM ascorbic acid, and 80 mM $FeCl_3$). Relative difference in the migration between the native and oxidized DNA was ensured on 1% agarose gel electrophoresis after staining with ethidium bromide. Gels were documented (Herolab, Wiesloch, Germany), and the intensity of the bands was determined (Easywin software). Protection to DNA was calculated on the basis of the DNA band corresponding to that of native in the presence and absence of 2 and 4 μg of MGFP and MGBP.

Statistical Analysis. Generally, the analyses were repeated three times, and all data are expressed as mean value ± standard deviation (SD). The comparisons between mean values were tested using Duncan's new multiple-range test at a level of $p \leq 0.05$ (*30*).

RESULTS AND DISCUSSION

Various dietary sources studied previously in our laboratory indicated the presence of diverse health beneficial phenolic compounds (*6*, *22*, *31*). The phenolic acids and their role in antiulcer and antioxidant activities in free and bound phenolic fractions of mango ginger warranted a thorough investigation. In this study, we report the potential ulcer preventive ability of free and bound phenolics of mango ginger by evaluating antioxidant, anti *H. pylori*, and inhibition of proton pump and DNA protective activities.

Total Phenolic Content. Total phenolic contents in MGFP and MGBP phenolic fractions were found to be 2.1 ± 0.20 and 1.9 ± 0.17 mg of GAE/g of dried, defatted mango ginger powder, respectively. Approximately 2−3% yield of phenolics was obtained in mango ginger phenolic fractions (**Table 1**). In the current study we have isolated the phenolic acids, which are devoid of curcumin and other bioactive compounds, in order to understand the contribution of phenolic acids in phenolic fractions of mango ginger.

Identification of Phenolic Acids in MGFP and MGBP. The phenolic acid composition in both MGFP and MGBP fractions of mango ginger was determined by RP-HPLC, and their relative percents are depicted in **Figure 1**. The phenolic acids present in MGFP were caffeic (26%), gentisic (24%), and ferulic (20%) followed by gallic (10%), cinnamic (7%), protocatechuic (7%), and small amounts of syringic (4%) and *p*-coumaric acids (2%). MGBP fractions contained ferulic (47%) and cinnamic acid (29%) as major phenolic acids; *p*-coumaric (11%), syringic (5%), caffeic (4%), vanillic (2%), gallic (1%), and gentisic (1%) acids were also present in small amounts. The amount of phenolic acids present in phenolic fractions of mango ginger is given in **Table 2** as milligrams per gram.

Inhibition of Gastric H^+,K^+-ATPase Activity by MGFP and MGBP. H^+,K^+-ATPase, the proton pump responsible for acid secretion in the stomach, is located in the gastric membrane vesicle and catalyzes the electroneutral exchange of intracellular H^+ and extracellular K^+ coupled with the hydrolysis of the cytoplasmic ATP (*12*). Hypersecretion of this enzyme in the stomach leads to acidity and ulcer. Therefore, this regulatory enzyme was found to be a pharmacological target for many ulcer drugs. Mango ginger phenolic fractions—MGFP and MGBP—inhibited gastric H^+,K^+-ATPase activity in a concentration-dependent manner (**Figure 2**). The concentration required to inhibit 50% of H^+,K^+-ATPase activity is designated IC_{50}, and MGFP and MGBP showed IC_{50} values of 2.2 ± 0.21 and 0.7 ± 0.08 μg/mL, respectively, compared to the IC_{50} of 19.3 ± 2.2 μg/mL of lansoprazole, a known proton pump inhibitor. The mango ginger phenolic fractions were found to be good inhibitors of the enzyme, and the inhibition could be due to the binding of phenolic acids to ATPase enzyme. We have determined the inhibition of H^+,K^+-ATPase by individual phenolic acids present in extracts that potentially inhibit the enzyme. Cinnamic acid showed maximum inhibitory effect with an IC_{50} of 15.1 μg/mL, followed by caffeic (IC_{50} = 27.1 μg/mL), ferulic (IC_{50} = 33.6 μg/mL), syringic (IC_{50} = 37.4 μg/mL), *p*-coumaric (IC_{50} = 39.7 μg/mL), protocatechuic (IC_{50} = 47.1μg/mL), gentisic (IC_{50} = 59.1 μg/mL), and gallic acid (IC_{50} = 132.1 μg/mL). Accordingly, in mango ginger phenolic fractions, MGBP inhibited H^+,K^+-ATPase better (0.7 ± 0.08 μg/mL) than MGFP (2.2 ± 0.21 μg/mL), and this correlates to the increased levels of cinnamic (237 mg/g) and ferulic acids (391.5 mg/g) (**Table 2**).

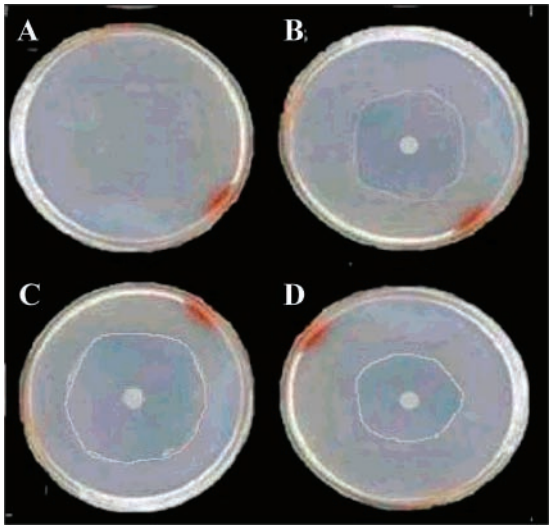


Figure 4. Effect of MGFP and MGBP on *H. pylori* growth as shown by the inhibitory zones in a disc diffusion method: (**A**) *H. pylori* control and treated with (**B**) amoxicillin, (**C**) MGBP, and (**D**) MGFP.

On the basis of the absolute amounts and potency of inhibition of H^+,K^+-ATPase activity of individual phenolic acids, the relative percentage contribution to H^+,K^+-ATPase inhibition in MGFP and MGBP is depicted in **Figure 3**. A total of 86% of the H^+,K^+-ATPase inhibition in MGFP is contributed by caffeic, gentisic, ferulic, and cinnamic acids. In MGBP, 70% of the PPA inhibition is contributed by cinnamic acid alone followed by 17% ferulic acid. It is indicated both in the literature and from our study that H^+,K^+-ATPase is up-regulated in ulcer condition. Inhibition of the same, therefore, would result in gastric protection or antiulcer property. The current study reveals that MGFP and MGBP have strong abilities to inhibit H^+,K^+-ATPase due to the presence of phenolic acids.

Inhibition of *H. pylori*. *H. pylori* is a Gram-negative, acid tolerant, microaerophilic bacterium that lives in the stomach and duodenum (*32*). The bacteria isolated from endoscopic

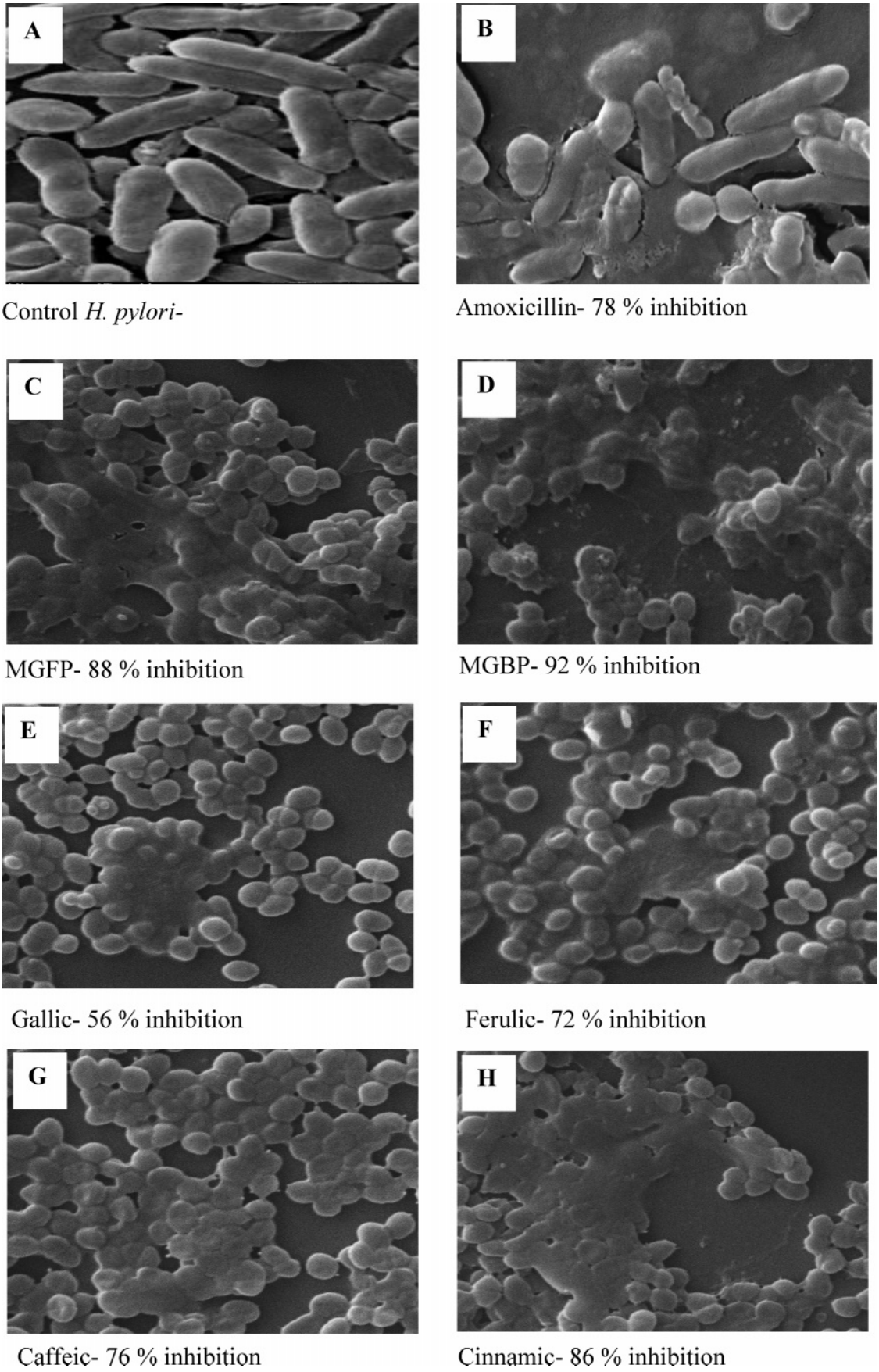


Figure 5. SEM view of *H. pylori* growth after treatment with MGFP and MGBP at 15K magnification: treated *H. pylori* with control (**A**), amoxicillin (**B**), MGFP (**C**), MGBP (**D**); *H. pylori* treated with pure phenolic acids gentisic (**E**), ferulic (**F**), caffeic (**G**), and cinnamic (**H**).

samples were Gram-negative, motile, and positive for urease, catalase, and oxidase tests (*33*). Furthermore, it was confirmed by the response to antibiotics, as it was resistant to antibiotics such as polymyxin B and was susceptible to amoxicillin. The

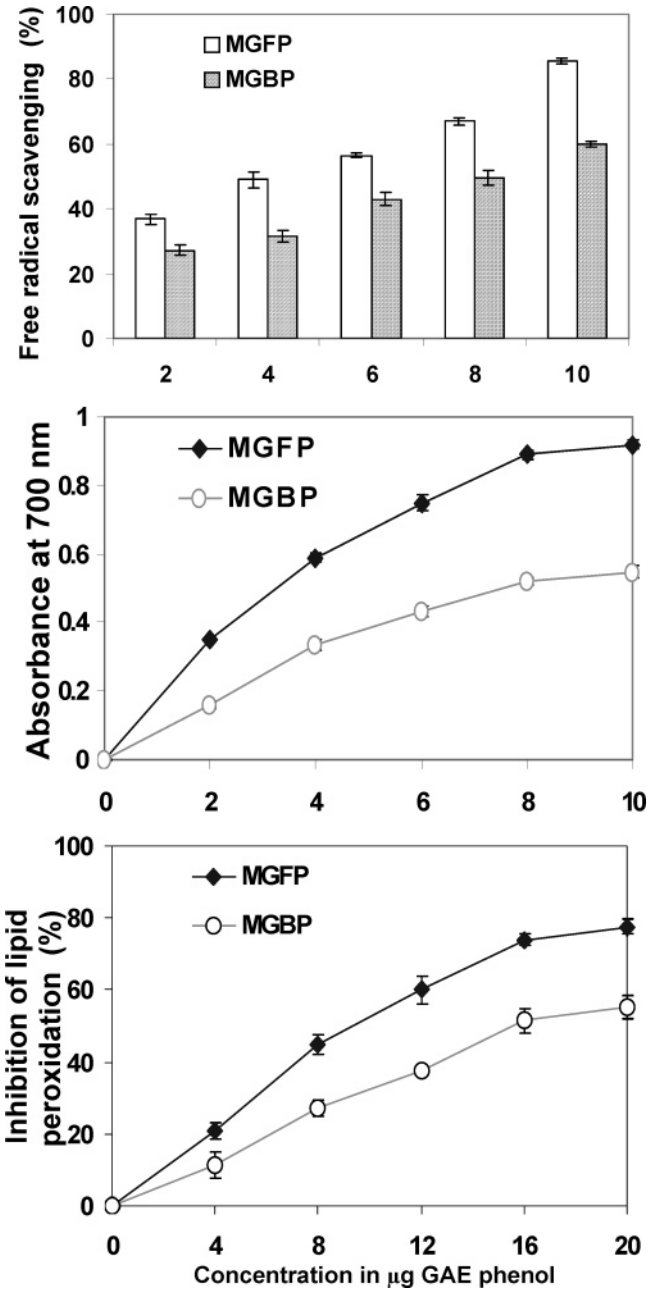


Figure 6. Antioxidant potency of MGFP and MGBP. Concentration of 2–10 μg of GAE/mL of MGFP and MGBP was examined for free radical scavenging (**A**), reducing power (**B**), and inhibition of lipid peroxidation as per the protocol described under Materials and Methods. All data are the mean ± SD of three replicates.

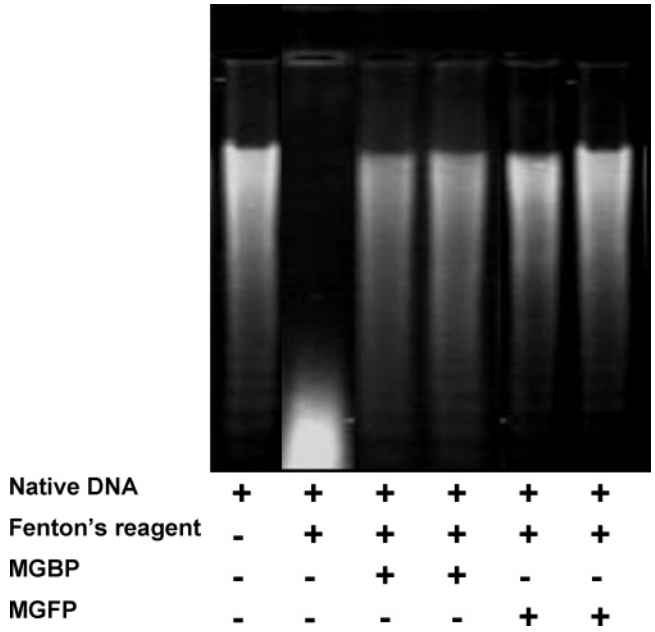


Figure 7. DNA protection ability of free (MGFP) and bound (MGBP) phenolics of ginger: 1 μg of native calf thymus DNA (lane 1); DNA treated with Fenton's reagent (lane 2); DNA pretreated with 2–4 μg of MGFB (lanes 2 and 3) and MGBP (lanes 3 and 4) were loaded onto the 1% agarose gel. Bands were visualized by staining with ethidium bromide, and in the transilluminator. Increased mobility represents DNA damage.

appearance of a characteristic white mucilaginous colony confirms the identity of bacteria as *H. pylori*. The antibacterial activity against *H. pylori* was assayed by using the agar diffusion method. Both phenolic fractions showed anti *H. pylori* activity. Both MGFP and MGBP showed a clear inhibition zone around the disk at a 10 μg/mL concentration, equivalent to that of a susceptible antibiotic, amoxicillin, at 10 μg/mL (**Figure 4**). To quantitate the inhibitory effect of *H. pylori*, the diameter of growth inhibition area was measured and expressed in millimeters. MGBP, MGFP, and amoxicillin showed inhibitory zones of 31 ± 2.8, 26 ± 2.4, and 36 ± 3.2 mm, respectively. Pure phenolic acids also showed *H. pylori* inhibition but to varying degrees (**Table 2**). Cinnamic acid appears to be the best inhibitor, with an inhibition zone of 34 ± 3.1 mm at 200 μg/disk followed by caffeic, ferulic, and syringic acid. Gallic acid showed the poorest activity of only 44%. Results are further supported by the observation of Vattem et al. (*34*), who suggested that phenolic phytochemicals such as cinnamic acid, cinnamaldehyde, coumarins, and flavanoids exhibit high anti *H. pylori* activity.

MIC. MIC values were determined according to the broth dilution method. The MIC values obtained confirm the significant ($p = 0.003$) anti *H. pylori* activity, MGFP MIC = 64 ± 6.1 μg/mL and MGBP MIC = 38 ± 2.2 μg/mL.

The relative percentage contribution of each phenolic acid in inhibiting *H. pylori* was calculated and is depicted in **Figure 3**. In MGFP, gentisic, caffeic, and ferulic acids contributed ~66% toward the inhibition of *H. pylori*, and although gallic acid is poorer in activity, due to its higher abundance, it contributed also significantly to *H. pylori* inhibition in MGFP. In MGBP, ferulic, cinnamic, and *p*-coumaric acids contributed ~83%. These phenolics were thought to exert their antimicrobial effect by causing (a) hyperacidification at the plasma membrane interface of the microorganism (*35*) or (b) intracellular acidification, resulting in disruption of the H^+,K^+-ATPase required for ATP synthesis of microbes (*36*) or (c) perhaps related to inactivation of cellular enzymes causing membrane permeability changes. The rate of inactivation of microbial cellular enzymes is dependent on the rate of penetration of phenolic antioxidants into the cell. In the case of *H. pylori*, it is possible that phenolics may be inactivating the urease enzyme, which is specifically expressed at its surface to neutralize hyperacidification to survive in the gastric environment of the stomach (*37*). The precise mechanism needs to be addressed.

SEM. The inhibitory effect of *H. pylori* by phenolic fractions of mango ginger and standard phenolic acids was confirmed by observing inhibition of *H. pylori* by electron microscopic observations. Untreated *H. pylori* showed uniform rod-shaped cells (**Figure 5A**), whereas the cells treated with mango ginger phenolic fractions—MGFP (**Figure 5C**) and MGBP (**Figure 5D**)—changed from helical form to coccoid and became necrotic.

A similar coccoid and necrotic form was also observed with *H. pylori* treated with amoxicillin (**Figure 5B**) and standard phenolic acids such as cinnamic (**Figure 5E**), caffeic (**Figure 5F**), ferulic (**Figure 5G**), and gallic acids (**Figure 5H**), showing alteration in *H. pylori* structure. In all of these treatments the coccoid form with blebs in the bacterial surface, the appearance of vacuoles and granules, and an area of low electron density in the cytoplasm were observed. These coccoid forms were known to result in the loss of infectivity (*38*). The lysis of *H. pylori* thus confirms the antimicrobial nature of MGFP and MGBP.

MGFP and MGBP Exhibited Multipotent Antioxidant Activity. Antioxidant potencies were determined to provide evidence that phenolic fractions also possess antioxidant activity, the action of which is essential to counteract the oxidative stress induced ulcers.

Free Radical Scavenging Activity. The effect of phenolic fractions on DPPH radical scavenging is thought to be due to their hydrogen-donating ability. DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. The reduction capability of DPPH radical is determined by the decrease in its absorbance at 517 nm induced by phenolic fractions. The decrease in absorbance of DPPH radical caused by phenolic fractions is due to the reaction between antioxidant molecules and radical, resulting in the scavenging of radical by hydrogen donation, and is visualized as a discoloration from purple to yellow (*39*). In the present study MGFP and MGBP showed concentration-dependent radical scavenging activity (**Figure 6A**). MGFP showed better radical scavenging activity with an IC_{50} of 2.2 ± 0.17 μg/mL compared to that of MGBP, 4.2 ± 0.36 μg/mL. The scavenging activity was directly attributed to their phenolic content, because we have shown antioxidant potencies of pure phenolic acids, which are the representative antioxidant components of mango ginger extracts.

Reducing Power Assay. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity (*40*). The reducing power of the sample increased with an increase in the concentration of phenolics. The reducing power of MGFP showed an approximate 2-fold increase in the total reducing power compared to MGBP (**Figure 6B**). MGFP and MGBP exhibited 193 ± 21 and 104 ± 8.6 units/g reducing abilities, respectively. These data may indicate that the phenolic fractions tested may act as reductones by donating electrons to free radicals and thereby converting free radicals to more stable products and terminating free radical chain reaction.

Inhibition of Lipid Peroxidation. Malondialdehyde (MDA) is a highly reactive species that is generated during lipid peroxidation and is found to be an important cause of cell membrane destruction and cell damage. It cross-links DNA with protein and thus damages the cells (*41*), disrupting its activity and leading to chronic diseases. In the present study we measured the potential of MGFP and MGBP to inhibit lipid peroxidation products (TBARS). The hydroxy radical generated through the Fenton reaction was scavenged by co-incubation of rat liver homogenate with various concentrations (4−20 μg of GAE/g of sample) of MGFP and MGBP. MGFP showed maximum inhibition of lipid peroxidation with an IC_{50} of 10.3 ± 0.91 μg of GAE/g sample compared to the IC_{50} of MGBP (15.6 ± 1.6 μg of GAE/g) (**Figure 6C**).

DNA Protection Activity. DNA fragmentation by Fenton's reagent was recovered with the treatment of MGFP and MGBP extracts prior to oxidative stress (as visualized by the increased electrophoretic mobility of DNA). A dose-dependent protection was observed by both free and bound phenolics of mango ginger at 2−4 μg of GAE, respectively (**Figure 7**). A significant (>80%, $p < 0.005$) protection to native DNA during oxidation in the presence of these fractions was observed. These results indicate that free and bound phenolics of mango ginger can quench free radicals and thereby may protect DNA against oxidative stress induced damage.

In conclusion, mango ginger (*C. amada*) contains significant amounts of phenolics as both free and bound forms. Both free and bound phenolic fractions of mango ginger were found to be antioxidant and effective in inhibiting H^+,K^+-ATPase activity and *H. pylori* growth. Furthermore, cinnamic and ferulic acids present in phenolic fractions of mango ginger may contribute significantly to H^+,K^+-ATPase as well as *H. pylori* growth inhibition, and other phenolic acids may contribute to antioxidant activity. Also, the antioxidant activity of phenolic acids and their esters depends on the number of hydroxy groups in the molecules, which would be strengthened by steric hindrance (*42*). Hydroxylated gallic acid was found to be very effective as an antioxidant. H^+,K^+-ATPase and *H. pylori* inhibition was higher in cinnamic derivatives that contained fewer hydroxy groups.

ABBREVIATIONS USED

MGFP, mango ginger free phenolics; MGBP, mango ginger bound phenolics; GAE, gallic acid equivalent; NSAID, non-steroidal anti-inflammatory drugs; TBA, 2-thiobarbituric acid; DPPH, 1,1-diphenyl-2-picrylhydrazyl.

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